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Role of nitric oxide/cyclic GMP and cyclic AMP in β_3 adrenoceptor-chronotropic response

Leonor Sterin-Borda *, Gustavo Bernabeo, Sabrina Ganzinelli, Lilian Joensen, Enri Borda

Pharmacology Unit, School of Dentistry, Buenos Aires University and Argentine National Research Council (CONICET), M.T. de Alvear 2142 - 4to. "B", 1122 AAH Buenos Aires, Argentina

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Abstract

In this study we determine different signaling pathways involved in β_3 adrenoceptor (β_3-AR) dependent frequency stimulation in isolated rodent atria. Promiscuous coupling between different G-proteins and β_3-AR could explain the multiple functional effects of β_3-AR stimulation. We examine the mechanisms and functional consequences of dual adenylate cyclase and guanylate cyclase pathways coupling to β_3-AR in isolated rodent atria. The β_3-AR selective agonists ZD 7114 and ICI 215001 stimulated in a dose-dependent manner the contraction frequency that significantly correlated with cyclic AMP (cAMP) accumulation. Inhibition of adenylate cyclase shifted the chronotropic effect to the right. On the other hand, the ZD 7114 activity on frequency was enhanced by the inhibition of nitric oxide synthase (NOS) and soluble guanylate cyclase. This countervailing negative chronotropic nitric oxide-cyclic GMP (NO-cGMP) significantly correlated with the increase on NOS activity and cGMP accumulation. Current analysis showed a negative cross talk between cAMP chronotropic and NO-cGMP effects by inhibition of phospholipase C (PLC), calcium/calmodulin (CaM), protein kinase C (PKC), NOS isoforms and Gi-protein on the effects of β_3-AR stimulation. RT-PCR detected both eNOS and nNOS in isolated rat atria. NOS isoforms performed independently. Only nNOS participated in limiting the effect of β_3-AR stimulation. In eNOS-KO (eNOS–/–) mice the chronotropic effect of β_3-AR agonists did not differ from wild type (WT) mice atria, but it was increased by the inhibition of nNOS activity. Our results suggest that the increase in frequency by β_3-AR activation on isolated rodent atria is associated to a parallel increases in cAMP. The nNOS-cGMP pathway negatively modulates β_3-AR activation. Multiple signal transduction pathways between G-protein and β_3-AR may protect myocardium from catecholamine-induced cardiotoxic effects.

Keywords: Heart contractility; β_3 Adrenoceptor; Nitric oxide; Nitric oxide synthase; Phospholipase C; Protein kinase C; Phosphoinositides hydrolysis

1. Introduction

The β_3-adrenoceptors (β_3-ARs) belong to the superfamily of G-protein coupled receptors. They can be found in several tissues including the cardiovascular system [1,2]. Promiscuous coupling between different G-proteins and β_3-ARs, provides a potential mechanisms to explain the different signal transduction pathways [3] that mediate the multifunctional effects of β_3-ARs stimulation [4].

Depending on the tissue, β_3-AR stimulation leads to functional effects that are either opposite or comparable to those produced by β_1 and β_2-ARs stimulation. For instance stimulation of β_3-ARs activates the pertussis toxin (PTX) sensitive Gi/o protein that mediates the decrease of cyclic AMP (cAMP) production [3]. However, other studies have shown that stimulation of β_3-AR activates adenylate cyclase and increases the intracellular cAMP concentration [4].

β_3-AR agonist activation mechanisms in the cardiovascular system are complex [5]. Functional observations using β_3-AR agonist have yielded conflicting results. Response to β_3-AR is different not only among species but also among the anatomical region within the myocardium [5]. Thus, in human and canine ventricles the β_3-AR agonist triggered inotropic negative effect [5] while in human atria preparation, no cardiodepressant effect was described in [6] although human atria [7] and ventricle [8] express this receptor subtype.

In rat atrium the effect of β_3-AR agonists has also shown substantial variability. For example, Kaumann and Molenaar [9] did not find β_3-AR agonist activity, while Cohen et al.

* Corresponding author. Fax: +54 11 4963 2767.
E-mail address: leo@farmaco.odon.uba.ar (L. Sterin-Borda).

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[10] showed tachycardia. Clearly, further research on potential differences between several preferential β_3-AR agonists is required to identify the functional role of β_3-AR in atria.

Moreover, the β_3-AR intracellular signal pathways that operate in cardiac tissue have not clear defined. In general, the elevation of intracellular cyclic GMP (cGMP) after nitric oxide synthase (NOS) activation through Gi/o protein has been associated with cardiac negative inotropic effect triggered by β_3-AR agonist [5].

In previous works, we provided evidence that the endogenous nitric oxide (NO) signaling cascade can modulate both muscarinic cholinergic and β adrenergic stimulation of isolated rat atria [11,12]. The increase of NO-cGMP synthesis contributes to the negative inotropic effect of muscarinic stimulation. On the other hand, it exerts a modulatory negative effect limiting the β adrenergic stimulation of rat atria. However, only few studies have focused on the regulation of spontaneous cardiac activity by β_3-AR. Thus, there is little information on the signal pathways involved in β_3-AR-mediated chronotropic effect on myocardium. Furthermore, the relation between the effect of NO-cGMP levels and the cAMP-coupled signal transduction mechanism that can modulate the β_3-AR chronotropic response has remained uncertain.

