Clinical Journal of Pharmacology and Pharmacotherapeutics

ISSN 2688-5530

Research Article

The α1A Adrenergic Receptor Inhibits Type 5 Adenylyl Cyclase in HL-1 Cardiomyocytes

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Abstract

Over the last few decades, the use of β-blockers has improved the clinical condition of patients with heart failure. However, the molecular mechanisms involved remain far from being completely understood. Adenylyl Cyclases (AC) are the major effectors of cardiac β-adrenergic receptors. Different functions have been attributed to their isoforms in relation to heart failure, with the AC5 isoform mediating harmful cardiac effects. To explore β-adrenergic receptor independent AC5 signaling, we pretreated HL-1 cardiomyocytes with norepinephrine in the presence of the β_1-selective antagonist metoprolol. This pretreatment inhibited subsequent isoproterenol-induced AC mediated cAMP accumulation. Results obtained using a α_1-adrenergic receptor selective agonist and antagonists, as well as kinase inhibitors, in the presence or absence of forskolin and pertussis toxin, revealed that the α_{1A} adrenergic receptor-CaMKII (calcium/calmodulin-dependent protein kinase II) pathway is involved in the inhibitory effect caused by norepinephrine pretreatment. Importantly, selective AC5 inhibition with SQ 22,536 decreased cAMP accumulation, and norepinephrine, in combination with metoprolol, was not able to further inhibit cAMP accumulation. This indicates a selective AC5 inhibition induced by α_{1A}-CaMKII signaling. Therapeutic implications are discussed.

Keywords: AC5; cAMP accumulation; α_{1A}-Adrenergic Receptor

Introduction

Over the last decade, the two major adenylyl cyclase isoforms expressed in the heart [1-3] (AC5 and AC6) have been extensively studied and different pathophysiological roles have been found to be associated with each. While targeted deletion of AC5 in mice was found to be associated with cardiac protection under pressure overload [4] and with increased survival [5], AC6 over expression exerted sustained beneficial effects on cardiac function [6]. Thus, selective AC5 inhibitors and AC6 activators have been proposed for the treatment of chronic heart failure [7].

Recently, the use of α_1 adrenergic receptor agonists has also been proposed for the treatment of heart failure [8]. Stimulation of α_1 adrenergic receptors mediates both inhibition and stimulation of cAMP accumulation, depending on the dose of agonist employed [9,10]. Whereas both α_{1A}- and α_{1B} adrenergic receptors mediate the stimulation of cAMP accumulation via G_s protein activation in the presence of high concentrations of agonist [10,11], it is not entirely clear which subtype of α_1 adrenergic receptor is involved in the inhibition of cAMP accumulation mediated by lower concentrations of agonist. Moreover, the absence of specific antibodies has hindered a detailed characterization of the AC isoform involved in this inhibition. Evidence of higher order signaling based on AC isoform complexing [12], suggests the possibility of specific regulation of these two AC isoforms, depending on the local receptor environment. In this regard, α_1 adrenergic receptors have been found in the proximity of muscle-A kinase anchoring protein β (mAKAPβ)-AC5 complexes in transverse T tubules [13,14]. Therefore, α_1 adrenergic receptor activation could in principle selectively regulate AC5 activity. Several mechanisms such as calcium concentration changes or protein kinase C (PKC) mediated inhibition may be involved in this regulation [15]. Thus, it is conceivable that the well-known beneficial effects of β-blockers such as metoprolol, in the clinical treatment of heart failure, may be due not only to the antagonism of β adrenergic receptors, but also to the inhibition of AC5 mediated by α_1 adrenergic receptors.

Therefore, the goal of the present study was to characterize the participation of α_1 adrenergic receptors in the regulation of AC5 activity, using a selective AC5 inhibitor (SQ 22,536) [16,17] as a pharmacological agent to distinguish between the two main cardiac AC isoforms. In addition, we also determined the subtype of α_1 adrenergic receptor and the downstream signaling pathway involved in the inhibition of norepinephrine-mediated cAMP accumulation in the presence of the β_1 adrenergic receptor antagonist metoprolol in HL-1 cardiomyocytes.

