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Alpha$_1$-adrenenoceptor stimulation inhibits cardiac excitation–contraction coupling through tyrosine phosphorylation of beta$_1$-adrenoceptor

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ABSTRACT

Adrenoceptor stimulation is a key determinant of cardiac excitation–contraction coupling mainly through the activation of serine/threonine kinases. However, little is known about the role of protein tyrosine kinases (PTKs) activated by adrenergic signaling on cardiac excitation–contraction coupling. A cytoplasmic tyrosine residue in β_1-adrenoceptor is estimated to regulate G_s-protein binding affinity from crystal structure studies, but the signaling pathway leading to the phosphorylation of these residues is unknown. Here we show α_1-adrenergic signaling inhibits β-adrenergically activated Ca^{2+} current, Ca^{2+} transients and contractile force through phosphorylation of tyrosine residues in β_1-adrenoceptor by PTK. Our results indicate that inhibition of β-adrenoceptor-mediated Ca^{2+} elevation by α_1-adrenoceptor-PTK signaling serves as an important regulatory feedback mechanism when the catecholamine level increases to protect cardiomyocytes from cytosolic Ca^{2+} overload.

1. Introduction

Catecholamines released from the sympathetic nervous system act on adrenoceptors (ARs) in heart to regulate heart inotropy and chronotropy [1–6]. In mammalian heart β_1-, β_2- and α_1-AR are the main AR subtypes expressed, with β_1-AR being the most abundant [4,5]. Acute activation of β-AR signaling controls cardiac excitation–contraction coupling through phosphorylation of various Ca^{2+}-handing proteins mainly by serine/threonine kinases such as protein kinase A (PKA), PKB, PKC, PKD and Ca^{2+}/calmodulin-dependent PK II [2,3,6], while chronic β-AR stimulation in response to stress strongly contributes to changes in contractility and to pathological remodeling, leading to hypertrophy and/or heart failure [7,8]. In cardiomyocytes, another AR subtype, α_1-AR is also expressed. Activation of α_1-AR also shows positive inotropic effects and contributes to pathological remodeling [2,5]. Although stimulation of the heart by either α_1- or β-ARs is associated with increases of ventricular contractile force, the molecular mechanisms underlying the mechanical response to each receptor stimulation are quite different [1,2,4]; β-AR stimulation induces the major contractile effect in the heart [1,3,9], while α_1-AR alone shows a moderate and delayed inotropic response [1,4,10,11]. Although modulation of excitation–contraction coupling and contribution to cardiac pathogenesis through both β-AR and α_1-AR has been extensively studied, little is known about the interactions (or cross talk) between these AR-subtypes' downstream signaling. Early studies from the 1960s to 1990s reported that an α_1-AR stimulation can inhibit the positive inotropic and chronotropic effects of β-AR stimulation [12–14]. However, the detailed mechanism underlying cardiac α_1-AR inhibition of β-AR signaling is still not well understood.

Here we showed that α_1-AR stimulation inhibits β-adrenergically activated Ca^{2+} transients (CaT) and contractile force through the inhibition of the Ca^{2+} current through L-type channels (I_{Ca}) by protein tyrosine kinase (PTK). We found that I_{Ca} inhibition is mediated through a decrease in cyclic adenosine monophosphate (cAMP) production concomitant with the phosphorylation of cytoplasmic tyrosine residues in β_1-AR by α_1-AR-PTK signaling. Interestingly, only a single cytoplasmic tyrosine residue exists in the cytoplasmic structures of β_1-AR, which is located in the 2nd cytoplasmic loop (C-loop) [15]. Because this single tyrosine residue in the 2nd C-loop is predicted to regulate G_s-protein binding affinity

Abbreviations: AR, adrenoceptor; PTK, protein tyrosine kinase; C-loop, cytoplasmic loop; CaT, Ca^{2+} transients; I_{Ca}, Ca^{2+} current through L-type channels; cAMP, cyclic adenosine monophosphate; Phe, phenylephrine; Iso, Isoproterenol; AC, adenylate cyclase; GPCR, G-protein coupled receptor; PTX, pertussis toxin.

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from observation in crystal structures [15], these results indicate that α_1-AR-mediated tyrosine phosphorylation of β_1-AR may reduce the binding affinity with G_s protein, followed by the inactivation of downstream signaling and the inhibition of cardiac excitation–contraction coupling. Our findings suggest that α_1-AR-PTK signaling serves as an important regulatory feedback mechanism when catecholamine levels increase to protect cardiomyocytes from cytosolic Ca^{2+} overload.