In this paper we demonstrate that β_3-AR agonist induces a positive chronotropic effect on isolated rat atria, and we examine the signaling events that participate in the positive chronotropic effect of β_3-AR activation. Concurrent analysis of the simultaneous activation of two independent signaling pathway (cAMP and NO-cGMP) emphasizes that the capacity of atria to respond to β_3-AR stimulation depends on the balance between two opposite second messengers (cAMP and cGMP), at least in concentrations likely to be generated endogenously within the myocardium.

Our data indicate that β_3-AR coupling to $G_i/_O$ stimulates NO-cGMP pathway restraining adenylate cyclase activation, which is, in the end, the molecule that produces the second messenger responsible in triggering the positive chronotropic effect of the agonist.

2. Materials and methods

2.1. Animals

Adult male Wistar strain rats (250–300 g), C57B1/6J wild type (WT) and homozygous for the $NOS3^{tm1Unc}$ targeted mutation (eNOS–/–) mice (3–5 months, Jackson Laboratories) were used. The animals housed in standard environmental conditions were fed with a commercial pelleted diet and water ad libitum. The experimental protocol followed the Guide to The Care and Use of Experimental Animals (DHEW Publication, NIH 80-23).

2.2. Atrial preparation for contractility

Rats and mice were killed by decapitation. The atria were carefully dissected from the ventricles, attached to a glass holder and immersed in a tissue bath containing Krebs–Ringer bicarbonate (KRB) solution gassed with 5% CO_2 in oxygen and maintained at pH 7.4 and 30 °C. KRB solution was composed as described previously in [11]. A preload tension of 750 and 500 mg was applied to the rat and mouse atria, respectively. Tissues were allowed to equilibrate for 1 hour. The frequency of the isolated atria were recorded by use of a force transducer coupled to an ink writing oscillograph. The frequency expressed as beat min^{-1} was evaluated while atria were beating spontaneously. Control values (= 100%) refer to the frequency before the addition of drugs. Cumulative dose response curves were obtained according previously report in [12]. A maximal effect was achieved within 8 min after each dose.

2.3. Measurement of total labeled inositol phosphates (PIs)

Rat atria were incubated for 120 min in 0.5 ml of KRB gassed with 5% CO_2 in O_2 with 1 μCi [myo-^{3}H]-inositol ([^{3}H]-MI) (Sp. Act. 15 Ci $mmol^{-1}$) from Dupont/New England Nuclear. LiCl (10 mM) was added for inositol monophosphate accumulation, according to the technique of Berridge et al. [13]. ZD 7114 was added 30 min before the end of the incubation period and the blockers 30 min before the addition of the agonist. Water-soluble IPs were extracted after 120 min incubation as previously described in [11].

2.4. Determination of NOS activity

NOS activity was measured in atria by production of [U–^{14}C]-citrulline from [U–^{14}C]-arginine according to the procedure described elsewhere [14] for rat atria. Briefly, after 20 min preincubation in KRB solution, atria were transferred to 500 μl of prewarmed KRB equilibrated with 5% CO_2 in O_2 in the presence of [U–^{14}C]-arginine (0.5 μCi). Appropriate concentrations of drugs were added and the atria were incubated for 20 min under 5% CO_2 in O_2 at 37 °C. Measurement of basal NOS activity in whole atria by the above mentioned procedure was inhibited 95% in the presence of 0.5 mM N^G-monomethyl-L-arginine (L-NMMA). The results (pmol g^{-1} tissue wet wt.) obtained for whole atria were expressed as the difference between values in the absence (252 ± 12; $N = 9$) and in the presence (12 ± 2, $N = 9$) of L-NMMA.

2.5. Cyclic GMP (cGMP) a cyclic AMP (cAMP) assay

Tissues were incubated in 1 ml KRB for 30 min and β_3 agonist (ZD 7114) was added in the last 5 min. When blockers were used, they were added 25 min before the addition of ZD 7114. After incubation, atria were homogenized in 2 ml of absolute ethanol and centrifuged at $6000 \times g$ for 15 min at 4 °C. Pellets were then rehomogenized in ethanol/water (2:1) and supernatants collected and evaporated to dryness as indicated above. Cyclic AMP or cyclic GMP in the residue was dissolved in 400 μl of 0.05 M sodium acetate buffer pH 6.2. Aliquots of 100 μl were taken for the nucleotide determination

using RIA procedure with a cyclic AMP^{3}H or cyclic GMP^{125}I - RIA KITS from Dupont New England Nuclear [14].

2.6. Protein kinase C (PKC) assay

PKC activity was assayed by measuring the incorporation of ^{32}P from gamma-^{32}P-ATP into histone H_1. Incubations were conducted for 30 min at 30 °C in a final volume of 85 μl. In final concentrations, the assay mixture contained 25 μM ATP (0.4 μCi), 10 mM Mg acetate, 5 mM β-mercaptoethanol, 50 μg of histone H_1, 20 mM HEPES pH 7.5 and unless otherwise indicated, 0.2 mM $CaCl_2$ and 10 μg ml^{-1} of phosphatidylserine vesicles. The incorporation of (^{32}P) phosphate into histone was linear for at least 20 min. The reaction was stopped by the addition of 2 ml of ice-cold 5% trichloroacetic acid, 10 mM H_3PO_4. The radioactivity restrained on GF/C glass-fiber filters after filtration was determined by counting the filters in 2 ml of scintillation fluid. PKC activity was determined after subtracting the incorporation in the absence of calcium and phospholipids. The data were expressed in picomol of phosphate into the substrate per minute and per milligram of protein (pmol min^{-1} mg $^{-1}$ prot).

