British Journal of Pharmacology (2000) 129, 448–456
www.nature.com/bjp

Regulation of β_1- and β_3-adrenergic agonist-stimulated lipolytic response in hyperthyroid and hypothyroid rat white adipocytes

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1 This study examined the effects of thyroid status on the lipolytic responses of rat white adipocytes to β-adrenoceptor (β-AR) stimulation. The β_1- and β_3-AR mRNAs and proteins were measured by Northern and saturation analyses, respectively. Glycerol production and adenyl cyclase (AC) activity induced by various non-selective and selective β_1/β_3-AR agonists and drugs which act distal to the receptor in the signalling cascade were measured in cells from untreated, triiodothyronine (T_3)-treated and thyroidectomized rats.

2 The β_3-AR density was enhanced (72%) by T_3-treatment and reduced (50%) by introduction of a hypothyroid state while β_1-AR number remained unaffected. The β_1- and β_3-AR density was correlated with the specific mRNA level in all thyroid status.

3 The lipolytic responses to isoprenaline, noradrenaline ($\beta_1/\beta_3/\beta_3$-AR agonists) and BRL 37344 (β_3-AR agonist) were potentiated by 48, 58 and 48%, respectively in hyperthyroidism and reduced by about 80% in hypothyroidism.

4 T_3-treatment increased the maximal lipolytic response to the partial β_3-AR (CGP 12177) and β_1-AR (xamoterol) agonists by 234 and 260%, respectively, increasing their efficacy (intrinsic activity: 0.95 versus 0.43 and 1.02 versus 0.42). The maximal AC response to these agonists was increased by 84 and 58%, respectively, without changing their efficacy.

5 In the hypothyroid state, the maximal lipolytic and AC responses were decreased with CGP (0.17 ± 0.03 versus 0.41 ± 0.08 μmol glycerol/10^6 adipocytes; 0.048 ± 0.005 versus 0.114 ± 0.006 pmol cyclic AMP min^{-1} mg^{-1}) but not changed with xamoterol.

6 The changes in lipolytic responses to postreceptor-acting agents (forskolin, enprofylline and dibutenyl cyclic AMP, $(Bu)_2$cAMP) suggest the modifications on receptor coupling and phosphodiesterase levels in both thyroid states.

7 Thyroid status affects lipolysis by modifying β_3-AR density and postreceptor events without changes in the β_1-AR functionality.

British Journal of Pharmacology (2000) **129,** 448–456

Keywords: [^{3}H]CGP 12177; xamoterol; BRL 37344; CGP 12177; β_1-adrenoceptor; β_3-adrenoceptor; adipocytes; lipolyse; hypothyroidism; hyperthyroidism

Abbreviations: β-AR, β-adrenoceptor; T_3, triiodothyronine; ISO, isoprenaline; NA, noradrenaline

Introduction

The β-adrenergic control of various functions in different tissues is markedly affected by altered thyroid states (Malbon *et al*., 1988). Thus, the thyroid status may modify lipolytic response by acting on the β-adrenoceptor (β-AR) level and/or by modifying the coupling efficiency and/or postreceptor components involved in lipolytic cascade. The mechanisms of these modifications in white adipocytes are not completely understood. In rodent white adipocytes, the lipolysis is mediated by β_1- and β_3-ARs (Van Liefde *et al*., 1992; Murphy *et al*., 1993; Germack *et al*., 1997). The β_3-AR subtype is the most abundant, and is predominantly involved in the functional activity of these cells (Murphy *et al*., 1993; Germack *et al*., 1997). The lipolysis induced by the β_3-AR selective agonist BRL 37344 (BRL) (Germack *et al*., 1996) and which do not differentiate between the three β-adrenoceptor subtypes such as isoprenaline (ISO), noradrenaline (NOR) and adrenaline (Malbon *et al*., 1978; Mills *et al*., 1986; Ben Cheikh *et al*., 1994) is enhanced in white adipocytes from hyperthyroid rats. Treatment of rats with triiodothyronine- (T_3-) treatment of rats increased β_3-AR number (Germack *et al*., 1996). Similar results were obtained in an *in vitro* study on 3T3-F442A adipocytes showing that the effect of thyroid hormones are exerted directly on the fat cells (El Hadri *et al*., 1996). By contrast, in hypothyroidism, both the lipolysis induced by catecholamines (Malbon *et al*., 1978; Mills *et al*., 1986; Ben Cheikh *et al*., 1994) and the cyclic AMP accumulation produced by another β_3-agonist, Cl 316,243 (Rubio *et al*., 1995) are decreased, as also level of β_3-AR binding by using one concentration of radioligand (Rubio *et al*., 1995). Nevertheless, a saturation binding study is necessary to validate the β_3-AR decrease and to compare the β_3- and β_1-AR density. The involvement of β_1-AR subtype in the modifications of lipolysis by thyroid status has not yet been evaluated, nor has the expression of β_1-AR mRNA been quantified *in vivo* in different thyroid states.

However, alterations in thyroid status have been shown to modify some postreceptor constituents in rat white adipocytes (Malbon *et al*., 1988). Thus, catecholamine-stimulated adenyl cyclase activity is reduced in adipocytes from hypothyroid rats and in 3T3L1-adipocytes maintained in T_3-depleted medium, while NaF- and forskolin-induced adenyl cyclase activities remain unchanged (Malbon *et al*., 1978; Elks & Manganiello, 1985). Few data on the postreceptor events are available in the

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hyperthyroid state. *In vivo*, short-term hyperthyroidism (less than 5 days) induces a paradoxical decrease in isoprenaline- and forskolin-stimulated adenyl cyclase activity (Mills *et al*., 1986; Rapiejko & Malbon, 1987) whereas *in vitro*, an increase was observed (Elks & Manganiello, 1985; El Hadri *et al*., 1996). Hyperthyroid and hypothyroid states can also decrease and enhance, respectively, the cyclic AMP phosphodiesterase activity (Van Inwegen *et al*., 1975; Elks & Manganiello, 1985; Ben Cheikh *et al*., 1994) and the expression of G-protein subunits, $G_{\alpha i}$ and G_{β} (Ros *et al*., 1988; Rapiejko *et al*., 1989; Milligan & Saggerson, 1990).

In light of these data, the consequences of the postreceptor alterations on lipolysis regulation in hyper- and hypothyroid rat white adipocytes remain unclear.

The aim of the present study was to examine in parallel the effects of thyroid status on β_1- and β_3-AR-induced lipolysis and on the associated postreceptor signalling events in rat white adipocytes. Thus, we investigated the effects of various non-selective and β_1- and β_3-AR selective agonists on lipolytic response. We studied as well the efficiency of the receptor-signalling coupling by comparing the effects of the agonists with those of drugs which act at sites in the signalling cascade distal to the receptors on lipolysis and on adenyl cyclase activity. We also examined the effects of thyroid state on β_1- and β_3-AR expression at the protein and mRNA levels.