2. Material and methods

An expanded Section 2 is in the online data supplement. The papillary muscles obtained from adult rat heart ventricles [9,11,16] were used for measuring CaT and tension with the injection of Ca^{2+}-sensitive photoprotein, aequorin [9,11,16]. Single adult rat ventricular myocytes [17–19] were used for measuring I_{Ca} by perforated patch-clamp [18,19] and measuring cAMP levels by competitive enzyme immunoassay [19]. HEK293T cells were used for detecting tyrosine phosphorylation of β_1-AR by co-transfection of HA-tagged human α_{1A}-AR and Flag-tagged β_1-AR. All results are shown as mean ± standard error (SE) or otherwise indicated. For multiple comparisons, one-way ANOVA or one-way repeated measure ANOVA followed by Bonferroni post hoc test was used with the significance level set at $P < 0.05$.

3. Results

3.1. α_1-AR stimulation inhibits CaT and contraction during β-AR stimulation

At first, we investigated whether α_1-AR stimulation changes Ca^{2+} handing during β-AR stimulation in tissue/cell levels. We examined intracellular Ca^{2+} kinetics and contractile properties simultaneously using aequorin-injected rat papillary muscle as we previously described [1,9,11,20] (Fig. 1). At first, papillary muscle was treated with β-AR agonist, 10 μM isoproterenol (Iso) for 5 min. Iso dramatically increased both CaT and tension [1,9] (Fig. 1A). Then, 100 μM phenylephrine (Phe) was applied in the continued presence of Iso. Indeed, Phe significantly inhibited Iso-stimulated peak tension and also decreased peak CaT (Fig. 1A) and this effect was abolished by removing Phe from the extracellular solution (Fig. 1). Thus, we confirmed that α_1-AR stimulation decreases β-adrenergically activated contractile force with decreasing peak CaT.

3.2. α_1-AR stimulation inhibits I_{Ca} during β-AR stimulation

Next, we hypothesized that the decrease in CaT by α_1-AR stimulation is due to the inhibition of I_{Ca}. Therefore, we next examined the effect of α_1-AR stimulation on I_{Ca} when β-AR is stimulated using perforated patch clamp. At first, isolated adult rat ventricular myocytes were treated with 100 nM Iso for 10 min and then, 100 μM Phe was applied in the continued presence of Iso (Fig. 2A–C). Indeed, Phe significantly inhibited the peak of I_{Ca} in the presence of Iso and this effect was abolished by removing Phe from the extracellular solution (Fig. 2A–C). This effect was not observed in the presence of an α_1-AR antagonist, 1 μM prazosin, confirming that this effect is mediated through α_1-AR stimulation (Fig. 2F and Supplementary Fig. 1A and D). In the heart β_1- and β_2-ARs are expressed with β_1-AR being the most abundant [4]. To address the question of which β-AR subtype is responsible for the increase of I_{Ca} by Iso, we investigated the effect of Iso in the presence of a β_2-AR selective antagonist, 100 nM ICI 118,551, for evaluating the effect of selective β_1-AR stimulation. Iso still increased I_{Ca} about 1.84-fold even in the presence of the β_2-AR selective antagonist (Supplementary Fig. 2). Next we tested the effect of β_2-AR selective stimulation on I_{Ca} by the combination of a β_2-AR relatively-selective agonist, 10 μM zinterol with β_1-AR selective antagonist, 300 nM CGP 20712A. Zinterol did not increase I_{Ca} amplitude in the presence of CGP 20712A, confirming that β_2-AR-G_s signaling is not functionally coupled to I_{Ca} in our preparations as previously reported ($n = 4$) (data not shown) [21]. Thus, β_1-AR is the dominant subtype responsible for the increase of I_{Ca} by Iso in our preparation. We next examined the effect of α_1-AR stimulation on I_{Ca} when β_1-AR is selectively stimulated (Supplementary Fig. 2). Phe significantly inhibited I_{Ca} in the presence of Iso and a β_2-AR selective antagonist, ICI 118,551, suggesting that α_1-AR signaling can inhibit β_1-adrenergically activated I_{Ca}.

