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Molecular characterization of the mouse β3-adrenergic receptor: relationship with the atypical receptor of adipocytes

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The gene encoding the murine β3-adrenergic receptor (β3AR) has been isolated. It translates into a polypeptide of 388 amino acid residues which shows 82% overall homology with the human β3AR. In Southern blot experiments, a probe derived from the murine β3AR gene hybridizes to a unique restriction fragment in the murine and human genomes. In both species, the β3AR gene is located on chromosome 8, in regions (8A2→8A4 in mouse, and 8p11→8p12 in man) which are conserved between mouse and man. The pharmacological profile of the mouse β3AR strongly resembles that of the human β3AR. It is characterized by a low affinity toward the radiolabelled β-adrenergic antagonist [^{125}I]Iodocyanopindolol and a low efficiency of other antagonists such as propranolol, ICI 118551 or CGP 20712A to inhibit cAMP production induced by isoproterenol. Another salient feature shared by the murine and the human β3ARs is the very potent effect of the lipolytic compound BRL 37344 on cAMP accumulation and the partial agonistic effect of the β1- and β2-adrenergic antagonists CGP 12177A, oxprenolol and pindolol. These properties are very close to those ascribed to the atypical βAR of rodent adipocytes. In addition, Northern blot analyses indicate that the β3AR gene is mainly expressed in mouse brown and white adipose tissues, suggesting that the murine β3AR described here is the atypical βAR involved in the control of energy expenditure in fat tissue.

Key words: adipose tissue/atypical β-adrenergic receptors/cAMP accumulation/catecholamines/chromosome localization

Introduction

Beta-adrenergic receptors (βARs) belong to the superfamily of integral membrane proteins that mediate their effects through coupling to guanine nucleotide regulatory proteins (G-proteins). Upon binding of catecholamines, βARs activate adenylate cyclase via the stimulatory G_s-protein, leading to the production of the second messenger cAMP. On the basis of their different sensitivity to a series of agonists and antagonists, and their different tissue distribution, two subtypes of βARs, the β1AR and the β2AR, have been distinguished. The cloning of the corresponding genes in several species (Dixon *et al.*, 1986; Emorine *et al.*, 1987; Frielle *et al.*, 1987; Allen *et al.*, 1988; Machida *et al.*, 1990) revealed that these two receptors exhibit an overall homology of 50% at the amino acid level, and share with other receptors coupled to G-proteins a similar structural organization characterized by seven hydrophobic regions interspersed with hydrophilic regions (reviewed by Strosberg, 1991; O'Dowd *et al.*, 1989a).

Unexpected effects of adrenergic drugs have suggested that 'atypical' βARs distinct from the β1 and β2 subtypes, may mediate the metabolic effects of catecholamines on a variety of tissues, including adipose tissue, soleus muscle, digestive tract, and heart (reviewed by Arch, 1989; Kaumann, 1989; Manara and Bianchetti, 1990; Zaagsma and Nahorski, 1990). Indeed, in these tissues, classical β-antagonists are inefficient in inhibiting agonist-induced β-adrenergic responses. In addition, compounds that selectively stimulate atypical βARs have been characterized. For example, BRL 37344 which is a weak agonist for the β1AR and the β2AR, behaves as a potent activator of lipolysis and thermogenesis in rodent fat cells (Arch *et al.*, 1984), and strongly inhibits rat colon motility (McLaughlin and MacDonald, 1990) and guinea-pig ileum contraction (Bond and Clarke, 1988). Furthermore, the β1- and β2-antagonist CGP 12177A has a partial agonistic effect on atypical βARs of adipose tissue (Mohell and Dicker, 1989; Granneman and Whitty, 1991).

The characterization of a human gene encoding a third βAR subtype ('β3AR') (Emorine *et al.*, 1989) has provided a structural basis for the existence of atypical βARs. The human β3AR is 50% homologous to the human β1AR and β2AR. Its pharmacological properties, clearly distinct from those of the β1AR and the β2AR, strongly evoke the unusual features of atypical βARs.

To analyse further the relationship between the β3AR and the atypical βARs, we have undertaken the cloning and molecular characterization of the murine equivalent of the human β3AR. Tissue distribution and pharmacological properties of the murine β3AR strongly suggest that this receptor is the atypical βAR involved in the control of energy expenditure in rodent fat cells.

Results

Structural and genetic analyses of the murine β3AR gene

A murine (NIH 3T3) genomic library was probed under moderate stringency hybridization conditions using the entire coding region of the human β3AR gene (Emorine *et al.*, 1989). Five clones hybridizing most strongly to the probe were found by restriction mapping to be either identical or overlapping. One of these clones containing a 14 kb insert was further analysed by sequencing a 2 kb *Bgl*II–*Bam*HI restriction fragment hybridizing to the probe. This fragment contains an intronless open reading frame specifying a