Methods

Animals and treatment

Age-matched euthyroid and thyroidectomized male Wistar rats (200–230 g) at age of 5 weeks were purchased from Iffa Credo (Lyon, France). Surgery was performed under ketamin/diazepan anaesthesia as described by Bouyard and Jadot (1997). The rats were separated into three experimental groups: thyroidectomized (hypothyroid), Triodothyronine (T3)-treated euthyroid (hyperthyroid) and control (euthyroid). The thyroidectomized rats received drinking water containing 0.9% $CaCl_2$ to preserve Ca^{2+} homeostasis and 0.03% methimazole to completely inhibit residual thryoid function for 20 days prior to sacrifice. Hyperthyroidism was induced by gastric administration of 0.5 mg T3 kg^{-1} daily for the last 7 days at 10.00 h. The final dose was given 24 h before sacrifice. During these 7 days, thyroidectomized and euthyroid rats received 0.3 ml of saline by gastric administration. In order to ascertain the effectiveness of treatment, we determined animal body weight and the concentration of thyroxine and T3 in serum. At the time of sacrifice, the body weight was 319 ± 2 g ($n=58$) for euthyroid rats, 235 ± 1 g ($n=99$) for hypothyroid rats, and 276 ± 2 ($n=77$) for hyperthyroid rats ($P<0.001$ versus euthyroid rats). The thyroxine and T3 concentrations were 31 ± 2 nmol l^{-1} and 5.8 ± 0.7 pM ($n=41$), respectively, for euthyroid rats, ND (not detectable) and 1.1 ± 0.2 pM ($n=67$) for hypothyroid rats, and ND and >85 pM ($n=48$) for hyperthyroid rats ($P<0.001$ versus euthyroid rats). The animals were fed *ad libitum*.

Isolation of adipocytes

Adipocytes were prepared accordingly to the method of Rodbell (1964) with minor modifications. Epididymal fat pads were removed, cut into small pieces and incubated with 0.1% collagenase in Krebs-Ringer buffer supplemented with 20 mM HEPES, 1.4 mM $CaCl_2$ and 3% bovine serum albumin, pH 7.4, for 60 min at 37°C in a shaking waterbath. After collagenase digestion, the viable adipocytes constitute the upper phase floating. The adipocytes were filtered through nylon cloth (mesh = 200 μm) and then washed with 30 ml of supplemented Krebs-Ringer buffer three times by centrifugation for 5 min at $400 \times g$. The washing discarded the rest of digestion subtracts with infranatant and sedimented stromal-vascular cells as well as the dead adipocytes.

The adipocyte suspension was divided into aliquots ($2-3 \times 10^{-5}$ cells 500 μl^{-1}) for lipolysis measurement and binding study, adenyl cyclase activity measurement or Northern analysis.

Lipolysis measurement

Adipocytes ($2-3 \times 10^5$ cells) were incubated in a final volume of 500 μl with graded concentrations of various lipolytic agents at 37°C in a shaking waterbath. After 90 min, the reaction was stopped by plunging the tubes into an ice bath. After centrifugation ($2000 \times g$ at 4°C for 10 min), glycerol released from adipocytes during the incubation was evaluated enzymatically (Glycerol assay kit; Boehringer, Mannheim, Germany) in an aliquot of the supernatant. Responses to the following drugs were evaluated: drugs that do not differentiate between β_1 and β_3 adrenoceptors (isoprenaline (ISO, $10^{-10}-10^{-5}$ M) and noradrenaline (NA, $10^{-9}-10^{-3}$ M), the selective β_3-agonist, BRL 37344 (BRL, $10^{-10}-10^{-5}$ M except for hyperthyroid conditions, $10^{-12}-10^{-6}$ M), selective β_3-partial agonist/β_1-, β_2-antagonist, CGP 12177 (CGP, $10^{-9}-10^{-4}$ M), selective β_1-partial agonist, xamoterol (XAM, $10^{-8}-10^{-3}$ M), adenyl cyclase activator, forskolin (FOR, $10^{-9}-10^{-3}$ M), phosphodiesterase inhibitor, enprofylline (ENP, $10^{-6}-3.10^{-2}$ M) and cyclic AMP analogue, ($(Bu)_2$cAMP, $10^{-5}-3.10^{-3}$ M).

Crude membrane preparation

Adipocytes were lysed at room temperature in hypotonic medium containing (mM): Tris-HCl 2, $MgCl_2$ 2.5, $KHCO_3$ 1, EGTA 100, pH 7.5, and the following protease inhibitors: 10 μg/ml leupeptin and 300 μM phenylmethylsulphonyl fluoride, then centrifuged for 15 min at $40{,}000 \times g$ at 15°C. The pellet was resuspended in 40 ml binding buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) or adenyl cyclase assay buffer (25 mM Tris-HCl, 1 mM EDTA, pH 7.5) and again centrifuged ($40{,}000 \times g$ for 15 min at 4°C). The pellet was resuspended in binding buffer or adenyl cyclase assay buffer to a final concentration of 2–3 mg protein ml^{-1} and stored at −80°C for subsequent adenyl cyclase assay and binding studies. Protein concentration was determined by the dye-binding assay using a commercial kit (Bio-Rad, München, Germany) and bovine serum albumin was used as standard.

Adenyl cyclase assay

Membranes (20–30 μg of protein) were preincubated with ISO ($10^{-8}-10^{-3}$ M), CGP ($10^{-10}-10^{-4}$ M), XAM ($10^{-10}-10^{-4}$ M) or FOR ($10^{-8}-10^{-3}$ M) for 15 min at 4°C. The assay was initiated by incubation of mixture in the presence of 1–2 μCi [α-^{32}P]ATP (mM): cyclic AMP 1, phosphocreatine, 0.5 unit creatine phosphokinase 10, GTP 0.1, ATP 0.2, $MgCl_2$ 5 in a final volume of 50 μl for 10 min at 35°C. The reaction was stopped by addition of 200 μl 1N HCl and heating at 95°C for 5 min. After ice-cooling and neutralization with 200 μl of 3.34 M imidazole, the radioactivity of [α-^{32}P]-cyclic AMP eluted from the alumina was measured using liquid scintillation β-counter (LS 6000SC, Beckman, U.S.A.).

Binding studies

Saturation studies were performed with 50–80 μg of crude membrane protein using [^{3}H]-CGP 12177 at concentrations ranging from 0.3 to 100 nM in a 96-well microplate. To the binding buffer containing 100 μM GTP, 50 μl ligand and 50 μl crude membrane were added to obtain a final volume of 200 μl. The binding experiments were performed in the presence of high concentration of GTP to shift all β_3-ARs into the low affinity state for the [^{3}H]-CGP 12177 since CGP 12177 is an agonist (Kent *et al*., 1980). The incubation was carried out in a shaking waterbath at 37°C for 30 min and then stopped by rapid filtration through glass fibre filters (Filtermats-Receptor Binding, Skatron, Lier, Norway) pre-wetted in binding buffer. A Skatron semi-automatic cell harvesting system (Skatron, Lier, Norway) with 4 s washing time was used. The radioactivity trapped by filters was measured using a liquid scintillation β-counter (LS6000SC, Beckman, U.S.A.). Non specific binding was determined in the presence of 100 μM propranolol as previously described (Germack *et al*., 1997).