3.3. Phenylephrine-induced I_{Ca} inhibition during β-AR stimulation is mediated through α_{1A}-AR subtype

In rat cardiomyocytes, two subtypes of α_1-AR, α_{1A}- and α_{1B}-AR are functionally expressed and each subtype has a distinct downstream signaling pathway [10,19]. Next we examined which subtype of α_1-AR is responsible for this mechanism. The α_{1A}-AR selective antagonist, 2 μM WB4101 [10,19], blocked this inhibitory effect by Phe (Supplementary Fig. 1C and D), but α_{1B}-AR selective antagonist, 100 nM L-765,314 [10,19] did not (Supplementary Fig. 1B and D). We also tested the effect of selective α_{1A}-AR agonist, 1 μM A61603 [10,19]. A61603 significantly inhibited I_{Ca} in the presence of Iso as in the case of Phe (Supplementary Fig. 1D). These results indicated that Phe-induced I_{Ca} inhibition during β-AR stimulation is mediated through α_{1A}-AR subtype, but not α_{1B}-AR. In adult rat cardiomyocytes, 5 subtypes of G proteins (G_S, G_q, $G_{i\text{-}2}$, $G_{i\text{-}3}$, G_o) are expressed [10,19,22]. We reported that α_{1A}-AR is mainly coupled to $G_{q\alpha}$-phospholipase C-PKC signaling and regulates I_{Ca} in the absence of β-AR stimulation [19] (Supplementary Fig. 3). However, inhibition of phospholipase C by 1 μM U73122 [19] did not block the Phe-induced inhibition of I_{Ca} during β-AR stimulation (Fig. 2F), indicating that other G-protein signaling (not $G_{q\alpha}$) is involved in this mechanism (Supplementary Fig. 4A).

3.4. Inhibitory effect of α_1-AR stimulation is acting upstream of adenylate cyclase activation

α_1-ARs are also known to couple with pertussis toxin (PTX)-sensitive G proteins ($G_{i/o}$), which inhibits cAMP production [23,24] (Supplementary Fig. 3). It is reported that α_1-AR activation antagonizes β-adrenergically stimulated cAMP levels through an increase in cAMP breakdown [25] or inhibition of cAMP synthesis in cardiac muscle [26]. Indeed, treatment with 100 nM Iso for 15 min strongly increased cAMP levels about 3-fold in isolated adult rat ventricular myocytes as we previously reported [19] and 100 μM Phe inhibited the β-adrenergically stimulated cAMP level (32.4 ± 2.58%, $n = 3$) as observed by a competitive enzyme immunoassay (see online Material and Methods). Therefore, we next hypothesized that a decrease in I_{Ca} might be due to the inhibition of β-adrenergically stimulated cAMP levels through the $G_{i/o}$ pathway. However, we found that inhibition of $G_{i/o}$ by PTX [19] did not block the Phe-induced inhibition of I_{Ca} during β-AR stimulation (Fig. 2F), indicating that (1) other signaling (not $G_{q\alpha}$ or $G_{i/o}$) may be involved and (2) this signaling might exert its effect through inhibiting upstream of cAMP production (Supplementary Fig. 4A). Therefore, we directly stimulated adenylate cyclase (AC) with 10 μM forskolin and observed the effect of Phe on I_{Ca} (Fig. 2F and Supplementary Fig. 4A–C). Phe was unable to inhibit the I_{Ca} activated by forskolin, suggesting that α_1-AR stimulation exerts its effect by inhibiting upstream of cAMP production through a non-$G_{q\alpha}$ and non-$G_{i/o}$ pathway.

