Regulation of β -adrenoceptor density and mRNA levels in the rat heart cell-line H9c2

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The regulation of the expression of β -adrenoceptors (β -ARs) is not thoroughly understood. We demonstrate that the rat heart cell-line H9c2 expresses both β_1 - and β_2 -ARs. In radioligand-binding experiments, the maximal binding capacity of (–)-[¹²⁵I]-iodocyanopindolol was determined as 18 ± 0.6 fmol/mg of protein with a K_D of 35.4 ± 4.1 pM. Competitive radioligand-binding experiments with subtype-specific β -antagonists reveal a subtype ratio of β_1 - to β_2 -ARs of 29%:71%. With competitive reverse-transcriptase PCR we found β_2 -mRNA to be up to 1600 times more frequent than β_1 -mRNA. Treatment of the H9c2 cell-line with the β -adrenergic agonist (–)-isoproterenol (10^{-6} M), the antagonist (–)-propranolol (10^{-6} M) and the glucocorticoid dexamethasone (500 nM) induces regulatory effects on both the β -AR protein and mRNA level. Isoproterenol treatment leads to down-regulation of the total receptor number by $56 \pm 4\%$, due to a decrease in β_2 -ARs, while maintaining the β_1 -AR number

constant. On the transcription level, both β_1 - and β_2 -mRNAs are decreased by 30% and 42% respectively. mRNA stability measurements reveal a reduced half-life of β_2 -mRNA from 9.3 h to 6.5 h after isoproterenol treatment. Incubation of cells with (–)-propranolol does not affect the amounts of β -ARs and their mRNAs. Dexamethasone induces a 1.8 ± 0.2 -fold increase in β -AR number over the basal level as well as a 1.9 ± 0.2 -fold increase in the amount of β_2 -mRNA. Because the half-life of β_2 -mRNA was unaffected by dexamethasone, the increased β_2 -mRNA level must be due to an enhanced transcription rate. The β_1 -mRNA levels are unchanged during dexamethasone-incubation of the cells. Our data clearly demonstrate that treatment of H9c2 rat heart cells with isoproterenol and dexamethasone induces alterations in the level of RNA stability as well as gene transcription, leading to altered receptor numbers.

INTRODUCTION

Beta-adrenoceptors (β -ARs) are members of a large family of transmembrane receptors which mediate their signal transduction via G-proteins. They play an important role in mediating the effects of catecholamine release on inotropic and chronotropic actions of the heart. Two isoforms of β -ARs exist in the human heart. They act like isoreceptors, as they may co-exist in the same cell and their effects are mediated via the same post-receptor pathway.

Tissue concentration of β -ARs is not a fixed number, and modulation of receptors is one of the mechanisms regulating the system of transmembrane signalling [1]. The amount of β -ARs present in a tissue or on a single cell can be influenced by drugs, hormones and physiological and pharmacological conditions. Receptor down-regulation and desensitization display protective effects to terminate over-stimulation by agonists in the target cell. While down-regulation refers to a decrease in receptor number after chronic agonist exposure, desensitization reflects a loss of responsiveness to receptor stimulation that may accompany or precede down-regulation [2]. For example, in asthma therapy, β -agonists show bronchodilatory effects, and chronic use of agonists leads to adaptation caused by down-regulation of β -ARs in lung, as determined by radioligand binding [3]. A decrease in β -ARs is concomitant with a decrease in the amount of receptor mRNA in target cells. This could be shown in DDT₁ MF-2 hamster cells [4] and S49 mouse lymphoma mutants [5], where chronic stimulation with isoproterenol led to down-regulation of β -ARs and their mRNA. Treatment of animals and patients with β -adrenergic antagonists often increases receptor number, possibly as a consequence of blocking tonic down-

regulation induced by ambient levels of agonist. For example, in rat hearts the non-selective β -adrenergic antagonist propranolol has been reported to increase the β -AR number [6], and in rat ventricles, lungs and lymphocytes, infusion of propranolol resulted in significant increases in the density of β -ARs [7]. In addition, the glucocorticoid dexamethasone increases the steady-state levels of β_2 -mRNA and receptor protein in DDT₁ MF-2 hamster cells [8]. It could be shown that increased transcription of the β_2 -AR gene is responsible for the increase in the amount of receptor mRNA [9,10].

Clinical data on the regulation and expression of β -ARs in heart tissue obtained from patients during heart surgery have to be discussed with care due to comedication, stress and age.

