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RESEARCH PAPER

Cross-regulation between β_1- and β_3-adrenoceptors following chronic β-adrenergic stimulation in neonatal rat cardiomyocytes

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Background and purpose: We have previously shown that β-adrenoceptors continuously stimulated with noradrenaline induces an increase in β_3-adrenoceptors ($G\alpha_i$PCRs) and a decrease in β_1-adrenoceptors ($G\alpha_s$PCRs) at functional, genomic and protein levels. This compensatory modification induced by noradrenaline is probably one of the consequences of cardiac depression observed in heart disease. Therefore, we investigated further the interaction between β_1- and β_3-adrenoceptors in neonatal rat cardiomyocytes.
Experimental approach: Functional studies were performed by cyclic adenosine monophosphate (cAMP) accumulation assays in cells untreated or treated with dobutamine and ICI 118551 (β_1-adrenoceptor) or CL-3162436243 (β_3-adrenoceptor) for 24 h in the presence or absence of protein kinase inhibitors. β-adrenoceptor and protein kinase expression was monitored by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and by Western blotting, respectively.
Key results: Chronic β_1- or β_3-adrenoceptor stimulation reduced β_1-adrenoceptor-mediated cAMP accumulation in association with a decrease in β_1-adrenoceptor mRNA and protein levels through protein kinase C (PKC), phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (p38MAPK) activation. In contrast, both treatments induced an increase in β_3-adrenoceptor expression and β_3-adrenoceptor-inhibited forskolin response through PKC, extracellular-signal-regulated kinases 1 and 2 (ERK1/2) and p38MAPK phosphorylation, although no β_3-adrenoceptor response was observed in untreated cells. ERK1/2 and p38MAPK were activated by both treatments. The modulation of β_1- or β_3-adrenoceptor function did not require stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) although chronic β_1-adrenoceptor stimulation activated SAPK/JNK. β_3-adrenoceptor treatment activated Akt although PI3K was not involved in β_3-adrenoceptor up-regulation.
Conclusion and implications: We show for the first time that chronic β_1- or β_3-adrenoceptor stimulation leads to the modulation of β_1- and β_3-adrenoceptors by a cross-regulation involving PKC, PI3K p38MAPK and MEK/ERK1/2 pathway, and through protein kinase A when β_1-adrenoceptors are chronically activated.
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Keywords: cardiomyocytes; heart; β_1-adrenoceptor; β_2-adrenoceptor; β_3-adrenoceptors; cAMP accumulation; up-regulation; down-regulation; MAP kinases; Akt

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular responsive kinase; GPCR, G-protein coupled receptor; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; p38MAPK, p38 mitogen-activated protein kinase; PTX, *Pertussis* toxin; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase

Introduction

Three β-adrenoceptors (β_1, β_2 and β_3-adrenoceptors; nomenclature follows Alexander *et al.*, 2008) are expressed in heart (Dzimiri, 1999; Rozec and Gauthier, 2006). These receptor

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subtypes belong to the G-protein coupled receptor (GPCR) superfamily and modulate cardiac function after stimulation by the catecholamines, noradrenaline and adrenaline (Dzimiri, 1999). Increases in heart rate and force of contraction are mediated mainly by β_1-adrenoceptors and to a lesser extent by β_2-adrenoceptors through a coupling to the G_s protein pathway (Dzimiri, 1999). In contrast, the β_3-adrenoceptor subtype coupled to G_i protein decreases cardiac contractility (Dzimiri, 1999; Rozec and Gauthier, 2006). It is well known that heart failure induces an increase in circulating cathecholamines, which leads to an alteration of cardiac function through a decrease in β_1-adrenoceptor density (Cohn *et al*., 1984; Dzimiri, 1999; Tilley and Rockman, 2006). In contrast, β_3-adrenoceptors were found up-regulated in failing human and animal hearts (Cheng *et al*, 2001; Moniotte *et al*., 2001; Morimoto *et al*., 2004; Zhang *et al*., 2005). Similarly, β_1-adrenoceptor expression was decreased and β_3-adrenoceptor level increased both in hearts from diabetic and from physically trained rats (Dinçer *et al*., 2001; Barbier *et al*., 2007). It is noteworthy that catecholamines, particularly noradrenaline, increase during physical exercise, and likewise during heart failure (Pagliari and Peyrin, 1995; Dzimiri, 1999; Tilley and Rockman, 2006). We have shown previously that chronic stimulation of cardiomyocytes with noradrenaline decreases β_1-adrenoceptor function and expression, and enhances β_3-adrenoceptor function (Germack and Dickenson, 2006). Interestingly, Thomas *et al* (1992) have reported a similar opposite regulation in adipocytes chronically stimulated with isoprenaline. In addition, β_1-adrenoceptor expression was lower in transgenic mice specifically overexpressing human β_3-adrenoceptor in the heart (Kohout *et al*., 2001) whereas β_3-adrenoceptor knockout mice exhibited an increase in β_1-adrenoceptor mRNA in adipose tissues (Susulic *et al*., 1995).

It is well known that β_1 and β_2-adrenoceptors are subject to desensitization, internalization and down-regulation following chronic exposure to agonists leading to a decrease in β-adrenoceptor responsiveness, whereas β_3-adrenoceptors are resistant to desensitization (Dzimiri, 1999; Rozec and Gauthier, 2006). Indeed, the β_1 and β_2-adrenoceptors contain consensus sites mainly in the C-terminal region that can be phosphorylated by G-protein receptor kinases (GRKs), mainly GRK2, and cAMP protein kinase A (PKA) that are involved in desensitization of the receptors. In contrast, the β_3-adrenoceptor subtype lacks phosphorylation sites preventing its desensitization by this mechanism (Dzimiri, 1999; Tilley and Rockman, 2006). β-adrenoceptor internalization requires the recruitment of β-arrestins and phosphoinositide 3-kinase (PI3K), which bind to phosphorylated β-adrenoceptors (Dzimiri, 1999; Naga Prasad *et al*., 2001; Nienaber *et al*., 2003). The down-regulation of GPCRs following persistent stimulation also involves transcriptional and post-transcriptional mechanisms leading to a decrease in mRNA and protein synthesis (Dzimiri, 1999). Headley *et al*. (2004) demonstrated that extracellular signal-regulated kinase (ERK1/2), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 mitogen-activated protein kinase (p38MAPK) pathways can modulate β_1-adrenoceptor mRNA stability. Finally, Li *et al*. (1998) showed that protein kinase C (PKC) induced the down-regulation of β_1-adrenoceptors at protein and mRNA levels in rat C6 glioma cells. Similarly, isoproterenol-induced adenylyl cyclase activity was impaired in human embryonic kidney (HEK) 293 cells overexpressing constitutively active PKC isoforms (α, βII, ε and ζ) and transfected with β_1- or β_2-adrenoceptors (Guimond *et al*., 2005).

Overall, these observations suggest that compensatory interactions between β_1- and β_3-adrenoceptors take place during chronic stimulation with the nonselective agonists, noradrenaline or isoprenaline. In addition, β_1-adrenoceptor desensitization and down-regulation involve PI3K and protein kinases whereas the implication of these kinases in β_3-adrenoceptor up-regulation is unknown. Therefore, we investigated the possible interaction between β_3-adrenoceptor up-regulation and β_1-adrenoceptor down-regulation at the functional and expression levels, and identified kinases implicated in the functional receptor modulation following chronic β_1- or β_3-adrenoceptor selective stimulation in neonatal rat cardiomyocytes.

In this study, we demonstrate for the first time that a cross-regulation at the expression and functional level between β_1- and β_3-adrenoceptors occurs following chronic receptor stimulation via the activation of PKC, PI3K, p38 MAPK and MEK/ERK1/2 pathways.

Methods

Cell culture

All animal care and experimental procedures complied with the UK Home Office Policy and approved by the Nottingham Trent University Ethical Committee. Neonatal ventricular myocytes were prepared from 1–4 day-old Wistar rats using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lornes laboratories, Reading, UK). As described previously (Germack and Dickenson, 2006), the cells were preplated three times for 30 min in a humidified incubator (95% air/5% CO_2 at 37°C) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% (v/v) foetal calf serum and penicillin/streptomycin (100 $U \cdot mL^{-1}$) in order to minimize fibroblast contamination. Cardiomyocyte-rich cultures (>90%) were plated onto fibronectin-coated plates at a final density of 1.25×10^5 $cells \cdot cm^{-2}$ in supplemented DMEM. For cAMP accumulation assay, the cells were plated onto 24-well plates and for RT-PCR and Western blotting analysis onto 6-well plates. After 3 days, confluent and spontaneously beating cells were serum-starved and untreated or treated with 10 μM dobutamine (a β_1-adrenoceptor selective agonist) and 1 μM ICI118551 (ICI, β_2-adrenoceptor selective antagonist) or 10 μM procaterol (a β_2-adrenoceptor selective agonist) and 1 μM CGP 20712A (CGP, β_1-adrenoceptor selective antagonist) or 2 μM CL-316243 (a β_3-adrenoceptor selective agonist) for 24 h. Because we did not observe any difference in the functional β_1-adrenoceptor down-regulation induced by chronic β_3-adrenoceptor stimulation in the presence and absence of propranolol at the concentration, which inhibits β_1 and β_2-adrenoceptors (data not shown), we did not combine CL-316243 treatment with an antagonist such as nadolol or propranolol to block β_1- and β_2-adrenoceptors. Similarly, Yoshida *et al*. (1994) showed that the treatment of

Table 1 Sequences of the specific primers for the three rat β-adrenoceptors (β_1-AR, β_2-AR, β_3-AR) and for β-actin

