α_1 -adrenergic Receptor Attenuates β_1 -adrenergic Modulation of I_{Kr} by Protein Kinase C-dependent Suppression of Adenylyl Cyclase in Ventricular Myocytes

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Abstract: The rapid delayed rectifier K^+ current (I_{Kr}) is critical for repolarization of the cardiac action potential. Previous studies have shown activated α_1 -adrenergic receptor (AR) attenuates β_1 -adrenergic regulation of I_{Kr} while the mechanisms involved are poorly understood. To evaluate how α_1 -adrenergic receptor affect β_1 -adrenergic modulation of I_{Kr} , whole-cell patch-clamp recordings were performed in isolated guinea-pig ventricular myocytes. Application of xamoterol, a selective β_1 -AR agonist, induced a negative shift in the activation curve and I_{Kr} current reduction by $40.50 \pm 6.66\%$ at the test pulse of +40 mV. Forskolin and 8-Br-cAMP also resulted in I_{Kr} reduction by $38.17 \pm 1.50\%$ and $24.65 \pm 3.37\%$, respectively. Phenylephrine, a selective α_1 -AR agonist, prevented the activation shift and I_{Kr} current reduction induced by xamoterol and forskolin, but not by 8-Br-cAMP. The effect of xamoterol or forskolin on I_{Kr} was also prevented by pretreatment with PDBu, a protein kinase C (PKC) activator, while the effect of cAMP on I_{Kr} can not, which was similar to pretreatment with phenylephrine. When cells were pretreated with chelerythrine, a specific PKC inhibitor, phenylephrine failed to prevent I_{Kr} reduction induced by xamoterol. Our data suggests that α_1 -adrenergic stimulation attenuates β_1 -adrenergic regulation of I_{Kr} , through PKC-dependent downregulation of adenylyl cyclase/cyclic AMP pathway.

Keywords: Adrenergic Receptors, I_{Kr} , Cross-talk, Protein Kinase C, Adenylyl Cyclase

1. Introduction

Human ether-a-go-go-related gene (hERG) potassium channels are crucial for cardiac action potential repolarization, conducting rapid delayed rectifier K^+ current (I_{Kr}) [1-3]. hERG mutations that reduce conductance or expression cause result in congenital long QT syndrome (LQTS) and inhibition of hERG channels by structurally diverse drugs, either antiarrhythmic or non-antiarrhythmic, may cause acquired LQTS or other arrhythmias [4-10].

Increasing evidence has shown that hERG/ I_{Kr} channels are regulated by a variety of G protein-coupled receptors (GPCRs), including adrenergic receptors. Adrenoreceptors (AR) are composed of more than nine subtypes, including: $\alpha_{1A,B,D}$, α_{2A-C} and β_{1-3} , of which β_1 -AR and α_1 -AR are the two principle ARs expressed in cardiomyocytes [11]. Sympathetic

regulation of cardiac hERG/ I_{Kr} current involves β -AR-dependent stimulation of adenylyl cyclase (AC) via the stimulatory G protein (Gs) and cAMP dependent activation of protein kinase A (PKA). Decrease of the hERG/ I_{Kr} current in response to β -AR is mediated by β_1 -AR and is the principal signaling mechanism contributing to an increase in ventricular arrhythmias during stress and exercise. Karle et al found that xamoterol, a specific β_1 -AR agonist, could cause 58% I_{Kr} tail current decrease and the effect were drastically reduced by PKA inhibitor KT5720. Tail current could also reduce by cAMP, forskolin and PKA catalytic subunit, indicated that I_{Kr} current is inhibited by β_1 -AR activation, via AC/cAMP/PKA-dependent pathways [12, 13]. Sympathetic regulation of cardiac hERG/ I_{Kr} current also involves

α_1 -adrenergic receptor (α_1 -AR), exerting its effects through protein kinase C (PKC) [14-16].