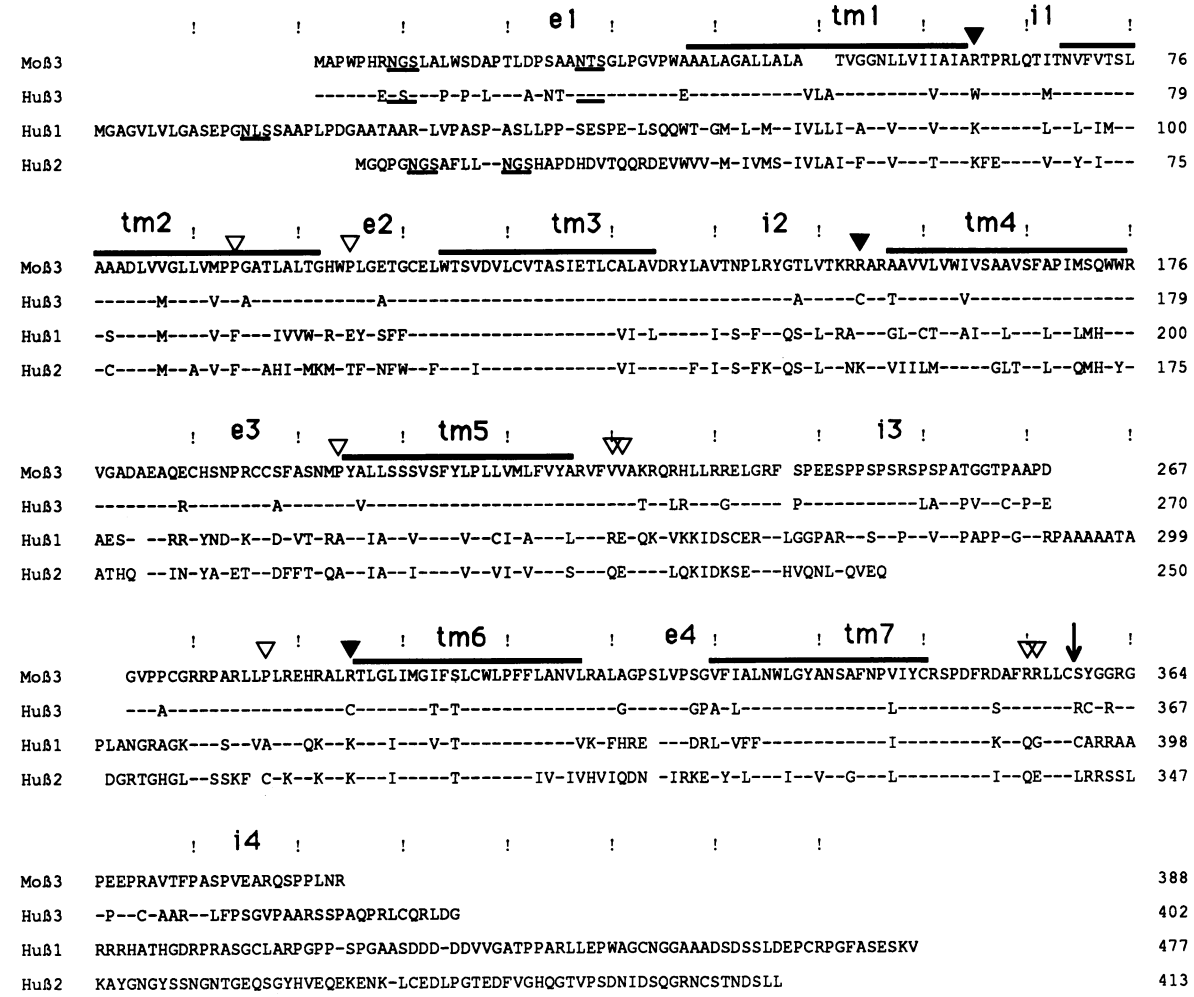

Fig. 1. Amino acid sequence comparison of the murine β3AR (Moβ3) with the human β3AR (Huβ3), β1AR (Huβ1) and β2AR (Huβ2). The position in the corresponding sequence of the amino acid residue at the end of each line is indicated on the right. An exclamation point (!) marks every 10th amino acid residue. Residues identical to the Moβ3 sequence are indicated by a dash. Gaps have been introduced to maximize homology. The seven transmembrane segments (tm1 – tm7) are highlighted by solid bars and alternate with extracellular (e1 – e4) and intracellular (i1 – i4) domains. Potential N-linked glycosylation sites are underlined. Residues that are conserved between Moβ3 and all the other β1ARs and β2ARs, but differ from Huβ3, are indicated by a black triangle. Amino acids that are shared between Moβ3 and Huβ3 but differ in the β1AR and β2AR sequences are indicated by an open triangle. An arrow indicates the cysteine residue after which the sequences of the βARs diverge. (EMBL data library accession number: X60438.)

polypeptide of 388 residues (Figure 1) which displays typical hallmarks of a G-protein coupled receptor, including seven hydrophobic regions and N-linked glycosylation motifs in the amino-terminal domain. This polypeptide exhibits 82% overall sequence homology with the human β3AR, but only 48–55% homology with β1ARs and β2ARs of different species (Dixon *et al.*, 1986; Emorine *et al.*, 1987; Frielle *et al.*, 1987; Allen *et al.*, 1988; Machida *et al.*, 1990). In the transmembrane domains, homology of the mouse receptor with the human β3AR reaches 93% while it is only 67% and 64% with the human β1AR and β2AR, respectively. Such homology indicates that the gene described here encodes a receptor of the β3 subtype, and we shall henceforth refer to the protein encoded by this gene as the murine β3AR. The carboxy-terminal tail of the murine β3AR is 11 residues shorter than that of the human β3AR, and the high homology between these two proteins ends abruptly just after the conserved cysteine residue (position 358 of the murine sequence) which was shown to anchor the carboxy-terminus of the β2AR in the membrane through palmitoylation (O'Dowd *et al.*, 1989b).

To analyse further the mouse β3AR gene at the genomic level, Southern blot experiments were performed at moderate stringency using probe A43 which encompasses the amino-terminal domain of the murine β3AR (Figure 2). Hybridization analyses of mouse genomic DNA cleaved with one of four restriction enzymes reveal in each case a single hybridizing fragment (3.6 kb *Bgl*II, 13 kb *Pst*I, 4.1 kb *Sac*I, 6.9 kb *Bam*HI). The sizes of these fragments correspond to those obtained by digesting the λ clone containing the murine β3AR gene (Figure 2C). Probe A43 does not hybridize to any other sequence in the mouse genome and therefore can be considered as specific for the murine β3AR gene. The same hybridization pattern (data not shown) is obtained by using two other DNA probes, encompassing the entire coding region and the tm3 to tm6 domains of the mouse β3AR respectively. In human genomic DNA (Figure 2B), probe A43 also reveals a single hybridizing sequence. The restriction fragment obtained with each of four different enzymes (4.8 kb *Bgl*II, 2.7 kb *Pst*I, 9.7 kb *Sac*I, 4.6 kb *Bam*HI) is of the size expected for the cloned human β3AR gene (Emorine *et al.*, 1991).

