Biochem. J. (2000) 347, 643–651 (Printed in Great Britain)

Differential adrenergic regulation of the gene expression of the β-adrenoceptor subtypes β_1, β_2 and β_3 in brown adipocytes

Tore BENGTSSON[1], Barbara CANNON and Jan NEDERGAARD

The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, S-106 91 Stockholm, Sweden

In brown adipocytes, fundamental cellular processes (cell proliferation, differentiation and apoptosis) are regulated by adrenergic stimulation, notably through β-adrenergic receptors. The presence of all three β-receptor subtypes has been demonstrated in brown adipose tissue. Due to the significance of the action of these receptors and indications that the subtypes govern different processes, the adrenergic regulation of the expression of the β_1-, β_2- and β_3-adrenoceptor genes was examined in murine brown-fat primary cell cultures. Moderate levels of β_1-receptor mRNA, absence of β_2-receptor mRNA and high levels of β_3-receptor mRNA were observed in mature brown adipocytes (day 6 in culture). Noradrenaline (norepinephrine) addition led to diametrically opposite effects on β_1- (markedly enhanced expression) and β_3-gene expression (full cessation of expression, as previously shown). β_2-Gene expression was induced by noradrenaline, but only transiently (< 1 h). The apparent affinities (EC_{50}) of noradrenaline were clearly different (7 nM for the β_1-gene and $\leqslant 1$ nM for the β_3-gene), as were the mediation pathways (solely via β_3-receptors and cAMP for the β_1-gene and via β_3-receptors and cAMP, as well as via α_1-receptors and protein kinase C, for the β_3-gene). The half-lives of the corresponding mRNA species were very short but different (17 min for β_1-mRNA and 27 min for β_3-mRNA), and these degradation rates were not affected by noradrenaline, implying that the mRNA levels were controlled by transcription. Inhibition of protein synthesis also led to diametrically opposite effects on β_1- and β_3-gene expression, but – notably – these effects were congruent with the noradrenaline effects, implying that a common factor regulating β_1-gene expression negatively and β_3-gene expression positively could be envisaged. In conclusion, very divergent effects of adrenergic stimulation on the expression of the different β-receptor genes were found within one cell type, and no unifying concept of adrenergic control of β-receptor gene expression can be formulated, either concerning different cell types, or concerning the different β-receptor subtype genes.

Key words: half-lives, mRNA, noradrenaline, transcription.

INTRODUCTION

In brown adipocytes, processes fundamental for cellular development are controlled by noradrenaline, including cell proliferation [1,2], apoptosis [3] and cellular differentiation [4,5]. These processes are, however, not all controlled via the same adrenergic receptor; rather, a marked functional segregation would seem to have occurred. Cell proliferation is stimulated via β_1-adrenoceptors [1] whereas enhanced differentiation, notably the expression of the tissue-specific uncoupling protein (UCP) 1 [6], is β_3-stimulated [4,5]. The distinctive adrenergic profiles for stimulation of proliferation and of differentiation in brown adipocytes are due to a differentiation-induced switch in the type of β-receptor mediating the noradrenaline response [1,7,8], from β_1 to β_3. Because of this central role of β-adrenoceptors in the control of fundamental processes of the brown adipocyte, and because of their differential association with tissue development, the control of the expression of these receptors is of considerable interest. We and others have previously reported that there is a dramatic down-regulation of β_3-receptor gene expression (in both extent and velocity) upon adrenergic stimulation of brown adipocytes both *in situ* and in culture [9–11]. Clearly, the differential effects of the different β-receptors on fundamental cellular processes prompted the question as to whether the expression of the different β-receptor genes was also under differential adrenergic control. In the present study we have analysed the adrenergic control of gene expression of β_1- and β_2-adrenergic receptors in primary cultures of brown adipocytes. To ascertain the validity of the observed differential regulation of β_1- and β_2-gene expression versus β_3-gene expression, we considered it necessary to re-examine, in parallel, the control of β_3-receptor gene expression. The results obtained (and extended) here were both qualitatively and quantitatively in accordance with our earlier observations [11].

We demonstrate in the present study that in the cultured brown adipocyte system, gene expression of the three β-adrenergic receptor subtypes is independently and differentially regulated by adrenergic stimulation. Studies under analogous *in vivo* conditions indicate that the validity of the results is not restricted to the cell culture system but that the differential control is a physiologically relevant phenomenon.

MATERIALS AND METHODS

Cell culture

For the cell culture studies, brown-fat precursor cells were isolated in principle as previously described [5] from 3-week-old mice that had been kept at the institute at 22 °C for 2–3 days before dissection. The mice were of an NMRI outbred strain

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; UCP, uncoupling protein.

[1] To whom correspondence should be addressed (e-mail tore.bengtsson@zoofys.su.se).

© 2000 Biochemical Society

from a local supplier (Eklund). The interscapular, axillary and cervical brown adipose tissue deposits were dissected out under sterile conditions. The tissue was carefully minced and transferred to the Hepes-buffered solution (pH 7.4) detailed by Néchad et al. [12], containing 0.2 % (w/v) crude collagenase type II (Sigma). Routinely, pooled tissue from six mice was digested in 10 ml of the Hepes-buffered solution. The tissue was digested for 30 min at 37 °C and vortex-mixed every 5 min. The digest was filtered through a 250 μm silk filter into sterile tubes. To allow the mature brown-fat cells and lipid droplets to float, the solution in sterile tubes was placed on ice for 15 min. The infranatant was filtered through a 25 μm silk filter and collected in 10 ml sterile tubes. The precursor cells were collected by centrifugation for 10 min at 700 ***g***, resuspended in Dulbecco's modified Eagle's medium (DMEM) and recentrifuged. The pellet was resuspended in a volume corresponding to 0.5 ml of cell culture medium/mouse dissected.

The cell culture medium consisted of DMEM supplemented with 10 % newborn calf serum (Life Technologies), 4 nM insulin and 10 mM Hepes and with 50 units/ml penicillin, 50 μg/ml streptomycin, and 25 μg sodium ascorbate per ml [13]. Aliquots of 0.5 ml of cell suspension were cultivated in 25 cm^2 tissue culture flasks (Life Technologies) with 4.5 ml of cell culture medium in each flask, or 0.2 ml of cell suspension in six-well culture dishes (Life Technologies) with 1.8 ml of cell culture medium in each well. The cultures were placed at 37 °C in a water-saturated atmosphere of 8 % CO_2 in air, in a Heraeus CO_2-auto-zero B5061 incubator. On days 1, 3 and 6 (if experiments were not performed on that day), the medium was discarded, the cells were washed with prewarmed DMEM, and fresh medium was added. Most experiments were performed with cells after 6–7 days in culture, i.e. at confluence. Detailed protocols are found in the description of each experiment.

At the end of each experiment, the medium was discarded and the cells were dissolved in 1 ml of an Ultraspec solution (Biotecx Laboratories, Houston, TX, U.S.A.); the manufacturer's procedure for RNA isolation was followed. The final pellet was suspended in 50 μl of 10 mM EDTA and the RNA extracted at 65 °C for 10 min with vortex-mixing every 2 min. RNA concentration was measured and absence of protein contamination was checked on a Beckman DU 50 spectrophotometer with readings at 260 nm and 280 nm. The ratio of 260/280nm was routinely higher than 1.7.