The adrenergic regulation of hERG is complex, primarily due to the reason that endogenous agonist catecholamines are non-selective and interact with multiple AR during emotional or physical stress. It has been proposed that α_1 - and β_1 -adrenergic signaling pathways may cross-talk in the regulation of hERG [17]. This study aimed to study the effects of α_1 -AR stimulation on β_1 -AR regulation on I_{Kr} and the underlying mechanism.

2. Material and Methods

2.1. Reagents

Na₂-ATP, EGTA, creatine phosphate, nifedipine, chelerythrine, forskolin, 8-bromoadenosin 3'5'-cyclic monophosphate, PDBu, phenylephrine and dofetilide were purchased from Sigma, xamoterol-hemifumarate from Santa Cruz (Dallas, TX, USA). The rest of reagents were purchased from Amresco.

2.2. Preparation of Guinea Pig Ventricular Myocytes

The Institutional Animal Care and Use Committee of the Nanjing Medical University approved the animal study protocols for this study. The procedures for preparing single left ventricular myocytes were carried out as described previously, using 350-400 g male adult guinea pigs [18].

2.3. Electrophysiology

All electrophysiological experiments were performed in a patch clamp chamber, with temperature automatically controlled at 37±0.5°C by a Warner TC-324B Temperature Controller (Warner, Hamden, CT, USA). The whole cell patch clamp method was employed for I_{Kr} recordings. The microelectrode amplifier used in this study was Axopatch 200B amplifier in combination with Digidata 1440A digitizer (Molecular Devices, Union City, CA). After transferring to the chamber, cardiac myocytes were perfused continuously with bath solution (140 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl₂, 1.4 mM MgSO₄ and 10 mM HEPES, pH 7.4) at 1-2 mL/min. The pipette solution for current recordings was 140 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 11 mM EGTA, 5 mM Na₂-ATP and 5 mM creatine phosphate (disodium salt) (pH 7.4). Chromanol and nifedipine, each at 0.01 mM, were applied to the bath solution to eliminate slowly activating delayed rectifier potassium currents and calcium currents, respectively. When filled with pipette solution, pipette resistances were approximately 3 -5 MΩ.

A two-step voltage stimulation protocol was used to

determine I_{Kr} as previously described [19]. Briefly, the potential was increased stepwise from -40 mV, at 10 mV steps, with a 225 ms duration to +40 mV, in order to activate currents and depolarize, and then was returned to -40 mV of duration 775 ms to induce large outward tail currents. The effects on peak tail currents of α_1 - and β_1 -AR agonists were tested by measuring I_{Kr} tail currents at 10 min, following by acute administration of drug treatment. K⁺ channel activation curves were determined using the single power Boltzmann equation: $I_{tail} = I_{tail, max} / [1 + \exp((V/0.5 - V)/k)]$, where I_{tail} , V , $V/0.5$ and k represent the tail current, the test pulse potential, the half-maximal activation voltage and the slope factor, respectively.

2.4. Statistical Analysis

SPSS v. 18.0 (SPSS Inc., Chicago, IL, USA) was employed for all statistical analyses. Experimental values were expressed as MEAN±SEM. The criteria for statistical significance was P<0.05. The unpaired Student's t test and one-way analysis of variance ANOVA were utilized to compare two samples or multiple samples, respectively.

3. Results

3.1. α_1 -AR Stimulation Attenuates β_1 -adrenergic Regulation on I_{Kr}

Application of xamoterol (10 μM), a selective β_1 -AR agonist, alone for 10 min induced a negative shift in activation curve with half-maximal activation voltage ($V_{0.5}$) changing from -4.91±2.99 to -11.46±4.23 and slope factor (k) changing from 14.29±2.26 to 16.24±2.17 (n=5; Figure 1A-B) and I_{Kr} current reduction 40.50±6.66% at a test pulse +40 mV (Figure 1E). Co-application of phenylephrine (1 μM), a selective α_1 -AR agonist, prevented the activation shift, half-maximal activation voltage ($V_{0.5}$) changing from -12.42±1.44 to -12.60±2.48 and slope factor (k) changing from 9.98±0.59 to 11.50±1.10 (Figure 1C-D) and I_{Kr} current reduction induced by xamoterol (Figure 1E, n=5, p<0.05).

