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Developmental expression and functional activity of β_1- and β_3-adrenoceptors in murine 3T3-F442A differentiating adipocytes

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Abstract

β_1- and β_3-adrenoceptor mRNA and protein expression, and contribution of each subtype to the catecholamine-sensitive adenylyl cyclase system were studied during the adipose conversion of the murine 3T3-F442A cell line. Northern and reverse transcriptase-polymerase chain reaction analyses indicated that emergence of β_3-adrenoceptor transcripts was concomittant with that of the gene encoding adipsin, a very late marker of adipose differentiation. Conversely, the induction of the β_1-adrenoceptor mRNA occurred early after cell commitment towards adipose conversion. Changes in β-subtype gene expression were accompanied by parallel modifications in receptor expression and function. ^{125}I-cyanopindolol saturation and competition binding experiments showed a 3-fold increase in β_1-adrenoceptor density in day 3 post-confluent cells. The β_3-subtype population became detectable later and represented ~95% of total β-adrenoceptors in day 8 and day 12 post-confluent cells. Adenylyl cyclase activity in response to the β_3-adrenoceptor-selective agonists CGP12177 (4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one), ICI201651 ([(*R*)-4-(2 hydroxy-3-phenoxypropylamino-ethoxy)-*N*-(2-methoxyethyl)phenoxy-acetamide]) and cyanopindolol was virtually absent in young adipocytes, but dramatically increased in mature cells. The respective contributions of the β_1- and the β_3-subtypes to the production of cAMP were resolved by an Eadie-Hofstee computer analysis of isoproterenol and norepinephrine concentration-response curve of adenylyl cyclase activity. Agonist response curves in the presence of β_1- and β_2-adrenoceptor antagonists indicated that the β_1-subtype accounted for the totality of β-adrenoceptor-mediated adenylyl cyclase activation in young adipocytes. In mature adipose cells ~90% of this response was due to an activation of the β_3-adrenoceptor. In addition, ~84% of the maximal norepinephrine-stimulated lipolysis was mediated by the β_3-adrenoceptor in fully differentiated adipocytes. The differentiation-dependent expression of β-subtypes in adipocytes is a biphasic process involving an initial and moderate induction of β_1-adrenoceptors followed by the emergence of a prominent β_3-adrenoceptor population. Compared analysis of both receptor occupancy and cAMP production shows that the β_3-subtype is more efficiently coupled to the adenylyl cyclase system than the β_1-adrenoceptor. Thus in mature adipose cells this receptor subtype represents the core of cAMP-dependent regulation of the lipolytic, antilipogenic and thermogenic effects of catecholamines.

Keywords: β-Adrenoceptor; Adipose differentiation; cAMP; Lipolysis

1. Introduction

β-Adrenoceptors are integral transmembrane receptors that mediate the effects of catecholamines through their coupling to a G protein complex that activates adenylyl cyclase and cAMP production. In turn, cAMP exerts a key role in processes such as cellular growth, differentiation and adaptations to environmental conditions. These processes are regulated in a tissue- and/or cell type-specific manner. Thus in adipocytes it is well documented that, essentially through the β-adrenoceptors, cAMP has pleiotropic effects in the control of adipocyte differentiation and metabolism. Previous studies have suggested that cAMP can trigger differentiation in adipogenic cell lines (Gaillard et al., 1989; Schmidt et al., 1990) or primary cultures of adipose precursors (Björntorp et al., 1980; Wiederer and Löffler, 1987). Others (Wang et al., 1992) have also

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documented that G_s α-subunit activity regulates 3T3-L1 adipose differentiation. In any case, this second messenger also plays a pivotal role in the metabolism of mature adipose cells. In addition to its involvment in the rapid activation of lipolysis, cAMP also modulates terminal differentiation by repressing the expression of a number of genes coding for essential proteins of the adipocyte, especially lipogenic enzymes. Thus in adipocytes cAMP decreases mRNA levels of adipose-specific markers which include lipoprotein lipase (Raynolds et al., 1990; Antras et al., 1991), fatty acid synthase (Paulauskis and Sul, 1988), glycerol-3-phosphate dehydrogenase (Dobson et al., 1987; Antras et al., 1991; Bhandari et al., 1991), type 4 glucose transporter GLUT4 (Kaestner et al., 1991) or adipsin (Antras et al., 1991). In brown adipose tissue which is involved in heat production, cAMP induces transcription of the gene coding for the uncoupling protein resulting in higher protein levels (Ricquier et al., 1986).

The human β_3-adrenoceptor gene was initially cloned, sequenced, and expressed in model cells by Emorine et al. (1989). The murine species homologue gene was similarly studied by Nahmias et al. (1991). Both in mouse and rat adipocytes, the β_3-adrenoceptor mRNA and protein seem particularly abundant (Nahmias et al., 1991; Granneman et al., 1991). Using the 3T3-F442A preadipose cell line, we have previously shown that preadipocytes possess exclusively a limited number of β_1-adrenoceptors, while mature adipocytes acquire a large β_3-adrenoceptor population ($> 90\%$ of total β-adrenoceptors). Acquisition of the β_3-adrenoceptor is correlated with a large increase in β-adrenergic sensitivity (Fève et al., 1990, 1991). β_2-Subtypes remain a negligible component, unless preadipocytes or adipocytes are exposed to glucocorticoids (Lai et al., 1982; Nakada et al., 1987; Fève et al., 1990). However, little is known about the exact time course of the β_3-adrenoceptor emergence during the adipose conversion, and the actual respective contributions of the different β-subtypes to the cAMP signaling pathway and to lipolysis.

In the present work, we have studied by molecular and pharmacological approaches the temporal emergence of β_3-adrenoceptor mRNA and protein during adipose conversion of 3T3-F442A cells. The β_3-adrenoceptor appears very late during the course of adipose conversion. It follows that of the β_1-subtype which is associated with early commitment. We have also evaluated the contribution of each receptor subtype to the adenylyl cyclase activation at different stages of differentiation. We clearly demonstrate that in young adipocytes, adenylyl cyclase activation is essentially under the control of the β_1-adrenoceptor while in mature adipocytes, the β_3-subtype becomes the quite prominent β-adrenoceptor component eliciting cAMP production and glycerol release.

2. Materials and methods

2.1. Cell culture

3T3-F442A preadipose cells (Green and Kehinde, 1976) were grown and differentiated in tissue culture dishes (Falcon) at 37°C in an atmosphere of air/CO_2 (90:10, v/v) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (High Bio Laboratories). Adipose conversion was achieved without further supplementation of the cell culture medium. Cell extracts were prepared at the indicated times.

2.2. RNA analysis

Total RNA was extracted by the method of Cathala et al. (1983). 10 μg RNA/lane was electrophoresed through a 1.5% agarose, 2.2 M formaldehyde gel. RNA was transferred to Nytran-plus membranes (Schleicher and Schuell). Prehybridization was carried out at 60°C for 30 min in the presence of 0.5 M sodium phosphate (pH 6.8), 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin and 1 mM EDTA (Church and Gilbert, 1984). Hybridization was performed in the same buffer for 16 h at 60°C in the presence of the heat-denatured probe (2×10^6 cpm/ml). Membranes were washed twice for 30 min at 60°C in 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate), 0.1% SDS, then once in 0.2 × SSC, 0.1% SDS for 15 min at 60°C. For quantitation, autoradiograms were analyzed by videodensitometric scanning (Vilber Lourmat Imaging). Probes were labeled by random priming with [α-^{32}P]dCTP (ICN Biomedicals) (Feinberg and Vogelstein, 1983). The β_3-adrenoceptor probe is a 305 bp amplification product of the cloned murine β_3-adrenoceptor gene (Nahmias et al., 1991) between a sense 5′-GCATGCTCCGTGGCCTCACGAGAA-3′ and an antisense primer 5′-CCCAACGGCCAGTGGCCAGTCAGCG-3′. cDNAs for adipsin (pAd-20), aP2 (pAd-5), glycerol-3-phosphate dehydrogenase (G3PDH) (pGPD-1) and β-actin (pAct-1) (Spiegelman et al., 1983) were generously given by Dr. Howard Green (Harvard Medical School, Boston, MA). The human lipoprotein lipase (LPL) cDNA probe (pLPL 35) (Wion et al., 1987) was a kind gift of Dr. Arjun Singh (Genentech, San Francisco, CA).

