

## Original Paper

# Mechanisms of $I_{hERG}/I_{Kr}$ Modulation by $\alpha 1$ -Adrenoceptors in HEK293 Cells and Cardiac Myocytes

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## Key Words

Phosphatidylinositol 4,5-bisphosphate • Src • PKA • hERG • PKC •  $I_{Kr}$

## Abstract

**Background:** The rapid delayed rectifier  $K^+$  current ( $I_{Kr}$ ), carried by the hERG protein, is one of the main repolarising currents in the human heart and a reduction of this current increases the risk of ventricular fibrillation.  $\alpha 1$ -adrenoceptors ( $\alpha 1$ -AR) activation reduces  $I_{Kr}$  but, despite the clear relationship between an increase in the sympathetic tone and arrhythmias, the mechanisms underlying the  $\alpha 1$ -AR regulation of the hERG channel are controversial. Thus, we aimed to investigate the mechanisms by which  $\alpha 1$ -AR stimulation regulates  $I_{Kr}$ . **Methods:**  $\alpha 1$ -adrenoceptors, hERG channels, auxiliary subunits minK and MIRP1, the non  $PIP_2$ -interacting mutant D-hERG (with a deletion of the 883-894 amino acids) in the C-terminal and the non PKC-phosphorylatable mutant N-terminal truncated-hERG (NTK-hERG) were transfected in HEK293 cells. Cell membranes were extracted by centrifugation and the different proteins were visualized by Western blot. Potassium currents were recorded by the patch-clamp technique.  $I_{Kr}$  was recorded in isolated feline cardiac myocytes. **Results:** Activation of the  $\alpha 1$ -AR reduces the amplitude of  $I_{hERG}$  and  $I_{Kr}$  through a positive shift in the activation half voltage, which reduces the channel availability at physiological membrane potentials. The intracellular pathway connecting the  $\alpha 1$ -AR to the hERG channel in HEK293 cells includes activation of the  $G\alpha_q$  protein, PLC activation and  $PIP_2$  hydrolysis, activation of PKC and direct phosphorylation of the hERG channel N-terminal. The PKC-mediated  $I_{Kr}$  channel phosphorylation and subsequent  $I_{Kr}$  reduction after  $\alpha 1$ -AR stimulation was corroborated in feline cardiac myocytes. **Conclusions:** These findings clarify the link between sympathetic nervous system hyperactivity and  $I_{Kr}$  reduction, one of the best characterized causes of *torsades de pointes* and ventricular fibrillation.

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## Introduction

The classical paradigm says that for an arrhythmia to be induced both a substrate and a trigger is needed. The human ether-a-go-go related gene (hERG) encodes the pore forming protein of the rapidly activated delayed rectifier  $K^+$  current ( $I_{Kr}$ ), one of the most important currents that contributes to the repolarisation of the human heart. Reduction of this current either by a mutation in the gene or by a blockade of the channel by drugs produce congenital and acquired Long QT Syndrome (LQTS) respectively, creating a substrate that increases the risk of ventricular fibrillation [1]. The clinical observation showed that, in patients with LQTS, arrhythmias are typically triggered during physical or emotional stress, suggesting a link between sympathetic stimulation and arrhythmias. Sympathetic stimulation exerts different proarrhythmic effects, such as an increase of  $I_f$  or L-type  $Ca^{2+}$  currents and a reduction of hERG potassium channel activity [2, 3]. In fact, activation of both  $\beta$ - and  $\alpha$ -adrenoceptors (-ARs) regulate  $I_{Kr}$ .

$\beta$ -AR stimulation has been proposed to regulate the Kv4.3 and hERG channels in cardiac myocytes [4] and heterologous systems inducing either an increase or a decrease of the hERG current. The proposed regulatory mechanisms of hERG included direct interaction of cAMP with the CNBD (cyclic nucleotide binding domain) of the channel, PKA-dependent phosphorylation and dynamic interaction with the 14-3-3 protein [5-8]. However, it was later demonstrated that cyclic nucleotides do not directly modulate hERG channels [9], and hERG-14-3-3 interaction has not been demonstrated in native cells. In fact, only an increase of  $I_{Kr}$  via the activation of PKA has been demonstrated in native ventricular cells [10]. Regarding  $\alpha 1$  adrenoceptors, although  $\alpha 1$ -AR activation also reduces native  $I_{Kr}$  and  $I_{hERG}$  in cardiac myocytes and in heterologous expression systems respectively, the specific mechanism remains elusive.

In 1999 it was reported for the first time a reduction in the  $I_{hERG}$  upon  $\alpha 1$ -AR activation, but the G protein involved was not determined [11]. Later works proposed that phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) consumption by PLC mediated hydrolysis might cause such reduction [12], probably involving a direct interaction between  $PIP_2$  and the C terminus of the channel [13]. However, the  $PIP_2$ -dependent regulation of hERG channel has been questioned since and is currently believed it is not essential for normal channel function [14, 15].

Simultaneously, other works proposed that the reduction of the  $I_{hERG}$  upon  $\alpha 1$ -AR stimulation was due to the phosphorylation of the hERG channel by PKA and PKC [16-18]. However, when mutation experiments in *Xenopus* oocytes showed that deletion of most of the consensus phosphorylation sites did not prevent the effect, authors suggested that hERG channels were modulated by PKA and PKC independently of direct phosphorylation [19]. It is important to note that not all the putative phosphorylation sites were mutated in that work and, therefore, the direct channel phosphorylation cannot be totally excluded.

In this work we aimed to investigate the molecular mechanism by which sympathetic activation of  $\alpha 1$ -AR regulates ventricular  $I_{Kr}$ . Thus, we analyzed the effect of  $\alpha 1$ -AR stimulation on hERG channel in Human Embryonic Kidney (HEK293) cells and on  $I_{Kr}$  in feline cardiac myocytes.