3.2. Phenylephrine Acts on Adenylyl Cyclase (AC) in the β_1 -adrenergic Signaling Cascade

As β_1 -AR couples to the Gs/AC/cAMP/PKA pathway, we tested whether treatment with forskolin (an AC activator) and membrane permeable 8-Br-cAMP could mimic the effects of β_1 -AR activation. As shown in Figure 2E, forskolin at 10 μM significantly decreased I_{Kr} amplitude 38.17±1.50% at +40 mV and caused a negative shift in the activation curve (Figure 2A-B), which was reversed when cells were pretreated with phenylephrine (Figure 2C-D).

Figure 1. Effects of β_1 -adrenergic regulation on I_{Kr} current in guinea pig cardiomyocytes with or without pre-activation of α_1 -adrenergic receptors. (A) Representative I_{Kr} tail currents when potential was returned to -40 mV from $+40$ mV with or without (basal) treatment with xamoterol. (B) Voltage-dependent activation I_{Kr} in controls and after treatment with $10 \mu\text{M}$ xamoterol. Tail current amplitudes at various potentials were normalized to the respective tail current values at $+40$ mV. (C) Representative I_{Kr} tail currents when the potential was returned to -40 mV from $+40$ mV with or without (basal) treatment with xamoterol when pre-treatment with phenylephrine. (D) Voltage-dependent I_{Kr} activation in control group and in the $10 \mu\text{M}$ xamoterol group with pre-activation of α_1 -adrenergic receptors. (E) Effects of xamoterol on I_{Kr} at the voltage of $+40$ mV with or without pre-activation of α_1 -adrenoceptor agonist phenylephrine. (Xamo: xamoterol; PE: phenylephrine; $n=5$, ** $p<0.01$).

Figure 2. Effects of forskolin on I_{Kr} current in guinea pig cardiomyocytes with or without pre-activation of α_1 -adrenergic receptors. (A, C) Representative I_{Kr} tail currents recorded at return pulse after depolarizing to $+40$ mV at baseline and after forskolin treatment without (A) or with (C) pre-activation of α_1 -adrenergic receptors. (B, D) Voltage-dependent I_{Kr} activation in control and treated with $10 \mu\text{M}$ forskolin without (B) or with (D) pre-activation of α_1 -adrenergic receptors. (E) Effects of forskolin on I_{Kr} at the voltage of $+40$ mV with or without pre-activation of phenylephrine. (Forsk: forskolin; PE: phenylephrine; $n=5$, ** $p<0.01$).

Similarly, treatment with 8-Br-cAMP at $500 \mu\text{M}$ reduced I_{Kr} tail current $24.65 \pm 3.37\%$ (Figure 3E) and caused a negative shift in the activation curve, with the half-maximal activation voltage ($V_{0.5}$) changing from -4.89 ± 0.64 to -10.36 ± 1.24 mV (Figure 3A-B). However, co-treatment with phenylephrine and 8-Br-cAMP caused a reduction in I_{Kr} by $33.97 \pm 6.70\%$, which was not significantly different from treatment with 8-Br-cAMP alone (Figure 3C-E). These results indicate that α_1 -AR exerts its inhibitory effects on β_1 -AR-mediated I_{Kr} current reduction by acting upstream of cAMP elevation.