For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of β-adrenoceptor expression, RNA was digested for 15 min at 37°C with 0.1 U of RNAse-free DNase I (RQ1 DNase, Promega) per μg of nucleic acid in 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$ in the presence of 0.05 U/μl of RNase inhibitor (RNAguard, Pharmacia). After phenol/chloroform extraction and ethanol precipitation, RNA (1 μg) was reverse-transcribed with moloney murine leukemia virus reverse transcriptase (MMLV RT) (200 U/μg, GIBCO BRL) in the presence of 10

μM random hexanucleotides, 2U/μl of RNase inhibitor, 400 μM of each dNTP in final volume of 20 μl consisting of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol. After a 1 h incubation at 42°C, MMLV RT was heat-inactivated. To ensure that subsequent amplification did not derive from contaminant genomic DNA a control without MMLV RT was included for each RNA sample. cDNAs were denatured for 5 min and submitted to 25 or 30 cycles of amplification (94°C, 1 min; 60°C, 2 min; 72°C, 2 min) followed by a final extention of 7 min at 72°C in a DNA thermal cycler 480 (Perkin-Elmer Cetus). PCR was performed in a 25 μl reaction containing 1 U *thermus aquaticus* (T*aq*) polymerase (Bioprobe), 125 μM each dNTP, 5% formamide, 125 nM both sense and antisense oligonucleotides. The buffer consisted of 20 mM Tris-HCl (pH 8.55), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ and 150 μg/ml bovine serum albumin. Sequences of sense and antisense oligonucleotides were: 5'-GGATCCAAGCTTTCGTGTGCACCGTGTGGGCC-3' and 5'-GGATCCAAGCTTAGGAAACGGCGCTCGCAGCTGTCG-3' for the β_1-adrenoceptor; 5'-ATGGCTCCGTGGCCTCAC-3' and 5'-CCCAACGGCCAGTGGCCAGTCAGCG-3' for the β_3-adrenoceptor. These sequences were derived from those of the human β_1-subtype cDNA (Frielle et al., 1987) and of the murine β_3-adrenoceptor gene (Nahmias et al., 1991). Amplification products had expected sizes of 289 bp and 308 bp for β_1- and β_3-adrenoceptors, respectively. They were separated on a 2% agarose gel and stained with ethidium bromide. In fully mature adipocytes (day 12 after confluence, corresponding to the optimal level of β_3-subtype mRNA expression), amplification with 25 cycles was linear up to 50 ng of RNA. Using 25 ng of RNA and 25 cycles of amplification, we were thus able to evaluate the relative levels of β_3-adrenoceptor mRNA at various times of differentiation. β_1-Subtype cDNA amplification was also performed in comparative and semi-quantitative conditions for analysis (30 cycles with 25 ng of RNA).

2.3. Binding experiments to membrane fractions

For ^{125}I-cyanopindolol binding experiments, cells were harvested and homogenized at 4°C in 1 mM EDTA, 25 mM Tris-HCl (pH 7.5). Homogenates were centrifuged for 10 min at $500 \times g$ at 4°C. The supernatant was then further centrifuged for 30 min at 4°C at $40\,000 \times g$. The pellet was resuspended in the homogenization buffer and stored at −80°C until it was used. Protein content was measured (Lowry et al., 1951) using bovine serum albumin as a standard. Binding assays were performed on membrane aliquots (40 μg of protein) incubated for 30 min at 37°C with ^{125}I-cyanopindolol (Amersham) with or without competing ligand in a final volume of 250 μl consisting of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM ascorbic acid and 100 μM GTP (Boehringer). After dilution with 3 ml of ice cold 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), separation of bound from free radioligand was achieved by vacuum filtration over Whatman GF/C filter paper followed by three washes with the same buffer. Saturation experiments were performed with ^{125}I-cyanopindolol concentrations ranging from 5 to 4000 pM. Competition experiments were carried out at 300 pM ^{125}I-cyanopindolol, in the absence or presence of various concentrations of the β_3-adrenoceptor-selective agonist BRL37344 (sodium-4-{2-[2-hydroxy-2-(3-chloro-phenyl)ethylamino]propyl}-phenoxyacetate sesquihydrate (*RR,SS* distereoisomer)) (Smith Kline Beecham Pharmaceuticals). Non-specific binding was determined in the presence of 100 μM (±)-propranolol (Sigma) and was usually 13 ± 1% of total binding at 300 pM ^{125}I-cyanopindolol. Data from saturation and competition binding experiments were analyzed with the non-linear least squares curve-fitting procedure of the EBDA/LIGAND program (Biosoft-Elsevier, Cambridge, England) (Munson and Rodbard, 1980).

2.4. Adenylyl cyclase assay

Adenylyl cyclase (EC 4.6.1.1) activity was determined as previously described (Fève et al., 1991). Briefly, the reaction was carried out for 10 min at 35°C and was initiated by the addition of crude membranes to a standard buffer containing 0.1 mM [α-^{32}P]ATP (1 μCi) (Amersham), 1 mM cAMP, 10 mM phosphocreatine, 0.5 unit creatine phosphokinase, 100 μM GTP (except when guanosine-5'-(3-*O*-thio)triphosphate (GTPγS) was used), 10 mM $MgCl_2$, 0.2 mM EDTA and 50 mM Tris-HCl (pH 7.5), with or without a β-adrenergic effector. (−)-Isoproterenol and (−)-norepinephrine were from Sigma. CGP12177 (4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one) and CGP20712A ((±)-(2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino)-3-(4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy)-2-propanol methane sulfonate) were generous gifts from Ciba-Geigy. ICI118551 (*erythro*-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) and ICI201651 ([(*R*)-4-(2hydroxy-3-phenoxypropylamino-ethoxy)-*N*-(2-methoxyethyl)phenoxy-acetamide]) were provided by ICI Pharma and cyanopindolol by Sandoz. For dose-response curve analysis, kinetic parameters of adenylyl cyclase activation (K_{act} and V_{max}) were calculated by a non-linear regression procedure (adapted from the EBDA/LIGAND program) according to a one- or two-site model. The validity of a one-site versus a two-site model was verified using an *F*-test. Data are presented as means ± S.E. of independent experiments.

2.5. Lipolysis experiments

Lipolysis was assessed in 6-well plates as glycerol release from adherent mature adipocytes (day 12 after confluence). Adipose cells were washed with Krebs-Ringer phosphate buffer supplemented with 20 mM Hepes (pH 7.4), 2% fatty acid free bovine serum albumin, 25 mM glucose, 1 mM ascorbate and 50 μg/ml $Na_2S_2O_5$. Cells were then incubated in 0.6 ml of this medium for 1 h at 37°C with increasing concentrations of norepinephrine, in the absence or the presence of the β_1-selective antagonist CGP20712A (0.1 μM). Glycerol was determined on 100 μl aliquots of the medium according to the procedure of Lin and Magasanik (1960). Reduction of NAD to NADH was recorded at 340 nM in the presence of glycerol dehydrogenase (EC 1.1.1.6) from *Enterobacter aerogenes* (Boehringer Mannheim). Reaction buffer consisted of 33 mM $(NH_4)_2SO_4$, 3 μM $MnCl_2$, 3.3 mM NAD and 100 mM $K_2CO_3/NaHCO_3$ (pH 10). Kinetic parameters of lipolysis activation by norepinephrine were calculated as for adenylyl cyclase experiments.