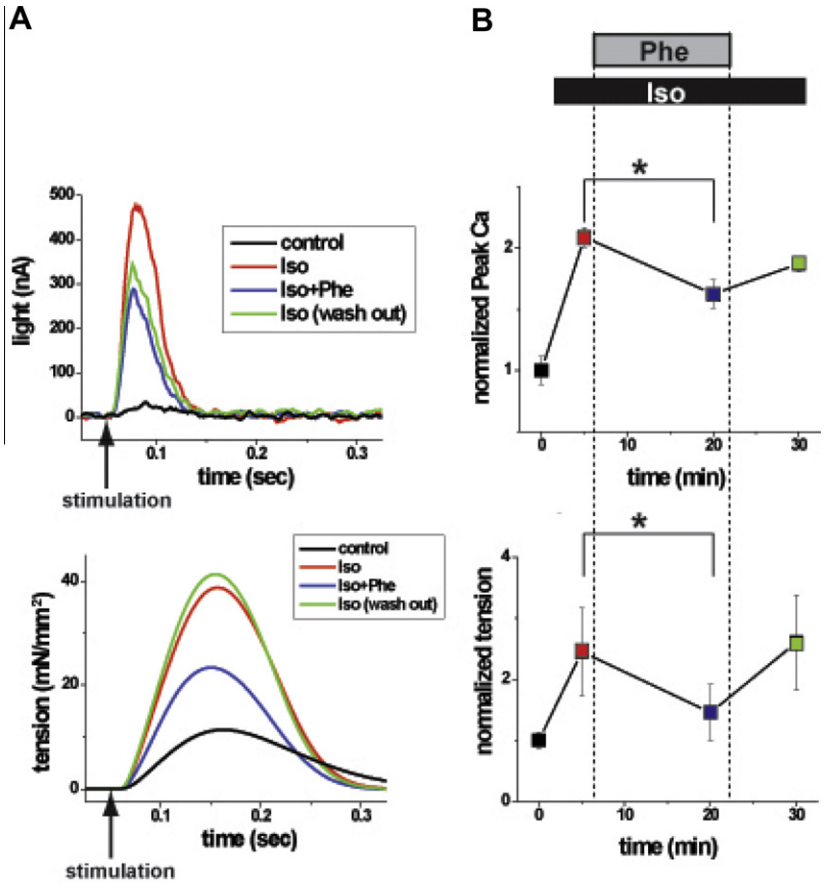

Fig. 1. α_1-AR stimulation inhibits β-adrenergically activated CaT and contractile force in rat papillary muscle. (A) Representative traces of CaT (upper traces) and isometric tension (lower traces) in rat papillary muscle. (B) Summarized data showing the effect of Phe in the presence of Iso on CaT (upper traces) and tension (lower traces).