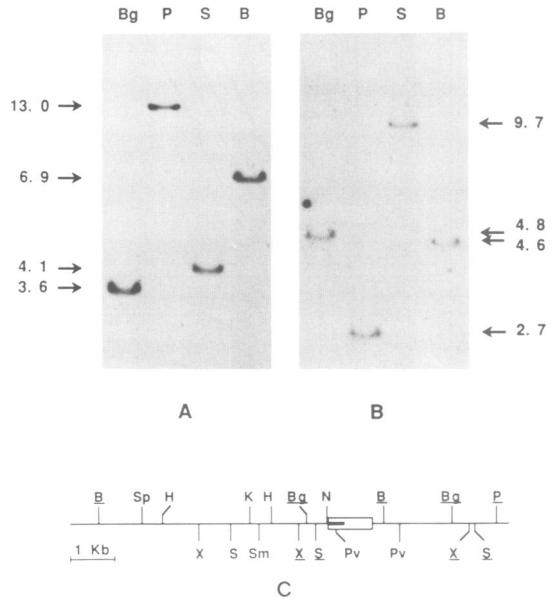

Fig. 2. Southern blot analysis of genomic DNA from (**A**) BALB/c mouse liver or (**B**) human epidermoid cell line (A431) using the murine β3AR probe A43. The sizes of the hybridizing fragments (in kb) are indicated on the left (mouse) and on the right (human) of the figure. (**C**) restriction map of one λ clone containing the murine β3AR gene. The coding region of the gene is boxed and the position of probe A43 is indicated by a bold line. The restriction enzymes used for the Southern blot are underlined. B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Nar*I; P, *Pst*I; Pv, *Pvu*II; S, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

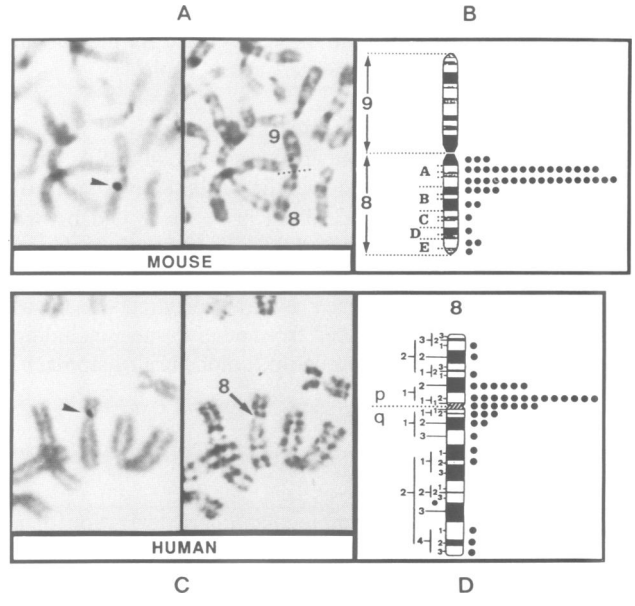

Fig. 3. Localization of the β3AR gene to mouse and human chromosome 8 by *in situ* hybridization. (**A**) and (**C**) Partial metaphase of mouse and human respectively, showing the specific site of hybridization to chromosome 8. Left, arrowheads indicate silver grains on Giemsa-stained chromosomes of a typical spread, after autoradiography. Right, chromosomes with silver grains were subsequently identified by R-banding. (**B**) and (**D**) Diagram of WMP mouse Rb (8;9) chromosome and human G-banded chromosome 8 respectively, illustrating the distribution of labelled sites.

Chromosome localization of the β3AR gene in mouse and man

The murine β3AR gene was assigned to the (8A2→8A4) region of chromosome 8 by *in situ* chromosomal hybridization (Figure 3) using probe A43. In the 150 metaphase cells examined, there were 278 silver grains associated with chromosomes: 46 silver grains (16.5%) were located on chromosome 8 and 32 of them (69.5%) mapped to the (8A2→8A4) region of this chromosome.

In situ chromosomal hybridization using a human β3AR-specific probe (Figure 3C) mapped the β3AR gene to human chromosome 8. There were 271 silver grains on 150 metaphase cells: 40 silver grains (14.7%) were located on chromosome 8 and 26 of them (65%) mapped to the (q11.1→p12) region of chromosome 8 with a maximum in the p11.2 band. Therefore the β3AR gene is most probably located in the (8p11.1→8p12) region of the human genome.

Pharmacology of the mouse β3AR expressed in CHO cells

To determine the pharmacological profile of the murine β3AR, the coding region of its gene was inserted into an expression vector, 3′ to the SV40 early promoter and 5′ to the polyadenylation signal of the hepatitis B antigen. This construct was introduced into Chinese Hamster Ovary (CHO) cells, which have also been used for expressing the human β1AR, β2AR and β3AR (Tate *et al.*, 1991).

Saturation binding of [^{125}I]Iodocyanopindolol (ICYP) and corresponding Scatchard analysis (Figure 4) indicate that the stably transfected CHO cell line ('CHO-Moβ3') expresses 200 000 ± 50 000 receptors per cell with a dissociation constant of 880 ± 88 pM (mean ± SEM, n = 3).

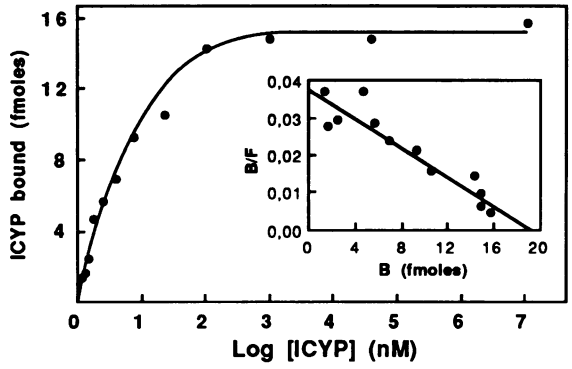

Fig. 4. Saturation isotherm of the specific binding of [^{125}I]ICYP to intact CHO-Moβ3 cells. The results shown are representative of one out of three independent experiments performed in duplicate. Inset, Scatchard plot of the same data.

