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Short communication

Receptor subtype involved in α_1-adrenergic receptor-mediated Ca^{2+} signaling in cardiomyocytes[1]

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Key words

α_1-adrenergic receptor; subtype; cardiomyocyte; Ca^{2+} signaling; A61603

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Abstract

Aim: The enhancement of intracellular Ca^{2+} signaling in response to α_1-adrenergic receptor (α_1-AR) stimulation is an essential signal transduction event in the regulation of cardiac functions, such as cardiac growth, cardiac contraction, and cardiac adaptation to various situations. The present study was intended to determine the role(s) of the α_1-AR subtype(s) in mediating this response. **Methods:** We evaluated the effects of subtype-specific agonists and antagonists of the α_1-AR on the intracellular Ca^{2+} signaling of neonatal rat ventricular myocytes using a confocal microscope. **Results:** After being cultured for 48 h, the myocytes exhibited spontaneous local Ca^{2+} release, sparks, and global Ca^{2+} transients. The activation of the α_1-AR with phenylephrine, a selective agonist of the α_1-AR, dose-dependently increased the frequency of Ca^{2+} transients with an EC_{50} value of 2.3 μmol/L. Blocking the α_{1A}-AR subtype with 5-methylurapidil (5-Mu) inhibited the stimulatory effect of phenylephrine with an IC_{50} value of 6.7 nmol/L. In contrast, blockade of the α_{1B}-AR and α_{1D}-AR subtypes with chloroethylclonidine and BMY 7378, respectively, did not affect the phenylephrine effect. Similarly, the local Ca^{2+} spark numbers were also increased by the activation of the α_1-AR, and this effect could be abolished selectively by 5-Mu. More importantly, A61603, a novel selective α_{1A}-AR agonist, mimicked the effects of phenylephrine, but with more potency (EC_{50} value =6.9 nmol/L) in the potentiation of Ca^{2+} transients, and blockade of the α_{1A}-AR by 5-Mu caused abolishment of its effects. **Conclusion:** These results indicate that α_1-adrenergic stimulation of intracellular Ca^{2+} activity is mediated selectively by the α_{1A}-AR.

Introduction

The α_1-adrenergic receptors (α_1-AR) play a key role in the modulation of sympathetic nervous system activity as well as a site of action for therapeutic agents, such as antihypertensive drugs. Three subtypes of the α_1-AR, α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR have been cloned and each has specific tissue distribution and pharmacological properties[1,2]. All subtypes of the α_1-AR are Gq protein-coupled receptors, which upon activation, catalyze the cleavage of polyphosphoinositide into dual signaling molecules, inositol 1,4, 5-trisphosphate (IP_3), and diacylglycerol via the activation of phospholipase C. IP_3 leads to the opening of IP_3 receptor channels at the endoplasmic/sarcoplasmic reticulum, and subsequently the release of intracellular Ca^{2+}, while the activation of protein kinase C is the downstream signaling pathway for diacylglycerol[3,4]. Through these signal transduction pathways, the intracellular responses upon α_1-AR stimulation are induced.

Accumulating studies have indicated that the α_1-AR system appears to play a role in cardiac growth, cardiac contraction, and cardiac automaticity in physiological condition[4–6], as well as in cardiac pathological processes, such as arrhythmogenesis or cardiac adaptation to various situations[5,7,8]. Although the exact underlying mechanisms have not been conclusively determined, the increase in intracellular Ca^{2+}

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signaling, a common event seen in α_1-AR stimulation, is considered to be a primary signaling pathway initiating acute as well as chronic cardiac function modulations by the α_1-AR[2,7–11]. For instance, the α_1-AR-mediated mobilization of Ca^{2+} from the sarcoplasmic reticulum contributes significantly to excitation–contraction coupling in atrial myocytes, and causes arrhythmogenic intracellular Ca^{2+} oscillations in the ischemic heart[7,9,10]. Additionally, α_1-AR-mediated Ca^{2+} signaling is essential for the activation of calmodulin-dependent protein kinase II and nuclear factor of activated T cells, both of which signal a hypertrophic program of cardiac gene expression[8,12,13].