	Primer	*PCR product Base pairs*	*Reference*
β_1-AR		368	
Forward	5′-TTCGGTAGACGTGCTATGTGTGAC-3′		
Reverse	5′-TCTTCTTCACCTGTTTCTGGGCCT-3′		
β_2-AR		328	
Forward	5′-TGCGTGATTGCAGTGGATCGCTAT-3′		
Reverse	5′-CTATCTTCTGCAGCTGCCTTTTGG-3′		
β_3-AR		202	Bensaid *et al.* (1993)
Forward	5′-AACTCTGCCTTCAACCCGCTCAT-3′		
Reverse	5′-TTCATGTGGGAAATGGACGCTCAC-3′		
β-actin		301	Germack and Dickenson (2006)
Forward	5′-CGTAAAGACCTCTATGCCAA-3′		
Reverse	5′-GGTGTAAAACGCAGCTCAGT-3′		

obese diabetic mice with CL-316243 induces anti-obesity and anti-diabetic effects through specifically β_3-adrenoceptors without involving β_1- and β_2-adrenoceptors (potency $\beta_1 : \beta_2 : \beta_3$ = 0:1:100 000). Finally, the CL-316243-inhibited forskolin response was only observed in noradrenaline-treated neonatal rat cardiomyocytes and not modified by the presence of propranolol (Germack and Dickenson, 2006). Therefore, these studies indicate that the treatment of cardiomyocytes with CL-316243 in the present investigation mediates its effects only through β_3-adrenoceptor stimulation. The signalling pathway involved in the regulation of β_1- and β_3-adrenoceptor cAMP responses following dobutamine/ICI or CL-316243 treatments was investigated in the absence or presence of protein kinase inhibitors (50 μM PD 98059, MEK1 inhibitor; 10 μM SB 203580, p38MAPK inhibitor; 10 μM SP 600125, SAPK/JNK inhibitor; 100 nM wortmannin, PI3K inhibitor; 1 μM KT 57201, PKA inhibitor; or 10 μM GF 109203, PKC inhibitor).

cAMP accumulation assay

Following the treatment of cardiomyocytes with dobutamine/ICI or procaterol/CGP or CL-316243 for 24 h, assays were carried out in serum-free DMEM in a humidified incubator (95% air/5% CO_2 at 37°C). Agonists as required by the experiments were added as described in the figure legends. The cells were incubated for 3 h in a humidified incubator (95% air/5% CO_2 at 37°C) with 500 μL of serum-free DMEM containing [^{3}H]adenine (2 μCi per well). [^{3}H]adenine-labelled cells were washed twice with Hanks/Hepes buffer and then incubated in 500 μL per well serum-free DMEM containing the cAMP phosphodiesterase inhibitor, rolipram (10 μM) for 15 min at 37°C in a humidified incubator. Agonists were added 5 min prior to the incubation with 1.5 μM forskolin (10 min). Antagonist or inhibitors were added 30 min before agonist. Incubations were terminated by the addition of 500 μL 5% trichloroacetic acid after removing the medium. [^{3}H]cAMP was isolated by sequential Dowex-alumina chromatography as previously described (Germack and Dickenson, 2006). After elution, the levels of [^{3}H]cAMP were determined by liquid scintillation counting.

Quantitative RT-PCR

Total RNA was extracted from untreated or treated cardiomyocytes for 24 h with dobutamine/ICI or procaterol/CGP or CL-316243 combining RNA isolation reagent, RNABee (AMS Biotechnology, Oxon, UK) and RNeasy Mini Kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg total RNA for the synthesis of cDNA using oligo d(T)15 (Promega, Southampton, UK) and Superscript II reverse transcriptase (Invitrogen Ltd, Praisley, UK) according to the manufacturer's instructions. Quantitative PCR was carried out with a Bio-Rad iCycler system using iQ SYBR green Supermix kit from Bio-Rad (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The PCR protocol used was 3 min initial denaturation at 95°C followed by 45 cycles of denaturation (20 s at 95°C), annealing (30 s at 65°C) and elongation (30 s at 72°C). For exact quantification, standard curves were generated by serial dilutions (1×10^6–1×10^2 single stranded cDNA molecules) for each target gene used as external standards. Standard cDNAs were prepared as followed: initial PCR was conducted with 1 μg cDNA in the presence of deoxyribonucleotides triphosphate (dNTPS) (1.25 mM), 200 ng of respective primers (Table 1) and 1.5 U *Taq* DNA polymerase (Promega, Southampton, UK). Thirty five cycles of the amplification steps involved 1 min denaturation at 94°C and 1 min annealing at 65°C and 1 min extension at 72°C. The PCR products were analysed using 1.5% agarose gel electrophoresis and extracted from the gel using QIAquick gel extraction kit (Qiagen Ltd, Crawley, UK). The purified products were again subjected to PCR and purified using QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK) followed by quantification by spectrophotometry (λ:260/280). β-actin mRNA was used as an internal standard to normalize β_1-, β_2- and β_3-adrenoceptor mRNA.

Western blot analysis

Cardiomyocytes untreated or treated with dobutamine/ICI or procaterol/CGP or CL-316243 for 24 h were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold hypotonic buffer [30 mM Tris-HCl, 5 mM MgCl2, 100 mM NaCl, 1 mM EGTA, 0.1 mM phenylmethylsulphonylfluoride (PMSF), 5 $\mu g \cdot mL^{-1}$ leupeptin] for G protein and β-adrenoceptor investigation. Cell membranes were collected by centrifugation for 30 min at 100 000 g and 4°C. The resulting plasma membrane pellets were dissolved in ice-cold detergent buffer [150 mM NaCl, 50 mM Tris.HCl, 5 mM ethylediaminetetraacetic acid (EDTA), 1% (v/v) IGEPAL

CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1 mM Na_3VO_4, 1 mM NaF, 1 mM benzamidine, 0.1 mM PMSF, 5 $\mu g \cdot mL^{-1}$ leupeptin]. For the study of the cell signalling, cardiomyocytes untreated or treated with dobutamine/ICI or CL-316243 for 24 h in the presence or absence of appropriate kinase inhibitors were washed with ice-cold PBS and lysed in ice-cold detergent buffer. Cell membrane homogenates or cell lysates were clarified by centrifugation (5 min; 9500× *g*) in an Eppendorf microcentrifuge. 100 µL of the cell lysate removed and stored at –20°C until required. Protein concentration was determined using Bio-Rad *DC* Protein assay (Bio-Rad laboratories, Hertfordshire, UK) with bovine serum albumin as the standard. Samples in lysis buffer were heated at 95°C in SDS-polyacrylamide gel electrophoresis (SDS/PAGE) sample buffer (v/v). Aliquots of the cell lysate (20–30 µg protein) were separated by SDS/PAGE (12% acrylamide gel), using a Bio-Rad Mini-Protean II system (1 h at 200 V). Proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot system (1 h at 100 V in 25 mM Tris, 192 mM glycine and 20% MeOH). Following transfer, the membranes were washed with Tris-buffered saline (TBS) and blocked for 1 h at room temperature in blocking buffer (TBS, 5% (w/v) skimmed milk powder, 0.1% Tween-20). Blots were then incubated for 24 h at 4°C with primary antibody in blocking buffer against $G\alpha_i$-protein, $G\alpha_s$-protein, phosphorylated ERK1/2, P38 MAPK, JNK or PKB at 1:1000 dilution or β_1-adrenoceptor at 1/200 dilution or β_2- or β_3-adrenoceptors at 1/100. The primary antibody was removed and the blot was extensively washed three times for 5 min in TBS/Tween 20. Blots were then incubated for 1 h at room temperature with the goat anti-rabbit secondary antibody coupled to horseradish peroxidase (DAKO Ltd, Cambridge, UK) at 1:1000 dilution in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as previously described and developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK) and quantified by densitometry using GeneGenius BioImaging System (Syngene, Synoptics Ltd, UK). The uniform transfer of proteins to the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain (Sigma Chemical Co.) prior to application of the primary antibody. In addition, replicate samples from each experiment were analysed on separate blots using an antibody (1:1000) that recognizes β-actin or unphosphorylated (total) ERK1/2, P38 MAPK, JNK and PKB.

Statistical analysis

Results are expressed as means ± SEM. Concentration-response curves were analysed by computer-assisted iteration using the GraphPad Prism (GraphPad software, San Diego, CA). Statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's test, and $P < 0.05$ was considered as the limit of statistical significance.

Materials

Bovine serum albumin, DMEM, foetal calf serum, fibronectin, forskolin, CL-316243, deoxynucleotide mix, IGEPAL CA-630 and leupeptin were all obtained from Sigma Chemical Co. (Poole, Dorset, UK). Trichloroacetic acid was purchased from Calbiochem (Nottingham, UK). Dobutamine, procaterol hydrochloride, ICI 118551, CGP 20712A, SB 203580, PD 98059, SP 600125, wortmannin, KT 5720 and GF 109203X were all from Tocris (Bristol, UK). β-actin, phospho-ERK1/2 (Thr^{202}/Tyr^{204}), ERK1/2, phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), SAPK/JNK, phospho-p38 MAPK (Thr^{180}/Tyr^{182}), p38 MAPK, phospho-PKB (Ser^{473}) and PKB antibodies were purchased from New England Biolabs (Hitchin, UK). Mouse β_1- (sc-568) and β_2- (sc-570) adrenoceptor antibodies were provided by Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Mouse β_3-adrenoceptor antibody was purchased from Alpha Diagnostic international (San Antonio, TX). G protein $\alpha_{i\text{-}2}$antibody was provided by Abcam (Cambridge, UK) and G protein α_s was from Chemicon (Eastleigh, UK). ([8-^{3}H]adenine was obtained from Amersham (Bucks, UK).

