Induction of β₃-Adrenergic Receptor Functional Expression following Chronic Stimulation with Noradrenaline in Neonatal Rat Cardiomyocytes

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ABSTRACT

This study aimed to characterize β₃-adrenergic receptors (ARs) in rat neonatal cardiomyocytes using the noradrenaline (NOR) properties to modulate the expression and function of the three β-ARs. We assessed the effect of NOR (physiological nonselective agonist), isoprenaline, and dobutamine (DOB, β₁-selective agonist), and procatheol (PROC, β₂-selective agonist) on cAMP accumulation using cardiomyocytes untreated or treated with 100 μM NOR for 24 h. The inhibition of forskolin-stimulated cAMP accumulation was determined using RT-PCR, reverse transcription-polymerase chain reaction and Western blotting. NOR pretreatment decreased the activation of cAMP induced by NOR, isoprenaline, and DOB, whereas PROC response was abolished. The inhibition of NOR response by CGP 20712A or ICI 118551 demonstrated that β₁- and β₂-ARs are down-regulated and that β₃-AR functional activity was also abolished in cardiomyocytes exposed to chronic stimulation. β₃-AR function was observed with NOR and ISO when β₁-/β₂-ARs were blocked and with both β₂-selective agonists in NOR-treated cells only. This response was completely inhibited by SR 59230A and involved G protein. Furthermore, the results from functional studies agree well with those from expression experiments. In conclusion, these data provide strong evidence that β₃-ARs are functionally up-regulated and coupled to G protein in rat neonatal cardiomyocytes following chronic exposure to NOR when β₁- and β₂-ARs are down-regulated.

Three β-adrenergic receptor subtypes (β₁, β₂, and β₃-AR) have been cloned and pharmacologically characterized (Strosberg, 1995). These different subtypes belong to the G protein-coupled receptor superfamily and modulate cardiac function after stimulation by the catecholamines, noradrenaline, and adrenaline. Increases in heart rate and force of contraction are mediated mainly by β₁-ARs and to a lesser extent by the β₂-AR (Dzimiri, 1999; Steinberg, 1999). These functional effects result from the sequential activation of stimulatory G proteins, adenylyl cyclase, and protein kinase A (PKA), leading to the phosphorylation of proteins involved in cardiac contractility (troponin I, L-type Ca²⁺ channels, and phospholamban) (Post et al., 1999). Previous studies have shown that the β₂-AR also couples to G protein in cardiomyocytes (Xiao, 2001). In contrast to β₁- and β₂-AR, activation of the β₃-AR induces a negative inotropic effect in different species, including human, dog, guinea pig, and rat

ABBREVIATIONS: β-AR, β-adrenergic receptor; PKA, protein kinase A; DMEM, Dulbecco’s modified Eagle’s medium; CL 316243 (CL), 1-[2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate; ICI 118551, 1-[(2,3-dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methyl-4-indole)amino]-2-butanol hydrochloride; CGP 20712A, 2-hydroxy-5-[2-[[2-hydroxy-3-[(1-methyl-4-trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate; SR 59230A, 1-(2-ethylphenoxy)-3-[[1S]-2,3,4-tetrahydro-1-naphthalenyl]amino-(2S)-2-propanol hydrochloride; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline; NOR, noradrenaline; ISO, isoprenaline; DOB, dobutamine; PROC, procatheol; PTX, pertussis toxin.
(Gauthier et al., 1998, 1999; Kitamura et al., 2000; Cheng et al., 2001; Moniotte et al., 2001; Morimoto et al., 2004). β1-AR-mediated decrease in cardiac contractility involves coupling to G_{i/o} protein(s) and activation of constitutively expressed endothelial nitric-oxide synthase and the subsequent generation of NO (Gauthier et al., 1998; Kitamura et al., 2000; Varghese et al., 2000; Brixius et al., 2004). Furthermore, the functional activity of ionic currents such as L-type Ca^{2+} and potassium I(Ks) are reduced by β2-AR stimulation, which also modulates cardiac contractility (Kitamura et al., 2000; Cheng et al., 2001; Bosch et al., 2002; Morimoto et al., 2004). Interestingly, β1-AR expression level increases in failing myocardium in human (Moniotte et al., 2001) and dog (Cheng et al., 2001), contributing to the depression of cardiac function as shown by Morimoto et al. (2004).

In adipocytes, chronic exposure to isoproterenol (Gauthier et al., 1998, 1999; Kitamura et al., 2000; Cheng et al., 2001), contributing to the depression of cardiac function as shown by Morimoto et al. (2004).