3. Results

3.1. Emergence of the β_1- and β_3-adrenoceptor mRNAs during 3T3-F442A adipose differentiation

Total RNA was prepared from growing (day −1), confluent (day 0), and differentiating 3T3-F442A cells. Northern analysis indicated the known kinetic patterns of induction or repression for the mRNAs of the control genes (Fig. 1A). The β-actin mRNA content dramatically decreased at confluence (day 0), while lipoprotein lipase mRNA steady state levels rapidly increased 24 h after cell commitment. Later aP2 and glycerol-3-phosphate dehydrogenase transcripts became detectable in developing adipocytes, and preceded the very late induction of adipsin mRNA. Emergence of the β_3-adrenoceptor mRNA paralleled that of adipsin. Hybridization with the β_3-subtype specific probe revealed the presence of three mRNA species: a major mRNA of 2.3 kilobases and two minor transcripts of 2.8 and 4.4 kilobases (Fève et al., 1991, 1994). These transcripts became detectable 5 days after confluence, but accounted at this stage for only 8% of the β_3-adrenoceptor mRNA content observed in fully mature adipocytes (Fig. 1B). A dramatic increase in β_3-adrenoceptor mRNA steady state levels occurred between day 5 and day 12.

β_3-Adrenoceptor gene expression was also studied in differentiating 3T3-F442A cells by RT-PCR, a very sensitive technique. Since β_3-subtype mRNA cellular content varied widely between preadipocytes and mature adipocytes, amplifications were carried out with

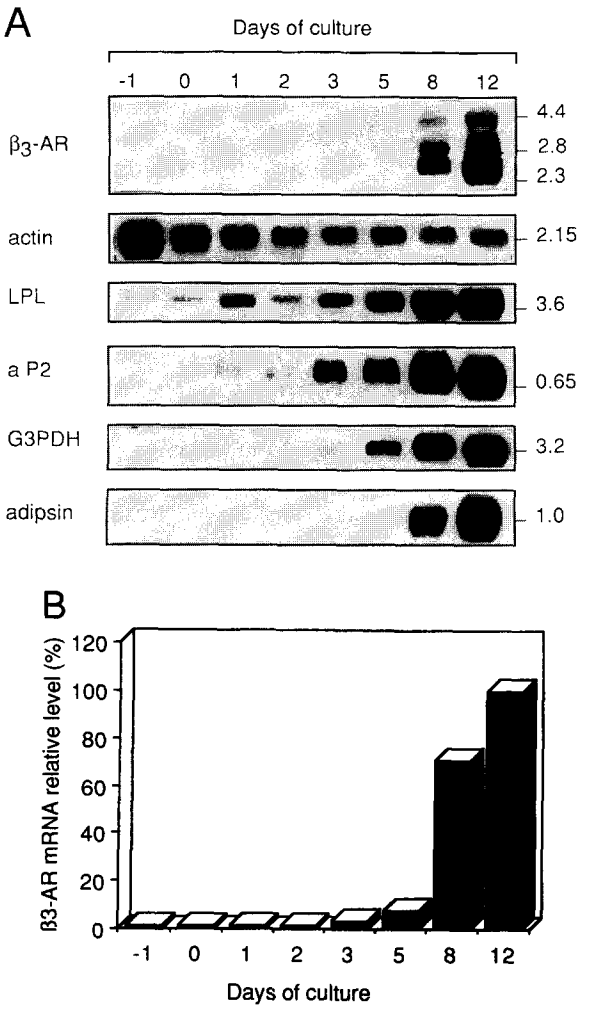

Fig. 1. Comparative time course in the steady-state levels of β_3-adrenoceptor mRNA and other adipose-specific transcripts during 3T3-F442A adipose differentiation. (A) Northern blots analysis was done on total RNA (10 μg/track) extracted from 3T3-F442A cells at the indicated times. Blots of the same RNA samples were hybridized to the β_3-adrenoceptor (β_3-AR), β-actin, lipoprotein lipase (LPL), glycerol-3-phosphate dehydrogenase (G3PDH) and adipsin cDNA probes. The size (kilobase) of each mRNA species is mentioned in the right margin. (B) Autoradiograms corresponding to hybridization with the β_3-adrenoceptor probe were scanned with a videodensitometer. β_3-Adrenoceptor mRNA steady-state levels were normalized to those measured in day 12 post-confluent adipocytes (arbitrarily considered as 100%).

different amounts of reverse-transcribed RNA and different numbers of cycles (see legend to Fig. 2). When performed in non-saturating conditions (i.e. 25 ng of reverse-transcribed RNA and 25 cycles), RT-PCR revealed a pattern of β_3-adrenoceptor mRNA expression quite similar to that observed by Northern blot analysis (Fig. 2A). β_3-Adrenoceptor mRNA became weakly detectable at day 5, was strongly induced at day 8 and reached its maximum level at day 12. However, when using conditions that saturated β_3-subtype cDNA amplification of fully mature adipocytes, low levels of

β_3-adrenoceptor could be measured before day 5 (Fig. 2B,C,D). Substantial levels of β_3-subtype mRNA could even be detected at confluence (Fig. 2D).

The pattern of β_1-adrenoceptor mRNA expression was also studied in the course of 3T3-F442A differentiation by RT-PCR. In preliminary experiments the optimal amounts of cDNA and number of cycles were determined. Amplification of 25 ng of reverse-transcribed RNA for 30 cycles proved to be the best semi-quantitative conditions. β_1-Adrenoceptor mRNA expression could be detected in preadipocytes; it increased by 70% just after confluence, was maximal at day 2 following confluence (80% increase) and became stable at ~20–30% above the initial levels at late stages of differentiation (Fig. 2E).

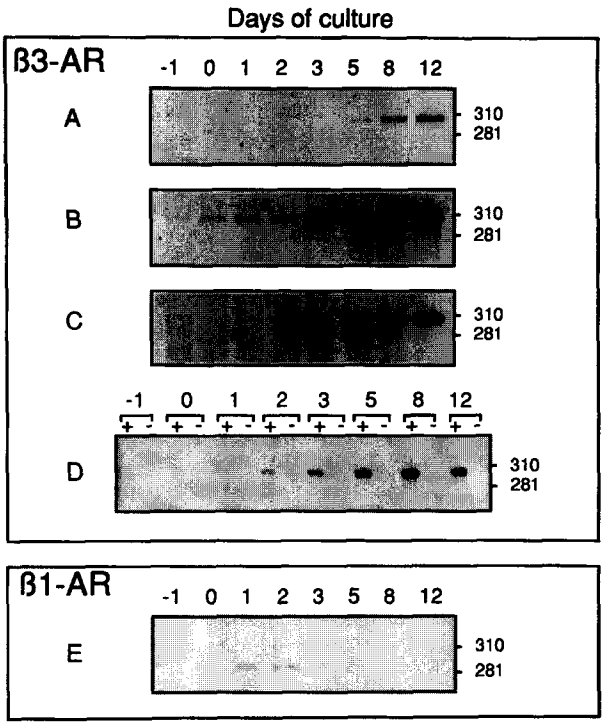

Fig. 2. RT-PCR analysis of β-adrenoceptor subtype mRNA expression during 3T3-F442A adipose differentiation. cDNAs were amplified during 25 (A,B) or 30 (C,D,E) cycles in the presence of *Taq* polymerase and primers specific for the β_3-adrenoceptor (β_3-AR) (A,B,C,D) or the β_1-adrenoceptor (β_1-AR) (E). cDNA content in the PCR assay corresponded to initial amounts of DNase I-treated RNA of 25 ng (A,C,E) or 100 ng (B,D). The resulting products were separated on a 2.5% agarose gel and stained with ethidium bromide. Panel D shows the amplification products derived from RNA treated (+) or not (−) with MMLV RT. Sizes (in base pairs) of molecular weight markers are indicated in the right margin. Videodensitometric scanning of the ethidium bromide-stained gel presented in panel A indicates that β_3-adrenoceptor mRNA content of day 5 and day 8 post-confluent cells represents 17 ± 2% and 67 ± 2% of that detected in day 12 post-confluent cells, respectively.