Citation: Garro MA, Urizar E, Lazkano A, Zubillaga E, Querejeta R. The α1A Adrenergic Receptor Inhibits Type 5 Adenylyl Cyclase in HL-1 Cardiomyocytes. Clin J Pharmacol Pharmacother. 2018; 1(1): 1003.

Publisher Name: MedText Publications LLC

Manuscript compiled: December 19th, 2018

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Material and Methods

Drugs

The following drugs were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA): the β_1-adrenergic receptor selective antagonist metoprolol tartrate, the non-selective adrenergic agonist norepinephrine bitartrate, the α_{1B}-adrenergic receptor antagonist Chloroethylclonidine (CEC), the non-selective α_1-adrenergic receptor antagonist prazosin hydrochloride, Pertussis Toxin (PTX) and forskolin. The following compounds were obtained from Tocris Bioscience (Ellisville, MI, USA): isoproterenol hydrochloride, a non-selective beta-adrenergic agonist, the α_{1A}-adrenergic receptor selective agonist A 61603, the α_{1A}-selective antagonists WB 4101 hydrochloride and RS 100329, the AC5 selective inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (THFA or SQ 22,536), the α_{1D}-selective adrenergic receptor antagonist BMY 7378 dihydrochloride, and the β_2-selective adrenergic receptor antagonist ICI 118,551 hydrochloride. All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Cell culture

HL-1 murine cardiomyocytes were a generous gift from Dr. W.C. Claycomb (Louisiana State Health Science Center, New Orleans, LA, USA), who first established and characterized this cell line [18]. These cells were handled exactly as described [19]. Briefly, all culture dishes and flasks were pre-coated with a gelatin-fibronectin substrate. Cardiomyocytes (passages 61-69) were cultured with a maintenance medium, which consisted of Claycomb media™ supplemented with 0.1 mM norepinephrine bitartrate (Sigma-Aldrich), 2 mM L-glutamine (Life technologies) and 10% fetal bovine serum (JRH Biosciences). This medium was changed every 24 hr. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

For the determination of cAMP levels, HL-1 cells were switched to a medium without norepinephrine for 5 days prior to the experiments to prevent receptor down regulation. Cells (10^6 cells/well) were harvested and plated in 6-well dishes the day before cAMP determination and a control group of cells (also 5 days without norepinephrine) were plated in parallel. In order to determine the effect of norepinephrine pretreatment in the presence of metoprolol, drugs were incubated for 10 min at 37°C prior to agonist (isoproterenol) stimulation.

cAMP accumulation experiments

HL-1 cardiomyocytes (10^6 cells/well) were washed three times with KHS buffer (118.4 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM KCl, 25 mM $NaHCO_3$ and 1.2 mM KH_2PO_4, pH 7.4). 1 ml KHS buffer containing the non-selective phosphodiesterase inhibitor IBMX (0.5 mM) was added to each well in order to pre-equilibrate the cardiomyocytes for 10 min at 37°C. To determine the effect of the different pharmacological agents (dissolved in 1 ml KHS+IBMX buffer) on cAMP accumulation, cells were incubated for 10 min with the corresponding drugs and washed for 10 min with KHS+IBMX prior to incubation with isoproterenol (10 min) to prevent undesirable interactions of the different drugs used. Termination of incubation and isolation of cAMP were carried out using a cAMP assay following the instructions of the manufacturer (Cayman Chemicals, Ann Arbor, MI, USA). cAMP levels were measured in triplicate.

Data analysis

Each value represented is the average of at least three independent experiments carried out in triplicate. Data are presented as mean ± SEM of the indicated number of experiments with statistical significance being evaluated using the unpaired Student *t*-test (two tailed). A p value <0.05 was considered to be statistically significant.

The concentration-response curves were analyzed by nonlinear regression using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, USA). Bottom, E_{max} and EC_{50} are expressed as the best fit ± SE provided by the fitting program. These SE values were not used for further statistical calculations. The parameters obtained from nonlinear regressions were compared by evaluating, using Snedecor's F-test, the goodness of fit to different models that shared one parameter. A p value <0.05 was considered to be statistically significant.

