Original Paper

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

Janire Urrutia^a Aintzane Alday^a Mónica Gallego^{a,b} L. Layse Malagueta-Vieira^{a,b}
Ivan Arael Aréchiga-Figueroa^c Oscar Casis^{a,b} José Antonio Sánchez-Chapula^c

^aDepartment of Physiology, Faculty of Pharmacy, University of the Basque Country UPV/EHU, Vitoria-Gasteiz; ^bLascaray Research Centre, University of the Basque Country UPV/EHU, Vitoria-Gasteiz, Spain; ^cUnidad de Investigación "Carlos Méndez" del Centro Universitario de Investigaciones Biomédicas, Universidad de Colima, Colima, México

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.

© 2016 The Author(s)
Published by S. Karger AG, Basel

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 β - and α -adrenoceptors (α -ARs) regulate I_{Kr} .

β -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 μ M of geneticin. Cell cultures were maintained in 5% CO_2 at 37°C. 4 μ 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 μ g of $\alpha 1$ -adrenoceptor cDNA (kindly provided by Dr. C. Hague, University of Washington) along with 0.2 μ 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 NaHCO₃, 0.42 NaH₂PO₄, 5.4 KCl, 1.8 CaCl₂, 1.05 MgCl₂, 11 glucose, and 10 taurine. The solution was equilibrated with 95% O₂ and 5% CO₂, pH 7.4. Nominally calcium-free solution was prepared by omitting CaCl₂ 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 KH₂PO₄, 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 Ω after filled with the internal solution. For HEK293 cells the internal solution was (in mM) 125 KCl, 5 MgCl₂, 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 KH₂PO₄, 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 MgCl₂, 10 HEPES-Na, 1.8 CaCl₂ and 10 glucose, pH 7.4 with NaOH. For cardiac myocytes the bathing solution was (in mM): 140 NaCl, 5.4 KCl, 0.1 CaCl₂, 0.4 CoCl₂, 1.0 MgCl₂, 10 HEPES and 11 glucose, pH adjusted to 7.4 with NaOH. HMR-1556 1 μ M was added to block I_{Ks} . For HEK-hERG cells terfenadine 1 μ M was added at the end of the experiment and the remaining endogenous current was subtracted.

To elicit I_{hERG} and I_{Kr} 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 $I = I_{\infty} / [1 + e^{(V_{1/2} - V)/k}]$, where I is the amplitude of the tail current, $V_{1/2}$ 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-PIP₂) was from Echelon Biosciences. Anti-PIP₂ 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 μ 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 μ 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 ($\Delta V_{1/2}$) 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 μM 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 (ΔV_h) induced by 20 μM 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, τ_{deact} was accelerated from 1331 ± 74 to 639 ± 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 ± 1.9 to 25.1 ± 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 μM 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 μM PE was added to hERG expressing HEK cells not transfected with the receptor, we still observed a faster tail current deactivation (1420 ± 98 vs 998 ± 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 μM PE shifted the $I_{hERG+minK}$ activation V_h to a similar extent than in the absence of the accessory protein (from 14 ± 4.1 to 27.7 ± 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 μM phenylephrine (Phen) in the presence of an anti- PIP_2 antibody in the internal solution. B) Displacement of the I_{hERG} activation half voltage (ΔV_h) caused by $\alpha 1$ -AR stimulation in cells with: the Gq protein blocker GPantagonist 2A 50 μM ; the PLC inhibitors Neomycin 1 mM, anti- PIP_2 antibody 80 nM and diC8- PIP_2 10 μM ; and the PKC blocker bisindolylmaleimide 1 (BIS-1) 10 μM . 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 ± 3 to 12.2 ± 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_2 hydrolysis and PKC activation. The involvement of the $G_{\alpha q}$ protein in the PE-induced I_{hERG} reduction was confirmed with 50 μM of the specific $G_{\alpha q}$ blocker GPant2A (RPPKQQDWFDWDM). In the presence of this blocker PE did not shift the activation half voltage of I_{hERG} (Fig. 3), suggesting that αq is the relevant G protein in this pathway.

