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Original Research

β-Arrestin2 regulates the rapid component of delayed rectifier K^+ currents and cardiac action potential of guinea pig cardiomyocytes after adrenergic stimulation

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Abstract: A decrease in the rapid component of delayed rectifier potassium current (IKr) during chronic heart failure (CHF) prolongs action potential (AP), and plays a key role in the pathogenesis of ventricular arrhythmias. β-Arrestin2 has been shown to restore the inotropic reserve of β-adrenergic regulation, but little or nothing is known about its effect on intrinsic channel. This study investigated the role of β-arrestin2 in the regulation of cardiac hERG/IKr potassium channel and AP during chronic adrenergic stimulation. Single left ventricular myocytes were isolated from guinea pig heart, and were transfected with adenovirus encoding β-arrestin2, or β-arrestin2 siRNA or an empty adenovirus. Cell cultures containing 10 nM isoproterenol, 1 nM phenylephrine or vehicle alone (control medium) were electro-physiologically examined after 48 h of incubation. Action potential duration at 50 and 90 % of repolarization (APD_{50} and APD_{90}) were measured using whole-cell patch-clamp recording**.** Sustained adrenergic stimulation significantly reduced the density of the IKr current ($p < 0.001$). β-Arrestin2 expression in cell cultures treated with isoproterenol or phenylephrine was significantly downregulated after adrenergic stimulation ($p < 0.001$). Overexpression of β-arrestin2 significantly attenuated isoproterenol or phenylephrine-induced reduction in IKr current. It also prevented the phenylephrine-induced prolongation of AP ($p < 0.05$ for APD_{50} and $p < 0.001$ for APD_{90}), but did not significantly affect AP profile after exposure of the cardiomyocytes to isoproterenol ($p > 0.05$). Therefore, Increased levels of β-Arrestin2 weaken dysregulation of IKr current and prevent excessive AP prolongation, making it an effective anti-arrhythmic strategy.

Key words: β-Arrestin2;Adrenergic stimulation; Ventricular arrhythmias; Action potential; Potassium channel.

Introduction

Ventricular arrhythmias are a common cause of sudden cardiac death among patients with chronic heart failure(CHF). Abnormal repolarization of cardiac action potential (AP) is thought to contribute to ventricular arrhythmogenesis (1–3). Rapid component of delayed rectifier potassium channel (IKr) is encoded by the human ether-a-go-go-related gene (hERG), and is most responsible for phase 3 repolarization (4, 5). So far, neural routes and humoral factors have been used to study IKr. Activation of β1-adrenergic receptor (ADRB1) results in the activation of adenylate cyclase which in turn catalyzes the conversion of ATP to cyclic AMP (cAMP), thereby increasing the intracellular concentration of the second messenger. The rise in the level of cAMP promotes phosphorylation of serine residues on K^+ channel, thereby reducing IKr density (6, 7). However, stimulation by α1-adrenergic agonist results in a decline in the amplitude of IKr channel in rabbit cardiomyocytes (8). It has been reported that β-arrestin2 may play a cardioprotective role in CHF (9–11). Similarly, a β-arrestin-mediated increase in IKr has been reported in hERG-HEK cells (12). However, little or nothing is known about the regulatory effect of β-arrestin2 on IKr channel under stress conditions. This study investigated the role of β-arrestin2 in the regulation of cardiac hERG/IKr potassium channel and AP during chronic adrenergic stimulation.

Materials and Methods

Materials

Collagenase type II was purchased from Worthington (USA). Adenoviral vectors (AdVs) were obtained from Applied Biological Materials Inc. (Canada). Medium-199 and fetal bovine serum (FBS) were products of Gibco (USA). Temperature control system (TC-324B) was purchased from Warner (USA). Patch-clamp amplifier (Axopatch 200B) was obtained from Axon (USA). Trizol reagent was a product of Invitrogen (USA). Reverse transcription kit was purchased from Takara (China); SYBR green PCR Master Mix and ABI-7900 real-time PCR detection system were products of Applied Biosystems (USA).