Results

The β_1-adrenergic receptor mediates isoproterenol-induced cAMP accumulation in HL-1 cardiomyocytes

The non-selective β-adrenergic agonist isoproterenol (10^{-6} M) stimulated cAMP accumulation in HL-1 cardiomyocytes to a similar extent in the presence or absence of the β_2-adrenergic receptor selective antagonist ICI 118,551 (2×10^{-8} M) (Figure 1). Similarly, inhibition of this accumulation by preincubation with norepinephrine in the presence of metoprolol was similar in the presence or absence of ICI 118,551 (Figure 1).

Norepinephrine pretreatment in the presence of metoprolol inhibits subsequent isoproterenol-induced cAMP accumulation in HL-1 cardiomyocytes. Effect of α_1 adrenergic receptor agonist and antagonists

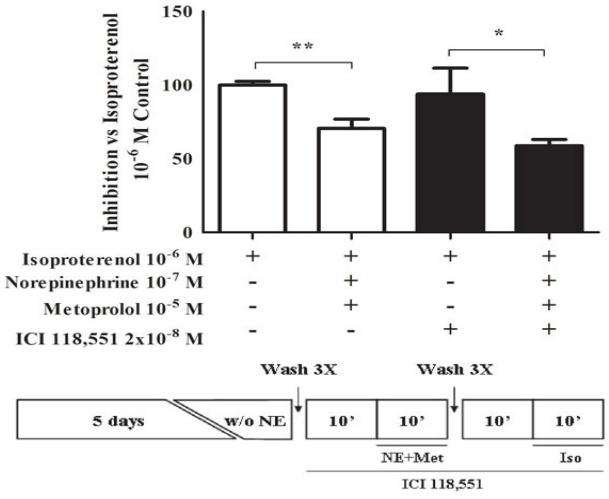

Figure 1: The β2 adrenergic receptor does not participate in the isoproterenol-induced stimulation of cAMP accumulation in HL-1 cardiomyocytes, nor does it mediate the inhibitory effect of norepinephrine preincubation (in the presence of metoprolol) on this cAMP accumulation. Cardiomyocytes were incubated at 37°C and prestimulated for 10 min with or without norepinephrine and metoprolol in the presence or absence of the β2-adrenergic receptor selective antagonist ICI 118,551. Finally, following washing, cardiomyocytes were stimulated for 10 min with the non-selective β-adrenergic agonist isoproterenol (10-6 M) as indicated in Materials and Methods. Data are expressed as mean ± SEM of three independent experiments performed in triplicate and are expressed as a percentage of isoproterenol-induced cAMP accumulation. *P<0.05; **P<0.01 vs. isoproterenol (10-6 M) stimulated cardiomyocytes (two-tailed unpaired Student t-test).
Abbreviations: NE: Norepinephrine; Met: Metoprolol; Iso: Isoproterenol; w/o: Without

Pretreatment with the endogenous adrenergic receptor agonist norepinephrine (10^{-7} M) in the presence of the β_1-adrenergic receptor antagonist metoprolol (10^{-5} M) inhibited isoproterenol-stimulated cAMP accumulation by 36 ± 6% (P<0.01) (Figure 2A). This inhibitory effect was blocked by the non-selective α_1 adrenergic receptor antagonist prazosin (10^{-6} M) (P<0.05) (Figure 2A). In order to identify which α_1 adrenergic receptor subtype mediated the inhibitory effect due to norepinephrine, we employed antagonists selective for the α_{1A} adrenergic receptor (WB 4101, 10^{-7} M), the α_{1B} adrenergic receptor (CEC, 10^{-5} M) and the α_{1D} adrenergic receptor (BMY 7378, 10^{-8} M). Only the α_{1A}-adrenergic receptor selective antagonist WB 4101 was able to revert the inhibitory effect of norepinephrine in the presence of metoprolol (P<0.05) (Figure 2A). On the other hand, the antagonists did not have any effect on isoproterenol induced cAMP accumulation when applied in the absence of norepinephrine. In order to further corroborate the involvement of the α_{1A} adrenergic receptor subtype in the inhibitory effect on cAMP accumulation, we performed another set of experiments using the selective agonist of the α_{1A} adrenergic receptor, A 61603 (5×10^{-9} M), and another selective antagonist of the α_{1A} adrenergic receptor (RS 100329, 10^{-9} M), as well as the same antagonists for the α_{1B} and α_{1D} adrenergic receptors as used above (Figure 2B). Inhibition of isoproterenol-induced cAMP accumulation by 34 ± 8% (P<0.05) was found using A 61603 which was only blocked by RS 100329 (P<0.05) (Figure 2B).