In the canonical pathway, $G_{\alpha q}$ protein-activated PLC hydrolyses PIP_2 producing the PKC activators 1,2-diacylglycerol (DAG) and inositol-1,4,5,-trisphosphate (IP_3). We blocked these steps with three chemical tools: neomycin and an anti- PIP_2 antibody, which block the access of PLC to PIP_2 ; and the non-hydrolysable PIP_2 analogue diC8- PIP_2 , 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 μM the activation V_h moved from 10.3 ± 3.4 to 25.9 ± 3 mV after adding phenylephrine ($n = 9$; $p < 0.001$). However, when PKC was inhibited by BIS-1 (10 μM) PE was not able to shift the activation half voltage of I_{hERG} to more positive potentials (from 11.2 ± 3.1 to 11.3 ± 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_2 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_2 and hERG channels [13]. Perfusion of the cells with 20 μM PE shifted the activation half voltage of I_{D-hERG} to more depolarized potentials (from 8.2 ± 3 to 20.1 ± 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_2 (10 μM) or the PKC blocker BIS-1 (10 μM) prevented the

Fig. 4. PIP₂ interaction with hERG channel is not relevant for regulation by $\alpha 1$ -AR. A) Expression of the presumably non PIP₂ 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 μ M phenylephrine (Phen) administration. C) Displacement of the I_{D-hERG} activation half voltage (ΔV_h) caused by PE in cells without ($\emptyset$) and with diC8-PIP₂ 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 μ 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 (ΔV_h) caused by 20 μ M phenylephrine in cells without ($\emptyset$) and with diC8-PIP₂ 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 μ 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 (ΔV_h) induced by $\alpha 1$ -AR stimulation in cells without ($\emptyset$) and with prazosin (1 μ M), diC8-PIP₂ 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 ± 4.4 to 11.3 ± 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-PIP₂ 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 β -blocker propranolol 100 nM to avoid any potential effect of PE on β -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 ± 1.1 to -1.1 ± 1.2 mV, n=10 paired cells; p < 0.01). The specific $\alpha 1$ -AR blocker prazosin (1 μ 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-PIP₂ 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 $\alpha 1$ -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 $\alpha 1$ -adrenergic regulation of I_{hERG} .

Jiang et al. found for the first time a reduction on I_{hERG} amplitude by $\alpha 1$ -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 PIP₂ that produces DAG and IP₃ 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 PIP₂ depletion and loss of PIP₂-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⁺ 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 IC₅₀, 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.

PIP₂ regulates inward rectifier potassium channels [44-50], voltage dependent Ca²⁺ channels [reviewed in 51], cardiac Na⁺/Ca²⁺ exchanger [52], KCNQ1 [53] and a role for PIP₂ in the regulation of I_{Kr}/I_{hERG} by $\alpha 1$ -AR has been proposed [12, 13, 54].

Negative phosphate groups of the PIP₂ 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 PIP₂ 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 chanelosome 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]. β -AR and α -AR are activated by noradrenalin, but although β -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.

Acknowledgements

This work was supported by Universidad del País Vasco UPV/EHU grants (PPM12/12 and PES12-33); Basque Government grant IT653-13 and MINECO (SAF2013-46708-R) to O.C.; and UPV/EHU grant (EHUA12/12) to M.G. The authors wish to thank Dr. E. Medei, from the University of Rio de Janeiro in Brasil, and Dr. A.A. Rodríguez-Menchaca, from the Universidad Autónoma de San Luis Potosí in México for the useful comments and critical review of the manuscript.

Disclosure Statement

The authors have nothing to disclose.