## Materials and Methods

### Cell culture and transfection

Human Embryonic Kidney cells and a cell line stably expressing hERG channel [20] (HEK-hERG; Kindly provided by Dr. J. Hancox, University of Bristol, with the permission of Dr. C.T. January, University of Wisconsin) were cultured in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin-amphotericin cocktail. HEK-hERG grew in the presence of 50  $\mu M$  of geneticin. Cell cultures were maintained in 5%  $CO_2$  at 37°C. 4  $\mu g$  of D-hERG cDNA (kindly provided by Dr. T.V. McDonald, Albert Einstein College of Medicine, NY) or NTK-hERG cDNA (kindly provided by Dr. M.C.

Sanguinetti, University of Utah) were transiently cotransfected with 1  $\mu$ g of  $\alpha 1$ -adrenoceptor cDNA (kindly provided by Dr. C. Hague, University of Washington) along with 0.2  $\mu$ g of GFP cDNA (kindly provided by Dr. M.T. Perez-Garcia, University of Valladolid) using Fugene 6 (Roche) following manufacturer instructions. Cells were used for electrophysiological studies and Western blot experiments 48-72 h after transfection.

#### Western blot

HEK293 cell membranes were isolated by centrifugation at 40000g, fractionated on 10 % SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences). Nitrocellulose membranes were blocked in TTBS solution (Tris-HCl 50 mM pH 7.5, NaCl 150 mM, Tween-20 0.05%) containing BSA 3%. Blots were incubated with primary antibodies anti-hERG (1:200; Santa Cruz Biotechnology); anti- $\alpha 1$ -AR (1:400; Oncogene Research Products); anti-minK (1:100; Santa Cruz Biotechnology); and anti-MIRP1 (1:200; Abnova). Secondary antibodies were conjugated with Horseradish Peroxidase and blots were developed using enhanced chemiluminescence (West Pico, Thermo Scientific).

#### Isolation of feline cardiomyocytes

Animal studies have been approved by the Ethics Committee for the Humane Use of Laboratory Animals of the University of Colima (México) and the experiments were conducted following the ethical standards laid down in the Declaration of Helsinki and its later amendments.

Single right ventricular myocytes were obtained from adult cats as described previously [21]. Hearts were mounted on a Langendorff apparatus and perfused for 5 min with normal Tyrode's solution, and then switched to a nominally calcium-free solution for an additional 5 min. Thereafter, the hearts were perfused for 30 min with a zero-calcium solution containing 1 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, MO) and 0.05 mg/ml protease XIV (Sigma-Aldrich). The enzymes were washed out by perfusion with a high-potassium, low chloride saline (KB medium) for 5 min [22]. The free wall of the right ventricle was dissected away from the rest of the heart, placed in beakers, and cut into small pieces. Single cells were maintained in a high-potassium, low chloride solution at 4°C for 2 h before use in electrophysiological experiments.

Tyrode's solution had the following composition (in mM): 125 NaCl, 24 NaHCO<sub>3</sub>, 0.42 NaH<sub>2</sub>PO<sub>4</sub>, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1.05 MgCl<sub>2</sub>, 11 glucose, and 10 taurine. The solution was equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, pH 7.4. Nominally calcium-free solution was prepared by omitting CaCl<sub>2</sub> from the Tyrode's solution. The high-potassium, low-chloride KB medium had the following composition (in mM): 80 potassium glutamate, 40 KCl, 20 taurine, 3 KH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 10 HEPES, and 0.2 EGTA. pH 7.4 with KOH.

#### Patch-clamp recordings

Macroscopic currents were recorded at room temperature (22°C) in the whole-cell configurations of the patch-clamp technique [23], using Axopatch-200B amplifiers (Molecular Devices). Borosilicate capillary glass were pulled obtaining a tip resistance of 1-3 M $\Omega$  after filled with the internal solution. For HEK293 cells the internal solution was (in mM) 125 KCl, 5 MgCl<sub>2</sub>, 5 EGTA-K, 10 HEPES-K and 5 Na-ATP adjusted to pH 7.2 with KOH. For cardiac myocytes the internal solution was (in mM): 90 potassium aspartate, 54 KCl, 10 KH<sub>2</sub>PO<sub>4</sub>, 5 HEPES, 0.1 EGTA, pH 7.3 with KOH.

Following the patch rupture, whole cell membrane capacitances were measured from integration of the capacitive transients elicited by voltage steps from -50 to -60 mV, which did not activate any time dependent membrane current. Series resistances were compensated 80% in order to minimize voltage errors and were checked regularly throughout the experiment to ensure that there were no variations with time. The voltage-clamp experimental protocols were controlled with the "Clampex" program of the "pClamp" software (Molecular Devices). HEK293 cells were perfused with the external solution containing (in mM): 136 NaCl, 4 KCl, 1 MgCl<sub>2</sub>, 10 HEPES-Na, 1.8 CaCl<sub>2</sub> and 10 glucose, pH 7.4 with NaOH. For cardiac myocytes the bathing solution was (in mM): 140 NaCl, 5.4 KCl, 0.1 CaCl<sub>2</sub>, 0.4 CoCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 10 HEPES and 11 glucose, pH adjusted to 7.4 with NaOH. HMR-1556 1  $\mu$ M was added to block I<sub>Ks</sub>. For HEK-hERG cells terfenadine 1  $\mu$ M was added at the end of the experiment and the remaining endogenous current was subtracted.