Northern blot analysis

Total RNA was isolated from epididymal white adipocytes from hyperthyroid, euthyroid and hypothyroid rats using RNA plus solution (Bioprobe Systems, Montreuil-sous-Bois, France). Fifty μg of total RNA were electrophoresed in 1.2% agarose formaldehyde-denaturing gel and transferred to a nylon membrane (Hybond N, Amersham) overnight in 10 × SSC, then fixed by baking at 80°C for 2 h. The blot was prehybridized in 50% formamide, 5 × SSPE, 5 × Denhart's solution, 0.5% sarcosyl and 125 μg/ml heat-denatured salmon sperm DNA at 42°C for at least 3 h. Then, the blot was hybridized with a ^{32}P-labelled specific probe and cyclophilin cDNA probe. The mouse 2 k.b.p. β_3-adrenoceptor probe (Nahmias *et al*., 1991) generously provided by Prof. D. Strosberg (Institut Cochin de Génétique Moléculaire, Paris, France), rat β_1-adrenoceptor probe 896 bp PstI DNA (Mashida *et al*., 1990) kindly provided by Dr Curtis A Machida (Oregon Regional Primate Research Center, OR, U.S.A.) and rat 700 bp cDNA cyclophilin probe (Danielson *et al*., 1988) generously offered by Dr James Douglas (Oregon Health Sciences University Portland, OR, U.S.A.) were labelled by random priming using [α-^{32}P]dCTP and multiprime labelling kit (both from Amersham). Blots were washed twice in 1 × SSC-0.5% SDS, one at room temperature and the other at 65°C, followed by washing in 1 × SSC-0.1% SDS at 65°C, and subjected to autoradiography. Specific band densities were quantified by densitometry (Biocom SA, Les Ulis, France). The amount of total RNA in each sample was internally standardized within each blot by correcting the specific mRNA levels according to the levels of cyclophilin mRNA.

Data and statistical analysis

Results are expressed as means ± s.e.mean. The binding parameters (K_d and B_{max}) were determined using LIGAND, a non-linear curve-fitting program (Munson & Rodbard, 1980). The statistical method given by the LIGAND program was used to determine whether the saturation curve was best fitted by a one- or two-site model. Concentration-response curves for glycerol release and adenyl cyclase activity were analysed by computer-assisted iteration using the GraphPad PRISM (San Diego, CA, U.S.A.). For other data, statistical significance was determined by analysis of variance (ANOVA) followed by Dunnet's test, and $P<0.05$ was considered as the limit of statistical significance.

Drugs and chemicals

Collagenase type II, bovine serum albumin (fraction V), GTP, (±)isoprenaline hydrochloride, (±)propranolol hydrochloride, N,O′-dibutyryl cyclic AMP ((Bu)$_2$cAMP) and 3-propylxanthine (Enprofylline) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). (±)Noradrenaline and forskolin, 7-deacetyl-7-(O-N-methylpiperazino)-γ-butyryl-,dihydrochloride were provided by Fluka Chemica-Biochemica (Buchs, Switzerland) and Calbiochem (San Diego, U.S.A.), respectively. Xamoterol fumarate was a gift from Zeneca (Cergy, France). BRL 37344 (sodium-4-{2′-[2-hydroxy-2-(3-chlorophenyl)-ethylamino]-propyl}phenoxyacetate sesquihydrate (RR, SS diastereoisomer) was provided by Smith Kline Beecham Pharmaceuticals (Epsom, U.K.). CGP 12177 ((±)(2-(3 -carbomyl- 4-hydroxyphenoxy)- ethylamino)-3-[4-(1-methyl-(4-trifluormethyl-2-imidazolyl)-phenoxy]-2 propanolmethane sulfone) was a gift from Ciba-Geigy (Basel, Switzerland). [^{3}H]-CGP 12177 (specific activity: 46 Ci mmol^{-1}) [α-^{32}P]-ATP (specific activity: 30 Ci mmol^{-1}) and [α-^{32}P]-dCTP (specific activity: 3000 Ci mmol^{-1}) were obtained from Amersham (Les Ulis, France).

Results

Effect of thyroid status on [^{3}H]CGP 12177 binding sites

The binding characteristics are shown in Table 1. LIGAND analysis indicated that the data fitted a two-site model ($P<0.05$) in three thyroid states. The low affinity binding sites in adipocyte membranes from euthyroid rats accounted for 92% of total binding sites and represented the β_3-AR subtype as we previously showed (Germack *et al*., 1997). The high affinity binding sites corresponded to the β_1-AR subtype. T_3-treatment induced a significant enhancement in β_3-AR density by 72% as compared to euthyroid control. Inversely, thyroidectomy decreased this population of sites by 50%. The β_1-AR number was not affected by thyroid status.

Effect of thyroid status on β_1- and β_3-AR mRNA

To further study the regulation β-AR expression by thyroid hormones, the level of specific mRNAs coding for β_1- and β_3-AR was assessed by Northern blot. Three specific β_3-AR transcripts of 2.1, 2.6 and 3.5 kilobases (kb) were identified (Figure 1a). The proportion of 2.1, 2.6 and 3.5 transcript in

Table 1 Effect of thyroid status on [^{3}H]-CGP 12177 binding sites of white adipocyte membranes

	β_1-AR		β_3-AR	
	K_d (nM)	B_{max} (fmol mg^{-1} *protein*)	K_d (nM)	B_{max} (fmol mg^{-1} *protein*)
Hyperthyroid	0.37 ± 0.10	38 ± 7	36 ± 10	528 ± 85*
Euthyroid	0.30 ± 0.09	26 ± 5	23 ± 3	307 ± 32
Hypothyroid	0.34 ± 0.14	32 ± 3	18 ± 7	153 ± 49*

The values are means ± s.e.mean of 4–5 separate experiments performed in triplicate. The dissociation constants (K_d) are expressed in nM and the receptor densities (B_{max}) in fmol of [^{3}H]-CGP 12177 bound per mg of membrane proteins. *$P<0.05$ versus euthyroid control.

adipocytes from euthyroid rats was 36±2, 33±3 and 31±1%, respectively. T_3-treatment and thyroidectomy did not modify this ratio (31±3, 39±8, 29±2% and 33±9, 31±6, 36±6%, respectively). In adipocytes from hyperthyroid animals, the level of 2.1-, 2.6- and 3.5-kb species significantly increased by 39, 84 and 47%, respectively (Figure 1b). Thyroidectomy decreased the level of these transcripts by 56, 55 and 45%. In contrast, thyroid status had no effect on the level of β_1-AR transcript (2.8 kb) (Figure 1b). Thus, these results are consistent with data obtained by saturation binding studies.

