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Pharmacological characterisation and signalling pathways of recombinant and endogenously expressed mouse β_3-adrenoceptors

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Abstract

The work presented in this thesis entitled "Pharmacological characterisation and signalling pathways of recombinant and endogenously expressed mouse β_3-adrenoceptors" investigated (i) the pharmacological properties and signal transduction pathways utilised by the mouse β_3-AR splice variants when expressed in CHO-K1 cells and (ii) the β-AR subtypes involved in mouse ileal smooth muscle relaxation in mice lacking the β_3-AR.

Chapter One provides an overview of β_3-ARs from gene and receptor structure and utilisation of signalling pathways, through to β_3-AR function and pharmacology. Chapter Two describes the methods used in the present studies to examine the pharmacological and signalling pathways utilised by the mouse β_{3a}-AR and β_{3b}-AR when expressed in CHO-K1 cells, and to examine ileum function in mice lacking β_3-ARs.

The experimental results for studies performed at assessing the pharmacology of the mouse β_{3a}- or the β_{3b}-AR transfected in CHO-K1 cells are described in Chapter 3. Stable cell lines expressing each of the β_3-AR isoforms at three different receptor density levels were identified by radioligand binding. Determination of the affinities or potency of several ligands (by radioligand binding, cytosensor microphysiometer or cAMP assays) showed that (i) the potency and efficacy of the β_3-AR agonist CL316243 were reduced with lowered receptor expression, and (ii) there was no difference in the pharmacology of the two splice variants expressed in CHO-K1 cells at similar receptor densities. The β_3-AR "antagonist" SR59230A was found to have agonist properties depending on the level of β_3-AR expression.

Chapter Four describes the signalling pathways utilised by the mouse β_3-AR splice variants in CHO-K1 cells. Studies show that both receptors couple to Gαs, but only the β_{3b}-AR appears to couple to Gαi. This coupling of the β_{3b}-AR to Gαi was not due to promiscuous coupling of the receptor, since cells expressing physiologically low levels of receptor were still able to couple to Gαi. Activation of the Erk1/2

pathway was not dependent on the ability of β_3-ARs to couple to Gαs or Gαi or activation of PKA, but appeared to involve the signalling proteins Src and PI3K.

Changes in ileal function in mice lacking β_3-ARs are described in Chapter Five. In mouse ileum, β_3-ARs and to a lesser extent β_1-ARs are the predominant adrenoceptor subtypes mediating smooth muscle relaxation. In β_3-AR KO mice, β_1-ARs functionally compensate for the lack of β_3-ARs, and this is associated with increased β_1-AR mRNA and levels of binding. Additionally, the β_3-AR antagonist SR59230A may not be as selective as previously reported, and also displays agonistic actions at the mouse β_3-AR in ileum.

Finally, the contribution of the current results to the existing literature is discussed in Chapter Six. The combined results in this thesis add to the knowledge of β_3-ARs in the literature and show that (a) the mouse β_3-AR splice variants share identical pharmacological properties but that differences in the C-terminal tail can affect intracellular signaling pathways utilised by these receptors, (b) the β_1-AR compensates for the lack of β_3-ARs in ileum from β_3-AR KO mice; (c) there is need for the further investigation of the pharmacological properties of the β_3-AR antagonist SR59230A which may not be as selective for the β_3-AR as previously reported and exhibited agonistic actions at both the cloned β_3-AR and that endogenously expressed in mouse ileum.

Declaration

I declare that the contents of this thesis have not previously been accepted for the award of any other degree or diploma in any other university or institution. Furthermore, this thesis contains no material previously published by another person except where due reference is made.

I certify that the writing of this thesis with the results, interpretation, opinions and suggestions presented are entirely my own work.

This thesis is less than 100,000 words, excluding tables, figure legends and references.

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Dana Sabine Hutchinson

December 2001

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Publications

During the course of this study, the following publications have been published or submitted for publication. Those publications which are not related to the work presented here in this thesis are denoted by (*).

Papers

Hutchinson, D.S., Bengtsson, T., Evans, B.A. & Summers, R.J. (accepted for publication). Mouse β_{3a}- and β_{3b}-adrenoceptors expressed in Chinese hamster ovary cells display identical pharmacology but utilise distinct signalling pathways. *Br. J. Pharmacol.*

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*Hutchinson, D.S., Evans, B.A. & Summers, R.J. (2000). β_3-Adrenoceptor regulation and relaxation responses in mouse ileum. *Br. J. Pharmacol.*, **129,** 1251-1259.

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Conference proceedings and attendance

Evans, B.A., Hutchinson, D.S., Papaioannou, M., Hamilton, S. & Summers, R.J. (1998). Cloning and expression of two isoforms of the β_3-adrenoceptor which are differentially expressed in mouse tissues. *Proc. Aust. Soc. Clin. Exp. Pharmacol. Toxicol.*, **5,** 130.

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expression and function in human SK-N-MC neuroblastoma cells. Keystone Symposia: Molecular Control of Adipogenesis and Obesity, Taos, New Mexico, U.S.A., Februaury 2000.

Hutchinson, D.S., Bengtsson, T., Papaioannou, M., Evans, B.A. & Summers, R.J. (2001). Different signalling properties of the mouse β_{3a}- and β_{3b}-adrenoceptor expressed in CHO-K1 cells. Joint meeting of ASCEPT, BPS, PSC and WPS, Vancouver, Canada, March 2001.

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Hutchinson, D.S., Papaioannou, M., Evans, B.A., Lowell, B. & Summers, R.J. (2000). Characterisation of ileal β-adrenoceptor mediated responses in β_3-adrenoceptor knock-out mice. British Pharmacological Society Winter Meeting, England, January 2000.

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Abbreviations

Abbreviations used in the text and figures are as follows:

aa	amino acid(s)
AC	adenylate cyclase
AD	adrenaline
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
AP-1	activator protein-1
APAT	aryloxypropanolaminotetralins
AR	adrenoceptor
ATP	adenosine 5'-triphosphate
βARK	β-adrenergic receptor kinase
BAT	brown adipose tissue
B_{max}	maximum density of binding sites
bp(s)	base pair(s)
BSA	bovine serum albumin
cAMP	cyclic AMP
cGMP	cyclic GMP
cDNA	complementary DNA
CHO-K1	Chinese hamster ovary-K1
cpm	counts per minute
c-r	concentration-response
CRE	cAMP response element
CREB	cAMP response element binding protein
Da	Dalton
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dr	dose-ratio

EC_{50}	concentration of compound which produces 50% of maximum effect
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Erk1/2	extracellular signal-regulated kinase 1/2
ES	embryonic stem
FBS	foetal bovine serum
FSE	fat specific element
Gab1	Grb2-associated binder 1
GDP	guanosine 5'-diphosphate
GLUT	glucose transporter
GMP	guanosine 5'-monophosphate
GPCR	G-protein coupled receptor
Grb2	growth factor-receptor-bound protein-2
GRE	glucocorticoid response element
GRK	G-protein coupled receptor kinase
GTP	guanosine 5'-triphosphate
HB-EGF	heparin binding-epidermal growth factor
HBS	Hepes-buffered saline
HEK293	human embryonic kidney 293
5-HT	5-hydroxytryptamine (serotonin)
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	concentration of compound which produces 50% of maximum effect
ICYP	[^{125}I]-cyanopindolol
IGFR	insulin-like growth factor receptor
IP_3	inositol trisphosphate
JNK	c-Jun N-terminal kinase
K_1	association rate constant
K_{-1}	dissociation rate constant
K_D	equilibrium dissociation constant
K_{obs}	observed association rate constant

