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# Agonist-induced Down-Regulation of the $\beta_2$-Adrenoceptor and Its mRNA in Human Mononuclear Leukocytes

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**ABSTRACT.** Agonist-mediated regulation of $\beta_2$-adrenoceptors in mononuclear leukocytes has been examined at the protein but not at the mRNA level. In the present study, incubation of mononuclear leukocytes with the β-agonist (−)-isoproterenol ($10^{-6}$ M) for up to 42 hr led to a maximum decrease in both $\beta_2$-adrenoceptor mRNA concentration and total receptor number of *ca.* 56 and 70%, respectively. The decrease in the mRNA level, however, was slower than for the protein level. After 4 hr of incubation with the β-agonist, the protein level decreased to a minimum of 65% of the initial amount, while an incubation of 8 hr was necessary to reach a similar decrease in the level of mRNA (69% of the initial level). Measurements of mRNA stability revealed a reduction in the half-life of $\beta_2$-adrenoceptor mRNA from 2.7 to 1.1 hr following 4 hr of incubation with (−)-isoproterenol. Our data clearly demonstrate that treatment of human mononuclear leukocytes with (−)-isoproterenol induces a $\beta_2$-adrenoceptor down-regulation together with a slower time course of mRNA down-regulation which is partly due to a reduction of mRNA stability. BIOCHEM PHARMACOL **56**;8:967–975, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** $\beta_2$-adrenoceptor; $\beta_2$-adrenoceptor mRNA; mononuclear leukocytes

β-Adrenoceptors are members of a large family of transmembrane receptors that mediate their signal transduction via G proteins. They play an important role in mediating the effects of catecholamine release on the inotropic and chronotropic actions of the heart.

The process by which cellular sensitivity to hormone and neurotransmitter stimulation becomes attenuated over time is called desensitization. Down-regulation of β-adrenoceptors is preceded by a desensitization of the adrenergic system and is a slow process lasting several hours [1]. Down-regulation can be caused by an increase in receptor degradation or reduced synthesis of the receptor [2]. The underlying mechanisms, which are complex, have been investigated for the β-adrenergic stimulation of the adenylylcyclase system. Adenylylcyclase is an enzyme (EC 4.6.1.1) that converts ATP to the signal molecule cyclic AMP, which acts as second messenger for a variety of hormones, neurotransmitters, and drugs. For example, in Chinese hamster fibroblast cells, the cyclic AMP analogue dibutyryl cAMP induces a time-dependent down-regulation of a number of $\beta_2$-adrenoceptors. This down-regulation is accompanied by a decline in the steady-state level of $\beta_2$-adrenoceptor mRNA [3]. Chronic stimulation with the β-agonist (−)-isoproterenol results in a decrease in β-adrenoceptor number and mRNA in $DDT_1$ MF-2 hamster cells [1], S49 mouse lymphoma mutants [4], and rat lung tissues [5]. In human, Bristow *et al.* [6] and Ungerer *et al.* [7] have shown that heart failure caused a decrease in $\beta_1$-adrenoceptor protein and mRNA while $\beta_2$-adrenoceptors were unchanged. In both dilated and ischemic heart failure, β-adrenergic receptor kinase mRNA levels were increased almost 3-fold and its activity was enhanced. Agonist-occupied β-adrenoceptors are uncoupled from $G_s$ proteins when they are phosphorylated by the β-adrenoceptor kinase. Regulation of β-adrenoceptors has been investigated in various systems including lymphocytes [8–10]. Changes in the amount of β-adrenoceptor protein after exposure to agonists are well known [9]. Nevertheless, few studies examining the regulation of β-adrenoceptor mRNA in combination with changes in receptor densities have been published. This has been examined in CH-$\beta_2$-hamster fibroblasts [3], $DDT_1$ MF-2 hamster cells [1], and S49 mouse lymphoma cells [4]. Studies of receptor density [11, 12] and mRNA [7] have been performed in human but exclusively in patients with heart failure, which is related to an increased level of catecholamines. These studies register

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only an end state of a long-lasting development. Mononuclear leukocytes that can be cultured provide a suitable human model to examine changes in β-receptor and mRNA due to agonist treatment.

Although the agonist-induced down-regulation of $\beta_2$-adrenoceptors in mononuclear leukocytes is well known, no reports concerning the time course of mRNA regulation during $\beta_2$-adrenoceptor down-regulation and agonist treatment exist. Furthermore, causes of down-regulation (either reduced mRNA stability or reduced transcription rate) of $\beta_2$ mRNA are still controversial.

We demonstrate the time-dependent influence of agonist treatment on human mononuclear leukocytes at both receptor protein and mRNA levels and attempt to ascertain causes of this regulation. To quantify the amounts of $\beta_2$-adrenoceptor mRNA, we used the competitive RT-PCR.* This method offers higher sensitivity than Northern blot analysis and permits molar quantification of low-abundance mRNA species.

## MATERIALS AND METHODS

### *Patients*

Subjects were healthy blood donors that had not been treated medically for at least 2 weeks. Five male subjects were included. The average age of the five subjects was 26 ± 4 years (N = 5). In detail, their ages were 26, 24, 28, 32, and 21 years. Peripheral venous blood (500 mL) was taken after 15 minutes' bed rest. Anticoagulated blood samples (1.6 mL of EDTA per mL blood) were centrifuged and the buffy coat separated from the erythrocyte concentrate. The buffy coat contains a concentrated mononuclear leukocyte suspension and thrombocytes.

### *Isolation and Culturing of Mononuclear Leukocytes*

Mononuclear leukocytes were isolated according to the protocol of Young and Steinman [13] from peripheral venous blood. Cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum (vol/vol), $NaHCO_3$ (44 mM), glucose (5.5 mM), glutamine (5 mM), nonessential amino acids (1%), sodium pyruvate (1 mM), and gentamicin (35 μg/mL) in a humidified atmosphere of 90% air and 10% $CO_2$ at 37°. All supplements were purchased from Life Technologies except glutamine (Biochrom). The cell density was maintained at $10^6$ cells/mL. (−)-Isoproterenol (Sigma) was added to a final concentration of $10^{-6}$ M. Isoproterenol was protected from oxidation by addition of ascorbic acid in a final concentration of $10^{-4}$ M.