References

- 1 Sanguinetti MC, Tristani-Firouzi M: hERG potassium channels and cardiac arrhythmia. *Nature* 2006;440:463-469.
- 2 Gordon E, Panaghie G, Deng L, Bee KJ, Roepke TK, Krogh-Madsen T, Christini DJ, Ostrer H, Basson CT, Chung W, Abbott GW: A KCNE2 mutation in a patient with cardiac arrhythmia induced by auditory stimuli and serum electrolyte imbalance. *Cardiovasc Res* 2008;77:98-106.
- 3 Priori S, Napolitano C, Paganini V, Cantu F, Schwartz P: Molecular biology of the long QT syndrome: impact on management. *Pacing Clin Electrophysiol* 1997;20:2052-2057.
- 4 Setién R, Alday A, Diaz-Asensio C, Urrutia J, Gallego M, Casis O: Mechanisms responsible for the trophic effect of beta-adrenoceptors on the $I_{(to)}$ current density in type 1 diabetic rat cardiomyocytes. *Cell Physiol Biochem* 2013;31:25-36.
- 5 Cui J, Melman Y, Palma E, Fishman GI, McDonald TV: Cyclic AMP regulates the HERG K⁺ potassium channel by dual pathways. *Curr Biol* 2000;10:671-674.
- 6 Kagan A, Melman YF, Krummerman A, McDonald TV: 14-3-3 amplifies and prolongs adrenergic stimulation of HERG K⁺ channel activity. *EMBO J* 2002;21:1889-1898.
- 7 Thomas D, Zhang W, Karle CA, Kathofer S, Schols W, Kubler W, Kiehn J: Deletion of protein kinase A phosphorylation sites in the HERG potassium channel inhibits activation shift by protein kinase A. *J Biol Chem* 1999;274:27457-27462.
- 8 Tutor AS, Delpón E, Caballero R, Gómez R, Núñez L, Vaquero M, Tamargo J, Mayor F Jr, Penela P: Association of 14-3-3 Proteins to $\beta 1$ -Adrenergic Receptors Modulates Kv11.1 K⁺ Channel Activity in Recombinant Systems. *Mol Biol Cell* 2006;17:4666-4674.
- 9 Brelidze TI, Carlson AE, Zagotta WN: Absence of direct cyclic nucleotide modulation of mEAG1 and hERG1 channels revealed with fluorescence and electrophysiological methods. *J Biol Chem* 2009;284:27989-27997.
- 10 Harmati G, Bányász T, Bárándi L, Szentandrassy N, Horváth B, Szabó G, Szentmiklósi JA, Szénási G, Nánási PP, Magyar J: Effects of β -adrenoceptor stimulation on delayed rectifier K(+) currents in canine ventricular cardiomyocytes. *Br J Pharmacol* 2011;162:890-896.
- 11 Jiang M, Dun W, Fan JS, Tseng G-NY: Use-Dependent 'Agonist' Effect of Azimilide on the HERG Channel. *J Pharmacol Exp Ther* 1999;291:1324-1336.
- 12 Bian J, Cui J, Mc Donald TV: HERG K⁺ channel activity is regulated by changes in phosphatidyl inositol 4, 5-bisphosphate. *Circ Res* 2001;89:1168-1176.
- 13 Bian JS, Kagan A, McDonald TV: Molecular analysis of PIP2 regulation of HERG and IKr. *Am J Physiol Heart Circ Physiol* 2004;287:H2154-H2163.
- 14 Kruse M, Hammond GR, Hille B: Regulation of voltage-gated potassium channels by PI(4,5)P2. *J Gen Physiol* 2012;140:189-205.
- 15 Kruse M, Hille B: The phosphoinositide sensitivity of the K(v) channel family. *Channels (Austin)* 2013;7:530-536.
- 16 Thomas D, Hammerling BC, Wimmer A-B, Wu K, Ficker E, Kuryshv YA, Scherer D, Kiehn J, Katus HA, Schoels W, Karle CA: Direct block of hERG potassium channels by the protein kinase C inhibitor bisindolylmaleimide I (GF109203X). *Cardiovasc Res* 2004;64:467-476.
- 17 Wang S, Xu DJ, Cai J, Huang Y, Zou JG, Cao KJ: Rapid component IKr of cardiac delayed rectifier potassium currents in guinea-pig is inhibited by $\alpha 1$ -adrenoreceptor activation via protein kinase A and protein kinase C-dependent pathways. *Eur J Pharmacol* 2009;608:1-6.
- 18 Wei Z, Thomas D, Karle DA, Kathöter S, Schenkel J, Kreye VA, Ficker E, Wible BA, Kiehn J: Protein Kinase A-mediated phosphorylation of HERG potassium channel in a human cell line. *Chin Med J* 2002;115:668-676.
- 19 Thomas D, Wu K, Wimmer AB, Zitron E, Hammerling BC, Kathofer S, Lueck S, Bloehs R, Kreye VA, Kiehn J, Katus HA, Schoels W, Karle CA: Activation of cardiac human ether-a-go-go related gene potassium currents is regulated by $\alpha 1A$ -adrenoceptors. *J Mol Med* 2004;82:826-837.
- 20 Zhou Z, Gong Q, Ye B, Fan Z, Makielski JC, Robertson GA, January CT: Properties of HERG channels stably expressed in HEK 293 cells studied at physiological temperature. *Biophys J* 1998;74:230-241.