Animals

For the studies in intact animals, 6-week-old male mice (NMRI strain; Eklund, Vallentuna, Sweden) were kept at +28 °C for at least 7 days with free access to food ('Standard rat and mouse feed', B&K Universal, Stockholm, Sweden) and water. The animals were thereafter either transferred to single cages at +4 °C (i.e. 3–5 °C) (cold exposure) or handled in the same way but kept at +28 °C (i.e. 27–29 °C) (control) for the indicated times. The animals were killed by CO_2, followed by decapitation, and the interscapular brown adipose tissue was excised. Homogenization in Ultraspec solution (Biotecx Laboratories) was performed with a Potter-Elvehjem homogenizer with a tightly fitting teflon pestle (10–15 strokes), and total RNA was isolated according to the manufacturer's total RNA isolation method.

Northern-blot analysis

Solutions containing RNA in 10 mM EDTA were freeze-dried in a SpeedVac. The RNA was then dissolved in 15 μl of RNA cocktail, consisting of 50 % (v/v) formamide, 5 mM Mops and 9 % (v/v) formaldehyde, and 5 μl of loading buffer consisting of 50 % (w/v) glycerol and 0.1 mg/ml Bromophenol Blue. The solution was incubated for 10 min at 65 °C and then chilled on ice. The samples were loaded on a gel [1.25 % agarose, 10 mM Mops, 6.2 % (v/v) formaldehyde and 20 μl of 1 mg/ml ethidium bromide]. The gel was run in 20 mM Mops buffer for 2–3 h at 4–5 V/cm. After electrophoresis, the 18 S–28 S rRNA bands were verified under UV-light to examine the samples for equal loading and to ensure that no degradation had occurred.

The RNA was blotted overnight from the gel to a Hybond-N membrane in 20 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). Three sheets of Whatman 3MM filter paper soaked in 20 × SSC were placed on top of the Hybond-N membrane. The gel and the Hybond-N membrane were examined under UV-light. The RNA was cross-linked to the Hybond-N membrane [UV Stratalinker 1800 (Stratagene) with the auto crosslink program]. The Hybond-N membrane was prehybridized with 10 ml of a solution containing 5 × SSC, 5 × Denhardt's solution (where 1 × Denhardt's solution is 2 % Ficoll 400/2 % polyvinylpyrrolidone/2 % BSA), 0.5 % SDS, 50 mM sodium phosphate, 50 % formamide and 100 μg/ml of degraded DNA from herring sperm (Sigma) in a hybridization oven (Hybaid) at 45 °C for 2 h. After this prehybridization, the Hybond-N membrane was transferred to a similar solution containing the denatured probe (see below) at a final concentration of (1–3) × 10^6 c.p.m./ml. The hybridization was carried out for at least 16 h at 45 °C. The Hybond-N membrane was then washed twice in 2 × SSC, 0.2 % SDS at 30 °C for 20–30 min and then twice in 0.1 × SSC, 0.2 % SDS at 50 °C for 45 min. The membrane was sealed in a plastic envelope and exposed to a PhosphorImager screen. The screens were analysed on a Molecular Dynamics PhosphorImager with the ImageQuant program. When the same membrane was analysed for several mRNA species, the previous probe was removed by pouring boiling 0.1 % SDS solution onto the membrane and letting it cool to room temperature.

β_1-, β_2- and β_3-adrenoceptor cDNA probes

The rat β_1-cDNA used was that previously characterized by Revelli et al. [9]. It was cloned into the *Eco*RI site of the PVZ_1 plasmid (size approx. 2.7 kb). The 1.5 kb fragment obtained by *Har*I digestion was used for hybridization. The β_2-probe was an 896 bp *Eco*RV–*Bst*EII fragment obtained from the human β_2-cDNA in pUC18 [14]. The β_3-probe originated from the A43 probe characterized previously [15]. A fragment of the A43 mouse β_3-adrenoceptor gene was subcloned into pUC18 at the *Xba*I site. This genomic DNA fragment has a length of 300 bp and corresponds to the 5′-coding region of the β_3-adrenoceptor from the initial ATG to the second transmembrane loop (TM2). To generate the β_3-adrenoceptor probe used in the present study, the plasmid was cut with the restriction enzymes *Bam*HI and *Sal*I to a length of 0.5 kb.

The probes were labelled with [α-^{32}P]dCTP using a DNA labelling kit (Boehringer) to an activity of (10–100) × 10^6 c.p.m./μg of DNA (and eluted in 200 μl of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA to 10000–60000 c.p.m./μl of stock solution).

Analysis of dose–response curves

For analysis of dose–response curves, the curve-fitting option of the KaleidaGraph 3.0 application (Synergy Software, Reading, PA, U.S.A.) was used. Monophasic dose–response data were analysed with the rearranged Michaelis–Menten equation:

$$V_A = basal + V_{max}/\{1 + (EC_{50}/[A])\}$$

where [A] is the concentration of adrenergic agent added, V_A is the response (mRNA level) observed at that concentration, *basal*

© 2000 Biochemical Society

is the basal mRNA level, and V_{max} is the estimated maximal increase in mRNA level. For the analysis of biphasic ('semi-bell-shaped') dose–response data, a model [16] for the interaction of a ligand with two different receptors, one stimulatory (S) and one inhibitory (I), was used:

$$V_A = basal + V_{max}(S)/\{1+(EC_{50}/[A])\} + V_{max}(I)/\{1+(IC_{50}/[A])\}$$

Chemicals

L-Noradrenaline bitartrate (arterenol), DL-propranolol and collagenase (type II) were obtained from Sigma. BRL-37344 was a gift from SmithKline Beecham Pharmaceuticals, CGP-12177 was a gift from Ciba-Geigy and ICI-89406 and ICI-118551 were gifts from Imperial Chemical Industries (AstraZeneca, Södertälje, Sweden). All adrenergic agents were freshly dissolved in water. Noradrenaline was dissolved in 0.125 mM sodium ascorbate (as in medium).

RESULTS

β-Adrenoceptor subtype gene expression in cell cultures

To study the possible differential regulation of the gene expression of the β-adrenoceptor subtypes, experiments were performed in primary cultures of mouse brown adipocytes. In these cell cultures, isolated undifferentiated brown-fat precursor cells grow to reach confluence. At the time of confluence (day 5–6 in culture), the cells have morphologically differentiated, contain multilocular lipid droplets and have the ability to show noradrenaline-induced expression of the gene for the tissue-specific mitochondrial protein UCP1 (thermogenin) [4,5]. At this point of innate differentiation, moderate levels of β_1-receptor mRNA and high levels of β_3-receptor mRNA may be encountered in the cells [8], and this stage was therefore selected for the examination of possible differential adrenergic regulation of the gene expression of these receptors.