### *Radioligand Binding Assays*

Washed mononuclear leukocytes were resuspended in Dulbecco's modified Eagle's medium resulting in a cell density of $2 \times 10^6$ cells per mL. Aliquots of 100 μL were used. Binding assays were carried out with (−)-[$^{125}$I]-iodocyanopindolol ((−)-[$^{125}$I]CYP; 5–120 pM; 2000 Ci/mmol; Amersham). Nonspecific binding and surface receptor number were assessed using (−)-propranolol ($10^{-6}$ M) and the hydrophilic ligand CGP-12177 ($10^{-6}$ M; Research Biochemicals Inc.), respectively. The results were analyzed by Scatchard plots [14]. Cell viability was monitored during the course of these experiments using trypan blue (Sigma).

### *Extraction of RNA*

Total RNA from mononuclear leukocytes was isolated by binding to a silica-based membrane using a microspin column technique according to the manufacturer's protocol ("RNeasy™", Qiagen). Remaining traces of DNA were digested in a final volume of 100 μL with 10 units of DNase I (FPLC*pure*, Pharmacia) in a buffer containing 10 mM of Tris-HCl, 10 mM of $MgCl_2$, 50 mM of KCl and 10 mM of dithiotreitol (DTT), pH 9.0 for 30 min at 37°. With an additional clean-up procedure, DNase I-treated RNA was recovered by adsorption on silica-based membranes as described for the total RNA extraction procedure.

RNA was free of genomic DNA as shown by the absence of a signal after PCR without reverse transcription. The concentration of isolated RNA was determined at 260 nm with a GeneQuant RNA/DNA Calculator (Pharmacia).

### *DNA Manipulation*

Quantifying specific mRNA species in RT-PCR experiments requires an internal standard RNA as competitor. Therefore, an artificially synthesized DNA sequence of 216 bp [15] containing the sequences complementary to the oligonucleotides used in the PCR (primer a: 5′-CGCTTC CATGTCCAGAACCTT-3′ / primer b: 5′-CTGTTCCA CGTGATATCCACT-3′) experiments was inserted into the *Sma*I restriction site of the plasmid pGEM-4Z. This plasmid allows *in vitro* transcription of inserts using the SP6 polymerase (Promega). Afterwards, a 338-bp *Taq*I fragment retained from pBR328 was inserted into the single *Xho*I restriction site of the 216-bp fragment resulting in plasmid pVT1. This plasmid containing the internal standard sequence provides a PCR product of a different length (479 bp) compared to the target PCR product (396 bp).

To determine the optimal amplification conditions for RT-PCR, we also cloned the corresponding $\beta_2$-adrenoceptor partial cDNA. The 396-bp PCR product of the $\beta_2$-ADR mRNA resulting from a PCR amplification with the primers described above was cloned into pIC20H [16], resulting in plasmid pNIC17. The pIC20H had been digested previously with *Eco*RV and incubated with *Taq*-polymerase (1U/μg plasmid/20 μL volume) in the presence of 2 mM dTTP for 2 hr at 70° [17]. The absence of any other nucleotides in the reaction mixture resulted in the addition of a single thymidine at the 3′ end of each fragment. The

* *Abbreviation:* RT-PCR, reverse transcriptase-polymerase chain reaction.

insert of pNIC17 was removed by *Sac*I and inserted into pGEM-4Z giving plasmid pNIC21.

Transformations in *Escherichia coli* JM109 [18] and DH5α [19] were performed according to Chung *et al.* [20]. Transformants were grown at 37° in/on dYT liquid/solid medium [21] supplemented with ampicillin (0.3 mM). Plasmid DNA was isolated according to the protocol of Qiagen [22].

RNA transcripts of the internal standard sequence from pVT1 and of the cloned partial cDNA from pNIC21 were prepared as run-off transcripts after linearization of the plasmids by *in vitro* transcription using a commercially available kit (Riboprobe Gemini System II, Promega). Subsequently, the DNA template was digested by adding 10 units of DNase I (FPLC*pure*, Pharmacia) to the reaction mixture and incubated for 30 min at 37°. After ethanol precipitation and two washing steps, the amount of internal standard RNA was determined at 260 nm. Absence of DNA was verified by PCR without reverse transcription which resulted in no signal.

### *Competitive RT-PCR*

Competitive RT-PCR, which shows the highest sensitivity in comparison to all other known quantification techniques [23], is used for quantification of low amounts of mRNA by RT and PCR amplification together with known amounts of competitor RNA. Using the internal standard RNA as competitor and specific DNA primers which fit the target $\beta_2$-adrenoceptor as well as the competitor sequence, we performed a modified procedure of the PCR aided transcript titration assay (PATTY) described by Becker-André and Hahlbrock [24]. Identical amounts of total cellular RNA (approx. 200 ng) were reverse-transcribed and amplified by PCR together with four different amounts of internal standard RNA reactions.

Synthesis of cDNA was carried out in a volume of 20 μL in a buffer containing 5 mM $MgCl_2$ (Perkin Elmer), 50 mM KCl (Perkin Elmer), 10 mM Tris–HCl, pH 8.3 (Perkin Elmer), 1 mM dNTPs (Pharmacia), 2.5 μM random hexamers (Pharmacia), 20 U of RNase inhibitor (Perkin Elmer), and 50 U of reverse transcriptase (SuperScript™ RNase H Reverse Transcriptase, Life Technologies). The reaction mixture was incubated at 42° for 20 min and thereafter at 99° for 5 min to inactivate enzymatic activity. PCR was performed at final concentrations of 2.5 mM $MgCl_2$ (Perkin Elmer), 50 mM KCl (Perkin Elmer), 10 mM Tris–HCl, pH 8.3 (Perkin Elmer), 0.2 mM dNTPs (Pharmacia), and 1.25 U of *AmpliTaq* DNA-polymerase (Pharmacia) in a total volume of 50 μL.