All 3 subtypes of α_1-AR have been detected at the levels of messenger RNA as well as protein in the heart[14,15]. How-ever, the subtype of the receptor in the mediation of cardiac function is not clear. Many studies have suggested that the α_{1A}- AR and α_{1B}-AR appear to play major roles in the heart[3,6,15,16]. More recently however, the α_{1A}-AR has been demonstrated to sufficiently induce cardiac arrhythmias and hypertrophy, while the α_{1B}-AR seems less important[7,17,18]. Furthermore, the avtivation of α_{1B}-AR even inhibits α_{1A}-AR mediated cardiac remodeling[19], but plays a crucial role in the generation of dilated cardiomyopathy[16]. As an increase in intracellular Ca^{2+} is the primary signaling transduction pathway for α_1-AR-mediated cardiac function[2,7–11], and the subtype involved is unclear, in this study we intended to identify the subtype of the α_1-AR involved in mediating intracellular Ca^{2+} signaling by using neonatal rat ventricular myocytes (NRVM), which express all 3 α_1-AR subtypes[14,15] and respond to α_1-AR stimulation markedly in the profiles of intracellular Ca^{2+} signaling and hypertrophic growth[8,20,21].

Material and methods

Isolation and culture of cardiomyocytes NRVM were isolated from 1–2-d-old Sprague-Dawley rats by enzymatic digestion with 0.1% trypsin and 0.03% collegenase, as previously described[20]. Then the myocytes were plated onto laminin-coated, 35 mm dishes at a density of 0.5×10^3–0.8×10^3 cells/mm^2 and cultured for 42 h in Dulbecco's modified Eagle's medium (DMEM) and Medium 199 (4:1) containing 10% fetal bovine serum, 4 mmol/L L-glutamine, 100 units/mL penicillin/streptomycin, and 0.1 mmol/L 5-bromo-2-deoxyuridine to inhibit fibroblast proliferation. Before use, the myocytes were further cultured for 6 h in serum-free DMEM to eliminate any influence of some factors in the serum.

Confocal Ca^{2+} imaging The cultured NRVM were loaded with 4 μmol/L Fluo-4/AM (Molecular Probes, Eugene, OR, USA) at 37 °C for 30 min, and were then washed with 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid *N*-(2-Hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES)-buffered salt solution (in mmol/L: NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10, and glucose 11, with pH 7.4 adjusted by NaOH) for 20 min. All the treatments for each dish were finished within 2 h.

Confocal images of fluo-4 fluorescence (excitation at 488 nm and emission detection at >515 nm) were obtained using a Leica SP2 inverted microscope equipped with a 63×, 1.3 numerical aperture, oil immersion objective. Time- lapsed (*xy*, 1.63 s/frame) or line-scan (*xt*, 2 ms/line, 0.15 μm/pixel) images were obtained with 1.5-μm axial resolution. Image data analysis used customer-devised routines coded in the Interactive Data Language Research System. All experiments were performed at room temperatures (22–24 °C).

Materials 5-Bromo-2-deoxyuridine, phenylephrine, 5-methylurapidil (5-Mu), chloroethylclonidine (CEC), BMY 7378 {8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro [4,5]decane-7,9-dione dihydrochloride}, propranolol, and prazosin were purchased from Sigma-Aldrich (St Louis, MO, USA). A61603 {N-[5-(4,5-dihydro-1*H*-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetra hydronaphthalen-1-yl]methanesulfonamide hydrobromide} was from Tocris (Ellisville, MO, USA).

Statistical analysis The data were analyzed and presented as mean±SEM. When appropriate, statistical comparison was carried out with 2-way paired or unpaired Student's *t*-test or χ^2 test. The accepted level of significance was $P<0.05$.

