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Interaction of amiodarone and triiodothyronine on the expression of β-adrenoceptors in brown adipose tissue of rat

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1 This study was undertaken to evaluate *in vivo* the influence of amiodarone on the effects of triiodothyronine (T3) in brown adipose tissue (BAT) which are independent of thyroid hormone synthesis and of the conversion of thyroxine (T4) to T3. Thyroidectomized rats were given a replacement dose of T3 (0.5 mg kg^{-1} p.o. daily for 3 days) with or without amiodarone (50 mg kg^{-1} p.o. daily for 1 week).

2 As assessed by RT–PCR, treatment of thyroidectomized rats with T3 caused a 2 fold decrease in β3-adrenoceptor (β3-AR) mRNA levels and a 2 fold increase in β1-AR mRNA levels.

3 Binding studies using [^{3}H]-CGP 12177 as a ligand showed that treatment of thyoidectomized rats with T3 resulted in a 70% decrease in β3-AR number and in an 80% increase in β1-AR in BAT membranes.

4 T3-treatment abolished the increase in BAT adenylyl cyclase (AC) activity induced by CGP12177 in thyroidectomized rats. It also decreased the amount of G_i protein (ADP-ribosylation) by 30%.

5 At variance with the literature on the heart, amiodarone administration did not inhibit the positive effect of T3 on β1-AR expression in BAT in thyroidectomized rats. However, it antagonized the effect of T3 on β3-AR number, but not on AC activity or on G_i expression.

6 These results indicate that the effects of thyroid hormones on the responsiveness of BAT to catecholamines involves both receptor and post-receptor mechanisms, they also suggest that interaction between amiodarone and thyroid hormones is highly tissue-specific and depends on the β-AR subtype.

Keywords: β1- and β3-adrenoceptors; amiodarone; triiodothyronine; adenylyl cyclase; G-proteins; brown adipose tissue; CGP 12177; ADP-ribosylation

Abbreviations: AC, adenylyl cyclase; β-AR(s), β-adrenoceptor(s); BAT, brown adipose tissue; PTX, pertussis toxin; T3, triiodothyronine

Introduction

Amiodarone is an iodinated benzofuran derivative that predominantly exerts class III antiarrhythmic effects (Singh & Vaughan-Williams, 1970). Its mechanism of action remains unclear. However, striking similarities between the effects of amiodarone and hypothyroidism on cardiac function have led to the conclusion that the cardiac effects of amiodarone are due, at least in part, to an interaction between the drug and the thyroid hormones (Singh & Vaughan-Williams, 1970; Talagic *et al*., 1989). Amiodarone is known to inhibit type I 5′-deiodinase, the enzyme responsible for peripheral conversion of thyroxine (T4) to the active triiodothyronine (T3) (Ceppi & Zaninovich, 1989; Dussaillant *et al*., 1994). Furthermore, it has been reported that amiodarone decreases the density of cardiac β-adrenoceptors (β-ARs) in euthyroid (Nokin *et al*., 1983), but not in hypothyroid rats (Yin *et al*., 1992) suggesting that some of its effects require the presence of thyroid hormones. Additionally, amiodarone antagonizes the increase in β-AR density observed in hearts of both hypothyroid and euthyroid rats treated with T3 (Hartong *et al*., 1990; Perret *et al*., 1992). Amiodarone bears a structural resemblance to the thyroid hormones, and it has been shown to inhibit, competitively and non-competitively, T3 binding to the thyroid hormone receptors (Norman & Lavin, 1989; Drvota *et al*., 1995). Taken together, these results have offered several possible mechanisms by which the antithyroid influences of amiodarone could be exerted. However, the effects of amiodarone on thyroid hormone action in other tissues, such as brown adipose tissue (BAT), which are under the control of thyroid hormones and the sympathetic system have not been examined, although interestingly, when given i.v., amiodarone accumulates in BAT with a tissue to serum ratio in excess of 1000 (Plomp *et al*., 1985).

BAT is an important thermogenic organ in which thermic activity is controlled by norepinephrine released through sympathetic innervation of the tissue. Although three subclasses of β-ARs are present in BAT (Nahmias *et al*., 1991; Granneman & Lahners, 1992), the β3-AR type predominate (Muzzin *et al*., 1992; Adli *et al*., 1997). These β-ARs are differentially regulated by thyroid hormones. Thus, in hyperthyroid rats, the number of β1-AR is increased and that of β3-AR is decreased (Rothwell *et al*., 1985; Rubio *et al*., 1995a; Adli *et al*., 1997), whereas in hypothyroid rats the opposite effects are observed (Revelli *et al*., 1991; Rubio *et al*., 1995b).

The purpose of this study was to evaluate *in vivo* the influence of amiodarone on the actions of T3 in BAT which are independent of thyroid hormone synthesis and of T4 to T3 conversion. To do this, we examined the effects of amiodarone in thyroidectomized rats receiving a replacement dose of T3

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and monitored the expression of β1 and β3-AR and the adenylyl cyclase (AC) cascade.