KO	knock-out
KSR-1	kinase suppressor of Ras-1
LAPS	light-addressable potentiometric sensor
LDL	low density lipoprotein
LPA	lysophosphatidic acid
MAPK	mitogen activated protein kinase
MEK	MAPK/Erk kinase
mRNA	messenger RNA
NA	noradrenaline
NAD	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide
NF-1	nuclear factor-1
NGFR	nerve growth factor receptor
PBS	phosphate buffered saline
PC12	pheochromocytoma-12
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGF	platelet-derived growth factor receptor
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-(4,5)-bisphosphate
PIP3	phosphatidylinositol-(3,4,5)-trisphosphate
PKA	protein kinase A
PKB	protein kinase B (also referred to as Akt)
PKC	protein kinase C
pEC_{50}	negative logarithm of the EC_{50}
pK_{act}	negative logarithm of the activation constant
pK_D	negative logarithm of the equilibrium dissociation constant
pK_i	negative logarithm of the affinity of a ligand
PLC	phospholipase C
PMSF	phenylmethylsulphonyfluoride
PTX	pertussis toxin
Ras-GEF	Ras-guanine nucleotide exchange factor
R_{max}	maximal relaxation

RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
SDS	sodium dodecyl sulfate
s.e.mean	standard error of the mean
SH	Src homology
TBS	Tris buffered saline
TBST	TBS-Tween-20
TK	tyrosine kinase
TKR	tyrosine kinase receptor
tm	transmembrane
tRNA	transfer RNA
WAT	white adipose tissue

General Introduction

Chapter 1

1.1 Classification of Adrenoceptors

The catecholamines noradrenaline (NA) and adrenaline (AD) act on a wide variety of tissues by activating α- and β-adrenoceptors (ARs). This original classification by Ahlquist (1948), differentiated the two receptor groups by classifying α-ARs as mediating most excitatory functions and β-ARs as mediating most inhibitory functions in the responses produced in several organ systems. Further sub-classification of β-ARs into two subtypes, β_1- and β_2-ARs, was based on rank potency orders for a series of agonists (Lands *et al.*, 1967). It was suggested that β_1-ARs mediated effects in adipose tissue (lipolysis), heart (force and rate) and jejunum (smooth muscle relaxation), while β_2-ARs mediated effects in uterus (relaxation), trachea (relaxation) and striated muscle (lactic acid production) (Arnold & Selberis, 1968; Lands *et al.*, 1967). Further sub-classification of β_1- and β_2-ARs was confirmed by the development of selective antagonists and agonists for both β_1- and β_2-ARs.

Originally the receptor responsible for effects mediated by catecholamines in adipose tissue was classified as a β_1-AR (Lands *et al.*, 1967). However, several studies with a series of β-AR antagonists and with non-catecholamine agonists suggested that the rat adipocyte receptor did not fit into the classical β_1-/β_2-AR division. It was suggested that a hybrid or atypical β-AR was responsible for lipolysis in adipose tissue and this receptor had low affinity for β_1- /β_2-AR selective and non-selective antagonists (De Vente *et al.*, 1980; Harms *et al.*, 1974). The β-AR mediating intestinal relaxation was also classified as a β_1-AR (Lands *et al.*, 1967). However a post-junctional receptor distinct from defined α- and β-ARs was observed in guinea-pig ileum that was resistant to blockade by several α- and β-AR antagonists (Bond *et al.*, 1986). Evidence illustrating the atypical responses in both adipose tissue and gastrointestinal tissues is extensively detailed in several reviews (Arch & Kaumann, 1993; Manara *et al.*, 1995). The main evidence in this large body of literature includes:

(a) Catecholamine responses in adipose and gastrointestinal tissues were resistant to blockade (or were weakly antagonised) by several β-AR antagonists
(b) Low stereoselectivity of β-AR agonist and antagonists enantiomers, unlike that observed for β_1- or β_2-AR responses
(c) Synthesis of a group of arylethanolamine compounds that selectively stimulated brown and white adipocytes and relaxed gastrointestinal smooth muscle, and whose responses were weakly (or not at all) antagonised by β_1- or β_2-AR selective antagonists and conventional non-selective β-AR antagonists
(d) Cloning of a β_3-AR that was found to possess atypical β-AR characteristics

This introduction gives an overview of β_3-ARs, their gene and receptor structure and signal transduction pathways, through to β_3-AR function and pharmacology.

1.2 Molecular cloning for identification of β-ARs

The β_2-AR was one of the first G-protein coupled receptor (GPCR) to be cloned (Dixon *et al.*, 1986) and provided new tools to identify receptors that had previously been difficult to identify by classical pharmacological approaches. Genes encoding the pharmacologically defined α_1-, α_2-, β_1- and β_2-ARs were isolated (Cotecchia *et al.*, 1988; Emorine *et al.*, 1987; Frielle *et al.*, 1987; Kobilka *et al.*, 1987b), and subsequently genes for additional α- and β-ARs, including the β_3-AR were cloned. The characterisation of a human gene encoding the β_3-AR from a human genomic deoxyribonucleic acid (DNA) library provided the structural basis for the existence of atypical β-ARs (Emorine *et al.*, 1989). Subsequent studies isolated genes encoding the β_3-AR from rat brown adipose tissue complementary DNA (cDNA) (Granneman *et al.*, 1991; Muzzin *et al.*, 1991), mouse genomic DNA (Nahmias *et al.*, 1991) and rat colonic cDNA (Bensaid *et al.*, 1993) libraries. The complete coding region of the β_3-AR has also been cloned from cow (Pietri-Rouxel *et al.*, 1995), rhesus monkey (Walston *et al.*, 1997), guinea-pig (Atgie *et al.*, 1996), dog (Lenzen *et al.*, 1998), sheep (Forrest & Hickford, 2000), goat (Forrest & Hickford, 2000), cat (Genbank accession number AF192488) and pig (Genbank accession number AF274007). Sequence homology diagrams for the mouse β_1-, β_2- and β_3-

ARs, and the mouse, rat and human β_3-ARs are shown in Figures 1.1 and 1.2 respectively.

The chromosomal localisation of the three β-AR genes in human and mouse has been established: for the β_1-AR, the chromosomal localisation is 10q24-26 (human) and 19 (mouse); the β_2-AR is located at 5q31-32 (human) and 11 (mouse); and the β_3-AR at 8p11-12 (human) and 8 (mouse) (Cohen *et al.*, 1993; Nahmias *et al.*, 1991; Yang-Feng *et al.*, 1990).

1.2.1 Gene structure and upstream promoters of the β_3-AR

The β_3-AR genes were originally assumed to consist of a single protein-coding exon (Emorine *et al.*, 1989; Nahmias *et al.*, 1991) like those encoding the β_1- and β_2-AR (Kobilka *et al.*, 1987a; Machida *et al.*, 1990). Closer inspection of the sequences of rat, mouse and human β_3-AR genes revealed potential donor splice signals at homologous sites near the 3' end of the coding block (Emorine *et al.*, 1989), which suggested the existence of introns in the β_3-AR gene.