Ten μL of cDNA aliquots were subjected to PCR using 20 pM of each primer with a denaturation step of 5 min at 94° followed by 30 cycles of denaturation at 94° for 30 sec, primer annealing at 62° for 1 min, and chain extension at 72° for 1 min. An aliquot of each PCR product was electrophoresed on a 2% agarose gel, stained with 1 μg/mL of ethidium bromide, and visualized by UV fluorescence. The ethidium bromide staining patterns were photographed on Polaroid 665 film and the intensities of the ethidium bromide-stained PCR products were measured with a densitometer (Hirschmann, Elscript 400). At the point of equivalence, the molarity of internal standard and $\beta_2$-adrenoceptor PCR product were equal. Because the point of equivalence is based on molar amounts of the target and competitor products, the intensities associated with the length of the standard signal were corrected by the factor 396/479 (target length/standard length). The point of equivalence is obtained by regression analysis of the corrected fluorescence intensities in a semilogarithmic diagram [24].

### *$\beta_2$-Adrenoceptor mRNA Stability*

The half-life of $\beta_2$-adrenoceptor mRNA was determined by the method of Rodgers *et al.* [25]. Cells were exposed to $10^{-6}$ M (−)-isoproterenol for 4 hr. Actinomycin D (Sigma), an inhibitor of RNA polymerase II, was added to a final concentration of 5 μg/mL to prevent further transcription. Total RNA was isolated after 0.5, 1, 2, 3, and 4 hr. The content of $\beta_2$-adrenoceptor mRNA in total cellular RNA was quantified by competitive RT-PCR. The results were expressed as percentage of control and were plotted on a logarithmic scale versus time. Points were connected using a linear fit.

## RESULTS

### *RT-PCR Optimization*

To obtain reliable quantification data, the reverse transcription and amplification efficiencies of the partial $\beta_2$-adrenoceptor and internal standard RNAs were compared and RT-PCR conditions varied until amplification efficiencies of patient RNA and internal standard RNA were equal. Therefore, run-off RNA from pNIC21 and the internal standard RNA were reverse-transcribed and amplified in the same reaction under varying conditions. The best amplification efficiency was obtained using an annealing temperature of 62°, 30 cycles, 2.5 mM $MgCl_2$, and 0.3 μM of each primer. Furthermore, it could be shown that 30 cycles of PCR still represented the exponential phase of amplification (data not shown).

The point of equivalence of the corrected UV fluorescence intensities of the PCR products (see Materials and Methods) was determined after gel electrophoresis. The UV fluorescence intensities were graphed as a function of the logarithm of the amount of competitor RNA initially added to the reaction (Fig. 1, a and b). The line of best fit was obtained using linear regression analysis for each sample. The RNA concentration at which the lines intersect represents the initial amount of target RNA in the assay.

In 5 different experiments, the initial level of the $\beta_2$-adrenoceptor mRNA was determined as $1.58 \pm 0.14 \times 10^8$ molecules per μg total RNA, which is equal to 200 ± 18 pg $\beta_2$-adrenoceptor mRNA per μg total RNA. The