Effect of thyroid status on lipolysis induced by various adrenergic agonists

As shown in Figure 2, the lipolytic responses to both full non selective β-AR agonists (NOR and ISO) and the selective β_3-agonist (BRL) were significantly enhanced in adipocytes from hyperthyroid rats and decreased in cells from hypothyroid rodents as compared to adipocytes from euthyroid animals. T_3-treatment increased the maximal response to NOR and ISO by 58 and 48%, respectively (Table 2). The enhancement of lipolytic activity observed with BRL was at a similar magnitude (48%) than the response obtained with catecholamines. Conversely, thyroidectomy induced a decrease of about 80% in the maximal lipolytic responses to the three agonists. EC_{50} of lipolysis stimulation by all three full β-agonists was also significantly modified by thyroid status. It was reduced in adipocytes from hyperthyroid rats and enhanced in the cells from hypothyroid animals (Table 2).

To assess the role of β_1- and β_3-AR subtypes in functional alterations, we studied the lipolytic responses to CGP, β_3-AR partial agonist/β_1-,β_2-AR antagonist (Van Liefde *et al*., 1992; Germack *et al*., 1997) and XAM, a partial and selective β_1-AR agonist (Germack *et al*., 1997). Even though the functional study with CGP has been suggested the presence of a putative β_4-AR in white adipocytes (Galitzky *et al*., 1997), no binding data and no cloning of β_4-AR gene have been reported up to

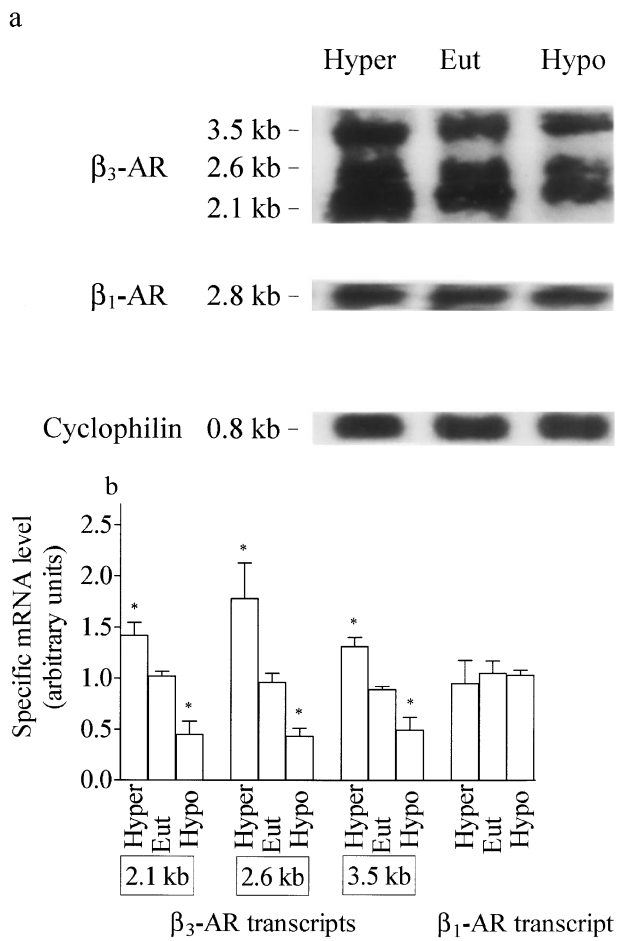

Figure 1 Northern analysis of β_1 and β_3-AR mRNA levels (a) in adipocytes from hyperthyroid (□), euthyroid control (△) and hypothyroid (○) rats. Blots were hybridized with ^{32}P-labelled DNA probes (3×10^6 d.p.m. ml^{-1}) for individual rat β-AR subtypes or for the ubiquitous cyclophilin to correct for fluctuations in the amount of RNA applied to the gel. The results from four independent observations per group are represented in (b). *, Significantly different from euthyroid control mRNA ($P<0.05$). (Hyper: hyperthroid, eut: euthyroid control and hypo: hypothyroid).

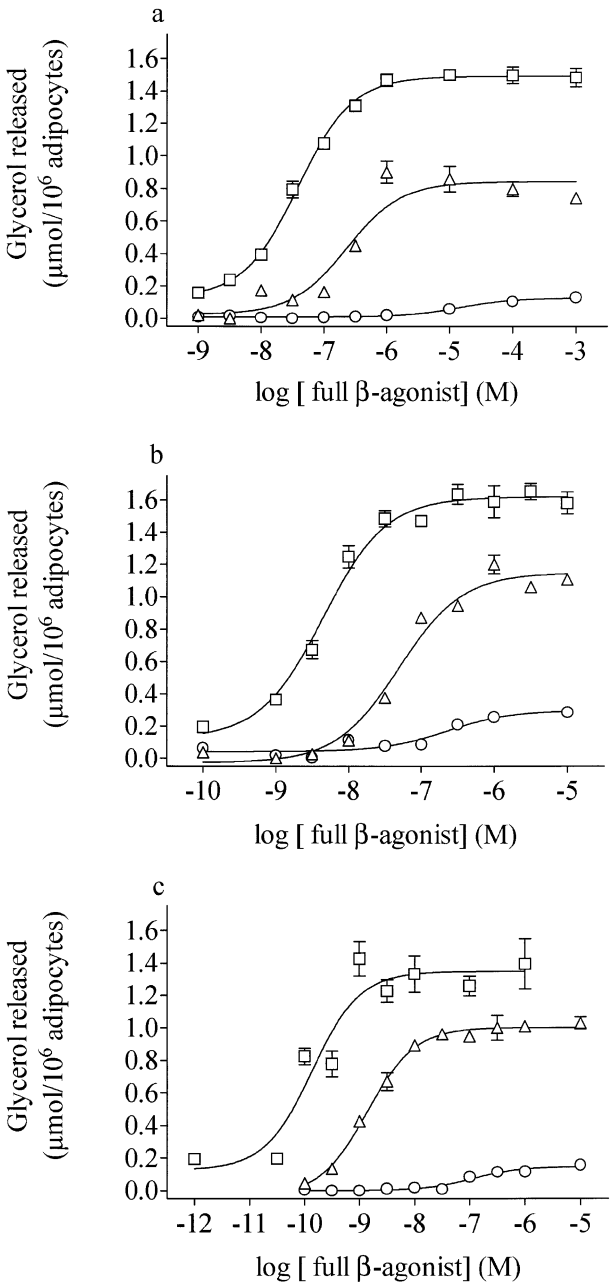

Figure 2 Concentration-response curves for stimulation of glycerol release from hyperthyroid (□), euthyroid control (△) and hypothyroid (○) rats, elicited by the full β-agonists, noradrenaline (a), isoprenaline (b) and the β_3-selective agonist BRL 37344 (c). Each curve is a representative experiment performed in triplicate. Each point is the mean±s.e.mean over basal lipolysis value. Standard deviations not shown are within the symbol.