Comparison of gene sequences and cDNA in a single species showed the existence of introns inside and downstream of the coding block. A schematic diagram showing the intron/exon structure of the mouse, rat and human β_3-AR genes is illustrated in Figure 1.3. The mouse β_3-AR gene is composed of 3 exons and 2 introns. The first exon (~1.3kb) contains the 5' untranslated region and the majority of the coding sequence (388 aa). The first intron is 463 base pairs (bps) in size. The second exon is small, containing 37 coding nucleotides that encode the last 12 aa residues of the receptor, and 31 nucleotides of the 3' untranslated region. The rest of the 3' untranslated region is located in the third exon. Two forms of the third exon of the mouse β_3-AR gene were reported, dependent upon two different potential splice sites which give rise to either a 680 or 593bp fragment (Van Spronsen *et al.*, 1993). The rat β_3-AR gene is very similar to that of the mouse. The human β_3-AR gene contains 2 exons. The first intron (~1.4kb) contains the 5' untranslated region and the majority of the coding region, comprising of 402 aa. The intron separating the two exons is ~1kb. The second exon (660bp) contains 19bp of coding region (corresponding to

Figure 1.1: Comparison between the amino acid (aa) sequences of the mouse β_1- (Jasper *et al.*, 1993), β_2- (Nakada *et al.*, 1989) and β_3-ARs (Nahmias *et al.*, 1991). Putative transmembrane (tm) regions are boxed. Dashes represent areas of homology with the mouse β_3-AR.

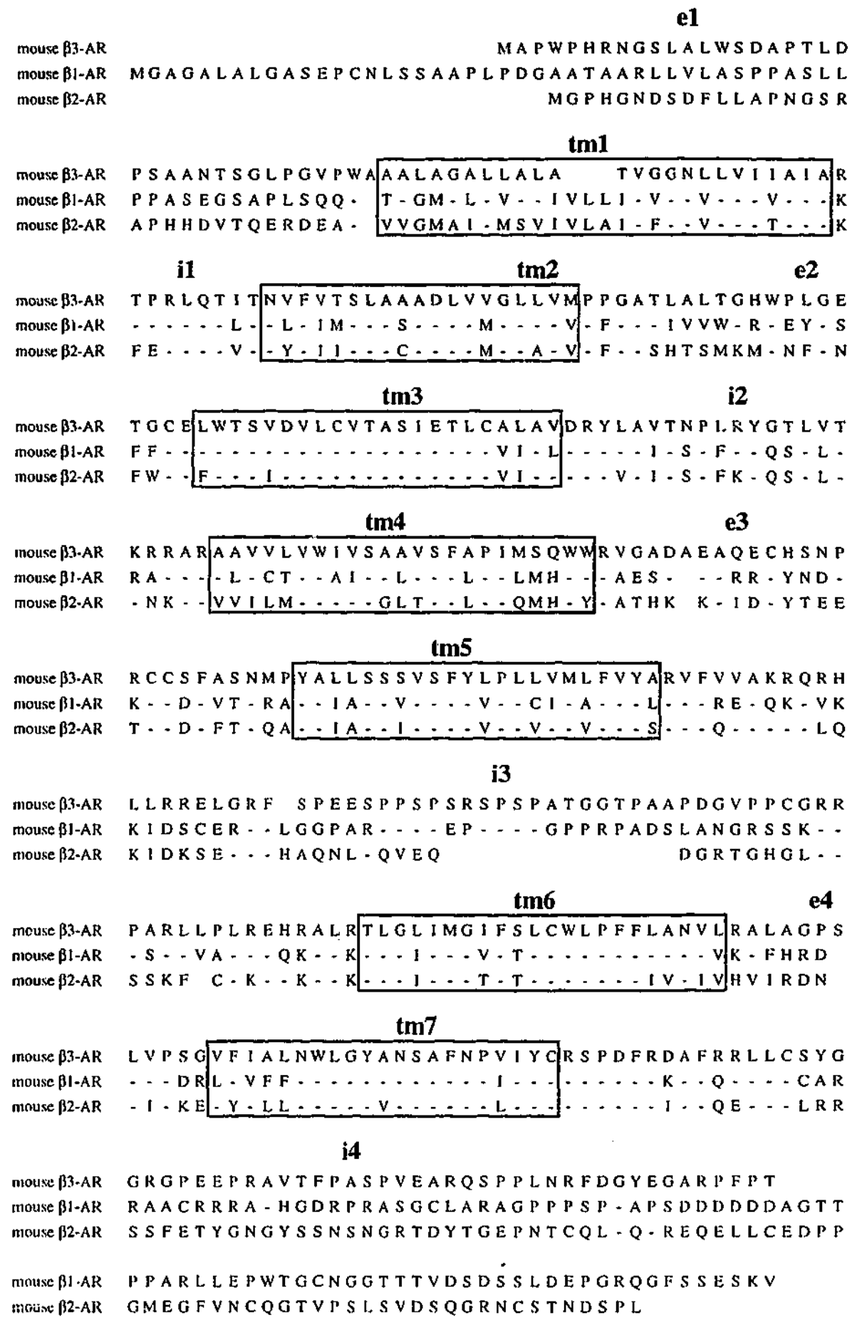


Figure 1.2: Comparison between the aa sequences of the mouse (Granneman *et al.*, 1992; Nahmias *et al.*, 1991; Van Spronsen *et al.*, 1993), rat (Bensaid *et al.*, 1993; Granneman *et al.*, 1991; Muzzin *et al.*, 1991) and human (Emorine *et al.*, 1989; Granneman *et al.*, 1992; Van Spronsen *et al.*, 1993) β_3-ARs. Putative tm regions are boxed. Dashes represent areas of homology with the mouse β_3-AR.

