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Cell type-specific regulation of β_2-adrenoceptor mRNA by agonists

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Abstract

Prolonged agonist stimulation of β_2-adrenoceptors results in receptor down-regulation which is often paralleled by a reduction of the corresponding mRNA. In this study, we investigated the agonist-dependent regulation of β_2-adrenoceptor mRNA in DDT_1-MF2 smooth muscle cells and C6 glioma cells. In DDT_1-MF2 cells the half-life of the mRNA was 12 h in monolayer compared to 2 h in suspension cultures. Under both conditions, the agonist isoproterenol reduced this half-life by a factor of 2. In contrast, in C6 glioma cells isoproterenol had no effect on the mRNA stability, even though it reduced mRNA levels by $\approx 50\%$. Isoproterenol-induced downregulation of β_2-adrenoceptor mRNA was completely blocked in C6 cells by the presence of a protein synthesis inhibitor, while this was not so in DDT_1-MF2-cells. These data show that β_2-adrenoceptor downregulation occurs via cell-type specific mechanisms. © 1997 Elsevier Science B.V.

Keywords: β_2-Adrenoceptor; Receptor down-regulation; mRNA half-life

1. Introduction

The β_2-adrenoceptor is a prototypical member of the large family of G-protein-coupled receptors and is subject to a complex regulation by hormones and other signalling molecules (Collins et al., 1991; Hadcock and Malbon, 1993; Lohse, 1993). Signalling via G-protein-coupled receptors can be affected in terms of both the number and the function of these receptors. Most importantly, receptor function varies in response to receptor stimulation. Repeated or prolonged stimulation results in a reduction of responsiveness, a process called agonist-induced receptor desensitization (Hausdorff et al., 1990; Lohse, 1993). Long-term desensitization often involves a significant reduction of receptor numbers, which is termed receptor downregulation. Several distinct molecular mechanisms on both mRNA and protein level contribute to receptor down-regulation, which may be operative to varying extents in different cell lines. To date there is evidence that the expression of the β_2-adrenoceptor gene can be regulated at the level of transcription (Collins et al., 1989, 1990), posttranscriptionally at the level of mRNA stability (Hadcock et al., 1989) or at the level of translation via a short peptide encoded within the 5′ untranslated region of the β_2-adrenoceptor gene (Parola and Kobilka, 1994).

However, there is little agreement whether these different mechanisms are operative in a single cell or whether they are cell-type specific, and to what extent they contribute to β_2-adrenoceptor downregulation. Furthermore, there are wide differences in the absolute values reported in the literature. Thus, the half-life of the β_2-adrenoceptor mRNA in the widely used smooth muscle cell line DDT_1-MF2 was determined by Hadcock et al. (1989) at ≈ 12 h and by Collins et al. (1989) at ≈ 45 min. Cell culture conditions and densities have not yet been investigated as a parameter involved in β_2-adrenoceptor regulation and might be a possible reason for such discrepancies.

Therefore, we set out to study agonist-induced regulation of the β_2-adrenoceptor mRNA under different culture conditions in two widely studied cell lines, hamster DDT_1-MF2 smooth muscle cells and rat C6 glioma cells (Collins et al., 1989, 1990; Hadcock et al., 1989; Hough and Chuang, 1990; Hosoda et al., 1995). We report that β_2-adrenoceptor mRNA is regulated in a cell-type specific manner via two different mechanisms and that the widely

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differing values for its half-life are due to the growth conditions of the cells and do not depend on the method of RNA quantification.

2. Materials and methods

2.1. Cell culture

Cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (Pan Systems), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all purchased from GIBCO). Monolayer cultures were inoculated with a density of 5×10^4 cells/ml and harvested at 60–70% confluence; suspension cultures were maintained at a cell density of about 5×10^5 cells/ml. 12 h after splitting, the cells were exposed to 10 μM (−)-isoproterenol (Sigma) or 10 μM forskolin (Sigma) for the times indicated. To block transcription or translation 5 μg/ml actinomycin D (Roth) or 0.5 μg/ml *Pseudomonas* exotoxin A (Sigma), respectively, were added 30 min after stimulation. At the indicated times the cells were harvested, washed with phosphate-buffered saline and stored at −20°C.

2.2. Radioligand binding assays

Crude cell membranes were prepared as described (Gabilondo et al., 1996) and incubated with 200 pM [^{125}I]cyanopindolol (^{125}I]CYP, New England Nuclear) at 37°C for 45 min to determine the number of β-adrenoceptors under standard saturating conditions. Non-specific binding was determined in the presence of 10 μM (−)-propranolol (Sigma).