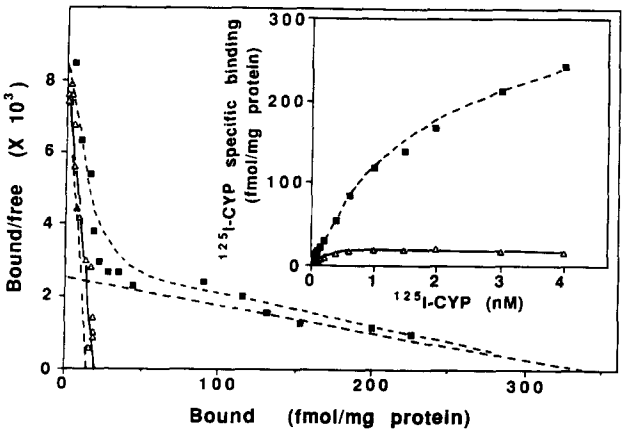

Fig. 3. Characterization of ^{125}I-cyanopindolol binding sites in day 5 and day 12 post-confluent 3T3-F442A cells. The figure shows the Scatchard plot of a typical ^{125}I-cyanopindolol (^{125}I-CYP) saturation experiment. Data are analyzed according to a one-site (day 5, △) or two-site (day 12, ■) model. The solid straight line represents the best fit for a one-site model in day 5 cells. The dashed curvilinear plot is the best fit for a two-site model in day 12 adipocytes. The corresponding binding components are indicated by the two dashed straight lines (inset, saturation curves of the corresponding experiment performed in day 5 (△) or day 12 (■) post-confluent cells). Binding parameters of three to four separate experiments are summarized in Table 1.

3.2. Emergence of the β_1- and β_3-adrenoceptor proteins during 3T3-F442A adipose conversion

Previous studies have indicated that the respective high affinity of the β_1- and β_2-adrenoceptors for ^{125}I-cyanopindolol and low affinity of the β_3-subtype for this radioligand allowed to distinguish these two populations of β-adrenoceptors (Fève et al., 1991). Therefore we performed ^{125}I-cyanopindolol saturation experiments in membrane fractions from 3T3-F442A cells at various times during the differentiation process. Scatchard analysis (Scatchard, 1949) of the data indicated that the β_3-sites (low affinity component) remained undetectable until day 5 after confluence (Fig. 3, Table 1). At day 8, we observed the appearance of a major β_3-subtype population (representing ~95% of total β-adrenoceptors). There was a further 1.5-fold increase in the β_3-adrenoceptor density between day 8 and 12 (233 ± 18 and 325 ± 48 fmol/mg of protein, respectively). During the course of adipose development, the pattern of expression of high affinity sites for ^{125}I-cyanopindolol was clearly biphasic. Preadipocytes (day 0) displayed a unique high affinity component for ^{125}I-cyanopindolol; at day 3 this component increased by a factor 3, but in mature adipocytes it only represented twice the level of that measured in preadipocytes.

Competition experiments of ^{125}I-cyanopindolol against the β_3-adrenoceptor-selective ligand BRL37344

Table 1
Characteristics of ^{125}I-cyanopindolol binding sites in differentiating 3T3-F442A cells

	^{125}I-Cyanopindolol binding sites			
	High affinity (β_1-subtype)		Low affinity (β_3-subtype)	
	K_D (pM)	B_{max} (fmol/mg)	K_D (pM)	B_{max} (fmol/mg)
Day 0	49.2± 9.6	8.4±1.2 (100)	–	none
Day 3	42.5± 9.7	24.0±3.3 (100)	–	none
Day 5	52.2± 8.8	17.9±2.0 (100)	–	none
Day 8	42.5± 8.9	12.7±0.6 (5.2)	2465±247	233±18 (94.8)
Day 12	39.8±13.5	15.5±1.3 (4.6)	2580±370	325±48 (95.4)

Membranes were prepared at different stages of cell development following confluence (day 0). Saturation experiments were carried out on these membranes within a wide range of ^{125}I-cyanopindolol concentrations (5–4000 pM). Scatchard (Scatchard, 1949) analysis of the data with the EBDA/LIGAND program allowed the calculation of the dissociation constants (K_D) of the high (β_1-adrenoceptor)- and the low (β_3-adrenoceptor)-affinity sites for ^{125}I-cyanopindolol, and the corresponding receptor densities (B_{max}). The relative contributions of each affinity component (%) are mentioned in parentheses. Results are presented as means±S.E. of three to four independent experiments.

Table 2
Characteristics of BRL37344 binding sites in 3T3-F442A differentiating cells

	BRL37344 binding sites			
	High affinity (β_3-subtype)		Low affinity (β_1-subtype)	
	K_i (μM)	% of B_{max}	K_i (μM)	% of B_{max}
Day 0	–	none	10.5±1.6	100
Day 3	–	none	10.4±0.9	100
Day 5	–	none	9.1±4.3	100
Day 8	0.24±0.12	94.2±0.7	8.8±1.6	5.8±0.7
Day 12	0.14±0.09	94.6±0.8	6.1±1.5	5.4±0.8

Membranes were prepared from 3T3-F442A cells cultures at the indicated times. Competition experiments were performed on membranes with 300 pM ^{125}I-cyanopindolol and varying concentrations of the β_3-adrenoceptor-selective agonist BRL37344. The inhibition constant (K_i) and the proportions of each binding class were derived from the analysis of the displacement curves with the EBDA/LIGAND program. % of B_{max} and K_i values are expressed as means±S.E. of three to four experiments.

were also performed on the same membrane preparations. At day 0, 3 and 5, displacement curves of ^{125}I-cyanopindolol by BRL37344 were monophasic with effective competition only at the highest concentrations of the β_3-adrenoceptor-selective agonist (Fig. 4). In contrast, the curves were clearly biphasic when performed on membranes prepared from day 8 or day 12 adipocytes. Computer analysis of these experimental data according to a one- or a two-site model confirmed that the β_3-adrenoceptor component became the prominent β-subtype expressed in mature adipocytes (~95% of total β-adrenoceptors) (Table 2). In agreement with previous reports (Fève et al., 1994; Krief et al., 1994), competition experiments with β_2-adrenoceptor-selective compounds did not reveal any β_2-adrenoceptor population (not shown).

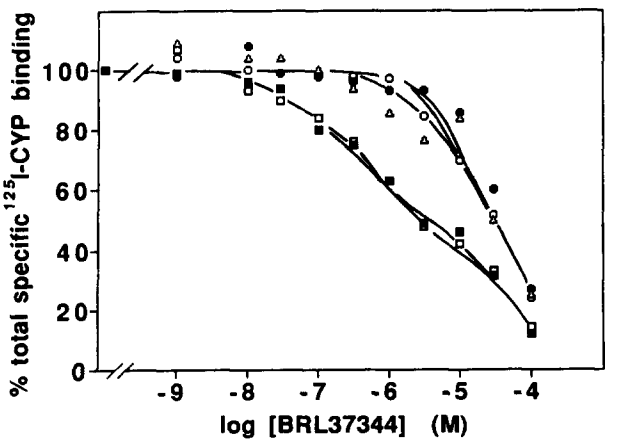

Fig. 4. Competition of BRL37344 for ^{125}I-cyanopindolol binding to 3T3-F442A cell membranes. 3T3-F442A cells were cultured as described in Materials and methods. Membranes were prepared at confluence (day 0,○) or at day 3 (●), 5 (△), 8 (□) or 12 (■) after confluence, and incubated together with 300 pM ^{125}I-cyanopindolol and the indicated concentrations of BRL37344. Results are expressed as the percentage of the specific ^{125}I-cyanopindolol (^{125}I-CYP) binding. The figure represents displacement curves from a typical experiment. Data from all experiments are analyzed with the EBDA/LIGAND program and presented in Table 2.