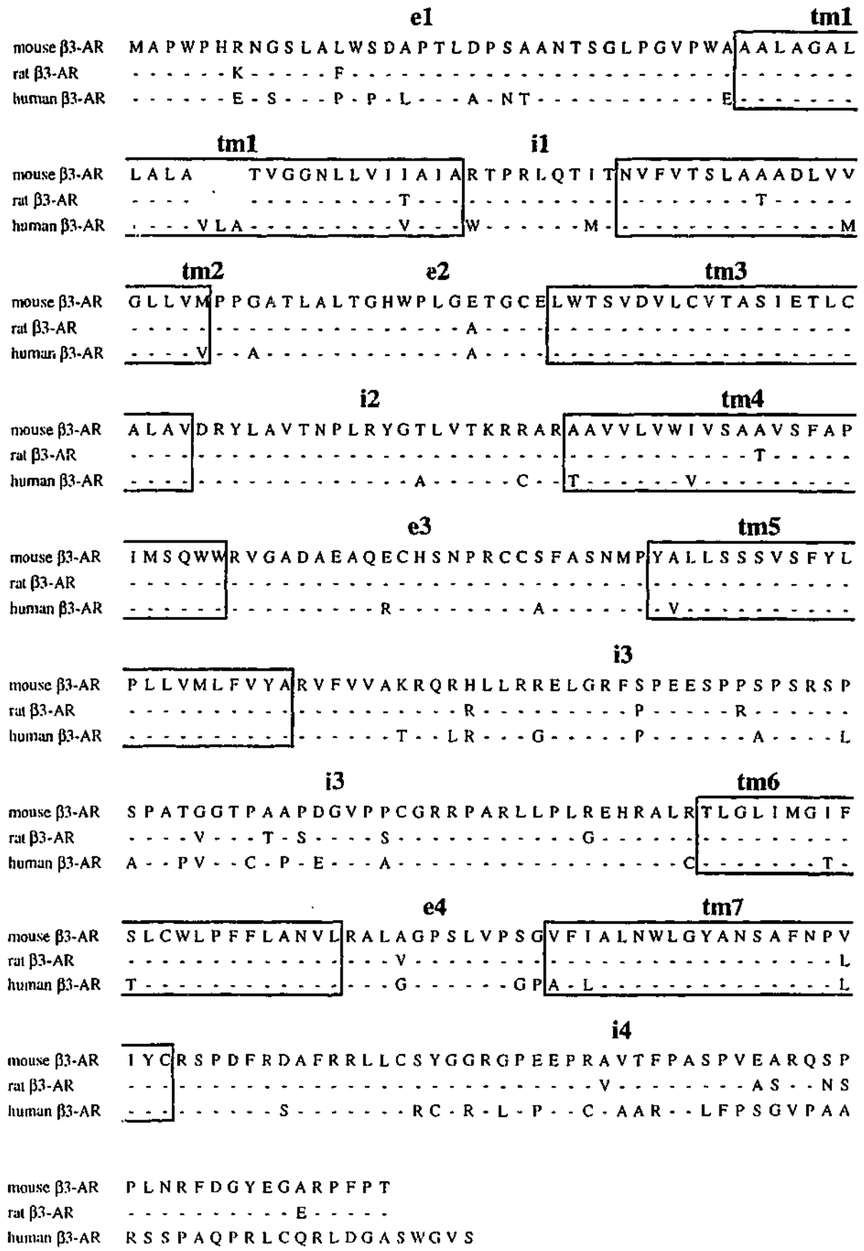
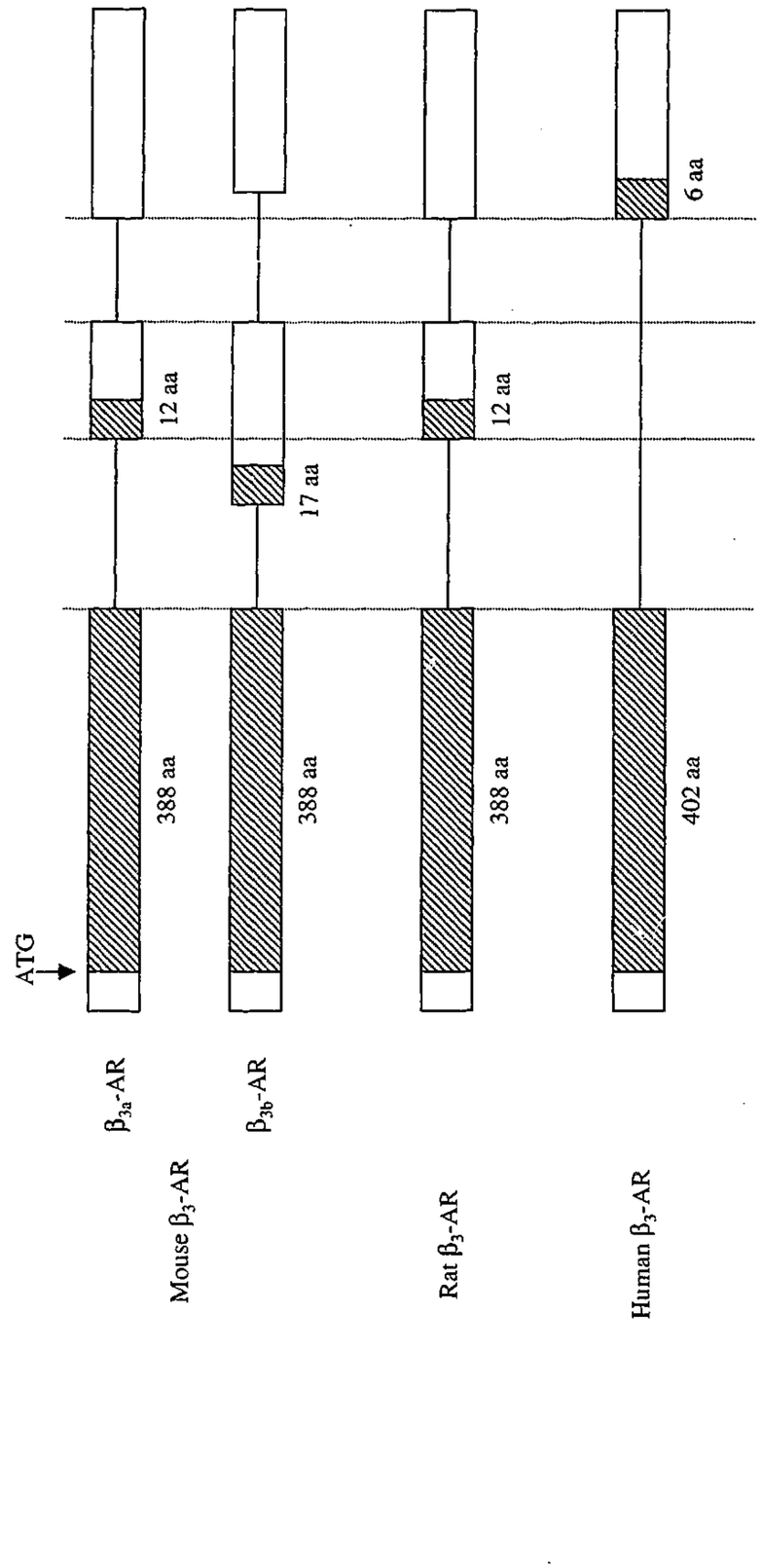


Figure 1.3: Schematic diagram showing alignment of the mouse (Evans *et al.*, 1999; Van Spronsen *et al.*, 1993), rat (Bensaid *et al.*, 1993; Granneman *et al.*, 1992) and human (Granneman *et al.*, 1992; Van Spronsen *et al.*, 1993) β_3-AR genes. Boxed areas indicate exons separated by introns. Shaded portions of exons represent coding regions. The vertical lines show alignment of exons and introns between the different β_3-AR genes. Diagram is not to scale.



the last 6 aa of the receptor) and the 3' untranslated region (Granneman *et al.*, 1993; Granneman *et al.*, 1992). Some regions of the intron in the human β_3-AR gene are high in homology to the mouse second exon. Van Spronsen *et al.* (1993) found no evidence that this region could be utilised in the same way as exon 2 of the mouse gene. However, another study showed that this region could be used like that of the mouse gene, which may result in the coding of a further 12 aa in the C-terminal tail, although there is no experimental evidence for this.

The transcription start sites for the mouse and human β_3-AR gene are located 100-200 nucleotides upstream of the ATG translational start codon (Granneman & Lahners, 1994; Van Spronsen *et al.*, 1993). The major start site in rat adipose tissue is 161 nucleotides upstream of the ATG codon but was found to be -109 and -123 nucleotides upstream in the rat gastric fundus (Granneman & Lahners, 1994). Two TATA boxes 25-30 nucleotides upstream of the transcription start site in the human gene are shown, whereas in the mouse gene, one poor TATA box approximation exists 5-20 nucleotides upstream of the mouse transcription start site. Upstream of these TATA boxes, 2 approximate CCAAT boxes exist in both the mouse and human gene. Other regulatory motifs include glucocorticoid response elements (GREs) in close proximity to the sequence recognised by the transcription factor activator protein (AP-1) in the human gene. Fat specific-elements (FSE) and cAMP response elements (CRE; one in mouse, four in human, none in rat) and close analogues for nuclear factor-1 (NF-1) exist in the mouse and human genes.