The H9c2 cell-line [11] derived from rat heart tissue may therefore be used as a model for molecular investigations in the β -AR signal-transduction pathway of the heart. Morphological, biochemical and electrophysiological characterization of H9c2 cells revealed that this cell-line has preserved elements of electrical and hormonal signalling pathways found in adult cardiac cells [12].

Our data are the first to report about β_1 - and β_2 -ARs in the H9c2 cell-line. Pharmacologically induced changes caused by β -adrenergic agonists and antagonists and the influence of glucocorticoids were investigated in a time-dependent manner with regard to both receptor protein and mRNA level.

MATERIALS AND METHODS

Cell culture

H9c2 (2-1) rat heart myoblasts [11] were obtained from the American Type Culture Collection (ATCC, Rockville, MD,

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; [¹²⁵I]CYP, (–)-[¹²⁵I]iodocyanopindolol; β -AR(s), β -adrenoceptor(s); RT, reverse transcriptase; IS, internal standard; PCR [a] and [b], PCR using outer primer pairs [a] or inner (nested) primer pairs [b]; β_1 - and β_2 -mRNA, mRNA of β_1 - and β_2 -adrenoceptors; nPCR, nested PCR; β -ARB protein, β -adrenergic receptor mRNA binding protein.

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U.S.A.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, glutamine (2 mM), non-essential amino acids [1% (v/v)] and gentamicin (100 mg/l) under an atmosphere of 5% CO₂ in air saturated with water vapour at 37 °C. All supplements were purchased from Gibco-BRL (Eggenstein, Germany) except glutamine (Biochrom, Berlin, Germany). The medium was replaced every 2 days. Cells were grown to confluence as monolayers and were split by treatment with trypsin. Stock solutions of (–)-isoproterenol, (–)-propranolol and dexamethasone (Sigma, Deisenhofen, Germany) were prepared in water and then diluted directly in DMEM. Untreated control cells were grown in the same DMEM to which only water was added.

Membrane preparation

Membranes were prepared as described [13]. In brief, cells were washed twice with PBS (10 mM CaCl₂/25 mM KCl/15 mM KH₂PO₄/5 mM MgCl₂/20 mM NaCl/8 mM Na₂HPO₄) at 37 °C and treated with buffer I [1 mM Tris/HCl/2 mM EDTA (pH 7.4, 4 °C)]. The cells were harvested and homogenized with 20 strokes of a manual Teflon-pestle homogenizer. The membrane suspension was adjusted to produce concentrations of 50 mM Tris/HCl/5 mM EDTA (pH 7.4, 4 °C) and membranes were washed twice at 40000 g for 30 min (4 °C).

Radioligand binding

Membranes were resuspended in 4 ml of buffer II [50 mM Tris/HCl/1 mM EDTA/10 mM MgCl₂ (pH 7.4, 30 °C)]. A 100 µl volume of this suspension was incubated with (–)-[¹²⁵I]iodocyanopindolol ([¹²⁵I]CYP; 5–80 pM, 2000 Ci/mmol; Amersham, Braunschweig, Germany) in 0.2 mM ascorbic acid in a total volume of 0.5 ml (adjusted with buffer II). The reaction mixture was incubated for 1 h at 30 °C in a shaking water-bath. Reactions were terminated by dilution in 5 ml of PBS followed by rapid vacuum filtration through Whatman GF/C filters. The filters were immediately washed with 10 ml and 5 ml of PBS and radioactivity was measured in a gamma counter at 75% efficiency (Berthold, Wildbad, Germany). All experiments were performed in triplicate. Specific binding of [¹²⁵I]CYP was defined as total binding minus non-specific binding. Non-specific binding, insensitive to competition by 1 µM propranolol, was typically less than 10% of total binding of 20 pM [¹²⁵I]CYP. Analysis of β -AR subtypes was done with competition-binding experiments using 17 different concentrations of a β_1 -selective (atenolol) and a β_2 -selective (ICI 118.551) adrenergic antagonist. Inhibition of specific 20 pM [¹²⁵I]CYP-binding by the respective subtype-selective antagonists was measured and the resulting inhibition curves were fitted by non-linear regression using the GraphPAD PRISM program (GraphPAD Software, San Diego, CA, U.S.A.).

Extraction of RNA

Total RNA from H9c2 cells was isolated by binding to a silica-based membrane using a microspin column technique according to the manufacturer's protocol ('RNeasy', Qiagen, Hilden, Germany). Remaining traces of DNA were digested in a total volume of 100 µl with 10 units of DNaseI (FPLCpure, Pharmacia, Freiburg, Germany) in a buffer containing 10 mM Tris/HCl/10 mM MgCl₂/50 mM KCl/10 mM dithiothreitol, pH 9.0 for 20 min at 37 °C. With an additional clean-up procedure, DNaseI-treated RNA was recovered by adsorption on silica-based membranes as described in the total RNA extraction procedure.