2.7. mRNA isolation and cDNA synthesis

Procedures were based on our earlier study [15]. Total RNA was extracted from rat atria and right ventricle slices by homogenization using guanidinium isothiocyanate method [16]. A 20 μl reaction mixture contained 2 ng of mRNA, 20 units of RNase inhibitor, 1 mM dNTPs and 50 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). First strand cDNA was synthesized at 37 °C for 60 min. In a selected tube, the reverse transcriptase was omitted to control for amplification from contaminating cDNA or genomic DNA.

2.8. Quantitative PCR

Quantitation of NOS and mRNA levels was performed by a method that involves simultaneous coamplification of both the target cDNA and a reference template (MIMIC) with a single set of primers [15]. MIMIC for nNOS and eNOS and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were constructed using a PCR MIMIC construction kit (Clontech Laboratories, Palo Alto, CA). The sequence of oligonucleotide primer pairs used for construction of MIMIC and amplification of nNOS and eNOS and G3PDH mRNA is listed in Table 1. Aliquots were taken from pooled first strand cDNA from the same group and constituted one sample for PCR. PCR MIMIC amplification was performed as previously describe in [15]. The internal control was the mRNA of the housekeeping gene G3PDH. PCR products were subjected to electrophoresis on ethidium bromide-stained gels. Band intensity was quantitated by densitometry using NIH Image software. Levels of mRNA were calculated from the point of equal density of the sample and MIMIC PCR products [15]. nNOS and eNOS mRNA levels were normalized with the levels of G3PDH mRNA present in each sample, which served to control for variations in RNA purification and cDNA synthesis.

2.9. Drugs

ZD 7114 hydrochloride, ICI 215001, SR 59230A hydrochloride, 1H-[1,2,4]-oxadiazola-[4,3-2]-quinoxaline-1-one (ODQ), L-NIO and *N*-propyl-L-arginine were purchased from Tocris Cookson Inc.; L-arginine, L-NMMA, PTX, trifluoperazine (TFP) and calphostin C from Sigma Chemical Company; 1-6-17β-3-methoxgestra-1,3,5 (10)-trien-17yl-aminohexyl-1-H-pirrole-2,5-dione (U-73122) and SQ 22536 from ICN Pharmaceuticals Inc. Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentration stated in the text.

2.10. Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P < 0.05$.

3. Results

As can be seen in Fig. 1A, the β_3-AR agonist-induced a concentration-dependent positive chronotropic effect at concentrations ranging from 1×10^{-9} to 1×10^{-5} M. This positive chronotropism was accompanied by an increase of endogenous cAMP production (Fig. 1B). The selective β_3-AR antagonist SR 59230A and the inhibitor of adenylate cyclase SQ 22536 could antagonize both the cAMP accumulation and the positive chronotropism, shifting the dose–response curves of ZD 7114 to the right (Fig. 1A, B). These results indicate that chronotropy and cAMP are direct effects triggered by the same receptor subtype.

The relative inhibitory strength of SR 53230A on the β_3-AR agonist-induced chronotropy was determined by increasing concentrations of the antagonist. The pA_2 value was 7.2 ± 0.20 (Fig. 2). Under these conditions neither atenolol

Table 1
Oligonucleotides of primers for PCR

Gene product	Sense	Antisense	Predicted size (bp)	Accession number
nNOS	5′ GCGGA GCAGA GCGGC CTTAT 3′	5′ TTTGGT GGGAG GACCG AGGG 3′	240	U 67309.1
eNOS	CGA GAT ATC TTC AGT CCC AAG C	GTG GAT TTG CTG CTC TAT AGG	164	U 0 2534.1
G3PDH	5′ ACCAC AGTCCA TGCCAT CAC 3′	5′ TCCAC CACCC TGTTG CTGTA 3′	452	XM 228169.1

Neuronal (nNOS) and endothelial (eNOS) NOS [32] and G3PDH [33].