1.2.1.1 Gene regulation of the β_3-AR

The β_3-AR gene is expressed throughout life in different mouse species, but β_3-AR levels vary during development and adipocyte differentiation. Differentiation of the mouse adipocyte cell 3T3-F442A line into mature adipocytes correlates with an increase of β_3-AR messenger RNA (mRNA) levels, with β-AR density and function changing from a predominantly β_1-AR population to a predominantly β_3-AR population (El Hadri *et al.*, 1996; Feve *et al.*, 1991). Similar results were obtained in primary mouse brown adipocytes grown in culture. In preadipocytes, only β_1-ARs are coupled to adenylate cyclase (AC), but during differentiation, there is a switch of

coupling from β_1-ARs to β_3-ARs, resulting in only functional β_3-ARs coupling to AC in mature adipocytes (Bronnikov *et al.*, 1999).

Obesity is correlated with lower mRNA levels and responsiveness of β_3-ARs in adipose tissue from C57BL/6J *ob/ob* mice and *fa/fa* Zucker rats (Collins *et al.*, 1994; Onai *et al.*, 1995), suggesting a role for β_3-ARs in obesity. Adrenalectomy in C57BL/6J *ob/ob* mice restores β_3-AR mRNA expression and response, suggesting that the hypercorticism associated with obesity in *ob/ob* mice represses β_3-AR expression and prevents normal β_3-AR function in white adipocytes from these animals (Gettys *et al.*, 1997).

Many factors influence the regulation of the β_3-AR, both in animals and cell culture. One potential factor that could affect β_3-AR regulation is catecholamines, since CREs identified in the promoter region of the β_2-AR have been implicated in transcriptional regulation of the β_2-AR. Positive auto regulation of the β_2-AR gene occurs via receptor mediated stimulation of AC, which leads to activation of cAMP response element binding protein (CREB) and stimulation of β_2-AR gene transcription (Collins *et al.*, 1990). The presence of CRE in the mouse and human β_3-AR promoter region suggested that β_3-AR expression is regulated by agonist stimulation. Several studies in adipose tissue have examined the effects of β-AR agonists on β_3-AR regulation. The consensus is that in the short term, β-AR agonists decrease and in the long term do not affect or even increase β_3-AR mRNA levels. Bengtsson *et al.* (1996) conducted a detailed study in primary brown adipocyte cell culture and showed that β-AR agonists and forskolin decreased β_3-AR mRNA. These effects were rapid, with more than 80% of β_3-AR mRNA lost within 2 h of agonist treatment. However with continued treatment, the levels of β_3-AR mRNA returned to control levels within 24 h, and it was postulated that the effect was associated with the production of a protein transcription factor. Other studies in several other models of β_3-AR mRNA regulation in adipose tissue all showed decreased β_3-AR mRNA levels following β_3-AR stimulation, which can be mimicked by 8-bromo-cAMP and forskolin (Feve *et al.*, 1992; 1994; Granneman & Lahners, 1992; 1994; Hutchinson *et al.*, 2000). This down regulation of β_3-AR

mRNA with agonist stimulation was not observed in mouse ileum, where treatment with a β_3-AR agonist or forskolin reduced responses to β_3-AR agonists without affecting β_3-AR number or mRNA levels (Hutchinson *et al.*, 2000), suggesting that distinct regulatory mechanisms are present in ileum as compared to adipose tissue.

Thyroid hormones appear to influence β-AR systems in a tissue or cell specific manner (Taylor, 1983) and therefore regulation of β-ARs may be tissue and receptor-specific. In brown adipose tissue (BAT), hypothyroidism increases β_3-AR expression, cyclic AMP (cAMP) and thermogenesis to β_3-AR agonists, which is associated with a reduction in β_1- and β_2-AR mRNA levels (Carvalho *et al.*, 1996; Chaudhry & Granneman, 1997; Ilyes & Stock, 1990; Revelli *et al.*, 1991; Rubio *et al.*, 1995a; 1995b), while hyperthyroidism down-regulates β_3-AR mRNA but increases β_1-AR mRNA levels (Adli *et al.*, 1997; Ilyes & Stock, 1990). In white adipose tissue (WAT), hypothyroidism decreases β_3-AR number and mRNA levels (Rubio *et al.*, 1995b), whereas hyperthyroidism increases β_3-AR binding, mRNA, responsiveness and sensitivity to β_3-AR agonists without affecting β_1-ARs (Fain *et al.*, 1997; Germack *et al.*, 1996; 2000; Wahrenberg *et al.*, 1994). In gastrointestinal tissues, hypothyroidism reduces β_3-AR responsiveness but this is not associated with changes in β_3-AR expression (Brown & Summers, 2001). Therefore it appears that thyroid hormone differentially affects the expression of β_3-ARs in BAT and WAT and further supports the notion that distinct regulatory mechanisms are present in ileum as compared to adipose tissues.

GREs have been identified in the 5' non-coding region of the human and mouse β_3-AR (Van Spronsen *et al.*, 1993). These response elements are in close proximity to AP-1 sites and sequences related to FSEs (Emorine *et al.*, 1991). It has been suggested that down-regulation of β_3-AR mRNA by dexamethasone may be due to negative interactions of the glucocorticoid receptor with AP-1 or FSE transcription factors (Feve *et al.*, 1992). The synthetic glucocorticoid, dexamethasone, decreases β_3-AR density and β_3-AR responsiveness in mouse 3T3-F442A adipocytes by reducing β_3-AR mRNA synthesis (Feve *et al.*, 1992). This is reversed by the glucocorticoid antagonist RU38486, suggesting that the glucocorticoid receptor is

the major mediator of dexamethasone inhibition of β_3-AR mRNA expression (Feve *et al.*, 1992). However, in gastrointestinal tissues, dexamethasone treatment of mice does not alter β_3-AR mRNA levels or function (Evans *et al.*, 1998; Hutchinson *et al.*, 2000).

Other regulatory influences on β_3-AR regulation include N-butyric acid (Krief *et al.*, 1994), phorbol esters (Feve *et al.*, 1995), and insulin (El Hadri *et al.*, 1997; Feve *et al.*, 1994).

1.2.2 Alternative splicing

Although many genes encoding GPCRs lack introns in their coding regions, others like the β_3-AR possess introns allowing the possibility of alternate splicing. A number of receptor isoforms result from alternate splicing, where the splice variants differ only in their C-terminal tail e.g. prostaglandin EP_3 receptor (Hasegawa *et al.*, 1996; Irie *et al.*, 1993; Jin *et al.*, 1997; Namba *et al.*, 1993), α_{1A}-AR (Chang *et al.*, 1998; Hirasawa *et al.*, 1995, Suzuki *et al.*, 2000), the 5-hydroxytryptamine (5-HT) receptor subtypes 5-HT_4 (Bach *et al.*, 2001; Gerald *et al.*, 1995; Mialet *et al.*, 2000) and 5-HT_7 (Heidmann *et al.*, 1997; Krobert *et al.*, 2001), somatostatin SSTR2 receptor (Reisine *et al.*, 1993; Schindler *et al.*, 1998; Vanetti *et al.*, 1993) and the endothelin ET_B receptor (Nambi *et al.*, 2000). The C-terminus is believed to contribute to the coupling efficiency between receptor and G-protein. The identification of splice variants that differ only in their C-terminal tails may suggest different interactions with second messenger signalling systems, and possible differences in regulatory properties. In most cases, the splice variants have identical pharmacological properties, and most splice variants show great differences in distribution profiles.