Results

Effect of chronic stimulation of β_1, β_2 or β_3-adrenoceptors on the functional response induced by the β-adrenoceptor subtypes in neonatal rat cardiomyocytes

We have shown previously that β_1-, β_2- and β_3-adrenoceptors are expressed in neonatal rat cardiomyocytes, and following chronic stimulation with noradrenaline, β_1- and β_2- are down-regulated whereas β_3-adrenoceptors are up-regulated (Germack and Dickenson, 2006). Therefore, we investigated the functional response induced by β_1-, β_2- and β_3-adrenoceptor stimulation following chronic stimulation of each subtype for 24 h (Figure 1, Table 2). Chronic stimulation of β_1-adrenoceptors was performed using 10 µM dobutamine (a β_1-adrenoceptor selective agonist) in the presence of 1 µM ICI118551 (ICI, a β_2-adrenoceptor selective antagonist). Continuous activation of β_2-adrenoceptors was carried out with 10 µM procaterol (a β_2-adrenoceptor selective agonist) and 1 µM CGP 20712A (CGP, a β_1-adrenoceptor selective antagonist). Finally, the pretreatment of cardiomyocytes with 2 µM CL-316243 (a β_3-adrenoceptor selective agonist) stimulated chronically β_3-adrenoceptors. The concentrations of agonists and antagonists were chosen according to their potency and maximal responses previously published (Germack and Dickenson, 2006). Following dobutamine/ICI (β_1) or CL-316243 (β_3) treatments, dobutamine-induced cAMP accumulation was decreased by 67% and 52%, respectively (Figure 1A) indicating an interaction between both β-adrenoceptor subtypes. In addition, the decrease in β_1-adrenoceptor response was significantly higher when the cells were treated with dobutamine/ICI. Chronic stimulation of β_2-adrenoceptors did not modify β_1-adrenoceptor maximal response although the sensitivity to dobutamine was shifted to the right and decreased by around 10 times suggesting an interaction also between β_1- and β_2-adrenoceptors. Indeed, pretreatment with dobutamine/ICI resulted in a significant decrease in β_2-adrenoceptor response by 69% and a decrease in sensitivity by 35 times whereas β_3-adrenoceptors pretreatment had no effect on procaterol-induced cAMP accumulation (Figure 1B). Chronic stimulation of β_2-adrenoceptors abolished procaterol response. As shown previously and in

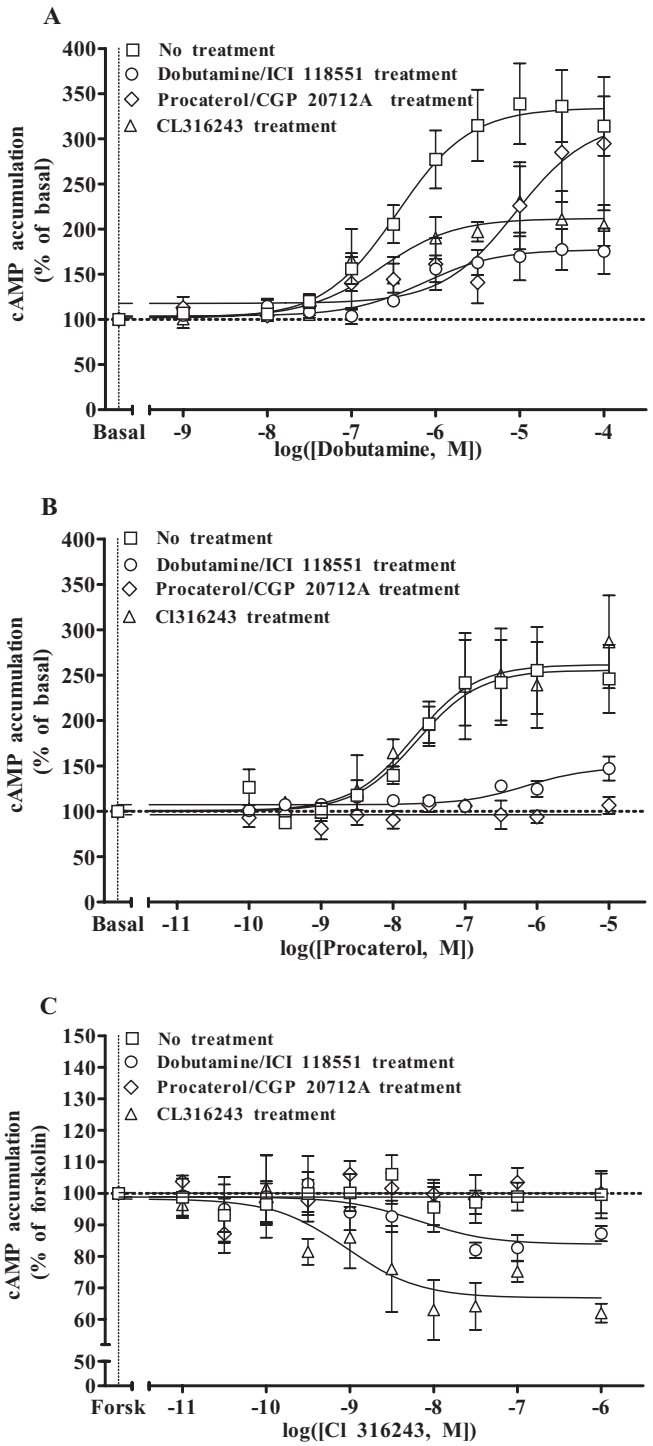


Figure 1 Activation of cAMP accumulation induced by dobutamine, β_1-selective agonist (A) and procaterol, β_2-selective agonist (B), and effect of the selective β_3-adrenoceptor agonist CL-316243 on forskolin (Forsk)-induced cAMP accumulation (C) in neonatal rat cardiomyocytes untreated, treated with 10 μM dobutamine in the presence of 1 μM ICI 118551, 10 μM procaterol in the presence of 1 μM CGP 20712A or 2 μM CL-316243 for 24 h. cAMP accumulation was measured as described under Methods. Data are expressed as percentage of the basal level of cAMP accumulation (100%, A, B) or as the percentage of 1.5 μM forskolin response in the absence of agonist (100%, C). Each point represents the mean ± SEM of four to six experiments performed in duplicate.

Figure 1C, β_3-adrenoceptors are not functional in normal physiological conditions in neonatal rat cardiomyocytes (Germack and Dickenson, 2006). Chronic stimulation of β_2-adrenoceptors did not induce functional β_3-adrenoceptor expression. In pretreated cardiomyocytes with dobutamine/ICI or CL-316243, CL-316243 induced a significant inhibition of forskolin-mediated cAMP accumulation without changing the potency between both treatments strengthening the assumption of a compensatory regulation between β_1- and β_3-adrenoceptors (Table 2). In addition, CL-316243 treatment induced a higher β_3-adrenoceptor response than β_1-adrenoceptor treatment.

Effect of chronic stimulation of β_1, β_2 or β_3-adrenoceptors on mRNA and protein expression of the β-adrenoceptor subtypes in neonatal rat cardiomyocytes

The functional studies suggest that a potential interaction exists between β_1- and β_2-adrenoceptors as well as β_1- and β_3-adrenoceptors. Therefore, we investigated whether this cross-regulation involves the modulation of the receptor expression by measuring the mRNA and protein level of the three receptors following the different treatments (Figures 2 and 3). The expression of the β-adrenoceptors in untreated cells by real time PCR (Figure 2A) displayed a $\beta_1/\beta_2/\beta_3$ ratio of 0.39/0.61/0.0008 indicating that β_2-adrenoceptors are predominantly expressed in neonatal rat cardiomyocytes as recently reported by Morisco *et al.* (2008) using binding studies. The $\beta_1/\beta_2/\beta_3$ mRNA ratio was 0.25/0.745/0.0017 or 0.24/0.75/0.0017 following β_1- or β_3-adrenoceptor treatments respectively. Although an increase in β_3-adrenoceptors was observed, β_3-adrenoceptor level remained very low, which may explain the absence of functional response in physiological conditions and a low functional activity (30%) following chronic stimulation. Chronic β_1- or β_3-adrenoceptor stimulation induced a decrease in β_1-adrenoceptor mRNA level by 47% and 50% respectively (Figure 2B). At protein level, β_1-adrenoceptor was reduced by 58% following β_1-treatment and 42% subsequent to β_3-adrenoceptor treatment (Figure 3A). β_2-adrenoceptor treatment had no effect on β_1-adrenoceptor expression (Figures 2B and 3A). A decrease in β_2-adrenoceptor expression by around 40% occurred at mRNA and protein level when cardiomyocytes were treated with procaterol/CGP whereas β_1- or β_3-adrenoceptor treatments had no effect (Figures 2C and 3B). No effect of chronic β_2-adrenoceptor stimulation on β_3-adrenoceptor mRNA and protein level was observed (Figures 2D and 3C). The amount of β_3-adrenoceptor transcripts was increased by 65% and 72%, and β_3-adrenoceptor protein level by 43% and 68% following chronic stimulation of β_1- or β_3-adrenoceptors respectively (Figures 2D and 3C). The observed bands corresponded to the expected molecular weight (Diebold *et al.*, 2001) and were modulated similarly to β-adrenoceptor mRNAs suggesting specific antibody binding. Overall, these data show that the modulation of receptor expression correlated well with the changes in β_1- and β_3-adrenoceptor functions.

Effect of chronic stimulation of β_1, β_2 or β_3-adrenoceptors on $G\alpha_s$ and $G\alpha_{i\text{-}2}$ protein in neonatal rat cardiomyocytes

Both, β_1- and β_3-adrenoceptor treatments induced a similar modulation of β_1-adrenoceptor or β_3-adrenoceptor expression,

Table 2 Effect of dobutamine/ICI118551, procaterol/CGP 20712A and CL-316243 treatments on dobutamine and procaterol-induced cAMP accumulation, and CL-316243-mediated inhibition of forskolin-induced cAMP response

Agonist		*Untreated*	*Treatment*		
			β_1 treatment	*β_2 treatment*	*β_3 treatment*
Dobutamine	Emax	234 ± 11	77 ± 4***	219 ± 28	112 ± 6***, #
β_1 response	*pD_2*	6.46 ± 0.18	6.15 ± 0.30	5.05 ± 0.32***	6.65 ± 0.30
Procaterol	Emax	156 ± 10	49 ± 2***	NR	162 ± 12###
β_2 response	*pD_2*	7.67 ± 0.27	6.16 ± 0.29***	–	7.71 ± 0.31
CL-316243	Imax	NR	23 ± 6	NR	30 ± 5#
β_3 response	*pIC_{50}*	–	8.04 ± 0.45	–	8.45 ± 0.23

Values are means ± SEM of four to six experiments performed in duplicate. The potencies of the agonists were evaluated by their EC_{50}, concentration of agonist inducing 50% of the maximal cAMP accumulation expressed as $-\log_{10} EC_{50}$ (*pD_2*) or their IC_{50}, concentration of agonist inducing 50% of inhibition expressed as $-\log_{10} IC_{50}$ (*pIC_{50}*). Emax is the maximal response expressed in % over basal response. Imax is the maximal percentage of inhibition.
*$P < 0.05$ and ***$P < 0.001$ versus untreated cells and #$P < 0.05$ and ###$P < 0.001$ versus β_1 treatment. NR means no response.