Enrichment of poly(A)⁺ mRNA from H9c2 cells was necessary to determine β_1 -AR mRNA levels due to the low abundance of this mRNA species in H9c2 cells. Poly(A)⁺ mRNA was isolated from cultured cells by adsorption on latex particles anchoring dT₃₀ oligonucleotides (Oligotex®, Qiagen, Hilden, Germany) in combination with the microspin column technique. Both RNA preparations were free from traces of remaining genomic DNA, as assessed by PCR without the reverse transcription step, where no signal was obtained (negative control). The concentration of isolated RNA was determined at 260 nm with a GeneQuant RNA/DNA Calculator (Pharmacia). The 260 nm:280 nm ratio of the solution was found to be 1.8:2.0, which reveals high purity of the RNA.

Preparation of internal standard RNA

In reverse-transcriptase PCR (RT-PCR) experiments for quantification of specific mRNA species one requires an internal-standard RNA as competitor. Therefore we amplified the β_1 - and β_2 -AR-specific sequences by RT-PCR and cloned the resulting fragments into the single *Sma*I restriction site of the pGEM-4Z plasmid (Promega, Madison, WI, U.S.A.). After sequencing the cloned region we removed the inner parts between the primer annealing sites and inserted pBR322 stuffer-fragments. The inserted stuffer-fragments provide PCR products of different lengths in comparison to their target PCR products. RNA transcripts of these internal-standard sequences were prepared as run-off transcripts by linearization at the *Eco*RI site located just 3' of the cloned β_1 and β_2 internal-standard sequences respectively. *In vitro* transcription was performed using a commercially available kit (Riboprobe Gemini System II, Promega, Madison, WI, U.S.A.). Subsequently, the DNA template was digested by adding 10 units of DNaseI (FPLCpure) to the reaction mixture and incubating for 20 min at 37 °C. After ethanol precipitation, the amount of internal standard RNA was determined at 260 nm. The absence of contaminating traces of remaining DNA template interfering in the competitive RT-PCR assay was verified by subjection of the RNA to PCR without reverse transcription, obtaining no signal.

Competitive RT-PCR

Competitive RT-PCR is used for quantification of low amounts of mRNA by its co-amplification of known amounts of added competitor RNA. β_1 - and β_2 -AR mRNA levels were determined using a modified procedure of the PCR aided transcript titration assay (PATTY) described by Becker-André and Hahlbrock [14]. In this titration assay, identical portions of total cellular RNA or poly(A)⁺ mRNA are spiked with decreasing amounts of internal standard RNA. After reverse transcription, the resulting cDNAs are amplified in a nested PCR assay where a competitive primer-annealing reaction between internal standard and target sequence takes place. The intensities of the ethidium-stained PCR products after gel electrophoresis were measured with a densitometer (Hirschmann Elscript 400; Germany).

cDNA synthesis was carried out in a 20 µl reverse transcription reaction containing 40 ng of poly(A)⁺ mRNA (β_1) or 200 ng of total RNA (β_2), and various amounts of the corresponding internal standard RNA. RNA was reverse transcribed in a buffer containing 5 mM MgCl₂, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1 mM dNTPs, 2.5 µM random hexamers, 20 units of RNase inhibitor and 100 units of RT. The reaction was carried out at 42 °C for 20 min. Thereafter, the mixture was incubated at 99 °C for 5 min to inactivate enzymic activity.

PCR was performed at final concentrations of 2 mM MgCl₂,

Table 1 Primers used in PCR resulting in the amplification products shown

PCR [b] provides a 349 bp product for β_1 -amplification and a 266 bp product for β_2 -amplification of the target cDNA sequence. PCR products with 384 bp and 322 bp were obtained by amplification of the cloned β_1 - and β_2 -internal standard sequences respectively. PCR [a] products are given in parentheses because they were subjected to PCR [b] after 13 cycles of amplification. (+) and (−) indicate sense and antisense respectively. [a]-Primers are used in PCR [a] and [b]-primers represent the inner primers for PCR [b]. All primers were purchased from Roth (Karlsruhe, Germany). PCR products: t, target; c, competitor.

