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Control of β_3-adrenergic receptor gene expression in brown adipocytes in culture

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Abstract

Brown adipose tissue is a mammalian thermogenic tissue. Its ability to dissipate energy as heat is due to a unique mitochondrial protein, uncoupling protein (UCP). Activation and expression of UCP is under control of the sympathetic nervous system acting through β-adrenergic receptors (AR). In this study we used Siberian hamster brown adipocytes differentiated in vitro to investigate the expression of the fat specific β_3-AR. Binding studies using the new labelled β_3-adrenergic ligand [^{3}H]SB 206606 showed a density of β_3-AR in brown adipocyte plasma membranes comparable to that measured in vivo. β_3-AR mRNA expression was very high in mature brown adipocytes and was started to be expressed during differentiation before UCP mRNA. Its half-life was approximately 50 min. Treatment of cells with non-specific β adrenergic agonists, specific β_3-adrenergic agonists, and dibutyryl cyclic AMP resulted in a marked down regulation of β_3-AR mRNA level within several hours.

Keywords: Siberian hamster; Brown adipose tissue; Receptor binding; Uncoupling protein

1. Introduction

Brown adipose tissue (BAT) is the main effector of non-shivering, cold-induced thermogenesis in rodents (Foster and Frydman, 1978). It also plays a crucial role in energy balance by dissipating excess energy intake as heat (Rothwell and Stock, 1979). It is known that many types of obesity in rodents are associated with a defective BAT function (Himms-Hagen, 1989). Production of heat by BAT is due to a mitochondrial protein, unique to brown adipocytes, called uncoupling protein (UCP) (for review see Klaus et al., 1991a) and is under the control of the sympathetic nervous system (Seydoux and Giradier, 1978) which acts through β-adrenergic receptors (AR) (Bukowiecki et al., 1981).

Only recently, primary cultures of brown adipocytes differentiating in vitro and expressing UCP could be developed, starting from mouse (Rehnmark et al., 1989, Kopecky et al., 1990), rat (Champigny et al., 1992) and Siberian hamster (*Phodopus sungorus*) (Klaus et al., 1991b) stromal vascular fraction. Primary cultures of mouse brown adipocytes were reported by Pavelka et al. (1993) to express another important BAT protein: type II iodothyronine 5'-deiodinase. Brown adipocytes in culture should be the most valuable tools for studying the metabolism of brown adipocyte and its control in vitro. Since, in BAT, thermogenesis is under control of the sympathetic nervous system, possible control of UCP expression in cultured brown adipocytes by β-adrenergic agonists has been studied. UCP expression was found to be strongly stimulated by various β-adrenergic agonists (Rehnmark et al., 1989, 1990; Kopecky et al., 1990; Klaus et al, 1991b; Champigny et al., 1992; Pavelka et al., 1993). Type II iodothyronine 5'-deiodinase was also stimulated by β-adrenergic agonists (Pavelka et al., 1993). Pharmacological data suggested that these stimulatory effects are mediated by the newly described β_3-AR (Rehnmark et al., 1990; Klaus et al, 1991b; Champigny et al., 1992; Pavelka et al., 1993).

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The gene of the β_3-AR has been cloned first from a human genomic library (Emorine et al., 1989). It was then also isolated from rat BAT cDNA (Granneman et al., 1991; Muzzin et al., 1991) and mouse genomic libraries (Nahmias et al., 1991). The β_3-AR was found to be expressed only in BAT and in white adipose tissue (WAT) in rodents (Muzzin et al., 1991; Nahmias et al., 1991), suggesting that it might represent a fat cell-specific β-AR subtype. Both the quantification of the β_3-AR mRNA by Northern blot analysis and of the receptor itself by ligand binding studies showed that the β_3-AR is not only specific for BAT and WAT, but is also predominant in these cells (Muzzin et al., 1991, 1992).

However, no direct study of the β_3-AR expression had so far been performed in primary cultures of brown adipocytes.