The α_{1A} adrenergic receptor-mediated inhibition of cAMP accumulation is G_i-independent

Preincubation of HL-1 cardiomyocytes with PTX (0.5 µg/ml) for 24 hrs did not block the α_{1A} adrenergic receptor-mediated inhibitory effect on cAMP accumulation (Figure 3). Although no differences in potency or efficacy were observed, pre-treating cardiomyocytes with PTX slightly increased the basal cAMP tone, but this increase was not statistically significant.

α_{1A} adrenergic receptor-induced cAMP inhibition is CaM-KII dependent and independent of β-adrenergic receptor desensitization

In order to identify the mechanism involved in α_{1A} adrenergic receptor-mediated inhibition, we employed several kinase inhibitors such as GF 109,203X (selective PKC inhibitor, 10^{-6} M), H 89 (selective PKA inhibitor, 3×10^{-6} M), AIP and KN-62 (selective inhibitors of

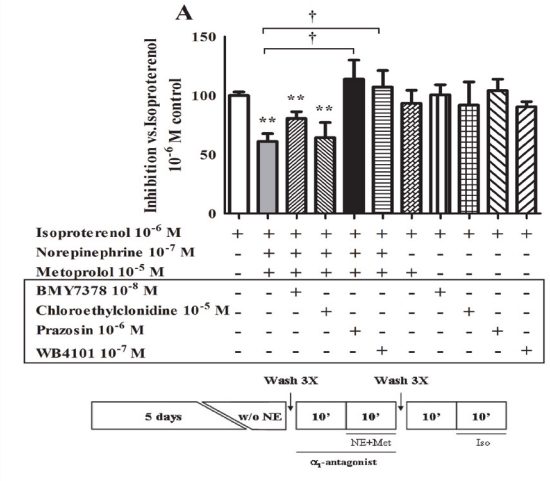

Figure 2A: The α1A adrenergic receptor inhibits isoproterenol-induced cAMP accumulation in HL-1 cardiomyocytes. (a) Effect of selective a1 adrenergic receptor antagonists on norepinephrine-mediated inhibition of cAMP accumulation in the presence of the β blocker metoprolol.

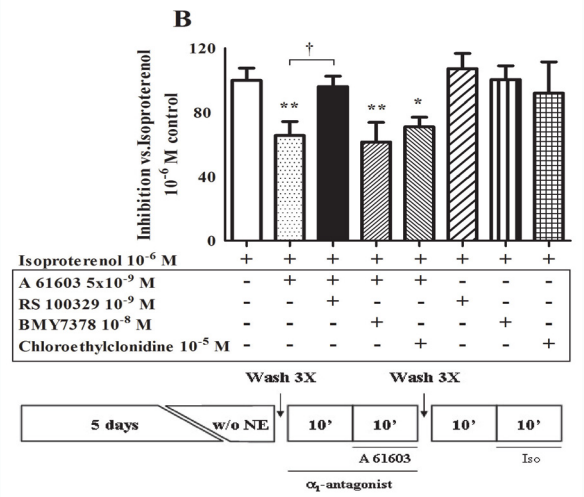

Figure 2B: (b) Effect of selective α1 adrenergic receptor antagonists on A 61603-mediated inhibition of isoproterenol (10-6 M) -induced cAMP accumulation. Cardiomyocytes (106 cells/well) were incubated in the same way as reported for Figure 1 and described in Materials and Methods. Data are expressed as mean ± SEM of at least four independent experiments performed in triplicate and are expressed as a percentage of isoproterenol induced cAMP accumulation. *P<0.05; **P<0.01 vs. isoproterenol stimulated cardiomyocytes; †P<0.05 vs. norepinephrine and metoprolol or A 61603 prestimulated cardiomyocytes (two-tailed unpaired Student t-test).
Abbreviations: NE: Norepinephrine; Met: Metoprolol; Iso: Isoproterenol; w/o: Without