To elicit I<sub>HERG</sub> and I<sub>Kr</sub> we applied depolarizing pulses from -40 to +70 in 10 mV increments for 2 s every 30 seconds from a holding potential of -80 mV. These pulses were followed by a pulse at -40 mV for 5 s to measure tail current. I-V curves were obtained by measuring the current at the end of the test pulses, normalized to cell capacitance and expressed as pA/pF. The peak of the tail current was normalized to the

maximum current and curves were fitted to Boltzmann relation  $I=1/[1+e^{(V_h-V)/k}]$ , where  $I$  is the amplitude of the tail current,  $V_h$  is a half-maximal activation,  $V$  is the applied membrane voltage and  $k$  is the slope factor. Clampfit 10.2 software was used for the analysis of currents. The deactivation kinetics were calculated by fitting the decay of tail currents to a monoexponential function.

#### Drugs

Phenylephrine (PE), propranolol and neomycin were from Sigma-Aldrich. GP antagonist 2A (GPant2A); and bisindolylmaleimide 1 (BIS-1) were from Calbiochem. 1-phosphatidyl-1D-myo-inositol-3,4-bisphosphate (diC8-PIP<sub>2</sub>) was from Echelon Biosciences. Anti-PIP<sub>2</sub> was from Santa Cruz Biotechnology. HMR-1556 was from Tocris.

#### Statistical analysis

Values are presented as mean  $\pm$  SEM. Student's t-test for paired data was used to compare the difference between two means.  $p < 0.05$  was considered significant.

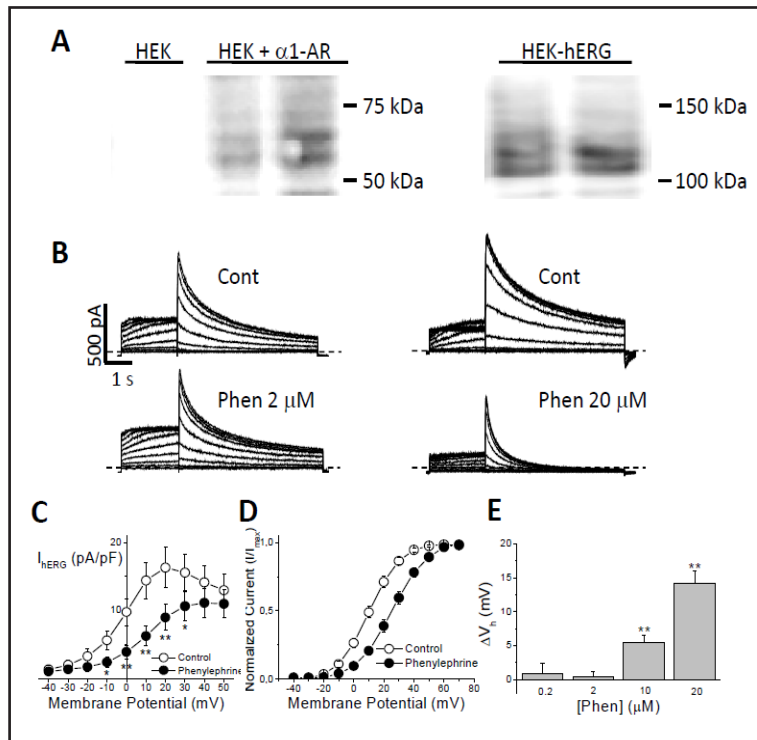
## Results

### Phenylephrine reduces $I_{hERG}$ in HEK293 cells

Cell lysates were immunoblotted to confirm the membrane expression of the  $\alpha 1A$ -adrenergic receptor and the hERG channel. The adrenoceptor, as well as the two bands, mature glycosylated and immature non-glycosylated, characteristic of the hERG channel proteins were detected only in transfected HEK-hERG cells (Fig. 1A).

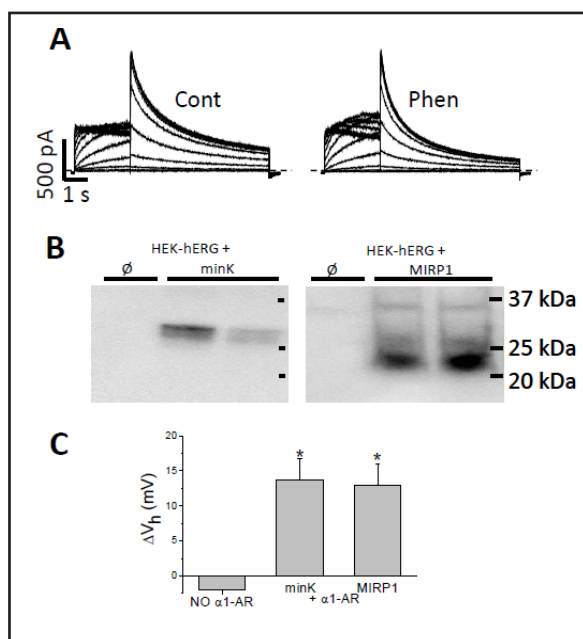
Transfection was functional and cells responded to receptor stimulation with phenylephrine (PE). Thus, hERG channel current was recorded before and five minutes after 20  $\mu$ M PE administration and a reduction on both activated  $I_{hERG}$  and tail current was observed (Fig. 1B). The current-voltage (I-V) relationship normalized to cell capacitance of the  $I_{hERG}$  at

**Fig. 1.**  $\alpha 1$ -AR stimulation reduces  $I_{hERG}$  and shifts the activation half voltage. A) Membrane expression of  $\alpha 1A$ -ARs in untransfected and transfected HEK293 cells (left) and channel expression in HEK293 cells stably expressing hERG (right); duplicates are shown. B) Representative hERG current recordings in two HEK-hERG cells transfected with the  $\alpha 1$ -adrenoceptor before (Cont) and after administration of 2 or 20  $\mu$ M phenylephrine (Phen). C) Current density-voltage curve and D) voltage dependence of activation in control and after  $\alpha 1A$ -AR stimulation. E) Concentration-dependence of the effect of phenylephrine on the voltage dependence of  $I_{hERG}$  activation. The displacement of the



activation half voltage ( $\Delta V_h$ ) is plotted against phenylephrine concentration. Mean  $\pm$  SEM;  $n = 7$ -37 paired cells. \* $p < 0.05$ ; \*\* $p < 0.01$ . Dashed lines indicate the zero current level.