The aim of this work was to study in cultured Siberian hamster brown adipocytes the density of the β_3-AR in the cell membrane using the newly described specific β_3-AR radioligand [^{3}H]SB 206606 (Muzzin et al., 1994), and the degree of β_3-AR mRNA expression in parallel with that of UCP during differentiation. Additionally, the expression of the β_3-AR gene upon exposure to β-adrenergic agonists was studied in the same cell model.

2. Materials and methods

2.1. Chemicals

All organic and inorganic chemicals were of analytical or molecular biology grade and were purchased from Merck (Darmstadt, Germany), Sigma (St-Louis, MO., USA), Fluka (Buchs, Switzerland), Gibco BRL (New York, NY, USA) or Boehringer (Mannheim, Germany). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from Amersham International (Amersham, Bucks, UK). Duralon-UV membranes and the Quik-Hyb hybridization mix were from Stratagene (La Jolla, CA, USA). BRL37344 ((*RR,SS*)-4-((2-(2-hydroxy-2-(3-chlorophenyl)ethyl)-amino)propyl)phenoxyacetic acid) and [^{3}H]SB 206606 ([^{3}H](*RR*)-4-((2-(2-hydroxy-2-(3-chlorophenyl)ethyl) amino)propyl)phenoxyacetic acid) (36.5 Ci/mmol) were gifts from SmithKline Beecham Pharmaceuticals (Epsom, UK), CGP 12177 ((−)-4-(3-t-butylamino-2-hydroxypropoxy)benzimidazol-2-one) from Ciba Geigy (Basel, Switzerland), and the active metabolite of D7114 ((*S*)-4-(2-hydroxy-3-phenoxypropylaminoethoxy)-*N*-(2-methoxyethyl)phenoxyacetamide) from ICI Pharmaceuticals, (Macclesfield, Cheshire, UK).

2.2. Animals

Siberian hamsters (*Phodopus sungorus*) were bred and kept at 23°C with 16 h of illumination per day and fed ad libitum with laboratory hamster chow. At the age of 4–6 weeks they were anesthetized with chloroform, killed by cardiac puncture and the axillary, suprasternal and interscapular brown adipose tissues (BAT) were rapidly excised. Hamsters of both sex were used and the brown fat of 6–10 animals was pooled for the preparation of a cell culture.

2.3. Cell culture

Brown preadipocytes were isolated and cultured as described before (Klaus et al., 1991b). Briefly, preadipocytes isolated by collagenase treatment were cultured in 50% DMEM and 50% Ham's nutritive medium supplemented with 9% fetal calf serum (FCS) at 37°C and 5% CO_2. Cells were inoculated in petri dishes (10 cm diameter) at approximately 1500–2000 cells/cm^2. Medium (10 ml per dish) was changed at days 1 and 3, when differentiation of adipocytes was induced by addition of 17 nM insulin and 1 nM triiodothyronine (T_3) which are important for terminal differentiation of this brown adipocyte culture system (Klaus et al., 1991b). No further change of cell medium took place and FCS was present throughout the experiment. Cells reached confluence around day 5 and lipid containing cells started to appear around day 6. The various drugs tested were added into the medium at day 9 or 10 of culture, when cells were fully differentiated unless otherwise indicated. All experiments were performed at least three times in independent cell cultures.

2.4. RNA analysis

At day 10 of culture, cells were rinsed with ice-cold phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and plates were stored at −20°C (no RNA degradation was observed during storage for up to 2 weeks). Total RNA from one 10 cm cell culture dish was extracted by the single step method of Chomczynski and Sacchi (1987). The yield of total RNA was usually between 50 and 100 μg per dish. Total RNA (20–30 μg) was electrophoresed in a 1% agarose gel containing formaldehyde and transferred to Duralon-UV membranes by capillary blotting. Equal loading of gels and transfer was checked visually by staining the membranes after the transfer with bromophenol blue. The rat UCP and β_3-AR cDNA probes used have been described in previous studies (Bouillaud et al., 1986; Muzzin et al., 1991). Additionally, a 418 bp cDNA fragment for Siberian hamster β_3-AR was obtained by PCR amplification of hamster DNA, using sense primer (TGGGACTCCTCGTAATGCCACCAC) matching position 249–273 and antisense primer (GGAACACTACGAGAAGCAGATAC) matching position 667–644 of the mouse β_3-AR (EMBL data bank: MMB3AR). This probe gave images identical to the rat β_3-AR cDNA probe and was used in some of the experiments. Specificity of these probes for the β_3-AR mRNA was checked by hybridization of blots with rat β_1 and β_2 adrenergic receptor cDNA probes, where no signal could be detected under similar hybridization conditions (not shown).