Methods

Animals and protocols

Age-matched thyroidectomized male Wistar rats (weight 100–120 g) purchased from Iffa Credo (Lyon, France) were used in these studies. Surgery was performed under Ketamin/Diazepam anaesthesia as described by Bouyard & Jadot (1977). Every effort was made to preserve the parathyroid glands, but as a precaution, rats were given 0.9% $CaCl_2$ to drink *ad libitum*. They were also given 0.03% metimazole in the drinking water after surgery for 5 weeks to inhibit completely any residual thyroid function. Rats were housed in a temperature controlled room ($22 \pm 1^{\circ}C$) on a fixed 12 : 12 h light-dark cycle and were fed on stock diet (U.A.R., 91600 Epinay sur Orge, France). They were divided in four groups: Control receiving 100 μl of saline, Amio receiving 100 μl of amiodarone, T3 receiving 100 μl of T3 and Amio + T3 receiving 100 μl of T3 and 100 μl of amiodarone. Amiodarone (50 mg kg^{-1}, a standard dose for rats experiments) was administered daily through a gastric tube for 1 week. T3 (0.5 mg kg^{-1}) was administered daily through a gastric tube for the last 3 days. The effects of the different treatments on thyroid status, were assessed by measurement of serum free triiodothyronine (FT3) using commercial radioimmunoassay kits (Behring, Marburg, Germany).

Tissue sampling and processing

Rats were killed by decapitation 24 h after the last doses of T3 and amiodarone were given. Interscapular BAT (IBAT) was carefully dissected out and immediately frozen in liquid nitrogen and kept at $-80^{\circ}C$ for further isolation of RNA or for preparation of membranes.

Preparation of plasma membranes

BAT plasma membranes were prepared according to Giacobino (1979). 300–500 mg of tissue were homogenized with glass Teflon homogenizer in 2 ml of ice-cold $CaCl_2$ (0.05 mM), $NaHCO_3$ (0.1 mM) and $MgSO_4$ buffer (pH 7.5) (0.02 mM). The homogenate was centrifuged at $1100 \times g$ for 10 min at 4°C, the supernatant fluid removed and the pellet resuspended and centrifuged a second time. The two supernatants were pooled and centrifuged at $35{,}000 \times g$ for 20 min at 4°C. The resulting pellet was homogenized in fresh buffer, layered on to 5 ml of 18.5% (w v^{-1}) metrizamide and centrifuged at $156{,}000 \times g$ for 90 min at 4°C. Plasma membranes were found in a layer above the metrizamide, they were collected and centrifuged at $180{,}000 \times g$ for 45 min at 4°C. The pellet was resuspended in 10 mM Tris/HCl (pH 7.4) buffer containing sucrose (25 mM) and EDTA (1 mM) and filtered on 60 μm pore size filter (Nybond HC60). Plasma membranes were frozen at $-80^{\circ}C$, until utilization for binding experiments.

Preparation of crude membranes

Membranes were prepared according to Grannemann & MacKenzie (1988). Briefly, 80–100 mg of tissue were homogenized in 2.5 ml of cold 25 mM Tris/HCl buffer (pH 7.4) containing sucrose (250 mM) and $MgCl_2$ (1 mM) and then filtered through glass wool. The filtrate was centrifuged at $1100 \times g$ for 10 min at 4°C, the supernatant fluid was removed and then centrifuged at $48{,}000 \times g$ for 10 min at 4°C. The resulting pellet was washed twice in fresh buffer. Finally, membranes were resuspended in 25 mM Tris/HCl (pH 7.5) buffer containing EDTA (1 mM).

[^{3}H]-CGP12177 binding to plasma membranes

In radioligand binding studies, membrane aliquots (40–50 μg) were incubated for 30 min at 37°C in a final volume of 200 μl containing (in mM) $MgCl_2$ 10, ascorbic acid 1, Tris/HCl (pH 7.5) 50, GTP (100 μM), and the indicated amount of [^{3}H]-CGP12177. The reaction was stopped by addition of 4.5 ml cold binding buffer immediately followed by filtration under vacuum through Whatman GF/C glass fibre filters pre-treated with polyethylenimine. Filters were washed with 15 ml cold binding buffer. The radioactivity trapped in the filters was measured in a liquid scintillation counter.

The binding of [^{3}H]-CGP12177 to β3-AR and β1-AR was determined according to Muzzin *et al*. (1992). A concentration of radiolabelled ligand close to the K_D of β3-AR (20 nM) was used in the absence or presence of 10 μM BRL 37344, which displaces the binding to β3-AR only. Even though the presence of a putative β4-AR in BAT has been recently suggested (Preitner *et al*., 1998), it has also been reported that BRL 37344 potency for this receptor was lower than for the β3-AR (Kaumann & Molnaar, 1996). Thus, specific binding to β3-AR is then defined as the difference between total binding, obtained in the absence of competing ligand, and the binding obtained in the presence of BRL 37344. Specific binding to β1-AR subtype in BAT can be quantified as the difference between [^{3}H]-CGP12177 binding in the presence of 10 μM BRL 37344 and the non-specific binding obtained in the presence of 100 μM (−) propanolol, a concentration sufficient to displace [^{3}H]-CGP12177 binding to all β-AR subtypes in BAT membranes (Muzzin *et al*., 1992).