**Fig. 2.** minK and MIRP1 are not required for  $\alpha 1$ -AR induced regulation of  $I_{hERG}$ . A) hERG Current recordings in a HEK-hERG cell not transfected with the  $\alpha 1A$ -adrenoceptor before (Cont) and after adding 20  $\mu M$  phenylephrine (Phen). B) Western blot of minK and MIRP1 in untransfected ( $\emptyset$ ) and transfected HEK-hERG cells. C) Displacement of the  $I_{hERG}$  activation half voltage ( $\Delta V_h$ ) induced by 20  $\mu M$  phenylephrine in HEK-hERG cells not transfected with  $\alpha 1$ -AR (NO  $\alpha 1$ -AR) and cotransfected with  $\alpha 1$ -AR plus either minK or MIRP1. Mean  $\pm$  SEM. n = 18, 7, 13 paired cells. \*p<0.01. Dashed lines indicate the zero current level.



the end of the 2 seconds pulse in control and PE stimulation shows current inhibition in a wide voltage range: between -10 to +30 mV. Because of the channel rectification properties, as the voltage increased the contribution of hERG channels to the total current become smaller and therefore the effect of PE was lower (Fig. 1C). Analysis of hERG tail currents showed that PE accelerates the current deactivation. When fitted to a monoexponential function,  $\tau_{deact}$  was accelerated from  $1331 \pm 74$  to  $639 \pm 44$  ms (n = 37 paired cells, p<0.0001). The voltage dependence of hERG activation was calculated from plots of tail current amplitudes (normalized to the peak current) versus voltage. PE administration caused a 14 mV positive shift in the activation half voltage ( $V_h$ ), from  $10.9 \pm 1.9$  to  $25.1 \pm 2.1$  mV (n = 37 paired cells; p < 0.001), and this effect was concentration-dependent, with an  $EC_{50} = 12 \mu M$  (Fig. 1D, E). PE concentrations higher than 40  $\mu M$  PE fully eliminated the  $I_{hERG}$ .

#### *$\alpha 1$ -AR stimulation shifts hERG channel activation $V_h$*

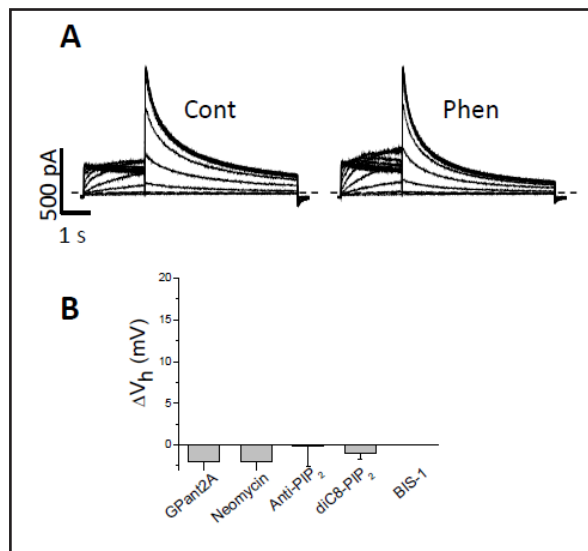
Since hERG channel has a great susceptibility to be blocked by many different drugs, we first tested whether the effects of PE on  $I_{hERG}$  were due to  $\alpha 1$ -AR stimulation or to a blockade of the channel. Thus, when 20  $\mu M$  PE was added to hERG expressing HEK cells not transfected with the receptor, we still observed a faster tail current deactivation ( $1420 \pm 98$  vs  $998 \pm 85$  ms; n = 18 paired cells, p<0.0001), suggesting a direct open channel blockade of the channel by PE. Conversely, the activation half voltage was not affected by phenylephrine in the absence of the receptor (Fig. 2). This result demonstrates that the positive shift in the hERG activation half voltage requires  $\alpha 1$ -AR stimulation. In conclusion, the mechanism responsible for the  $I_{hERG}$  reduction after the  $\alpha 1$ -AR activation is the positive displacement of the activation half voltage because, at a given membrane potential, the current amplitude is expected to be reduced.

#### *Neither minK nor MIRP1 modulate the $\alpha 1$ -AR regulation of hERG channels*

Although the real hERG partners in native systems is a matter of debate, in heterologous expression systems hERG channels can interact with the accessory proteins minK and MIRP1 [24]. We wondered whether these proteins could affect the  $\alpha 1$ -AR-induced  $I_{hERG}$  reduction, so we transfected HEK-hERG cells with the receptor plus one of the accessory subunits and tested the effects of PE. Membrane expression of minK and MIRP1 was confirmed by Western blot (Fig. 2B).

Current recordings showed that 20  $\mu M$  PE shifted the  $I_{hERG+minK}$  activation  $V_h$  to a similar extent than in the absence of the accessory protein (from  $14 \pm 4.1$  to  $27.7 \pm 3.1$  mV; n =

**Fig. 3.**  $\alpha 1$ -AR induced  $I_{hERG}$  regulation depends on PLC and PKC activation. A) hERG current recordings in a HEK-hERG cell transfected with the  $\alpha 1A$ -adrenoceptor in control (Cont) and after administration of 20  $\mu M$  phenylephrine (Phen) in the presence of an anti-PIP<sub>2</sub> antibody in the internal solution. B) Displacement of the  $I_{hERG}$  activation half voltage ( $\Delta V_h$ ) caused by  $\alpha 1$ -AR stimulation in cells with: the Gq protein blocker GPantagonist 2A 50  $\mu M$ ; the PLC inhibitors Neomycin 1 mM, anti-PIP<sub>2</sub> antibody 80 nM and diC8-PIP<sub>2</sub> 10  $\mu M$ ; and the PKC blocker bisindolylmaleimide 1 (BIS-1) 10  $\mu M$ . Mean  $\pm$  SEM. n = 9, 9, 8, 11, 18 paired cells. Dashed lines indicate the zero current level.