- 21 Sánchez-Chapula JA, Salinas-Stefanon E, Torres-Jácome J, Benavides-Haro DE, Navarro-Polanco RA: Blockade of currents by the antimalarial drug chloroquine in feline ventricular myocytes. *J Pharmacol Exp Ther* 2001;297:437-445.
- 22 Isenberg G, Klöckner U: Calcium tolerant ventricular myocytes prepared by preincubation in a "KB Medium". *Pflügers Arch* 1982;395:6-18.
- 23 Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 1981;391:85-100.
- 24 McCrossan ZA, Abbott GW: The minK-related peptides. *Neuropharmacology* 2004;47:787-821.
- 25 Mironov SL: Metabotropic glutamate receptors activate dendritic calcium waves and TRPM channels which drive rhythmic respiratory patterns in mice. *J Physiol* 2008;586:2277-2291.
- 26 Smith KE, Browne L, Selwood DL, McAlpine D, Jagger DJ: Phosphoinositide Modulation of Heteromeric Kv1 Channels Adjusts Output of Spiral Ganglion Neurons from Hearing Mice. *J Neurosci* 2015;35:11221-11232.
- 27 Liu L, Heneghan JF, Mitra-Ganguli T, Roberts-Crowley ML, Rittenhouse AR: Role of PIP2 in regulating versus modulating Ca^{2+} channel activity. *J Physiol* 2007;583:1165-1166.
- 28 Roberts-Crowley ML, Mitra-Ganguli T, Liu L, Rittenhouse AR: Regulation of voltage-gated Ca^{2+} channels by lipids. *Cell Calcium* 2009;45:589-601.
- 29 Barros F, del Camino D, Pardo LA, Palomero T, Giraldez Channels T, De la Peña P: Demonstration of an inwardly rectifying K^{+} current component modulated by thyrotropin-releasing hormone and caffeine in GH3 rat anterior pituitary cells. *Pflügers Arch* 1997;435:119-129.
- 30 Cayabyab FS, Schlichter LC: Regulation of an ERG K^{+} current by Src tyrosine kinase. *J Biol Chem* 2002;277:13673-13681.
- 31 Thomas D, Zhang W, Wu K, Wimmer AB, Gut B, Wendt-Nordahl G, Kathöfer S, Kreye VA, Katus HA, Schoels W, Kiehn J, Karle CA: Regulation of HERG potassium channel activation by protein kinase C independent of direct phosphorylation of the channel protein. *Cardiovasc Res* 2003;59:14-26.
- 32 Cockerill SL, Tobin AB, Torrecilla I, Willars GB, Standen NB, Mitcheson JS: Modulation of hERG potassium currents in HEK-293 cells by protein kinase C. Evidence for direct phosphorylation of pore forming subunits. *J Physiol* 2007;581:479-493.
- 33 Spector PS, Curran ME, Zou A, Keating MT, Sanguinetti MC: Fast inactivation causes rectification of the IKr channel. *J Gen Physiol* 1996;107:611-619.
- 34 Heath BM, Terrar DA: Protein kinase C enhances the rapidly activating delayed rectifier potassium current, IKr , through a reduction in C-type inactivation in guinea-pig ventricular myocytes. *J Physiol* 2000;522:391-402.
- 35 Gallego M, Setién R, Puebla L, Boyano-Adán C, Arilla E, Casis O: $\alpha 1$ -Adrenoceptors stimulate a $G_{\alpha s}$ protein and reduce the transient outward K^{+} current via a cAMP/PKA-mediated pathway in the rat heart. *Am J Physiol Cell Physiol* 2005;288:C577-C585.
- 36 Brown JH, Buxton ILO, Brunton LL: $\alpha 1$ -adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. *Circ Res* 1985;57:532-537.
- 37 Graham RM, Perez DM, Hwa J, Piascik MT: $\alpha 1$ -adrenergic receptors subtypes. Molecular structure, function, and signalling. *Circ Res* 1985;78:737-749.
- 38 Nishizuka Y: The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 1988;334:661-665.
- 39 Gallego M, Fernández D, Ahyayauch H, Casis E, Casis O: Reduced calmodulin expression accelerates transient outward potassium current inactivation in diabetic rat heart. *Cell Physiol Biochem* 2008;22:625-634.
- 40 Zhang D-Y, Wang Y, Lau C-P, Tse H-F, Li G-R: Both EGFR kinase and Src-related tyrosine kinases regulate human ether-à-go-related gene potassium channels. *Cell Signal* 2008;20:1815-1821.
- 41 Barros F, Gomez-Varela D, Vilorio CG, Palomero T, Giraldez T, De la Peña P: Modulation of human erg K^{+} channel gating by activation of a G protein-coupled receptor and protein kinase C. *J Physiol* 1998;511:333-346.
- 42 Murray AJ: Pharmacological PKA inhibition: all may not be what it seems. *Sci Signal* 2008 DOI: 10.1126/scisignal.122re4.
- 43 Kiehn J, Karle C, Thomas D, Yao X, Brachmann J, Kübler W: HERG potassium channel activation is shifted by phorbol esters via protein kinase A-dependent pathways. *J Biol Chem* 1998;273:25285-25291.