RNA analysis

Total RNA was extracted from BAT by the CsCl (5.7 M) method and treated for 15 min at 37°C with 0.3 unit of ribonuclease (Rnase) free deoxyribonuclease (Dnase) I (RQI Dnase; Promega) per microgram of nucleic acid in Tris/HCl (pH 7.9) (40 mM), NaCl (10 mM), $MgCl_2$ (6 mM), and $CaCl_2$ (10 mM) in the presence of placental RNase inhibitor (Promega). After phenol/chloroform extraction and ethanol precipitation, RNA (0.8–1 μg) was reverse-transcribed with Maloney murine leukaemia virus M-MLV (RT) (400 units μg^{-1}) in the presence of 10 μM random hexanucleotides, placental Rnase inhibitor at 2 units μl^{-1} and each dNTP at 400 μM in a final volume of 40 μl consisting of Tris/HCl at pH 8.3 (50 mM), KCl (75 mM), $MgCl_2$ (3 mM), and dithiotreitol (10 mM). To ensure that subsequent amplification did not derive from contaminant genomic DNA, a control without M-MLV (RT) was included for each RNA sample. cDNAs were denatured at 95°C for 5 min and submitted to either 25 (β3-AR and β-actin) or 33 (β1-AR and β-actin) cycles of amplification (94°C, 5 min; 94°C, 15 s; 60°C, 15 s; 72°C, 15 s) followed by 7 min of final extension at 72°C in a temperature cycler (GeneAmp PCR System 9600; Perkin-Elmer). PCR was performed in a 50 μl reaction sample volume containing 1.5 units of Taq DNA polymerase, each dNTP at 125 μM and 125 nM both sense and antisense oligonucleotides. The buffer consisted of (in mM) KCl 50, Tris/HCl (pH 8.3) 10, $MgCl_2$ 3 and 0.1 mg ml^{-1} gelatin. Sequences of the sense and

antisense oligonucleotides were: 5′-ATGGCTCCGTGGCCT-CAC-3′ and 5′-CCCAACGGCCAGTGGCCAGTCAGCG-3′ for the β3-AR, 5′-TCGTGTGCACCGTGTGGGCC-3′ and 5′-AGGAAACGGCGCTCGCAGCTGTCG-3′ for β1-AR and 5′-GAGACCTTCAACACCCC-3′ and 5′-GTGGTG-GTGAAGCTGTAGCC-3′ for β-actin. Amplification products had expected sizes of 308, 265 and 236 base pairs for β3-AR, β1-AR and β-actin respectively. They were separated on a 2% agarose gel and visualized by ethidium bromide staining. cDNA amplification of β3-AR and β-actin with 25 cycles was linear up to 150 and 100 ng RNA respectively and that of β1-AR with 33 cycles was linear up to 300 ng RNA.

Adenylyl cyclase assay

AC (EC 4.6.1.1) activity was measured as previously described (Charon *et al*., 1995). Membranes (20–30 μg) were incubated at 35°C in a final volume of 50 μl containing (in mM) [α^{32}P]-ATP 0.2 (PB 171; Amersham Co), cyclic AMP 1, phosphocreatine 10, $MgCl_2$ 5, EDTA 0.2, Tris/HCl (pH 7.5) 50, GTP (5 μM) and 0.5 unit of creatine phosphokinase. The assay was initiated by the addition of membranes and terminated after 10 min. The [α^{32}P]-cyclic AMP was separated from AMP by column chromatography on alumina. The [α^{32}P]-cyclic AMP eluted with 3 ml of 50 mM imidazole was quantified by liquid scintillation spectroscopy. AC activity was expressed as the amount of cyclic AMP formed min^{-1} mg^{-1} of protein.

Activity of the AC catalytic subunit was estimated in the presence of the 2 μM Mn^{2+}, 100 mM guanosine 5′-[β-thio]diphosphate (GDPγs) and without Mg^{2+} (to inhibit G_s activity) in basal conditions, or after stimulation by 100 μM diterpene forskolin. Another drug, which exerts its actions directly through G_s, i.e. NaF, was used to estimate G_s activity. Furthermore, isoprenaline a non specific β-AR agonist with pD2 values for AC activation in BAT of 7.2 and 5.8 for β1/β2 and β3-AR respectively (Charon *et al*., 1995) and CGP12177A a β1/β2-AR antagonist but a partial β3-AR agonist (pD2 = 6.0) were used to activate AC.