3.3. Contribution of the β_1- and β_3-adrenoceptor subtypes to the cAMP signaling pathway during 3T3-F442A adipose differentiation

To determine the functional consequence of the β_3-adrenoceptor emergence during adipocyte development, adenylyl cyclase activity was measured on crude membranes of 3T3-F442A cells at various times of adipose conversion. We tested adenylyl cyclase activity in response to a maximal (100 μM) concentration of isoproterenol, CGP12177, ICI201651 or cyanopindolol. Isoproterenol is known to stimulate the three β-subtypes, while CGP12177, ICI201651 and cyanopindolol solely activate the β_3-subtype, and are antagonists at the β_1- and β_2-adrenoceptors (Blin et al., 1993). In agreement with saturation and competition binding experiments, a clear activation of adenylyl cyclase by these drugs became detectable 8 days after confluence, but was inconstantly and weakly found at day 5 (Fig. 5).

Isoproterenol and norepinephrine are about 100 times more potent (EC_{50}) in stimulating adenylyl cyclase in Chinese hamster ovary cells expressing β_1-adrenoceptor than in those expressing the β_3-subtype (Granneman et al., 1991; Chaudhry et al., 1992; Liggett, 1992; Emorine et al., 1994). Dose-response curves of adenylyl cyclase activity in response to isoproterenol and norepinephrine were performed in membranes

Table 3
Relative potency of (−)-isoproterenol and (−)-norepinephrine for stimulating adenylyl cyclase activity in differentiating 3T3-F442A cells

	(−)-Isoproterenol		(−)-Norepinephrine	
	EC_{50}	V_{max}	EC_{50}	V_{max}
Day 5	0.27 ± 0.04	16.7 ± 2.1	0.80 ± 0.03	13.4 ± 2.1
Day 8	0.94 ± 0.12	35.7 ± 6.4	2.57 ± 0.06	29.6 ± 3.4
Day 12	1.15 ± 0.20	64.7 ± 7.9	3.46 ± 0.20	58.9 ± 7.2

Adenylyl cyclase activity in response to varying concentrations of isoproterenol or norepinephrine was measured in membranes from day 5, day 8 and day 12 post-confluent cells. The EC_{50} (μM) value corresponds to the concentration of agonist giving half-maximal adenylyl cyclase activation. The V_{max} value (pmol cAMP/min per mg of protein) is the maximal agonist-stimulated adenylyl cyclase activity. Data are presented as means ± S.E. of three to four independent experiments.

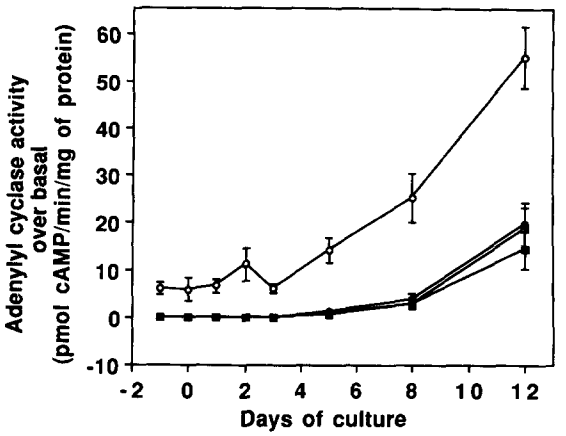

Fig. 5. Emergence of β_3-adrenoceptor coupling to the adenylyl cyclase system during 3T3-F442A adipose differentiation. At the indicated times, crude membranes were prepared and tested for isoproterenol (○)-, CGP12177 (●)-, ICI201651 (□)- or cyanopindolol (■)-stimulated (100 μM) adenylyl cyclase activity. The figure represents the means ± S.E. of three experiments performed in triplicate.

from day 5, 8 and 12 post-confluent cells. Eadie-Hofstee analysis of the data according to a one- or two-site model allowed to determine the kinetic parameters of the β_1 (high affinity)- and β_3 (low affinity)-adrenoceptor components in stimulating adenylyl cyclase (Hofstee, 1952). As compared to day 5 post-confluent cells, we observed a sharp increase in the V_{max} values of isoproterenol- or norepinephrine-stimulated adenylyl cyclase activity in day 8 or day 12 post-confluent adipocytes (Table 3). Interestingly, the corresponding EC_{50} values increased in parallel. This increased efficacy (V_{max}) but decreased potency (EC_{50}) of isoproterenol and norepinephrine to stimulate adenylyl cyclase activity reflected the increased involvement of the β_3-adrenoceptor in the catecholamine-sensitive pathway during the course of the differentiation process (Table 4). In day 5 post-confluent cells, the β_1-adrenoceptor was the unique subtype that promoted cAMP production in response to isoproterenol and norepinephrine. This pattern was dramatically reversed in day 8 and day 12 post-confluent adipocytes. In these cells, Eadie-Hofstee representation of isoproterenol- or norepinephrine-stimulated dose-response curves clearly visualized the biphasic nature of adenylyl cyclase activation, resolved in a minor high affinity β_1-adrenoceptor component and a major low affinity β_3-subtype one (Fig. 6A,B). The β_3-adrenoceptors accounted for ~ 90% of maximal adenylyl cyclase activity, while the contribution of the β_1-component (in terms of V_{max} value or in % of the total V_{max}) became low (8–11%) (Table 4). Additional experiments with the β_1-adrenoceptor (CGP20712A)- and the β_2-adrenoceptor (ICI

Table 4
Kinetic parameters of adenylyl cyclase activation by (−)-isoproterenol, (−)-norepinephrine and BRL37344 in differentiating 3T3-F442A cells

		(−)-Isoproterenol		(−)-Norepinephrine		BRL37344	
		K_{act}	V_{max}	K_{act}	V_{max}	K_{act}	V_{max}
Day 5							
	β_1	0.27 ± 0.04	16.7 ± 2.1 (100)	0.80 ± 0.03	13.4 ± 2.1 (100)	1.83 ± 0.57	3.1 ± 0.1 (100)
	β_3	–	0 (0)	–	0 (0)	0	0 (0)
Day 8							
	β_1	0.12 ± 0.01	3.1 ± 0.6 (8.7)	0.49 ± 0.07	3.3 ± 0.6 (11.3)	–	0 (0)
	β_3	3.58 ± 0.50	32.6 ± 0.6 (91.3)	17.90 ± 5.00	26.3 ± 0.6 (88.7)	0.09 ± 0.01	26.6 ± 0.8 (100) [a]
Day 12							
	β_1	0.12 ± 0.03	5.2 ± 0.6 (8.0)	0.47 ± 0.05	5.6 ± 0.4 (9.5)	–	0 (0)
	β_3	5.43 ± 1.11	59.5 ± 0.6 (92.0)	18.04 ± 4.46	53.3 ± 0.4 (90.5)	0.10 ± 0.02	52.3 ± 5.2 (100) [a]

Adenylyl cyclase activity was measured in membranes from day 5, day 8 or day 12 post-confluent 3T3-F442A cells in response to increasing concentrations of isoproterenol, norepinephrine or BRL37344. Hofstee analysis (Hofstee, 1952) of each dose-response curve was performed in the EBDA program for best fit according to a one-site or two-site model. The K_{act} (μM) and the V_{max} (pmol cAMP/min per mg of protein) values are mentioned for each activation component, while their relative contribution (%) for adenylyl cyclase maximal activation is given in parentheses. Results are expressed as means ± S.E. of three to four separate experiments. [a] Best fit to a single component (see text and legend to Fig. 6).