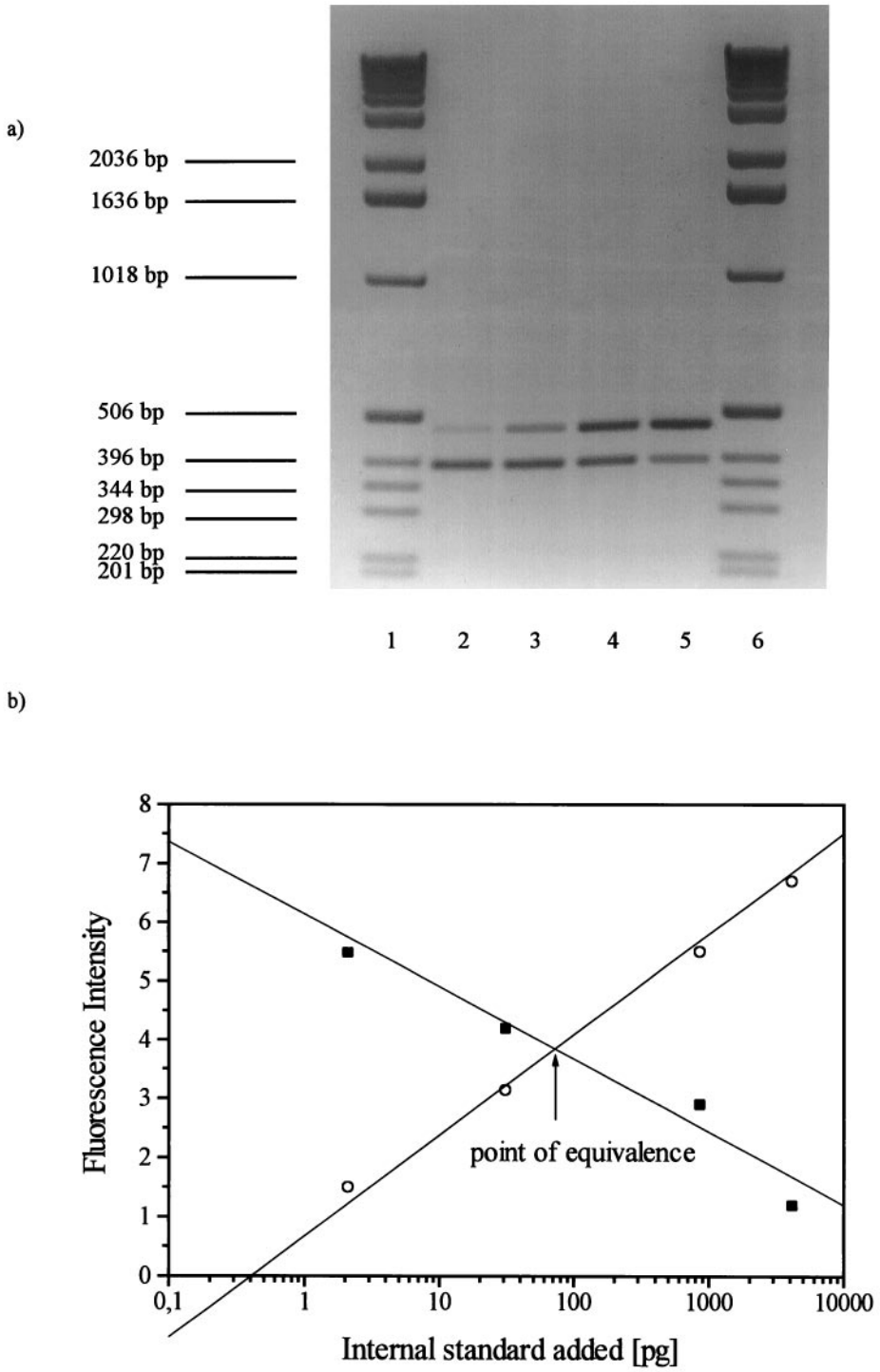


**FIG. 1.** Example of a competitive RT-PCR for quantification of $\beta_2$-adrenoceptor mRNA. Two hundred ng total RNA was subjected to RT-PCR together with 2.1 pg, 31 pg, 856 pg, and 4.1 ng internal standard RNA. Target (RNA of patients) and competitor RNA were reverse-transcribed and amplified resulting in two PCR products of different lengths (competitor: 479 bp / target: 396 bp). (a) Separation of PCR products by 2% agarose gel electrophoresis. Lanes 1 and 6 show the molecular weight marker (1 kb DNA ladder, Life Technologies). Lanes 2 to 5 represent an aliquot of the four PCR assays. (b) Evaluation of the fluorescence intensities. Fluorescence intensities of target (■) and internal standard (○) were graphed against the logarithm of the amount (in pg) of internal standard RNA. The lines were drawn from a linear regression analysis of the data points. The RNA concentration at which the lines intersect (point of equivalence; the number of molecules of target and competitor are equal) represents the target RNA that was initially present in the assay. In five independent RT-PCR experiments using the same total RNA preparation, we determined the $\beta_2$-adrenoceptor mRNA to be 200 ± 18 pg, i.e $1.58 \pm 0.14 \times 10^8$ molecules per μg of total RNA.

coefficient of variation of five independent PCR reactions using the same total RNA preparation was 13%.

### *Effect of (−)-Isoproterenol Treatment on $\beta_2$-Adrenoceptor Protein and mRNA Amount*

Saturation binding analysis of mononuclear leukocyte receptors with (−)-[$^{125}$I]-iodocyanopindolol revealed a β-adrenoceptor density of 2210 ± 225 binding sites per cell and a dissociation constant of 15.7 ± 1.6 pM. Cell viability was monitored during the course of these experiments and revealed a cell viability of 92–96%. Scatchard transformation of the data showed that (−)-[$^{125}$I]-iodocyanopindolol was bound to a homologous class of binding sites as inferred from the linearity of the Scatchard plot. Under basal conditions, the number of surface receptors was determined to be 90% of the total receptor number.

Figure 2 shows the results of total receptor number (A), surface receptor number (B), and mRNA amount (C) in control and (−)-isoproterenol-treated ($10^{-6}$ M) mononuclear leukocytes. Treatment of mononuclear leukocytes with vehicle (control) led to a parallel and continuous decrease in total receptor number (Fig. 2A) as well as $\beta_2$-adrenoceptor mRNA (Fig. 2C), but the number of surface receptors (Fig. 2B) decreased to a lower level. Within 8 hr, the number of total receptors per cell declined to 84 ± 10% and the mRNA amount decreased to 74 ± 11% of the initial amount. The number of surface receptors plunged rapidly in the period between 0 and 4 hr following treatment with vehicle, then stayed constant between 4 and 8 hr, reaching a final level of 55 ± 7% of the basal number at the end of the incubation time of 8 hr. The levels of total receptor number varied only slightly, while the surface receptor numbers and mRNA amounts did not change between 8 and 42 hr of incubation.

Treatment of mononuclear leukocytes with $10^{-6}$ M (−)-isoproterenol for 4 hr led to a decrease in total receptor number to 51 ± 5%, in surface receptor number to 37 ± 6%, and in mRNA amount to 73 ± 7% of the basal amounts (Fig. 3A–C). The number of total receptors stayed constant in the time period from 4 to 12 hr and continuously decreased to 16 ± 6% of the basal amount by the end of the incubation time of 42 hr. Surface receptor number did not change in the incubation period between 4 and 16 hr; this was followed by a period of continuous decrease, reaching a level of 16 ± 7% of the basal receptor number after 42 hr of incubation. The $\beta_2$-adrenoceptor mRNA decreased to 44 ± 7% of the basal mRNA amount during the first 8 hr of incubation but then did not change between 8 and 42 hr.

Figure 3 shows the results following (−)-isoproterenol treatment. These data result purely from (−)-isoproterenol incubation because the vehicle effect has already been subtracted. Between 0 and 4 hr, the total receptor number declined, reaching 65 ± 10% of the initial number. Between 4 and 24 hr, the number of receptors decreased slightly, followed by a phase from 24 to 42 hr where the total receptor number stayed almost constant (Fig. 3). After 4 hr of incubation with (−)-isoproterenol, the level of $\beta_2$-adrenoceptor mRNA remained at 88 ± 12% of the initial amount. This indicated a lower rate of change in mRNA levels compared to the rapid decrease in total receptor number during the same time period of 0 to 4 hr following treatment. By 8 hr, however, the mRNA level had dropped to 69 ± 11% of the initial amount, similar to the remaining percentage of total receptor number (66 ± 5%) at this time. (Fig. 3).

### *mRNA Stability*

In order to investigate the role of post-translational regulation in this phenomenon, the stability of $\beta_2$-adrenoceptor mRNA with or without (−)-isoproterenol treatment of mononuclear leukocytes was assessed. After a lag phase of 30 min, the mRNA amount in isoproterenol-treated cells declined more rapidly than in control cells. Half-life measurements of $\beta_2$-adrenoceptor mRNA after a preincubation period of 4 hr with and without (−)-isoproterenol showed that (−)-isoproterenol treatment reduced the $\beta_2$-adrenoceptor mRNA half-life from 2.7 hr to 1.1 hr (Fig. 4).