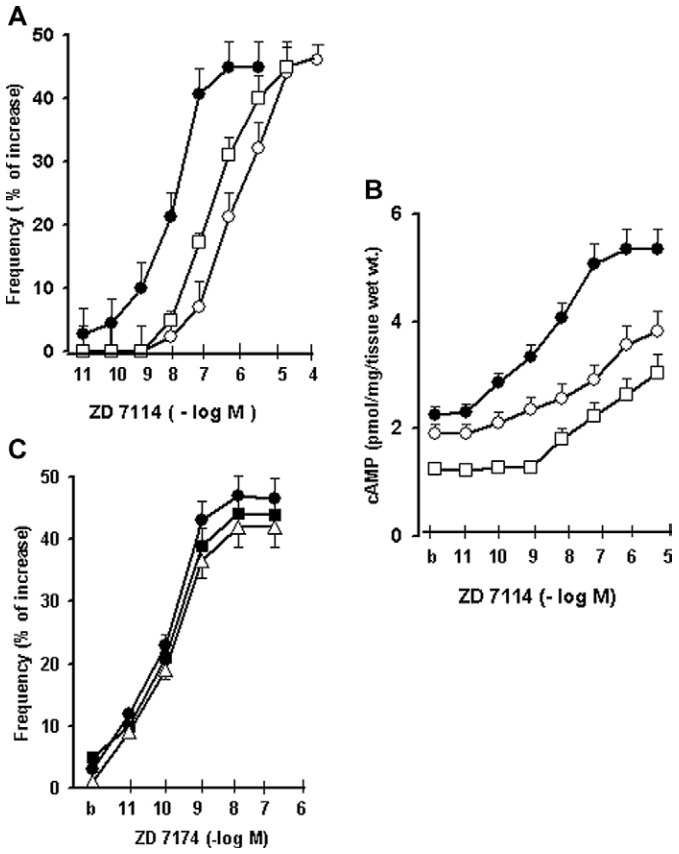

Fig. 1. A and C: Increase in frequency. B: Increase in cAMP of rat atria by increasing concentrations of ZD 7114 (●). The inhibitory action of SR 59230A (1×10^{-7} M) (○), SQ 22536 (5×10^{-6} M) (■), butoxamine (5×10^{-7} M) (□), and atenolol (5×10^{-7} M) (△) are also shown. Basal frequency was 145 ± 12. Values represent the mean ± S.E.M. of seven different determinations in each group. Cyclic AMP assays were performed by duplicates. Tissues were incubated for 30 min in presence or absence of different blockers and then ZD 7114 was added. Values are expressed as percentage of changes calculated by comparison with the absolute values prior to the addition of ZD 7114. No effects were observed with SR 59230A or SQ 22536 at the concentration used.

nor butoxamine modified the EC_{50} of the agonist (EC_{50}: ZD 7114, $1.42 \pm 0.18 \times 10^{-8}$ M; ZD 7114 + atenolol $1.53 \pm 0.20 \times 10^{-8}$ M; ZD 7114 + butoxamine, $1.53 \pm 0.20 \times 10^{-8}$ M) (Fig. 1C). Another β_3-AR agonist ICI 215001 triggered both positive chronotropy and increased cAMP production. Half maximal effect (EC_{50}) was reached at $3.6 \pm 0.14 \times 10^{-8}$ M,

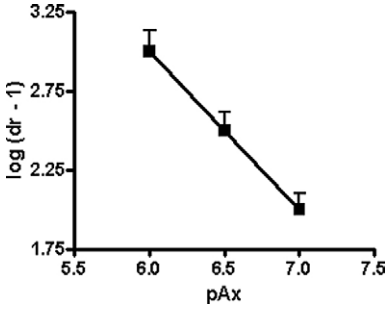

Fig. 2. Schild plots of SR 59230A antogonism of ZD 7114 mediated positive chronotropic effect on rat isolated atria. Logs of dose ratios –1 are plotted as a function of the pAx of the antagonist. Results are shown in one of four experiments with similar results.

and the maximal stimulation was 43.2 ± 3%; $N = 5$ for frequency and 96.2 ± 7%; $N = 5$ for cAMP production.

To determine if the endogenous NO participates in the chronotropic effect of ZD 7114, isolated rat atria were incubated with particular NOS isoform inhibitors. As can be seen in Fig. 3A the inhibition of NOS or nNOS activities by L-NMMA (2×10^{-6} M) or *N*-propyl-L-arginine (5×10^{-6} M), respectively, enhanced the stimulatory action of ZD 7114 on frequency. These effects were higher at low agonist concentrations. In contrast, the inhibition of eNOS by L-NIO (5×10^{-6} M) had no effect. As control, L-arginine (2×10^{-5} M) reversed the L-NMMA effect (data non shown). Fig. 3B also shows that ZD 7114 at low concentrations increased NOS activity above basal values. This activity was significantly higher at low concentrations and decreased thereafter. Similarly, ICI 215001 increased the NOS activity with maximal effect 158 ± 27%; $n = 5$ at 3×10^{-10} M. Moreover the NOS activity triggered by ZD 7114 was prevented by *N*-propyl-L-arginine (5×10^{-6} M) but not by L-NIO (5×10^{-6} M) It is noteworthy that both eNOS and nNOS inhibitors, were used at 5×10^{-6} M. At this concentration they inhibited about 68% of basal NOS activity, but did not modify the basal frequency of contraction. Contrasting sharply, the nNOS inhibitor was able to modify the ZD 7114 effects.

Fig. 4 demonstrates that under identical experimental conditions a significant correlation ($\alpha = 0.05$) between ZD 7114 stimulated chronotropy and modulation of cAMP and NOS was found (Pearson *r*: cAMP: 0.9908; NOS: –0.9114). These results indicate that β_3-AR activation-induce increase in frequency of contraction as a result of the increase in cAMP accumulation and NOS activity inhibition.

To investigate the possibility that NOS activation through β_3-AR-stimulation could increase cGMP production, the effect of a selective inhibitor of NO-sensitive guanylate cyclase ODQ (5×10^{-5} M) was explored. Fig. 5A shows that ODQ enhanced the chronotropic effect of ZD 7114. Moreover, the effect of β_3-AR agonist on frequency was studied in parallel regarding its

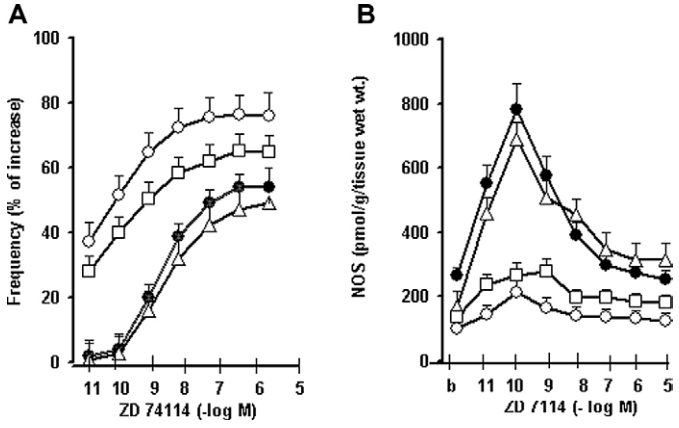

Fig. 3. Effects of increasing concentration of ZD 7114 on frequency (A) and NOS activity (B) on rat isolated atria. The effects of ZD 7114 alone (●) or in the presence of 2×10^{-6} M L-NMMA (□) or 5×10^{-6} *N*-propyl-L-arginine (○) or 5×10^{-6} M L-NIO (△) are shown. Basal frequency was: 142 ± 10. Values represent the mean ± S.E.M. of six different determinations in each group. NOS activity assays were performed by duplicates. For other details see legend of Fig. 1.