Furthermore, in adipocytes, chronic exposure to isoproterenol induces an increase in the positive inotropic response to β1- and β2-ARs, and an increase in the negative inotropic activity induced by the β1-AR, resulting probably in the impairment of the cardiac function as shown by Morimoto et al. (2004). In contrast, β1-ARs are down-regulated, and both β1- and β2-ARs are uncoupled from G_{i/o} proteins (Dzimir, 1999). It is widely accepted that heart failure induces an increase in the activity of the sympathetic nervous system, leading to an elevation in circulating catecholamines levels (Dzimir, 1999; Steinberg, 1999). We can assume that the consequences of elevated catecholamines are a decrease in the positive inotropic response to β1- and β2-ARs, and an increase in the negative inotropic activity induced by the β1-AR, resulting probably in the impairment of the cardiac function as shown by Morimoto et al. (2004).

Materials and Methods

Materials. Bovine serum albumin, Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, fibronectin, forskolin, pertussis toxin, β,β-norepinephrine hydrochloride, (-)-propranolol, hydrochloride, CL-316243 (CL), deoxynucleotide (dNTP) mix, IOPAL CA-630, octylphenoxypolyethoxyethanol, and leupeptin were all obtained from Sigma Chemical (Poole, Dorset, UK). Trichloracetic acid was purchased from Calbiochem (Nottingham, UK). Procatelol hydrochloride, xamoterol hemifumarate, isoproterenol hydrochloride, BRL 37344 (BRL, ICI 118551, CGP 20712A, and SR 59230A were all from Tocris Cookson Inc. (Bristol, UK). RQ1 RNase-free DNase, RNasin RNase inhibitor, dithiothreitol (molecular grade), random primers, Moloney murine leukemia virus reverse transcriptase, and TaqDNA polymerase were all purchased from Promega (Southampton, UK). (8-β)Adenosine was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Rat β1- (sc-568), β2- (sc-570), and β3- (sc-1473)-adrenergic receptor antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture. Neonatal ventricular myocytes were prepared from 1- to 4-day-old Wistar rats using the neonatal cardiomyocyte isolation system (Worthington Biochemicals, Lornes Laboratories, Reading, UK) as described previously (Germack and Dickenson, 2004). The cells were preplated three times for 30 min in a humidified incubator (95% air, 5% CO2 at 37°C) in DMEM supplemented with 2 mM L-glutamine, 10% (v/v) calf serum and penicillin/streptomycin (100 U/ml) to minimize fibroblast contamination. Cardiomyocyte-rich cultures (>90%) were plated onto fibronectin-coated plates at a final density of 1.25 × 10^5 cells/cm² in supplemented DMEM. For cAMP accumulation assay, the cells were plated onto 24-well plates; and for RT-PCR and Western blotting, cells were plated onto 20-cm² dishes. After 3 days, confluent and spontaneously beating cells were serum starved overnight before the experiments and treated or not treated with 100 μM noradrenaline.

CAMP Accumulation Assay. After serum starvation of cardiomyocytes (overnight), assays were carried out in serum-free DMEM in a humidified incubator (95% air, 5% CO2 at 37°C). Agonists, antagonists, and/or inhibitors according to the experiments were added as described in the figure legends. The cells were incubated for 3 h in a humidified incubator (95% air, 5% CO2 at 37°C) with 500 μM of serum-free DMEM containing [3H]adenine (2 μCi/well). [3H]Adenine-labeled cells were washed twice with Hank’s/HEPES buffer and then incubated in 500 μl/well serum-free DMEM containing the cyclic AMP phosphodiesterase inhibitor rolipram (10 μM) for 15 min at 37°C in a humidified incubator. Agonists were added 5 min before 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 of 5% trichloroacetic acid after removing the medium. [3H]Cyclic AMP was isolated by sequential Dowex-alumina chromatography as described previously (Dickenson and Hill, 1998). After elution, the levels of [3H]cyclic AMP were determined by liquid scintillation counting.

RT-PCR. Total RNA was extracted from cardiomyocytes treated or not treated with 100 μM noradrenaline using RNA isolation reagent, RNAlwit (Ambion, Eurotech, Huntingdon, UK). Total RNA was purified by chloroform/water extraction and isopropanol precipitation. All RNA preparations were treated with RQ1 DNase (1 U/μl) for 20 min at 37°C in the presence of RNasin (40 U/μl) and dithiothreitol (100 mM).