Western-blot analysis of G_s protein

Twenty μg of crude membranes were electrophoresed on an SDS polyacrylamide gel [12% (w v^{-1})], electroblotted and incubated with a 1/1000 dilution of an anti-$G_s\alpha$ protein antibody. The immune complex was detected by autoradiography after incubation with ^{125}I-labelled Protein A.

ADP-ribosylation by pertussis toxin

Two μg of Pertussis toxin (PTX) was preactivated with dithiothreitol (0.4 M) for 30 min at room temperature. Membranes (50–100 μg) were incubated at 37°C for 30 min in a final volume of 200 μl of reaction mix containing Tris/HCl (pH 7.4) (20 mM), NaCl (100 mM), ATP (0.2 mM), GTP (1 mM), [^{32}P]-NAD (3 μM), phosphoenol-pyruvate (10 mM), 0.5 unit ml^{-1} pyruvate-kinase, 0.5 unit ml^{-1} myokinase, thymidine (10 mM), 0.1% Lubrol and activated PTX. The

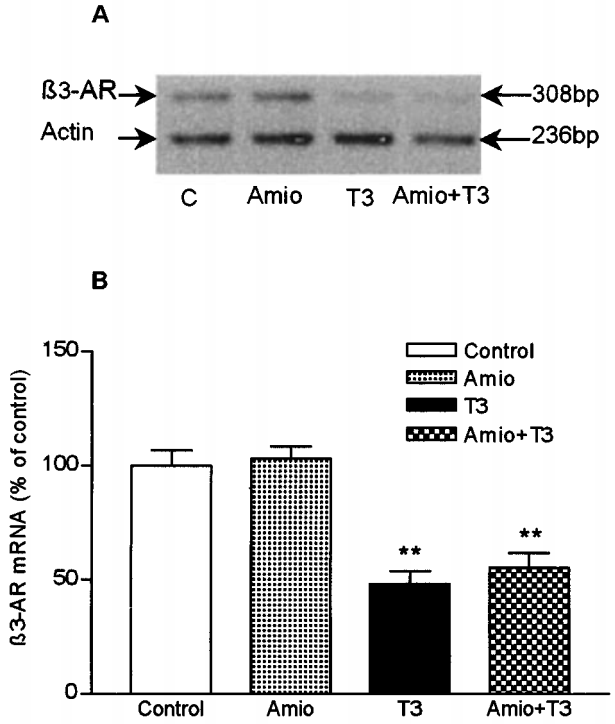

Figure 1 Effects of T3 and amiodarone (Amio) on β3-AR mRNA levels in thyroidectomized rats. Total RNA was extracted from BAT and digested with DNase I. β3-AR and β-actin cDNA amplification was carried out in a non-saturating conditions (25 cycles; 80 ng cDNA). The resulting products were separated on a 2% agarose gel and visualized by ethidium bromide staining and analysed by video scanning. (A) corresponds to a typical RT–PCR experiment. Position of β3-AR and β-actin PCR products are given on the left. The sizes of cDNA(s) in base pairs (bp) are indicated on the right. (B) represents the means ± s.e.mean of five RT–PCR analyses. β3-AR mRNA levels were normalized to β-actin mRNA content and are expressed as the percentage of the level detected in control. $^{**}P<0.01$ T3 or Amio + T3 vs control rats.

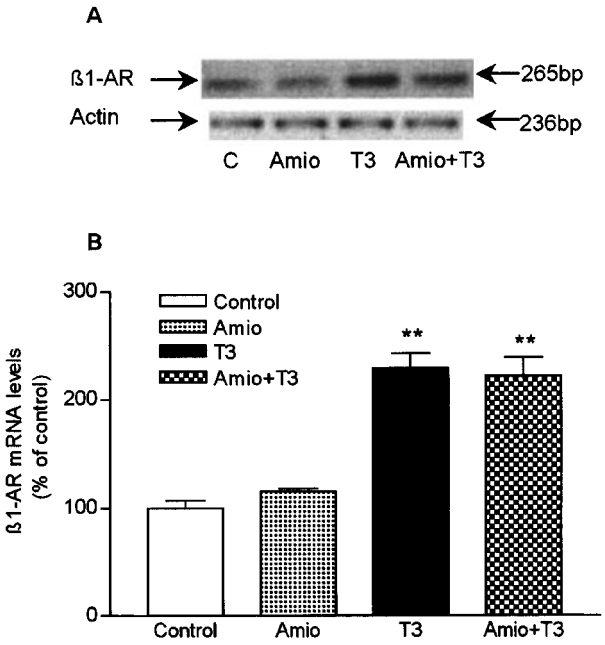

Figure 2 Effects of T3 and amiodarone (Amio) on β1-AR mRNA levels in thyroidectomized rats. Total RNA was extracted from BAT and digested with DNase I. cDNA content corresponds to initial amounts of DNase I-treated RNA of 200 ng for the β1-AR and 25 ng for β-actin. The resulting products were separated on a 2% agarose gel and visualized by ethidium bromide staining and analysed by video scanning. (A) corresponds to a typical RT–PCR experiment. Position of β1-AR and β-acton PCR products are given on the left. The sizes of cDNA(s) in base pairs (bp) are indicated on the right. (B) represents the means ± s.e.mean of five RT–PCR analyses. β1-AR mRNA levels were normalized to β-actin mRNA content and are expressed as the percentage of the level detected in control. $^{**}P<0.01$ T3 or Amio + T3 vs control rats.