The presence of β_1-receptor mRNA in these cells was confirmed in the Northern-blot displayed in the top panel of Figure 1 (0 h lane). The size of the β_1-transcript was 2.6 kb, in good agreement with what has been observed in a variety of tissues, including brown adipose tissue [17–20].

There was little or no expression of the β_2-adrenergic receptor gene in unstimulated brown adipocytes [although this receptor was expressed in the brown adipocyte sample tissue (rightmost lane)].

The β_3-gene was well expressed. The estimated size of the β_3-adrenergic receptor major transcript was 2.4 kb (top panel of Figure 1), in good agreement with previous reports for mouse β_3-receptors [8,11,15,21]. In the following we have used only the major band of 2.4 kb for compilation of results.

As similar labelling procedures were used for the different receptors, the intensity of the signals versus background can be used as a semiquantitative estimation of mRNA levels. It is then clear that the β_3-mRNA levels were much higher than the β_1-mRNA levels, and that the innate expression level of the β_2-gene was orders of magnitude lower than that of the β_1- or β_3-genes.

Noradrenaline-induced differential regulation of β-adrenoceptor subtype gene expression in cultured brown adipocytes

To examine possible differential adrenergic control of expression of the three β-receptor subtypes, the cultured brown adipocytes were treated with noradrenaline for various times, and the levels of β-adrenoceptor mRNA in the total RNA determined by Northern-blot analysis, as exemplified in the Northern-blot in the top panel of Figure 1.

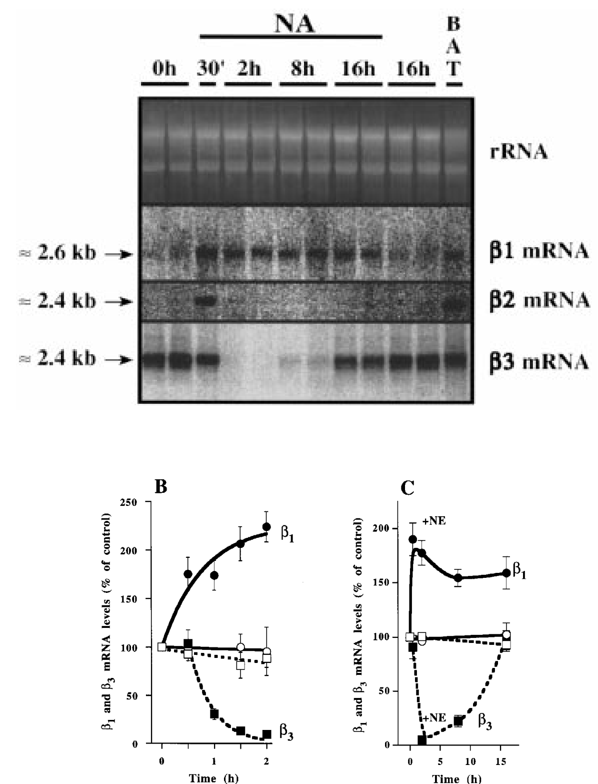

Figure 1 Influence of noradrenaline on β_1-, β_2- and β_3-receptor mRNA levels in primary cultures of mouse brown adipocytes

(Top panel) Confluent cultures of brown adipocytes (day 6 in culture) were treated with 0.1 μM noradrenaline (NA/NE) for the times indicated. Total RNA was isolated as described in the Materials and methods section. Samples (15 μg) were analysed by Northern-blot analysis, by hybridization with the β-adrenoceptor probes described in the Materials and methods section. 'BAT' indicates 10 μg of total RNA from brown adipose tissue from one control mouse (22 °C). (Bottom left panel) Compilation of the results from short-time experiments principally similar to those exemplified in the top panel. The results are the means ± S.E.M. of two independent cell culture series. Within each series the experiments were performed in independent duplicate cell culture wells. ●, β_1-mRNA levels; ■, β_3-mRNA levels in cells treated with 0.1 μM noradrenaline. ○, β_1-mRNA levels; □, represents β_3-mRNA levels in cells treated only with vehicle. The line for the β_1-mRNA levels was drawn after reiterative fitting to the function: $V_t = basal + V_{max} \cdot [1-\exp(-\tau \cdot t)]$, where V_t is the mRNA level at time t, V_{max} is the maximal increase in mRNA level, and τ is the time constant. The fitting yielded a V_{max} of 127 ± 24% of initial β_1-mRNA levels and a half-time of 33 ± 15 min. The line for the β_3-mRNA levels was drawn after reiterative fitting to the function: $V_t = basal \cdot \exp[\tau \cdot (t - t_l)]$, where t_l is the lag time. The fitting yielded a half-time of 19 ± 5 min for the β_3-mRNA levels, with a lag time of 30 min. (Bottom right panel) Compilation of results from experiments similar to those exemplified in the top panel. In each experiment, the mean level of β_1- and β_3-mRNA in untreated wells at zero time was set to 100% and the other values given relative to this. The results are the means ± S.E.M. of three cell culture series (different from the bottom left panel). Within each series the experiments were performed in independent duplicate cell culture wells. The same symbols are used as in the bottom left panel. The lines were drawn with the 'smooth' option in the KaleidaGraph application for Macintosh.

Quite in contrast to the adrenergically induced down-regulation reported previously for β_3-receptors in brown adipocytes *in situ* or in primary culture and in adipocyte-like cell lines [9–11,21,22] (which is also confirmed below), noradrenaline addition led to a rapid increase in the β_1-mRNA level (bottom left panel of Figure 1). After as little as 7.5 min, there was a detectable increase in β_1-receptor mRNA levels (Table 1), and the level was further increased during the first 2 h (bottom left panel of Figure 1) of adrenergic stimulation, after which the level

© 2000 Biochemical Society

was maintained at approx. double the initial level for at least 16 h (bottom right panel of Figure 1). Fitting of the data points from the bottom left panel of Figure 1 to an exponential time function yielded a half-time of 33 min for the increase of the β_1-receptor mRNA levels.

Notably, the β_2-adrenergic receptor gene, which was not expressed at all in unstimulated brown adipocytes, was transiently induced by noradrenaline. After 15 min of noradrenaline stimulation, β_2-adrenergic receptor mRNA could be detected, and after 30 min the expression had reached a significant level (Table 1 and the top panel of Figure 1). The size of the β_2-adrenoceptor transcript was 2.4 kb. This is similar to the size previously reported in adipose cells [18,20]. However, after 2 h of stimulation with noradrenaline, the β_2-adrenergic receptor mRNA had again become undetectable (Table 1 and the top panel of Figure 1). The control of β_2-expression has not been further studied here.

Also in the present study, β_3-adrenergic receptor gene expression was rapidly, extensively and transiently down-regulated by noradrenaline, as previously reported by us and others [9–11,21,22]. It may be noted that in the present experiments, with a more detailed time resolution, a slight but statistically significant increase in the β_3-receptor mRNA levels during the initial lag phase was detected (Table 1), before the rapid decrease (half-time 19 min) in mRNA levels.

Thus, the three β-receptor subtypes displayed markedly different responses in the adrenergic regulation of their gene expression.