Reverse transcription was performed with 40 μg of total RNA for the synthesis of cDNA using hexadeoxynucleotide random primers (540 ng/ml), RNasin (40 U/μl), dNTP (5 mM), and Moloney murine leukemia virus reverse transcriptase (200 U/ml) for 90 min at 42°C. Polymerase chain reaction was conducted with 1 μl of cDNA for β2-actin, 3 μl for β1- and β2-ARs, and 5 μl for β3-AR in the presence of dNTPS (1.25 mM), 200 ng of respective primers, and 1.5 U of TaqDNA polymerase. Following polymerase chain reaction, the samples were denatured for 5 min at 95°C. The amplification steps involved 1-min denaturation at 94°C for β1- and β2-AR cDNA and 1.5 min for β3-AR and β-actin; 1 min annealing for β1-, β2-, and β3-AR and 1.5 min for β3-AR; and 1 min extension at 72°C for β1- and β2-AR and β-actin and 2 min for β3-AR cDNA. Annealing temperatures, sequences of the primers and amplification cycle number are given in Table 1. The RT-PCR products were analyzed using 1.5% agarose gel electrophoresis. β-Actin mRNA was used as an internal standard. The RT-PCR products were quantified by densitometry using GeneGenius BioImaging system (Syngene; Synoptics Ltd., Cambridge, UK) and normalized to the signal of β-actin.

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Western Blot Analysis. Cardiomyocytes treated or not treated with 100 μM noradrenaline were washed with ice-cold phosphate-buffered saline and lysed in ice-cold hypotonic buffer (30 mM Tris-HCl, 5 mM MgCl₂, 100 mM NaCl, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin). Cell membranes were collected by centrifugation for 30 min at 70,000g and 4°C. The resulting plasma membrane pellets were dissolved in detergent buffer ([150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% (v/v) IGEPAL (TBS, 5% (w/v) skimmed milk powder, and 0.1% Tween 20]). Blots were then incubated overnight at 4°C with the primary antibody. Following removal of the secondary antibody, blots were extensively washed as described above and developed using the enhanced chemiluminescence detection system (GE Healthcare) and quantified by densitometry using GeneGenius BioImaging System (Syngene).

Statistical Analysis. Results are expressed as means ± S.E. Concentration-response and inhibition-response curves were analyzed by computer-assisted iteration using Prism (GraphPad Software Inc., San Diego, CA). Statistical significance was determined by Student’s t test, and P < 0.05 was considered as the limit of statistical significance.

Results

Effect of Nonselective, β₁-, and β₂-Selective Agonists on cAMP Accumulation in Neonatal Rat Cardiomyocytes Untreated or Treated with 100 μM Noradrenaline. It is well known that chronic stimulation of β-ARs causes a decrease in β-adrenergic response by down-regulation of the β-ARs (Dzimiri, 1999; Steinberg, 1999). Indeed, as shown in Fig. 1 and Table 2, the response to noradrenaline (NOR; physiological α- and β-adrenergic agonist) and isoprenaline (ISO; nonselective β-adrenergic agonist) was significantly reduced by 61 and 77%, respectively, following the pretreatment with 100 μM NOR for 24 h. The desensitization of the functional response depends mainly on the alteration of both, β₁- and β₂-ARs (Dzimiri, 1999; Steinberg, 1999).

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR Product</th>
<th>Annealing Temperature</th>
<th>No. of Cycles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₁-AR</td>
<td>Forward 5′-CTCACGCACCCTTCCTATCCTCTCATG-3′</td>
<td>60</td>
<td>33</td>
<td>Podlowski et al. (1998)</td>
</tr>
<tr>
<td>β₁-AR</td>
<td>Reverse 5′-GAGCCGCTCGGCTGATCGTGTCG-3′</td>
<td>60</td>
<td>33</td>
<td>Podlowski et al. (1998)</td>
</tr>
<tr>
<td>β₂-AR</td>
<td>Forward 5′-CTCCTCCTCAGCGTCAAGGTCG-3′</td>
<td>60</td>
<td>40</td>
<td>Bensaid et al. (1993)</td>
</tr>
</tbody>
</table>

Fig. 1. Activation of cAMP accumulation by nonselective β agonists noradrenaline (A) and isoprenaline (B) in neonatal rat cardiomyocytes untreated () or treated with 100 μM noradrenaline (○) for 24 h. cAMP accumulation was measured as described under Materials and Methods. Data are expressed as percentage of the basal level of cAMP accumulation (100%). Each point represents the mean ± S.E. of three to four experiments.
Therefore, we determined the stimulation of cAMP induced by dobutamine (DOB; β₁-selective agonist) and procaterol (PROC; β₂-selective agonist) in untreated and treated cardiomyocytes (Fig. 2; Table 2). Pretreatment with 100 μM NOR resulted in a significant decrease in DOB-induced cAMP production by 81% without change in potency and abolished the PROC response (Fig. 2B; Table 2). The β₂-AR subtype is also coupled to PTX-sensitive G proteins in addition to the classical G<sub>s</sub>/cAMP/PKA pathway (Xiao, 2001). Therefore, β₂-AR-mediated G<sub>s</sub> activation can counteract G<sub>i</sub>-mediated cAMP production. This dual pathway may explain the low cAMP response to PROC stimulation in untreated cells and the absence of stimulation in treated cardiomyocytes. To determine whether the β₂-AR response involves G<sub>i</sub> coupling, cAMP accumulation was performed in the presence of PTX in treated and untreated cells. Pretreatment with PTX did not modify PROC-induced cAMP production (Fig. 2B; Table 2), showing the absence of β₂-AR coupling to G<sub>i</sub> proteins in neonatal rat cardiomyocytes. In addition, these results indicate that the nonselective agonist response in NOR-treated cardiomyocytes involves the β₁-AR subtype rather than the β₂-AR.