Primer	Sequence	PCR products	Conc. (pmol/ μ l)
[a] β_1 (+)	5'-CATCGTGCTGCTCATCGTAGT-3'	(t: 392 bp)	3
[a] β_1 (−)	5'-AGTGCATGAGGATGGGCAGGAA-3'	(c: 427 bp)	
[b] β_1 (+)	5'-AAGGACACCGCGGGAGAT-3'	t: 349 bp	20
[b] β_1 (−)	5'-GCAACGTGTTGGTGATCGTG-3'	c: 384 bp	
[a] β_2 (+)	5'-TTCTGGTGCGAGTTCTGGACT-3'	(t: 363 bp)	10
[a] β_2 (−)	5'-CTGGAAGACCTGGAATAGAC-3'	(c: 419 bp)	
[b] β_2 (+)	5'-TGATGTGTTGTCGTCACAGC-3'	t: 266 bp	50
[b] β_2 (−)	5'-TAGCATAGGCCTGGTTCGTGA-3'	c: 322 bp	

50 mM KCl, 10 mM Tris/HCl (pH 8.3), 0.2 mM dNTPs and 1.25 units of *AmpliTa*q DNA polymerase in a total volume of 50 μ l. In the first PCR amplification (PCR [a], using outer primer pairs) 1 μ l of each primer was added, and 1 μ l of the corresponding inner primers were used for a second nested PCR reaction (PCR [b]). The nucleotide sequences of the primers for amplification of β_1 - and β_2 -targets and internal standard cDNAs and their concentrations are shown in Table 1.

PCR [a] was performed with a 6 μ l aliquot of cDNA incubated for 5 min at 94 °C following 13 cycles of denaturation at 94 °C for 30 s, primer annealing at 62 °C for 1 min and chain extension at 72 °C for 1 min. Then 4 μ l of PCR [a] was subjected to PCR [b] for 30 cycles using the same temperature profile as described above. dNTPs and random hexamers were purchased from Pharmacia, RT from Gibco-BRL (SuperScript® RNase H reverse transcriptase, Gibco-BRL, Eggenstein, Germany) and all other RT-PCR additives were obtained from Perkin-Elmer (Überlingen, Germany).

A 10 μ l aliquot of the resulting PCR products was electrophoresed on a 2% agarose gel, stained with ethidium bromide (1 μ g/ml) and visualized by UV fluorescence. The gels were photographed with a polaroid positive/negative film (polaroid 665), and the resulting densities of both PCR products were measured with a densitometer. Since the equivalence point of this reaction is based on molar amounts of target and competitor, the fluorescence associated with the length of the standard signal is corrected by a factor (target [bp] ÷ standard [bp]) which enables direct comparison of the fluorescence intensities in the target band with the measured fluorescence intensity of the internal-standard band. The point of equivalence is obtained by regression analysis in a semilogarithmic diagram [14].

Receptor mRNA stability

The half-life of β_2 -AR mRNA was determined by the method of Rodgers et al. [15]. Cells were pre-incubated with isoproterenol (10^{-6} M), dexamethasone (500 nM) or vehicle for 4 h. Actinomycin D at a final concentration of 5 μ g/ml was added to prevent further transcription. Total RNA was isolated after 4, 8 and 12 h. The content of β_2 -AR mRNA in total cellular RNA was quantified by competitive RT-nested PCR (RT-nPCR).

RESULTS

Radioligand-binding experiments

Saturation-binding analysis of [^{125} I]CYP with H9c2 cell membranes revealed a K_D of 35.4 ± 4.1 pM and a B_{max} of 18 ± 0.6 fmol/mg of protein (mean $\pm$ S.D., $n = 5$). Scatchard transformation of the data showed that [^{125} I]CYP is bound to a homologous class of binding sites as inferred from the linearity of the Scatchard plot. Binding of [^{125} I]CYP to β -ARs in H9c2 cell membranes was dose-dependently inhibited by antagonists that are highly selective for β_1 - and β_2 -ARs respectively (Figure 1). The inhibition curves were biphasic, indicating more than one

Figure 1 Characterization of β -AR subtypes in H9c2 cells by competitive inhibition of [^{125} I]CYP

Binding of 20 pM of the radioligand was inhibited by increasing amounts of a β_1 -selective (atenolol) or a β_2 -selective (ICI 118,551) antagonist. Analysis of competition with ICI 118,551 (▼) revealed that $71 \pm 6\%$ of total β -ARs were of the β_2 -subtype, while competition with the β_1 -selective antagonist atenolol (□) displayed a fraction of $29 \pm 6\%$ for β_1 -binding sites. The results displayed are means $\pm$ S.D. from three (atenolol) and four (ICI 118,551) independent experiments respectively, each assayed in triplicate.