Accumulation of cAMP is stimulated following incubation of CHO-Moβ3 cells with isoproterenol, norepinephrine, and epinephrine (Figure 5A). This effect is stereospecific since (−)-isoproterenol is 20 times more potent than (+)-isoproterenol (Table I). The agonist BRL 37344 is 10 times more potent than (−)-isoproterenol for inducing cAMP accumulation (Figure 5B, Table I). The β1- and β2-antagonists CGP 12177A, oxprenolol and pindolol behave as partial agonists of the murine β3AR, with maximal stimulation of cAMP accumulation being 77%, 45% and 36% respectively, of that of (−)-isoproterenol. For other β-antagonists such as propranolol (non-selective), ICI 118551 (β2-selective) and CGP 20712A (β1-selective) (Figure 5C), up to micromolar concentrations are necessary to inhibit cAMP accumulation induced by (−)-isoproterenol. The

pharmacological properties of the murine β3AR expressed in CHO cells are very close to those described for the human β3AR expressed in the same cell type (Table I).

Tissue distribution of the β3AR in mouse

Northern blot hybridization analyses of total RNA from various mouse tissues to the specific probe A43 (Figure 6) reveal a major band of 2.2 kb in both white and brown adipose tissue. In differentiated 3T3-F442A adipocytes, a major transcript of similar size (2.2 kb) as well as a minor species of 2.8 kb were detected. No hybridization signal was apparent in total RNA prepared from other tissues, including femoral muscle, lung, liver, ileum, colon, brain, stomach, heart and kidney.

Discussion

This report describes the molecular characterization of a mouse gene isolated by homology with the coding region of the human β3AR gene. As for other βAR genes, this murine gene is intronless. It encodes a polypeptide of 388 amino acid residues which presents the features characteristic of βARs, including the conserved amino acids identified as crucial for catecholamine binding in the β2AR (Strader *et al.*, 1989).

The high amino acid sequence homology (82%) between this murine polypeptide and the human β3AR is reminiscent of the homology (87%) between the human and the murine β2ARs, and therefore indicates that the receptor described here is of the β3 subtype. The homology between the two β3ARs is even higher in the transmembrane regions that confer binding selectivity (Kobilka *et al.*, 1988; Marullo *et al.*, 1990) and in the intracytoplasmic segments implicated in signal transduction (O'Dowd *et al.*, 1989a). Several residues located in these functional domains are shared by the two β3ARs but are not found in the sequences of other βARs. For example, four proline residues (positions 90, 101, 200 and 281), as well as two valine (positions 226 and 227) and two arginine (positions 354 and 355) residues are unique to the β3ARs and may specify the properties of this receptor subtype (Emorine *et al.*, 1991).

The two β3ARs have a short carboxy-terminal tail which lacks both the canonical site for phosphorylation by a cAMP-dependent kinase implicated in the desensitization of the β2AR and the numerous hydroxyl residues phosphorylated by the βAR kinase after exposure to agonists (Hausdorff *et al.*, 1990). This observation thus suggests that regulation of β3AR sensitivity may differ from that of other βAR subtypes.

The murine and the human β3ARs are genetically related

In Southern blot analyses of the human genome, a specific probe derived from the murine β3AR gene hybridizes to the β3AR gene, but not to other β-adrenergic sequences. Analyses of mouse genomic DNA using a probe derived from the human β3AR gene (Emorine *et al.*, 1991) also revealed, with every restriction enzyme used, a single hybridizing fragment whose size corresponds to that of the murine gene described here. These cross hybridization data indicate that the murine β3AR gene is the genetic counterpart of the human β3AR gene.

This conclusion is reinforced by the localization of the

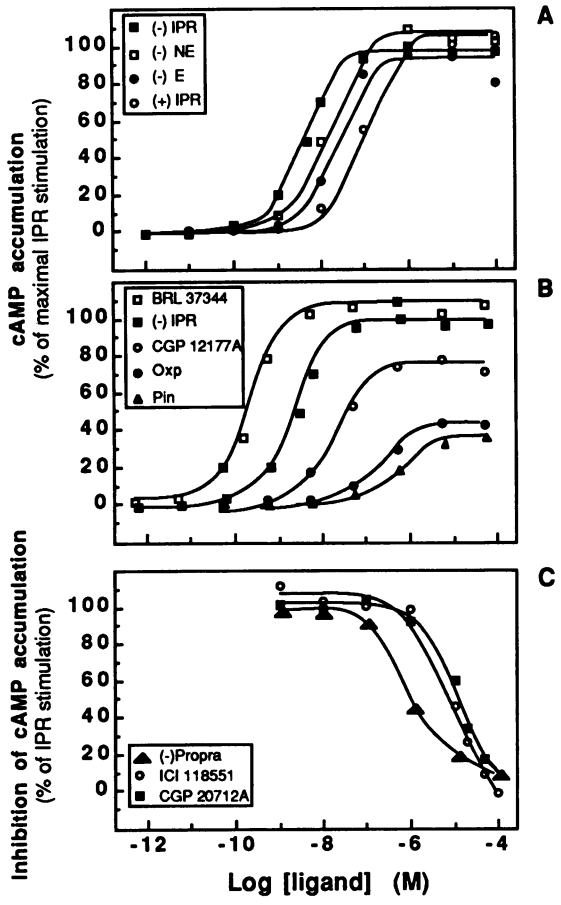

Fig. 5. cAMP accumulation following exposure of CHO-Moβ3 cells to (**A**) classical β-agonists, (**B**) atypical β-agonists and (**C**) β-antagonists in the presence of 5 nM (−)-isoproterenol. IPR, isoproterenol; NE, norepinephrine; E, epinephrine, Oxp, oxprenolol; Pin, pindolol; propra, propranolol. The results are expressed as the mean percentage (n = 2−3) of the response induced by (−)-isoproterenol: 1 μM in (A) and (B), and 5 nM in (C).

β3AR gene in the 8A2→8A4 region, and in the 8p11.1→8p12 region, of mouse and human chromosome 8, respectively. Each of these regions is part of a segment which in both species carries the genes for DNA polymerase β, plasminogen activator, ankyrin, glutathione reductase, lipoprotein lipase and defensin 1 (Davisson *et al.*, 1990). Assignment of the β3AR gene to a chromosomal segment which is conserved between mouse and man strongly suggests that the two β3AR genes are homologues.