Table 2 Effect of thyroid status on white adipocyte lipolysis induced by various adrenergic agonists

	Maximal repsonse E_{max} (μmol 10^6 *adipocytes*)			EC_{50} ($-log_{10}$ EC_{50})		
	Hyperthyroid	*Euthyroid*	*Hypothyroid*	*Hyperthyroid*	*Euthyroid*	*Hypothyroid*
Full agonists						
Noradrenaline	1.33±0.08*	0.85±0.03	0.15±0.05*	8.36±0.48*	6.59±0.08	5.30±0.48*
Isoprenaline	1.41±0.06*	0.95±0.07	0.18±0.05*	9.14±0.40*	7.36±0.05	6.69±0.24*
BRL 37344	1.49±0.07*	1.01±0.07	0.22±0.05*	9.96±0.17*	8.72±0.22	7.62±0.19*
Partial agonists						
CGP 12177	1.37±0.32*	0.41±0.08	0.17±0.03*	7.13±0.03*	6.84±0.08	5.92±0.11*
Xamoterol	1.44±0.32*	0.40±0.05	0.35±0.02	6.45±0.14	6.69±0.18	5.64±0.32*

The values are means±s.e.mean of 3–5 separate experiments performed in triplicate. The concentration of agonists including 50% of maximal lipolysis (EC_{50}) expressed in $-\log_{10}$ EC_{50}. The maximal response, E_{max} is lioplysis at the maximum effective dose of agonist minus basal lipolysis (hyperthyroid: 0.88±0.04*, euthyroid: 0.70±0.04, hypothyroid: 0.50±0.03* μmol 10^6 adipocytes). *$P<0.05$ versus euthyroid control.

date to support this hypothesis. Moreover, it is clear now that the β_3-AR subtype plays the prominent role in lipolysis in rat white adipocytes (Van Liefde *et al*., 1992; Murphy *et al*., 1993; Germack *et al*., 1997). Thus, these two agonists, CGP and XAM, provide the necessary tools to examine functional selectivity as well as partial property in different thyroid states. The maximal responses to the partial agonists in adipocytes from euthyroid animals were about 2.5 fold weaker than to the full agonist response using ISO like reference, as expected (intrinsic activity: 0.43 for CGP and 0.42 for XAM). The lipolytic responses to CGP and XAM were enhanced by T_3-treatment as compared to cells from euthyroid rats (Figure 3) and the maximal responses did not differ significantly from those triggered by the full agonists (intrinsic activity: 0.97 for CGP and 1.02 for XAM versus 0.43 and 0.42, respectively; Table 2). These two agonists appeared to act as full agonists in adipocytes from hyperthyroid rats. Thus T_3-treatment increased their efficacy. However, while the T_3-treatment reduced the EC_{50} for CGP, it failed to modify the EC_{50} for XAM.

In hypothyroid conditions, the maximal lipolytic response to CGP was decreased by 59%, whereas the maximal response to the β_1-AR selective agonist was unchanged (Table 2; Figure 3). Furthermore, the CGP E_{max} value was similar to those for the full agonists. Nevertheless, the EC_{50} for both partial agonists was decreased (Table 2). These data suggest that modification of the lipolytic response observed in hypothyroid state could be due to an alteration in the β_3-AR lipolytic pathway.

Effect of thyroid status on lipolysis induced by postreceptor-acting agents

To further characterize the mechanism of alterations induced by thyroid status on lipolysis, we studied the effects of adenyl cyclase (AC) activation by FOR, phosphodiesterase (PDE) inhibition by ENP and protein kinase A (PKA) activation by the cyclic AMP analogue $(Bu)_2$cAMP, each of which is implicated in lipolysis pathway. The ENP is non selective PDE inhibitor with higher potency for PDE III and PDE IV (Ukena *et al*., 1993), the first being prominently expressed in white adipocytes (Degerman *et al*., 1997). In all three thyroid states, FOR, ENP and $(Bu)_2$cAMP stimulated lipolysis (Figure 4). In adipocytes from hyperthyroid rats, the maximal lipolytic responses to FOR was enhanced by 58% as compared to cells from euthyroid animals, whereas the response to ENP and $(Bu)_2$cAMP was unaffected (Table 3). Furthermore, EC_{50} for FOR and ENP was significantly increased, while the $(Bu)_2$cAMP EC_{50} was not affected, suggesting a thyroid hormone effect upstream from PKA.

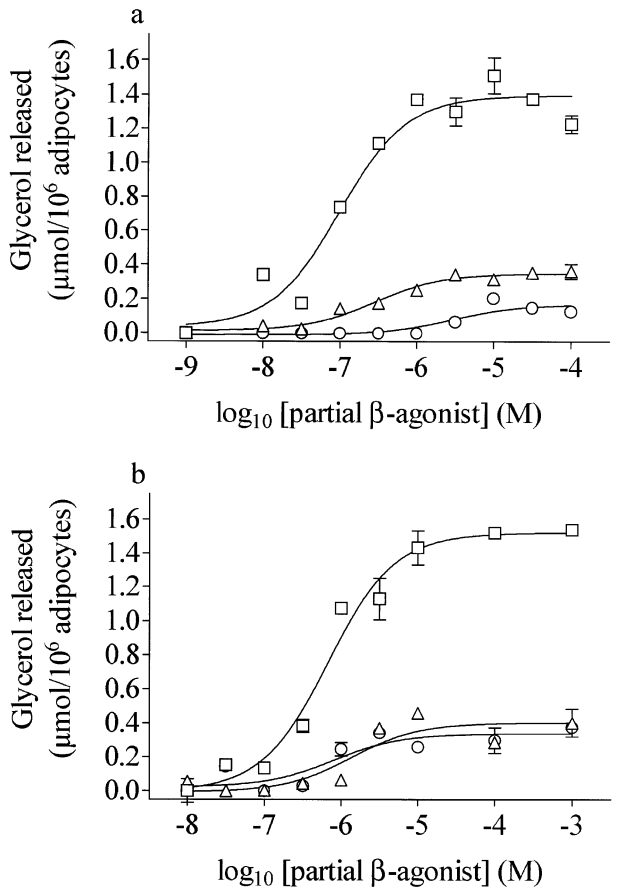

Figure 3 Concentration-response curves for stimulation of glycerol release from hyperthyroid (□), euthyroid control (△) and hypothyroid (○) rats, elicited by the partial agonists, the β_3-selective agonist CGP 12177 (a) and the β_1-selective agonist xamoterol (b). Each curve is a representative experiment performed in triplicate. Each point is the mean±s.e.mean over basal lipolysis value. Standard deviations not shown are within the symbol.

In hypothyroidism, lipolytic responses to FOR, ENP and $(Bu)_2$cAMP were unchanged compared to those from euthyroid rats, but the EC_{50} for these three agents were reduced (Table 3).