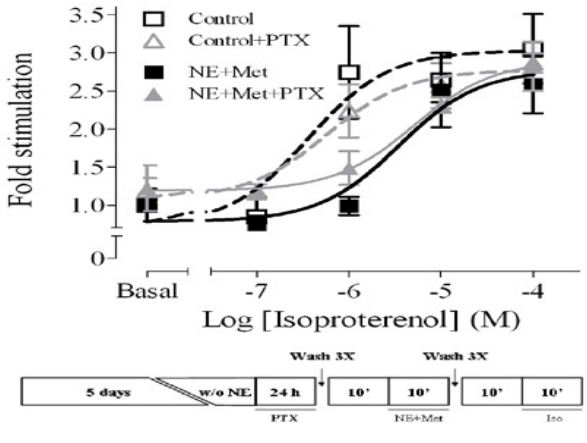

Figure 3: α2-adrenergic receptors or Gi/o proteins do not mediate the observed effects. Concentration-response curves for isoproterenol after short-term (10 min) pretreatment with norepinephrine (NE) and metoprolol (Met) preceded by 24 hr pretreatment with PTX (pertussis toxin) (0.5 µg/ml) vs. basal control in HL-1 cardiomyocytes. Symbols represent mean ± SEM of three independent experiments performed in triplicate and are expressed as a percentage of control basal values (17 ± 3.6 pmol cAMP/mg of cell protein). EC50, Emax and bottom values were: control (3.7 × 10-7 M, 3 fold stimulation and 0.72 respectively); control+PTX (5.8 × 10-7 M, 2.8 fold stimulation and 1.1 respectively); Met+NE (3.5 × 10-6 M, 2.8 fold stimulation and 0.78 respectively); Met+NE+PTX (5.1 × 10-6 M, 2.9 fold stimulation and 1.2 respectively).

the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), 10^{-6} and 10^{-5} M respectively). Both selective CaMKII inhibitors blocked the inhibition pointing to the involvement of this kinase in the α_{1A} adrenergic receptor-mediated inhibitory effect (P<0.05) (Figure 4A). Again, when given alone no kinase inhibitor had any effect on cAMP accumulation.

Complementary experiments were performed with forskolin (10^{-6} and 10^{-5} M) in order to directly activate adenylyl cyclase and to see if norepinephrine-induced cAMP inhibition in the presence of metoprolol was maintained. As shown in Figure 4B, α_{1A} adrenergic receptor activation decreased forskolin increased cAMP accumulation by 34 ± 9% and 46 ± 5% (P<0.05) at the two forskolin concentrations respectively.

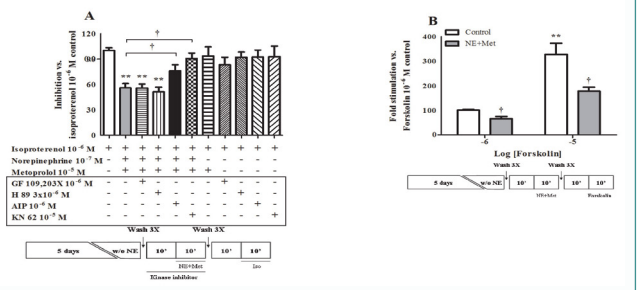

Figure 4: CaMKII mediates the α1A adrenergic receptor inhibition of cAMP accumulation in HL-1 cardiomyocytes. (a) Effect of selective kinase inhibitors on norepinephrine-mediated inhibition (10 min) of cAMP accumulation in the presence of metoprolol (Met). Cardiomyocytes (106 cells/well) were incubated as indicated in Figure 1 and in Materials and Methods. (b) Effect of norepinephrine (NE) and metoprolol (Met) pretreatment (10 min) on forskolin-stimulated cAMP accumulation. Data are expressed as mean ± SEM of at least four independent experiments performed in triplicate and are expressed as a percentage of isoproterenol (10-6 M) (a) or forskolin (10-6 M) (b) induced cAMP accumulation. **P<0.01 vs. isoproterenol (10-6 M) or forskolin (10-6 M) stimulated cardiomyocytes; †P<0.05 vs. norepinephrine and metoprolol (a) or forskolin (b) prestimulated cardiomyocytes (two-tailed unpaired Student t-test).