Despite this, several splice variants show great divergence in signalling pathways compared to the previously characterised receptor. The most interesting example is that of the mouse prostaglandin EP_3 receptor. The EP_3 receptor gene is alternatively spliced to generate four different isoforms that differ only in their C-terminal tail (EP_{3A}, EP_{3B}, EP_{3C}, and EP_{3D}) (Namba *et al.*, 1993). When expressed in Chinese

hamster ovary-K1 (CHO-K1) cells, the differences in the C-terminus did not appear to alter ligand-binding specificity. However the four isoforms couple to different second messenger systems (Namba *et al.*, 1993). Other splice variants of the EP_3 receptor, $EP_{3\alpha}$, $EP_{3\beta}$ and the $EP_{3\gamma}$, couple to different G-proteins ($EP_{3\alpha}$ and $EP_{3\beta}$ to $G\alpha i$, $EP_{3\gamma}$ to $G\alpha s$ and $G\alpha i$) (Irie *et al.*, 1993; Negishi *et al.*, 1996). These later splice variants are distinct from the splice variants reported by Namba *et al.* (1993).

1.2.2.1 Alternative splicing of the β_3-AR

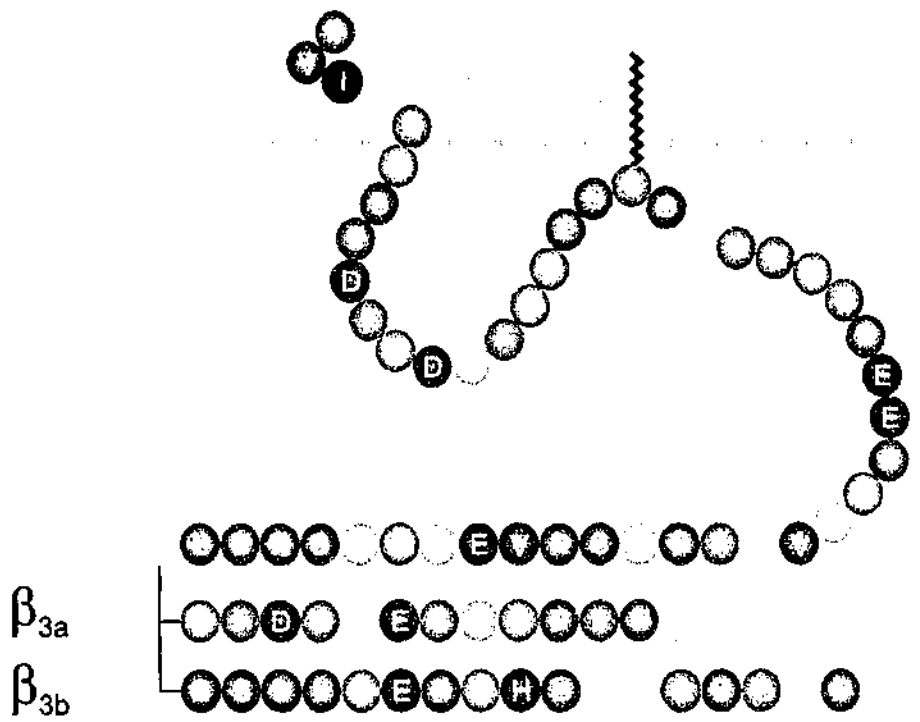
The discovery of introns in the β_3-AR gene (Granneman *et al.*, 1992) increased the search for multiple isoforms of the β_3-AR that would result in the translation of multiple functional receptor proteins with different C-terminals (Bensaid *et al.*, 1993; Granneman *et al.*, 1992; 1993). Previous studies in mice showed that the use of alternate splice acceptor sites results in the generation of two β_3-AR transcripts which differ in the 3' untranslated regions (Granneman & Lahners, 1995; Van Spronsen *et al.*, 1993), but encode the same protein. These transcripts have different tissue expression in WAT and BAT, with the shorter transcript predominant in WAT, and the longer transcript in BAT, but were not differentially regulated (Granneman & Lahners, 1995; Van Spronsen *et al.*, 1993). The use of primers in the 3' untranslated region has demonstrated that there are at least three other transcripts of the mouse β_3-AR (Evans *et al.*, 1999).

The mouse β_3-AR gene contains two introns that lead to alternate splice isoforms (Evans *et al.*, 1999). Alternate splicing of the mouse β_3-AR within the coding region results in the production of mRNA encoding a β_3-AR variant, termed the β_{3b}-AR The β_{3b}-AR is produced by alternate splicing at a novel acceptor site 100bp upstream from the previously characterised start of exon 2. The β_{3b}-AR has an unique C-terminal tail, with 17 aa (SSLLREPRHLYTCLGYP) that differ from the 13 in the known β_{3a}-AR (RFDGYEGARPFPT) (Figure 1.4, 1.5). There is differential expression of the two isoforms in mouse tissues, with lowest proportion of transcripts of the β_{3b}-AR in BAT, and the highest in hypothalamus (Evans *et al.*, 1999).