Effect of noradrenaline concentration on β-adrenoceptor gene expression

Although contrasting expression responses (up- or down-regulation) of the β_1- and β_3-receptor genes were observed, it could be envisaged that the same intracellular processes controlled these responses. One initial possibility to examine whether this was the case, was to examine if the two responses displayed identical or different sensitivity to adrenergic stimulation.

The dose–response curve for the noradrenaline-stimulated increase in β_1-mRNA levels is depicted in Figure 2. The response, which had an EC_{50} value of 7 nM (uncertainty interval 6–8 nM), did not appear to display simple Michaelis–Menten kinetics. Instead a semi-bell-shaped appearance of the dose–response curve may be envisaged. Such unusual dose–response curves are not without precedence in studies of cultured brown adipocytes. They have been observed for the adrenergic control of the expression of the UCP1 gene ([4,5,23]; and the present study, results not shown), and for adrenergic control of cAMP accumulation [8]. That the noradrenaline-induced increase in expression of the β_1-receptor displayed such a dose–response curve is thus in itself an indication that the effect is mediated via the cAMP pathway (see also below).

The dose–response curve for the noradrenaline-induced decrease in β_3-mRNA levels deviated markedly from the dose–response curve for β_1-expression. The response displayed simple Michaelis–Menten kinetics. In agreement with [11] the EC_{50} value for the down-regulation of the β_3 mRNA levels was 1 nM (uncertainty interval 0.7–1.3 nM), i.e. nearly an order of magnitude lower than that for the β_1-gene effect.

Thus, the adrenergic regulation of β_1- and β_3- gene expression not only displayed contrasting effects (up- versus down-regulation) and different temporal kinetics, but also such different receptor kinetics that it may be assumed that the adrenergic receptors involved, and/or the intracellular mediation, were non-identical.

Table 1 Short-term differential influence of noradrenaline on the mRNA levels of the three β-adrenergic receptors in primary cultures of mouse brown adipocytes

After 6 days in culture, cells were treated with 0.1 μM noradrenaline for the indicated times, after which 20 μg of RNA was analysed by Northern-blot analysis by hybridization with the β-adrenoceptor probes, as described in the Materials and methods section. Data are means ± S.E.M. from two cell culture series each performed in independent duplicate cell culture wells. The mean levels of β_1- and β_3-mRNAs in untreated wells were set to 100% and the other values given relative to this. The highest value of β_2-mRNAs was set to 100% and the other values given relative to this. The early increase in β_3-receptor expression is statistically significant ($P < 0.01$; Student's paired t test).

	β_1-mRNA level (%)	β_2-mRNA level (%)	β_3-mRNA level (%)
Control	100 ± 0	0	100 ± 0
7.5 min	117 ± 2	3 ± 2	124 ± 8
15 min	137 ± 9	25 ± 8	125 ± 6
30 min	195 ± 5	100 ± 0	104 ± 3
120 min	251 ± 25	0	3 ± 1

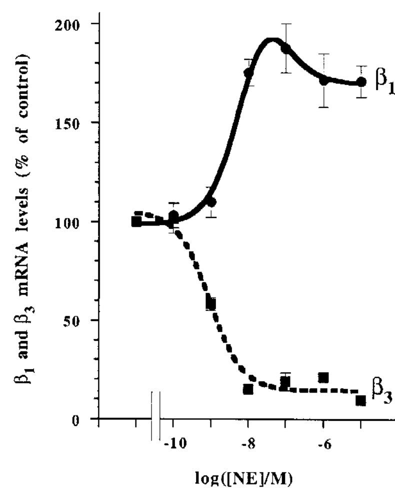

Figure 2 Dose–response curves for the effects of noradrenaline on β_1-and β_3-adrenergic receptor mRNA levels in cultures of brown adipocytes

Confluent cultures of brown adipocytes (day 7 in culture) were treated for 2 h with the indicated concentrations of noradrenaline (NE). Total RNA was isolated and analysed as described in the Materials and methods section. The values are means ± S.E.M. of two cell culture series, one performed in triplicate and one performed in quadruplicate in independent cell culture wells. Untreated wells were set to 100% for each probe and cell culture series and the other values given relative to this. ●, β_1-mRNA levels; ■, β_3-mRNA levels. The β_1-mRNA levels were analysed for Michaelis–Menten kinetics, using a model for the interaction of a ligand with two different receptors, one stimulatory and one inhibitory, as described in the Materials and methods section. Prior analysis with simple Michaelis–Menten kinetics or with Michaelis–Menten kinetics with a free Hill coefficient yielded worse correlation values. The results of the β_3-mRNA levels were fitted with the general curve-fitting procedure for Michaelis–Menten kinetics with one stimulatory receptor. More complex kinetic models did not increase the correlation value.

Characterization of the adrenergic receptor and the intracellular mediators involved in the adrenergic control of β-receptor gene expression

Based on the indication that different receptors and/or intracellular pathways may be involved for the two genes, the nature of the adrenergic receptor and of the intracellular mediation was studied (Table 2).

© 2000 Biochemical Society

Table 2 Influence of adrenergic agents on β_1- and β_3-receptor mRNA levels in cultures of brown adipocytes

After 6 days in culture, cells were treated with the indicated agonists for 2 h, after which time total RNA was isolated and analysed as described in the Materials and methods section. Data are means ± S.E.M. from the number of cell culture series indicated. Within each series, the experiments were performed in independent duplicate cell culture wells. The agonists noradrenaline (NA), adrenaline, isoprenaline and BRL-37344 were added at a concentration of 0.1 μM, the agonists cirazolin and CGP-12177 were added at a concentration of 1 μM, the antagonists prazosin, yohimbine and D,L-propranolol were added at a concentration of 10 μM and the antagonists ICI-89406 and ICI-118551 were added at a concentration of 1 μM (all antagonists were added 10 min before noradrenaline addition). Concentrations of 1 μM forskolin, 1 μM A23187, 1 mM 8-bromo-cAMP, and 500 ng/ml PMA were added. $[Ca^{2+}]_i$ and [cAMP], intracellular concentrations of Ca^{2+} and cAMP, respectively.