cDNA probes were labelled by random priming with [α-^{32}P]dCTP to a specific radioactivity of approximately 1×10^9 dpm/μg DNA. RNA blots were hybridized for 1 h at 68°C in the Quick-Hyb hybridization mix, then washed in a solution of 0.1× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS (pH 7.0) at 60°C for 15 min and exposed to Kodak X-AR film at −70°C. Size estimates for the RNA species were established by comparison with a RNA ladder.

2.5. Binding studies

At day 10 of culture, the cells were rinsed in ice-cold PBS and harvested by scraping with a rubber policeman. Cells originating from 15 plates (10 cm diameter) were pooled and transferred into 45 ml of ice-cold lysis buffer (5 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4). They were then homogenized at 1800 rev./min using a Potter Elvehjem homogenizer (Teflon pestle; 8 up and down strokes; clearance 0.3 mm) and plasma membranes were prepared as described previously by Giacobino (1979). Protein concentrations were determined by the method of Lowry et al. (1951).

The conditions chosen to study the binding of [^{3}H]SB 206606 to Siberian hamster brown adipocyte plasma membrane were those determined in a previous study in which the binding of [^{3}H]SB 206606 to rat plasma membranes was characterized (Muzzin et al., 1994). Membranes (25 μg protein) were incubated in 50 mM Tris–HCl, 10 mM $MgCl_2$ (pH 7.4); total volume 0.5 ml, containing the indicated concentrations of [^{3}H]SB 206606 at 37°C for a period of time sufficient to reach equilibrium, i.e. 30 min. Ascorbic acid (1 mM) and 1 μM pyrocatechol-3,5-disulfonic acid (Tiron) were added to the incubation medium in order to protect the catechol group of the β-adrenergic agonist (Durand et al., 1979). Despite the fact that it is presently not known if the β_3-AR mediates its action via a classical G protein, GTP (50 μM) was systematically added to the incubation medium. The binding of the radioligand to the membranes was stopped by dipping the tube into an ice-bath and was determined by filtration at 4°C using a Brandel M-24 R apparatus.

In previous experiments it was found that the specific binding of [^{3}H]SB 206606 (40 nM) to rat BAT plasma membranes could be totally displaced by (−)-isoproterenol (5 mM) (Muzzin et al., 1994). In the binding experiments performed in this study, specific [^{3}H]SB 206606 binding was defined as the difference between the total binding obtained in the absence of competing ligand and the non-specific binding obtained in the presence of concentrations of (−)-isoproterenol which were 10^5-fold those of [^{3}H]SB 206606. Each assay was performed in duplicate. The non-specific binding was found to increase linearly with increasing concentrations of [^{3}H]SB 206606. The quantitative parameters (K_d and B_{max}) were determined by Scatchard-plot analysis using the LIGAND program (Munson and Rodbard, 1980).