Figure 1.4: Nucleotide and corresponding aa sequence of the mouse β_3-AR gene (exon 1 and 2, intron 1 and adjacent sequences). Diagram shows the splice donor-1 (nucleotide 1732) and acceptor-1 (nucleotide 2193) sites utilised in β_{3a}-AR mRNA. The novel splice acceptor site utilised in β_{3b}-AR mRNA (nucleotide 2090) is also shown. Evans *et al.* (1999) demonstrated that exon 2 in this mRNA continues through the coding and 3'-untranslated regions found in β_{3a}-AR mRNA. Also boxed are the intron B donor-2 site (nucleotide 2263) and three potential acceptor sites within the 3'-untranslated region, acceptor-2◊ (nucleotide 2458), acceptor-2 (nucleotide 2517) and acceptor-2* (nucleotide 2606) (Evans *et al.*, 1999). Nucleotide numbers correspond to those in the mouse β_3-AR Genbank entry (accession number X72862, locus MMB3A). Amino acids are shown in one-letter code over the second nucleotide of each codon.

```
                                                M  A    P  W  P    H  R  N    G  S  L  A
 544 cttcccaccc caggcgccac acgagATGGC TCCGTGGCCT CACAGAAACG GCTCTCTGGC
       L  W  S    D  A  P    T  L  D  P    S  A  A    N  T  S    G  L  P  G
 604 TTTGTGGTCG GACGCCCCTA CCCTGGACCC CAGTGCAGCC AACACCAGTG GGTTGCCAGG
       V  P  W    A  A  A    L  A  G  A    L  L  A    L  A  T    V  G  G  N
 664 AGTACCATGG GCAGCGGCAT TGGCTGGGGC ATTGCTGGCG CTGGCCACGG TGGGAGGCAA
       L  L  V    I  I  A    I  A  R  T    P  R  L    Q  T  I    T  N  V  F
 724 CCTGCTGGTA ATCATAGCCA TCGCCCGCAC GCCGAGACTA CAGACCATAA CCAACGTGTT
       V  T  S    L  A  A    A  D  L  V    V  G  L    L  V  M    P  P  G  A
 784 CGTGACTTCA CTGGCCGCAG CTGACTTGGT AGTGGGACTC CTCGTAATGC CACCAGGGGC
       T  L  A    L  T  G    H  W  P  L    G  E  T    G  C  E    L  W  T  S
 844 CACATTGGCG CTGACTGGCC ATTGGCCCTT GGGCGAAACT GGTTGCGAAC TGTGGACGTC
       V  D  V    L  C  V    T  A  S  I    E  T  L    C  A  L    A  V  D  R
 904 AGTGGACGTG CTCTGTGTAA CTGCTAGCAT CGAGACCTTG TGCGCCCTGG CTGTGGACCG
       Y  L  A    V  T  N    P  L  R  Y    G  T  L    V  T  K    R  R  A  R
 964 CTACCTAGCT GTCACCAACC CTTTGCGTTA CGGCACGCTG GTTACCAAGC GCCGCGCCCG
       A  A  V    V  L  V    W  I  V  S    A  A  V    S  F  A    P  I  M  S
1024 CGCGGCAGTT GTCCTGGTGT GGATCGTGTC CGCTGCCGTG TCCTTTGCGC CCATCATGAG
       Q  W  W    R  V  G    A  D  A  E    A  Q  E    C  H  S    N  P  R  C
1084 CCAGTGGTGG CGTGTAGGGG CAGATGCCGA GGCACAGGAA TGCCACTCCA ATCCGCGCTG
       C  S  F    A  S  N    M  P  Y  A    L  L  S    S  S  V    S  F  Y  L
1144 CTGTTCCTTT GCCTCCAACA TGCCCTATGC GCTGCTCTCC TCCTCCGTCT CCTTCTACCT
       P  L  L    V  M  L    F  V  Y  A    R  V  F    V  V  A    K  R  Q  R
1204 TCCCCTCCTT GTGATGCTCT TCGTCTATGC TCGAGTGTTC GTTGTGGCTA AGCGCCAACG
       H  L  L    R  R  E    L  G  R  F    S  P  E    E  S  P    P  S  P  S
1264 GCATTTGCTG CGCCGGGAAC TGGGCCGCTT CTCGCCCGAG GAGTCTCCGC CGTCTCCGTC
       R  S  P    S  P  A    T  G  G  T    P  A  A    P  D  G    V  P  P  C
1324 GCGCTCTCCG TCCCCTGCCA CAGGCGGGAC ACCCGCGGCA CCGGATGGAG TGCCCCCCTG
       G  R  R    P  A  R    L  L  P  L    R  E  H    R  A  L    R  T  L  G
1384 CGGCCGGCGG CCTGCGCGCC TCCTGCCACT CCGGGAACAC CGCGCCCTGC GCACCTTAGG
       L  I  M    G  I  F    S  L  C  W    L  P  F    F  L  A    N  V  L  R
1444 TCTCATTATG GGCATCTTCT CTCTGTGCTG GCTGCCCTTC TTCCTGGCCA ACGTGCTGCG
       A  L  A    G  P  S    L  V  P  S    G  V  F    I  A  L    N  W  L  G
1504 CGCACTCGCG GGGCCCTCTC TAGTTCCCAG CGGAGTTTTC ATCGCCCTGA ACTGGCTGGG
       Y  A  N    S  A  F    N  P  V  I    Y  C  R    S  P  D    F  R  D  A
1564 CTATGCCAAC TCCGCCTTCA ACCCGGTCAT CTACTGCCGC AGCCCGGACT TTCGCGACGC
       F  R  R    L  L  C    S  Y  G  G    R  G  P    E  E  P    R  A  V  T
1624 CTTCCGTCGT CTTCTGTGTA GCTACGGTGG CCGTGGACCG GAGGAGCCAC GCGCAGTCAC
       F  P  A    S  P  V    E  A  R  Q    S  P  P    L  N
1684 CTTCCCAGCC AGCCCTGTTG AAGCCAGGCA GAGTCCACCG CTCAACAGGT aggggacacg
1744 agcgggggac cggagtctct gggtggggac gtctctgtct ctatttttga gtttggagat
1804 tgggggaggg gaagatgtag atgggggtgc ggtgtgtgtg tgggtggggg gtggcctttg
1864 tcttgagagg acagaaaaga ggtaggaact aaaacgggcc ctttctcttc ttggatccaa
1924 tccctgggtc tgaagcaaaa gggaggaagg ggataattgc gcaccttagg accaggtgac
1984 ccccacaggc agttgctgct cttccggcag gtttctgacc tctctggtcg cctctagttt
                                                                S  S  L  L  R
2044 ggggtttgtt tgtttttgtt tgtttgtttg tttgttttgt ttttttAGTT CCCTTCTTCG
       E  P  R    H  L  Y    T  C  L  G    Y  P  *
2104 GGAACCCAGG CATCTCTATA CCTGTCTGGG ATATCCATAG acagcaatgg acttccctag
                                          R  F  D  G    Y  E  G    A  R  P
2164 tcctcggcct cagtcccgct ctctctcaaA GGTTTGATGG CTATGAAGGT GCGCGTCCGT
     F  P  T  *
2224 TTCCCACGTG Aagggccgtg aagatccagc aaggaagctg tgagttggct tggagttgct
2284 ttcctccctc agggactgga ttagaactat agggtgggac ttgggggga gggagggtgc
2344 aggatggacc ctatgggatt tgggggtgga gtagagggat gcgggaatgg tccctatatc
2404 cttgaaaagt gaatatgctt ttcagggttc ctgaatcact tccctcttcc ttccagtgct
2464 tgatccccat cttcttgact ggttgcccca agaaatattg tttccgtttt tgccaggactt
2524 ctggggattt ttttttttcct ccagaaagac aagcaacggc tatggatgca acatttttat
2584 aatgcctttg atttctactc agagtgagtc ccctggaacc tcaactctcc aacgctccag
2644 aaccgatgac tagaccacga ggtgtaaggg aaatcttacc aaatgggttt caccgtcctc
```

Figure 1.5: Schematic diagram showing aa sequence of the C-terminal tail of the mouse β_{3a}- and β_{3b}-AR.


I
D
D
E
E
E
β_{3a}
β_{3b}

1.2.3 Tissue localisation of the β_3-AR

Molecular biology techniques have been used to investigate the tissue distribution of β_3-AR mRNA. Studies which used a cDNA probe based on the human β_3-AR sequence showed by Northern analysis β_3-AR mRNA in various rat tissues, including adipose tissue, liver, soleus muscle and ileum (Emorine *et al.*, 1989). However it is likely that the cDNA probe used in these early studies was non-specific and produced artefacts. Subsequent studies have shown in all species examined, that β_3-AR mRNA is present in WAT, BAT and gastrointestinal tissues (Evans *et al.*, 1996; Granneman *et al.*, 1991; 1993; Krief *et al.*, 1993; Nahmias *et al.*, 1991). In rats, levels of β_3-AR mRNA in WAT and BAT is 4-5 times higher than β_1-AR mRNA (Muzzin *et al.*, 1991). In humans, β_3-AR expression has been described by RT/PCR in heart, prostate, bladder, gall bladder and various gastrointestinal tissues such as colon and ileum (Berkowitz *et al.*, 1995; Granneman *et al.*, 1993; Krief *et al.*, 1993), but not in human skeletal muscle, lung, liver, kidney, thyroid or lymphocytes (Berkowitz *et al.*, 1995; Krief *et al.*, 1993). A similar pattern of β_3-AR mRNA expression was observed in the rat (Evans *et al.*, 1996). β_3-AR mRNA was also detected in the human neuroblastoma cell line SK-N-MC (Berkowitz *et al.*, 1995; Granneman *et al.*, 1992). Although early studies failed to detect β_3-AR mRNA in human brain (Berkowitz *et al.*, 1995), β_3-AR mRNA has been detected in various rat (Summers *et al.*, 1995) and human brain regions (Rodriguez *et al.*, 1995; Summers, unpublished observations). Levels in rat were highest in the hippocampus, cerebral cortex and striatum, and lower in the hypothalamus, brainstem and cerebellum (Summers *et al.*, 1995).