Figure 2 Down-regulation of β -AR number in isoproterenol-treated H9c2 cells

Cells were incubated with vehicle (untreated, □) or with 10^{-6} M isoproterenol (treated, ▼) for the indicated times and were then harvested for membrane preparation. Saturation-binding analysis of [^{125} I]CYP shows a decline of $56 \pm 4\%$ in total receptor number after 12 h of treatment. The data are displayed as means $\pm$ S.D. of three separate experiments each performed in triplicate.

Table 2 Change in the amounts of β_1 - and β_2 -receptors after agonist treatment

Cells were incubated for 0, 4, 8, 12 and 24 h with (—) isoproterenol (10^{-6} M). The amounts of β_1 - and β_2 -receptors were determined by competitive radioligand binding using the β_2 -selective antagonist ICI 118.551. The results displayed are the mean values $\pm$ S.D. of three experiments each performed in triplicate.

Time of incubation (h)	β_1 -receptor (fmol/mg of protein)	β_2 -receptor (fmol/mg of protein)
0	5.5 $\pm$ 0.4	12.9 $\pm$ 0.6
4	5.8 $\pm$ 0.8	7.4 $\pm$ 0.9
8	5.7 $\pm$ 0.8	4.5 $\pm$ 0.4
12	5.2 $\pm$ 0.9	2.2 $\pm$ 0.5
24	5.3 $\pm$ 0.6	2.1 $\pm$ 0.9

Figure 3 Influence of dexamethasone on β -AR number in H9c2 cell membranes

H9c2 cells were incubated for the indicated times with vehicle (untreated, □) or with 500 nM dexamethasone (treated, △). Cells were harvested for radioligand-binding experiments as described. Saturation-binding analysis of [^{125} I]CYP shows an 1.8 ± 0.2 -fold increase in total receptor number after 12 h of treatment. The results displayed are the means $\pm$ S.D. of three to four separate determinations each performed in triplicate.

population of binding sites. A ratio of $71 \pm 6\%$ to $29 \pm 6\%$ ($n = 4$) of high- to low-binding-affinity sites was obtained for the β_2 -selective antagonist ICI 118.551. IC_{50} values of $1.1 \pm 0.3 \mu\text{M}$ and $1.7 \pm 0.6 \text{ nM}$ were found for β_1 - and β_2 -ARs respectively. With atenolol (β_1 -antagonist) the ratio was reversed, confirming the coexistence of β_1 - and β_2 -ARs in this cell-line.

Treatment of the cells with 10^{-6} M isoproterenol for up to 24 h induced a continuous decrease in the amount of [^{125} I]CYP bound to H9c2 membranes (Figure 2), while total receptor number in untreated cells remained almost constant. After 12 h of challenge with isoproterenol, total receptor number was down-regulated by $56 \pm 4\%$. This new steady-state level remained constant during the next 12 h.

Competitive-binding experiments performed at the same time-intervals revealed that the decrease in total receptor number caused by challenge with isoproterenol was primarily due to an 80% decrease in β_2 -AR number, while β_1 -AR number was unchanged (Table 2). Treatment of H9c2 cells with the β -adrenergic antagonist propranolol (10^{-6} M) for 4, 8, 12 and 24 h had no effect on β -AR density as determined by radioligand binding (results not shown).

Figure 4 Down-regulation of β_2 -mRNA in isoproterenol-treated H9c2 cells

Cells were incubated with vehicle (untreated, □) or 10^{-6} M isoproterenol (treated, △) for the indicated times. Total RNA was isolated for determination of β_2 -mRNA levels by the competitive RT-nPCR method. Treatment with isoproterenol induced a sharp decrease in the β_2 -mRNA level within 2 h, which reached a steady-state level of $42 \pm 7\%$ below the initial level after 4 h of incubation. The values are given as a percentage of the control ($t = 0$) and represent means $\pm$ S.D. of three separate experiments each performed in triplicate.

When cells were challenged with 500 nM dexamethasone, total binding of [^{125} I]CYP increased, after a delay of 8 h, from an initial B_{max} of 17.3 ± 0.8 fmol/mg of protein to a new maximum of 32 ± 4 fmol/mg of protein after 12 h of dexamethasone incubation. The new steady-state level showed a 1.8 ± 0.2 -fold increase in comparison to untreated cells and remained constant for the next 12 h (Figure 3). This upregulation of total receptor number is due to the increase in the β_2 -AR subtype alone, demonstrated by the shift of receptor subtype ratio $\beta_2:\beta_1$ from 71%:29% to 86%:14% after dexamethasone stimulation (results not shown).

Quantification of β -AR mRNAs

Analysis of the mRNA levels of β_1 - and β_2 -ARs required the use of competitive RT-nPCR. This technique offers higher sensitivity than Northern blot analysis and permits molar quantification of low-abundance or rare mRNA species.