7 paired cells;  $p < 0.01$ ). As expected, MIRP1 shifted hERG activation  $V_h$  to more negative potentials. However, when PE was added to the cells the approximately 14 mV shift in  $I_{hERG+MIRP1}$  activation half voltage was again observed (from  $-1.2 \pm 3$  to  $12.2 \pm 3.6$  mV;  $n = 13$  paired cells;  $p < 0.01$ ). As the effect of PE on the activation  $V_h$  of  $I_{hERG+minK}$ ,  $I_{hERG+MIRP1}$  and  $I_{hERG}$  is basically identical, we can conclude that minK and MIRP1 do not alter the hERG channel response to the  $\alpha 1$ -ARs stimulation (Fig. 2C).

#### Intracellular pathway mediating the $\alpha 1$ -AR modulation of hERG

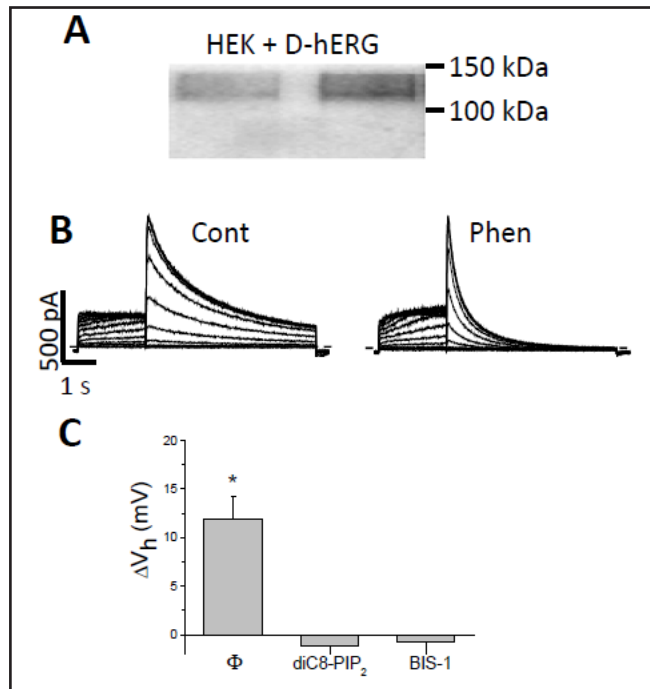
The  $\alpha 1$ -adrenoceptor belongs to a G-protein coupled receptors family classically associated with G $\alpha_q$  dissociation, phospholipase C (PLC) activation, PIP<sub>2</sub> hydrolysis and PKC activation. The involvement of the G $\alpha_q$  protein in the PE-induced  $I_{hERG}$  reduction was confirmed with 50  $\mu M$  of the specific G $\alpha_q$  blocker GPant2A (RPKPQQDWFWDWM). In the presence of this blocker PE did not shift the activation half voltage of  $I_{hERG}$  (Fig. 3), suggesting that  $\alpha q$  is the relevant G protein in this pathway.

In the canonical pathway, G $\alpha_q$  protein-activated PLC hydrolyses PIP<sub>2</sub> producing the PKC activators 1,2-diacylglycerol (DAG) and inositol-1,4,5,-trisphosphate (IP<sub>3</sub>). We blocked these steps with three chemical tools: neomycin and an anti-PIP<sub>2</sub> antibody, which block the access of PLC to PIP<sub>2</sub>; and the non-hydrolysable PIP<sub>2</sub> analogue diC8-PIP<sub>2</sub>, a putative competitive inhibitor of phospholipases [25-28]. The presence of each of the inhibitors prevented the PE-induced shift in  $I_{hERG}$  activation half voltage (Fig. 3), indicating the involvement of PLC.

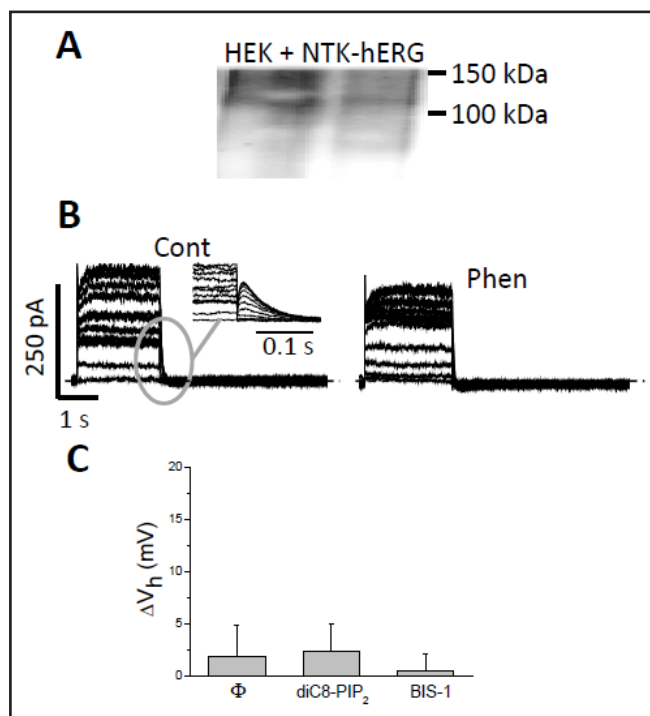
hERG channels are modulated by Ser/Thr kinases like PKA and PKC and by Tyrosine kinases such as Src [19, 29, 30]. Scansite database reveals 1 putative phosphorylation site for Src (Y1009) and 18 sites for PKA and/or PKC in the channel sequence. Our results showed that the effect of  $\alpha 1$ -AR stimulation on  $I_{hERG}$  does not depend on tyrosine kinases mediated phosphorylation. In the presence of the Tyr-kinase inhibitor genistein 50  $\mu M$  the activation  $V_h$  moved from  $10.3 \pm 3.4$  to  $25.9 \pm 3$  mV after adding phenylephrine ( $n = 9$ ;  $p < 0.001$ ). However, when PKC was inhibited by BIS-1 (10  $\mu M$ ) PE was not able to shift the activation half voltage of  $I_{hERG}$  to more positive potentials (from  $11.2 \pm 3.1$  to  $11.3 \pm 3$  mV;  $n = 18$  paired cells, ns; Fig. 3). Thus, PKC has a key role in the regulation of the hERG channel after  $\alpha 1$ -AR stimulation.