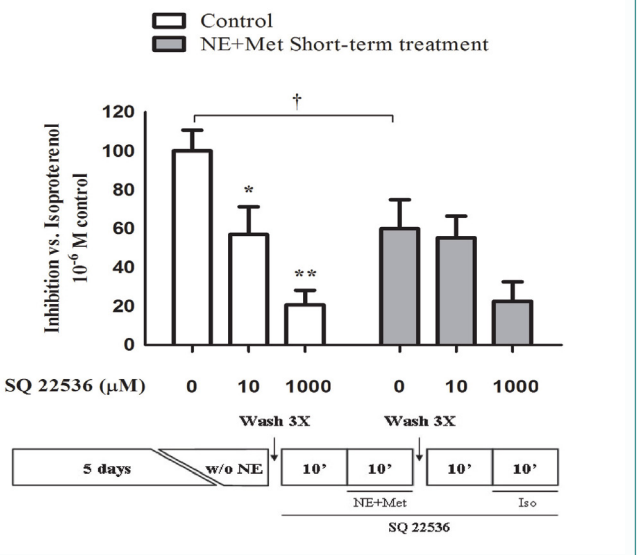

Figure 5: Effect of pretreatment with norepinephrine (NE) and metoprolol (Met) on the inhibition mediated by SQ 22,536 (a selective AC5 inhibitor) of cAMP accumulation induced by isoproterenol (10-6 M) in HL-1 cardiomyocytes. Cardiomyocytes (106 cells/well) were incubated as explained in Figure 1 and in Materials and Methods. Data are expressed as mean ± SEM of at least four independent experiments performed in triplicate and are expressed as a percentage of isoproterenol (10-6 M) induced cAMP accumulation. *P<0.05; **P<0.01 vs. isoproterenol stimulated cardiomyocytes; †P<0.05 vs. untreated control cardiomyocytes (two-tailed unpaired Student t-test).

α_{1A} adrenergic receptor prestimulation mediates selective AC5 inhibition in HL-1 cardiomyocytes

We identified which adenylyl cyclase subtype mediated isoproterenol-induced cAMP accumulation in non-prestimulated cardiomyocytes using the selective inhibitor of AC5, SQ 22,536. When 10 μM SQ 22,536 was employed, a concentration which selectively inhibits AC5 [16,17], the effect of isoproterenol was reduced by 43 ± 14% (P<0.05), whereas with 1 mM SQ 22,536, inhibition reached 82 ± 10% in control cells (P<0.01) (Figure 5). Prestimulation of α_{1A} adrenergic receptors induced a 40 ±15% (P<0.05) inhibition when compared to non-prestimulated cardiomyocytes. 10 μM SQ 22,536 did not enhance this inhibition further (45 ± 11%; N.S. *vs.* 0 μM SQ 22,536) (Figure 5).

Discussion

The results obtained in the present study are indicative of an α_{1A} adrenergic receptor-mediated inhibition of cAMP accumulation induced by the non-selective beta-adrenergic agonist isoproterenol in HL-1 cardiomyocytes. The absence of effect by the β_2 adrenergic receptor antagonist ruled out β_2 adrenergic receptor involvement. In the same way, it is remarkable that none of the tested antagonists, when applied alone, including metoprolol, inhibited cAMP accumulation, indicating the absence of residual antagonism on the isoproterenol effect or of inverse agonism described for metoprolol [20] in our system. Pretreatment of HL-1 cardiomyocytes with PTX did not have any effect on α_{1A} adrenergic receptor-mediated inhibition, ruling out the involvement of α_2-adrenergic receptors or $G_{i/o}$ proteins in this effect. Importantly, results obtained with the α_{1A} adrenergic receptor subtype-selective agonist, A 61603, resembled the inhibition induced by norepinephrine in the presence of metoprolol. Moreover, direct activation of cAMP by forskolin was inhibited to the same extent by norepinephrine in the presence of metoprolol, excluding the participation of β_1 adrenergic receptor desensitization in this inhibitory effect.