## DISCUSSION

The basal number of $\beta_2$-adrenoceptors in human mononuclear leukocytes, the percentage of surface receptors, and the dissociation constant reported in this work are consistent with the values found by others [26, 27]. The basal $\beta_2$-adrenoceptor mRNA level was 200 ± 18 pg, i.e. 1.58 ± 0.14 × $10^8$ molecules per μg total RNA. Using *in situ* hybridization, Liebl *et al.* [28] detected 1290 $\beta_2$-adrenoceptor mRNA molecules per cell in human mononuclear leukocytes. Assuming a total RNA amount of 30 to 500 μg per $10^7$ mammalian cells, this corresponds to 2.6 × $10^7$ to 4.3 × $10^8$ molecules per μg total RNA, i.e. to values consistent with those reported here.

Treatment with (−)-isoproterenol led to a rapid decrease in total cellular receptors. A reduction of 30% within 3 hr and a further reduction of approximately 80% within 24 hr of incubation with the same agonist have also been observed in Chinese hamster fibroblast cells expressing wild-type human $\beta_2$-adrenoceptors [3]. As expected, (−)-isoproterenol treatment caused an immediate and more effective decrease in surface receptor number in comparison to total receptor number. This may be due to the nonphysiological environment of the mononuclear leukocytes in culture. The reduction in surface receptor number may be a result of the well-known internalization process [29]. It must be noted, however, that vehicle treatment had a greater effect on surface receptor number than on total receptor number. This meant that the actual reduction in surface receptor number was less than that of the total receptor number following (−)-isoproterenol treatment. The decreases in mRNA level and total receptor number were significantly correlated (Passing–Bablok regression, $y = 11.9 \times -333.3$;

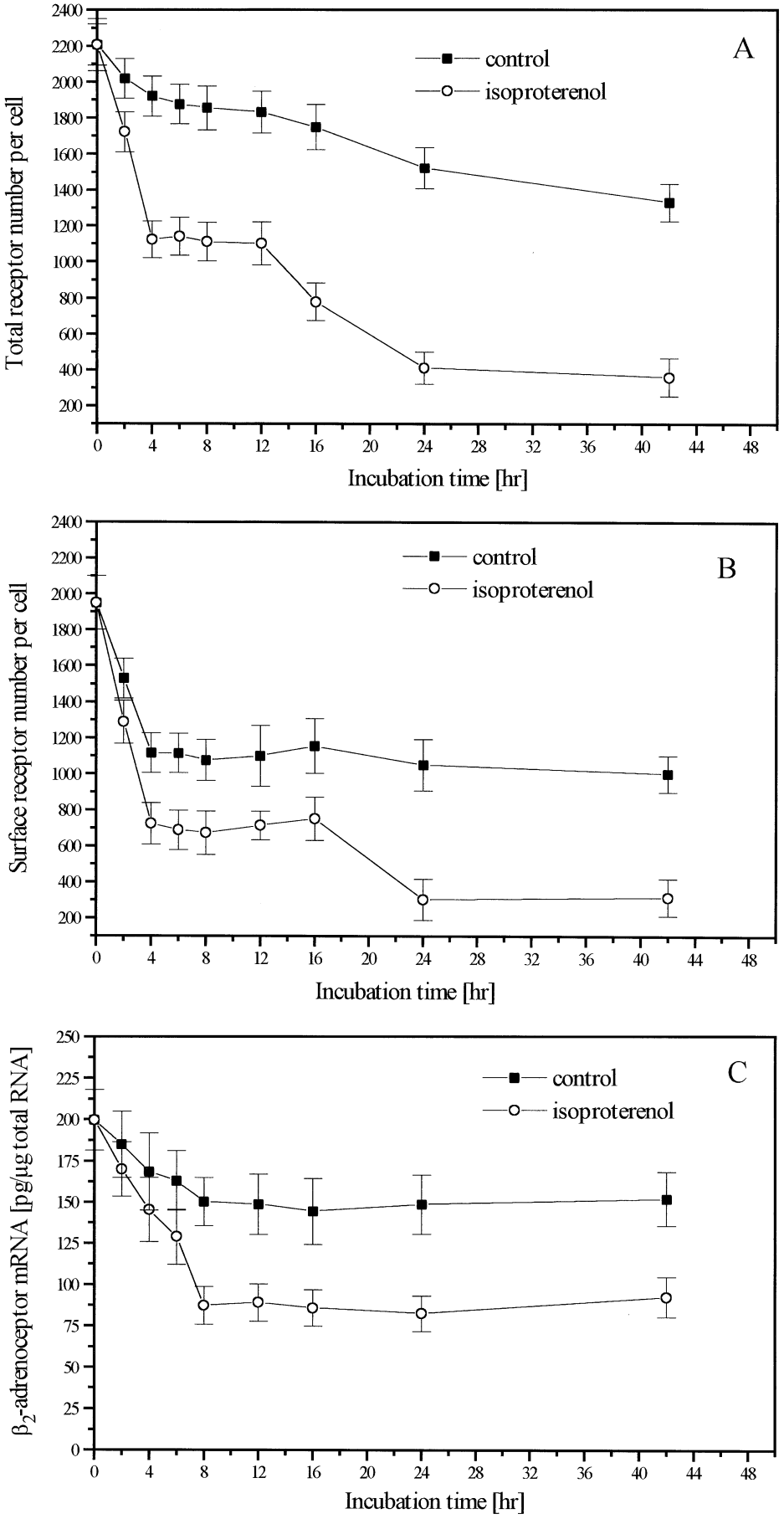


FIG. 2. Effect of (−)-isoproterenol treatment on total $\beta_2$-adrenoceptor protein and mRNA amounts. Mononuclear leukocytes were incubated with vehicle (control, ■) and $10^{-6}$ M (−)-isoproterenol (○) for the indicated times, harvested, and subjected to saturation binding analysis of (−)-[$^{125}$I]-iodocyanopindolol and to RNA isolation for further quantification analysis by RT-PCR. (A) Total receptor number. Data are the results of saturation binding assays using (−)-propranolol ($10^{-6}$ M) to determine nonspecific binding in untreated (control) and isoproterenol-treated mononuclear leukocytes. The data for total receptor protein given as total receptor number per cell are displayed as the means ± SEM of three separate experiments each performed in duplicate. (B) Surface receptor number. Surface receptor number as assessed using the hydrophilic ligand CGP-12177 ($10^{-6}$ M). The data for surface receptor protein given as surface receptor number per cell are displayed as the means ± SEM of three separate experiments each performed in duplicate. (C) mRNA amount. The values of $\beta_2$-adrenoceptor mRNA were determined using RT-PCR, including an internal standard for quantitation. The values are given in pg per μg total RNA and represent the means ± SEM of two separate experiments each performed in duplicate.