reaction was stopped by centrifugation. The pellet was washed with ice cold buffer (20 mM Tris/HCl) and then solubilized in Laemmli sample buffer. The labelled proteins were resolved on SDS-polyacrylamid gels [12% (w v^{-1})] and electroblotted onto nitro-cellulose. Blots were exposed to X-ray film and the resulting autoradiograms were quantitated by densitometry.

Statistical analysis

Results are expressed as means ± s.e.mean. The level of significance in the difference between groups was calculated according to the analysis of variance (ANOVA) test. Statistical significance was determined by using the Tukey test.

Drugs and chemicals

Pertussis toxin, isoprenaline hydrochloride, (−) propranolol hydrochloride, GTP, ATP, phosphocreatine, creatine kinase, myokinase were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Forskolin was from Calbiochem-Behring (Terochem Laboratories, Rexdale, Ontario, Canada). 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 ((−)-4-(3-t-butyl amino-2-hydroxy-propoxy) benzimidazole-2-one) was a gift from Ciba-Geigy (Basel, Switzerland). [^{3}H]-CGP12177 (specific activity: 46 Ci $mmol^{-1}$), [^{32}P]-ATP (30 Ci $mmol^{-1}$) and ^{125}I-labelled protein A were obtained from Amersham (Les Ulis, France). [^{32}P]-NAD and anti $G_s\alpha$ protein antibody were purchased from NEN (Dupont de Nemours, France). Sense and anti-sense oligonucleotides were from Eurogentec.

Results

Effects of T3 and amiodarone on β-AR mRNA and protein level

To evaluate the effects of T3 and amiodarone on β-ARs gene expression, β3 and β1-AR mRNA(s) were examined by RT–PCR. Preliminary experiments were performed to ensure that amplification was carried out in the linear range for β-AR and β actin (see the experimental section). Under these conditions, we were able to compare the relative amounts of specific mRNA. The results presented in Figure 1A and B show that thyroidectomized rats responded to T3 with a 50% decrease in the β3-AR mRNA concentration ($P<0.01$ vs saline control). These results are in good agreement with those reported by previous studies from our own and other laboratories in which T3 was injected into hypothyroid (Rubio *et al*., 1995a) or euthyroid rats (Adli *et al*., 1997). By contrast amiodarone treatment had no effect on the β3-AR mRNA concentration in thyroidectomized rats given either saline or T3. We also examined the effects of T3 and amiodarone on the β1-AR mRNA concentration in BAT (Figure 2). Treatment with T3 resulted in a 2 fold induction in the β1-AR mRNA concentration but amiodarone was without effect (Figure 2).

To determine whether changes in β-AR mRNA affected the number of β-ARs, the density of β1 and β3-AR was assessed by the binding of [^{3}H]-CGP12177 to rat BAT membranes. The data presented in Figure 3 revealed that thyroidectomized rats responded to T3 treatment with a marked decrease in the