2.3. Northern analyses

Total RNA was isolated according to Chomczynski and Sacchi (1987), separated on formaldehyde gels and transferred to nylon membranes (QIAGEN) by downward alcaline capillary transfer (Chomczynski, 1992). Single stranded, digoxigenin-labeled DNA probes were prepared as previously described (Danner and Lohse, 1996). The primers used for the preparation of the β_2-adrenoceptor probe were *β_2 AR.seq2* 5′-GGGCAACCCGGGAAC-3′ and *β_2 AR.rev4* 5′-GGCCTCCTGAAAGACCCTGG-3′ spanning a 675 bp fragment immediately downstream of the start codon. For the synthesis of an α_B-crystallin-specific probe a 468 bp fragment (position 619–1087 of the rat coding sequence) was amplified with the primers *cry.seq* 5′-CCCTTCTTTCCTTTCCACTCCCC-3′ and *cry.rev* 5′-CAGCAGGCTTCTCTTCACGGGT-3′. Hybridization was done at 37°C for 24 h in 50% formamide, 5 × SSC, 3 × Denhardt's solution, 0.5% SDS, 0.2% sodium-laurylsarcosinate and 5% dextrane sulfate. Chemiluminescent detection was performed using the DIG Luminescent Detection Kit (Boehringer-Mannheim). The signals on the X-ray films were analyzed densitometrically.

2.4. Quantitative reverse transcription polymerase chain reactions

1 μg total RNA was transcribed into cDNA using an oligo $(dT)_{15}$ primer (Boehringer-Mannheim) according to Sambrook et al. (1989). 1/10 of the total cDNA obtained was used as template in the polymerase chain reactions. The assay mix contained 2.5 U *Taq* polymerase (Boehringer-Mannheim), 1 μM respective primers mentioned above, 200 μM dNTPs plus 0.5 μCi [α-^{32}P]dCTP, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$ in a volume of 100 μl. The PCR products were separated on agarose gels and their ^{32}P content was quantitated using a Phospho-imager essentially as described (Engelhardt et al., 1996). Control templates not subjected to reverse transcription and known amounts of PCR products encoding the respective sequences were amplified to monitor the accuracy and efficiency of this method.

3. Results

Since the expression of β_2-adrenoceptors has been shown to depend on cell density in certain cell types (Schleifer et al., 1989; Szentendrei et al., 1992; Baeyens and Cornett, 1993), we initially attempted to define the effects of cell culture conditions on the total RNA content/cell. At a confluency of over 80% the cellular RNA concentration determined as μg RNA/mg cellular protein decreased to 30% of the initial value (data not shown). Therefore, care was taken that monolayer cultures were harvested at a maximal confluence of 60–70% and that suspension cultures were maintained at cell densities of about 5×10^5 cells/ml to minimize this phenomenon known as 'in contact-inhibition'.

In order to elucidate a potential influence of the quantification method on the β_2-adrenoceptor mRNA concentrations determined, we compared Northern analysis and quantitative PCR in DDT_1-MF2 cells grown as monolayer cultures (Fig. 1). β_2-adrenoceptor down-regulation was induced by stimulating the cells with 10 μM isoproterenol for the times indicated. Total RNA preparations were analyzed simultaneously with digoxigenin-labeled probes covering either a 675 bp fragment immediately downstream of the β_2-adrenoceptor start codon and a 468 bp α_B-crystallin-specific sequence used as an internal standard. Two transcripts were detected, one of about 2.2 kb specific for the β_2-adrenoceptor mRNA as confirmed by an experiment with only the β_2-adrenoceptor probe and one of about 0.9 kb specific for α_B-crystallin (Fig. 1A). As shown in Fig. 1B, densitometric quantification of the