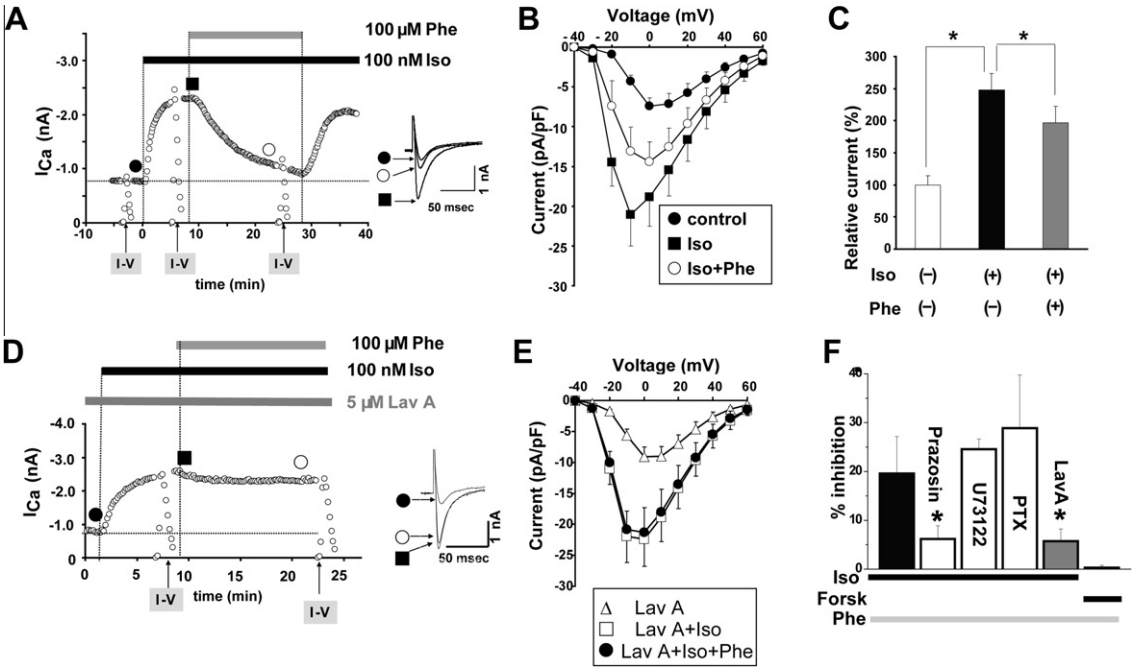

Fig. 2. α_1-AR signaling inhibits β-adrenergically activated I_{Ca} through PTK in rat isolated ventricular myocytes. (A) Typical time course of I_{Ca} during 100 μM Phe stimulation in the presence of 100 nM Iso. Holding potential was set at −40 mV and I_{Ca} was evoked by 0 mV depolarization pulse at 1 Hz. (B) Summarized data of the effect of Phe in the presence of Iso on *voltage-current* (*I–V*) relationships. (C) Summarized data of the effect of Phe on Iso-activated I_{Ca} at 0 mV depolarization pulse. $^*P < 0.05$. (D) Typical time course of I_{Ca} during 100 μM Phe stimulation in the presence of 100 nM Iso and PTK inhibitor 5 μM lavendustin A (LavA). (E) Summary data of the effect of Phe in the presence of Iso and LavA on *I–V* relationships. (F) Summarized data of the inhibitory effect of Phe on Iso- or 10 μM forskolin (Forsk)-activated I_{Ca} in the absence or presence of various inhibitors. $^*P < 0.05$.

3.5. PTK inhibition antagonizes the inhibitory effect of α_1-AR stimulation

Because Harvey's group previously reported that α_1-AR stimulation did not inhibit the I_{Ca} and I_{Cl} in guinea-pig cardiomyocytes activated by histamine (which is another receptor coupled with G_s like β-ARs) [27,28], we hypothesized that α_1-AR signaling may act directly at the level of the β_1-AR rather than G_s protein (Supplementary Fig. 5). In addition, our data clearly showed that α_1-AR signaling downstream of $G_{q\alpha}$ and $G_{i/o}$ are not involved in the inhibitory effect on β-adrenergically stimulated I_{Ca} (Supplementary Fig. 4A). Therefore, another signaling pathway associated with α_1-AR might be involved such as PTK activity, which is activated through βγ subunits of G_q [29] (Supplementary Fig. 5). Therefore, we next tested whether PTK activity is involved in the signaling pathway for the I_{Ca} inhibition. In the presence of a general PTK inhibitor, 5 μM lavendustin A, Phe-induced inhibition of I_{Ca} during Iso stimulation was completely abolished (Fig. 2D–F). We also tested another general PTK inhibitor, 50 μM genistein, and found that this also abolished the inhibitory effect of α_1-AR stimulation on β-adrenergically stimulated I_{Ca} (data not shown). We concluded that α_{1A}-AR signaling inhibits β_1-adrenergically activated I_{Ca} through PTK activity, which possibly inhibits the β_1-AR signaling at the level of receptor (Supplementary Fig. 5).

3.6. α_1-AR stimulation phosphorylates tyrosine residues in β_1-AR

We next addressed whether β_1-AR can be directly phosphorylated by PTKs upon α_1-AR stimulation because a cytoplasmic tyrosine residue in β_1-AR is predicted to regulate G_s protein binding affinity from the observation in crystal structures [15]. We overexpressed human HA-tagged α_{1A}-AR and Flag-tagged β_1-AR into HEK293T cells and stimulated the cells by 100 nM Iso for 5 min and then added 100 μM Phe in the continued presence of Iso for an additional 15 min. We found that β_1-AR showed tyrosine phosphorylation by α_1-AR stimulation using general anti-phosphotyrosine antibody [30] (Fig. 3A). Collectively, these data indicate that α_1-AR stimulation signals for phosphorylation of tyrosine residues in β_1-AR, which may reduce the binding affinity of G_s to β_1-AR and decrease the cAMP levels.

4. Discussion

In this study, we identified a detailed molecular mechanism of α_1-adrenergic modulation of β-adrenergic signaling focusing on excitation–contraction coupling regulation by PTK (Fig. 4). Our data shows that α_1-AR signaling inhibits β-adrenergically activated CaT and contractility (Fig. 1) through inhibition of L-type Ca^{2+} channel activity (I_{Ca}) (Fig. 2). This effect requires PTK activity activated by α_{1A}-AR-$G_{q\beta\gamma}$ pathway, and is mediated through inhibition at the upstream portion of β_1-AR signaling (Fig. 2). Moreover, we found that PTK activated by α_1-AR signaling phosphorylates the tyrosine residue of β_1-AR, which may desensitize downstream signaling by decreasing the binding affinity of G_s (Fig. 3). Inhibition of β-AR signaling by α_1-AR stimulation may serve as an important regulatory feedback mechanism when catecholamine levels increase under pathophysiological conditions.