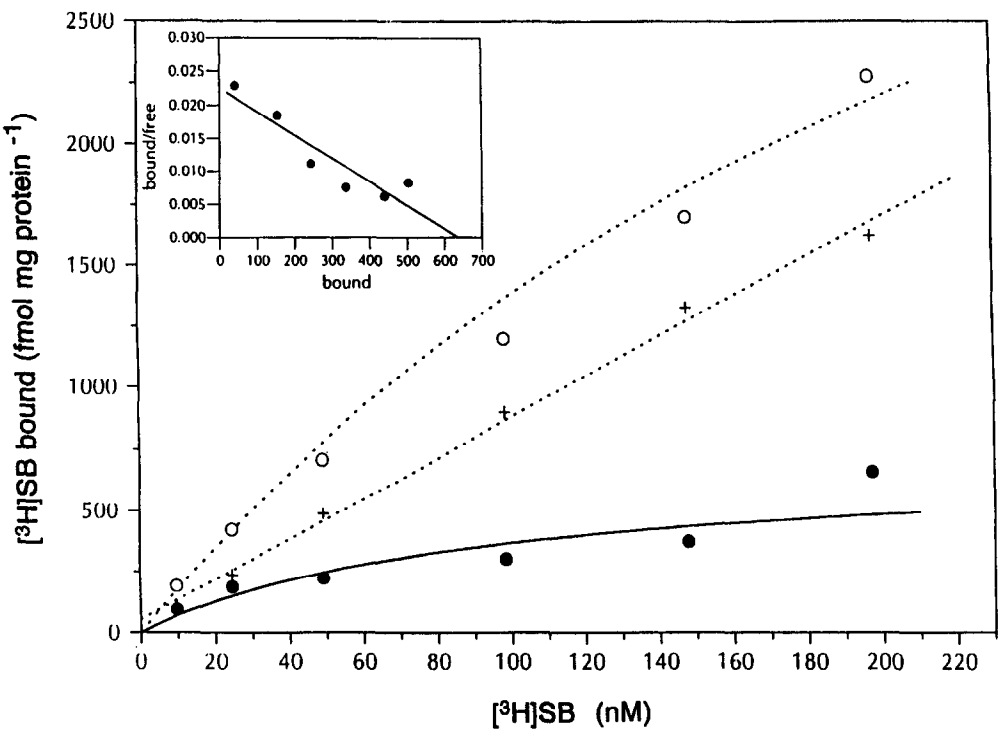

Fig. 1. Total (O), non-specific (+) and specific (●) binding of [^{3}H]SB 206606 ([^{3}H]SB) to Siberian hamster brown adipocyte plasma membranes as a function of increasing ligand concentrations. The results illustrated are from one representative experiment and are expressed as fmol of ligand bound per mg of cell membrane protein. Inset: Scatchard analysis of the data of the representative experiment.

3. Results

The tritiated *RR* enantiomer of the β_3-AR agonist BRL 37344, called [^{3}H]SB 206606 was reported to bind selectively to the β_3-AR (Muzzin et al., 1994). Fig. 1 shows the results of binding studies performed using this new radioligand on Siberian hamster brown adipocyte plasma membranes. The specific binding of [^{3}H]SB 206606, measured in a range of concentrations varying from 10 nM to 200 nM, was found to be saturable. The non-specific binding value, obtained in the presence of a concentration of (−)-isoproterenol exceeding 30 000 times that of the labeled ligand, increased linearly with increasing concentrations of the ligand. It had a mean value of 70% of the total binding value. Ligand binding analysis indicated the presence of a single population of binding sites with K_d and B_{max} values of 93 ± 11 nM and 749 ± 124 fmol/mg of protein ($n = 3$), respectively (values ± SE).