Results

In fluo-4-loaded NRVM, due to spontaneous action potentials as the trigger, rhythmic and spontaneous Ca^{2+} oscillations, were observed at a rate of 6.02±0.58 min^{-1} (n=12 experiments). Phenylephrine (PE, 10 μmol/L), a non-subtype specific agonist of the α_1-AR[17,19,22], increases the frequency of the spontaneous Ca^{2+} transients (Figure 1A, upper panel), which was completely blocked with 1 μmol/L prazosin, an α_1-AR antagonist, but not the β-AR antagonist propranolol at 1 μmol/L. This effect of PE is dose-dependent with an EC_{50} value (the concentration for inducing 50% of maximal response) of 2.3 μmol/L (Figure 1B). To determine the role of the α_1-AR subtypes in $[Ca^{2+}]_i$ regulation, we then examined the effects of subtype-specific antagonists on PE-mediated Ca^{2+} signal. As shown in Figure 1 (1A, bottom panel, 1C), pretreatment of myocytes with 5-Mu for 10 min, a specific inhibitor of the α_{1A}-AR[23], caused dose-dependent

suppression of the stimulatory response to PE (10 μmol/L) with an IC_{50} value (the concentration for 50% inhibition of agonist-induced response) of 6.7 nmol/L, and a complete abolishment was seen at a concentration of 30 nmol/L. In contrast, pretreatment of the cells for 30 min with CEC to inhibit the α_{1B}-AR showed no influence, except that CEC at higher concentration (30 μmol/L) induced a 33.5% inhibition of PE-enhanced Ca^{2+} transients (Figure 1D). The alkylating agent CEC primarily inactivates the α_{1B}-AR, but studies have shown that this compound can also produce partial inactivation of the other subtypes, especially α_{1A}-AR, with prolonged exposure at high concentrations[24,25]. Thus, the partial blockade of the PE effect by CEC at higher concentration is most likely due to its non-specific inhibition of other subtypes. Nevertheless, blockade of the α_{1D}-AR with BMY 7378 (0.1 μmol/L)[26,27] demonstrated no any influence in the PE effect (Figure 1D). Presently, the α_{1D}-AR expressing much less than other subtypes in cardiomyocytes is functionally unknown in the heart[3,15]. These findings provide clues that the α_{1A}-AR, not the α_{1B}-AR and α_{1D}-AR, may be the primary mediator of PE-regulated spontaneous Ca^{2+} transients.

At present, the determination of the role of the α_{1A}-AR versus the α_{1B}-AR subtypes in mediating physiological responses to α_1-adrenergic stimulation is difficult because of the paucity of highly selective antagonists specific for one subtype over the other. Therefore, to further discriminate the α_{1A}-AR from other subtypes, we investigated subtype specific agonists in this protocol. So far no specific com-