Agent	Receptor(s) or intracellular mediator stimulated	β_1-mRNA level (%)	*n*	β_3-mRNA level (%)	*n*
Control		100 ± 0	4	100 ± 0	5
Noradrenaline	$\alpha_1\alpha_2\beta_1\beta_2\beta_3$	162 ± 12	4	8 ± 2	5
Noradrenaline + prazosin	$\alpha_2\beta_1\beta_2\beta_3$	167 ± 24	2	5 ± 3	2
Noradrenaline + yohimbine	$\alpha_1\ \beta_1\beta_2\beta_3$	159 ± 30	2	5 ± 2	2
Noradrenaline + ICI-89406	$\alpha_1\alpha_2\ \beta_2\beta_3$	174 ± 18	2	6 ± 4	2
Noradrenaline + ICI-118551	$\alpha_1\alpha_2\beta_1\ \beta_3$	188 ± 17	2	7 ± 2	2
Noradrenaline + propranolol	$\alpha_1\alpha_2\ (\beta_3)$	90 ± 4	3	23 ± 5	3
Cirazoline	α_1	100 ± 13	3	31 ± 6	4
Cirazoline + Prazosin		88 ± 15	2	95 ± 7	3
NA + Propranolol + Prazosin		115 ± 20	2	48 ± 4	3
Adrenaline	$\alpha_1\alpha_2\beta_1\beta_2\beta_3$	163 ± 15	2	3 ± 2	2
Isoprenaline	$\beta_1\beta_2\beta_3$	149 ± 14	4	6 ± 2	5
BRL-37344	β_3	147 ± 7	2	8 ± 5	2
CGP-12177	β_3	120 ± 11	2	8 ± 3	3
A23187	$[Ca^{2+}]_i$	82 ± 12	4	90 ± 9	5
PMA	PKC	78 ± 23	2	16 ± 4	3
Forskolin	$[cAMP]_i$	181 ± 12	2	19 ± 6	2
8-Bromo-cAMP	$[cAMP]_i$	169 ± 3	1	10 ± 1	1

β_1

Concerning β_1-adrenergic receptor gene expression, the subtype-selective α-adrenergic antagonists prazosin (α_1) or yohimbine (α_2) both failed to prevent the noradrenaline-induced up-regulation of β_1-gene expression (Table 2). Therefore, subtype-selective β-adrenergic antagonists, ICI-89406 (β_1) and ICI-118551 (β_2), were tested. However, these antagonists also failed to prevent the noradrenaline-induced up-regulation of β_1-gene expression. In contrast, the subtype-nonselective β-adrenergic antagonist propranolol, added in sufficiently high concentration (100-fold excess) that it should also be able to inhibit the β_3-receptor (which has a much lower affinity for propranolol than the β_1/β_2-receptors [24,25]), prevented the noradrenaline effect. Thus the receptor involved was apparently of the β_3-subtype, but as no established β_3-antagonist is available, we could not investigate the effect of selective inhibition of the β_3-adrenergic receptors. Instead, agonist effects were examined. Treatment with the α_1-receptor agonist cirazoline had no effect on β_1-receptor expression. However, adrenaline, as well as the subtype-nonselective β-adrenergic agonist isoprenaline, the β_3-adrenergic receptor selective agonist BRL-37344 and the absolute β_3-agonist CGP-12177 (which is an antagonist on β_1- and β_2-receptors [26]) all mimicked the effect of noradrenaline. These agonist experiments therefore confirm that the effect could be brought about by stimulation of β_3-receptors.

Concerning the intracellular mediation of the adrenergic signal controlling β_1-receptor gene expression, three established intracellular mediators were tested: Ca^{2+}, protein kinase C (PKC) and cAMP. The Ca^{2+}-ionophore A23187 failed to induce an up-regulation of β_1-receptor mRNA, and, similarly, treatment with the PKC activator phorbol 12-myristate 13-acetate (PMA) did not induce an up-regulation of β_1-receptor gene expression. However, the adenylate cyclase activator forskolin increased β_1-receptor gene expression, as did treatment with 8-bromo-cAMP (a cAMP analogue). Thus, up-regulation of β_1-receptor mRNA was brought about by an activation of β_3-receptors and by subsequent increases in intracellular cAMP levels, whereas α_1 receptors and the corresponding second messengers were without effect.

β_3

Prazosin or yohimbine also failed to prevent the noradrenaline-induced down-regulation of β_3-gene expression (Table 2). Thus, a β-adrenergic stimulation was sufficient to down-regulate β_3-gene expression. The subtype selective β-adrenergic receptor blockers, ICI-89406 and ICI-118551, similarly failed to prevent the noradrenaline-induced down-regulation. However, in contrast to what was observed above concerning β_1-expression, propranolol also failed to *fully* prevent the noradrenaline effect on β_3-receptor gene expression. Thus, a discrepancy between receptor types involved in β_1- and β_3-gene expression was implied through these antagonist experiments. This was confirmed through agonist experiments. Treatment with the α_1-agonist cirazoline resulted in a down-regulation of β_3-receptor mRNA levels (although to a lesser extent than with noradrenaline). This effect of cirazoline could be completely blocked by the α_1-receptor antagonist prazosin, confirming that this down-regulation was indeed α_1-adrenoceptor mediated. As expected [11], the β_3-agonists BRL-37344 and CGP-12177 also down-regulated the β_3-adrenergic receptor mRNA levels. Thus down-regulation of β_3-receptor mRNA could be mediated via both β_3- and α_1-receptor activation.

Concerning the intracellular mediation of the adrenergic signal controlling β_3-receptor gene expression, the Ca^{2+}-ionophore A23187 failed to induce a decrease in β_3-mRNA (Table 2).

© 2000 Biochemical Society

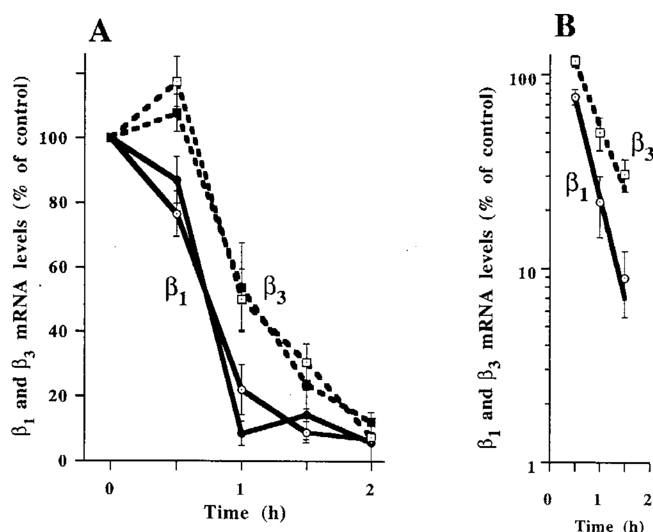

Figure 3 Effect of inhibition of RNA synthesis

(**A**) Confluent cultures of brown adipocytes (day 6 in culture) were treated with the indicated drugs. ○, β_1-mRNA levels; □, β_3-mRNA levels, in cells treated with 1 μg/ml actinomycin. ●, β_1-mRNA levels; ■-■, represents β_3-mRNA levels, in cells treated with 0.1 μM noradrenaline +1 μg/ml actinomycin. Untreated wells at zero time were set to 100% for each probe and the other values given relative to this. (**B**) The rate of decline of mRNA levels due to inhibition of RNA synthesis. The points are from (**A**) but only those relevant for the calculation of β_1-mRNA half-life (○) and β_3-mRNA half-life (□) are depicted, here on a semilogarithmic scale. The line is drawn for best-fit to first-order kinetics, i.e. *basal*·[exp($-\tau\cdot$t)]. The curve was fitted with the general curve-fit procedure in the KaleidaGraph application for Macintosh. The half-life was estimated to be 17±1 min for the β_1-mRNA and 27±4 min for the β_3-mRNA.