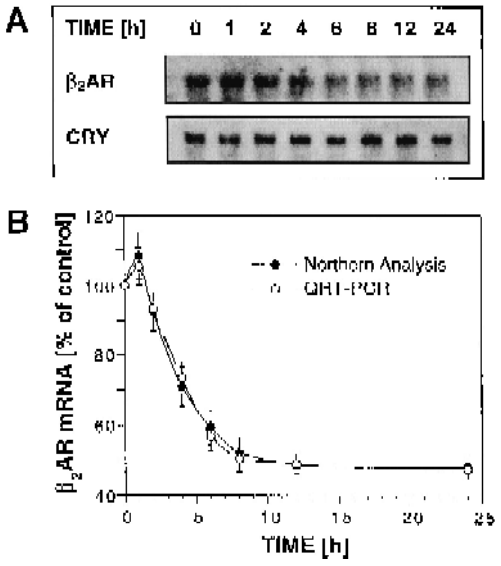

Fig. 1. β_2-adrenoceptor mRNA down-regulation in DDT_1-MF2 smooth muscle cells. (A) Northern analysis of cells treated with 10 μM isoproterenol for the times indicated using probes specific for the β_2-adrenoceptor and for α_B-crystallin as an internal standard, respectively. (B) Time-course of β_2-adrenoceptor mRNA down-regulation determined by two independent methods. The results for the β_2-adrenoceptors are standardized against the respective α_B-crystallin values and expressed as % of control. Data are means $\pm$ SEM of three experiments.

β_2-adrenoceptor mRNA levels, standardized against the respective α_B-crystallin levels, revealed a slight increase 1 h after stimulation; after 24 h these levels were reduced by about 50% compared to control values. To validate these results, we performed quantitative PCR as a second method to determine β_2-adrenoceptor mRNA concentrations. RNA was reverse transcribed and fragments specific for the β_2-adrenoceptor and α_B-crystallin were amplified from the cDNA using the same primers as for the preparation of the probes. The amount of ^{32}P incorporated was measured using a Phospho-imager. The results were in excellent agreement with the data from the Northern analyses (Fig. 1B), with a correlation coefficient of 0.997. Therefore, we conclude that the method applied does not have an influence on the mRNA levels determined.

The values for β_2-adrenoceptor mRNA half-lives in DDT_1-MF2 cells published so far were obtained by using either cells grown as monolayers and solution hybridization assays to quantitate mRNA levels (Hadcock et al., 1989) or suspension cultures and Northern analyses (Collins et al., 1989). The results from the initial experiments of this study suggested a predominant role for the culture conditions on the regulation of the β_2-adrenoceptor mRNA. Therefore, we compared monolayer and suspension cultures by measuring β_2-adrenoceptor mRNA half-lives in Northern analyses (Fig. 2A) and β-adrenoceptor numbers in radioligand binding assays (Fig. 2B). In monolayer cultures the mRNA half-life of untreated control cells was determined to be $\approx$ 12 h. Upon stimulation with 10 μM isoproterenol it decreased to $\approx$ 6.5 h, which parallels the results obtained by Malbon and coworkers (Hadcock et al., 1989). Surprisingly, in suspension cultures the β_2-adrenoceptor mRNA half-lives were reduced by a factor of six without changes in the regulatory pattern: in control cells it was about 2 h, in stimulated cells a $\approx$ 50% reduction was observed resulting in a half-life of $\approx$ 50 min, quite similar to the one measured by Collins et al. (1989). We then investigated whether the shortened mRNA half-life in suspension culture results in altered receptor densities. In monolayer cultures the receptor number decreased upon 12 h stimulation from 190 ± 15 fmol/mg membrane protein to 44 ± 12 fmol/mg (23% of control), in suspension cultures from 175 ± 14 fmol/mg to 27 ± 9 fmol/mg (15% of control) (Fig. 2B). Thus, the culture conditions caused a marked reduction in β_2-adrenoceptor mRNA stability, but