Effect of thyroid status on adenyl cyclase activity induced by forskolin and β-AR agonists

To understand better the mechanism of alterations in the lipolytic response at the postreceptor level, adenyl cyclase

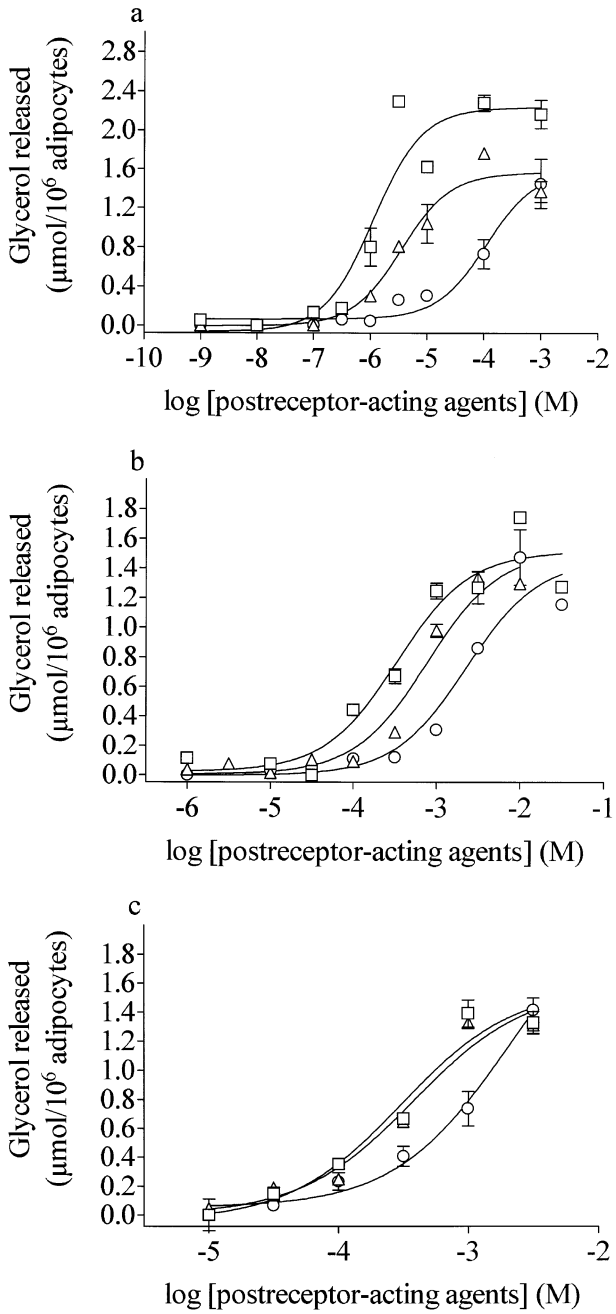

Figure 4 Concentration-response curves for stimulation of glycerol release from hyperthyroid (□), euthyroid control (△) and hypothyroid (○) rats, elicited by postreceptor-acting agents, the adenyl cyclase activator, forskolin (a), the phosphodiesterase inhibitor enprofylline (b) and the protein kinase A activator $(Bu)_2$cAMP (c). Each curve is a representative experiment performed in triplicate. Each point is the mean ± s.e.mean over basal lipolysis value. Standard deviations not shown are within the symbol.

activation by ISO, CGP, XAM and forskolin (FOR) was assessed (Figure 5 and Table 4).

The dose-response curves for stimulation of adenyl cyclase by ISO were biphasic and best described by a two-site model ($P<0.05$) in all three thyroid states (Figure 5a). The curves could thus be dissected into high and low affinity components corresponding to β_1-AR and β_3-AR subtypes, respectively as reported by others (Chaudhry *et al*., 1994; Gettys *et al*., 1995). In line with evidence that β_3-AR subtype is predominantly involved in the functional lipolytic activity (Murphy *et al*., 1993; Germack *et al*., 1997), 61 ± 9% (0.226 pmol cyclic AMP min^{-1} mg^{-1} of protein) corresponded to β_3-AR stimulation and the remaining 39 ± 6% (0.145 pmol cyclic AMP - min^{-1} mg^{-1} of protein) of the AC activity stimulated by ISO in adipocytes from euthyroid rats was due to β_1-ARs. T_3-treatment did not modify the proportion of AC activity due to β_1-AR and β_3-AR, 32 ± 5% (0.201 pmol cyclic AMP min^{-1} mg^{-1} of protein) and 68 ± 11% (0.434 pmol cyclic AMP min^{-1} mg^{-1} of protein), respectively. Nevertheless, the maximal Ac activity in response to ISO was enhanced by 70% in hyperthyroid state (Table 4). In the case of hypothyroidism, the contribution of each β-AR subtype represented about one half of the AC activation (β_1-AR: 48 ± 8 and β_3-AR: 52 ± 9%) while, the maximal response was reduced by 61% (Table 4). No changes in K_{act} for β_1- and β_3-Ars were observed in all thyroid states (Table 4).

The dose-response curves for partial agonists CGP and XAM were monophasic (Figure 5b,c). The maximal AC activity was enhanced by 84% for CGP and 58% for XAM in adipocytes from hyperthyroid rats (Figure 5b,c, Table 4). Interestingly, the efficacy of CGP and XAM to stimulate AC was not modified by T_3-treatment (intrinsic activity: 0.48 versus 0.50 for CGP and 0.34 versus 0.30 for XAM) whereas this parameter was increased in lipolysis measurement. Thyroidectomy decreased the maximal CGP-induced stimulation of AC by 58%, whereas the XAM effect was unchanged (Table 4). Furthermore, only K_{act} for CGP was significantly affected whatever the thyroid status, arguing for the involvement of β_3-ARs in lipolysis regulation in hyper- and hypothyroidism.

T_3-treatment enhanced the AC stimulation by FOR, without a modification of K_{act} (Figure 5d; Table 4). This increase in AC activity was lower (23%) than the rise observed with the three β-agonists. FOR-induced AC activity and K_{act} for FOR were not affected by hypothyroid state (Table 4).

Discussion

The present study shows that the alterations in catecholamine induced lipolysis induced in white adipocytes by alterations in thyroid status involve the modifications in both the β_3-AR expression and postreceptor events but not in the β_1-AR

Table 3 Effect of thyroid status on white adipocyte lipolysis induced by postreceptor-acting agents

	Maximal repsonse E_{max} (μmol 10^6 *adipocytes*)			EC_{50} ($-log_{10}$ EC_{50})		
	Hyperthyroid	*Euthyroid*	*Hypothyroid*	*Hyperthyroid*	*Euthyroid*	*Hypothyroid*
Forskolin	1.96 ± 0.15*	1.24 ± 0.18	1.21 ± 0.20	6.13 ± 0.36*	5.08 ± 0.17	4.35 ± 0.17*
Enprofylline	1.52 ± 0.12	1.49 ± 0.12	1.42 ± 0.24	3.79 ± 0.10*	3.21 ± 0.07	2.45 ± 0.19*
$(Bu)_2$cAMP	1.48 ± 0.19	1.47 ± 0.15	1.44 ± 0.11	3.68 ± 0.17	3.63 ± 0.11	3.14 ± 0.06*

The values are means ± s.e.mean of 3–5 separate experiments performed in triplicate. The concentration of agonists including 50% of maximal lipolysis (EC_{50}) expressed in $-\log_{10}$ EC_{50}. The maximal response, E_{max} is lipolysis at the maximum effective dose of agonist minus basal lipolysis (hyperthyroid: 0.88 ± 0.04*; euthyroid: 0.70 ± 0.04, hypothyroid: 0.50 ± 0.03* μmol 10^6 adipocytes). *$P<0.05$ versus euthyroid control.