Isolation of cardiomyocytes

Single left ventricular myocytes were isolated from Guinea pig heart using standard methods, with slight modification (13). Male Guinea pigs weighing 300 – 400 g (mean weight = 350 ± 30 g) were euthanized with pentobarbital (30 mg/kg body weight) and anticoagulated with sodium heparin (100 IU/mL). The heart was quickly excised, and the ascending aorta was mounted on a Langendorff equipment for retrograde coronary perfusion. The preparation was perfused first with oxy-

genated Tyrode's solution (143 mM NaCl, 5.4 mM KCl, 0.25 mM NaH_2PO_4, 5 mM HEPES, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5.6 mM glucose) at 37 °C until the efflux was blood-free (100 % O_2, pH 7.35). Then, the solution was changed to Ca^{2+}-free Tyrode's solution within 5 min. The perfusion was sustained with the same buffer containing 140 U/mL collagenase type II and 1 % bovine serum albumin (BSA). The left ventricular tissue mass was gently separated from the softened heart, minced, and then initially incubated in King's B (KB) medium supplemented with 85 mM KOH, 30 mM KCl, 30 mM KH_2PO_4, 3 mM $MgSO_4$, 10 mM HEPES, 0.5 mM EGTA, 20 mM taurine, 10 mM glucose, and 50 mM L-glutamic acid (pH 7.4) at room temperature for 40 min. The cells were concentrated using natural settling, and the resultant precipitate was removed for culturing. The whole cell isolation procedure was performed under aseptic conditions.

Adenoviral infection of cell cultures

Full-length β-arrestin2 cDNA and β-arrestin2-siRNA cDNA fragment (β-arrestin2 siRNA, CAATATGCAGACATCTGCCTCTTCA) were inserted into adenoviral vectors (AdVs) which were then packaged in human embryonic kidney 293 cells (HEK293). An empty vector expressing only green fluorescent protein (GFP) was used as control. Cardiomyocytes were transfected with adenovirus encoding β-arrestin2, or β-arrestin2 siRNA or an empty adenovirus at a multiplicity of infection (MOI) of 40 (or 50 for AdV-siRNA). The transfection efficiency was then determined.

Cell culture

Trypsinized cells were seeded in culture plates precoated with laminin (20 μg/mL) at a density of 1 x 10^4 cells/mL) in Medium-199 supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. After every 4 h, any dead or unadhered cells were washed away leaving behind a homogeneous layer of rod-shaped myocytes that adhered to the plates. Similarly, the medium was changed every two days and replaced with fresh medium containing 10 nM isoproterenol, 1 nM phenylephrine or vehicle alone (control medium). The culture medium was electro-physiologically examined after 48h.

Whole-cell patch-clamp recording

After 48 h of preparation, the medium was removed and the myocytes were transferred to a plexiglass chamber with constant perfusion of the extracellular solution. The entire procedure was carried out at 37 ± 0.5 °C with the help of a temperature control system. Pipettes with tip resistances of 3 – 6 mΩ were filled with internal solution and connected to a patch-clamp amplifier. For IKr recording, the pipette and bath solutions were prepared according to standard procedures (14, 15). Nifedipine (10 μM) and chromanol 293B (10 μM) were added to block calcium ion channels and the slow component of delayed rectifier potassium channel, respectively. The test pulse protocol was carried out thus: from a holding potential of −40 mV, currents were activated with a variable test pulse from −40 to +40 mV, followed by a return pulse to −40 mV to evoke outward tail currents. The mean currents were normalized to the cell capacitance and expressed as current densities to eliminate interference of different cell sizes. Action potentials (APs) were elicited using whole-cell current-clamp mode for 5 ms with twice-threshold pulses, and were recorded at 1 Hz. Action potential duration (APD) at 50 and 90 % of repolarization (APD_{50} and APD_{90}) were then measured.

Assessment of the effect of β-arrestin2 on isoproterenol or phenylephrine-induced IKr tail currents

To assess the effect of β-arrestin2 on IKr, isolated myocytes were cultured in a medium containing AdV-β-arrestin2 (β-arr2) or AdV-β-arrestin2 siRNA (β-arr2 siRNA) to either overexpress or knock down β-arrestin2. Normal cardiomyocytes infected with empty adenovirus (GFP) were used as control. Whole-cell current-clamp recording was performed 2 days after transfection.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Trizol RNA extraction reagent was used to extract total RNA from cells in each group, while cDNA synthesis kit was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the mRNA expressions of the genes. Variation in the cDNA content was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The cycling conditions for cDNA synthesis involved an initial 30-min incubation at 95 °C, followed by 40 cycles of 95 °C for 5 sec and 57 °C for 20 sec. The PCR reaction mixture (20 μL) consisted of 6.4 μL of dH_2O, 1.6 μL of gene-specific primer (10 μM), 2 μL of synthesized cDNA and 10 μL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of the proteins.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (20.0). Nonlinear curve fitting was performed using Clampfit routine in pCLAMP (Chebyshev algorithm). Groups were compared using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of isoproterenol (a β-AR agonist) and phenylephrine (an α1-AR agonist) on IKr tail currents and β-arrestin2 expression in cultured ventricular myocytes