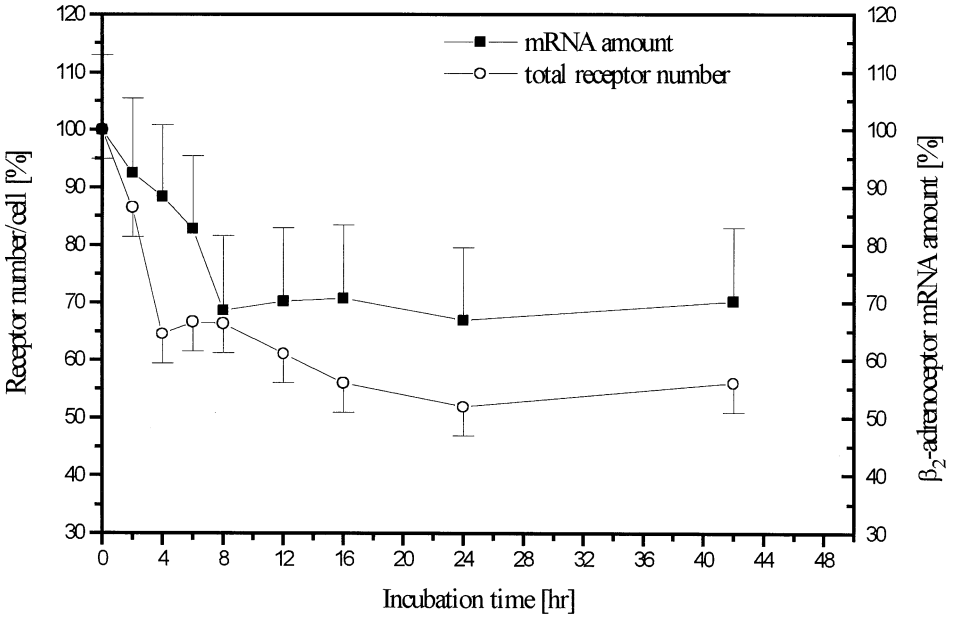


**FIG. 3. Effect of (−)-isoproterenol treatment on total $\beta_2$-adrenoceptor protein and RNA amounts. Mononuclear leukocytes were incubated with vehicle (untreated) and $10^{-6}$ M (−)-isoproterenol (treated) for the indicated times, harvested, and subjected to saturation-binding analysis of (−)-[$^{125}$I]-iodocyanopindolol and to RNA isolation for further quantification analysis by RT-PCR. Data shown represent only the effect of (−)-isoproterenol incubation, since the vehicle effect has been subtracted. The values of $\beta_2$-adrenoceptor mRNA (■) are given in pg per μg total RNA and represent the means ± SEM of two separate experiments each performed in duplicate. The data for receptor protein (○) given as total receptor number per cell are displayed as the means ± SEM of three separate experiments each performed in duplicate.**

$r = 0.85$; $P < 0.001$). This is reminiscent of the situation in S49 lymphoma cells [4]. Moreover, receptor number down-regulation caused by isoproterenol preceded the decrease in mRNA concentration, as observed in DDT$_1$ MF-2 hamster cells [1]. Hence, the decreased receptor density might be due firstly to internalization followed by receptor degradation and secondly to reduced mRNA level leading to reduction of receptor translation. In contrast, in Chinese hamster fibroblast cells treated with the cyclic AMP analogue dibutyryl cAMP [3] and in H9c2 rat heart cells treated with isoproterenol [30], the decrease in mRNA level clearly occurred before the reduction in $\beta_2$-adrenoceptor number. In the first case, dibutyryl cAMP did not cause internalization of receptors and the down-regulation was caused by increased receptor phosphorylation and reduced mRNA concentration [3].

Reports from the clinical investigation of patients with heart failure, a condition involving increased catecholamine levels, are controversial with regard to β-adrenoceptor number and mRNA level. Bristow *et al.* [6], Engelhardt *et al.* [31], and Ungerer *et al.* [7] found no difference in the amount of $\beta_2$-adrenoceptor protein and mRNA between failing and nonfailing human ventricular myocardium. Conversely, Borst *et al.* [32] showed that a chronically increased catecholamine level in heart failure was correlated with a reduction in $\beta_2$-adrenoceptor number and mRNA amount in the myocard. Brodde *et al.* [33] and Steinfath *et al.* [34] demonstrated that $\beta_2$-adrenoceptors decreased in ischemic cardiomyopathy and mitral valve