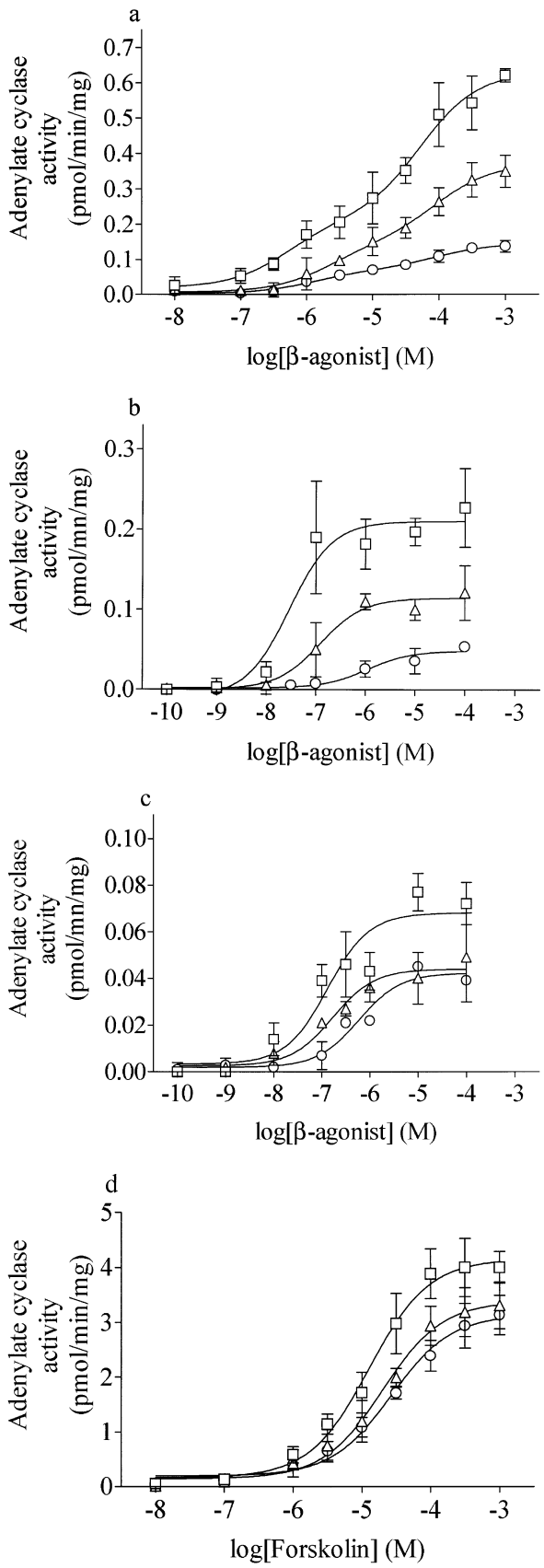

Figure 5 Concentration-response curves for adenyl cyclase activity in purified membranes from hyperthyroid (□), euthyroid control (△) and hypothyroid (○) rats, elicited by isoprenaline (a), CGP 12177 (b), xamoterol (c) and forskolin (d). The results illustrated are means over basal value (vertical lines show standard deviation) of 3–4 experiments performed in duplicate. Standard deviations not shown are within the symbol.

expression/activity. Our findings of elevated (72%) and decreased (50%) β_3-AR expression in hyper- and hypothyroidism, respectively, without modification in the β_1-AR density (Table 1), support the idea that β_3-AR is a preferential target for the effects of thyroid hormone variations. The modulation of β_3-AR density according to thyroid status was well correlated with modifications in the β_3-AR mRNA level. The unchanged β_1-AR number was associated with an unaltered specific transcript level. Concerning β_3-AR, three transcripts of 2.1, 2.6 and 3.5 kb were identified in agreement with previous studies in mice white adipocytes (Collins *et al*., 1994; Arbeery *et al*., 1995). Up- and down-regulation of β_3-AR transcripts and proteins in hyper- and hypothyroidism reflect, at least in part, the mechanism by which the lipolytic activity could be controlled in white adipocytes. This mechanism seems to be tissue-specific since cardiac β_1-AR level is enhanced by T_3 (Bahouth, 1991) and depressed in hypothyroidism (Revelli *et al*., 1991) as well as in brown adipocytes (Revelli *et al*., 1991; Rubio *et al*., 1995) for the last state. Furthermore, in contrast to white adipocytes, T_3-treatment decreased and hypothyroidism increased β_3-AR expression in brown adipocytes (Rubio *et al*., 1995; Adli *et al*., 1997).

Accordingly to changes in β_3-AR level, the maximal lipolytic response and sensitivity (EC_{50}) to catecholamines (NOR and ISO) were increased in adipocytes from T_3-treated and decreased in cells from hypothyroid animals (Figure 2; Table 2) in agreement with other report (Malbon *et al*., 1978; Mills *et al*., 1986; Ben Cheikh *et al*., 1994). To evaluate the role of β_1- and β_3-AR subtypes in lipolytic alterations according to thyroid status, we have used BRL, full selective β_3-AR agonist, CGP, β_1/β_2-AR antagonist and a partial selective β_3-AR agonist at higher concentrations (Van Liefde *et al*., 1992; Germack *et al*., 1997) and XAM, partial and highly selective β_1-AR agonist in rat white adipocytes (Germack *et al*., 1997) (Figure 2c and 3; Table 2). The maximal lipolytic response to BRL was increased in adipocytes from hyperthyroid rats similarly to the responses induced by catecholamines as previously shown (Malbon *et al*., 1978; Mills *et al*., 1986; Ben Cheikh *et al*., 1994; Germack *et al*., 1996). On the contrary, the maximal response to BRL decreased in adipocytes from hypothyroid rats (Figure 2c; Table 2) as it has been observed using another selective β_3-AR agonist, CL 316,243 (Rubio *et al*., 1995). These data indicate that thyroid status modulates catecholamine-induced lipolysis through the modifications at β_3-AR level. The lipolysis produced by CGP and XAM in hyperthyroid state (Figure 3; Table 2) was strongly enhanced leading to the increase in their efficacy (intrinsic activity: 0.95 versus 0.43 and 1.02 versus 0.42, respectively). Nevertheless, their efficacy in AC activation was unchanged although the maximal AC activity generated by these partial agonists was increased (CGP: 85%; XAM: 58%), (Figure 5; Table 4). Since β_1-AR density was unaffected by thyroid status, the modulation of XAM-stimulated AC activity indicates that thyroid hormones may regulate lipolytic function by modulating the coupling between the receptor and AC as previously assumed by others (Mills *et al*., 1986; Rapiejko & Malbon, 1987). The rank order of increase in maximal AC activity, CGP (84%) > ISO (70%) > XAM (58%) > FOR (23%) (Figure 5; Table 4), evidences the important role of β_3-AR in lipolysis regulation by T_3. In hypothyroid state, the regulation of functional activity also depends on the β_3-AR expression. Indeed, lipolysis induced by CGP but not by XAM was reduced (Figure 3; Table 2). Furthermore, the lipolysis impairment was associated with a decrease in AC stimulation by catecholamines and β_3-AR selective agonist, but not by β_1-AR agonist- and FOR (Figure 5; Table 4). It is noteworthy