Figure 3. Effects of 8-Br-cAMP on I_{Kr} current in the guinea pig cardiomyocytes with or without pre-activation of α_1 -adrenergic receptors. (A, C) Typical original I_{Kr} tail currents when the potential was returned to -40 mV from $+40$ mV with or without (basal) treatment of 8-Br-cAMP ($500 \mu\text{M}$), a membrane-permeable analogue of cAMP, without (A) or with (C) pre-activation of α_1 -adrenergic receptors. (B, D) Voltage-dependent activation of I_{Kr} in controls and in the presence of 8-Br-cAMP without (B) or with (D) pre-activation of α_1 -adrenergic receptors. (E) Effects of 8-Br-cAMP on I_{Kr} at the voltage of $+40$ mV with or without pre-activation of phenylephrine. (cAMP: 8-Br-cAMP; PE: phenylephrine; $n=5$, $**p<0.01$).

3.3. Effects of α_1 -AR on β_1 -adrenergic Regulation on I_{Kr} are PKC Dependent

PDBu, a PKC activator, also prevented the effects of xamoterol or forskolin on I_{Kr} , in a similar manner to phenylephrine, but not the effects of 8-Br-cAMP (Figure 4A). However, when cells were pretreated with the PKC inhibitor chelerythrine at $1 \mu\text{M}$, phenylephrine failed to prevent xamoterol-induced reduction in I_{Kr} (Figure 4B).

Figure 4. The role of PKC in the regulation of α_1 -AR stimulation attenuate β_1 -adrenergic regulation on I_{Kr} . (A) Effects of xamoterol or forskolin or cAMP on I_{Kr} at the voltage of $+40$ mV with or without pre-activation of PKC activator PDBu ($n=5$ of each group, $*p<0.05$, $**p<0.01$). (B) Effects of PKC inhibitor chelerythrine on phenylephrine attenuation of β_1 -adrenergic regulation on I_{Kr} at the voltage of $+40$ mV. ($n=5$, $**p<0.01$ vs xamoterol group, $\#p<0.05$ vs xamoterol (PE) group).

4. Discussion

In the presented study, we confirmed that activation of β_1 -ARs reduced I_{Kr} tail current in native cardiac myocytes, which could be mimicked by forskolin treatment and a membrane permeable cAMP analog, suggesting β_1 -AR regulate I_{Kr} through Gs/AC/cAMP pathway. Furthermore, we provided evidence that α_1 -AR, when activated, attenuates β_1 -AR mediated reduction of I_{Kr} , by PKC dependent suppression of AC.

It is well established that cardiac K^+ channels are regulated

by β -AR and α -AR [20-25]. β -AR couples to the classical Gs-AC-cAMP-PKA signaling pathway, promoting phosphorylation of hERG at multiple serine residues [12, 13]. In the presented study, β_1 -AR stimulated by xamoterol reduced the I_{Kr} tail current, as did the AC activator forskolin and a membrane permeable cAMP analog (8-bromo-cAMP). In the presence of xamoterol, forskolin and 8-bromo-cAMP, there was a small shift in $V_{1/2}$ to a more negative potential. These results suggest that the reduction in I_{Kr} induced by β_1 -adrenergic stimulation is due to increases in intracellular cAMP.

We have previously found that α_1 - or β_1 -AR, when stimulated separately, reduces I_{Kr} currents in guinea pig

cardiomyocytes [19]; however, simultaneous activation of α_1 -AR and β_1 -AR did not generate significant inhibitory effects [26]. These findings suggest activation of α_1 -AR may prevent I_{kr} reduction mediated by β_1 -AR stimulation; however, the underlying mechanisms remain unknown. In the present study, we found that a selective α_1 -AR agonist could prevent forskolin-mediated, but not cAMP-analog-mediated, I_{kr} reduction in left ventricular myocytes, strongly suggesting α_1 -AR affects β_1 -AR signaling cascades upstream of cAMP elevation.