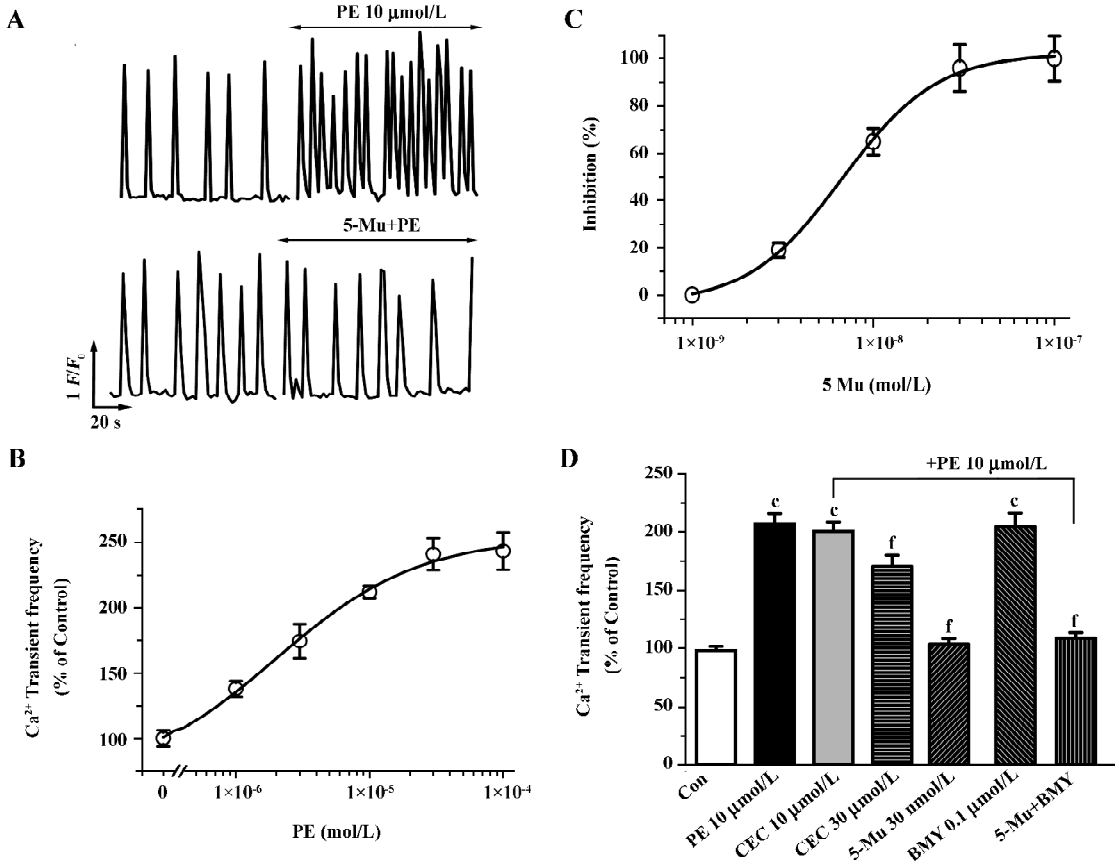

Figure 1. Effects of different α_1-AR subtype blockade on PE-induced potentiation of spontaneous Ca^{2+} transients in neonatal rat ventricular myocytes. (A) in myocytes loaded with the Ca^{2+} indicator fluo-4, increased spontaneous Ca^{2+} oscillations were observed 3 min after 10 μmol/L PE treatment, and were abolished by the pretreatment of cells with 30 nmol/L 5-Mu for 10 min. (B) dose-dependence of PE effect on spontaneous Ca^{2+} oscillation frequency (EC_{50}=2.3 μmol/L). Data were expressed as the percentage of the control. n=5–9 separated experiments for each PE concentration. (C) concentration-dependent inhibition of 5-Mu on PE induced enhancement of spontaneous Ca^{2+} transients (IC_{50}=6.7 nmol/L). n=6–7 separated experiments for each point. (D) effects of CEC, 5-Mu, BMY 7378, and 0.1 μmol/L BMY 7378+30 nmol/L 5-Mu (pretreated cells for 30, 10, 10 and 10 min, respectively) on PE-potentiated spontaneous Ca^{2+} transients. Data are presented as the percentage of the vehicle control (con, H_2O). ^cP<0.01 *vs* con; ^fP<0.01 *vs* PE group. n=6–8 experiments.