Pharmacological comparison of the murine and the human β3ARs

Comparison of the pharmacological properties of the murine and the human β3ARs (Table I) helps to define distinctive features common to these two receptors. The radiolabelled β-adrenergic antagonist [^{125}I]ICYP binds to the murine and the human β3ARs with a similar affinity which is 10−20 times lower than that found for the β1AR and β2AR (Tate *et al.*, 1991). In the same way, antagonists such as propranolol, ICI 118551 and CGP 20712A, are weak blockers of isoproterenol-induced cAMP accumulation. Like its human counterpart, the murine β3AR can be activated by the β1- and β2-antagonists CGP 12177A, oxprenolol and pindolol. Finally, both receptors display a low stereoselectivity, and a characteristic potency order for full agonists, since the lipolytic selective compound BRL 37344

Table I. Stimulation and inhibition of cAMP accumulation in CHO-Moβ3 and CHO-Huβ3 cells

Ligands	CHO-Moβ3		CHO-Huβ3[a]	
	I.A. (% of IPR)	K_{act} or K_i (nM)	I.A. (% of NE)	K_{act} or K_i (nM)
Full agonists				
BRL 37344	107.4 ± 8.1	0.4 ± 0.1	87.2 ± 15.4	5.9 ± 1.3
(−)-Isoproterenol	100.0 ± 10.3	4.5 ± 1.8	90.2 ± 2.1	3.9 ± 0.4
(−)-Norepinephrine	105.6 ± 5.6	12.9 ± 4.2	100	6.3 ± 0.7
(−)-Epinephrine	90.6 ± 2.8	23.0 ± 0.3	100.4 ± 3.8	49.2 ± 5.3
(+)-Isoproterenol	104.0 ± 10.2	99.0 ± 44.0	97.3 ± 10.9	111.0 ± 1.0
Partial agonists				
CGP 12177A	74.9 ± 7.9	40.5 ± 8.7	67.6 ± 2.0	138.7 ± 44.3[b]
Oxprenolol	43.8 ± 6.1	535.3 ± 79.3	53.3 ± 6.8	76.7 ± 12.6
Pindolol	35.1 ± 3.1	998.7 ± 187.0	55.0 ± 5.2	153.0 ± 12.0
Antagonists				
Propranolol		450.0 ± 221.2		N.D.
ICI 118551		4968.9 ± 137.4		770.0 ± 80.0
CGP 20712A		6424.6 ± 583.6		6700.0 ± 870.0

Intrinsic activity (I.A.) represents the percentage of the maximal stimulation achieved by each agonist, relative to the maximal effect of (−)-isoproterenol, with basal and maximal isoproterenol-induced cAMP concentrations being 25 and 500 pmol per million cells, respectively. K_{act} values (± SEM) correspond to the concentration of agonist giving 50% of the maximal stimulation. K_i values (± SEM) were calculated according to Cheng and Prusoff (1973), with IC_{50} values defined as the concentration of antagonist required to induce a half-maximal inhibition of (−)-isoproterenol (5 nM) stimulation. The values are means of two to three separate experiments performed in duplicate.

[a] I.A. and K_{act}/K_i values for the CHO-Huβ3 are from Emorine *et al.* (1989) except for CGP 12177A.

[b] In our initial studies of the human β3AR, we had reported that CGP 12177A had no detectable effect on the β3 subtype. This was based on the observation that at 100 μM, this compound had no influence on cAMP accumulation induced by 5 nM isoproterenol in CHO-Huβ3 cells. The effect of CGP12177A on CHO-Huβ3 cells has now been reassessed, and expressed relative to the maximal isoproterenol stimulation.

is at least as potent as (−)-isoproterenol, while it is 10−100 times less active for β1 and β2ARs (Tate *et al.*, 1991).

Besides these common properties which characterize the β3 subtype, minor differences can be found between the murine and the human β3ARs. These variations are mainly quantitative and are likely to be due to the structural differences observed between the two receptors. In the transmembrane domains, which are involved in ligand binding, 12 substitutions occur between the two receptors. Except for two changes (alanine to threonine and isoleucine to threonine, at positions 153 and 297 of the murine sequence, respectively), the other replacements appear to be conservative. However, as is the case for the β2AR (Strader *et al.*, 1989) a single substitution could modify the pharmacological properties of the β3AR. The most striking differences between the mouse and the human β3ARs are found in the intracytoplasmic loops, close to the transmembrane regions, where three arginine residues (at positions 61, 150 and 289 of the murine β3AR) are substituted for a tryptophan, a cysteine and a second cysteine, respectively, in the human receptor. It is worth noting that at corresponding positions, basic residues also occur among all the β1ARs and β2ARs sequenced until now. The presence of cysteines therefore appears to be specific for the human β3AR. As already reported for rhodopsin (Al Saleh, *et al.*, 1987) these cysteine residues may be involved in an intracellular disulphide bond that would impose a structural constraint on the human β3AR.

The murine β3AR is the atypical receptor of adipocytes

The pharmacological profile of the murine β3AR strongly evokes that described for the atypical βAR mediating lipolysis in rat adipose tissue. For example, the potent agonistic effect of BRL 37344 on the β3AR is reminiscent