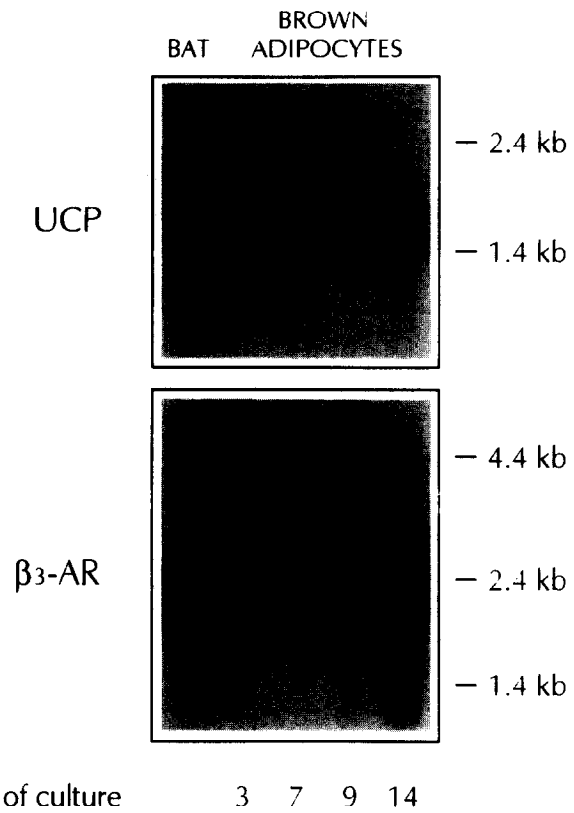

Fig. 2. Expression of the uncoupling protein (UCP) and of the β_3-adrenergic receptor (AR) mRNA in cultured Siberian hamster brown adipocytes at various stages of differentiation. The signals of cultured cell mRNA are compared with that of rat brown adipose tissue (BAT). As indicated in the figure, cells were harvested at days 3, 7, 9 and 14 of culture. 20 μg of total RNA were electrophoresed, transferred to membrane filters and hybridized with ^{32}P-labelled UCP or β_3-AR probes (see Section 2). The figure shows representative autoradiograms. The duration of exposure of Kodak X-AR films to hybridized Northern blots was 2–4 days. The positions of molecular size markers are shown in kb (0.24–9.5 kb RNA ladder).

As seen in Fig. 2, the β_3-AR mRNA was easily detected in rat BAT and cultured Siberian hamster brown adipocytes by total RNA Northern blot analysis. Three messengers with sizes of 1.8, 2.4 and 3.3 kb, respectively, were detected, the 2.4 kb species being the most abundant. This corresponds well to findings in rat adipose tissue (Muzzin et al., 1991). In culture the β_3-AR mRNA level changed with differentiation, being low in preadipocytes and increasing dramatically after confluence (which occurs around day 5). As also shown in Fig. 2, the β_3-AR mRNA level in fully differentiated cells, was in fact higher than that in BAT of rat. It can also be seen in Fig. 2 that the onset of UCP mRNA expression was delayed with respect to that of β_3-AR mRNA expression, no UCP mRNA being detected in preadipocytes. Unstimulated UCP mRNA expression in cultured brown adipocytes was as high as that in BAT of rat (kept at thermoneutrality), with highest level at day 9. This high, transient UCP mRNA expression in cultured brown adipocytes is typical for the Siberian hamster (Klaus, unpublished results).

Addition of actinomycin D into the culture medium resulted in a rapid disappearance of the β_3-AR mRNA, indicating a high turn-over of β_3-AR mRNA (Fig. 3). By fitting an exponential curve to the data, the half-life of the messenger could be calculated to be 52 min. UCP mRNA level in the same experiments was found to decrease much more slowly than β_3-AR mRNA with a half-life of about 24 h (not shown).

As seen in Fig. 4, addition of (−)-isoproterenol resulted in a marked decrease of the β_3-AR mRNA level to about 40 and 20% of control value after 4 and 8 h, respectively. Comparable results were obtained using (−)-norepinephrine, an specific β_3-AR agonist, the active metabolite of D7114 and dibutyryl cyclic AMP (db cAMP). The autoradiographs in this figure are representatives of several independently performed experiments. In all cases, β_3-AR mRNA levels were reduced to about 20–30% of control level within 8 h.

Fig. 5 shows the effects produced in 8 h on the β_3-AR mRNA level by increasing concentrations of the active metabolite of D7114 and of CGP 12177. It can be seen that both drugs induced a decrease in the β_3-AR mRNA level which was already observed at a concentration of 100 nM and reached a maximum at 1 μM. The fact that CGP 12177, which is a β_1-, β_2-adrenergic antagonist and a β_3-adrenergic agonist (Mohell and Dicker, 1989) induced a down-regulation of the β_3-AR comparable to that induced by the active metabolite of D7114, suggests that the effect observed was mostly mediated by interaction of the drugs with the β_3-AR.