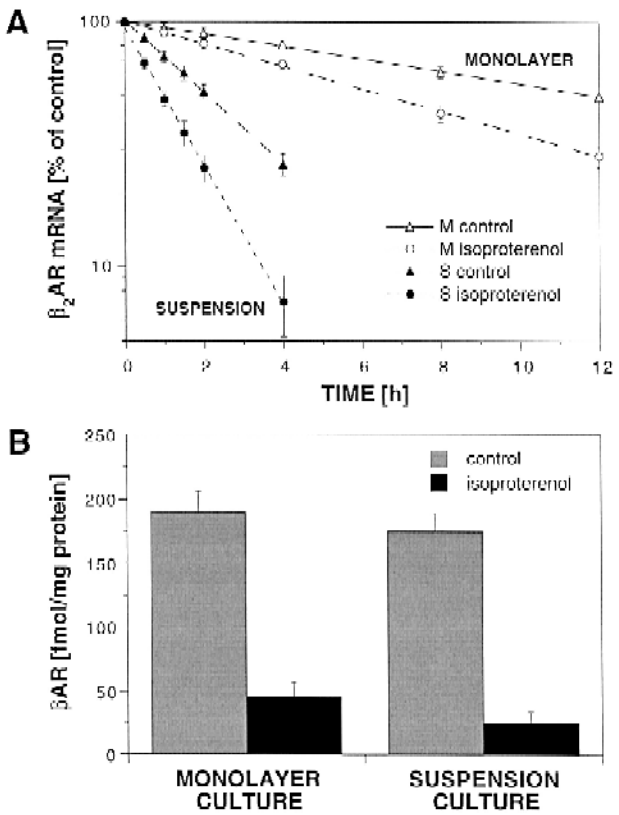

Fig. 2. Effects of culture conditions on agonist-regulation of β_2-adrenoiceptors in DDT1-MF2 smooth muscle cells. Cells were grown in monolayer (M) or suspension (S) culture as described. (A) Determination of the β_2-adrenoceptor mRNA half-lives of control and stimulated cells. Cells were treated with 5 μg/ml actinomycin D 30 min after stimulation ($t = 0$) with 10 μM isoproterenol (or medium for controls) for the times indicated. β_2-adrenoceptor mRNA levels were quantitated by Northern analyses. Data are means $\pm$ SEM of three experiments. (B) β-adrenoceptor numbers expressed in fmol receptor/mg crude membrane protein in control cells and after 12 h stimulation with isoproterenol determined by radioligand binding assays. Data are means $\pm$ SEM of three experiments.

Table 1
Regulation of β-adrenoceptors in C6 glioma cells

Agonist	β_2 AR mRNA	β_2 AR numbers		
	% of control	fmol/mg	fmol/mg	% of control
	12 h	0 h	12 h	12 h
Isoproterenol	48 ± 5 [a]	121 ± 11	32 ± 12	26 ± 5 [a]
Forskolin	50 ± 6 [a]	126 ± 12	64 ± 9	51 ± 7 [b,c]

β_2-adrenoceptor mRNA levels and β-adrenoceptor numbers were determined in control cells and after 12 h incubation with 10 μM isoproterenol or forskolin, respectively. mRNA levels were measured by Northern analyses, receptor numbers were quantitated by [^{125}I]CYP binding as described. The results are expressed as % of control and are means $\pm$ SEM of three experiments. Statistical comparisons were made by means of an unpaired *t*-test.
[a] $P < 0.01$; [b] $P < 0.05$ (12 h versus control); [c] $P < 0.05$ (isoproterenol versus forskolin)

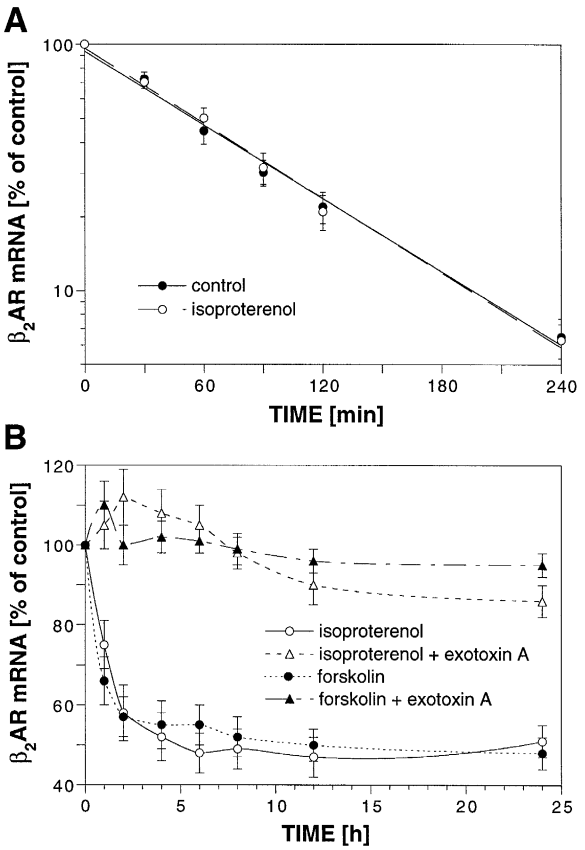

Fig. 3. Regulation of β_2-adrenoceptor mRNA in C6 glioma cells. (A) Determination of the β_2-adrenoceptor mRNA half-lives of control and stimulated cells. Cells were treated with 5 μg/ml actinomycin D 30 min after stimulation ($t = 0$) with 10 μM isoproterenol (or medium for controls) for the times indicated. β_2-adrenoceptor mRNA levels were quantitated by Northern analyses. Data are means $\pm$ SEM of three experiments. (B) Agonist- and forskolin-mediated regulation of β_2-adrenoceptor mRNA. Cells were incubated with either 10 μM isoproterenol or 10 μM forskolin for the times indicated. To inhibit protein synthesis they were additionally treated with 0.5 μg/ml exotoxin A 30 min after stimulation. The results are presented as % of control and are means $\pm$ SEM of three experiments.

this difference was not reflected in the level of receptor expression.