Effect of β₁- and β₂-Selective Antagonists on NOR-Induced cAMP Accumulation in Neonatal Rat Cardiomyocytes Untreated or Treated with 100 μM Noradrenaline. To characterize further the β-AR subtypes involved in NOR-induced cAMP accumulation, we studied the inhibition of 1 μM NOR response by the selective β₁-antagonist CGP 20712A and the β₂-antagonist ICI 118551 in cardiomyocytes untreated or treated with 100 μM NOR (Fig. 3). As expected, NOR pretreatment induced a decrease in 1 μM NOR response by 72% (treated, 66 ± 7% versus untreated, 239 ± 11%; n = 7; P < 0.001). As shown in the Fig. 3A, CGP 20712A inhibited by 58 ± 4% NOR-induced cAMP accumulation in untreated cells. Following the pretreatment, NOR-induced cAMP response was fully inhibited (91 ± 9% versus untreated; P < 0.01), indicating that the residual NOR response (42%) observed in untreated cells may involve β₂-AR stimulation. Pretreatment with 100 μM NOR did not modify the CGP 20712A potency evaluated by its IC<sub>50</sub> expressed as −log<sub>10</sub> IC<sub>50</sub> (pIC<sub>50</sub>), treated 7.83 ± 0.15 (14.7 nM; n = 3) versus untreated 7.72 ± 0.21 (19.1 nM; n = 3). Interestingly, ICI 118551 exhibited a significant biphasic inhibition curve in untreated cardiomyocytes (P < 0.01; Fig. 3B). The first population of receptors with a high inhibition potency (pIC<sub>50</sub> = 8.39 ± 0.43; 4.1 nM; n = 4) accounted for 32 ± 7% of the total inhibition. The second part of the curve elicited 68 ± 14% inhibition response with a pIC<sub>50</sub> value of 5.24 ± 0.30 (5.8 μM; n = 4). Interestingly, the inhibition curve following NOR treatment was monophasic. The ICI

![Graph](https://via.placeholder.com/150)

**Fig. 2.** Activation of cAMP accumulation by dobutamine, β₁-selective agonist (A) and procaterol, β₂-selective agonist (B) in neonatal rat cardiomyocytes untreated (○) or treated with 100 μM noradrenaline (□) for 24 h. cAMP accumulation was measured as described under Materials and Methods. Data are expressed as percentage of the basal level of cAMP accumulation (100%). Each point represents the mean ± S.E. of four experiments.

### TABLE 2

Effect of nonselective and β₁- and β₂-selective agonists on cAMP accumulation in newborn rat cardiomyocytes treated or untreated with 100 μM noradrenaline.

<table>
<thead>
<tr>
<th>β-Adrenergic Agonist</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (−log&lt;sub&gt;10&lt;/sub&gt; EC&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>K&lt;sub&gt;max&lt;/sub&gt; (% over basal response)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>Nonselective agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>6.91 ± 0.11</td>
<td>6.54 ± 0.09</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>7.63 ± 0.10</td>
<td>7.51 ± 0.43</td>
</tr>
<tr>
<td>β₂-Selective agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dobutamine</td>
<td>6.27 ± 0.12</td>
<td>6.13 ± 0.19</td>
</tr>
<tr>
<td>β₁-Selective agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Procaterol</td>
<td>7.45 ± 0.10</td>
<td>NR</td>
</tr>
<tr>
<td>Procaterol + PTX</td>
<td>7.18 ± 0.37</td>
<td>NR</td>
</tr>
</tbody>
</table>

NR, no response.

*P < 0.05, **P < 0.01, and ***P < 0.001 versus untreated cells.
118551 potency was 5.63 ± 0.30 (2.34 μM; n = 4) corresponding to the low-potency population with an inhibition response of 93 ± 6% (P < 0.01 versus untreated), suggesting a complete down-regulation of the β2-AR subtype in accordance with PROC-induced cAMP accumulation (Fig. 2B). These data indicate that cAMP accumulation induced by NOR involved mainly β1-AR subtype and weakly β2-ARs, which is completely down-regulated following a chronic stimulation.