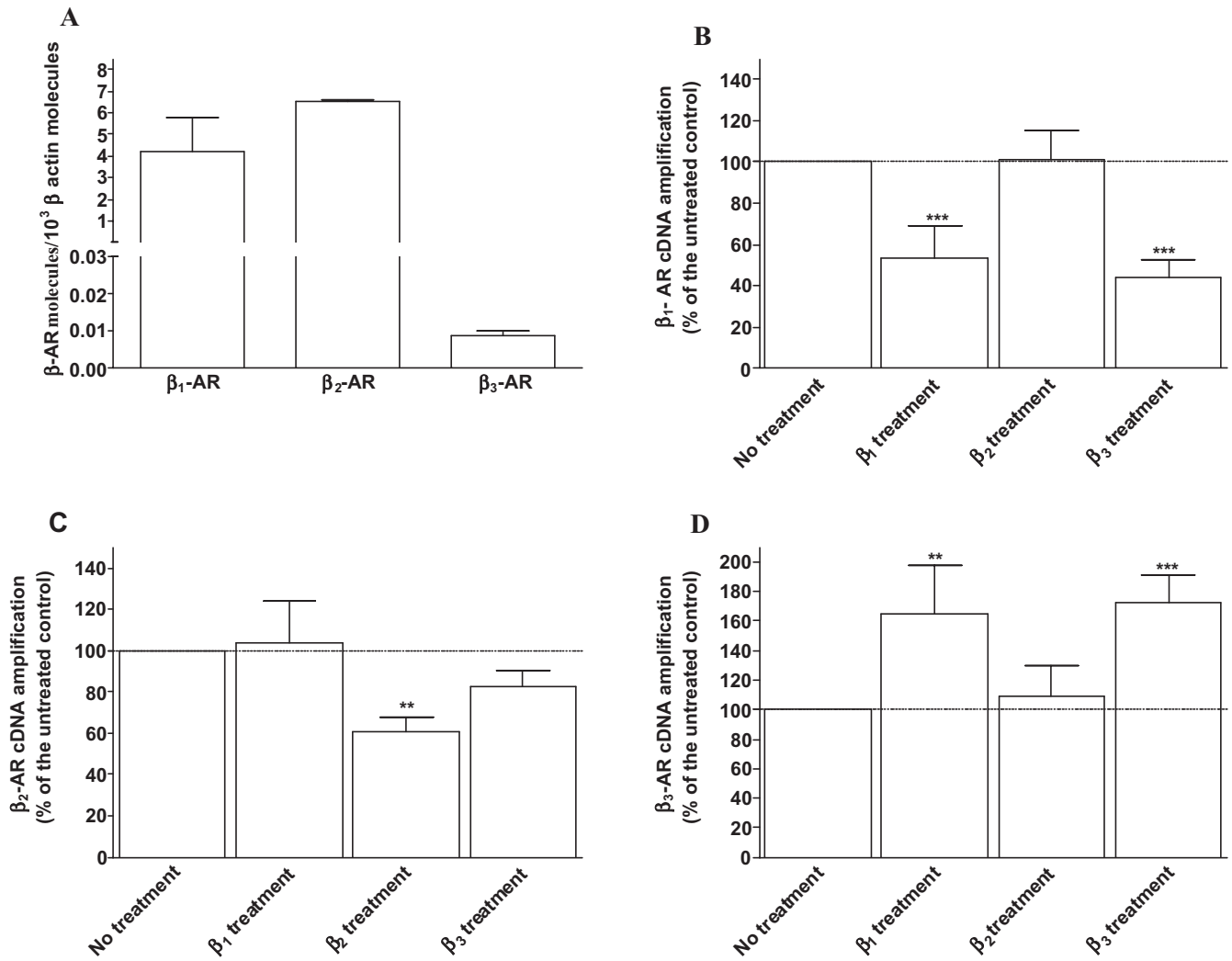


Figure 2 Quantitative expression of β-adrenoceptors in untreated neonatal rat cardiomyocytes (A) and expression of β_1- (B), β_2- (C) and β_3-adrenoceptors (D) mRNA obtained from untreated (control), treated with 10 μM dobutamine in the presence of 1 μM ICI 118551 (β_1 treatment), 10 μM procaterol in the presence of 1 μM CGP 20712A (β_2 treatment) or 2 μM CL-316243 (β_3 treatment) for 24 h. Total RNA was prepared, reverse transcription and real time PCR were carried out as described under Methods. Values were expressed as β-adrenoceptor molecules $\times 10^{-3}$ β actin molecules in A and normalized to β-actin molecules and expressed as the percentage of untreated cardiomyocytes (100%) in B, C and D. Each point represents the mean ± SEM of five to six independent experiments. **$P < 0.01$ and ***$P < 0.001$ versus untreated control.

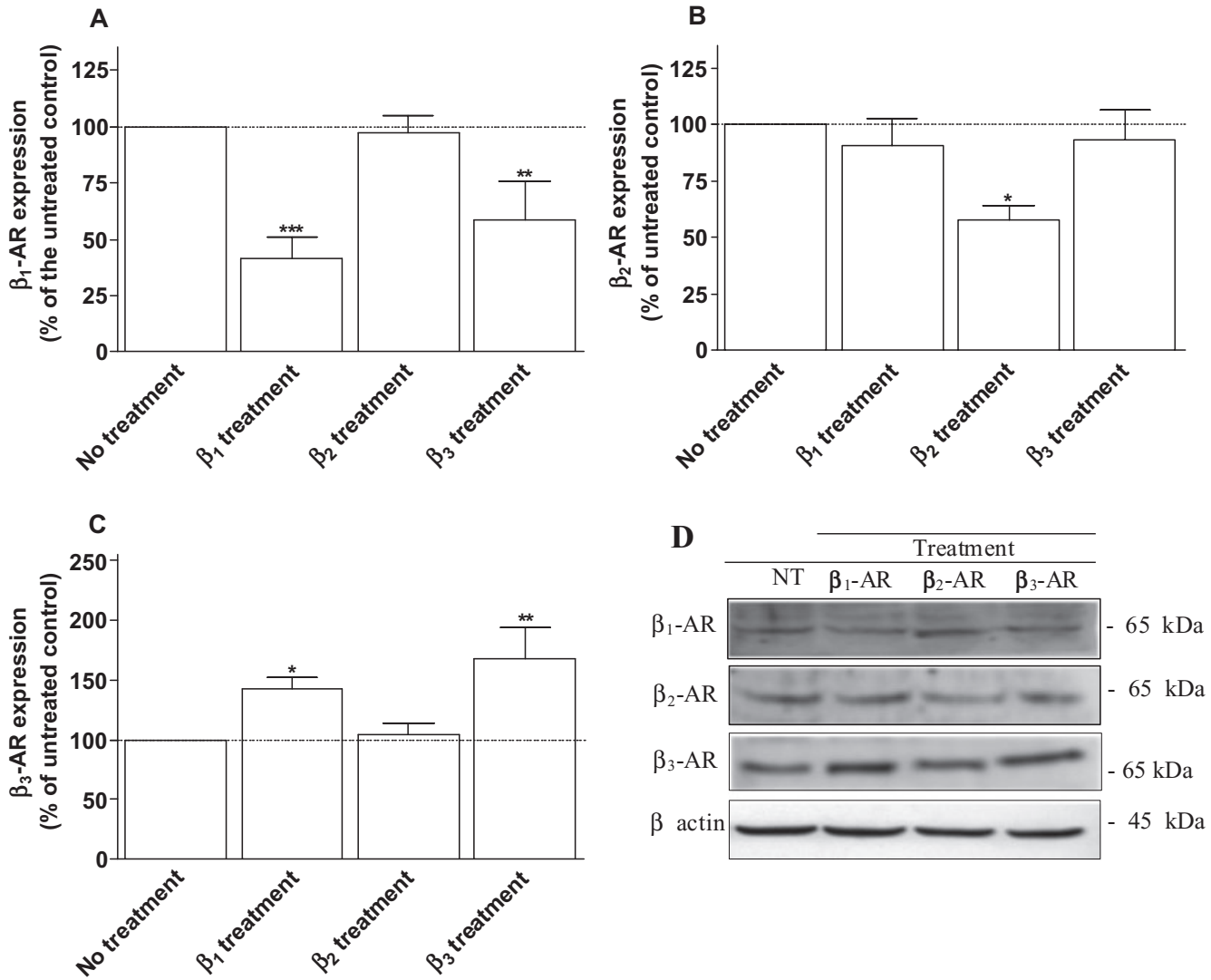


Figure 3 Expression of β_1- (A), β_2- (B) and β_3-adrenoceptors (C) obtained from neonatal rat cardiomyocytes untreated (control), treated with 10 μM dobutamine in the presence of 1 μM ICI 118551 (β_1 treatment), 10 μM procaterol in the presence of 1 μM CGP 20712A (β_2 treatment) or 2 μM CL-316243 (β_3 treatment) for 24 h. Cell membrane homogenates were monitored by Western blotting for β_1-, β_2- and β_3-adrenoceptors as described under Methods. The same samples were also analysed on separate blots using an antibody that recognizes β actin to confirm equal loading on each lane. Representative immunoblots for each proteins are shown in panel C. Data are expressed as the percentage of untreated cardiomyocytes (100%) following the calculation of β-adrenoceptor/β-actin ratio for each lane. The combined results (panel A, B and C) obtained from densitometric analysis of blots represent the mean ± SEM of five to seven independent experiments. *$P < 0.05$ and **$P < 0.01$ versus untreated control.

whereas the decrease in β_1-adrenoceptor and increase in β_3-adrenoceptor functional responses were higher in cardiomyocytes treated with dobutamine/ICI and CL-316243 respectively. These results indicate that the modulation of β_1- and β_3-adrenoceptor function may also involve modifications in receptor and G protein coupling.