In order to answer the question whether mRNA destabilization is a general mechanism for β_2-adrenoceptor regulation or whether cell type-specific pathways exist, we chose C6 glioma cells as a second cell line. Upon stimulation with 10 μM isoproterenol their β_2-adrenoceptors were down-regulated in C6 glioma cells by 52% on mRNA level and by 74% on the protein level, respectively (Table 1). These data are comparable to those obtained with DDT_1-MF2 cells. On the other hand, the determination of the β_2-adrenoceptor mRNA half-life suggested a completely distinct mode of regulation. The half-life was about 55 min and was entirely independent from agonist-stimulation (Fig. 3A). This agrees with findings obtained in the same cell line by Hosoda et al. (1995). Incubation with 10 μM forskolin, which directly stimulates adenylyl cyclase, resulted in a 50% loss of receptor mRNA, which parallels the results obtained with isoproterenol (Fig. 3B), but only a 49% reduction of receptor protein (Table 1). This indicates that β_2-adrenoceptor mRNA down-regulation is mediated, at least mostly, by the cAMP system.

A pattern of mRNA regulation distinct from that found in DDT_1-MF2 cells was also observed when C6 cells were treated with exotoxin A to block de novo protein synthesis. When exotoxin A was added 30 min after stimulation with either isoproterenol or forskolin, a short-lived 10% increase in β_2-adrenoceptor mRNA levels became visible, and there was an almost complete blockade of mRNA down-regulation (Fig. 3B). These results agree with those obtained by Hosoda et al. (1995), who in contrast to this study pretreated the cell with exotoxin A for 16 h before exposition to agonist. In contrast, exotoxin A had only minor effects on the downregulation of the β_2-adrenoceptor mRNA in DDT_1-MF2 cells. Stimulation with either

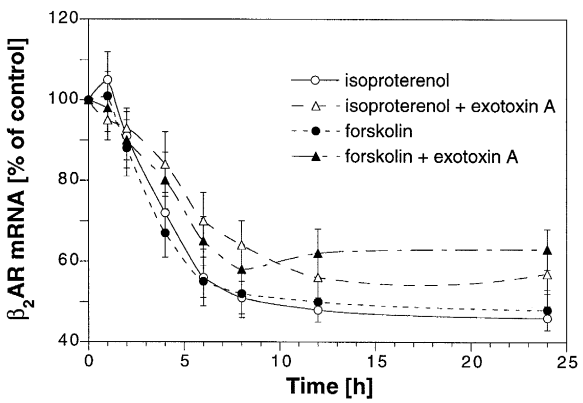

Fig. 4. Down-regulation of β_2-adrenoceptor mRNA in DDT_1-MF2 smooth muscle cells in the presence of exotoxin A. Cells were incubated with either 10 μM isoproterenol or 10 μM forskolin for the times indicated. Exotoxin A was added in a concentration of 0.5 μg/ml 30 min after stimulation. β_2-adrenoceptor mRNA levels were determined by Northern analyses. The results are expressed as % of control and are means $\pm$ SEM of three experiments.

isoproterenol or forskolin for 12 h reduced the β_2-adrenoceptor mRNA levels by about 40% (Fig. 4).

4. Discussion

The aim of this study was to investigate the role of different cell lines and of the cell culture conditions in agonist-induced β_2-adrenoceptor downregulation, and to attempt to reconcile the wide discrepancies in absolute values reported for the β_2-adrenoceptor mRNA half-life (Collins et al., 1989; Hadcock et al., 1989).