In four different experiments the initial level of the β_1 -mRNA present was determined as 8.4 ± 0.9 pg of internal standard (IS) RNA/ μg of poly(A) $^+$ mRNA, which is equal to $2.9 \pm 0.3 \times 10^7$ molecules/ μg of poly(A) $^+$ mRNA. The β_2 -mRNA level was 133 ± 27 pg of IS-RNA/ μg of total RNA ($n = 4$), which is equal to $4.7 \pm 1 \times 10^8$ molecules/ μg of total RNA. Because mRNA in eukaryotic cells represents about 1–5% of the total RNA, these data show that β_2 -mRNA is about 300–1600 times more frequent than β_1 -mRNA in H9c2 cells.

Challenge with isoproterenol was characterized by a progressive decline in the β_2 -mRNA level within the first 2 h of treatment (Figure 4). After 4 h a new steady-state level, which was $42 \pm 7\%$ below the β_2 -mRNA level of untreated cells, developed and remained unchanged for the next 6 h. This time-course of down-regulation on the mRNA level precedes down-regulation on the receptor-protein level (Figure 2). When cells were treated with propranolol (10^{-6} M) for 4, 8, 12 and 24 h, no change in the β_2 -mRNA level was observed (results not shown).

Treatment of H9c2 cells with 500 nM dexamethasone induced a sharp increase (2.3 ± 0.3 -fold) in the β_2 -mRNA level after 4 h of incubation, reaching a new steady-state level after 12 h. This was

Figure 5 Up-regulation of β_2 -mRNA in dexamethasone-treated H9c2 cells

Cells were incubated with vehicle (untreated, $\square$) or 500 nM dexamethasone (treated, $\triangle$) for the indicated times. Total RNA was isolated for determination of β_2 -mRNA levels by the competitive RT-nPCR method. Treatment with dexamethasone induced a sharp increase in β_2 -mRNA level within 2 h, reaching a level 1.9 ± 0.2 -fold greater than the basal level after 12 h of treatment. The result, expressed as a percentage of control ($t = 0$), are the means $\pm$ S.D. of three to five experiments.

Figure 6 β_2 -mRNA stability is reduced in response to isoproterenol

Cells were incubated with vehicle (untreated, $\square$), 10^{-6} M isoproterenol ($\triangle$) or 500 nM dexamethasone (∇). They were harvested at the indicated times for isolation of total RNA, and subsequent measurement of β_2 -mRNA was carried out as described in the text. Treatment with isoproterenol reduced the initial half-life of β_2 -mRNA from 9.3 to 6.5 h. In contrast dexamethasone had no influence on the β_2 -mRNA stability. The results, expressed as a percentage of the control ($t = 0$), represent the means $\pm$ S.D. of three to four separate experiments, each performed in triplicate.

1.9 ± 0.2 times the basal level and remained constant for the next 12 h (Figure 5). This time-course reveals that a β_2 -mRNA increase precedes up-regulation of total receptor number in dexamethasone-treated H9c2 cells (Figure 3).

The β_1 -mRNA level was found to be decreased by 30% after challenge with 10^{-6} M isoproterenol. The initial level of $2.9 \pm 0.3 \times 10^7$ copies/ μ g of poly(A)⁺ mRNA was reduced to $2.1 \pm 0.2 \times 10^7$ copies/ μ g poly(A)⁺ mRNA within 24 h of treatment. In contrast, the β_1 -mRNA level was found to be unchanged during treatment with dexamethasone for 24 h (results not shown).

The stability of β_2 -mRNA was assessed in H9c2 cells challenged with isoproterenol, dexamethasone or vehicle, by using the

technique of Rodgers et al. [15] which takes advantage of the RNA polymerase II inhibitor, actinomycin D. These experiments probed whether or not post-transcriptional regulation played some role in the effects of isoproterenol and dexamethasone on β_2 -mRNA levels (Figure 6). Half-life measurements of β_2 -mRNA showed that isoproterenol treatment reduced the initial half-life from 9.3 to 6.5 h. Dexamethasone had no effect on the β_2 -mRNA half-life.

DISCUSSION

Signal transduction is a basic process in cell communication within a single cell or tissue. In the heart, β_1 - and β_2 -ARs mediate positive inotropic and chronotropic effects by activation of their intracellular second-messenger system, adenylate cyclase. Receptor regulation has been investigated in human lymphocytes [16] as well as in cell-lines such as DDT₁ MF-2 hamster vas deferens cells [4,8]. Both the human and the cell-culture-model systems display receptors of the β_2 -subtype only, and changes in the β_2 -receptor number of human lymphocytes only mirror changes in tissue β_2 -receptor number [16]. Investigations in human myocardium on β -AR regulation and gene expression have to be discussed with regard to *in vivo* factors such as haemodynamic changes or sympathetic nervous system activity.