Therefore, we investigated the expression of $G\alpha_s$ and $G\alpha_{i\text{-}2}$ proteins in neonatal rat cardiomyocytes following β_1-, β_2- or β_3-adrenoceptor treatment using Western blotting (Figure 4). $G\alpha_s$ protein level was not significantly modified in cardiomyocytes following the three treatments (Figure 4A). Inversely, $G\alpha_{i\text{-}2}$ protein was increased by 44% when β_1-adrenoceptors were chronically stimulated and by 94% and 112% following β_2- or β_3-adrenoceptor treatments respectively (Figure 4B). These results suggest that the higher inhibition of the response to forskolin induced by β_3-adrenoceptor stimulation following CL-316243 treatment, compared with dobutamine/ICI treatment involves a better coupling to $G\alpha_{i\text{-}2}$ protein.

Effect of kinase inhibitors on the down-regulation of β_1-adrenoceptor functional response induced by chronic stimulation of β_1 or β_3-adrenoceptors in neonatal rat cardiomyocytes

PI3K, PKA, PKC have been shown to be involved in the process of β_1-adrenoceptor down-regulation (Li *et al.*, 1998; Dzimiri, 1999; Naga Prasad *et al.*, 2001; Nienaber *et al.*, 2003). In addition, ERK1/2, SAPK/JNK and p38 seem to be also involved in the regulation of β-adrenoceptor expression

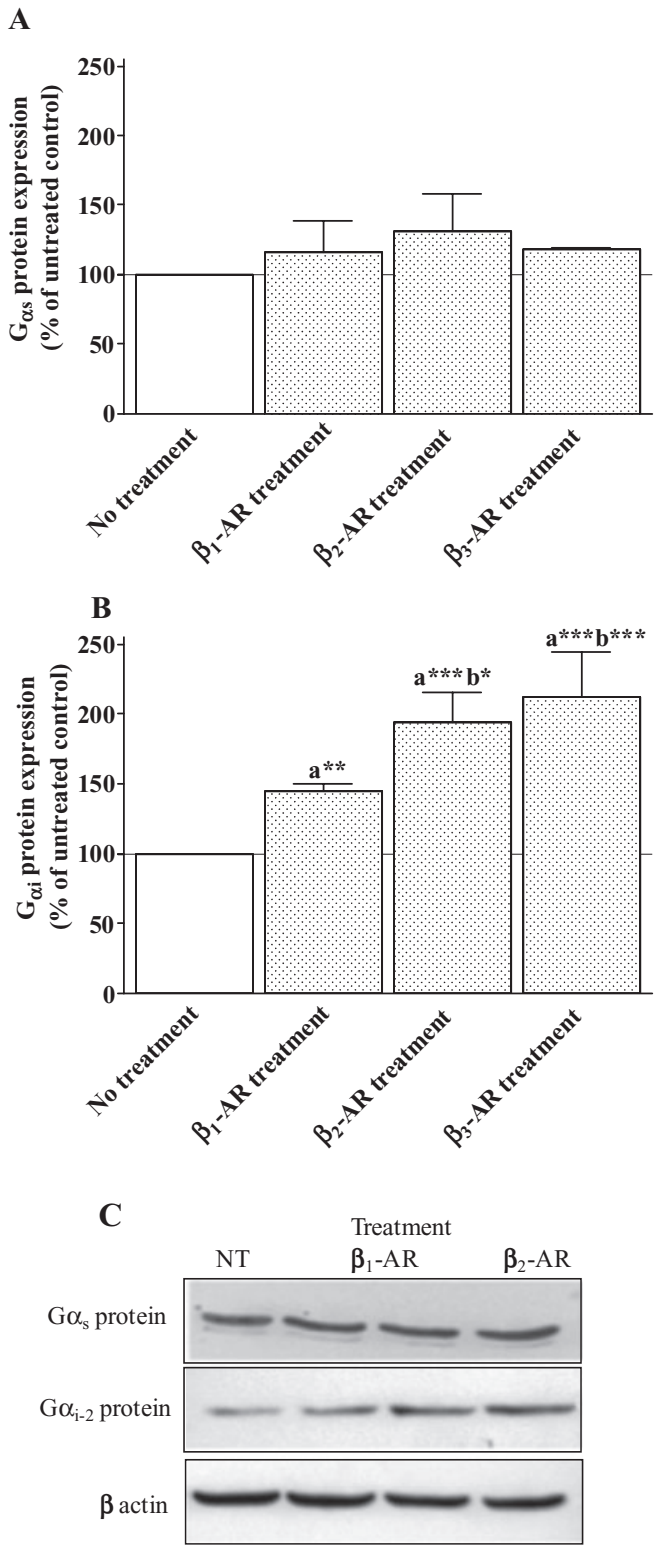
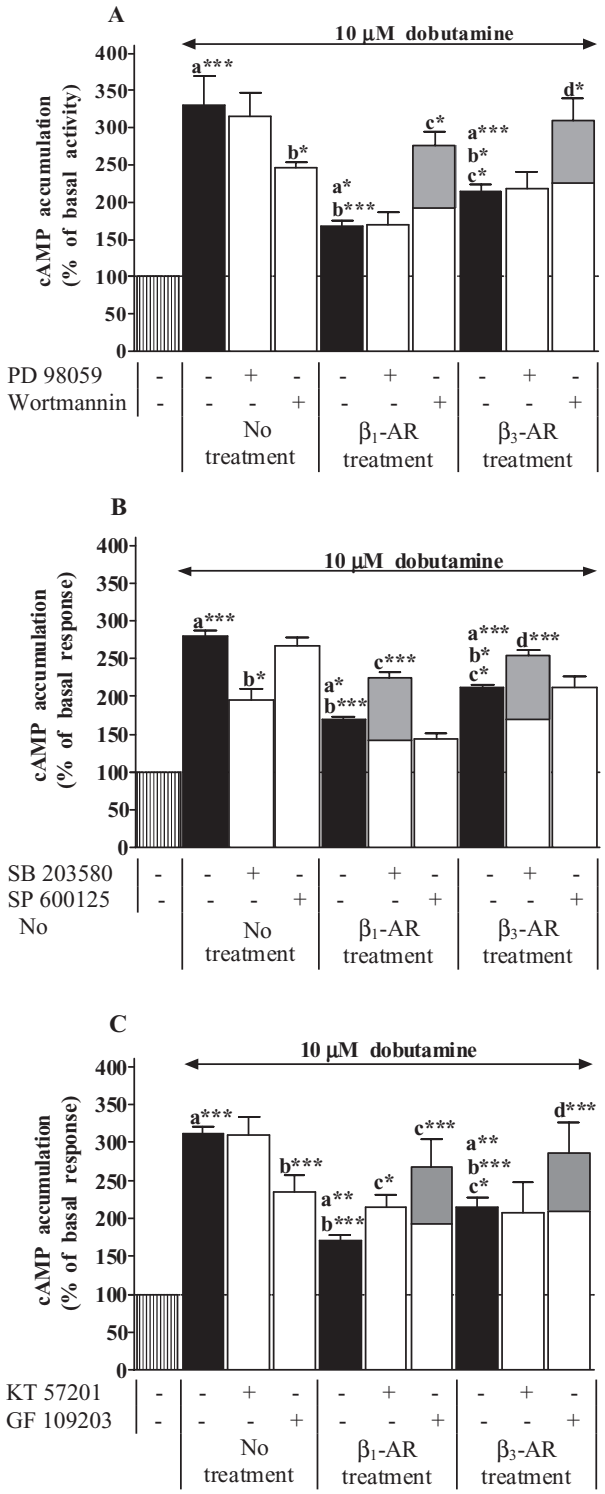


Figure 4 Expression of $G\alpha_s$ and $G\alpha_i$ proteins obtained from neonatal rat cardiomyocytes untreated (control), treated with 10 μM dobutamine in the presence of 1 μM ICI 118551 (β_1 treatment), 10 μM procaterol in the presence of 1 μM CGP 20712A (β_2 treatment) or 2 μM CL-316243 (β_3 treatment) for 24 h. Cell membrane homogenates were monitored by Western blotting for $G\alpha_s$ (A) and $G\alpha_i$ (B) proteins as described under Methods. The same samples were also analysed on separate blots using an antibody that recognizes β actin to confirm equal loading on each lane. Representative immunoblots for each proteins are shown in panel C. Data are expressed as the percentage of untreated cardiomyocytes (100%) following the calculation of β-adrenoceptor/β-actin ratio for each lane. The combined results (panels A and B) obtained from densitometric analysis of blots represent the mean ± SEM of four to six independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, (a) versus untreated control and (b) versus β_1-adrenoceptor treatment.