Since earlier studies measuring β_2-adrenoceptor mRNA half-lives differed in cell types used, growth conditions and the methods used for RNA quantification, we first validated our mRNA quantification by comparison of two independent methods, Northern analysis, which had also been used by Collins et al. (1989) and quantitative PCR. The latter technique has been used repeatedly in our laboratory (Ungerer et al., 1993, 1994; Engelhardt et al., 1996) and has been shown to be three orders of magnitude more sensitive than solution hybridisation assays, the method used by Hadcock et al. (1989). Using this approach we were able to circumvent possible problems in Northern analyses caused by hybridization stringency, i.e. incomplete binding of the probe to its target. However, the two methods revealed almost identical results strengthening our hypothesis that the growth conditions are the critical parameter for differing mRNA half-lives. Indeed, the β_2-adrenoceptor mRNA half-lives determined in monolayer and suspension cultures, respectively, differed by a factor of 6, 12 h versus 2 h, respectively. Upon stimulation both values were reduced by $\approx 50\%$. It is tempting to speculate that cell-to-cell contact phenomena, which are more prominent in monolayer cultures, are responsible for the reduced mRNA half-life suspension cultures. Interestingly, this transcriptional phenomenon has no consequences on protein level since measuring β-adrenoceptor numbers revealed no significant difference between monolayer and suspension cultures indicating that β_2-adrenoceptor levels are obviously regulated by transcriptional and posttranscriptional mechanisms.

In order to test if these mechanisms vary depending on the cell type used as may be inferred from different studies (Collins et al., 1989, 1990; Hadcock et al., 1989; Hough and Chuang, 1990; Hosoda et al., 1995), we compared DDT_1-MF2 and C6 glioma cells using the cell culture conditions established in our initial experiments. Upon stimulation with isoproterenol, both cell lines down-regulate their β_2-adrenoceptor mRNAs by $\approx 50\%$ and the β-adrenoceptor numbers by $\approx 75\%$, respectively, but they differ completely in their regulation of β_2-adrenoceptor mRNA stability. In C6 glioma cells, the β_2-adrenoceptor half-life remained unchanged upon agonist-stimulation whereas in DDT_1-MF2 cells it was reduced by 50%, indicating distinct regulatory pathways. In DDT_1-MF2 cells, β_2-adrenoceptor regulation seems to occur at the posttranscriptional level via regulation of mRNA stability. Using this cell line, two proteins have been identified which appear to bind to the 3′ untranslated region of the β_2-adrenoceptor mRNA (Port et al., 1992; Pende et al., 1996).

In contrast, β_2-adrenoceptor mRNA regulation in C6 glioma cells does not involve destablilization of the mRNA consistent with the results of Hosoda et al. (1995). The extent of receptor and mRNA downregulation in these cells is, however, identical to that observed in DDT_1-MF2 cells. Moreover, a similar degree of β_2-adrenoceptor mRNA downregulation was induced with isoproterenol and forskolin. In agreement with other studies, in which a decrease in the rate of β_2-adrenoceptor gene transcription by about 25% was determined (Hough and Chuang, 1990; Hosoda et al., 1995), these data indicate that in C6 cells β_2-adrenoceptor downregulation occurs at the transcriptional level via the activation of the cAMP system. Interestingly, this type of downregulation appears to require the presence of a short-lived protein component, since it was completely blocked by treating the cells with exotoxin A. No such protein component seems to be required for the β_2-adrenoceptor mRNA destabilization in DDT_1-MF2 cells. This mechanism of mRNA destabilization, which leads to a reduction in the half-life by 50%, can fully account for the 50% decrease in the steady state levels of this mRNA, suggesting that it is at least the major mechanism of β_2-adrenoceptor mRNA downregulation in these cells.

Forskolin and isoproterenol caused a similar reduction in mRNA levels both in DDT_1-MF2 and in C6 glioma cells. This indicates that both types of regulation are essentially mediated by cAMP. In contrast, the downregulation of the β_2-adrenoceptor protein itself was larger when induced by isoproterenol than by forskolin. This supports the notion that receptor downregulation is at least in part cAMP-independent (Su et al., 1980; Allen et al., 1989) and that the agonist-occupied receptor may be preferentially degraded (Bouvier et al., 1989) The degradation of the receptor protein causes a further 2-fold reduction of receptor levels, i.e. is of similar magnitude as the reduction of the steady-state mRNA levels in the two cell lines.

In summary, β_2-adrenoceptor mRNA levels appear to be regulated by distinct mechanisms. Agonist-induced downregulation of β_2-adrenoceptor mRNA occurs via cAMP, but is mediated by transcriptional effects in C6 glioma cells and by mRNA destabilization in DDT_1-MF2 cells. In addition to this agonist-induced regulation, the β_2-adrenoceptor mRNA stability is very significantly affected by the cell culture conditions. The mechanisms mediating the latter effects remain to be unravelled. Taken together, these data show that a series of mechanisms are involved in regulating β_2-adrenoceptor expression, and that these are utilized in a distinct manner by different types of cells.

Acknowledgements

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