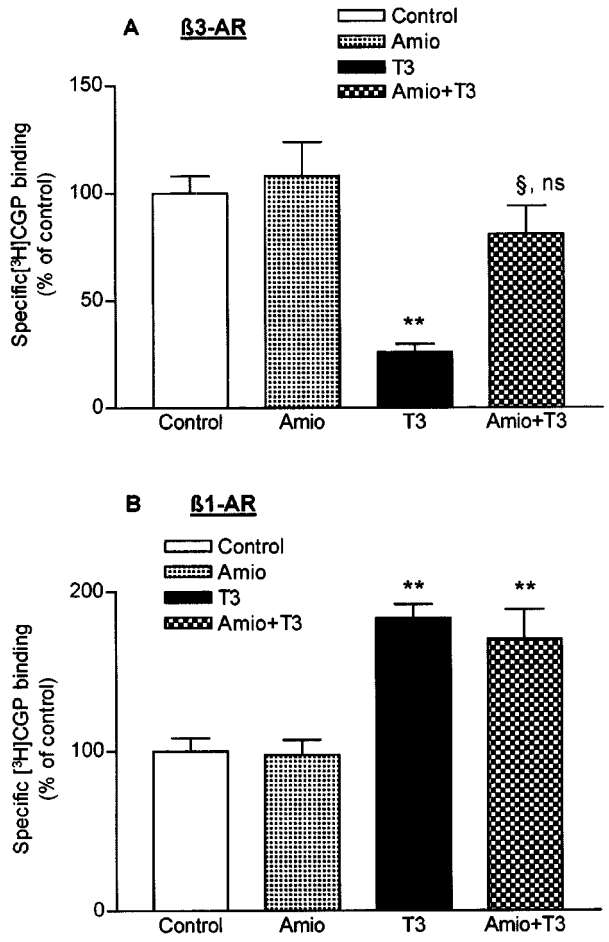

Figure 3 Effects of T3 and amiodarone (Amio) on the density of β1 and β3-AR in BAT plasma membranes from thyroidectomized rats. The binding values were calculated in fmol of [^{3}H]-CGP12177 bound/mg of plasma membranes. Specific binding to β3-AR is defined as the difference between total binding obtained in the absence of competing ligand, and the binding obtained in the presence of 10 μM BRL 37344 (A). Specific binding to β1-AR is the difference between [^{3}H]-CGP12177 binding in the presence of 10 μM BRL 37344 and in the presence of 100 μM propranolol (B). The results are means ± s.e.mean of four experiments. Results are expressed as percentage of Control and the values obtained in Amio, T3, and Amio + T3 rats were compared with those obtained in control rats. $^{**}P<0.01$ T3 or Amio + T3 vs Control; §$P<0.01$ Amio + T3 vs T3; ns: not significant Amio + T3 vs Control rats.

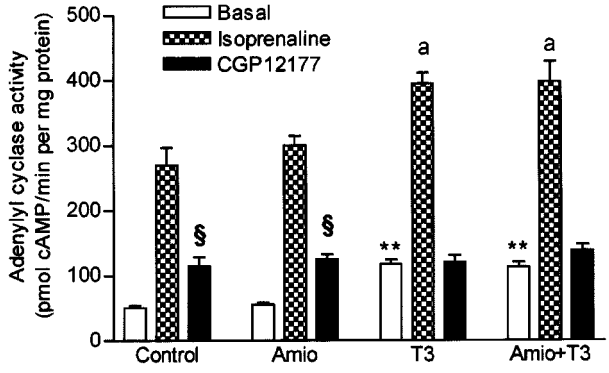

Figure 4 Adenylyl cyclase (AC) activity in basal conditions and after maximal stimulation by different β-agonists. AC activity in BAT membranes from thyroidectomized rats treated with T3 and/or Amio (Amio = amiodarone) was determined in the absence or in the presence of 100 μM agonist isoprenaline or CGP12177. Results are presented as means ± s.e.mean of ten experiments. ** and $^{a}P<0.01$ T3 or Amio + T3 vs Control for basal and isoprenaline stimulated AC activities respectively; §$P<0.01$ CGP12177 vs basal.

number of β3-AR (−70%, $P<0.01$ vs saline control) (Figure 3A) and a concomitant increase in that of β1-AR (+80%, $P<0.01$ vs saline control) (Figure 3B). Amiodarone alone had no effect on the density of β-AR subtypes in thyroidectomized rats (Figure 3A and B). However, in the treated group, amiodarone significantly increased the number of β3-ARs (+215%, $P<0.01$), whereas it did not affect the density of β1-AR. Thus, the impaired β3-AR expression resulting from T3 treatment was completely corrected by amiodarone.

Efffects of T3 and amiodarone on AC activity

To determine the functional consequences of the differential regulation of the β-AR subtypes by T3 and amiodarone, AC activity was measured in BAT crude membranes in basal conditions and in response to isoprenaline 100 μM or CGP12177 100 μM. These concentrations were previously shown to produce maximal stimulation in BAT (Charon *et al*., 1995; Adli *et al*., 1997). While isoprenaline is a non selective β-AR agonist, CGP12177 is a $\beta1/\beta2$-AR antagonist (Staehelin *et al*., 1983), but has agonist properties at the β3-AR site (Mohell & Dicker, 1989; Langin *et al*., 1991), and thus addresses β3-AR and the putative β4-AR (Preitner *et al*., 1998)

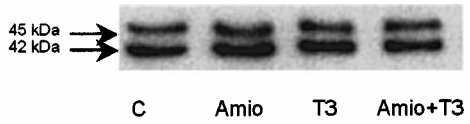

Figure 5 Effects of T3 and amiodarone (Amio) on $G_s\alpha$ protein isoforms in BAT from thyroidectomized rats treated with T3 and/or Amio. Result of one representative Western blot analysis of 20 μg protein as described in Methods section.