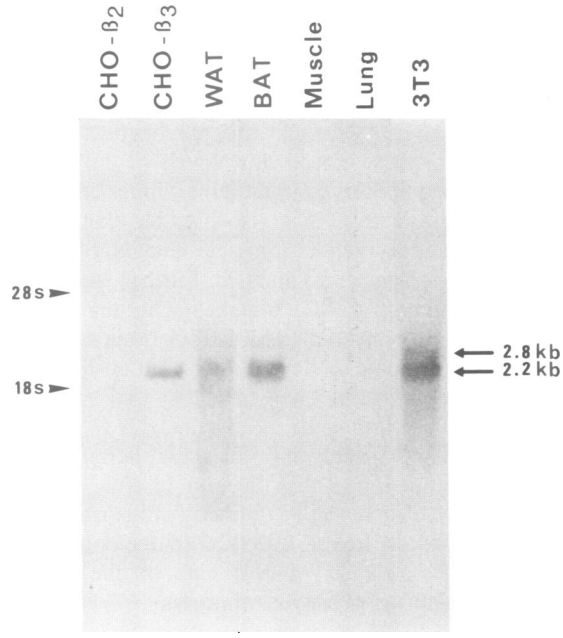

Fig. 6. Northern blot hybridization of probe A43 to total RNA (30 μg) from mouse tissues or from 3T3-F442A adipose cell line (day 7 of differentiation). WAT, white adipose tissue; BAT, brown adipose tissue. The sizes of the hybridizing transcripts are indicated in kb on the right, and the position of 28S and 18S rRNA bands is shown on the left side of the figure. The hybridization patterns obtained with RNA from mouse heart, kidney, stomach, brain, ileum or colon were the same as that for muscle and lung. Five μg of total RNA from CHO-β2 and CHO-β3 (cell lines transfected with the human β2AR and β3AR genes, respectively) were used to assess the specificity of the probe.

of its efficiency for stimulation of cAMP accumulation, lipolysis and thermogenesis in rat fat cells (Arch *et al.*, 1984; Arch, 1989). The β3AR-specific agonist CGP 12177A has

also been shown to stimulate lipolysis (Mohell and Dicker, 1989) and adenylate cyclase activity (Granneman and Whitty, 1991) in rodent fat cells. Moreover, low pA2 values of (−)-propranolol, ICI 118551 and CGP 20712A for antagonism of lipolysis and isoproterenol-induced cAMP accumulation have been reported in rat adipocytes (Bojanic *et al.*, 1985; Arch, 1989; Hollenga and Zaagsma, 1989). In addition to this pharmacological correlation, Northern blot analyses of total RNA reveal that the β3AR gene is highly expressed in white and brown adipose tissues of mouse and in 3T3-F442A adipocytes, as well as (our unpublished results) in brown adipose tissue of rat and hamster. Additional evidence that the β3AR corresponds to the atypical βAR of adipocytes is provided by studies performed on the murine 3T3-F442A cell line (Fève *et al.*, 1991). In these cells, adipose differentiation correlates with the emergence of an atypical βAR pharmacologically and genetically related to the β3AR.

The existence of atypical βARs has also been reported in rat soleus muscle (Challiss *et al.*, 1988) and colon (Bianchetti and Manara, 1990; McLaughlin and MacDonald, 1990), and in guinea-pig ileum (Bond and Clarke, 1988; Blue *et al.*, 1990). Minor pharmacological differences between these systems have been recently discussed (Zaagsma and Nahorski, 1990) and may reflect either species differences or the existence of multiple receptor subtypes. However, the apparent inconsistencies between these different reports may now be reevaluated in the light of the present work, since the murine and the human β3AR also diplay such minor pharmacological differences, even when compared in identical assays.

No β3AR mRNA was detected in mouse femoral muscle, ileum and colon, but this might be explained by the expression of the β3AR being restricted to a few specialized cells in these tissues. Indeed, low levels of β3AR mRNA have already been observed in rat femoral muscle and ileum (Emorine *et al.*, 1989). It is also possible that the tissue distribution and thus the physiological function of the β3AR differs from one species to another. Further studies of the murine and human β3AR, together with the molecular characterization of atypical βARs from tissues other than adipose will contribute to elucidate the relationship between these receptors.

The molecular cloning of the murine β3AR provides a valuable tool for analysing the regulation and the physiological function of this receptor *in vivo*. Moreover, the availability of genetically defined strains of mice with metabolic disorders (Leiter, 1989) offers the opportunity to investigate the possible involvement of the β3AR in pathologies, such as obesity or diabetes.

Material and methods

Materials

BRL 37344: sodium-4-{2′-[2-hydroxy-2-(3-chlorophenyl) ethylamino]-propyl}phenoxyacetate sesquihydrate (RR.SS diastereoisomer) was a gift from Smith Kline Beecham. CGP 12177A: (−)-4-(3-*t*-butyl amino-2-hydroxypropoxy) benzimidazole-2-one, and CGP 20712A: (±)-(2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino)-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-2-propanolmethane sulphonate, were gifts from Ciba-Geigy. ICI 118551: erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropyl-aminobutan-2-ol, and (−)-propranolol were from Imperial Chemical Industries. (−)-isoproterenol and (+)-isoproterenol, (−)-epinephrine, (−)-norepinephrine, (±)-oxprenolol and (±)-pindolol were purchased from Sigma.

Isolation and sequencing of the murine β3AR gene

All the methods used for recombinant DNA procedures are from Ausubel *et al.* (1987). A 1.3 kb *Nco*I−*Bam*HI DNA fragment encompassing the entire coding region of the human β3AR gene (Emorine *et al.*, 1989) was used as a probe to screen a mouse NIH 3T3 genomic library in the lambda FIX™II vector (Stratagene). The nitrocellulose replica filters of the library were prehybridized for 16 h at 42°C in a buffer containing 8 mM Tris−HCl (pH 7.5), 40% formamide, 4×SSC, 5×Denhardt's, 0.2% SDS, 50 mM sodium phosphate and 100 μg/ml heat-denatured salmon sperm DNA. Hybridization was performed for 16 h at 42°C in the same buffer containing the probe (2×10^6 c.p.m./ml) ^{32}P-labelled by the random priming method to a specific activity of 10^9 c.p.m./μg. Final washes were at 45°C in 0.1×SSC and 0.05% SDS.

A 2 kb *Bgl*II−*Bam*HI restriction fragment hybridizing to the probe was subcloned in both orientations into M13mp18. Nested deletions were created with the exonuclease III and sequencing was performed by the dideoxy chain termination method using [α-^{35}S]dATP (800 Ci/mmol, Amersham) and *Thermophilus aquaticus* (*Taq*) polymerase (Taquence kit, USB). Sequence analyses were carried out using the CITI2 (University Paris V, Paris, France) computer software facilities.