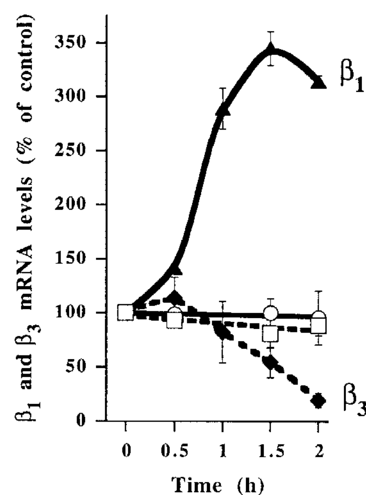

Figure 4 Divergent influence of a translational inhibitor on the levels of β_1- and β_3-adrenergic receptor mRNAs in cultures of brown adipocytes

Confluent cultures of brown adipocytes (day 6 in culture) were treated with the translational inhibitor cycloheximide (50 μM) or treated only with vehicle. Untreated wells at zero time were set to 100% for each probe and the other values given relative to this. The results are the means±S.E.M. of two cell culture series. Within each series the experiments were performed in independent duplicate cell culture wells. ▲, β_1-mRNA levels; ◆, β_3-mRNA levels, in cells treated with cycloheximide. ○, β_1-mRNA levels; □, represents β_3-mRNA levels in cells treated only with vehicle. The lines were drawn with the 'smooth' option in the KaleidaGraph application for Macintosh.

However, treatment with PMA brought about down-regulation of the β_3-receptor mRNA. Forskolin also repressed β_3-receptor mRNA, as did treatment with 8-bromo-cAMP. Down-regulation of the β_3-receptor mRNA may therefore clearly be brought about by activation of two different receptors and pathways: through β_3-receptors and cAMP, and through α_1-receptors and activation of PKC.

The adrenergic effect on gene expression is not due to alterations in β-receptor mRNA stability

To determine the stablity of the β-receptor transcripts, the RNA-synthesis inhibitor actinomycin D was used at a concentration previously shown not to be detrimental to the cells during short-term treatment [27,28]. The turnover of both transcript species was very rapid. The half-life of the β_1-receptor mRNA was 17 min (uncertainty interval 16–18 min) (Figures 3A and 3B), and that of the β_3-receptor mRNA was 27 min (uncertainty interval 23–31 min) (Figures 3A and 3B) (the latter in agreement with what we have reported earlier [11]).

Up-regulation of the β_1-receptor mRNA levels by noradrenaline could be due to a decrease in the rate of degradation of the β_1-mRNA or to an increase in transcription of the β_1-receptor gene. It was therefore investigated whether noradrenaline influenced the turnover of the transcript. The half-life after treatment with actinomycin plus noradrenaline was 15 min (uncertainty interval 10–20 min) (Figure 3A). Thus, the β_1-receptor mRNA half-life after actinomycin plus noradrenaline was identical to that after treatment with actinomycin alone (17 min). The mRNA level was therefore most likely fully determined by regulation of transcription.

Similarly, the half-life of the β_3-adrenergic receptor mRNA after treatment with actinomycin and noradrenaline (Figure 3A) was calculated to be 28 min (uncertainty interval 27–29 min) and thus not significantly different from that after treatment with actinomycin alone. This indicates that the down-regulation was mainly due to cessation of transcription, as concluded previously [11].

Influence of inhibition of protein synthesis on β-receptor mRNA levels

A distinctive dependence on ongoing protein synthesis has been reported for β_3-receptor gene expression [11]. To examine whether a similar dependence existed for β_1-receptor gene expression, the brown adipocytes were treated with cycloheximide. However, in direct contrast to the pronounced negative effect of this treatment on β_3-gene expression (confirmed here, Figure 4), β_1-adrenergic receptor mRNA levels increased markedly as an effect of protein synthesis inhibition and after 1.5 h reached levels more than 3-fold higher than initial levels (Figure 4) (i.e. even higher than that seen after noradrenaline stimulation).

Physiological regulation of β-adrenoceptor gene expression in intact animals

To clarify the physiological relevance of the above observations in brown adipocyte cultures on adrenergic regulation of β-receptor gene expression, analogous experiments were performed *in vivo*. Cold exposure of animals leads to a physiologically-induced release of noradrenaline within the tissue which stimulates the cells, and this adrenergic stimulation continues unabated during prolonged demands for thermogenesis [29]. Consequently we also investigated whether the physiologically induced adrenergic stimulation would lead to differential regu-

© 2000 Biochemical Society

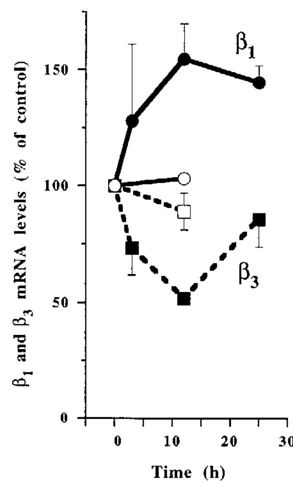

Figure 5 Levels of β_1- and β_3-adrenergic receptor mRNAs in brown adipose tissue of mice exposed to cold

Total RNA was isolated from the brown adipose tissue of 6-week-old mice which had been preacclimatized to 28 °C for 7 days and were then maintained at 28 °C (open symbols) or were placed at 4 °C (filled symbols) for the indicated times. A sample of 20 μg of RNA was analysed by Northern-blot analysis, by hybridization with the β-adrenoceptor probes as described in the Materials and methods section. The mean value at 28 °C was set to 100% for each probe and the other values expressed relative to this. Each point represents the mean $\pm$ S.E.M. from four mice. ●, β_1-mRNA levels; ■, β_3-mRNA, in mice placed at 4 °C. ○, β_1-mRNA levels; □, β_3-mRNA levels, in mice maintained at 28 °C for the indicated times.

lation of the gene expression of the β-receptors on the cells in the intact tissue.

As seen in Figure 5, acute exposure of mice to cold led indeed to an increase in mRNA levels of the β_1-adrenergic receptor in brown adipose tissue. The level was measurably increased after 2 h in the cold and it reached 150% of control values after 12 h. The levels remained elevated for at least 25 h. No significant differences were observable in the β_2-adrenergic receptor mRNA levels (results not shown), but, in agreement with our earlier observations [11], a decrease in the levels of mRNA for the β_3-adrenergic receptor was observed after 2 h in the cold. The levels were further reduced to half of control values after 12 h in the cold, but subsequently returned towards control levels (Figure 5).

It was therefore concluded that the differential effects of adrenergic stimulation on expression of the β-receptor subtype genes observed and analysed in brown adipocyte cultures above were not phenomena restricted to this *in vitro* system but that these events do occur during physiological stimulation of the tissue, with a time-course not much different from that observed in the cell culture system.

DISCUSSION

In the present study, we examined the adrenergic regulation of the expression of the three β-adrenergic receptor subtype genes in brown adipocytes. We found that the expression of each gene was distinctively regulated within this single cell type, as summarized below for each receptor. It is therefore clear that no general principle for the adrenergic control of the expression of all the β-adrenoceptor genes exists. Even for each subtype, cell type-specific adrenergic regulation seems to prevail.