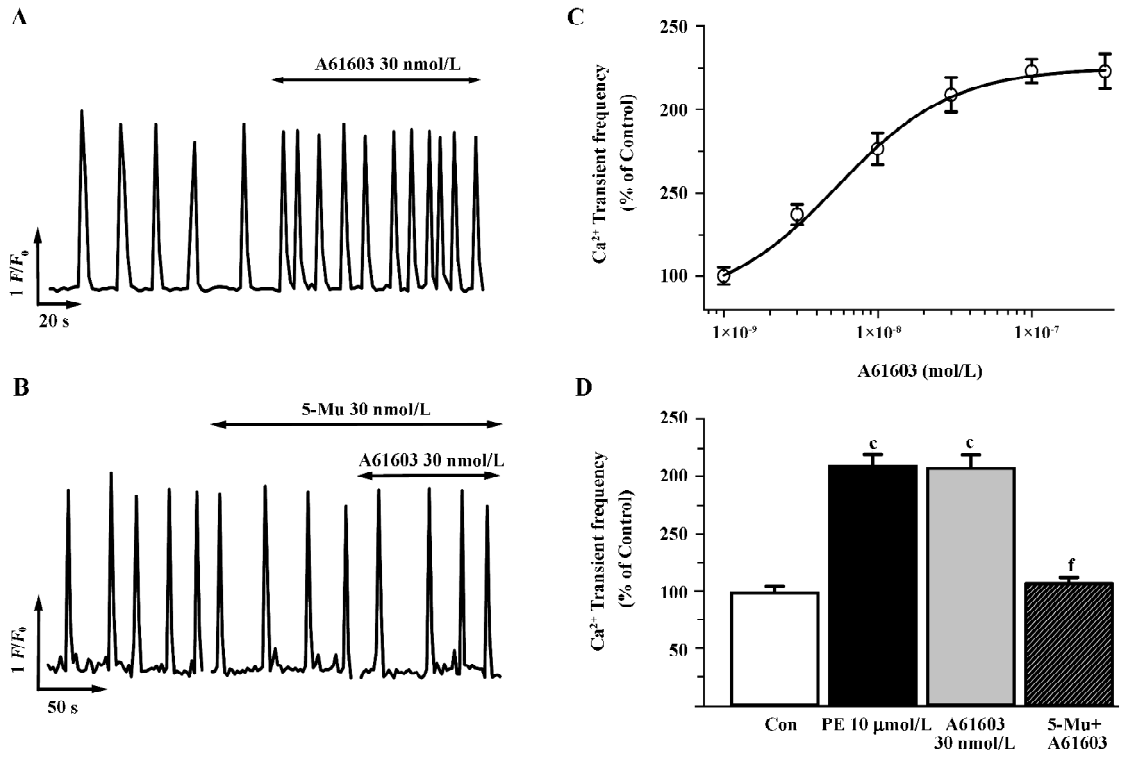

Figure 2. Effect of A61603 on spontaneous Ca^{2+} oscillations in neonatal rat ventricular myocytes. Increased spontaneous Ca^{2+} oscillations were observed 3 min after 30 nmol/L A61603 treatment (A), and were abolished by 30 nmol/L 5-Mu pretreatment of myocytes for 10 min (B). (C) dose-response effect of A61603 on spontaneous Ca^{2+} transients (EC_{50}=6.9 nmol/L). n=7–8 separated experiments for each point. (D) comparable effects of PE (10 μmol/L) and A61603 (30 nmol/L) on spontaneous Ca^{2+} transients and abolishment of these effects by 30 nmol/L 5-Mu, pretreatment of cells for 10 min. Data are presented as the percentage of the vehicle control (con, H_2O). ^cP<0.01 *vs* con; ^fP<0.01 *vs* A61603 group. n=6–7 experiments.

pound for the α_{1B}-AR or the α_{1D}-AR subtype is available, so we examined the effects of A61603, the recently described potent α_{1A}-adrenergic agonist[28], and compared the dose-response characteristics of PE and A61603. As shown in Figure 2 (2A,2C), A61603 induced a dose-response increase in spontaneous Ca^{2+} transients with an EC_{50} value of 6.9 nmol/L, indicating a 330-fold greater potency for PE (2.3 μmol/L). Furthermore, we tried to find the concentration of A61603 at which stimulated Ca^{2+} transients with a similar potency to that of PE so as to evaluate and compare the inhibitory effect of 5-Mu. We observed that 30 nmol/L A61603 potentiated the rate of Ca^{2+} transients with an almost same potency as that of 10 μmol/L PE (2.11 and 2.09 times that of the control, respectively; Figure 2C,2D). Similar as its effect on PE (Figure 1D), 5-Mu (30 nmol/L), pretreatment of cells for 10 min, abolished the increment in Ca^{2+} transients induced by 30 nmol/L A61603 (Figure 2B,2D), while no effect was observed in 10 μmol/L CEC- or 0.1 μmol/L BMY 7378-treated cells (data not shown).