Only a minor part of the cell-lines presently available are known to express both β_1 - and β_2 -ARs, and their receptor regulation has been investigated. It has been reported that mouse 3T3-L1 cells [17] and 3T3-F442A cells [18] express β_1 - and β_2 -ARs but display different regulation of their expression during adipogenesis. Further reports show that C6 rat glioma cells also express β_1 - and β_2 -ARs [19–21].

Our investigations have focused on dynamic changes of β -ARs in cells derived from heart tissue. We have detected and classified adrenergic receptors of the β_1 - and β_2 -subtype in the H9c2 (2-1) cell-line. Furthermore, we have determined steady-state levels of the β -AR-mRNA species and the receptor-protein level during treatment with the β -agonist isoproterenol, the antagonist propranolol and the synthetic glucocorticoid dexamethasone.

The percentages of β -ARs we found in the H9c2 cell-line (71% β_2 :29% β_1) are different from those in the human heart. Receptors of the β_2 -subtype represent about 20% of the total β -AR population in ventricular myocardium [22] and 30% in the atria [23]. In the atrio-ventricular conducting system however, up to 50% of the ARs are of the β_2 -subtype [24], which is close to the subtype ratio that we determined in H9c2 cells. In rat heart tissue the percentages of β_1 - and β_2 -ARs were found to be $73 \pm 8\%$ and $27 \pm 8\%$ respectively [25]. The reason for the reversed ratio in our rat heart cell-line is unknown. One can suppose that embryonic rat heart cells, being the origin of our cell-line, may display a different subtype distribution than heart tissue of adult rats. Moreover, there might be a change in β -AR subtype distribution when establishing the cell-line.

Isoproterenol induced a down-regulation of β -ARs in rat heart H9c2 cells, extending the previous report in DDT₁ MF-2 hamster vas deferens cells of a decline in β -ARs in response to isoproterenol [4,5]. Our H9c2 cells displayed an interesting phenotypic response to isoproterenol, shifting the ratio of β -AR subtypes β_1 : β_2 from 29:71 to 75:25. This inversion of receptor ratio is due to the strong decrease in β_2 -AR number, while the β_1 -receptor number remained unchanged.

Comparisons with other studies show inhomogeneous results. The decrease in β -ARs in C6 rat glioma cells after isoproterenol treatment is due to down-regulation of both β_1 - and β_2 -subtypes [26], and C6BU1 cells show a faster decline in β_2 -AR than in β_1 -AR number [27]. Because in our *in vitro* cell model the β_2 -AR is

the predominant subtype its down-regulation by isoproterenol treatment is the main reason for total β -AR down-regulation.

Reduced levels of β_1 - and β_2 -receptor mRNAs can be indicative of agonist-induced down-regulation of both receptor subtypes. Using the RT-PCR technique, which is a very sensitive method for the specific determination of low amounts of mRNA, we found down-regulation of β_2 -mRNA by 42 % and a reduction in β_1 -mRNA levels by 30 %. These results are consistent with reports on S49 mouse lymphoma [5] and DDT₁ MF-2 hamster smooth-muscle cells [10] after treatment with isoproterenol, which led to a new steady-state β_2 -mRNA level after 24 h, which was 35 % (S49 cells) and 50 % (DDT₁ MF-2 cells) below the level of control cells. A decline in β_1 -mRNA by 50 % in rat C6 glioma cells has been reported by Hosoda et al. [26].

Because β_1 -ARs in our cell model represent only 29 % of the total receptor number, putative slight alterations of this receptor subtype on the protein level might not be detectable by the competitive radioligand-binding technique.

A further field of investigation was to determine the effects caused by the non-selective β -antagonist propranolol on β -receptor regulation. In rat hearts Glaubiger and Lefkowitz [6] determined a 2-fold increase in β -AR number after propranolol treatment. Aarons and Molinoff [7] reported that the influence of a β -antagonist is able to increase the β -AR number in heart, lung and lymphocytes of rats after 7 days of treatment. In contrast to this observation, in membranes prepared from BC3H-1 muscle cells and in intact S49 mouse lymphoma cells, which both contain a pure population of β_2 -receptors, propranolol failed to increase the receptor number and elicited a substantial 20–70 % decrease in receptor number after 16–20 h of treatment [28]. In our studies, H9c2 (2-1) cells were incubated with propranolol for 24 h without any changes in either the total β -receptor level or in the β_2 -mRNA level. Therefore no further investigations on the β_1 -mRNA level during propranolol treatment were performed. We conclude that *in vitro* cell-culture models fail to monitor up-regulatory effects during antagonist treatment. A possible explanation may be the limited stimulation time (maximally 24 h) for cell-lines, which might be too short to elicit up-regulatory effects. Another reason could be the absence of catecholamines which lead, *in vivo*, to a tonic down-regulation of β -ARs, which are antagonized by propranolol treatment [29].