Taken together, these results demonstrate that α_{1A} adrenergic receptor signaling regulates the norepinephrine-induced cAMP decrease in HL-1 cardiomyocytes in the presence of metoprolol. In this sense, α_{1A} adrenergic receptor is present in ventricular cardiomyocytes [21]. However, classically, it has been assumed that α_{1B} adrenergic receptor-G_i protein-mediated inhibition of AC activity underlies this effect [9,22]. In agreement with these studies, mice over expressing α_{1B} adrenergic receptor showed an impairment of β adrenergic receptor signaling that was reversed by PTX pretreatment [23]. However, the absence in that study of any effect of PTX pretreatment in control mice points to a putative activation of a PTX-sensitive inhibitory G protein coupling that may have resulted from elevated levels of the α_{1B} adrenergic receptor. Furthermore, it has been reported that α_{1B} adrenergic receptor expression decreases with aging, whereas the overall contribution of the α_{1A} adrenergic receptor is increased in the adult heart [24]. In this sense, the adult model of murine HL-1 cardiomyocytes used in the present study could model more appropriately the conditions present in aged cardiomyocytes, in which α_1 and β_1 adrenergic receptor interactions take place. On the other hand, moving downstream in the signaling cascade, we found that selective CaMKII kinase inhibitors, but

not PKC or PKA inhibitors, reversed the inhibition of AC activity induced by α_{1A} adrenergic receptors, demonstrating the involvement of this CaMKII kinase. The well-characterized CaMKII mediated L-type calcium current [25] could enhance cytoplasmic calcium to reach submicromolar levels, thereby inducing the isoform-specific inhibition of AC5 activity. In this sense, it has been reported that Ca^{2+} entry via L-type channels causes a pronounced inhibition of adenylyl cyclases in chick myocytes [26].

The main AC isoforms expressed in cardiomyocytes are AC5 and AC6 [1-3]. The results obtained in the present study using a concentration of an AC inhibitor, SQ 22,536, reported to selectively inhibit AC5 activity (10 μM) [16,17], uncovered a prominent role of the AC5 isoform in the inhibitory effect of α_{1A} adrenergic receptors on cAMP accumulation. However, a report using membranes of Sf9 insect cells over expressing different recombinant AC isoforms questioned the selectivity of SQ 22,536 in inhibiting AC5 isoform [27]. In this study, the results obtained in 3 experiments using 10 μM of SQ 22,536 showed a non-significant increased inhibition of AC5 with respect AC6 isoform. Similarly, another study [28] using cardiac membranes from mice did not obtain results supporting the selectivity of SQ 22,536 on AC5 activity. However, it is surprising that in that study the maximum levels of enzyme activity did not differ between wild-type and knock-out cardiac membranes for AC5. In this sense, the results obtained by other study [29] could help to clarify these results, since they show that the AC5 is located mainly in the t-tubular region where its influence on I_{CaL} is restricted by phosphodiesterase. The isolation procedure of cardiac membranes and the biological differences between mammals and insects could explain the absence of selectivity of SQ 22,536 found in these reports.

The selective inhibition of AC5 by α_{1A} adrenergic receptors could be of clinical relevance when a selective inhibition of AC5 is required in order to avoid the deleterious effects of this isozyme in cardiac function, since reduction of AC5 activity has been shown to improve myocardial function in mice [5]. Accordingly, α_1 adrenergic receptor agonists have recently been proposed for the treatment of heart failure [8].

In conclusion, norepinephrine pretreatment in the presence of metoprolol *selectively* inhibited an AC5-mediated stimulation of cAMP production by an α_{1A} adrenergic receptor-related mechanism in HL-1 cardiomyocytes. The existence of a selective inhibition of AC5 by the α_{1A} adrenergic receptor could be of relevance to the clinical outcomes obtained after metoprolol treatment of patients with heart failure. This study reveals the potential which these drugs may have for the successful clinical treatment of human heart failure.

Highlights

The α_{1A} adrenergic receptor inhibits cAMP accumulation induced by isoproterenol in HL-1 cardiomyocytes.

The inhibition of cAMP accumulation induced by norepinephrine in the presence of metoprolol is CaMKII dependent.

The α_{1A} adrenergic receptor - CaMKII signaling pathway selectively inhibits AC5 activity.

Funding

This work was supported by the Gobierno Vasco [GV2005111012 to M.A.G.] and the Universidad del País Vasco/Euskal Herriko Unibertsitatea [EHU 07/30 to M.A.G.]. A.L. holds a fellowship from the Gobierno Vasco [BFI-2010-380].

Acknowledgement

The authors would like to thank Dr. Sergio Barrondo and Dr. Javier J. Meana for their valuable suggestions. The authors also wish to thank Bioentelechia Translations for improving the English of this paper.

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