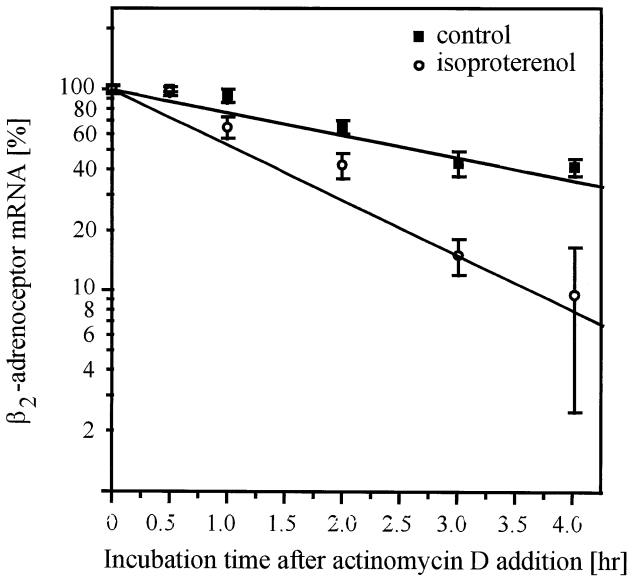


**FIG. 4. Reduction of $\beta_2$-mRNA stability in response to isoproterenol. Cells were preincubated with vehicle (untreated, ■) or $10^{-6}$ M (−)-isoproterenol (treated, ○) for 4 hr. They were harvested at the indicated times (0.5, 1, 2, 3, and 4 hr) after addition of actinomycin D (5 μg/mL). The results are expressed as percentage of control and are plotted on a logarithmic scale versus time. The half-life of $\beta_2$-adrenoceptor mRNA was derived from the slope of the line. Treatment with (−)-isoproterenol reduced the initial half-life of $\beta_2$-adrenoceptor mRNA from 2.7 to 1.1 hr. The results ([%] of control, t = 0; mean ± SEM) represent the means of two separate experiments each performed in triplicate.**

disease but not in idiopathic dilated cardiomyopathy, where the $\beta_2$-adrenoceptor function appeared to be reduced. Phosphorylation of adrenoceptors may also be associated with desensitization since β-adrenergic receptor kinase activity and mRNA level are markedly increased in heart failure [35]. Finally, Ratge *et al.* [36] and Smiley *et al.* [37] have reported that cardiac surgery results in significant down-regulation and desensitization of the $\beta_2$-adrenoceptor system of lymphocytes that may parallel alterations in other organs.

The process of mRNA down-regulation may be composed of a lowered transcription rate as well as a reduced mRNA stability. We have found a reduction in the mRNA half-life after (−)-isoproterenol treatment that may partially explain the receptor down-regulation. In $DDT_1$ MF-2 hamster vas deferens cells, a reduction in the half-life of receptor mRNA after (−)-isoproterenol treatment was involved in a similar mRNA decrease, but the translation rate was unaffected [38]. Conversely, the transcription rate of the $\beta_2$-receptor gene was altered, but the mRNA stability was unchanged in rat glioma cells after treatment with the same agonist [39]. The $\beta_2$-adrenoceptor gene is subject to transcriptional regulation from a variety of inputs including cyclic AMP [40–42]. The role of the transcription rate in $\beta_2$-adrenoceptor down-regulation in our system remains to be investigated. Nevertheless, in human mononuclear leukocytes, as in other cell types [43], the mRNA level is likely to be one of the most important points of control for agonist-induced receptor down-regulation.

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## References


1. Hadcock JR and Malbon CC, Down-regulation of beta-adrenergic receptors: Agonist-induced reduction in receptor mRNA levels. *Proc Natl Acad Sci USA* **85:** 5021–5025, 1988.
2. Lohse MJ, Molecular mechanisms of membrane receptor desensitization. *Biochim Biophys Acta* **1179:** 171–188, 1993.
3. Bouvier M, Collins S, O'Dowd BF, Campbell PT, deBlasi A, Kobilka BK, MacGregor C, Irons GP, Caron MG and Lefkowitz RJ, Two distinct pathways for cAMP-mediated down-regulation of the $\beta_2$-adrenergic receptor. *J Biol Chem* **264:** 16786–16792, 1989.
4. Hadcock JR, Ros M and Malbon CC, Agonist regulation of beta-adrenergic receptor mRNA. Analysis in S49 mouse lymphoma mutants. *J Biol Chem* **264:** 13956–13961, 1989.
5. Mak JC, Nishikawa M, Shirasaki H, Miyayasu K and Barnes PJ, Protective effects of a glucocorticoid on down-regulation of pulmonary beta 2-adrenergic receptors *in vivo*. *J Clin Invest* **96:** 99–106, 1995.
6. Bristow MR, Minobe WA, Raynolds MV, Port JD, Rasmussen R, Ray PE and Feldman AM, Reduced $\beta_1$ receptor messenger RNA abundance in the failing human heart. *J Clin Invest* **92:** 2737–2745, 1993.
7. Ungerer M, Böhm M, Elce JS, Erdmann E and Lohse MJ, Altered expression of β-adrenergic receptor kinase and $\beta_1$-adrenergic receptors in the failing human heart. *Circulation* **87:** 454–463, 1993.
8. Brodde O-E, Beckeringh JJ and Michel MC, Human heart β-adrenoceptors: A fair comparison with lymphocyte β-adrenoceptors? *TIPS* **8:** 403–407, 1987.
9. Brodde O-E, Michel MC, Gordon EP, Sandoval A, Gilbert EM and Bristow MR, Beta-adrenoceptor regulation in the human heart: Can it be monitored in circulating lymphocytes? *Eur Heart J* **10** (suppl B) 2–10, 1989.
10. Haen E, The peripheral lymphocyte as clinical model for receptor disturbance: Asthmatic disease. *Bull Eur Physiopathol Respir* **23:** 539–541, 1987.
11. Brodde O-E, Die Rolle adrenerger alpha und beta-Rezeptoren in der Pathogenese von Hypertonie und Herzerkrankungen. *Internist* **29:** 397–413, 1988.
12. Mancini DM, Martin JF, Fischberg D, Molinoff PB and Wilson JR, Characterization of lymphocyte beta-adrenergic receptors at rest and during exercise in ambulatory patients with chronic congestive heart failure. *Am J Cardiol* **63:** 307–312, 1989.
13. Young JW and Steinman RM, Accessory cell requirements for the mixed leukocyte reaction and polyclonal mitogens, as studied with a new technique for enriching blood dendritic cells. *Cell Immunol* **111:** 167–182, 1988.
14. Scatchard G, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51:** 660–672, 1949.
15. Höhnel K, Ratge D, Giray J, Lauk C, Rose T, Hellberg K and Wisser H, Non-isotopic competitive reverse transcription polymerase chain reaction coupled with high-performance liquid chromatography to measure $\beta_2$-receptor messenger RNA in human heart. *Eur J Clin Chem Biochem* **34:** 411–417, 1996.
16. Marsh JL, Erfle M and Wykes EJ, The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. *Gene* **32:** 481–485, 1984.
17. Marchuk D, Drumm M, Saulino A and Collins FS, Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res* **19:** 1154, 1990.
18. Yanisch-Perron C, Vieira J and Messing J, Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119, 1995.
19. Hanahan D, Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166:** 557–580, 1983.
20. Chung CT, Niemela SL and Miller RH, One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci USA* **86:** 2172–2175, 1989.
21. Sambrook J, Fritsch EF and Maniatis T, Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor NY, 1989.
22. Qiagen Plasmid Handbook. Qiagen, Hilden, 1993.
23. Price T, Aitken J, Head J, Mahendroo M, Means G and Simpson E, Determination of aromatase cytochrome P450 messenger ribonucleic acid in human breast tissue by competitive polymerase chain reaction amplification. *J Clin Endocrin Metab* **74:** 1247–1252, 1992.
24. Becker-André M and Hahlbrock K, Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res* **17:** 9437–9446, 1989.
25. Rodgers JR, Johnson ML and Rosen JM, Measurement of mRNA concentration and mRNA half-life as a function of hormonal treatment. *Methods Enzymol* **109:** 572–592, 1985.
26. O'Hara N and Brodde O-E, Identical binding properties of (±)- and (−)-[$^{125}$I] iodocyanopindolol to $\beta_2$-adrenoceptors in intact human lymphocytes. *Arch Int Pharmacodyn Ther* **272:** 24–39, 1984.
27. DeBlasi A, Maisel AS, Feldman RD, Ziegler MG, Fratelli M, DiLallo M, Smith DA, Lai CC and Motulsky HJ, *In vivo*