DNA probe and hybridization analyses

A 310 bp DNA fragment ('A43') corresponding to the N-terminus of the mouse β3AR (Figure 2C) was produced by polymerase chain reaction using as template 5 ng of the λ clone containing the mouse β3AR gene. Thirty cycles of amplification (93°C for 1.5 min, 55°C for 2 min and 72°C for 2 min) were performed using 2.5 U *Taq* polymerase (Cetus) in 100 μl of buffer containing 10 mM Tris−HCl (pH 8.4), 3 mM $MgCl_2$, 0.05% Tween 20, 0.05% NP40, 10% dimethylsulphoxide, 5% formamide, 125 μM of each deoxynucleotide triphosphate and 125 pM of each primer. Sense (5′-GCTCCGTGGCCTCACGAGAA-3′) and antisense (5′-CCCAACGG-CCAGTG GCCAGTCAGCG-3′) primers corresponded to amino acids 2−8 and 98−106, respectively of the human β3AR sequence (Emorine *et al.*, 1989). The amplified 310 bp DNA fragment was purified by electrophoresis through an acrylamide gel, ^{32}P-labelled by random priming to a specific activity of 10^9 c.p.m./μg, and used as a probe in Southern and Northern blot experiments.

For Southern blot hybridization analysis, single restriction enzyme digests of genomic DNA (10 μg) were performed, and the samples electrophoresed through a 0.8% agarose gel and blotted onto nylon (Hybond N+, Amersham) filters. Prehybridization and hybridization procedure was as described above and final washes were at 45°C in 0.1×SSC, 0.05% SDS.

For Northern blot hybridization analyses, total RNA extracted from mouse tissues or cultured cells (Chomczynski and Sacchi, 1987) was denatured in Glyoxal-DMSO, electrophoresed through a 1% agarose gel and transferred onto a nylon membrane (Hybond N+, Amersham). Prehybridization, hybridization and washing were as described above, except that hybridization was performed in the presence of 10% dextran sulphate.

In situ hybridization

For chromosomal localization of the β3AR gene in mouse, *in situ* hybridization was performed on metaphase spreads of concanavalin A-stimulated lymphocytes from a WMP male mouse, in which all the autosomes except number 19 are in the form of metacentric Robertsonian translocations. For localization of the β3AR gene in man, metaphase spreads were from phytohaemagglutinin-stimulated human lymphocytes. In both cases the lymphocytes were cultured at 37°C for 72 h, with 5-bromodeoxyuridine (60 μg/ml) added for the final 7 h of culture.

Specific probes were the 310 bp A43 fragment of the mouse β3AR gene, and a 206 bp (*Acc*I−*Apa*LI) DNA fragment encompassing the third intracytoplasmic loop of the human β3AR (Emorine *et al.*, 1991). These DNA fragments were subcloned into the pUC19 plasmid vector, tritium labelled by nick-translation to a specific activity of 10^8 d.p.m./μg, and used at a final concentration of 25 ng/ml of hybridization solution. Hybridization to metaphase spreads, post-hybridization washes, emulsion autoradiography, R-banding and silver grain analysis were carried out as previously described (Mattei *et al.*, 1985).

Constructions, cell culture and transfections

For expression in eukaryotic cells, a 1365 bp *Nar*I−*Bam*HI restriction fragment from the mouse β3AR gene (Figure 2C), was inserted under the control of the SV40 early promoter into a plasmid vector containing the 3′ untranslated region of the gene for the hepatitis-B surface antigen, and the murine dihydrofolate reductase (DHFR) gene as a selectable marker (Larsky *et al.*, 1984). The resulting construct contained 15 bp of 5′ untranslated region, 1164 bp of coding region and 186 bp of 3′ non-coding region of the mouse β3AR gene.

CHO cells, deficient in DHFR, were grown in Ham's F12 medium (Seromed) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 15 μg/ml glycine, 9.5 μg/ml hypoxanthine and 1.46 μg/ml thymidine. Transfection with 5 μg of the expression vector was performed by the calcium phosphate precipitation method as described (Tate *et al.*, 1991). Stable transformants expressing the DHFR gene were screened for expression of βAR by stimulation of cAMP accumulation with 1 μM (−)-isoproterenol. One clone giving the highest stimulation factor was subcloned by limiting dilution, and is referred to as 'CHO-Moβ3'.

cAMP accumulation assay

Preconfluent cells (5×10^5) were incubated at 37°C for 20 min in 0.5 ml of Hanks' buffer containing 20 mM HEPES (pH 7.4), 1 mM ascorbic acid, 1 mM isobutylmethylxanthine and various concentrations of agonist. After boiling for 5 min and centrifugation (4000 r.p.m., 10 min, 4°C), the amount of cAMP produced was determined using the Amersham cAMP assay kit. For studies on the inhibition of cAMP accumulation, cells were preincubated at 37°C for 10 min with antagonist before addition of 5 nM (−)-isoproterenol and incubation for another 20 min period. Computer analysis of the data was carried out using the Graph-PAD program (copyright 1987 by M.J.Motulsky).

ICYP binding assay

Preconfluent cells (10^5) were incubated for 1 h at 37°C in 0.5 ml of Hanks' buffer containing 20 mM HEPES (pH 7.4), 1 mM ascorbic acid, 2 μM desipramine, 0.05% bovine serum albumin and 70−7000 pM (−)-[^{125}I]ICYP (2000 Ci/mmol, Amersham). Non-specific binding was determined as that occurring in the presence of 100 μM (−)-isoproterenol. Incubations were terminated by dilution in cold phosphate buffered saline and bound ligand was separated from free [^{125}I]ICYP by filtration through Whatman GF/C filters soaked in 0.3% polyethyleneimine. The filters were washed three times with cold phosphate buffered saline, and the radioactivity retained was measured using a LKB 1282 Compugamma counter. Values the EBDA program (Biosoft−Elsevier, Cambridge, UK) (Munson and Rothbard, 1980).