A typical IKr tail current of Guinea pig ventricular myocytes is shown in Figure 1A. This current was completely blocked by 1 μM dofetilide, a specific IKr blocker (Figure 1B). Myocytes cultured in isoproterenol-treated medium showed reduced IKr tail current amplitudes relative to the control group (n=8, $p < 0.001$; Figure 1C). The mean current density of IKr at +40 mV was significantly reduced (n=8, $p < 0.001$; Figure 1D). A decrease in IKr was observed in phenylephrine-treated cardiomyocytes, and the current density dropped to 0.81 ± 0.08 (n=8, $p < 0.001$; Figure 1D). β-Arrestin2 has been reported previously to restore inotropic reserves of

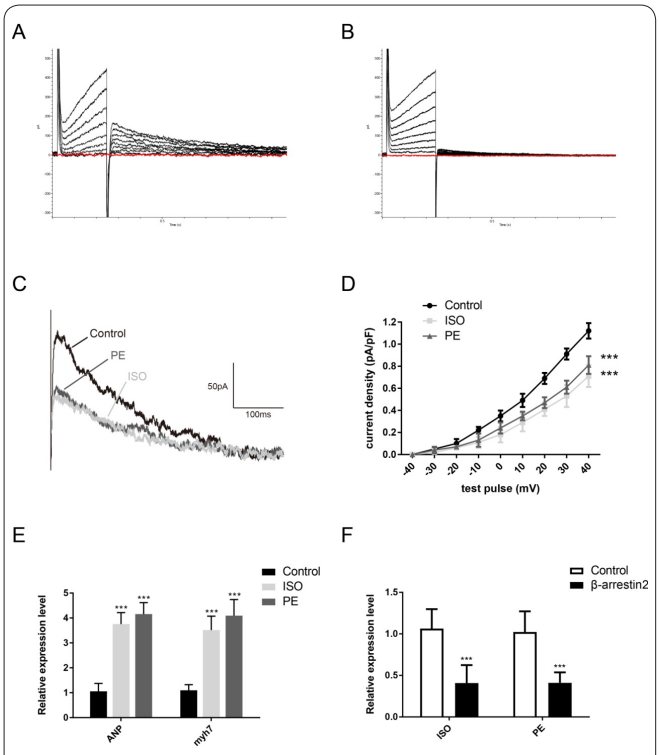

Figure 1. Effects of isoproterenol and phenylephrine on IKr tail currents and β-arrestin2 expression in cultured ventricular myocytes. (A): Whole-cell patch-clamp recordings of original IKr tail currents without dofetilide application; **(B):** Whole-cell patch-clamp recordings of original IKr tail currents with dofetilide application; **(C):** Representative tail traces of IKr inhibited by isoproterenol or phenylephrine in isolated myocytes; **(D):** IKr density-voltage relationships under control conditions or in the presence of isoproterenol or phenylephrine; **(E):** The atrial natriuretic peptide (ANP) and alpha myosin heavy chain (myh7) mRNA levels as measured using qRT-PCR; and **(F):** Levels of expression of β-arrestin2 mRNA in isoproterenol or phenylephrine-treated cardiomyocytes. ISO = isoproterenol; PE = phenylephrine. $^{***}p <$ 0.001, when compared with control group (untreated cells), n=8 cells.