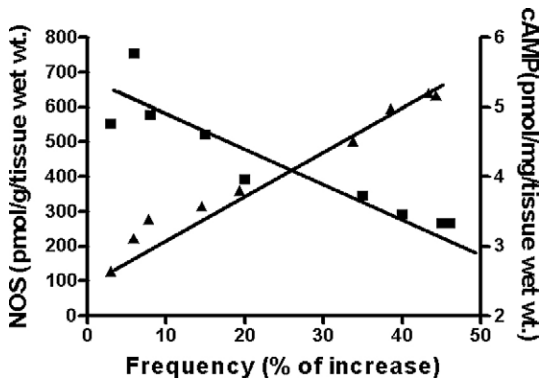

Fig. 4. Correlation in the modulatory effect of ZD 7114 from 1×10^{-11} to 1×10^{-5} M on frequency, cAMP and NOS activity. Frequency was plotted as a function of either NOS (■) ($P < 0.0043$) or cAMP (▲) ($P < 0.0001$). Values are mean of six experiments of each group. For other details see legend of Fig. 1.

ability to increase cGMP production. Fig. 5B shows that ZD 7114 increased the production of cGMP in a concentration dependent manner, reaching maximal effect at 1×10^{-10} M and decreasing thereafter. The specific β_3-AR blocker SR 592302A, prevented the increment of cGMP production

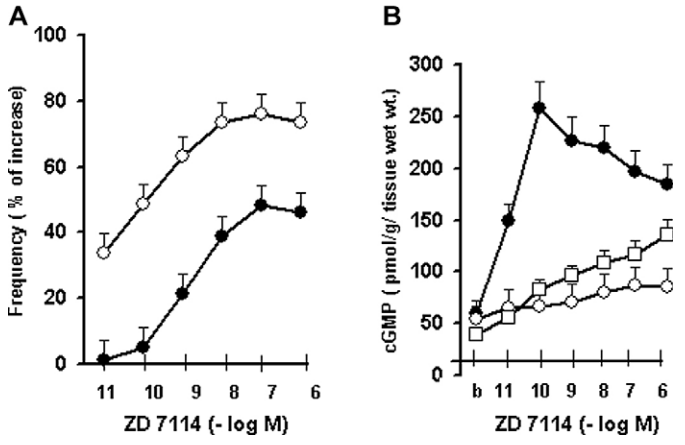

Fig. 5. Effect of 5×10^{-5} M ODQ (○) and SR 59230A (1×10^{-7} M) (□) on the dose–response curve of ZD 7114 (●) upon frequency (A) and cGMP accumulation (B). Basal frequency was: 148 ± 11. Values are mean ± S.E.M. of five different experiments in each group. Enzymatic activity was performed by duplicate. For other details see legend of Fig. 1.

(Table 2); as also did the NO-sensitive guanylate cyclase inhibitor (Fig. 5B). The increase in cGMP (+289 ± 27%; $N = 5$) was also observed when using ICI215001 with maximal effect at 3×10^{-10} M concentration.

To verify the nature of the mechanism by which the activation of β_3-AR increase cGMP synthesis and NOS activity, rat atria were incubated with several inhibitors of the enzymatic pathways involved in the receptor dependent activation of NO and cGMP synthesis. Fig. 6 shows that either 5×10^{-6} M U 73319 (PLC inhibitor) (A), 5×10^{-9} M calphostin C (PKC inhibitor) (A) or 5×10^{-6} M TFP (calcium calmodulin inhibitor) (C) enhanced the chronotropic effect of ZD 7114. Additional studies were performed in order to assess if β_3-AR is coupled to PI turnover and activates PKC in rat atria. As can be seen in Fig. 6, β_3-AR activation translocated PKC (B) and increased PI turnover (D) in a concentration-dependent manner, being the maximal effect at 1×10^{-10} M and decreasing thereafter. The ZD 7114 enzymatic effects were abolished by 1×10^{-7} M SR 59230A, indicating the β_3-AR specificity. The same Fig. 6 shows that 5×10^{-6} M U 73319 and 5×10^{-9} M calphostin C prevented the stimulation of PI turnover and PKC translocation induced by β_3-AR activation. No inhibitory agents at the concentrations used had any effect upon basal values of frequency as previously reported in [14].

In order to determine whether the enzyme activities are dependent on each other, atria were incubated with different enzymatic pathways inhibitors involved in β_3-AR activation. Table 2 shows that the inhibition of PLC by U-73122 (5×10^{-6} M), CaM by TFP (5×10^{-6} M), PKC by calphostin C (5×10^{-9} M), nNOS by *N*-propyl-L-arginine (5×10^{-6} M), and guanylate cyclase by ODQ (5×10^{-5} M) prevented the stimulatory action of the agonist on NOS activity and cGMP production, while they increased the production of cAMP. Atria pretreated with PTX ($1–3 \times 10^{-6}$ M) exhibited decrease in NOS activity and cGMP accumulation with a strong increase in cAMP. As control, the action of the specific β_3-AR blocker SR 59230A (5×10^{-7} M) on NOS, cGMP and cAMP is demonstrated.