**Effect of Nonselective and β3-Selective Agonists on Forskolin-Induced cAMP Accumulation in Neonatal Rat Cardiomyocytes Untreated or Treated with 100 μM Noradrenaline.** The β3-AR coupled to G<sub>io</sub> protein is up-regulated in heart disease where NOR level is increased (Cheng et al., 2001; Moniotte et al., 2001) and in rat adipocytes following chronic agonist exposure (Thomas et al., 1992). However, at present it is not known whether the β3-AR is up-regulated in rat heart following agonist exposure. Therefore, we determined the effect of the nonselective agonists NOR and ISO and the selective β3-AR agonists BRL and CL on forskolin-induced cAMP accumulation in untreated and treated neonatal rat cardiomyocytes with 100 μM NOR for 24 h (Figs. 4, B and C, 5, 6, and 7).
We used 1.5 μM forskolin to study β₂-AR stimulation. This concentration was under the maximal response of forskolin-induced cAMP accumulation as illustrated in Fig. 4A. In untreated and treated cells, no significant inhibition was observed using both nonselective agonists. Indeed, NOR potentiated forskolin-induced cAMP accumulation (E\textsubscript{max} of 829 ± 80% over the forskolin response; n = 3) with a pEC\textsubscript{50} value of 7.23 ± 0.18 (Fig. 4, B and C). Pretreatment with 100 μM NOR induced a significant decrease in the maximal response (E\textsubscript{max} of 115 ± 38%; n = 4; P < 0.001) without change in the potency (7.13 ± 0.19). Similarly shown in Fig. 5, ISO stimulated a marked increase in forskolin-induced cAMP accumulation (E\textsubscript{max} of 1883 ± 293%; n = 4), which was significantly down-regulated after treatment with NOR (E\textsubscript{max} of 257 ± 56%; n = 4; P < 0.01). The pretreatment did not modify the pEC\textsubscript{50} (treated cells, 7.23 ± 0.40 versus untreated cells, 7.80 ± 0.09). Interestingly, in the presence of 0.5 μM propranolol (nonselective β-AR antagonist), a concentration that inhibits β₁/β₂-AR responses (Strosberg, 1995; Germack et al., 1997), NOR-induced inhibition of forskolin-stimulated cAMP accumulation (Fig. 4; Table 3). ISO also inhibited the forskolin-induced cAMP response in treated cardiomyocytes after incubation with 1 μM CGP 20712A and ICI 118551, selective β₁- and β₂ antagonists, respectively (Fig. 5; Table 3). Since the percentage of inhibition with both nonselective agonists was similar, this would seem to exclude an effect of α\textsubscript{1b}-AR in NOR-mediated inhibition of the forskolin response. This adrenergic subtype highly expressed in neonatal cardiomyocytes is negatively coupled to calcium channel via G\textsubscript{i} protein and therefore could inhibit adenylyl cyclase activity (Deng et al., 1996, 1998). These data suggest that the functional expression of the β₂-AR in neonatal rat cardiomyocytes after treatment with 100 μM NOR.

In untreated cells BRL potentiated forskolin-induced cAMP accumulation (645 ± 34% over forskolin response; n = 4) with a pEC\textsubscript{50} value of 6.03 ± 0.07 (Fig. 6). In contrast, the other selective β₂-AR agonist CL had no effect on forskolin-induced cAMP accumulation. Since the affinity of the β₂-AR for BRL in functional assays is around 1 nM (Germack et al., 1997, 2000), the low potency would indicate the involvement β₁/β₂-ARs. Following 24-h pretreatment with NOR, the effects of BRL on forskolin-stimulated cAMP accumulation were biphasic. At concentrations below 0.1 μM, BRL inhibited forskolin-stimulated cAMP accumulation by 17 ± 1% with a potency of 6.47 ± 0.99 (n = 4). The potency was similar to the pEC\textsubscript{50} without treatment and the response was decreased by 97% (P < 0.001), suggesting a down-regulation of β₁/β₂-ARs. Finally, 0.5 μM propranolol had no significant effect on BRL-mediated inhibition of forskolin cAMP accumulation (Fig. 6; Table 3). However, propranolol inhibited BRL-induced cAMP responses in untreated cells, indicating

![Fig. 5](image-url)  
**Fig. 5.** Effect of isoprenaline on forskolin-induced cAMP accumulation in neonatal rat cardiomyocytes untreated (□) or treated with 100 μM noradrenaline for 24 h in the absence (○) or presence of 1 μM CGP 20712A and ICI 118551 (●). cAMP accumulation was measured as described under Materials and Methods. Data are expressed as the percentage of 1.5 μM forskolin response in the absence of agonist (100%). Each point represents the mean ± S.E. of four independent experiments. B corresponds to the enlargement of the frame in A.