following chronic stimulation (Headley *et al.*, 2004). As β_2-adrenoceptor treatment did not show any interaction at both expression and functional levels between β_2- and β_1-adrenoceptors, we investigated the signalling pathway involved in the down-regulation of the β_1-adrenoceptor following chronic stimulation of β_1- and β_3-adrenoceptors. In order to determine the mechanisms implicated in β_1-adrenoceptor down-regulation, cardiomyocytes were treated for 24 h with dobutamine/ICI or CL-316243 in the presence or absence of inhibitors of MAP kinase (MEK1, 50 μM, PD 98059), PI3K (100 nM, wortmannin), p38 MAPK (10 μM, SB 203580), SAPK/JNK (10 μM, SP 600125), PKA (1 μM, KT 57201) and PKC (10 μM, GF 109203). As shown in Figure 5, wortmannin, SB 203580 and GF 10920 produced a decrease in dobutamine-induced cAMP accumulation by 25%, 30% and 24% in untreated cells respectively. The inhibition of β_1-adrenoceptor response in untreated cardiomyocytes was likely to occur following dobutamine/ICI and CL-316243 treatments. Therefore, to consider only the effect of both treatments on the modulation of β_1-induced cAMP accumulation, the functional values obtained in the treated groups were corrected from the inhibitory effect of wortmannin, SB203580 or GF 10920 observed in untreated cells (decrease in untreated cells + value of treated cardiomyocytes for each individual experiments), as explained in the legend of Figure 5. PD 98059 and SP 600125 had no effect on the functional down-regulation induced by both β_1- or β_3-adrenoceptor treatments indicating that MEK and SAPK/JNK are not implicated in this process. Wortmannin, SB 203580 and GF 109203 reversed the inhibition of dobutamine response following β_1-adrenoceptor treatment by 64%, 32% and 58%, and by 44%, 20% and 34% when cardiomyocytes were treated with CL-316243 (β_3-adrenoceptor treatment) respectively. The PKA inhibitor, KT 57201 counteracted the decrease in β_1-adrenoceptor function by 26% in the cells treated with dobutamine/ICI and did not modify β_1-induced cAMP accumulation in CL-316243 treated cardiomyocytes.

Effect of kinase inhibitors on the up-regulation of β_3-adrenoceptor functional response induced by chronic stimulation of β_1 or β_3-adrenoceptors in neonatal rat cardiomyocytes

β_3-Adrenoceptor agonist-induced inhibition of responses to forskolin was investigated when cardiomyocytes were treated for 24 h with dobutamine/ICI or CL-316243 in the presence or absence of inhibitors of MEK1 (50 μM, PD 98059), PI3K (100 nM, wortmannin), p38 (10 μM, SB 203580), SAPK/JNK (10 μM, SP 600125), PKA (1 μM, KT 57201) and PKC (10 μM, GF 10920). As shown in Figure 6, PD 98059, SB 203580 and



GF 10920 inhibited β_1- or β_3-adrenoceptor treatment-induced up-regulation of β_3-adrenoceptor functional response indicating the involvement of MEK, p38 and PKC in the regulation of the β_3-adrenoceptor subtype. KT 57201 also abolished the increase in β_3-adrenoceptor response when the cells were treated with dobutamine/ICI but not with CL-316243. No effect of wortmannin and SP 600125 was observed indicating that PI3K and SAPK/JNK do not regulate β_3-adrenoceptor up-regulation.

Figure 5 Effect of kinase inhibitors on dobutamine-induced cAMP accumulation in neonatal rat cardiomyocytes untreated, treated with 10 μM dobutamine in the presence of 1 μM ICI 118551 (β_1 treatment), or 2 μM CL-316243 (β_3 treatment) for 24 h. In addition, untreated and treated cells were incubated in panel A with 50 μM PD 98059 (MEK inhibitor) or 100 nM wortmannin (phosphoinositide 3-kinase inhibitor), in panel B with 10 μM SB 203580 (p38 MAPK inhibitor) or 10 μM SP 600125 (JNK inhibtor) and in panel C with 1 μM KT 57201 (PKA inhibitor) or 10 μM GF 109203 (PKC inhibitor). cAMP accumulation was measured as described under Methods. Data are expressed as the percentage of the basal level of cAMP accumulation (set at 100%, striped bars). Black and open bars represent cAMP accumulation induced by dobutamine in the absence and presence of protein kinase inhibitors in all groups respectively. Wortmanin (A), SB 203580 (B) and GF 109203 (C) elicited a significant decrease in cAMP accumulation induced by dobutamine in untreated cardiomyocytes. Grey filled bars represent the data corrected from the effect of these inhibitors on dobutamine response in untreated cardiomyocytes in order to consider only the effect of β_1- or β_3-adrenoceptor treatments on the modulation of β_1-adrenoceptor response (decrease in untreated cells + value of treated cardiomyocytes for each individual experiments). Each bar represents the mean ± SEM of six to seven independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, (a) versus basal level, (b) versus dobutamine response in untreated cells, (c) versus dobutamine response in β_1-adrenoceptor-treated cells and (d) versus dobutamine response in β_3-adrenoceptor-treated cells.

Effect of chronic stimulation of β_1 or β_3-adrenoceptors on ERK1/2, p38, SAPK/JNK and Akt activation in neonatal rat cardiomyocytes

The functional study indicates that PI3K, MEK, p38 but not SAPK/JNK are involved in the modulation of the receptors following the different treatments. Consequently, chronic stimulation of β_1- and β_3-adrenoceptors should lead to the activation of p38, extracellular signal regulated-kinase (ERK1/2, down-stream MEK target) and Akt (down-stream PI3K target). Therefore, we investigated the activation of ERK1/2, p38, SAPK/JNK and Akt by either dobutamine/ICI or CL-316243 treatments. Chronic stimulation of β_1- or β_3-adrenoceptor subtypes induced ERK1/2 phosphorylation by 17% and 49% respectively (Figure 7A). Significantly, lower ERK1/2 activation was obtained when the cells were treated with dobutamine/ICI compared with chronic β_3-adrenoceptor stimulation. Both treatments activated p38 by around 60%, which correlated well with the involvement of this kinase in the regulation of β_1- and β_3-adrenoceptor functional response following dobutamine/ICI or CL-316243 treatments (Figure 7B). Chronic β_1-adrenoceptor stimulation produced an increase in SAPK/JNK activation by 241% whereas β_3-adrenoceptor treatment had no effect (Figure 7C). Interestingly, Akt was phosphorylated by 149% when cardiomyocytes were treated with CL-316243 and not with dobutamine/ICI (Figure 7D) indicating a possible involvement of the β_3-adrenoceptor subtype in cell survival.

Discussion and conclusions

We have previously shown that chronic β-adrenoceptor stimulation with noradrenaline induces a decrease in

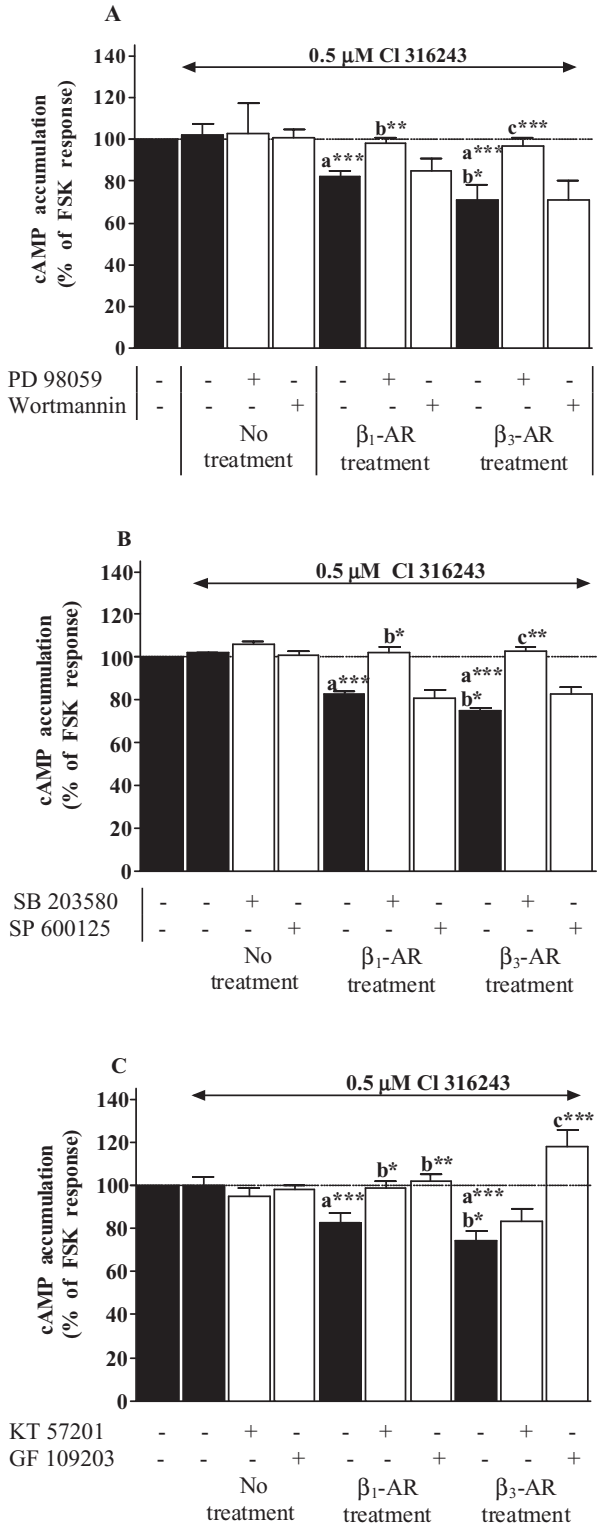


Figure 6 Effect of kinase inhibitors on CL-3162436243-inhibited forskolin response in neonatal rat cardiomyocytes untreated, treated with 10 μM dobutamine in the presence of 1 μM ICI 118551 (β_1 treatment), or 2 μM CL-316243 (β_3 treatment) for 24 h. In addition, untreated and treated cells were incubated in panel A with 50 μM PD 98059 (MEK inhibitor) or 100 nM wortmannin (phosphoinositide 3-kinase inhibitor), in panel B with 10 μM SB 203580 (p38 MAPK inhibitor) or 10 μM SP 600125 (JNK inhibtor) and in panel C with 1 μM KT 57201 (PKA inhibitor) or 10 μM GF 109203 (PKC inhibitor). cAMP accumulation was measured as described under Methods. Data are expressed as the percentage of 1.5 μM forskolin (FSK) response in the absence of agonist (100%, striped bars). Black and open bars represent the inhibition of FSK-mediated cAMP accumulation induced by CL-316243 in the absence and presence of protein kinase inhibitors in all groups respectively. Each bar represents the mean ± SEM of six to seven independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, (a) versus CL-316243 response in untreated cells, (b) versus CL-316243 response in β_1-adrenoceptor treated cells and (c) versus CL-316243 response in β_3-adrenoceptor treated cells.