Using specific oligonucleotide primers, RT-PCR amplified products showed bands of the predicted size for eNOS and

Table 2
Influence of inhibitors on ZD 7114 effects on NOS activity and cyclic nucleotides accumulation

Drugs	NOS (pmol g^{-1} per tissue wet wt.)	Cyclic GMP (pmol g^{-1} per tissue wet wt.)	Cyclic AMP (pmol mg^{-1} per tissue wet wt.)
None	265 ± 13	59 ± 4	2.25 ± 0.18
ZD 7114	752 ± 19*	251 ± 11*	2.87 ± 0.20*
ZD 7114 + U-73122	272 ± 15	87 ± 6	4.98 ± 0.28**
ZD7114 + calphostin C	298 ± 16	51 ± 5	5.92 ± 0.25**
ZD 7114 + TFP	350 ± 18	81 ± 3	4.69 ± 0.27**
ZD 7114 + N-pa	210 ± 15	52 ± 5	4.52 ± 0.22**
ZD 7114 + ODQ	710 ± 25*	18 ± 2	5.36 ± 0.27**
ZD 7114 + SR 59230A	258 ± 12	62 ± 4	2.18 ± 0.20
ZD 7114 + PTX	296 ± 20	82 ± 4	6.85 ± 0.35**

Values are mean ± S.E.M. of five experiments in each group performed in duplicate. Enzyme activities were measured after incubation for 30 min atria with or without enzymatic inhibitors and with additional 10 min with ZD 7114. The following concentrations were used: 1×10^{-10} M ZD 7114, 5×10^{-6} M U-73122, 5×10^{-9} M calphostin C, 5×10^{-6} M TFP, 5×10^{-6} M *N*-propyl-L-arginine (*N*-pa), 5×10^{-5} M ODQ, 5×10^{-7} M SR 59230A and *pertussis* toxin (PTX) 1- 3×10^{-6} M. Values are mean ± S.E.M. of five experiments performed by duplicated. *$P < 0.001$ comparing with basal values (none); **$P < 0.001$ comparing with ZD 7114 alone.

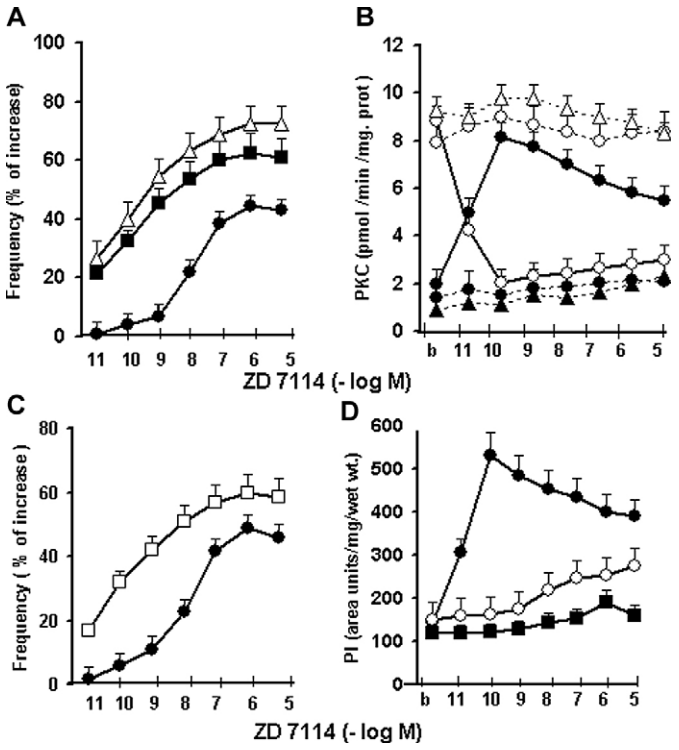

Fig. 6. Dose–response curves of ZD 7114 alone (●) or in the presence of: (A) 5×10^{-6} M U-73122 (■) and 5×10^{-9} M calphostin C (△) or (C) 5×10^{-6} M TFP (□) on frequency. (A) The translocation of PKC (B) from cytosol (●) to membrane (○); alone (full line) or in the presence of SR 59230A (dotted line). The action of 5×10^{-9} M calphostin C on cytosolic PKC (▲) or membrane PKC (△) is also shown. (D) Stimulation of PI turnover by ZD 7114 alone (●) or in the presence of SR 59230A (○) or 5×10^{-6} M U-73122 (■). Values are mean ± S.E.M. of six different experiments in each group. Enzymatic activities were performed by duplicate. For other details see legend of Fig. 1.

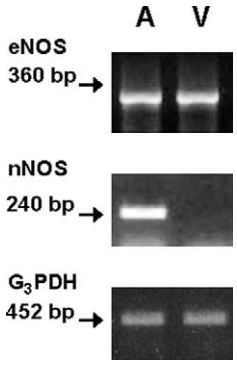

Fig. 7. RT-PCR product for eNOS (upper panel) and nNOS (lower panel) obtained from rat atria (A) and ventricle (V). Tissues were incubates for 1 hour in KRB on identical experimental condition that was described for contractile and enzymatic assays, and then mRNA isolation and DNA synthesis were extracted and determined as described in Section 2.

nNOS detected in atria. On the other hand, the ventricle showed expression of constitutive eNOS but no nNOS (Fig. 7).