4. Discussion

Brown adipocytes of mouse, rat and Siberian hamster cultured under controlled conditions can differentiate in vitro and express the unique mitochondrial protein UCP

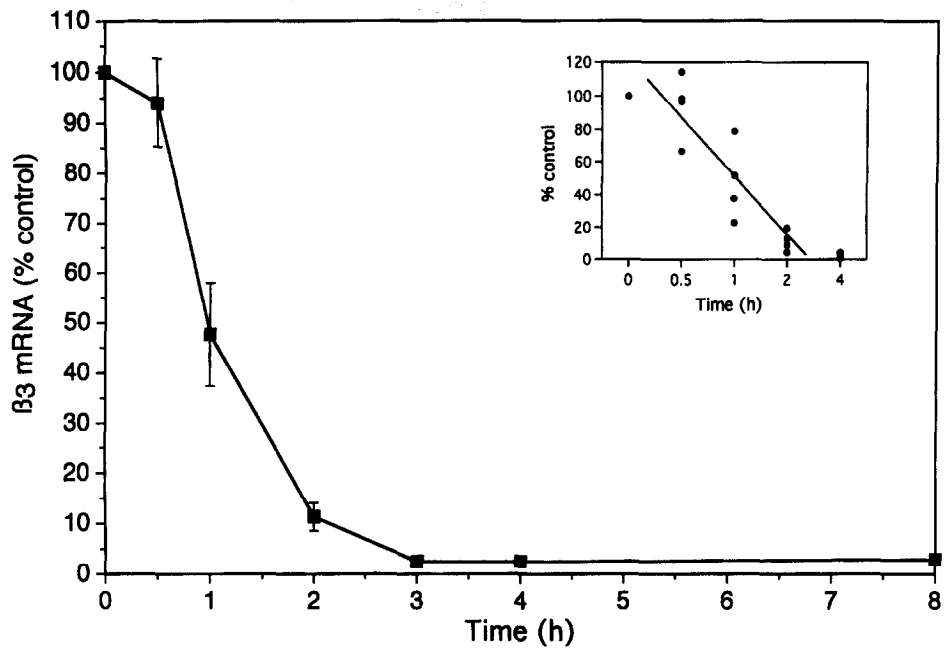

Fig. 3. β_3-Adrenergic receptor mRNA half-life in cultured Siberian hamster brown adipocytes. Differentiated brown adipocytes were treated with actinomycin D (120 ng/ml) at time 0, and harvested for RNA extraction 0.5, 1, 2, 4 and 8 h later. Total RNA (20 μg) was electrophoresed, transferred to membrane filters and hybridized with ^{32}P-labelled β_3-AR probes. Autoradiograms were analyzed by scanning photodensitometry. The results are expressed in percent of the control value at time 0. Each point is the mean ± SE of 2–4 independently performed experiments. Inset: the original data plotted on a log time scale (up to 4 h).

(Rehnmark et al., 1989, Kopecky et al., 1990; Klaus et al. 1991b; Champigny et al., 1992). Although previous studies clearly demonstrated functional β_3-ARs in cultured cells, for the first time our study demonstrates directly the existence of the β_3-AR in brown adipocytes in culture.

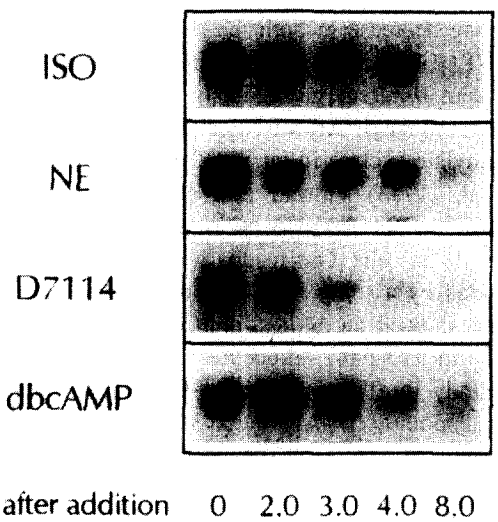

Fig. 4. Effects of (−)-isoproterenol (ISO), (−)-norepinephrine (NE), the active metabolite of D7114 (1 μM each), and of dibutyryl cAMP (db cAMP, 1 mM) on β_3-adrenergic receptor mRNA expression in cultured Siberian hamster brown adipocytes as a function of time. Differentiated brown adipocytes were treated at time 0, and harvested for RNA extraction 2, 3, 4 and 8 h later. Total RNA (20 μg) was electrophoresed, transferred to membrane filters and hybridized with ^{32}P-labelled β_3-AR probes. The figure shows the 2.4 kb signal in representative autoradiograms. The duration of exposure of Kodak X-AR films to hybridized Northern blots was 2–7 days.