**TABLE 3**  
Effect of 100 μM noradrenaline treatment on nonselective and β₂-selective agonist-mediated inhibition of forskolin-induced cAMP accumulation in newborn rat cardiomyocytes in the presence or absence of β-antagonists

<table>
<thead>
<tr>
<th>β-Adrenergic Agonist</th>
<th>IC\textsubscript{50} (nM)</th>
<th>I\textsubscript{max} (%)</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>Treated + Antagonists</td>
<td>Treated</td>
</tr>
<tr>
<td>Nonselective agonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>7.95 ± 0.30</td>
<td>8.39 ± 0.34</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td>8.39 ± 0.34</td>
<td>8.75 ± 0.18</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>BRL 37344</td>
<td>9.52 ± 0.59</td>
<td>9.41 ± 0.32</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>CL 316243</td>
<td>7.79 ± 0.48</td>
<td>7.85 ± 0.18</td>
<td>36 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of four to six experiments performed in duplicate. The potencies of the agonists were evaluated by their IC\textsubscript{50}, concentration of agonist inducing 50% inhibition expressed in \(-\log\textsubscript{10} IC\textsubscript{50}\). I\textsubscript{max} is the maximal percentage of inhibition. Cardiomyocytes were treated with 100 μM noradrenaline for 24 h. Propranolol (0.5 μM) was used in the experiments with NOR and the β₂-AR agonists, CGP 20712A and ICI 118551 (1 μM) were used altogether in the experiments with ISO.
strongly an activation of \(\beta_1/\beta_2\)-ARs. Similarly, CL decreased forskolin-induced cAMP production in treated cells and 0.5 \(\mu\)M propranolol did not change significantly the functional parameters (Fig. 7; Table 3). Not only the functional expression of the \(\beta_2\)-AR subtype but also its coupling to \(G_i\) were strengthened by using the selective \(\beta_2\)-AR antagonist SR 59230A (Fig. 8A) and pertussis toxin (PTX; Fig. 8B), respectively, which both abolished BRL- and CL-induced inhibition of forskolin response in NOR-treated cardiomyocytes.

\(\beta_1\), \(\beta_2\), and \(\beta_3\)-AR mRNA and Protein Expression in Neonatal Rat Cardiomyocytes Untreated or Treated with 100 \(\mu\)M Noradrenaline. To confirm the functional expression of the \(\beta_3\)-AR subtype in neonatal rat cardiomyocytes and its up-regulation after 24-h incubation with 100 \(\mu\)M NOR, mRNA and protein expression of the three \(\beta\)-AR subtypes were examined by RT-PCR and Western blotting.
respectively (Figs. 9 and 10). Both $\beta_1$ and $\beta_2$-AR mRNA ($n = 6$) and protein expression ($n = 4$) were significantly reduced, following NOR treatment, by around 40 and 55% of the control, respectively. The $\beta_2$-ARs seemed more sensitive to down-regulation than the $\beta_1$-AR, which may explain the absence of the $\beta_2$-AR response after treatment with NOR. In contrast, $\beta_3$-AR mRNA and protein levels increased by 156 and 169% of the control ($n = 5; P < 0.05$) in treated cardiomyocytes, respectively. These data are in agreement with the functional studies showing a down-regulation of $\beta_2/\beta_3$-AR response and an up-regulation of the $\beta_3$-AR following NOR treatment for 24 h.