118551)-selective antagonists further confirmed the nature of the adenylyl cyclase activation components at different stages of adipose differentiation. Norepinephrine-stimulated dose-response curves performed in the absence or the presence of 0.1 μM CGP20712A or ICI118551 are shown in Fig. 7. This concentration of 0.1 μM CGP20712A was choosen because it corresponds to a value ~20–50 times higher than the previously reported inhibition constant (K_i) of the β_1-adrenoceptor for this antagonist, but is ~50–100 times lower than the K_i values of the β_2- and β_3-subtypes for this drug (Fève et al., 1990, 1991, 1994; Krief et al., 1994). Otherwise the concentration of 0.1 μM ICI118551 is ~100 times higher than the K_i value of the β_2-adrenoceptor for this compound, is similar to that of the β_1-subtype, and is ~50–100 times lower than that of the β_3-adrenoceptor for this β_2-subtype-selective antagonist (Fève et al., 1990, 1991, 1994; Krief et al., 1994). As illustrated in day 5 post-confluent 3T3-F442A cells (Fig. 7A), the β_1-adrenoceptor-selective antagonist CGP20712A provoked a dramatic rightward shift of the norepinephrine dose-response curve (> to one order of magnitude). As expected the β_2-adrenoceptor-selective antagonist ICI118551 had little effect on the dose-response curve, since at the concentration of 0.1 μM it elicited a 3-fold increase of the apparent K_{act} value for norepinephrine-stimulated adenylyl cyclase activation. Computer analysis of the data strongly supported that the adenylyl cyclase response in day 5 post confluent cells corresponded to a single high affinity component, typically β_1 in nature. Similar experiments in day 12 adipocytes (Fig. 7B) showed that the high affinity component of norepinephrine-stimulated dose-response curve was reduced from 10.0 ± 0.8% to 2.3 ± 1.2% in the presence of CGP20712A (t-test, $P = 0.01$, $n = 4$), but only to 6.9 ± 0.7% in the presence of ICI118551 ($P = 0.1$, $n = 4$). Otherwise the two antagonists did not elicit any change in the maximal stimulation and had no significant effect on the low-affinity (β_3-adrenoceptor) component of the adenylyl cyclase activation by norepinephrine. We also examined activation of adenylyl cyclase by BRL37344, a β_3-adrenoceptor-selective agonist which stimulates the β_1- and β_2-subtypes with a much lower potency (Emorine et al., 1989; Fève et al., 1991; Nahmias et al., 1991; Granneman et al., 1991). In day 5 post-confluent cells BRL37344 exhibited a low potency and a poor intrinsic activity (~20% of isoproterenol effect) with respect to the low-affinity component (β_1-adrenoceptors) (Table 4). In day 8 and day 12 post-confluent adipocytes the dose-response curve to BRL37344 was best fit to a single high affinity component corresponding to β_3-subtype activation (Table 4, Fig. 6C). The β_3-adrenoceptor function accounted for the entire BRL37344-stimulated adenylyl cyclase activity and obscured any detectable β_1-subtype-mediated adenylyl cyclase activation. Finally, we studied activation of adenylyl cyclase by varying con-

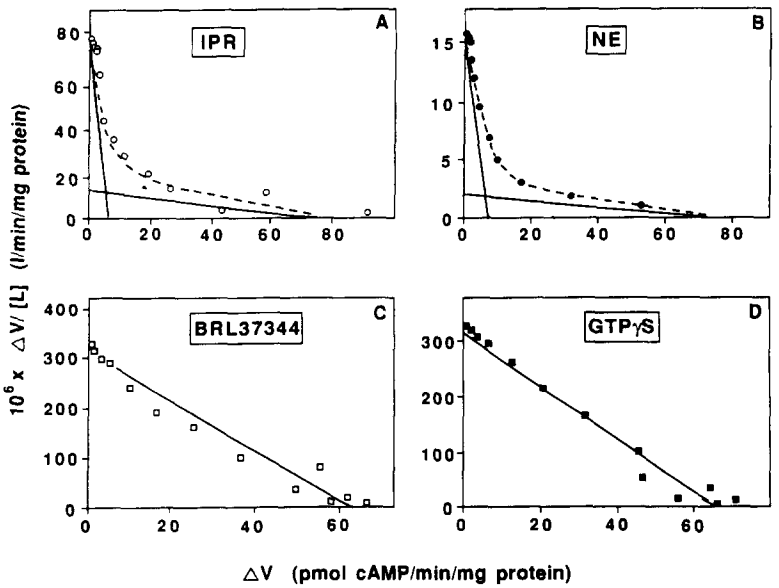

Fig. 6. Eadie-Hofstee analysis of isoproterenol-, norepinephrine-, BRL37344- and GTPγS-dose-response curves in mature adipocytes. Adenylyl cyclase activity over basal (ΔV) was determined in day 12 post-confluent adipocytes in the presence of various concentrations of isoproterenol (IPR) (A,○), norepinephrine (NE) (B,●), BRL37344 (C,□) and GTPγS (D,■). The Eadie-Hofstee analysis of the data was performed with the EBDA/LIGAND program according to a one- or two-site model. The figure shows the representation of a typical experiment. The dashed curves are the curvilinear best fits for a two-site model, while activation components are indicated with the solid straight lines (panels A and B). K_{act} and V_{max} values of each activation component for three to four independent experiments are presented in Table 4. Computer analysis of BRL37344 dose-response curve (panel C) indicated that the one-site fit gave a better fit than the two-site model ($F = 5.89$; $P = 0.02$).

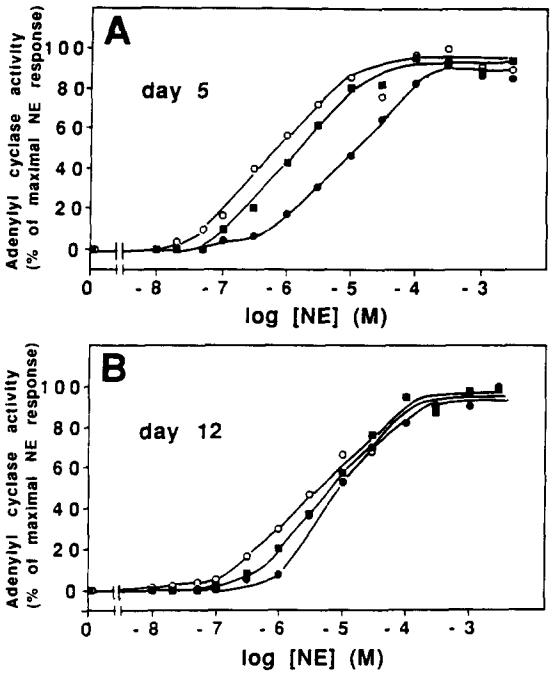

Fig. 7. Effect of β_1- or β_2-adrenoceptor-selective antagonists on norepinephrine-stimulated adenylyl cyclase activity. Dose-response curve of stimulation by norepinephrine (NE) was performed in day 5 (panel A) or day 12 (panel B) post-confluent cells in the absence (○) or the presence of 0.1 μM CGP20712A (●) or 0.1 μM ICI118551 (■). Each curve was computer-anlyzed according to a one- or two-site model and the respective V_{max} and K_{act} values were calculated. The figure shows the typical profile of one observation (among four independent experiments). Maximal adenylyl cyclase activity over basal was 15.9±2.1 and 56.6±2.3 pmol cAMP/min/mg of protein in day 5 and day 12 cells, respectively. Basal activitiy was 7.1±0.9 and 8.8±0.6 pmol cAMP/min per mg of protein in day 5 and day 12 post-confluent cells, respectively.