β_1-Adrenergic receptor gene expression

The β_1-adrenoceptor gene was innately expressed in the mature brown adipocytes under study. Adrenergic stimulation rapidly increased mRNA levels for the β_1-receptor. This increase was mediated through β_3-receptors and through an elevation of cAMP levels. The increased mRNA level was not due to prolongation of mRNA half-life.

An augmenting effect of β-adrenergic stimulation on β_1-gene expression has been noted in several different systems [30–32]. However, in the C6 glioma cell lines used previously [31–33], this initial induction was rapidly followed by a phase of repression of gene expression. Only in non-cell-line systems, such as that used here and the explant-cultured pineal gland cells used by Carter [30], is a persistent increase in β_1-gene expression observed.

The up-regulation of β_1-gene expression was mediated via β_3-receptors, i.e. it was heterologous. It could be fully mimicked by other agents able to increase cAMP levels, i.e. receptor occupancy was not necessary for the induction of expression, and its dose–response curve was similar to that for other cAMP-mediated gene expressions (UCP1 [5]) and for cAMP accumulation itself [8].

Compared to the effect of adrenergic stimulation, the β_1-adrenergic receptor mRNA levels were augmented even more rapidly, and to a larger extent, when the cells were treated with the protein synthesis inhibitor cycloheximide. This may indicate that a negative, short-lived proteinaceous factor regulates β_1-adrenoreceptor gene expression. This could fit with the fact that the β_1-adrenoceptor gene has inhibitory elements in the promoter region [34–36]. As anticipated from the cell culture studies, cold exposure of mice led to a persistent increase in the β_1-mRNA levels in brown adipose tissue. This is probably due to the chronic noradrenaline release occurring in the tissue under these conditions. The prediction would be that this would also result in an increased level of the β_1-receptor. Indeed, in ligand binding studies in which CGP-12177 was used as a ligand for β_1-/β_2-receptors [37], cold acclimatization was reported to lead to an increase in the density of β_1-/β_2-receptors in brown adipose tissue. Thus the observations on β_1-gene expression in cell cultures seem to correspond well to both tissue gene expression and final receptor level.

β_2-Adrenergic receptor gene expression

In the cultured primary brown adipocytes studied here, the β_2-adrenoceptor gene was not innately expressed, at least not to an extent detectable in a Northern-blot (in contrast to what has been reported in a brown-adipocyte-like cell line [20]). It was only expressed very transiently after acute noradrenaline treatment. The physiological significance of this transient expression is not easily understood; it is unlikely that it would result in the synthesis of a physiologically significant number of β_2-receptors and it may therefore presently be considered a regulatory coincidence. That the expression is induced, not repressed, by noradrenaline in these cells, is principally in accordance with what is observed in the vas-deferens-like cell line DDT$_1$MF-2 [38] but in contrast to what is observed in the C6 glioma cell line [31]. Thus, the effect of adrenergic stimulation on β_2-receptor gene expression is apparently also cell-type specific.

The apparent absence of β_2-gene expression and thus of functional β_2-receptors on the brown adipocytes is principally in agreement with the absence of any reported β_2-receptor-mediated effects of noradrenaline in cultured primary brown adipocytes or in isolated mature brown-fat cells (e.g. [39]) (a β_2-effect has only been reported in the brown-adipocyte-like cell line also reported to innately express the β_2-receptor [20]).

© 2000 Biochemical Society

However, we observed β_2-mRNA in intact brown adipose tissue, and equilibrium binding studies with adrenergic ligands have previously indicated that β_2-receptors exist in the tissue [40,41]. The absence of β_2-mRNA in the brown adipocyte cultures and the presence of β_2-receptors in the tissue would therefore imply that the β_2-receptors observed in the tissue were probably not localized to the parenchymal cells. Indeed, not only the brown adipocytes, but also the blood vessels of the brown adipose tissue, are intensely innervated from the sympathetic nervous system. The blood vessels are specifically innervated by sympathetic neurons which co-store neuropeptide Y [42]. Vasoconstriction in the tissue is induced via α_1-adrenoceptors and vasodilation via β-receptors [43]. It is established in several blood vessel systems that β_2-receptors may be coupled to vasodilation (e.g. [44]) and this may also be the case in brown adipose tissue. Thus in brown adipose tissue, the expression of β_2-receptors may be restricted to the blood vessels.

β_3-Adrenergic receptor gene expression

In agreement with our earlier observations [8,11], the β_3-adrenoceptor gene was well expressed in the mature mouse brown adipocytes studied here, and noradrenaline addition led to a rapid and absolute, although transient, cessation of gene expression. This effect was mediated via both β_3- and α_1-adrenoceptors, probably mediated through cAMP and PKC, respectively, and was not due to accelerated mRNA degradation.

That β-adrenergic stimulation leads to repression of β_3-gene expression has been established in several cell systems [10,11,21,22] (but see [45]). This could be considered a typical homologous desensitization process but it is only homologous in that it is mediated via cAMP generated by the β_3-receptor; no receptor occupancy is needed. The potent α_1-induced repression of β_3-gene expression demonstrated here has not been observed previously.

The significance of short-lived proteinaceous factor(s) for β-adrenoceptor gene expression

In the brown adipocytes studied here, β_1-expression was augmented whereas the β_3-expression was repressed as an effect of protein synthesis inhibition. This implies that both genes are under the control of short-lived proteinaceous factors. Notably these are the same effects as those observed upon noradrenaline addition. It may therefore be suggested that, for both genes, the noradrenaline effects may be related to these putative proteinaceous factors. Indeed, a unifying concept could be envisaged, in which the expression of a [(brown-)adipocyte-]specific factor would become innately induced at the moment of the differentiation switch in these cells [8]. This factor would lead to the induction of β_3-gene transcription and may be related to the inhibition of β_1-gene transcription that occurs with time [8]. Inhibition of the synthesis of this factor would, of course, reverse the situation, and if noradrenaline did this, it would elicit opposite effects on the expression of the two genes. Compelling as such a unifying hypothesis/factor would be, a closer study of the temporal kinetics of the effects of differentiation, of noradrenaline and of cycloheximide on the expression of the two genes reveals that the match is not perfect, and only continued studies of β-receptor gene expression in these cells can reveal whether such a factor exists. However, it is clear that the brown adipocyte system is of principal interest in the elucidation of the regulation of β-adrenoceptor gene expression, in that these are one of only few cell types displaying the ability to entopically express all three β-adrenergic receptors.

This study was supported by the Swedish Natural Science Research Council and the Swedish Cancer Society. The β_1-clone was a gift from Dr J.-P. Giacobino (Department of Medical Biochemistry, University of Geneva, Geneva, Switzerland), and the β_2- and β_3-clones were generously given by Dr A. D. Strosberg (Laboratoire d'Immunopharmacologie Moleculaire, CNRS-UPR415 and Universite Paris VII, Institut Cochin de Genetique Moleculaire, Paris, France).