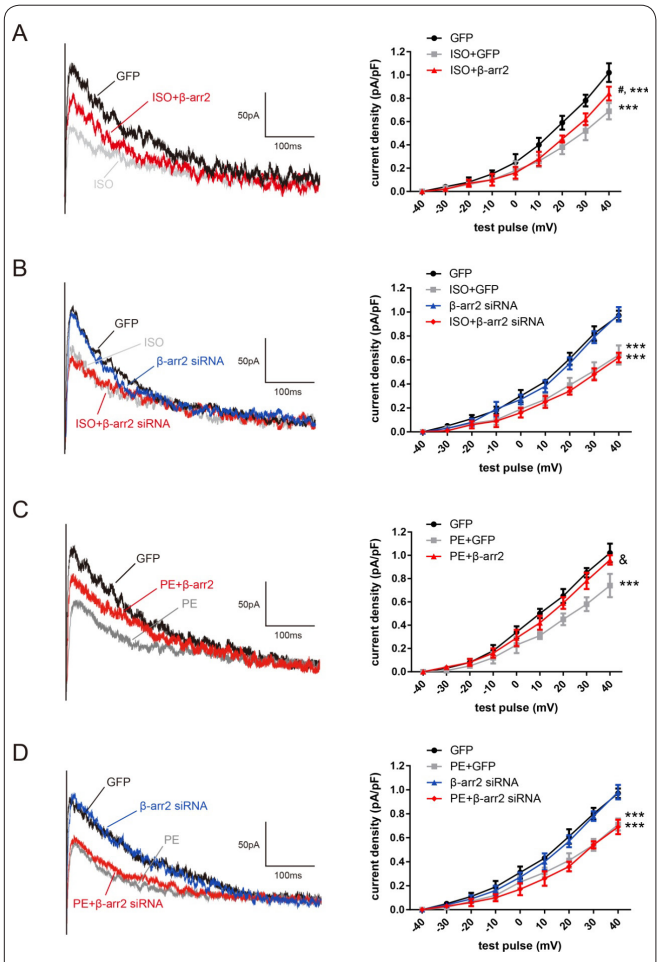

Figure 2. Effect of β-arrestin2 on isoproterenol or phenylephrine-induced IKr tail currents. (A): Typical original IKr tail currents recorded at return pulse after depolarizing to +40 mV at basal conditions and after application of isoproterenol and Adv-β-arrestin2; **(B):** Representative traces of IKr tail currents in cardiomyocytes treated with isoproterenol and β-arrestin2 siRNA transfection; **(C):** Typical original IKr tail currents recorded at return pulse after depolarizing to +40 mV after treatment with phenylephrine and phenylephrine + AdV-β-arrestin2; and **(D):** Representative traces of IKr tail currents in cardiomyocytes treated with phenylephrine with or without β-arrestin2 siRNA transfection. The IKr tail current densities (pA/pF) measured at different membrane voltages of the groups are shown beside the corresponding representative traces. ISO = isoproterenol; PE = phenylephrine. n=8 in each group, $^{***}p$<0.001 vs. GFP, $^{\#}p$<0.001 vs ISO+GFP, $^{\&}p$<0.001 vs. PE+GFP.

β-adrenergic regulation in mice (16) but has not been explored during IKr regulation to date. Therefore, we further explored its function. Similarly, level of expression of β-arrestin2 mRNA was significantly reduced in cultured guinea pig myocytes stimulated with isoproterenol or phenylephrine (Figures 1E and 1F). The level of expression of β-arrestin2 was significantly correlated with the presence of adrenergic agitation.

Effect of β-arrestin2 on isoproterenol or phenylephrine-induced IKr tail currents

The IKr density was significantly increased in the AdV-β-arr2-treated group (0.84 ± 0.06), when compared with isoproterenol group (0.69 ± 0.07) (n=8, $p < 0.001$; Figure 2A). Similarly, a significant decrease in IKr was observed in phenylephrine-treated cardiomyocytes, but this was mitigated via upregulation of β-arrestin2 expression (n=8, $p < 0.001$; Figure 2C). However, the knockdown of β-arrestin2 did not significantly affect the IKr density (0.98 ± 0.06), when compared with control group (0.97 ± 0.04) (n=8, $p > 0.05$; Figures 2B and 2D). These studies suggest that β-Arrestin2 may antagonize isoproterenol or phenylephrine -induced inhibition of IKr tail currents.