**Discussion**

We show for the first time in this report that the $\beta_3$-AR subtype coupled to Gi, is up-regulated and functional after chronic stimulation with noradrenaline when $\beta_1$- and $\beta_2$-ARs are down-regulated in neonatal rat cardiomyocytes. $\beta_1$- and $\beta_2$-ARs regulate cardiac function by increasing the rate and the force of contraction after stimulation by the catecholamines (noradrenaline and adrenaline) via $G_i/cAMP/ PKA$ signaling pathway (Dzimiri, 1999; Steinberg, 1999). It is also well established that $\beta_1$- and $\beta_2$-ARs are down-regulated after a chronic stimulation. As expected, in this study, 24-h NOR pretreatment induced a decrease in NOR (physiological agonist) and ISO ($\beta$-nonselective agonist) response by 61 and 77%, respectively (Fig. 1; Table 2). In addition, cAMP production induced by the $\beta_1$-selective agonist (dobutamine) was decreased by 81% in treated cardiomyocytes, whereas the $\beta_2$ response using procaterol was abolished (Fig. 2; Table 2). The inhibition study using CGP 20712A ($\beta_1$-selective antagonist) and ICI 118551 ($\beta_2$-selective antagonist) (Fig. 3) supports the findings obtained in agonist-induced cAMP response. Indeed, 1 mM NOR-induced cAMP accumulation was partially blocked by CGP 20712A in untreated cardiomyocytes, whereas NOR response was abolished following 24-h exposure to NOR without change in pIC$_{50}$, indicating the same population of receptors. Interestingly, the biphasic inhibition of NOR response by ICI 118551 indicated the presence of a minor population of receptors (32 ± 7% of inhibition) with high potency in untreated cells, which disappeared following NOR pretreatment. These data indicate that not only $\beta_2$-AR is more sensitive to down-regulation than $\beta_1$-ARs but also $\beta_2$-AR subtype represents the minor population of $\beta$-ARs in normal physiological conditions in neonatal cardiomyocytes. This last finding is in agreement with the dose-response curves using $\beta_1$- and $\beta_2$-selective agonists previously discussed. Indeed, the maximal response induced by the $\beta_2$-
selective agonist was lower by 57% than β₁-AR maximal stimulation in untreated cells, indicating clearly that the β₁-ARs are mainly expressed in neonatal cardiomyocytes in normal physiological conditions as already shown in heart from different species (Steinberg, 1999). Likewise, the expression of β₁- and β₂-ARs evaluated by RT-PCR and Western blotting (Figs. 9 and 10) revealed that β₁-ARs are highly expressed and resistant to a chronic NOR stimulation compared with β₂-ARs. Susuki et al. (1992) have shown that β₁- and β₂-ARs expressed in Chinese hamster fibroblast at the same expression level were also differently regulated following 24-h pretreatment with isoprenaline. β₂-ARs are also coupled to PTX-sensitive Gi protein in addition to the classical Gs/cAMP/PKA pathway in adult rat and mouse cardiomyocytes (Xiao et al., 1995, 1999) and human atrium (Kilts et al., 2000). However, in the present study, PTX pretreatment did not modify proproterol response in untreated or treated cells, suggesting that β₂-ARs are not coupled to PTX-sensitive Gi protein in neonatal rat cardiomyocytes as already shown in these cells (Vitalyi et al., 2003). Overall, these results demonstrate that β₁- and β₂-ARs are involved in Gi/cAMP/PKA signaling pathway and differentially downregulated in neonatal cardiomyocytes.

In contrast to β₁- and β₂-ARs, the β₃-AR induces a decrease in cardiac contractility in different species, including human, dog, guinea pig, and rat (Gauthier et al., 1999; Kitamura et al., 2000; Cheng et al., 2001; Morimoto et al., 2004). A coupling of β₃-ARs to G_{i/o} protein and the activation of endothelial nitric-oxide synthase mediate this negative inotropic effect (Gauthier et al., 1998; Kitamura et al., 2000; Varghese et al., 2000; Brixius et al., 2004). Therefore, the modulation of cardiac contractility by the β₃-ARs seems to involve the inhibition of the adenylyl cyclase and the decreased in intracellular cAMP content. In contrast, this subtype controls lipolysis in rat white adipocytes (Van Liefde et al., 1992; Germack et al., 1997) and thermogenesis in brown adipocytes (Atgie et al., 1997) through Gs protein activation. However, a dual coupling of the β₁-ARs to Gs and G₁ has been reported in white adipocytes (Chaudhry et al., 1994) and 3T3-F442A adipocytes (Soeder et al., 1999). β₃-ARs are also expressed in gastrointestinal smooth muscles and mediate relaxation through Gs protein and cAMP-independent mechanism (Horinouchi et al., 2003). To determine the physiological significance of β₃-ARs, we determine the effect of NOR on forskolin-induced cAMP accumulation (Fig. 4; Table 3). NOR produced an inhibition of forskolin response in NOR-treated cardiomyocytes only when propranolol was added and used at the concentration, which inhibits β₁- and β₂-ARs. Similarly, forskolin-mediated cAMP accumulation was inhibited by ISO in the presence of the selective β₁- and β₂-antagonists following a chronic stimulation with NOR (Fig. 5; Table 3). Gauthier et al. (1998) have also reported that NOR induced a negative inotropic effect when α- and β₁/β₂-ARs were inhibited in human heart. Similarly, β₁-AR decreased potassium channel activity in the presence of β₁- and β₂-antagonists in guinea pig cardiomyocytes (Bosch et al., 2002). These observations indicate that β₃-ARs are not involved in the regulation of the cardiac function in normal physiological conditions (neonatal cardiomyocytes; this study) or that this subtype plays a minor role in the modulation of the functional response (human and guinea pig). NOR and ISO pIC₅₀ values were in the same range as the corresponding potencies for cAMP accumulation observed in rodent β₃-ARs expressed in Chinese hamster ovary cells (Strosberg, 1997), suggesting the expression of β₃-ARs in neonatal cardiomyocytes when β-ARs are continuously activated. The inhibition of forskolin.