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References

Allen,J.M., Baetge,E.E., Abrass,I.B. and Palmiter,R.D. (1988) *EMBO J.*, **7**, 133−138.

Al Saleh,S., Gore,M. and Akhtar,M. (1987) *Biochem. J.*, **246**, 131−137.

Arch,J.R.S. (1989) *Proc. Nut. Soc.*, **48**, 215−223.

Arch,J.R.S., Ainsworth,A.T., Cawthorne,M.A., Piercy,V., Sennitt,M.V., Thody,V.E., Wilson,C. and Wilson,S. (1984) *Nature*, **309**, 163−165.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. Eds (1987) *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York.

Bianchetti,A. and Manara,L. (1990) *Br. J. Pharmacol.*, **100**, 831−839.

Bojanic,D., Jansen,J.D., Nahorski,S.R. and Zaagsma,J. (1985) *Br. J. Pharmacol.*, **84**, 131−137.

Bond,R.A. and Clarke,D.E. (1988) *Br. J. Pharmacol.*, **95**, 723−734.

Blue,D.R., Bond,R.A., Adham,N., Delmendo,R., Michel,A.D., Eglen,R.M., Whiting,R.L. and Clarke,D.E. (1990) *J. Pharm. Exp. Ther.*, **252**, 1034−1042.

Challiss,R.A.J., Leighton,B., Wilson,S., Thurlby,P.L. and Arch,J. (1988) *Biochem. Pharmacol.*, **37**, 947−950.

Cheng,Y. and Prusoff,W.H. (1973) *Biochem. Pharmacol.*, **22**, 3099−3108.

Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156−159.

Davisson M.T., Lalley,P.A., Peters,J., Doolittle,D.P., Hillyard,A.L. and Searle,A.G. (1990) *Cytogenet. Cell. Genet.*, **55**, 434−456.

Dixon,R.A.F., Kobilka,B.K., Strader,D.J., Benovic,J.L., Dohlman,H.G., Frielle,T., Bolanowski,M.A., Bennett,C.D., Rands,E., Diehl,R.E., Mumford,R.A., Slater,E.E., Sigal,I.S., Caron,M.G., Lefkowitz,R.J. and Strader,C.D. (1986) *Nature*, **321**, 75−79.

Emorine,L.J., Marullo,S., Delavier-Klutchko C., Kaveri,S.V., Durieu-Trautmann,O. and Strosberg,A.D. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6995−6999.

Emorine,L.J., Marullo,S., Briend-Sutren,M.-M., Patey,G., Tate,K., Delavier-Klutchko,C. and Strosberg,A.D. (1989) *Science*, **245**, 1118−1121.

Emorine,L.J., Fève,B., Pairault,J., Briend-Sutren,M.-M., Marullo,S., Delavier-Klutchko,C. and Strosberg,A.D. (1991) *Biochem. Pharmacol.*, **41**, 853−859.

Fève,B., Emorine,L.J., Lasnier,F., Blin,N., Baude,B., Nahmias,C., Strosberg,A.D. and Pairault,J. (1991) *J. Biol. Chem.*, in press.

Frielle,T., Collins,S., Daniel,K.W., Caron,M.G., Lefkowitz,R.J. and Kobilka,B.K. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7920−7924.

Granneman,J.G. and Whitty,C. (1991) *J. Pharmacol. Exp. Ther.*, **256**, 421−425.

Hausdorff,W.P., Caron,M. and Lefkowitz,R.J. (1990) *FASEB J.*, **4**, 2881−2889.

Hollenga,C. and Zaagsma,J. (1989) *Br. J. Pharmacol.*, **98**, 1420−1424.

Kaumann,A.J. (1989) *Trends Pharmacol. Sci.*, **10**, 316−320.

Kobilka,B.K., Kobilka,T.S., Daniel,K., Regan,J.W., Caron,M.G. and Lefkowitz,R.J. (1988) *Science*, **240**, 1310−1316.

Larsky,L.A., Dowbenko,D., Simonsen,C.C. and Berman,P.W. (1984) *Biotechnology*, **2**, 527−530.

Leiter,E.H. (1989) *FASEB J.*, **3**, 2231−2241.

Machida,C.A., Bunzow,J.R., Searles,R.P., Van Tol,H., Tester,B., Neve,K.A., Teal,P., Nipper,V. and Civelli,O. (1990) *J. Biol. Chem.*, **265**, 12960−12965.

McLaughin,D.P. and MacDonald,A. (1990) *Br. J. Pharmacol.*, **101**, 569−574.

Manara,L. and Bianchetti,A. (1990) *Trends Pharmacol. Sci.*, **11**, 229−230.

Marullo,S., Emorine,L.J., Strosberg,A.D. and Delavier-Klutchko,C. (1990) *EMBO J.*, **9**, 1471−1476.

Mattei,M.G., Philip,N., Passage,E., Moisan,J.P., Mandel,J.L. and Mattei,J.F. (1985) *Human Genetics*, **69**, 268−271.

Mohell,N. and Dicker,A. (1989) *Biochem. J.*, **261**, 401−405.

Munson,P.J. and Rodbard,D. (1980). *Anal. Biochem.*, **107**, 220−239.

O'Dowd,B.F., Lefkowitz,R.J. and Caron,M. (1989a) *Annu. Rev. Neurosci.*, **12**, 67−83.

O'Dowd,B.F., Hnatowich,M., Caron,M.G., Lefkowitz,R.J. and Bouvier,M. (1989b) *J. Biol. Chem.*, **264**, 7564−7589.

Strader,C.D., Sigal,I.S. and Dixon,R.A.F. (1989) *FASEB J.*, **3**, 1825−1832.

Strosberg,A.D. (1991) *Eur. J. Biochem.*, **196**, 1−10.

Tate,K.M., Briend-Sutren,M.M., Emorine,L.J., Delavier-Klutchko,C., Marullo,S. and Strosberg,A.D. (1991) *Eur. J. Biochem.*, **196**, 357−361.

Zaagsma,J. and Nahorski,S.R. (1990) *Trends Pharmacol. Sci.*, **11**, 3−7.

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