Effect of β-arrestin2 on AP of isoproterenol (or phenylephrine)-treated cardiomyocytes

Typical APs in isoproterenol-treated myocytes had shortened plateaux and triangular shapes, when compared with those of control group (Figure 3A), but phenylephrine –treated myocytes produced prolonged AP ($p <$ 0.001), as shown in Figures 3B and 3C). Isoproterenol significantly decreased early phase APD ($p < 0.001$), but phase 3 repolarization and terminal repolarization time were not significantly affected ($p > 0.05$). However, phenylephrine stimulation caused a prolongation of both APD_{50} and APD_{90} ($p < 0.001$). The APDs were significantly shortened in phenylephrine group overexpressing β-arrestin2, when compared with those of myocytes treated with phenylephrine alone ($p < 0.01$). There was no significant difference in the APDs of myocytes

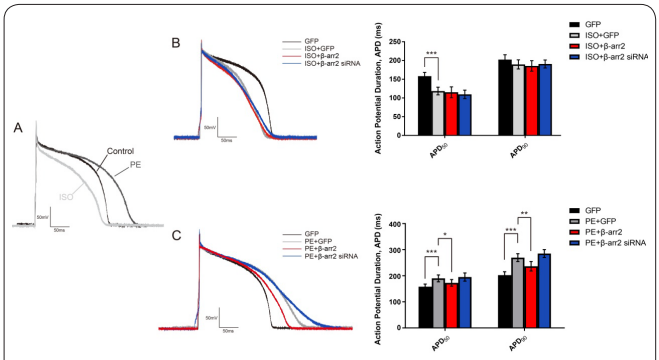

Figure 3. Effect of β-arrestin2 on AP of isoproterenol (or phenylephrine) -treated cardiomyocytes. (A): Representative AP traces in myocytes cultured for 48 h with vehicle or isoproterenol or phenylephrine; (**B and C):** Representative AP recordings from isoproterenol or phenylephrine -treated myocytes transfected with an adenovirus vector (left, n=8) and summarized data for the APD measured at 90% and 50% of repolarization (APD_{90} and APD_{50}) (right, n=8). ISO = isoproterenol; PE = phenylephrine.$^{*}p <$ 0.05, when compared with PE + GFP; $^{**}p < 0.01$, when compared with PE + GFP; $^{***}p < 0.001$, when compared with GFP.

Table 1. Action potential parameters of cultured ventricular myocytes.

Group	APD_{50} (ms)	APD_{90} (ms)
GFP	158.13 ± 10.03	202.38 ± 12.96
ISO + GFP	118.50 ± 10.36^{c}	189.50 ± 12.18
ISO + β-arr2	115.25 ± 14.42	185.50 ± 14.03
ISO + β-arr2 siRNA	109.63 ± 11.24	190.63 ± 10.63
PE + GFP	190.00 ± 13.14^{c}	269.38 ± 15.01^{c}
PE + β-arr2	172.88 ± 12.65^{a}	236.63 ± 17.98^{b}
PE + β-arr2 siRNA	195.13 ± 15.63	285.13 ± 14.92

ISO = isoproterenol; PE = phenylephrine. n=8 in each group, $^{a}p < 0.05$, when compared with PE+GFP; $^{b}p < 0.01$, when compared with PE+GFP; $^{c}p < 0.001$, when compared with GFP group.

between the isoproterenol group treated with β-arrestin2 and those treated with β-arr2 siRNA ($p > 0.05$; Table 1).

Discussion

The IKr channel regulates physiological repolarization in myocytes (17, 18). Its modulation is essential for controlling APD and QT interval in different pathological conditions (4). This study investigated the role of β-arrestin2 in the regulation of cardiac hERG/IKr potassium channel and AP during chronic adrenergic stimulation. The results showed that ventricular β-arrestin2 was significantly reduced during α1- and β-adrenergic activation, an indication that it might contribute to changes in the IKr current and APs during the course of adrenergic stimulation. β-Arrestin2 may be a new target for restoration of hERG/IKr function and identification of potential therapeutic strategies for long-QT syndrome (LQTS).

It has been reported that hERG/IKr potassium channels are modulated by α1- and β-adrenergic stimulation, and provide a mechanism for the pathophysiology behind increased incidence of arrhythmias under conditions of stress (6, 8). The results of this study suggest that isoproterenol or phenylephrine may be capable of reducing IKr currents via stimulation of α1- and β-Ars. These findings are in agreement with the results of previous studies (15, 19). At present β-adrenergic regulation of IKr is still debatable. Studies on rabbits showed that IKr currents were slightly increased after acute exposure to isoproterenol (20). However, studies using guinea pigs revealed a reduction in IK currents sustained by β-adrenergic stimulation (21). In this study, the down-regulation of IKr currents may be due to an adaptive intracellular regulatory effect rather than direct, membrane-delimited cell signaling.