To confirm that PIP<sub>2</sub> does not directly affect the channel modulation, HEK293 cells were cotransfected with the  $\alpha 1$ -AR plus the D-hERG mutant. This mutant has a deletion of the 883-894 amino acids in the C-terminal, the main site of interaction between PIP<sub>2</sub> and hERG channels [13]. Perfusion of the cells with 20  $\mu M$  PE shifted the activation half voltage of  $I_{D-hERG}$  to more depolarized potentials (from  $8.2 \pm 3$  to  $20.1 \pm 3$  mV;  $n = 11$  paired cells;  $p < 0.001$ ; Fig. 4), same as was observed with the wild type channel. On the contrary, addition of the non-hydrolysable analogue diC-8-PIP<sub>2</sub> (10  $\mu M$ ) or the PKC blocker BIS-1 (10  $\mu M$ ) prevented the

**Fig. 4.** PIP<sub>2</sub> interaction with hERG channel is not relevant for regulation by  $\alpha 1$ -AR. A) Expression of the presumably non PIP<sub>2</sub> interacting mutant D-hERG in transfected HEK293 cells (duplicates are shown). B)  $I_{D-hERG}$  recordings in a HEK cell coexpressing the D-hERG and the  $\alpha 1$ -AR, before (Cont) and after 20  $\mu$ M phenylephrine (Phen) administration. C) Displacement of the  $I_{D-hERG}$  activation half voltage ( $\Delta V_h$ ) caused by PE in cells without ( $\emptyset$ ) and with diC8-PIP<sub>2</sub> or bisindolylmaleimide 1. Mean  $\pm$  SEM. n = 11, 7, 8 paired cells. \*p<0.01. Dashed lines indicate the zero current level.

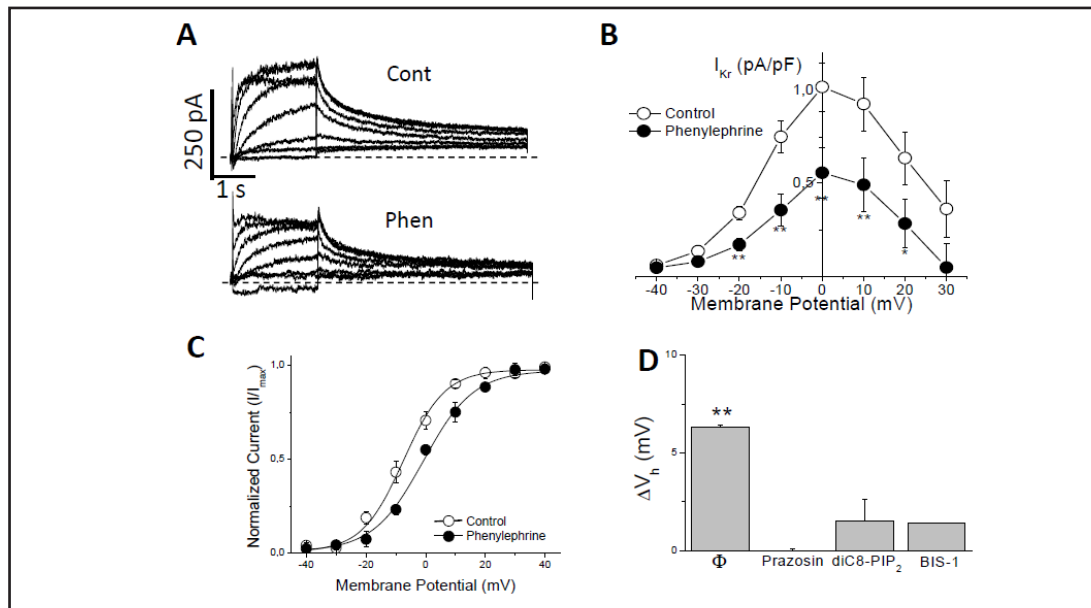


**Fig. 5.** hERG regulation by  $\alpha 1$ -ARs is dependent on PKC phosphorylation. A) Expression of the non PKC-phosphorylatable mutant NTK-hERG in transfected HEK293 cells. B)  $I_{NTK-hERG}$  recordings in a HEK cell coexpressing the NTK-hERG and the  $\alpha 1$ -AR, before (Cont) and after 20  $\mu$ M phenylephrine (Phen) administration. Since NTK-hERG tail deactivation kinetics are faster than WT, the inset shows an amplification of the tail currents. C) Displacement of the  $I_{NTK-hERG}$  activation half voltage ( $\Delta V_h$ ) caused by 20  $\mu$ M phenylephrine in cells without ( $\emptyset$ ) and with diC8-PIP<sub>2</sub> or bisindolylmaleimide 1 (BIS-1). Mean  $\pm$  SEM. n = 10, 6, 7 paired cells. Dashed lines indicate the zero current level.



effect of PE on the deletion mutant, supporting the role of PKC-dependent phosphorylation of the channel (Fig. 4C).