REFERENCES

1 Bronnikov, G., Houstek, J. and Nedergaard, J. (1992) J. Biol. Chem. **267**, 2006–2013
2 Yamashita, H., Sato, N., Kizaki, T., Oh-ishi, S., Segawa, M., Saitoh, D., Ohira, Y. and Ohno, H. (1995) Cell Growth Differ. **6**, 1457–1462
3 Lindquist, J. M. and Rehnmark, S. (1998) J. Biol. Chem. **273**, 30147–30156
4 Kopecky, J., Baudysová, M., Zanotti, F., Janíková, D., Pavelka, S. and Houstek, J. (1990) J. Biol. Chem. **265**, 22204–22209
5 Rehnmark, S., Néchad, M., Herron, D., Cannon, B. and Nedergaard, J. (1990) J. Biol. Chem. **265**, 16464–16471
6 Nedergaard, J. and Cannon, B. (1992) in New Comprehensive Biochemistry vol. 23: Molecular Mechanisms in Bioenergetics (Ernster, L., ed.), pp. 385–420, Elsevier, Amsterdam
7 Nisoli, E., Tonello, C., Landi, M. and Carruba, M. O. (1996) Mol. Pharmacol. **49**, 7–14
8 Bronnikov, G., Bengtsson, T., Kramarova, L., Golozoubova, V., Cannon, B. and Nedergaard, J. (1999) Endocrinology **140**, 4185–4197
9 Revelli, J.-P., Muzzin, P. and Giacobino, J.-P. (1992) Biochem. J. **286**, 743–746
10 Klaus, S., Muzzin, P., Revelli, J.-P., Cawthorne, M. A., Giacobino, J.-P. and Ricquier, D. (1995) Mol. Cell. Endocrinol. **109**, 189–195
11 Bengtsson, T., Redegren, K., Strosberg, A. D., Nedergaard, J. and Cannon, B. (1996) J. Biol. Chem. **271**, 33366–33375
12 Néchad, M., Kuusela, P., Carneheim, C., Björntorp, P., Nedergaard, J. and Cannon, B. (1983) Exp. Cell Res. **149**, 105–118
13 Néchad, M., Nedergaard, J. and Cannon, B. (1987) Am. J. Physiol. **253**, C889–C894
14 Emorine, L. J., Marullo, S., Delavier-Klutchko, C., Kaveri, S. V., Durieu-Trautmann, O. and Strosberg, A. D. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6995–6999
15 Nahmias, C., Blin, N., Elalouf, J.-M., Mattei, M. G., Strosberg, A. D. and Emorine, L. J. (1991) EMBO J. **10**, 3721–3727
16 Rovati, G. E. and Nicosia, S. (1994) Trends Pharmacol. Sci. **15**, 140–144
17 Frielle, T., Collins, S., Daniel, K. W., Caron, M. G., Lefkowitz, R. J. and Kobilka, B. K. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7920–7924
18 Fève, B., Emorine, L. J., Briend-Sutren, M. M., Lasnier, F., Strosberg, A. D. and Pairault, J. (1990) J. Biol. Chem **265**, 16343–16349
19 Collins, S., Daniel, K. W., Rohlfs, E. M., Ramkumar, V., Taylor, I. L. and Gettys, T. W. (1994) Mol. Endocrinol. **8**, 518–527
20 Rohlfs, E. M., Daniel, K. W., Premont, R. T., Kozak, L. P. and Collins, S. (1995) J. Biol. Chem. **270**, 10723–10732
21 Fève, B., Piétri-Rouxel, F., El Hadri, K., Drumare, M. F. and Strosberg, A. D. (1995) J. Biol. Chem. **270**, 10952–10959
22 Granneman, J. G. and Lahners, K. N. (1995) Am. J. Physiol. **268**, C1040–C1044
23 Champigny, O., Holloway, B. R. and Ricquier, D. (1992) Mol. Cell. Endocrinol. **86**, 73–82
24 Arch, J. R. S. (1989) Proc. Nutr. Soc. **48**, 215–223
25 Arch, J. R. S. and Kaumann, A. J. (1993) Med. Res. Rev. **13**, 663–729
26 Mohell, N. and Dicker, A. (1989) Biochem. J. **261**, 401–405
27 Puigserver, P., Herron, D., Gianotti, M., Palou, A., Cannon, B. and Nedergaard, J. (1992) Biochem. J. **284**, 393–398
28 Picó, C., Herron, D., Palou, A., Jacobsson, A., Cannon, B. and Nedergaard, J. (1994) Biochem. J. **302**, 81–86
29 Young, J. B., Saville, E., Rothwell, N. J., Stock, M. J. and Landsberg, L. (1982) J. Clin. Invest. **69**, 1061–1071
30 Carter, D. A. (1993) Endocrinology **133**, 2263–2268
31 Hough, C. and Chuang, D.-M. (1990) Biochem. Biophys. Res. Commun. **170**, 46–52
32 Hosoda, K., Feussner, G. K., Rydelek-Fitzgerald, L., Fishman, P. H. and Duman, R. S. (1994) J. Neurochem. **63**, 1635–1645
33 Fitzgerald, L. R., Li, Z., Machida, C. A., Fishman, P. H. and Duman, R. S. (1996) J. Neurochem. **67**, 490–497
34 Searles, R. P., Midson, C. N., Nipper, V. J. and Machida, C. A. (1995) J. Biol. Chem. **270**, 157–162
35 Tseng, Y. T., Waschek, J. A. and Padbury, J. F. (1995) Biochem. Biophys. Res. Commun. **215**, 606–612
36 Bahouth, S. W., Cui, X., Beauchamp, M. J., Shimomura, H., George, S. T. and Park, E. A. (1997) Mol. Pharmacol. **51**, 620–629
37 Raasmaja, A. and York, D. A. (1988) Biochem. J. **249**, 831–838

© 2000 Biochemical Society

38 Collins, S., Bouvier, M., Bolanowski, M. A., Caron, M. G. and Lefkowitz, R. J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 4853–4857
39 Zhao, J., Unelius, L., Bengtsson, T., Cannon, B. and Nedergaard, J. (1994) Am. J. Physiol. **267**, C969–C979
40 Rothwell, N., Stock, M. and Sudera, D. (1985) Am. J. Physiol. **248**, E397–E402
41 Levin, B. E. and Sullivan, A. C. (1986) J. Pharmacol. Exp. Ther. **236**, 681–688
42 Cannon, B., Nedergaard, J., Lundberg, J. M., Hökfelt, T., Terenius, L. and Goldstein, M. (1986) Exp. Cell Res. **164**, 546–550
43 Flaim, K., Horwitz, B. and Horowitz, J. (1977) Am. J. Physiol. **232**, R101–R109
44 Fujimoto, S. and Itoh, T. (1996) Gen. Pharmacol. **27**, 1355–1359
45 Thomas, R. F., Holt, B. D., Schwinn, D. A. and Liggett, S. B. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4490–4494

Received 21 September/13 January 2000; accepted 16 February 2000

© 2000 Biochemical Society