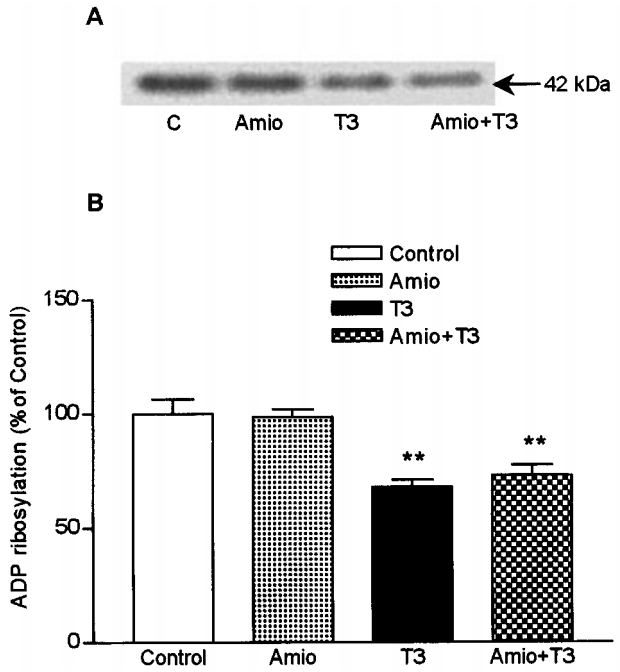

Figure 6 Effects of T3 and amiodaraone (Amio) on G_i protein. (A) typical autoradiogram of [^{32}P]-ADP-ribosylated membrane proteins resolved on SDS-polyacrylamide gel. BAT membranes (50 μg) from thyroidectomized rats treated with T3 and/or Amio were incubated with pertussis toxin for 30 min at 37°C as described under Methods section. (B) results are presented as means±s.e.mean of five membrane preparations that were processed separately, and are expressed as the percentage of the level detected in control. $^{**}P<0.01$, T3 or Amio+T3 vs Control rats.

coupling to the AC system. In thyroidectomized rats, T3-treatment produced a significant increase (2.3 fold) in basal AC activities (vs saline) but amiodarone was without effect (Figure 4). In control rats, isoprenaline produced a more pronounced increase in AC activity (5 fold), which was unaffected by amiodarone treatment. In both groups (i.e. control and amiodarone treated rats) the responses to isoprenaline were additive with those of T3. By contrast CGP12177, produced a modest increase (2.3 fold) in AC activities in control and amiodarone treated rats. These responses were unaffected by treatment with T3 (Figure 4).

To determine whether T3 regulates catecholamines responsiveness, essentially through the modulation of β-AR, AC activity was also measured in the four groups in the presence of the G protein or AC effectors.

Effects of T3 and amiodarone on the AC catalytic subunit

Activity of the AC catalytic subunit was assessed by using forskolin in the presence of Mn^{2+} and without Mg^{2+}. Neither T3 nor amiodarone administration had a significant effect on AC activated by forskolin (950±66, 990±83, 1320±152 and 1400±150 pmol cyclic AMP min^{-1} mg^{-1} protein for control, Amio, T3 and Amio+T3 respectively, mean of five experiments).

Effects of T3 and amiodarone on G_s protein activity and quantity

The ability of G_s to stimulate AC activity was investigated at 10 mM NaF. Results were expressed as relative responses: stimulated/basal. In our experimental conditions, NaF produced a 6–7 fold increase in AC activity for all groups (control 6.6±0.29, Amiodarone 7.2±0.47, T3 5.7±0.66 and T3+Amiodarone 5.7±0.25 mean of five experiments). Thus, in thyroidectomized rats treated with T3 and/or amiodarone, there was no significant change in the activity of G_s as compared to the saline-treated controls. Western analysis of $G_s\alpha$ in BAT (Figure 5) revealed the presence of two bands corresponding to 42 and 45 kDa. In good agreement with G_s activity, these results clearly show that the $G_s\alpha$ concentration was similar in all groups (five experiments).

Effects of T3 and amiodarone on the G_i protein

Since β3-AR has been shown to couple with G_i (Chaudry *et al*., 1994), we also determined the relative amounts of G_i in BAT membranes from the four groups. The ability of PTX to ADP-ribosylate the α-subunit of G_i was evaluated and results presented in Figure 6A show that, as expected, PTX catalyzed ADP ribosylation of a 42 kDa peptide. Membranes from T3-treated rats with or without amiodarone incorporated less ADP-ribose than did the membrane from control rats (Figure 6B). These findings suggest that, in BAT, the G_i level is modulated by thyroid hormones, but amiodarone did not counteract this effect.

Discussion

The present study shows for the first time that amiodarone opposes the down-regulation of β3-AR induced in thyroidectomized rats by T3. Indeed, treatment with T3 alone produced a dramatic decrease in β3-AR density which was restored to control levels by treatment with amiodarone. These results are

at variance with those we previously observed in white adipose tissue (Germack *et al*., 1996) and strengthens our conclusion concerning the tissue specificity of the interaction between thyroid hormones, β-ARs and amiodarone. It has been proposed that the hypothyroid-like effects of amiodarone reflect inhibition of thyroid hormone synthesis in response to iodine load (Burger *et al*., 1976), and/or a decrease in the peripheral T4 to T3 conversion (Sogol *et al*., 1983; Pekary *et al*., 1986). Since thyroid hormones synthesis and T4 to T3 conversion were ablated in our model, our results suggest that the effect of amiodarone on β3-AR is due to its antagonistic influence on the actions of T3 at the cellular level. Since β3-AR mRNA levels were not affected by amiodarone in T3 treated rats, the antagonistic effect of amiodarone might be either translational or post translational.