In previous works the four PKA phosphorylation sites and 17 of the 18 consensus PKC phosphorylation sites in hERG were mutated to alanine and the channel modulation by the  $\alpha 1$ -AR was similar to WT channels [19, 31]. However, the T74, located in the Per-Arnt-Sim (PAS) domain of the channel, cannot be deleted or mutated to alanine without the loss of function of the channel, showing that this is a key residue in the channel function. Interestingly, the T74 was demonstrated to be phosphorylated by PKC [32]. So we then



**Fig. 6.**  $\alpha$ 1-AR stimulation reduces  $I_{Kr}$  by a PKC-phosphorylation mediated mechanism. A)  $I_{Kr}$  recordings in a feline ventricular myocyte in control (Cont) and after application of 20  $\mu$ M phenylephrine (Phen). B) Current density-voltage curve and C) voltage dependence of activation before and after  $\alpha$ 1-AR stimulation. D) Displacement of the  $I_{Kr}$  activation half voltage ( $\Delta V_h$ ) induced by  $\alpha$ 1-AR stimulation in cells without ( $\emptyset$ ) and with prazosin (1  $\mu$ M), diC8-PIP<sub>2</sub> or bisindolylmaleimide 1 (BIS-1). Mean  $\pm$  SEM. n = 10, 5, 6, 5 paired cells. \*  $p < 0.05$ ; \*\* $p < 0.01$ . Dashed lines indicate the zero current level.

cotransfected HEK293 cells with the  $\alpha$ 1-AR and the NTK-hERG channel, a mutant whose 2-354 amino acids containing the PAS domain and the T74 have been removed [33]. Although both activation and deactivation kinetics are faster in NTK-hERG than in WT hERG, the activation half voltage is similar and was not regulated by PE (from  $9.5 \pm 4.4$  to  $11.3 \pm 3.4$  mV; n=10, ns). The absence of effect of  $\alpha$ 1-AR stimulation on the NTK-hERG activation  $V_h$  is again consistent with a major role of PKC in this regulation pathway. As expected, diC8-PIP<sub>2</sub> or BIS-1 did not affect the  $I_{NTK-hERG}$  activation  $V_h$  after PE exposure (Fig. 5).

#### *$\alpha$ 1-Adrenergic modulation of $I_{Kr}$ in feline cardiac myocytes*

In order to test whether the results obtained in HEK-hERG cells could be extrapolated to native cardiac cells we used isolated feline ventricular cardiomyocytes. All the experiments with ventricular myocytes were performed in the presence of the  $\beta$ -blocker propranolol 100 nM to avoid any potential effect of PE on  $\beta$ -ARs. As previously observed in transfected HEK293 cells, in feline native cardiomyocytes the stimulation of the  $\alpha$ 1-AR with PE reduced the  $I_{Kr}$  activated current and shifted the activation half voltage to more depolarized potentials (from  $-7.4 \pm 1.1$  to  $-1.1 \pm 1.2$  mV, n=10 paired cells;  $p < 0.01$ ). The specific  $\alpha$ 1-AR blocker prazosin (1  $\mu$ M) abolished the effect on  $I_{Kr}$  indicating that is dependent on receptor activation (Fig. 6). The relevance of PKC in the intracellular pathway was tested using diC-8-PIP<sub>2</sub> and BIS-1 and, as expected, they abolished the PE effect on  $I_{Kr}$ . Therefore, PKC has a critical role on  $I_{Kr}$  regulation upon  $\alpha$ 1-AR stimulation in ventricular myocytes.

## Discussion

### *Mechanisms of $I_{hERG}$ reduction upon $\alpha$ 1-AR stimulation*

In this work, the activation of the  $\alpha$ 1-AR with phenylephrine reduces the  $I_{hERG}$  amplitude as previously published [12, 13, 19, 31, 34]. The experiments performed in hERG-HEK cells not expressing  $\alpha$ 1-AR show that the shift in the activation  $V_h$  to more positive potentials is



the cause of  $I_{hERG}$  reduction upon  $\alpha 1$ A-AR stimulation, because there will be fewer available channels at any physiological membrane potential. The regulatory proteins minK and MIRP1 are not relevant for the  $\alpha 1$ A-adrenergic regulation of  $I_{hERG}$ .

Jiang et al. found for the first time a reduction on  $I_{hERG}$  amplitude by  $\alpha 1$ A-AR stimulation; however the authors did not identify the G protein involved [11]. Although this receptor can activate  $G\alpha s$  protein and the cAMP/PKA pathway in cardiac myocytes [35], the most extensively documented biochemical responses to  $\alpha 1$ -AR stimulation in the heart is  $G\alpha q$  protein activation of phospholipase C-mediated hydrolysis of PIP<sub>2</sub> that produces DAG and IP<sub>3</sub> and leads to PKC activation by these second messengers [36-38]. Our results show that the regulation of hERG current is mediated by the canonical  $G\alpha q$ -PLC intracellular signalling pathway. It has been proposed that the effect of PLC activation on  $I_{hERG}$  upon  $\alpha 1$ -AR stimulation might be caused by PIP<sub>2</sub> depletion and loss of PIP<sub>2</sub>-hERG interaction [12, 13]. However, other authors suggest that the channel modulation lies on DAG production and PKC-dependent phosphorylation [19, 31]. As will be discussed below, our experiments with deletion mutants support the latter.

#### *Implication of Protein Kinases*

The most common mechanism to regulate K<sup>+</sup> channels is reversible phosphorylation [35, 39]. The Tyrosine kinase Src shifts the activation half voltage of both  $I_{Kr}$  and  $I_{hERG}$  [30, 40]. In our work, however, inhibition of Tyrosine kinases did not prevent the shift in the activation  $V_h$  induced by  $\alpha 1$ -AR stimulation, suggesting that they are not involved in this regulatory pathway.