β_1-adrenoceptors and an increase in β_3-adrenoceptors at the functional, genomic and protein levels (Germack and Dickenson, 2006). For the first time, the present study provides evidence of a cross-regulation at the expression and functional level between β_1- and β_3-adrenoceptors following chronic receptor stimulation via PI3K, PKC, p38 MAPK and MEK/ERK1/2 pathway in neonatal rat cardiomyocytes.

Changes in cardiac rate and force are triggered by β_1- and β_2-adrenoceptors acting via the G_s/cAMP/PKA signalling pathway whereas β_3-adrenoceptors mediate a decrease in contractility through a coupling to G_i protein (Dzimiri, 1999; Rozec and Gauthier, 2006). It is well established that β_1- and β_2-adrenoceptors are down-regulated following chronic stimulation (Dzimiri, 1999). As expected, in this study, chronic β_1-adrenoceptor treatment led to a decrease in β_1-adrenoceptor-induced cAMP accumulation and continuous β_2-adrenoceptor stimulation abolished β_2-adrenoceptor response, which was well correlated to the receptor expression. Interestingly, β_2-adrenoceptor treatment decreased sensitivity to dobutamine without changing β_1-adrenoceptor expression. Similarly, chronic β_1-adrenoceptor stimulation reduced β_2-adrenoceptor maximal response and efficiency with no alteration in β_2-adrenoceptor level. These results indicate that the modification of β_1- and β_2-adrenoceptor functional response without modification of receptor expression may reflect an interaction between both subtypes at the membrane level. Indeed, Zhu *et al.* (2005) have demonstrated that adult cardiomyocytes elicit not only a colocalization and physical association between β_1- and β_2-adrenoceptors, but also a functional synergy in cAMP and contractile responses. The disruption of β_1- and β_2-adrenoceptor heterodimerization may also contribute to the decrease in cardiac function in addition to receptor down-regulation in heart diseases where catecholamines are elevated (Dzimiri, 1999; Tilley and Rockman, 2006). Chronic β_3-adrenoceptor stimulation also produced a decrease in β_1-adrenoceptor response and expression. In contrast, this treatment induced an increase in β_3-adrenoceptor function and expression as observed in cardiomyocytes continuously exposed to β_1-adrenoceptor treatment. It is noteworthy that the relative abundance of the β_3-adrenoceptor in untreated neonatal rat cardiomyocytes was similar to the relative β_3-adrenoceptor level observed in healthy human hearts using also real-time PCR ($\beta_1/\beta_2/\beta_3$ ratio: neonatal rat 0.39/0.61/0.0008 vs. human 0.71/0.28/0.0012; Moniotte *et al.*, 2007). Sepsis is also associated with impairment in β-adrenoceptor response and myocardial dysfunction (Silverman *et al.*, 1993; Muller-Werdan *et al.*, 2006). Interestingly, the relative proportion of the β_3-adrenoceptor increased

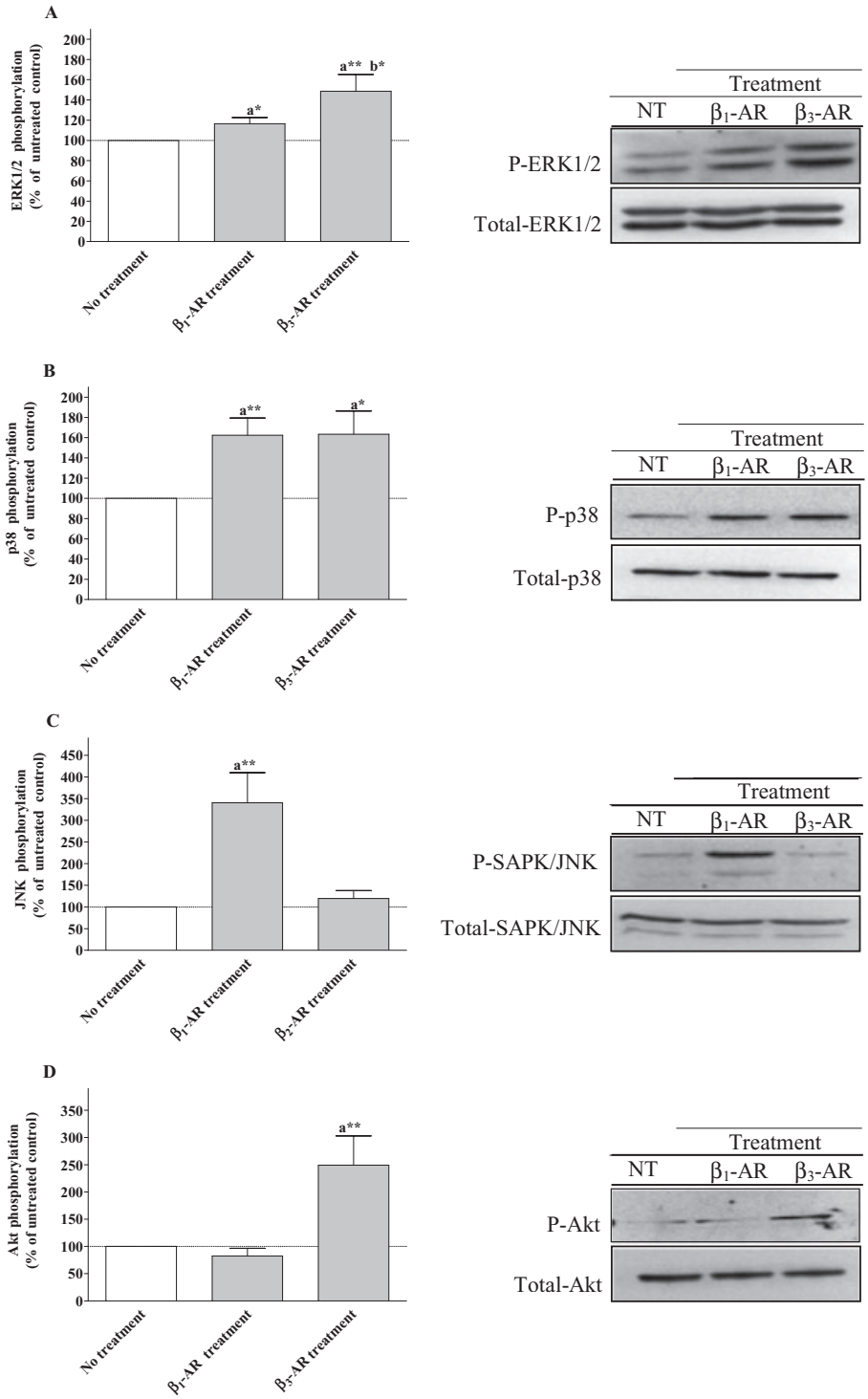


Figure 7 Effect of β_1-adrenoceptor and β_3-adrenoceptor treatments on ERK1/2, p38 MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and Akt activation in neonatal rat cardiomyocytes. The cells were untreated or treated with 10 μM dobutamine in the presence of 1 μM ICI 118551 (β_1-AR treatment), or 2 μM CL-316243 (β_3-AR treatment) for 24 h. Following the treatments, cell lysates were analysed by Western blotting as described under Methods using phospho-specific antibodies. The same samples were also analysed on separate blot using an antibody that recognizes both unphosphorylated (total) and phosphorylated kinases to confirm equal loading on each lane. Data are expressed as percentage of the untreated control following the calculation of the phosphorylated/total ratio for each lane. The combined results (panel A for ERK1/2, panel B for p38 mitogen-activated protein kinase, panel C for SAPK/JNK and panel D for Akt) obtained from densitometric analysis of blots, represent the mean ± SEM of for to six independent experiments. *$P < 0.05$ and **$P < 0.01$, (a) versus untreated control, (b) versus β_1-adrenoceptor treated cells.

in human hearts from septic patients, from 0.0012 to 0.0024 (Moniotte *et al.*, 2007) as observed in our study in neonatal rat cardiomyocytes following β_1- or β_3-adrenoceptor treatments (0.0008 to 0.0017). In addition, Moniotte *et al.* (2007) when using cytokines in murine cardiomyocytes as a model of sepsis showed a decrease in contractility by around 45% following β_3-adrenoceptor stimulation. All together, these data indicate that even when it is up-regulated but expressed at low level, the β_3-adrenoceptor may regulate cardiac function in pathological situation. Overall, our results clearly indicate that changes in β_1- and β_3-adrenoceptor regulation following chronic stimulation involves a cross-talk between both subtypes as observed in failing human and animal hearts (Cheng *et al*, 2001; Moniotte *et al.*, 2001; Morimoto *et al.*, 2004; Zhang *et al.*, 2005).