Impaired myocardial β-AR signaling is known to play a role in ventricular arrhythmias. β-Arrestins were first identified in a process called desensitization which promotes recycling and degradation of G protein-coupled receptors such as β-Ars, involved in physiological and pathophysiological conditions (11, 22). Furthermore, the regulatory effects of β-arrestins clearly go beyond the realm of 7TMRs and involve several other classes of membrane proteins such as ion channels, transporters, receptor tyrosine kinases and cytokine receptors (23).β-Arrestin2 interacts transiently and weakly with β-AR, and is able to restore inotropic reserve of β-adrenergic regulation during left ventricular remodeling after myocardial infarction (16). The results of this study revealed significant association between decreased β-arrestin2 expression and adrenergic stimulation (both β and α1) in adult guinea pig myocytes. Previous studies have focused on the functions of β-arrestin2 as it relates to myocardial infarction and left ventricular structural remodeling (24, 25). However, little or nothing is known about the potential involvement of β-arrestin2 in the production of ionic channel currents in myocytes. It was only recently that UNC9994 (a β-arrestin2 selective agonist) was reported to modulate G protein-coupled inward rectifier potassium channel in *Xenopus* oocytes (26). A β-arrestin-mediated increase in hERG/IKr has been reported in human embryonic kidney cells (12). It is important to note that, while heterologous expression systems are powerful due to their adaptability to molecular manipulation, they may not exactly generalize the actual signaling pathways. Studies on the role of β-arrestin2 in current regulation under conditions of stress are scanty. In the present study, β-arrestin2 significantly enhanced IKr, and mimicked the modification associated with CHF.

A consensus exists that CHF is related to a prolonged cardiac APD (27). A dysfunction in hERG/IKr potassium channel prolongs the duration of AP, and increases the risk of fatal arrhythmia (28). During adrenergic stimulation, changes in AP depend on the balance between the increased inward current such as the L-type calcium current, and an opposing increased outward current such as IKr. In this study, α1- and β-adrenergic stimulation showed large differences in shaping the AP. α1-Agonists have been reported to prolong/shorten or exert no effect on APD; the effect is dependent on the species, time of exposure, agonist type and other factors (29). On the other hand, studies have shown that β-adrenergic stimulation may shift the balance in APD and the systolic-diastolic interval interaction (30). The AP restitution curve is steepened by increased intracellular Ca^{2+} concentration (31). In this study, chronic stimulation with phenylephrine produced significant prolongation of ADP. However, chronic stimulation

with isoproterenol marginally shortened the AP plateau and produced a triangular shape. The tendency for this alteration differed with regard to β-arrestin2 overexpression. β-Arrestin2 shortened the phenylephrine-induced APD extension, but had no effect on APD after exposure of the cells to isoproterenol. This discrepancy may be attributed to a regulatory effect of the type of adrenoceptor occupying the cardiac ion channels. The phenylephrine-induced reduction in IKr may contribute less current during the AP plateau, which can functionally result in prolongation of APD. The results of this study suggest that enhanced β-arrestin2 expression may produce dramatic changes in IKr characteristics. These results are in agreement with those of previous studies (12). It is likely that enhanced β-arrestin2 expression will mitigate AP prolongation during α1-adrenoceptor stimulation. β-Adrenergic stimulation affects several ion transport pathways (32). Strong Ca^{2+}-related events rather than remodeling of IKr currents may alter the shape of AP curve (33–35). Therefore, augmentation of IKr caused by β-arrestin2 may not be sufficient to counteract the electrophysiology of ventricular myocytes during β-adrenergic stimulation.

The limitations of this study were: (1) The course of chronic adrenergic stimulation was performed in cultured myocytes; (2) inability to investigate the precise molecular mechanism underlying the effect of β-arrestin2 on IKr current and APD; and (3) the use of a single factor to investigate the incidence of arrhythmias.

Enhancement in β-arrestin2 level weakens the dysregulation of IKr current and prevents excessive APD prolongation, making it an effective anti-arrhythmic strategy.

Conflict of interest

No conflict of interest is associated with this work.

Acknowledgments

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