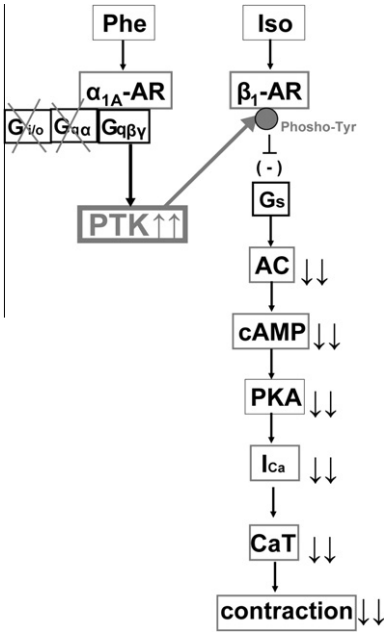

Fig. 4. Working model: α_{1A}-AR stimulation inhibits cardiac excitation–contraction coupling through tyrosine phosphorylation of β_1-AR. Schematic diagram of the proposed intracellular mechanism underlying the inhibitory effect of α_{1A}-AR signaling on β_1-AR signaling.

4.1. Crosstalk signaling between α_1- and β-adrenergic stimulation in cardiac excitation–contraction coupling

It is well reported from 1960s that α_1-AR agonist can inhibit the positive inotropic and chronotropic effects of β-ARs stimulation [12–14,31,32]. From the 1990s, the α_1-AR inhibitory effect was found not only in β-AR-mediated increase in contractile force [14], but also I_{Ca} [26,27,33] and I_{Cl} [28,34,35]. A suggested mechanism for this inhibition was α_1-AR activation antagonizing β-AR stimulated cAMP levels in cardiac muscle [25] possibly through PTX-sensitive G-proteins [23,24], but Hool et al. showed that G_i protein is not involved in this mechanism [34]. Chen et al. reported that PKC is involved in α_1-AR inhibitory effect on β-adrenergically stimulated I_{Ca} [33], but Oleksa et al. showed that PKC activation does not mimic this effect [28]. Belevych et al. first reported that broad PTK inhibitors could antagonize the α_1-AR inhibitory effect [27]. Overall, the information from prior research related to the mechanism underlying cardiac α_1-AR inhibition of β-AR signaling

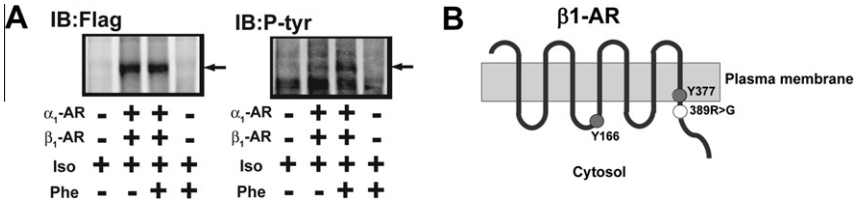

Fig. 3. α_1-AR stimulation phosphorylates tyrosine residues in β_1-AR. (A) HEK293T cells transfected with human HA-tagged α_{1A}-AR and Flag-tagged β_1-AR were stimulated with or without 100 μM Phe in the presence of 100 nM Iso. Western blot was performed on same membrane with anti-Flag (left) and aniti-phosphotyrosine (right) antibodies. (B) Schematic structure of human β_1-AR showing the location of a cytoplasmic tyrosine residue (Y166). Y377 is located at the boarder of the transmembrane domain and cytoplasmic C-term tail. 389R>G is the location of a polymorphism found in human.

is widely scattered and lacking continuity to understand the whole picture of excitation–contraction coupling under adrenergic stimulation. In this study, we confirmed that α_1-AR signaling inhibits both CaT and contractility during β-AR stimulation through I_{Ca} inhibition (Fig. 4). Furthermore, we clearly identified a detailed signaling pathway in which the α_{1A}-AR-PTK pathway phosphorylates β_1-AR, which inhibits downstream signaling (G_s-AC-cAMP-PKA), followed by a decrease in I_{Ca}, CaT and contractility (Fig. 4).