regulation of β-adrenergic receptors on human mononuclear leucocytes. Assessment of receptor number, location, and function after posture change, exercise, and isoproterenol infusion. *J Clin Endocrin Metab* **63:** 847–853, 1986.
28. Liebl B, Pachmann K, Römer U, Haen E, Hallek M and Emmerich B, Quantitation of mRNA for the $\beta_2$-adrenoceptor gene in human mononuclear leucocytes by *in situ* hybridization with fluorochrome-labeled cloned DNA. *J Recept Res* **11:** 473–482, 1991.
29. Insel PA, Adrenergic receptors: Evolving concepts and clinical implications. *N Engl J Med* **334:** 580–585, 1996.
30. Dangel V, Giray J, Ratge D and Wisser H, Regulation of β-adrenoceptor density and mRNA levels in the rat heart cell line H9c2. *Biochem J* **317:** 925–931, 1996.
31. Engelhardt S, Böhm M, Erdmann E and Lohse MJ, Analysis of β-adrenergic mRNA levels in human ventricular biopsy specimens by quantitative polymerase chain reactions: Progressive reduction of $\beta_1$-adrenergic receptor mRNA in heart failure. *J Am Coll Cardiol* **27:** 146–154, 1996.
32. Borst MM, Schwencke C, Beuthien W, La Rosée P, Marquetant R and Strasser RH, Downregulation pulmonaler β-adrenerger Rezeptoren in der Herzinsuffizienz: eine mögliche Ursache des "Asthma cardiale"? *Z Kardiol* **84** (suppl 1), 28, 1995.
33. Brodde O-E, Daul A, Michel MC and Zerkowski H-R, Bedeutung von $\beta_2$-Adrenozeptoren bei Herzinsuffizienz. *Z Kardiol* **81** (suppl 4), 71–78, 1992.
34. Steinfath M, Lavicky J, Schmitz W, Scholz H, Doring V and Kalmar P, Changes in cardiac beta-adrenoceptors in human heart diseases: Relationship to the degree of heart failure and further evidence for etiology-related regulation of beta 1 and beta 2 subtypes. *J Cardiothorac Vasc Anesth* **7:** 668–673, 1993.
35. Summers RJ, McMartin LR, Kompa A and Molenaar P, Signalling pathways in cardiac failure. *Clin Exp Pharmacol Physiol* **22:** 874–876, 1995.
36. Ratge D, Wiedemann A, Kohse KP, Hellberg K and Wisser H, Magnitude and kinetics of alterations in plasma catecholamines and leukocyte β-adrenergic receptors in response to anaesthesia and surgery. *J Clin Chem Clin Biochem* **28:** 391–398, 1990.
37. Smiley RM, Pantuck CB, Chadburn A and Knowles DM, Down-regulation and desensitization of the beta-adrenergic receptor system of human lymphocytes after cardiac surgery. *Anesth Analg* **77:** 653–661, 1993.
38. Hadcock JR, Wang H-Y and Malbon CC, Agonist-induced destabilization of β-adrenergic receptor mRNA. *J Biol Chem* **264:** 19928–19933, 1989.
39. Hosoda K, Fitzgerald LR, Vaidya VA, Feussner GK, Fishman PH and Duamn RS, Regulation of beta-2-adrenergic receptor mRNA and gene transcription in rat C6 glioma cells: Effects of agonist, forskolin and protein synthesis inhibition. *Mol Pharmacol* **48:** 206–211, 1995.
40. Collins S, Quarmby VE, French FS, Lefkowitz RJ and Caron MG, Regulation of the beta2-adrenergic receptor and its mRNA in the rat ventral prostate by testosterone. *FEBS Lett* **233:** 173–176, 1988.
41. Collins S, Altschmied J, Herbsman O, Caron MG, Mellon PL and Lefkowitz RJ, A cAMP response element in the $\beta_2$-adrenergic receptor gene confers transcriptional autoregulation by cAMP. *J Biol Chem* **265:** 19330–19335, 1990.
42. Emorine LJ, Marullo S, Delavier-Klutchko C, Kaveri SV, Durieu-Trautmann O and Strosberg AD, Structure of the gene for human beta2-adrenergic receptor expression and promoter characterization. *Proc Natl Acad Sci USA* **84:** 6995–6999, 1987.
43. Morris BJ, Control of receptor sensitivity at the mRNA level. *Mol Neurobiol* **7:** 189–205, 1993.