![Fig. 10. Expression of β₁-, β₂-, and β₃-AR protein obtained from untreated (control, C) and treated (T) with 100 μM noradrenaline for 24 h neonatal rat cardiomyocytes. Cell membrane homogenates were monitored by Western blotting for β₁-, β₂-, and β₃-ARs as described under Materials and Methods. The immunoblots are representative of the experiments summarized in the graph. Data are expressed as the percentage of untreated cardiomyocytes (100%). Each point represents the mean ± S.E. of four to five independent experiments. *P < 0.05; and **P < 0.01 versus untreated control.](4002727725815-1.png)
response by BRL and CL, both β2-selective agonists, was in the similar range in cardiomyocytes treated with NOR in absence or presence of propranolol to prevent β1- and β2-ARs effect (Figs. 6 and 7; Table 3). Moreover, BRL potentiated forskolin response with a low activity in untreated cardiomyocytes contrary to CL, which had no effect. Furthermore, following NOR pretreatment, BRL dose-response curve was biphasic and displayed a down-regulation of the low-potency component, indicating that this agonist lacks selectivity at concentrations >100 nM and stimulates β1- and β2-ARs. In addition, in this study, the potency order of BRL > ISO > NOR in NOR-treated cells was characteristic of the β2-AR subtype as reported previously (Strosberg, 1997; Atgie et al., 1997; Germack et al., 1997; Horinouchi et al., 2003). Interestingly, CL is more potent in adipocytes than in human heart to stimulate β2-ARs (Atgie et al., 1997; Umekawa et al., 1997, 1999; Gauthier et al., 1999) as we also found in neonatal cardiomyocytes. Rodent β2-AR gene contains two introns, which by alternate splicing can generate two variants, which differ at the C-terminal region (Strosberg, 1997). The different expression of one of both isoforms can explain the pharmacological difference between adipocytes and cardiomyocytes. The inhibition of forskolin by BRL and CL was abolished using the β2 selective antagonist SR 59230A (Fig. 8) in NOR-treated cells. Furthermore, PTX treatment also counteracted completely both agonist responses (Fig. 8), indicating clearly the involvement of G protein in β2-AR transduction pathway in neonatal rat cardiomyocytes as shown in human, dog, and guinea pig heart (Gauthier et al., 1999; Kitamura et al., 2000; Cheng et al., 2001; Morimoto et al., 2004). Results from functional studies agree well with those from RT-PCR and Western blotting (Figs. 9 and 10), showing an up-regulation of the β2-AR subtype following NOR pre-treatment. Overall, these data provide strong evidence that β2-ARs are functionally expressed after being up-regulated following a chronic stimulation in neonatal cardiomyocytes. Similarly, Thomas et al. (1992) demonstrated that adipocytes continuously stimulated with isoprenaline display an enhancement in β2-AR level and a reduction of β1-AR subtype. Although no β2-AR functional response was observed in untreated neonatal cardiomyocytes, the present data may partially explain the up-regulation of β2-ARs described in failing myocardium (Cheng et al., 2001; Moniotte et al., 2001), especially as circulating catecholamines are elevated in heart failure (Dzimirri, 1999; Steinberg, 1999). Morimoto et al. (2004) demonstrated that the functional up-regulation of the β2-ARs contributes to the depression of the cardiac function in cardiomyocytes from dog with pacing-induced cardiac heart failure. Therefore, the regulation of cardiac contractility by catecholamines in heart disease indicate an opposite compensatory modifications between β/β2- and β2-ARs. Kohout et al. (2001) have reported that β1-AR expression was lower in transgenic mice displaying cardiac specific overexpression of the human β2-AR. In contrast, β2-AR knockout mice exhibited an increase in β1-AR mRNA in white and brown adipose tissue (Susulic et al., 1995). Finally, β1-AR subtype decreases and β2-AR level increases in hearts from diabetic rats explaining partially the impairment in cardiac function in chronic diabetes (Dinger et al., 2001).

In conclusion, we demonstrate for the first time that the β2-AR subtype, which was not functional in neonatal rat cardiomyocytes under normal physiological conditions, was functionally up-regulated following chronic exposure to noradrenaline and inhibited cAMP accumulation through G1 protein coupling, whereas, β1- and β2-ARs were down-regulated. The rank order of functional expression of β-ARs is β1 > β2 in physiology conditions and β1 > β2 in chronic β-AR stimulation. Neonatal rat cardiomyocytes represent a suitable in vitro model to study further the role of β2-AR subtype in heart disease.

References
Podlofski S, Luther HP, Morwinski R, Muller J, and Wallukat G (1996) Agonistic anti β2-adrenoceptor receptor autoantibodies from cardiomyopathy patients reduce...


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