To settle the role of nNOS in the regulation of atria β_3-AR activation, we used genetically engineered mice deficient in eNOS (eNOS–/– mice). Fig. 8 shows that there was no difference in the agonists stimulatory action in atria from eNOS–/– mice or atria from WT mice. ZD-7114 and ICI-215001 increased the spontaneously beating atria in a concentration dependent manner, with maximal stimulation at about 40%. The EC_{50} was reached at 1.2×10^{-8} M. Moreover, the nNOS inhibitor *N*-propyl-L-arginine (5×10^{-6} M) enhanced the stimulatory action of the agonists in both eNOS–/– and WT mice. There was no significant difference in basal frequency of atria from both groups of mice (see legend of Fig. 8). To give evidence that the effect is associated with nNOS, Fig. 8C, D, shows that atria β_3-AR activation increased NOS activity above basal values in both eNOS–/– and WT mice. This activity was significantly higher at low concentrations and decreased thereafter. Moreover the NOS activity stimulated by ZD 7114 was prevented by *N*-propyl-L-arginine (5×10^{-6} M) but not by L-NIO (5×10^{-6}); indicating the specificity of the reaction.

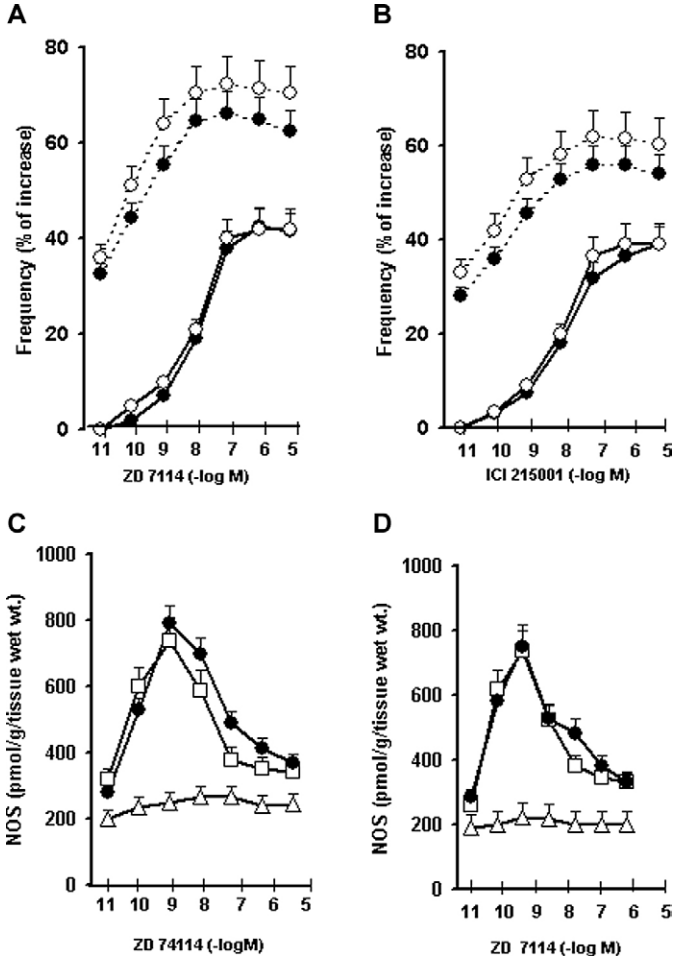

Fig. 8. Chronotropic effects triggered by ZD 7114 (A) and ICI 215001 (B) on WT mice (●) or eNOS–/– mice (○); in absence (full line) or in presence of 5×10^{-6} M *N*-propyl-L-arginine (dotted line). Mice atria frequency is expressed in % of basal values before addition of the first concentration of the agonists taken as 100%. Basal values are: 332 ± 18, WT mice ($n = 7$); and 310 ± 15, eNOS–/– mice ($N = 5$). In the presence of *N*-propyil-L-arginine are: 340 ± 12 WT mice ($N = 7$) and 328 ± 9 eNOS–/– mice ($n = 5$). NOS activity triggered by ZD 7114 alone (●) or in the presence of 5×10^{-6} M *N*-propyl-L-arginine (△) or in the presence of 5×10^{-6} M L-NIO (□) on WT mice (C) or on eNOS–/– mice (D). Points indicate the mean ± S.E.M.

4. Discussion

The current study provides pharmacological evidence for the existence of functional β_3-AR in isolated rodent atria and

indicates that both cAMP and NO-cGMP production are involved in the β_3-AR mediated positive chronotropic effect of the agonists. Our data give an insight into the mechanisms related to NO and cGMP mediated effect.

Results suggest the existence of a countervailing negative chronotropic NO and cGMP-mediated mechanism that limit the effect of β_3-AR stimulation through adenylate cyclase activation in isolated rat atria. The results indicate that PLC, PKC and NOS activation modulate intracellular cGMP and cAMP levels.

Two lines of evidence support these conclusions. On one hand, the concentration response curves of ZD 7114 acting on β_3-AR shifted to the right when adenylate cyclase activity was inhibited. On the other hand, the ZD 7114 action on frequency was enhanced when NOS or soluble guanylate cyclase were inhibited. Both these effects were receptor-mediated actions demonstrated by virtue of blockade by the selective β_3-AR antagonist.

The mechanism by which the agonist stimulation restrain the positive chronotropy seems to involve an increase of PI hydrolysis which intermediates would turn on calcium/calmodulin and PKC. Two lines of evidence support this statement. On the first hand, we showed that ZD 7114 chronotropic effect was enhanced by PLC, calcium mobilization or PKC inhibition. On the other hand, the activation of β_3-AR led to a parallel increase of PI accumulation, PKC translocation, NOS activity and cGMP accumulation.