- 44 Baukrowitz T, Schulte U, Oliver D, Herlitze S, Krauter T, Tucker SJ, Ruppersberg JP, Fakler B: PIP2 and PIP as determinants for ATP inhibition of KATP channels. *Science* 1998;282:1141-1144.
- 45 Fan Z, Makielski JC: Anionic phospholipids activate ATP-sensitive potassium channels. *J Biol Chem* 1997;272:5388-5395.
- 46 Liou HH, Zhou SS, Huang CL: Regulation of ROMK1 channel by protein kinase A via a phosphatidylinositol 4,5-bisphosphate-dependent mechanism. *Proc Natl Acad Sci USA* 1999;96:5820-5825.
- 47 Rohacs T, Chen J, Prestwich GD, Logothetis DE: Distinct specificities of inwardly rectifying K(+) channels for phosphoinositides. *J Biol Chem* 1999;274:36065-36072.
- 48 Shyng SL, Nichols CG: Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science* 1998;282:1138-1141.
- 49 Xie LH, Horie M, Takano M: Phospholipase C-linked receptors regulate the ATP-sensitive potassium channel by means of phosphatidylinositol 4,5-bisphosphate metabolism. *Proc Natl Acad Sci USA* 1999;96:15292-15297.
- 50 Zhang H, He C, Yan X, Mirshahi T, Logothetis DE: Activation of inwardly rectifying K+ channels by distinct PtdIns (4,5)P2 interactions. *Nat Cell Biol* 1999;1:183-188.
- 51 Roberts-Crowley ML, Mitra-Ganguli T, Liu L, Rittenhouse AR: Regulation of voltage-gated Ca2+ channels by lipids. *Cell Calcium* 2009;45:589-601.
- 52 Berberian G, Forcato D, Beaugé L: Key role of a PtdIns-4,5P2 micro domains on ionic regulation of the mammalian heart Na+/Ca2+ exchanger. *Cell Calcium* 2009;45:546-553.
- 53 Matavel A, Lopes C MB: PKC activation and PIP2 depletion underlie biphasic regulation of IKs by Gq-coupled receptors. *J Mol Cell Cardiol* 2009;46:704-712.
- 54 Kubo T, Ding WG, Toyoda F, Fujii Y, Omatsu-Kanbe M, Matsuura H: Phosphatidylinositol4-phosphate 5-kinase prevents the decrease in the HERG potassium current induced by Gq protein-coupled receptor stimulation. *J Pharmacol Sci* 2015;127:127-134.
- 55 Huang CL, Feng S, Hilgemann DW: Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gbetagamma. *Nature* 1998;391:803-806.
- 56 Wilde AA, Jongbloed RJ, Doevendans PA, Duren DR, Hauer RN, van Langen IM, van Tintelen JP, Smeets HJ, Meyer H, Geelen JL: Auditory stimuli as a trigger for arrhythmic events differentiate HERG-related (LQTS2) patients from KVLQT1-related patients (LQTS1). *J Am Coll Cardiol* 1999;33:327-332.
- 57 Kamarck T, Jennings JR: Biobehavioral factors in sudden cardiac death. *Psychol Bull* 1991;109:42-75.
- 58 Lown B: Sudden cardiac death: biobehavioural perspective. *Circulation* 1987;76:186-196.
- 59 Schwartz PJ, Priori SG, Spazzolini C, Moss AJ, Vincent GM, Napolitano C, Denjoy I, Guicheney P, Breithardt G, Keating MT, Towbin JA, Beggs AH, Brink P, Wilde AA, Toivonen L, Zareba W, Robinson JL, Timothy KW, Corfield V, Wattanasirichaigoon D, Corbett C, Haverkamp W, Schulze-Bahr E, Lehmann MH, Schwartz K, Coumel P, Bloise R: Genotype-phenotype correlation in the long-QT syndrome: gene-specific triggers for life-threatening arrhythmias. *Circulation* 2001;103:89-95.
- 60 Mow T, Frederiksen K, Thomsen MB: Assessment of anti-arrhythmic activity of antipsychotic drugs in an animal model: influence of non-cardiac $\alpha 1$ -adrenergic receptors. *Eur J Pharmacol* 2015;748:10-17.