Table 5
Kinetic parameters of lipolysis activation by (−)-norepinephrine in mature adipocytes

	−CGP20712A		+CGP20712A	
	K_{act} (nM)	V_{max} (%)	K_{act} (nM)	V_{max} (%)
β_1-Adrenoceptor	1.20±0.37	16.0±7.3	0.61± 0.07	2.9±3.1 [a]
β_3-Adrenoceptor	14.20±7.50	84.0±7.3	28.40±17.90	97.1±3.1 [a]

Lipolysis experiments were performed directly on adherent day 12 post-confluent 3T3-F442A adipocytes in response to increasing concentrations of norepinephrine, in the absence or the presence of CGP20712A (100 nM). Glycerol production was measured as mentioned in Materials and methods. Hofstee analysis (Hofstee, 1952) of each norepinephrine dose-response curve was performed in the EBDA/LIGAND program for best fit according to a one- or two-site model. The apparent K_{act} values (nM) of β_1- and β_3-adrenoceptors for norepinephrine and the relative contribution (%) of each activation component are indicated. The basal and maximal lipolytic activities were similar in the absence or the presence of CGP20712A, and were 381±29 and 1156±57 nmol NADH/2 h per well, respectively. Results are expressed as means±S.E. of five separate experiments. [a] $P < 0.05$, CGP20712A-exposed cells versus CGP20712A-untreated cells.

centrations of GTPγS, a non-hydrolysable analog of GTP which directly stimulates the G_s complex. As expected the concentration-response curve was resolved in a monophasic component (V_{max} = 79.2 ± 10.6 pmol cAMP/min per mg of protein; K_{act} = 0.67 ± 0.38 μM) (Fig. 6D).

Taken together, the results of our adenylyl cyclase measurements in membranes from 3T3-F442A cells at various stages of their differentiation showed the very late emergence of β_3-adrenoceptor coupling to the adenylyl cyclase system, and its prominent contribution to adenylyl cyclase activation in mature adipose cells.

3.4. Contribution of the β_1- and β_3-adrenoceptors to lipolysis in mature adipocytes

In order to definitively assess the role of β_3-adrenoceptors in the control of lipolysis we studied whether the contribution of this β_3-subtype for glycerol production in intact cells in response to norepinephrine. In fully mature adipocytes (day 12 after confluence), glycerol release was measured in response to increasing concentrations of norepinephrine, in the absence or the presence of the β_1-adrenoceptor-selective antagonist CGP20712A (0.1 μM). Though β_3-adrenoceptor contribution to lipolysis was less important than for cAMP production, computer analysis of norepinephrine dose-response curve indicated that about 85% of the maximal glycerol release was ensured by this subtype (Table 5). The β_1-adrenoceptor-selective antagonist did not cause a significant rightward shift of the norepinephrine dose-response curve (EC_{50} values: 5.8 ± 1.0 nM and 6.9 ± 0.7 nM in cells not exposed and exposed to CGP20712A, respectively; $P = 0.20$, $n = 5$), in agreement with previous results in rat adipocytes (Hollenga and Zaagsma, 1989). The presence of CGP20712A reduced the contribution of the high-affinity component (β_1) of norepinephrine-stimulated lipolysis from 16% to 3%, but had no effect on maximal glycerol production.

4. Discussion

β-Adrenoceptors are essential mediators for the acquisition of an increased adrenergic sensitivity during adipose conversion and play a central role in the regulation of adipocyte metabolism. Differentiation-dependent β-adrenoceptor gene expression takes place in a coordinated multistep process that depends upon environmental factors, such as hormones, nutrients or drugs. Thus, in 3T3-F442A cells, β_3-adrenoceptor gene expression can be induced by cAMP (Thomas et al., 1992), but is repressed at a transcriptional level by glucocorticoids (Fève et al., 1992), butyrate (Krief et al., 1994), insulin (Fève et al., 1994) and phorbol esters

(Fève et al., 1995). In various cell systems these effectors, with the exceptions of insulin and phorbol esters, are also able to modulate β_1- and/or β_2-adrenoceptor gene expression (Fève et al., 1990; Henneberry et al., 1977; Collins et al., 1988, 1989; Hadcock and Malbon, 1988; Hadcock et al., 1989). The aim of our study was to determine the contribution of each β-subtype to the cAMP signaling pathway during murine adipocyte development. For this purpose the differentiation has been allowed to proceed in a medium supplemented with serum alone, without addition of pharmacological effectors. Although these culture conditions might not totally mimic the physiological conditions of adipose tissue maturation in vivo, our approach prevents misleading conclusions drawn from the interplay between the differentiation program and the intrinsic effect of a drug supplementation (Guest et al., 1990).

Onset of adipose conversion is accompanied by profound changes in the expression of several early genes: decrease in β-actin mRNA and increase in lipoprotein lipase mRNA levels. This early step is followed by terminal differentiation which is characterized by the induction of late markers such as glycerol-3-phosphate dehydrogenase, aP2 and very late markers such as adipsin. Our results confirm the kinetics of expression of these various mRNA species, as reported in previous studies (Spiegelman et al., 1983; Ailhaud et al., 1992; Spiegelman and Farmer, 1982; Bernlohr et al., 1984; Vannier et al., 1985). Overall, induction of β_3-adrenoceptor mRNA accompanies appearance of adipsin mRNA. The β_3-subtype can thus be considered as a very late marker of terminal differentiation. It is tempting to speculate that one or several events of terminal differentiation, for instance adipose-specific transcription factors, regulate both adipsin and β_3-adrenoceptor expression. However such a link remains quite hypothetical. The very low β_3-adrenoceptor mRNA levels detected in preadipocyte by RT-PCR are likely to correspond to a minimal transcription of the gene without any significant protein synthesis or coupling to the adenylyl cyclase system. The large changes in β_3-adrenoceptor gene expression observed during adipose conversion are accompanied by parallel modifications in β_3-subtype protein levels. In ^{125}I-cyanopindolol saturation experiments, the low affinity component which corresponds to the β_3-sites becomes only detectable at day 8 after confluence. This result is in agreement with the competition experiments of ^{125}I-cyanopindolol against BRL37344 which reveal the presence of a high affinity component for the β_3-adrenoceptor-selective agonist at day 8 and day 12 post-confluent cells, but not earlier. Presence of β_3-sites belongs to the phenotype of terminally differentiated adipocyte. By contrast, the presence of the β_1-adrenoceptor does not represent per se an adipocytic character, since this receptor subtype is present in preadipocyte. β_1-Sites are only moderately induced following commitment and are overcome by the considerable β_3-subtype population present in mature adipocytes.

Numerous studies (reviewed by Arch and Kaumann, 1993) have given support to the idea that in most rodent species, the β_3-adrenoceptor is the main subclass involved in the control of lipolysis, oxygen consumption, cAMP accumulation or adenylyl cyclase activity. These conclusions have always been drawn from a set of functional pharmacological specificities that can be summarized as follows: low efficiency of classical β_1/β_2-adrenoceptor antagonists or of β_1- and β_2-adrenoceptor-selective antagonists to inhibit agonist-stimulated cAMP production or lipolysis, high potency of a panel of new β-adrenoceptor agonists such as BRL37344, and 'paradoxical' partial agonistic properties of β_1-/β_2-adrenoceptor antagonists, such as CGP12177 and pindolol derivatives (Arch and Kaumann, 1993; Emorine et al., 1994). These studies have generally provided a schematic pharmacological pattern of the biological responses observed in a tissue that mainly expresses the β_3-subtype. A recent study (Collins et al., 1994) has determined that 77% of epinephrine-sensitive adenylyl cyclase activity in murine white adipocytes is driven through the β_3-adrenoceptor, the residual one being under β_1- and/or β_2-subtype control. In young 3T3-F442A adipocytes, an Eadie-Hofstee analysis of isoproterenol, norepinephrine and BRL37344 concentration-response curves only identifies a β_1-subtype-mediated component; this is in agreement with the absence of any detectable β_3-site in binding experiments. In mature adipocytes (in contrast to the monophasic character of adenylyl cyclase activation by GTPγS) (Fig. 6), isoproterenol and norepinephrine concentration-response curves are resolved into two components. Such a picture suggests a receptor-related but not a G protein-related phenomenon as the basis of the kinetic differences. Overall, the β_3-adrenoceptor component overcomes its β_1-subtype counterpart, and it represents $\sim$90% of the total response to these two β-adrenoceptor agonists. These data correspond reasonably well with those reported in the study of Collins et al. (1994). Moreover, they are quite consistent with binding experiments in which β_3-sites represent $\sim$95% of the total β-adrenoceptor population of 3T3-F442A mature adipocytes. In these cells, the absence of any detectable β_1-adrenoceptor-mediated adenylyl cyclase activation by BRL37344 (Fig. 6, Table 4) is likely to be due to the magnitude of the β_3-subtype population. It is also explained by the low intrinsic activity of BRL37344 on the β_1-subtype, as assessed by its low efficacy compared to that of isoproterenol and norepinephrine in young adipocytes expressing only β_1-subtypes (Table 4).