The results presented in this study are consistent with previous data that demonstrated *in vivo* a reduced density of β3-AR and an increased number of β1-AR in BAT after T3 treatment (Rothwell *et al*., 1985; Rubio *et al*., 1995a; Adli *et al*., 1997). The present work also shows that amiodarone did not affect β1- and β3-AR gene expression in thyroidectomized rats in the absence of T3. These findings, together with previous work (Talagic *et al*., 1989; Yin *et al*., 1992), reinforce the idea that *in vivo* amiodarone does not have a direct action of β-ARs and that some of its effects, particularly its antiadrenergic effects, may require the presence of T3. Like the cardiac β1-AR subtype, the BAT β1-AR subtype was up-regulated by T3 treatment. However, at variance with the findings in the heart, our data clearly establish that amiodarone does not inhibit the positive effect of T3 on β1-AR in BAT. Thyroid hormones regulate cardiac β1-AR expression by controlling the rate of transcription of the β1-AR gene (Latham *et al*., 1987; Bahouth, 1991). It is also well known that the control of transcriptional activity involves the interaction of T3 receptor complexes with specific responsive elements located in the 5′ flanking region of targeted genes. Thus, amiodarone could interfere with the activation of the thyroid hormone-responsive gene *via* the nuclear thyroid hormone receptor. Indeed, several reports have shown that amiodarone and its main metabolite, desethylamiodarone, inhibit the binding of T3 to its nuclear receptor and, thus, decrease thyroid hormone-dependent gene expression in various tissues, including the heart (Franklyn *et al*., 1987; Hartong *et al*., 1987; Paradis *et al*., 1991). Recently, desethylamiodarone has been shown to inhibit the binding of T3 to α1 and β1 thyroid hormone receptors by competitive and non-competitive mechanisms respectively (Bakker *et al*., 1994; Van Beeren *et al*., 1995). However, the reasons for the different effects of amiodarone on β1-AR in heart and in BAT are unclear. It could be that the effects of T3 on β1-AR in the heart and in BAT are mediated *via* different subtypes of T3 receptors, coreceptors and/or comodulators and, thus, amiodarone might interfere with the binding of T3 to varied elements in different ways and in a tissue-specific manner.

In our work, studies of AC activity demonstrated that in thyroidectomized rats treated with T3 but not amiodarone, changes in β3-AR mRNA levels correlated well with changes in β3-AR density and coupling to the AC system. Neither G_s activity nor AC catalytic subunit activity was affected by T3 treatment, but the concentration of G_i proteins was decreased. These findings are in good agreement with previous work (Adli *et al*., 1997) showing that the effects of thyroid hormones on catecholamines responsiveness involve both receptor and post-receptor mechanisms.

The increase in basal AC activity observed after treatment with T3 is consistent with the negative effect of this hormone on G_i level also reported in this study. It is well known that β3-AR accounts for 80% of total β-ARs in BAT (Musin *et al*., 1992; Adli *et al*., 1997), suggesting that AC activity in response to maximal stimulation by isoprenaline, is essentially modulated through β3-AR. The normal response of AC to isoprenaline observed in T3-treated rats despite the down-regulation of β3-AR might be attributed to the concomitant up-regulation of β1-AR observed in this group.

CGP12177 is a β1/β2-AR antagonist (Staehelin *et al*., 1983) but has agonist properties at the β3-AR (Mohell & Dicker, 1989; Langin *et al*., 1991), thus in control thyroidectomized rats, the increase in AC activity obtained with CGP12177 may reflect an action at the β3-AR while after treatment with T3, the absence of a response to CGP12177 correlates well with the reduction in β3-AR expression. The interpretation of the data in rats treated with amiodarone plus T3 is more complex. In this group, although amiodarone increased β3-AR expression there was no corresponding increase in isoprenaline or CGP12177 induced AC activity. These findings suggest that the receptors are not functional, possibly because they are not coupled with AC, as it has been reported in rat myocardiac cells after T3-treatment (Disatnik & Shainberg, 1991).

In conclusion, this study is the first to show an antagonistic effect of amiodarone on T3 action in the expression of the β3-AR subtype in BAT. Unlike cardiac β1-AR, amiodarone did not inhibit the positive effect of T3 on β1-AR brown adipocytes. Moreover, this drug did not counteract the effect of T3 on G_i protein levels. Thus, the interaction between amiodarone and T3 remains unclear, but seems to be highly tissue-specific. Additional studies will be required to clarify the interaction between amiodarone and T3, and their respective roles in the regulation of β3-AR gene expression in BAT.

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