Binding data performed using the new labelled β_3-adrenergic ligand [^{3}H]SB 206606 characterized by Muzzin et al. (1994) showed a density of β_3-AR in Siberian hamster adipocyte plasma membranes which was as high as that measured in rat BAT (Muzzin et al., 1991, 1994). The level of expression of the β_3-AR mRNA in the cells in culture was also studied and found to be even higher than that in rat BAT. Thus, the model of Klaus et al. (1991b) expresses not only UCP but also the β_3-AR at a high level, comparable to that observed in rat BAT in vivo.

It was also found in this study that, during differentiation in vitro, the β_3-AR started to be expressed before UCP. This result suggests that β_3-AR stimulation by catecholamines during cell development might turn on and stimulate UCP expression. It is noteworthy that, in bovine perirenal fat, the appearance of β_3-AR mRNA in fetal life precedes that of UCP mRNA (Casteilla et al., 1994). Although in our culture system insulin and T_3 were necessary for terminal differentiation, they did not seem to have a direct effect on β_3-AR mRNA expression (not shown).

The half-life of the β_3-AR mRNA determined in this study is around 50 minutes, a result similar with that obtained for the β_2-AR mRNA in DDT1MF-2 cells (Collins et al., 1989) but dissimilar with that of 12 h obtained for the β_2-AR mRNA in the same cells by a different research group (Hadcock et al., 1989).

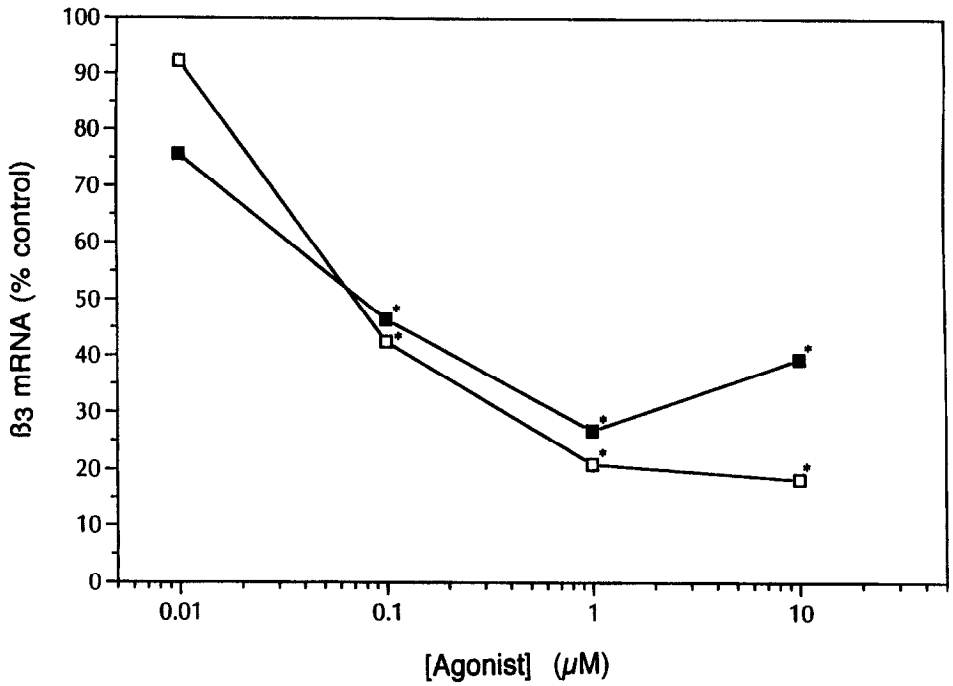

Fig. 5. Effects of the active metabolite of D7114 (■) and of CGP 12177 (□) on β_3-AR mRNA expression in cultured Siberian hamster brown adipocytes as a function of increasing drug concentrations. Cells were treated at time 0 and harvested for RNA extraction 8 h later. Total RNA (20 μg) was electrophoresed, transferred to membrane filters and hybridized with ^{32}P-labelled β_3-AR probes. Autoradiograms were analyzed by scanning photodensitometry. The results are expressed in percent of the control value obtained in the absence of drug. Each point is the mean of 2–3 independently performed experiments. *The value is significantly different from the control ($P < 0.005$).