The synthetic glucocorticoid dexamethasone has been shown to elevate the rate of β_2 -AR gene transcription through a glucocorticoid response element in the 5'-flanking region of the gene [30]. Further reports confirmed these observations. In DDT₁ MF-2 hamster smooth-muscle cells, glucocorticoid treatment increased the β_2 -AR number 2.2 ± 0.4 -fold [9]. It has also been reported that glucocorticoids affect not only β_2 - but also β_1 - and β_3 -receptor number. Both β_1 - [3,31] and β_3 -receptor subtypes [18] have been shown to be down-regulated by glucocorticoids. In C6 rat glioma cells, treatment with dexamethasone led to an increase in β_2 -receptor number, but was associated with a simultaneous reciprocal decrease in β_1 -receptors [19]. In summary, little or no alteration in total receptor number was observed. In fact no further reports exist of a link between the counter-regulatory effects of β_1 - and β_2 -receptors in the same cell during dexamethasone treatment. Our investigations on the H9c2 cell-line showed a 1.8 ± 0.2 -fold up-regulation of the total β -AR number after 12 h of dexamethasone challenge. This is almost equivalent to a selective 2-fold increase in β_2 -AR number, taking into account that the receptor subtype ratio during dexamethasone treatment was shifted from 71 %:29 % (β_1 : β_2) to 86 %:14 %. In contrast to the observations in C6 rat glioma cells, we could not detect any changes in β_1 -AR number.

Similarly to other systems, β_2 -mRNA was up-regulated

1.9 ± 0.2 -fold in H9c2 cells after dexamethasone treatment. Increased β_2 -mRNA levels were reported as a consequence of increased transcription activity of the β_2 -receptor gene [30]. Contradictory findings have been reported for the regulation of β_1 -mRNA during glucocorticoid treatment in rat C6 glioma cells. While it has been suggested that inhibitory elements that reduce the transcription rate of the β_1 -receptor gene are responsible for diminished β_1 -mRNA levels [20], this mechanism must be explored in further studies. Our results with the H9c2 cell-line, which showed no alterations in the β_1 -mRNA level during 24 h of dexamethasone treatment, are therefore in good agreement with observations about unaltered β_1 -mRNA levels in rat C6 glioma cells after dexamethasone treatment for 24 h [19].

Destabilization of pre-existing mRNA provided one basis for isoproterenol-induced down-regulation of β_2 -AR mRNA, which explains the faster decrease (4 h) to its new steady-state level than that of the receptor (12 h).

Port et al. [32] identified a 35 kDa β -adrenergic receptor mRNA-binding protein (β -ARB protein) which is involved in agonist-induced destabilization of β -AR mRNA. They have shown that the amount of β -ARB protein correlates inversely with the decrease in β -AR mRNA during treatment with agonists in DDT₁-MF2 hamster cells. In contrast, glucocorticoids decrease the amount of β -ARB protein. This may be one explanation for the unchanged half-life of β_2 -mRNA we found during dexamethasone treatment. We therefore conclude that receptor up-regulation is due to enhanced gene transcription, as inferred from Northern blot analysis in C6 rat glioma cells [19].

Apart from the AUUUA destabilization pentamer, β -ARB protein also requires flanking U-rich domains for RNA recognition [33]. The precise role of the β -ARB protein remains to be established. A further report shows that β -ARB is also involved in destabilization of the mRNA of other G-protein-linked receptors, e.g. the thrombin receptor [34].

Our cell-culture model showed characteristic mechanisms in β -AR regulation, although the β -AR subtype distribution was not as expected in cells derived from heart tissue. The kinetic studies of receptor-protein levels and mRNA levels revealed that during isoproterenol and dexamethasone treatment, down- and up-regulation of the mRNAs precedes changes on the protein level.

The kinetic studies on β -AR mRNA levels in comparison to β -receptor number present during pharmacological treatment will probably offer new possibilities for future therapeutic approaches acting on gene expression rather than on altered receptor function.

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