Although several works propose a role for PKC in the modulation of hERG channels in response to  $G\alpha q$  protein coupled receptors [19, 29, 32, 41], one paper using the PKC inhibitor chelerythrine excluded PKC involvement in  $\alpha 1$ -AR modulation of hERG [12]. In that study cells were incubated extracellularly for one hour with a chelerythrine concentration barely above the  $IC_{50}$ , so probably neither the incubation time nor the concentration used were enough to cause a significant blockade. We have administrated the PKC blocker bisindolylmaleimide-1 in the intracellular solution to ensure inhibition of the kinase and then found that  $\alpha 1$ -AR stimulation does not shift the activation half voltage of  $I_{hERG}$ . Thus, PKC has a key role in the regulation of the hERG channel after  $\alpha 1$ -AR stimulation.

Thomas et al. suggested that  $\alpha 1$ -AR regulation of hERG channel was mediated by both PKA and PKC by a mechanism independent of direct channel phosphorylation [19], since PKA inhibition prevented the effect of  $\alpha 1$ -AR stimulation on  $I_{hERG}$  but mutation of the four PKA phosphorylation sites did not. It is currently accepted that KT5720, the PKA blocker used, can also inhibit PKC at physiological ATP concentrations [42] and therefore involvement of PKA is not sustained. That work also ruled out the direct phosphorylation of the channel by PKC because mutation of 17 out of 18 phosphorylation sites to alanine did not prevent the  $\alpha 1$ -AR effect on hERG [19]. Authors could not exclude the T74 site, located in the PAS domain, as the target for PKC-dependent phosphorylation of hERG channel after  $\alpha 1$ -AR stimulation because mutation of that residue resulted in non-functional channels. In this sense, it is currently known that PKC modulates hERG channels [29, 31, 41, 43] precisely by direct phosphorylation of the T74 site [32]. Whereas point mutation or deletion of T74 causes channel loss of activity [19, 32], the NTK-hERG mutant lacks the full PAS domain and is functional enough to allow exploring the modulation by PKC [32, 33]. In the present work,  $\alpha 1$ -ARs stimulation does not modulate  $I_{NTK-hERG}$ , supporting a role for direct channel phosphorylation by PKC.

PIP<sub>2</sub> regulates inward rectifier potassium channels [44-50], voltage dependent Ca<sup>2+</sup> channels [reviewed in 51], cardiac Na<sup>+</sup>/Ca<sup>2+</sup> exchanger [52], KCNQ1 [53] and a role for PIP<sub>2</sub> in the regulation of  $I_{Kr}/I_{hERG}$  by  $\alpha 1$ -AR has been proposed [12, 13, 54].

Negative phosphate groups of the PIP<sub>2</sub> head interact with positively charged amino acids of the channel proteins [45, 55] and the deletion or substitution of the 883-894 amino acids in the hERG channel C-terminal eliminates the apparent main interaction site between PIP<sub>2</sub> and hERG [13]. We used the D-hERG mutant (deletion of C-terminal 883-894 amino acids) in

order to test whether  $PIP_2$  might be involved in the  $\alpha 1$ -AR regulation of  $I_{hERG}$ . D-hERG mutant responds to  $\alpha 1$ -AR stimulation and behaves like the WT despite the lack of the apparent  $PIP_2$  interaction site. On the contrary, PKC inhibition fully abolishes  $\alpha 1$ -adrenergic modulation of D-hERG, which is again consistent with a relevant role of direct phosphorylation by PKC in this pathway.

#### Limitations of the study

Although PE induces a positive shift in the activation  $V_h$  in both HEK cells and feline cardiac myocytes, the smaller effect of PE in feline myocytes can be explained by different factors such as a lower  $\alpha 1$ -AR expression. Feline myocytes are native cells, whereas in HEK cells the receptor was transfected and overexpressed. It might also be differences in efficiency of coupling between the components of the intracellular pathway (receptor-Gq proteins; Gq-PLC; PLC-PKC;...) or a different stoichiometry between PKC and ERG channels.

Feline  $I_{Kr}$  is not identical to transfected  $I_{hERG}$ . In addition to the possible differences between feline and human ERG channel, it is well accepted that hERG channel resembles most but not all the characteristics of human  $I_{Kr}$  and that accessory subunits would be required to create the channelosome responsible for native  $I_{Kr}$ . However, we found that the main candidates, minK or MIRP1, do not modify the effect of Phenylephrine on hERG.

#### Translational perspective

Our results in native, feline, cardiomyocytes reproduce what was observed in transfected cells. Thus,  $\alpha 1$ -AR stimulation reduces the  $I_{Kr}$  activated and tail currents by shifting the voltage-dependence of activation to more depolarized potentials in a mechanism that requires PLC and PKC activation and PKC-dependent channel phosphorylation.

One common trigger of arrhythmia in LQT2 patients seems to be emotional stress and high and unexpected noise [56]. Symptomatic patients that suffer lethal and no lethal cardiac events are related with an increase in the sympathetic tone [3, 57-59], which means a clear relation between sympathetic nervous system and arrhythmias. In fact, auditory stimulus-induced arrhythmias are almost exclusively associated with mutations in KCNH2, the gene encoding hERG [2].  $\beta$ -AR and  $\alpha$ -AR are activated by noradrenalin, but although  $\beta$ -ARs are saturated by moderate sympathetic activation,  $\alpha 1$ -ARs activate in situations of sympathetic hyperactivity such as emotional or physical stress. In this sense, an anti-arrhythmic effect of  $\alpha 1$ -AR blockade has been demonstrated in an animal model of LQT2 [60]. Here, we clarified the link between sympathetic nervous system hyperactivity and  $I_{Kr}$  reduction, one of the best characterized causes of *torsades de pointes* and ventricular fibrillation.

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#### Disclosure Statement

The authors have nothing to disclose.

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