As spatial temporal Ca^{2+} sparks or waves constitute the elementary events of Ca^{2+} signaling in response to α_1-adrenergic stimulation inside the cells, we then investigated the characteristics of Ca^{2+} sparks mediated by PE and A61603 in NRVM. With the aid of the line-scan confocal imaging of NRVM (Figure 3A, inset), we found that Ca^{2+} sparks occurred at a frequency of 1.56±0.2/100 μm·s or 1.27±0.15/100 μm·s in the control condition (in the absence of PE or A61603, respectively). PE (10 μmol/L) and A61603 (30 nmol/L) elicited a spark increase by 2.0- and 2.3-fold, respectively (Figure 3A, 3B), while the amplitude, width, and duration of the Ca^{2+} sparks were not altered by either treatment of the agonists. Consistent with the data in global Ca^{2+} transients, the local Ca^{2+} release responses to PE and A61603 could be abolished by 30 nmol/L 5-Mu (Figure 3B, 3C), but not by 10 μmol/L CEC or 0.1 μmol/L BMY 7378 treatment (data not shown). Therefore, the similar responses to A61603 and PE from local Ca^{2+} release to global Ca^{2+} transients, and a complete abolishment of both effects by specific α_{1A}-AR antagonist,

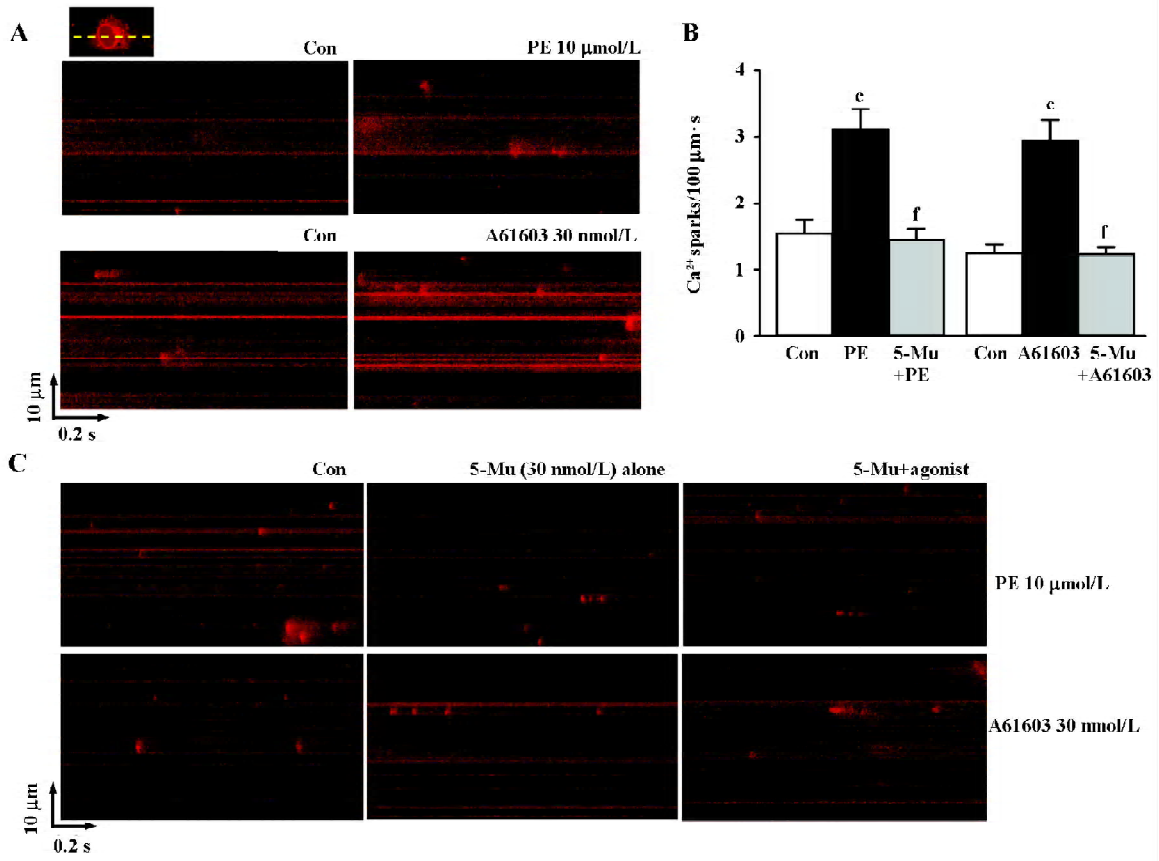

Figure 3. Modulation of local Ca^{2+} sparks by PE and A61603 in neonatal rat ventricular myocytes. (A) typical line-scan images of Ca^{2+} sparks under control conditions (con) and in the presence of PE (10 μmol/L, 3 min, upper panel) or A61603 (30 nmol/L, 3 min, bottom panel). Inset: dashed line shows the position of confocal line-scanning across the nucleus and the cytosol where the images were taken. (B) statistics of spark numbers (events/100 μm·s) from line-scan images in A for the control, PE or A61603, and 5-Mu+PE or A61603 groups as indicated. n=24–30 cells. $^{c}P<0.01$ *vs* control (con). $^{f}P<0.01$ *vs* PE or A61603 group, respectively. (C) typical line-scan images of Ca^{2+} sparks under control condition (con) in the presence of 5-Mu (10 min) alone, and PE or A61603 after 5-Mu treatment.

strongly suggest that the α_{1A}-AR subtype plays a major role in the α_1-AR-associated regulation of intracellular Ca^{2+} signaling in NRVM.

Discussion

Increases in intracellular Ca^{2+} signaling have been implicated to be an essential signal transduction event in the regulation of cardiac functions by α_1-AR stimulation[2,7–11,20,21]. However, the subtype involved is not clear. The present study demonstrates that the stimulatory responses of spontaneous Ca^{2+} oscillations to α_1-AR activation were greatly sensitive to and selectively abolished by the α_{1A}-AR antagonist, but not by antagonism of the α_{1B}-AR or the α_{1D}-AR subtype (Figure 1). Additionally, A61603, the novel α_{1A}-AR-selective agonist, exhibited a 330-fold greater potency than PE in stimulating spontaneous Ca^{2+} transient