α_1 -adrenoceptors predominantly couples with Gq, which stimulates phosphatidyl inositol specific phospholipase C (PLC), eventually leading to activation of protein kinase C (PKC) through diacylglycerol (DAG) and calcium channels in the endoplasmic reticulum via 1, 4, 5-inositol-trisphosphate (IP3). Besides α_1 -adrenergic signaling, PKC has been reported to be involved in β_1 -adrenergic pathways and PKC plays an important role in adrenergic signaling crosstalk [27-30]. Our results show that chelerythrine, a specific PKC inhibitor, may prevent the effects of α_1 adrenergic on β_1 -adrenergic regulation of I_{kr} . Furthermore, we showed that the effects of α_1 -adrenergic receptors attenuated β_1 -adrenergic modulation of I_{kr} is mediated by PKC. We further investigated PDBu, an activator of PKC, which led to a significant decrease in the sensitivity of I_{kr} to β_1 -adrenergic stimulation. Akin to the α_1 -adrenoceptor agonist phenylephrine, PDBu also prevented the decrease in I_{kr} tail current by the AC activator forskolin, but had no effect on the 8-Br-cAMP effect on I_{kr} . These findings support the hypothesis that the reduction of α_1 -adrenergic receptor attenuated β_1 -adrenergic action on I_{kr} is due to PKC dependent inhibition of AC. Importantly, it has been reported that PKC phosphorylates and inhibits AC6, desensitizing the A2a-adenosine receptor [31].

The mechanism underlying α_1 - and β -adrenergic interaction on cardiac muscle has been studied in some detail in the rat heart. Boutjdir, Restivo, Wei & El-Sherif showed that, in rat ventricular myocytes, α_1 -adrenoceptor stimulation exerted an inhibitory effect on Ca^{2+} current, that was enhanced by β -adrenergic stimulation [32]. However, α_1 -adrenoceptor stimulation also suppressed the facilitatory action of forskolin on Ca^{2+} current, though it was ineffective on the current that was enhanced directly by intracellular dialysis of cyclic AMP. These authors suggested that this α_1 -action was mediated by inhibiting adenylate cyclase activity. Barrett S and Karliner JS also found that stimulation of α_1 -adrenoceptors decreased cAMP accumulation and adenylyl cyclase activation, stimulated by a selective beta-adrenoceptor agonist in neonatal rat ventricular myocytes [33]. We showed that α_1 -AR activation attenuated the effects on I_{kr} of AC activator forskolin, but not 8-Br-cAMP, suggesting α_1 -AR attenuates β_1 -adrenergic regulation on I_{kr} by suppressing adenylyl cyclase activity.

As already demonstrated, significant delays in repolarization due to I_{kr} downregulation prolong cellular action potentials and QT-interval, thereby increasing the risk of arrhythmias, such as polymorphic ventricular tachycardia (PVT), arising from early afterdepolarizations [34]. The

inhibition of β_1 induced I_{kr} reduction by α_1 -AR is likely to have pathophysiological and therapeutic significance in heart disease. It is widely accepted that beta-blockers are effective for the treatment of systolic heart failure. Carvedilol is a non-selective blocker drug targeting both β - and α_1 -adrenoreceptors, and is efficacious for treating congestive heart failure. Carvedilol, different from β blockers, may possess pleiotropic physiological properties. Recently, Rain and Rada concluded that patients treated with carvedilol may have lower mortality rates than those treated with metoprolol or bisoprolol; however, carvedilol is superior to bisoprolol or metoprolol in decreasing hospitalization risk [35].

5. Conclusion

Stimulation of α_1 -AR with phenylephrine attenuates β_1 -adrenergic action on I_{kr} in guinea pig cardiomyocytes. The effect of phenylephrine was blocked by the PKC inhibitor chelerythrine. Phenylephrine, and the PKC activator PDBu, could reverse the effects of xamoterol and forskolin on I_{kr} , but could not reverse the effects of 8-Br-cAMP on I_{kr} . These results suggest that α_1 -AR exerts its effects on β_1 -adrenergic modulation of I_{kr} through PKC-dependent inhibition of AC.

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