The efficiency that each intracellular signal display at different agonist concentrations appear to be a pivotal factor in the differential regulatory activity on both the positive and the restraining effect of the chronotropic response. Thus, here we observed that at low concentrations the scarce ZD 7114 chronotropic effect correlated to low cAMP accumulation and high NOS activity. On the other hand, at high concentrations the efficient chronotropic effect triggered by the agonist correlated with high cAMP accumulation and low NOS activity. The contractile inverse effects at higher NO exposure have been described in sepsis [17]. In that case, large concentration of NO was proposed as the cause of cardiac function depression through cAMP and protein kinase G mediated desensitization of cardiac myofilaments.

The effects of NO endogenously produced by other regulators of myocardial function, has been demonstrated when using other agonists on isolated rat atria. Thus, direct stimulation of A_1 adenosine receptors [18], M_2 muscarinic acetylcholine receptors [11] and cannabinoid CB_1 receptor [14] decreased contractility associated with PI accumulation, stimulation of NOS and increased production of cGMP. However, the effect of these agonists differ from the β_3-AR agonist, since the later is the only one, among them that stimulates adenylate cyclase activity triggering positive chronotropic effect. In this way, overlapping effects between both signaling systems (NOS-cGMP and cAMP) occurs only when β_3-AR is activated. In fact, in this work we show that the inhibition of PLC, CaM, PKC, NOS and soluble guanylate cyclase increase the production of cAMP induced by β_3-AR stimulation, suggesting a negative cross talk between the cyclic nucleotides. Furthermore, the results obtained when using PTX suggest that in rat atria β_3-AR is constitutively coupled to both G_s and G_i, since inhibition of G_i function leads to increased cAMP production and decreased NOS-cGMP. The present results agree with others described in smooth muscle [19] and in adipocytes [3] in which the β_3-AR is constitutively coupled to more than one G-protein. In adipocytes, G_i activation resulted in the attenuation of β_3-AR-mediated stimulation of adenylate cyclase and served to initiate additional signal transduction pathways, as well [3]. Similarly, PTX treatment abolished the β_3-AR stimulation on NO-dependent cGMP increase in human ventricular biopsies [27]. Thus, our data suggest that the promiscuous coupling of the β_3-AR in atria permits the simultaneous transduction of two independent signaling pathways: cAMP elevation and NOS-cGMP elevation. The first one is related to an increasing atrium rate, while the last one is related to the restrain of cAMP mediated positive chronotropism.

Various experimental models have shown that the inhibition of the NO pathway augmented the stimulatory effects of catecholamines on the heart [12,20] and conversely, the activation of NO pathway attenuated them [11,21,22]. In addition, the intracellular increase in cGMP induced by NO decreased the frequency on spontaneous beating cardiac myocytes [20,23].

But, the effects of NOS isoform on pacemaker cells are more difficult to dissect. Analysis of mice phenotype genetically deficient in one or several isoforms of NOS showed that basal heart rate is unchanged in most eNOS–/–mice but some nNOS–/– mice exhibited increase in frequency [24]. When using nNOS–/– myocytes or after acute preferential nNOS inhibition in WT myocytes [25,26], evidence was provided that cardiomyocytes nNOS inhibited calcium influx. Here we show that the basal frequency of isolated atria preparation from eNOS–/– mice did not differ from that of WT mice. This suggests that the eNOS is not obligatory for control of atria frequency. These eNOS–/– mice showed neither alteration in G_i/G_o proteins nor up-regulation of other NOS isoforms [26]. Moreover, the fact that action of β_3-AR agonist on atria from eNOS–/– mice was increased by the inhibition of nNOS activity, supports the independent role of each NOS isoform and suggests that nNOS mediates the mechanism that limits the β_3-AR chronotropic response.

Accordingly, in this paper, although both eNOS and nNOS enzymes are constitutively expressed in rat atria, the β_3-AR activity was affected only by nNOS. On the other hand, in ventricle the nNOS appears not to be expressed constitutively. The lack of nNOS in ventricle probably is accountable for the discrepancy between β_3-AR stimulation in atria and ventricle. In previous studies [27] it has been suggested that the eNOS isoform was involved in the negative inotropic effect of β_3-AR stimulation on human ventricle. Similarly, mice over-expressing large amounts of eNOS exhibited depression in ventricle function [28]. In nNOS–/– ventricle myocytes, β_3-AR agonist effect was unchanged while it was absent in eNOS–/– myo-

cytes. This supports the linkage of eNOS with the ventricular β_3-AR signaling mechanism, discarding the relation to nNOS [24].

Strikingly, calphostin C known to inhibit PKC activity was able to inhibit the NOS-cGMP signaling pathway triggered by β_3-AR activation. This suggests that PKC is an important activator of nNOS in atria. The requirement of CaM and PKC activation observed for the β_3-AR agonist-induced stimulation of the NO-cGMP mediated pathway in rat atrium, revealed in this paper, is consistent with reports that show CaM kinase and PKC-mediated phosphorylation and regulation of NOS [29,30].

Unlike the β_1 and β_2 adrenergic receptor subtypes, β_3-AR lack regulatory phosphorylation sites for G-protein receptor kinases [31] and could be relatively resistant to agonist-induced desensitization. Further research will be needed to identify the functional consequences of the alterations in the balance between the opposing inotropic influence of the β_3-AR subtypes in cardioneuromyopathy.

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