Based on the weak rightward shift of the norepinephrine- or isoproterenol-stimulated dose-response curve for lipolysis in the presence of β_1-adrenoceptor-selective antagonists, previous studies have already suggested the presence of only a minor population of functional β_1-adrenoceptors in rat adipocytes (Wilson et al., 1984; Bojanic et al., 1985; Hollenga and Zaagsma, 1989). These β_1-adrenoceptors seem essentially efficient at low agonist concentrations (Van Liefde et al., 1992). In 3T3-F442A adipocytes, by delineation of the high- and low-affinity activation components, we calculate that the β_3-adrenoceptors mediate about 85% of the maximal norepinephrine-stimulated lipolysis. These results support the view that the β_3-adrenoceptor is the main subtype involved in lipolysis of rodent adipocyte, despite the fact that functional β_1-adrenoceptors could significantly contribute to glycerol production, essentially at low catecholamine concentrations.

Assuming the absence of cooperative interactions between β_1- and β_3-adrenoceptors, we have calculated the respective receptor occupancy and contribution to adenylyl cyclase activation of each β-subtype at several concentrations of the endogenous catecholamine, norepinephrine (Fig. 8). At 5.5 μM norepinephrine, β_1- and β_3-adrenoceptor occupancies are similar (β_3/β_1 'binding' ratio = 1), but the β_3-subtype contribution in cAMP production is 2.5-fold higher than that of the β_1-adrenoceptor (β_3/β_1 'cAMP' ratio > 1). In the same way, at a norepinephrine concentration giving a comparable adenylyl cyclase activation by the two subtypes (1.6 μM) (β_3/β_1 'cAMP' ratio = 1), β_3-adrenoceptor occupancy by the catecholamine is 2.7-fold lower than that of the β_1-subtype (β_3/β_1 'binding' ratio < 1). This analysis strongly suggests that the β_3-adrenoceptor has a better ability than the β_1-adrenoceptor to stimulate adenylyl cyclase. Such β-subtype-selective differences may be related to variations in the coupling to the G_s complex. Green et al. (1992) have evaluated the properties of the cloned human β_1- or β_2-adrenoceptor expressed in fibroblastic cell lines and have shown that a greater degree of agonist-promoted coupling occurs between G_s and β_2-subtype, than between G_s and β_1-subtype. Alternatively, the differential ability of the β_1- and β_3-adrenoceptor to stimulate the adenylyl cyclase system may depend on their preferential coupling to specific α_s subunit isoforms. In reconstitued systems, it is now clear that many G protein-coupled receptors preferentially interact with specific α_s, α_i or α_0 isoforms (Hepler and Gilman, 1992). Finally, whether the adrenoceptor is of the β_1- or β_3-subtype might determine the extent to which the receptor activates an adenylyl cyclase system. In a recent work, in which Levy et al. (1993) coexpressed cloned β_1- and β_2-adrenoceptors in permanent cell lines at various β_1/β_2 ratios, it has been demonstrated that the more

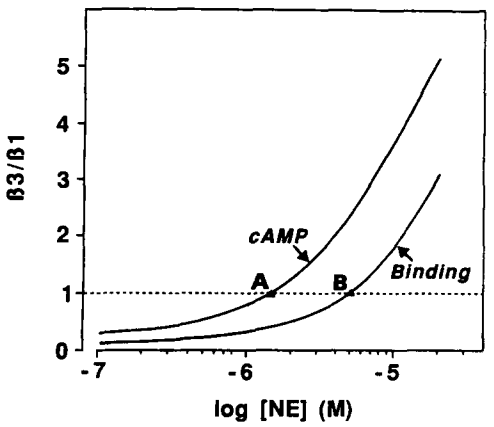

Fig. 8. Theoretical β_3/β_1 ratios for receptor occupancy and cAMP production in 3T3-F442A mature adipocytes stimulated by norepinephrine. At a given norepinephrine concentration ([NE]), β_1- or β_3-adrenoceptor occupancy was determined according to the equation: $B = (B_{max} \times [NE])/(KD + [NE])$, where B (fmol/mg of protein) represents the number of sites occupied by norepinephrine, B_{max} the total density of the related subtype (see Table 1, day 12 post-confluent adipocytes), and K_D the dissociation constant of β_1- or β_3-adrenoceptor for norepinephrine (Fève et al., 1991). Respective contributions of β_1- or β_3-adrenoceptor in adenylyl cyclase activation were calculated with the following equation: $V = (V_{max} \times [NE])/(K_{act} + [NE])$, where V (pmol cAMP/min/mg of protein) is the contribution of the β-subtype at this norepinephrine concentration, V_{max} the maximal adenylyl cyclase activation caused by this subtype and K_{act} the activation constant of each β-subtype for norepinephrine (see Table 4). β_3/β_1 ratios for receptor occupancy (*Binding* curve) and adenylyl cyclase activation (*cAMP* curve) were plotted against norepinephrine concentration. The dashed horizontal line visualized the β_3/β_1 ratio = 1. The intercept (A) between the dashed line and the *cAMP* curve, corresponding to an equal contribution of the β_1- and β_3-adrenoceptors to cAMP production, is located at a norepinephrine concentration of 1.6 μM. The intercept (B) between the dashed line and the *Binding* curve corresponds to an equal β_1- and β_3-adrenoceptor occupancy and is located at a norepinephrine concentration of 5.5 μM.

effective stimulation of adenylyl cyclase through β_2-adrenoceptors than through β_1-subtypes is an inherent property of the receptor and not of the cell in which it is expressed. We cannot also exclude that beyond their coupling to a G_s complex, each β-subtype preferentially stimulates different adenylyl cyclase isoforms. Whatever mechanism is involved, the increased potency of the β_3-adrenoceptor to activate this transducing pathway and the magnitude of its density circumvent its lower affinity for norepinephrine (see Tables 4 and 5) than that of the β_1-subtype, and highlight the importance of the β_3-adrenoceptor in the control of cAMP production and lipolysis in adipocytes.

The pattern of β-adrenoceptor subtype emergence described in this study is reminiscent of the situation encountered in cultured mouse brown adipocyte precursors (Bronnikov et al., 1992). The noradrenergic control of brown fat cell proliferation that occurs in the early stages of culture is primarily mediated through

the β_1-adrenoceptors, while appearance of coupled β_3-adrenoceptors takes place later and seems efficient in promoting gene expression for the differentiation-specific uncoupling protein thermogenin. Despite this similarity in the β-adrenoceptor subtype ontogeny between 3T3 adipocytes and brown primary adipocytes, the absence of thermogenin expression in the cell line does not support the brown nature of this preadipocytic model. It is conceivable that the differentiation-dependent expression of the adipocyte β-adrenoceptor system corresponds to a two-step physiological process. The early induction of β_1-adrenoceptors could be required for the noradrenergic control of the initial steps of adipose conversion, including the regulation of cell growth and of commitment-related events. Subsequently, the emergence in terminally differentiated adipose cells of the prominently expressed β_3-adrenoceptor is likely to play a central role in the noradrenergic modulation of cAMP-dependent biological processes.

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