Table 4 Effect of thyroid status on adenyl cyclase activity of white adipocyte membranes

	Maximal activity V_{max} (pmol cAMP min^{-1} mg^{-1})			K_{act} ($-log10\ K_{act}$)		
	Hyperthyroid	*Euthyroid*	*Hypothyroid*	*Hyperthyroid*	*Euthyroid*	*Hypothyroid*
β_1				6.30±0.27	5.65±0.20	5.87±0.25
Isoprenaline	0.629±0.021*	0.371±0.012	0.146±0.008*			
β_3				4.27±0.14	4.04±0.16	4.03±0.27
CGP 12177	0.210±0.015*	0.114±0.006	0.048±0.005*	7.53±0.24*	6.91±0.15	5.97±0.25*
Xamoterol	0.068±0.007*	0.043±0.002	0.042±0.003	6.88±0.27	6.77±0.15	6.23±0.18
Forskolin	4.15±0.11*	3.38±0.09	3.14±0.11	4.90±0.07	4.70±0.07	4.57±0.08

The values are means±s.e.mean of four separate experiments performed in duplicate. The K_{act}, value corresponds to the concentration of compounds giving half-maximal adenyl cyclase activity expressed in $-\log_{10} K_{act}$. The maximal activity, V_{max} is activity at the maximum effective dose of compound minus basal activity (hyperthyroid: 0.35±0.04*; euthyroid: 0.25±0.04, hypothyroid: 0.15±0.02* pmol cAMP min^{-1} mg^{-1} of protein). *$P<0.05$ versus euthyroid control.

that the reduction in maximal AC response to ISO and CGP was similar, 61 and 58%, respectively and in accordance with the decrease in β_3-AR density. In adipocytes from hyperthyroid rats, the sensitivity (EC_{50}) to XAM was unaltered in lipolytic and AC activity measurements while it was enhanced with CGP. In hypothyroid state, lipolytic sensitivity to both agonists was reduced while this parameter in AC stimulation remained unchanged with XAM. The differences in functional sensitivity between the β_1-AR and β_3-AR agonists in hyperthroid and hypothyroid state may be, at least partly, explained on the level of coupling of β_3-ARs to G-proteins. Indeed, it has been reported that β_3-ARs can be coupled to the α subunits of G_s as well as G_i proteins (Chaudhry *et al*., 1994; Begin-Heick, 1995) which mediate the stimulatory and inhibitory pathways, respectively, whereas β_1-ARs are coupled to G_2 protein. Thus, activation of β_3-ARs by agonists is controlled by a dual pathway. In addition, the expression of $G_{i\alpha}$ subunits was decreased in hyperthyroidism and increased in hypothyroid state without modification of the $G_{s\alpha}$ level in adipocytes (Ros *et al*., 1988; Rapiejko *et al*., 1989; Milligan & Saggerson, 1990). In the light of these data, β_3-ARs could be mainly coupled to $G_{s\alpha}$ in hyperthyroid state and predominantly to $G_{i\alpha}$ in hypothyroidism leading to an increase and a decrease in functional sensitivity to catecholamines, respectively.

The existence of postreceptor alterations in hyper- and hypothyroidism is further strengthened by the study of lipolysis induced by selective postreceptor-acting agents, ENP, FOR and $(Bu)_2$cAMP, which act as selective phosphodiesterase (PDE) inhibitor, AC and protein kinase A (PKA) activators respectively (Figure 4; Table 3). The ability of FOR to increase the lipolysis was enhanced in adipocytes from hyperthyroid rats and unaltered in cells from hypothyroid animals in accordance with AC activity results. In both thyroid states, the maximal lipolytic response produced by PDE inhibitor and PKA activator was unchanged. These data argue for the changes in functional response located not only at the β_3-AR level in hyper- and hypothyroidism, but also, in hyperthyroid state, at the AC activity level. In hyperthyroidism, the lipolytic sensitivity (EC_{50}) to FOR and ENP but not to $(Bu)_2$cAMP was increased. The lipolysis stimulation by β-AR agonists results from synthesis of cyclic AMP by AC, as well as from degradation of this nucleotide by PDE, forming a feedback regulation of cyclic AMP accumulation (Degerman *et al*., 1997). It was reported that PDE activity was decreased in hyperthyroid adipocytes (Elks & Manganiello, 1985; Ben Cheikh *et al*., 1994). All these data indicate that hyperthyroidism can effect the lipolytic response not only by enhancement of β_3-AR density and by modifications of receptor-cyclase coupling but also by regulation of cyclic AMP production and degradation. In cells from hypothyroid rats, a decrease in lipolytic sensitivity (EC_{50}) to FOR, ENP and $(Bu)_2$cAMP indicates the modifications downstream from AC in the lipolytic pathway. Since hypothyroidism is associated with enhanced PDE activity in adipocytes (Elks & Manganiello, 1985; Ben Cheikh *et al*., 1994) and unaltered PKA activity (Van Inwegen *et al*., 1975), we postulate that the alteration in sensitivity (EC_{50}) of lipolysis to catecholamines could also involve the regulation of hormone-sensitive lipase (HSL) activity.

In conclusion, the alteration in lipolytic response to catecholamines arises from the changes in both, β_3-AR density and postreceptor events, in hyperthyroidism whereas it is only due to changes in the β_3-AR number in hypothyroidism. The modification of the sensitivity to catecholamines in hypo- and hyperthyroidism involves the postreceptor component changes. Probable modulations of β_3-AR coupling and PDE activity are common to both thyroid states. Moreover, hypothyroidism leads to some changes in the lipolytic pathway downstream from PKA. The modifications in functional response and sensitivity (EC_{50}) are not related to variations in the β_1-AR level. The regulation of β_1- and β_3-ARs coupling as well as HSL activity in different thyroid states needs to be evidenced.

We thank Prof. D. Strosberg and Dr Curtis for generously providing β_3- and β_1-adrenoceptor hybridization probes. We also thank Dr James Douglas for providing the cDNA cyclophilin probe. We are indebted to C. Charon and A. Arnoux for advice on the adenylyl cyclase assay. Berhing Laboratory is gratefully acknowledged for its gift of FT4 kits. D. Anger and F. Gourbault are sincerely thanked for their skilful technical assistance. This work was partially supported by grants from the Direction de la Recherche et des Etudes Doctorales (DRED) of the French Education Ministry.

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(Received February 19, 1999
Revised October 1, 1999
Accepted October 15, 1999)