Structural determinants involved in agonist-induced receptor desensitization have been characterized in the β_2-AR cytoplasmic tail: they consist of serine and threonine which are potential phosphorylation sites for the β-AR receptor kinase and a consensus sequence for phosphorylation by protein kinase A (Bouvier et al., 1988; Hausdorff et al., 1989). The cytoplasmic tail of human and rat β_3-AR lacks these structural determinants (Emorine et al., 1989; Granneman et al., 1991; Muzzin et al., 1991) It was thus expected that the β_3-AR could be less prone than the β_2-AR to agonist desensitization.

It was indeed reported by Granneman (1992) that the β_3-AR of isolated rat white adipocytes is not desensitized by acute exposure (60 min) to (−)-isoproterenol or to the β_3-adrenergic agonist BRL 37344. It was also found that the human β_3-AR stably expressed in Chinese hamster fibroblasts (CHO) does not display short-term (30 min) agonist promoted desensitization or sequestration (Ligett et al., 1993; Nantel et al., 1993). In the present study, the effect of exposure to β-adrenergic agonists on the β_3-AR mRNA expression in Siberian hamster brown adipocytes was studied. It was found that exposure to various β-adrenergic agonists resulted in a marked decrease in β_3-AR mRNA level. The decrease of β_3-AR mRNA by β-adrenergic agonists (Fig. 4) was very fast after an initial lag time of 1–2 h. Already after 3 h a significant decrease could be observed. However, no complete disappearance of the messenger was observed even after long-term treatment.

Our observations that the β_3-AR does not resist long-term down-regulation are in agreement with results previously obtained in vivo: Revelli et al. (1992) administering the β_3-adrenergic agonist Ro 16-8714 observed a strong decrease in both the β_3-AR and its mRNA in rat BAT. Granneman and Lahners (1992), administering various β-adrenergic agonists, also showed a strong decrease in the β_3-AR mRNA and in the β_3-AR activation of adenylate cyclase in rat WAT and BAT. Finally, Unelius et al. (1993) showed that cold-acclimation induces a physiological desensitization of β_3-adrenergic response in brown fat cells. Our results were also very recently confirmed by a study from Granneman and Lahners (1994) who showed an agonist-induced down-regulation of β_3-ARs in 3T3-F442 adipocytes. Altogether this contradicts a study by Thomas et al. (1992) who reported an up-regulation of β_3-AR in 3T3-F442A adipocytes during chronic (up to 30 h) exposure to high concentration (100 μM) of (−)-isoproterenol. The reason for this discrepancy, which is also discussed by Granneman and Lahners (1994), is not entirely clear. It might rest in differences in the characteristics of the cells used or in the experimental protocol.

The results of the present study suggest that the structural determinants of acute desensitization (Bouvier et al., 1988; Hausdorff et al., 1989) are not involved in long-term down-regulation. Similar conclusions were reached in the recent study of Proll et al. (1993) who showed that β-AR receptor kinase and protein kinase A consensus

sites are not required for long-term (24 h) down-regulation of the β_2-AR in L cells.

The present study also suggests that in Siberian hamster brown adipocytes, the agonist-induced down-regulation of the β_3-AR is mediated by cAMP. This finding is in agreement with the results of experiments performed in vivo which suggested that the down-regulation of the β_3-AR observed in rat BAT upon administration of β-adrenergic agonists could be mediated by cAMP (Granneman and Lahners, 1992) and is also confirmed by a recent in vitro study on 3T3-F442 adipocytes (Granneman and Lahners, 1994). Our study suggests further that the regulation of the β_3-AR in brown adipocytes is not different from that in white adipocytes.

Acknowledgments

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