The up-regulation of β_3-adrenoceptor function following β_3-adrenoceptor treatment was higher by 30% compared with chronic β_1-adrenoceptor activation. In addition, both treatments did not produce a significant difference in the increase in β_3-adrenoceptor expression suggesting an enhancement in the coupling between β_3-adrenoceptors and $G\alpha_i$ protein. Indeed, the overexpression of $G\alpha_i$ protein in neonatal cardiomyocytes reduced isoprenaline-activated adenylyl cyclase, which was restored in the presence of *Pertussis* toxin (PTX) (Rau *et al*, 2003). Furthermore, the increase in $G\alpha_i$ protein level in addition to β-adrenoceptor down-regulation led to the desensitization of functional response, which was improved with PTX in failing rat heart (Kompa *et al.*, 1999; Xiao *et al.*, 2003). These works indicate that the function mediated by β-adrenoceptor is also regulated by coupling to $G\alpha_i$ protein when this protein is highly expressed. Although three isoforms $G\alpha_{i\text{-}1,\ 2\ \text{and}\ 3}$ have been identified, $G\alpha_{i\text{-}2}$ is mainly expressed in the heart and up-regulated during heart failure (El-Armouche *et al.*, 2003). In addition, the inhibition of cardiac $G\alpha_{i\text{-}2}$ increased infarct size and apoptosis in transgenic mice expressing a $G\alpha_{i\text{-}2}$ inhibitor peptide, when the mice were subjected to ischemia/reperfusion indicating an important role of this isoform in cardioprotection. In our study, $G\alpha_{i\text{-}2}$ expression was increased by both, β_1- or β_3-adrenoceptor treatments as observed in neonatal cardiomyocytes chronically treated with noradrenaline and in failing rat heart (Reithmann *et al.*, 1990; Kompa *et al.*, 1999; Xiao *et al.*, 2003). Interestingly, cardiomyocytes chronically stimulated with CL-316243 displayed a further increase in $G\alpha_{i\text{-}2}$ protein level compared with β_1-adrenoceptor treated cells by 32%. We can assume that an enhancement in β_3-adrenoceptor coupling to $G\alpha_i$ protein may explain the difference in β_3-adrenoceptor functional response between both, β_1- and β_3-adrenoceptor treatments. It is noteworthy that β_2-adrenoceptor treatment also increased $G\alpha_{i\text{-}2}$ protein as much as β_3-adrenoceptor treatment without inducing β_3-adrenoceptor functional response. This implies that the up-regulation of β_3-adrenoceptor function requires an enhancement of the receptor expression taking place at the genomic level and an increase in $G\alpha_i$ protein as observed with chronic β_1-adrenoceptor stimulation. In contrast, chronic β_1-adrenoceptor stimulation led to a lower decrease in β_1-adrenoceptor response by 31% compared with chronic β_3-adrenoceptor activation suggesting that the increase in $G\alpha_i$ protein did not affect β_1-adrenoceptor response as previously reported in cardiomyocytes from failing heart (Xiao *et al.*, 2003).

β_1-Adrenoceptor desensitization requires receptor phosphorylation by GRK2 (agonist dependent, homologous regulation) and PKA (agonist independent, heterologous regulation), which alters the coupling between the receptor and $G\alpha_s$ protein, leading to the reduction of β_1-adrenoceptor functional response (Dzimiri, 1999; Tilley and Rockman, 2006). In the present study, the PKA inhibitor, KT 57 201 partially blocked the functional β_1-adrenoceptor down-regulation following chronic β_1-adrenoceptor stimulation, whereas this inhibitor had no effect on β_3-adrenoceptor treatment-induced decrease in β_1-adrenoceptor response. β_1-adrenoceptors desensitization by PKA may explain the difference in β_1-adrenoceptor down-regulation observed between chronic β_1- and β_3-adrenoceptor stimulation. Interestingly, the up-regulation of β_3-adrenoceptor response was counteracted by KT 57201 only following chronic β_1-AR activation. These data confirm previous reports that β_3-adrenoceptors do not activate the $G\alpha_s$/PKA signalling pathway in cardiomyocytes (Germack and Dickenson, 2006; Rozec and Gauthier, 2006). In our study, the PKC inhibitor, GF 109203, also reversed β_1-adrenoceptor responsiveness following both, β_1- or β_3-adrenoceptor treatments. As previously shown, PKC mediates heterologous desensitization of β-adrenoceptors. Indeed, pretreatement of rat C6 glioma cells with phorbol 12-myristate 13-acetate (PMA, a PKC activator) produced β_1-adrenoceptor down-regulation at protein and mRNA levels (Li *et al.*, 1998). Guimond *et al.* (2005) have reported that overexpression of PKC isoforms (α, βII, ε and ζ) in HEK 293 cells transfected with β_1-adrenoceptors resulted in the decrease in β_1-adrenoceptor-induced adenylyl cyclase activity. Finally, PKC produced by the activation of angiotensin II receptors and overexpression α_{1B}-ARs reduced heart rate and inotropic response induced by β_1-ARs respectively (Schwartz and Naff, 1997; Lemire *et al.*, 1998). The PKC inhibitor also impaired the functional β_3-adrenoceptor up-regulation in chronic β_1- or β_3-adrenoceptor-treated cardiomyocytes. It is noteworthy that Gβγ subunits from GPCRs activate PI3K (Salazar *et al.*, 2007) that in turn targets PKC ε, η, ζ and λ (Toker and Cantley, 1997). These observations may explain PKC activation mediated by chronic β_1- or β_3-adrenoceptor stimulation observed in our investigation.

We found in the present study that β_1- or β_3-adrenoceptor treatments in the presence of the PI3K inhibitor wortmannin restored β_1-adrenoceptor function in cardiomyocytes indicating the recruitment of PI3K by either chronic β_1- or β_3-adrenoceptor stimulation in the regulation of β_1-adrenoceptors. Indeed, β-adrenoceptor desensitization and down-regulation require PI3Kγ recruitment through the interaction with GRK2 (Naga Prasad *et al.*, 2001; Ribas *et al.*, 2007). In addition, cardiac overexpression of inactive PI3Kγ in mice prevented β-adrenoceptor down-regulation and desensitization to chronic isoprenaline administration, and preserved β-adrenoceptor-induced left ventricle contractility and adenylyl cyclase activity (Nienaber *et al.*, 2003). Although the heart expressed two PI3K isoforms, PI3Kα and PI3Kγ the former form is mainly involved in the regulation of GPCRs (Salazar *et al.*, 2007). In contrast to β_1-adrenoceptor down-regulation, wortmannin had no effect on β_3-adrenoceptor up-regulation

induced by both, β_1- or β_3-adrenoceptor treatments. As shown previously, short term β_3-adrenoceptor stimulation induced ERK1/2 activation through $G_{i/o}$/PI3K pathway in β_3-adrenoceptor transfected CHO/K1 cells and human lung epithelial-derived cell line A549 (Gerhardt *et al*., 1998; Robay *et al*., 2005). In addition, Akt was also activated by the $G_{i/o}$/PI3K pathway in β_3-adrenoceptor transfected cells (Gerhardt *et al*., 1998) as observed in our study where chronic β_3-adrenoceptor but not β_1-adrenoceptor treatments induced Akt phosphorylation. Liu *et al*. (2005) showed that Akt binds to GRK2 and this association inhibits Akt activity. This regulation may explain why chronic β_1-adrenoceptor stimulation did not induce Akt phosphorylation while PI3K was activated. Overall, these data indicate that the chronic stimulation of both, β_1- or β_3-adrenoceptors, activates PI3K leading to β_1-adrenoceptor down-regulation. In addition, continuous β_3-adrenoceptor treatment triggered Akt activation suggesting the involvement of this subtype in cardioprotection. Indeed, the PI3K/Akt pathway is well known to induce cell viability through an antiapoptotic effect (Matsui and Rosenzweig, 2005).

Chronic β-adrenoceptor stimulation activates several members of the MAPK family including p38 MAPKs, SAPK/JNK and ERK1/2, which may be involved in the regulation of the β-adrenoceptor receptors (Palfi *et al*., 2005; Kim *et al*., 2006). The p38 MAPK inhibitor, SB 203580 inhibited the functional β_1- and β_3-adrenoceptor changes induced by β_1- or β_3-adrenoceptor treatments. In addition, both treatments activated p38 MAPK as previously suggested by Peter *et al*. (2007) regarding β_1-adrenoceptors. Indeed, chronic β_1-adrenoceptor stimulation enhances p38 MAPK activation in left ventricle from transgenic mice overexpressing β_1-adrenoceptors. In contrast to SB 203580, the SAPK/JNK inhibitor, SP 6000125 did not modify the functional β_1- and β_3-adrenoceptor response modulated by both β_1- or β_3-adrenoceptor treatments. However, chronic β_1-adrenoceptor stimulation activated SAPK/JNK as observed in rat heart following continuous isoprenaline administration (Palfi *et al*., 2005). Chronic β_1- or β_3-adrenoceptor stimulation also mediated ERK1/2 activation. Soeder *et al*. (1999) have shown that continuous β_3-adrenoceptor stimulation leads to ERK1/2 activation through the $G\alpha_i$ pathway in 3T3F442A adipocytes as observed in the present study in neonatal cardiomyocytes. However, Robidoux *et al*. (2006) showed that β_3-adrenoceptors activated ERK1/2 through a PKA-dependent and PKA-independent pathway in 3T3-L1 cells, the former involving src and epidermal growth factor receptor kinase. This seems to indicate that different pathways inducing ERK1/2 activation may be cell type dependent. However, only β_3-adrenoceptor up-regulation was sensitive to PD 98059 (MEK inhibitor). It is well known that MAPKs are involved in the regulation of transcription factors and gene expression (Edmunds and Mahadevan, 2004). The modulation of β_1- and β_3-adrenoceptor mRNA levels suggests the involvement of a transcriptional regulation in the change of receptor expression following chronic β-adrenoceptor stimulation. Therefore, β_1-adrenoceptor down-regulation and β_3-adrenoceptor up-regulation should involve the activation of transcription factors like ICER or cAMP response element binding protein via MAPK stimulation. Further studies are required to investigate possible transcriptional mechanism involved in the cross-regulation between β_1- and β_3-adrenoceptors.

In conclusion, we have presented novel data showing that chronic stimulation of β_1- or β_3-adrenoceptors leads to an opposite regulation of the receptors at the expression and functional levels. The cross-talk between β_1- and β_3-adrenoceptors occurs via the activation of PKC, PI3K, p38 MAPK and MEK/ERK1/2 pathway, and through PKA when β_1-adrenoceptors are continuously stimulated.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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