4.2. Tyrosine phosphorylation of β_1-AR by α_1-AR stimulation and its possible role in adrenergic signaling

β_1-AR signaling is strongly down-regulated in pathophysiological conditions [5] through enhanced activity of a serine/threonine kinase such as G-protein coupling receptor (GPCR) kinase-2, which phosphorylates agonist-occupied β-ARs, leading to homologous desensitization and impaired signaling through these receptors despite the continued presence of agonist [36]. β_2-AR can be phosphorylated by PTKs on tyrosine residues located in 2nd C-loop and the C-terminal tail leading to receptor internalization in non-cardiac cells [37,38]. However, there are no reports on β_1-AR desensitization by tyrosine residue phosphorylation and the upstream signaling responsible for the tyrosine phosphorylation. In human β_1-AR, there are 9 tyrosine residues. Interestingly human β_1-AR has only one tyrosine at the cytoplasmic region based on recently reported crystal structure, which is Y166 (Y149 in turkey) in the 2nd C-loop [15] (Fig. 3B). It is reported that this single tyrosine residue in the 2nd C-loop is likely to have a key role in binding with G_s proteins [15]. It is also shown in another GPCR, muscarinic M5 receptor that 2nd C-loop has a key role in G-protein coupling [39]. Human Y377 is located at the end of the 5th transmembrane domain and just before the C-term tail (the boarder of transmembrane domain and cytoplasmic C-term). This tyrosine residue might also be phosphorylated by cytosolic PTKs (Fig. 3B). Interestingly this Y377 is located in close proximity to a polymorphism (389R>G) (Fig. 3B) shown to have major functional effects in humans, due to a reduction of basal cAMP production [40]. This polymorphic variant of β_1-AR is found in 25–40% of the human population and has been shown to influence the risk of ventricular arrhythmia and survival rate after heart failure in patients [40]. Therefore, these studies supports our idea that α_1-AR-mediated tyrosine phosphorylation of Y166/Y377 of β_1-AR mediates decreased G_s binding affinity, thereby down-regulating β_1-AR-G_s-AC-cAMP-PKA signaling. Future studies will address which tyrosine residue (Y166/Y377) is the α_1-AR signaling-specific phosphorylation site and ultimately responsible for the antagonism of ß$_1$-AR downstream signaling.

4.3. α_1-AR-mediated PTK activations and their role on cardiac excitation–contraction coupling

Serine/threonine protein kinases are activated by either β- or α_1-AR stimulation. Upon activation they phosphorylate various Ca^{2+}-handling proteins and regulate cardiac functions including excitation–contraction coupling [2–5]. The effect of serine/threonine protein kinases on excitation–contraction coupling is widely studied, however little is known about the role of PTKs on excitation–contraction coupling. G_qPCRs including α_1-AR are known to activate serine/threonine protein kinases and various non-receptor or receptor-type PTKs [29,41,42]. The PTKs downstream of G_qPCRs regulate transcription factors, leading to cardiac hypertrophy [29,43]. PTKs activated by G_qPCRs are well studied as components for nuclear signal transduction, but little information is available about the effect of PTKs on cardiac Ca^{2+} handling. Only several reports including this current report showed that general PTK inhibitors increase L-type Ca^{2+} channel activity and cardiomyocyte contractility, consequently showing a gain of excitation–contraction coupling [27,44]. Further studies are needed (1) to specify the type of PTK that contributes to excitation–contraction coupling and (2) to determine target proteins of this PTK in cardiomyocytes other than β_1-AR.

In summary, the current results show that α_1-AR stimulation inhibits cardiac excitation–contraction coupling during β_1-AR stimulation. This effect is mediated through tyrosine phosphorylation of β_1-AR by α_{1A}-AR-PTK signaling, which may reduce the binding affinity with G_s protein, followed by the uncoupling of downstream β_1-AR signaling. Our findings suggest that inhibition of β_1-AR signaling by α_{1A}-AR-PTK signaling serves as an important regulatory feedback mechanism for protecting the cardiomyocytes from cytosolic Ca^{2+} overload when catecholamine levels increase. Furthermore, inhibition of α_{1A}-AR-PTK signaling may lead to the development of novel pharmacological managements for the treatment of cardiac dysfunction in heart failure patients, where β_1-AR signaling is down regulated.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.072.

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