activity (Figure 2). This is in agreement with the findings that A61603 produces 340- and 330-fold greater potency than PE in stimulating sarcolemmal Na-H exchange activity in rat ventricular myocytes and in inducing contraction of the rat vas deferens, respectively. These physiological activities of α_1-AR activation are further confirmed to be mediated by α_{1A}-AR selectively[22,28]. Furthermore, Ca^{2+} sparks, an important event of the local Ca^{2+} releasing activity, were also stimulated by PE and A61603 with almost an equal potency with that in stimulating Ca^{2+} transients. This response to PE or A61603 is consistently abolished by the α_{1A}-AR antagonist, but not by α_{1B}-AR or α_{1D}-AR inhibition (Figure 3). Taking these results together, these observations provide supportive evidence that α_1-adrenergic stimulation of Ca^{2+} signaling activity is mediated selectively by the α_{1A}-AR subtype.

Our previous study and those of others have shown that stimulated intracellular Ca^{2+} signaling plays an important role in the induction and perpetuation of cardiac hypertrophy by α_1-AR activation[11,12,20,21]. Importantly, studies have shown that the α_{1A}-AR subtype is sufficient in inducing hypertrophy in cultured cardiac myocytes[17,18] and is important in the development of the heart[6]. Thus, combined with the previous reports, the present study suggests that α_{1A}-AR-mediated Ca^{2+} signaling response may assume greater significance in hypertrophy formation. Additionally, Ca^{2+} signal abnormity has also been suggested to play a key role in triggering ectopic automaticity in physiological as well as pathological circumstances, such as cardiac ischemia and heart failure[7,9,10] and α_1-AR activation appears to be one of the important underlying mechanisms in this regard[7,29–31]. Interestingly, the α_{1A}-AR has been suggested to be the crucial arrhythmogenic subtype by both *in vivo* and *in vitro* studies[7, 9]. Therefore, taken these results together, the finding that the α_{1A}-AR is the major subtype in intracellular Ca^{2+} signaling regulation during α_1-AR activation may provide significant information for the functional roles of the α_1-AR subtype and an alternate insight into the potential therapeutic candidates in heart remodeling and arrhythmias, particularly in humans as the α_{1A}-AR appears to be the predominant subtype expressed in the human ventricular myocardium[32].

In summary, the present study has shown that the α_{1A}-AR is the predominant subtype in regulating intracellular Ca^{2+} signaling for the α_1-AR activation of neonatal rat ventricular myocytes, which may provide potential information for more specific drug development to hinder the cardiac remodeling process.

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