Identification of β_3-AR mRNA in heart and skeletal muscle (Berkowitz *et al.*, 1995; Evans *et al.*, 1996; Krief *et al.*, 1993; Summers *et al.*, 1995) has been attributed to β_3-AR mRNA present in intrinsic fat deposits in these tissues (Evans *et al.*, 1996). The presence of significant levels of adipsin mRNA with low amounts of β_3-AR mRNA makes this likely since adipsin is confined to adipose tissue and the myelin sheath of nerves (Cook *et al.*, 1987).

The first antibody raised against the human β_3-AR (an anti-peptide polyclonal antibody; Guillaume *et al.*, 1994) could only identify β_3-ARs in CHO-K1 cells transfected with the human β_3-AR (maximum density of binding sites (B_{max}) 140 fmol/10^6 cell, or 90,000 receptors/cell; Tate *et al.*, 1991) or in human gall bladder which contains high levels of β_3-AR mRNA (Berkowitz *et al.*, 1995; Krief *et al.*, 1993). A further study with this same antibody localised β_3-ARs to vascular and non-vascular smooth muscle in the human gastrointestinal tract (Anthony *et al.*, 1998), and in neuroendocrine cells in the human pancreas and duodenum (Anthony *et al.*, 2000). A monoclonal human β_3-AR antibody detects β_3-ARs in adipose tissue, gall bladder, colon and prostate, which confirmed previous mRNA results, however, β_3-ARs were also detected with this antibody in right atrium (Chamberlain *et al.*, 1999). Commercial polyclonal mouse/rat- or human-reactive β_3-AR antibodies have recently been available from Santa Cruz Biotechnology, although data on the specificity of these antibodies is not yet available.

1.2.4 Structural features of the β_3-AR

The β_3-AR is 408, 400 and 388 aa long in human, rat and mouse respectively. As seen in Figure 1.2, the β_3-AR sequence is conserved with high homology between these three species, with homology even higher in the transmembrane regions (rat and human β_3-AR's are 70% identical and 98% similar in their predicted tm regions; Granneman *et al.*, 1991). In comparison, the human β_3-AR shares only 51% and 46% identity with the human β_1- and β_2-AR aa sequences respectively (Emorine *et al.*, 1989).

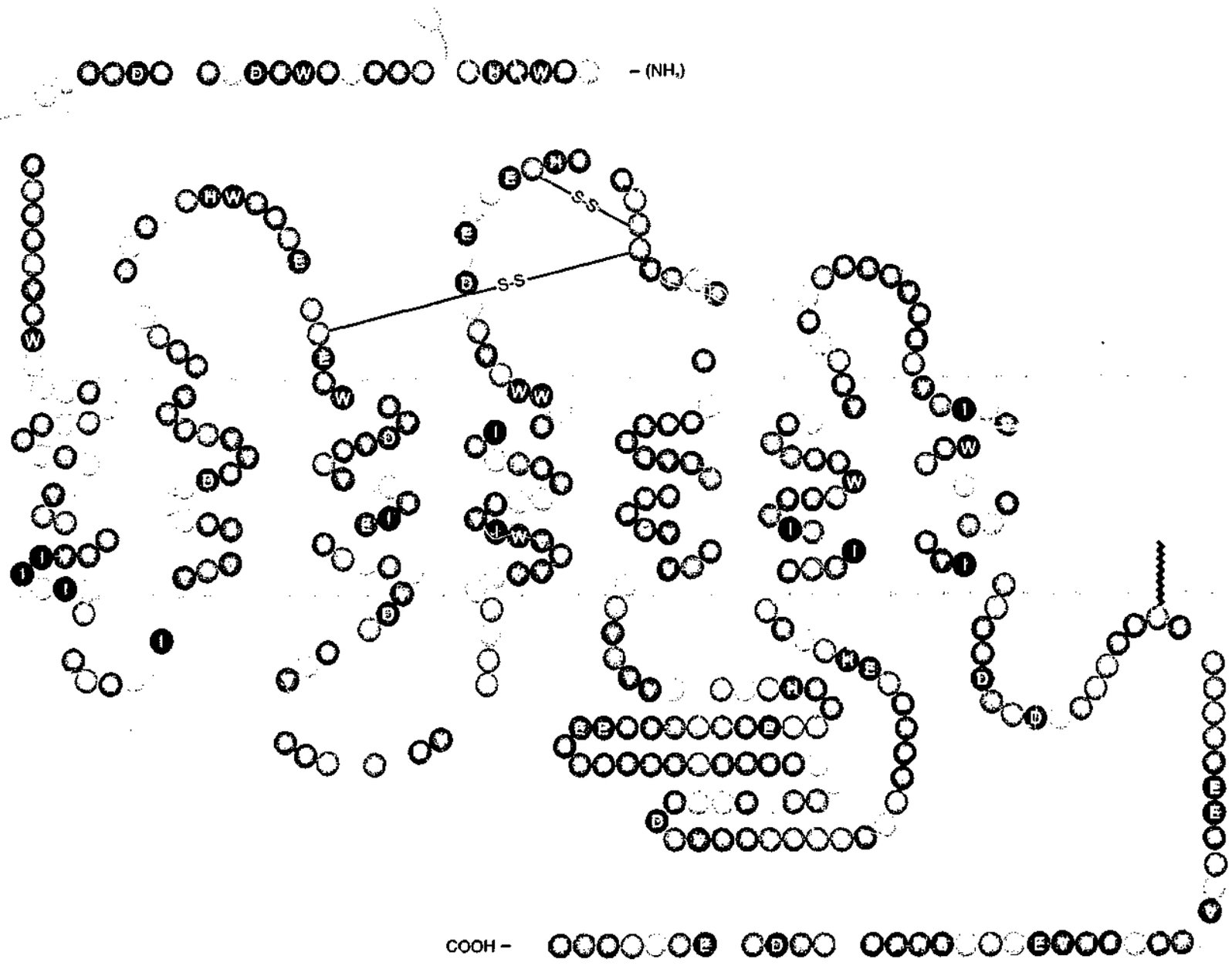
The β_3-AR consists of a single glycosylated polypeptide chain which like the β_1- and β_2-ARs, contains 7 hydrophobic tm α-helices of 21 to 27 aa, separated by hydrophilic segments that make up the intracellular and extracellular loops of the receptor. The N-terminal region of the β_3-AR is extracellular and contains two Asn-X-Ser/Thr consensus sites for N-glycosylation in human, mouse and rat. The C-terminus is intracellular and displays a Cys residue 14 residues distal to the tm7 domain that can be palmitoylated. The ligand-binding pocket of the β_3-AR is formed

in the hydrophobic seven tm domains within the core of the β_3-AR. Figure 1.6 illustrates the structure of the β_3-AR.

1.2.4.1 Ligand binding site of the β_3-AR

Several aa residues in the human β_2-AR have been identified as forming the binding pocket and/or are important in binding properties. These include Asp113 (tm3), Thr164 (tm4), Ser165 (tm4), Ser204 (tm5), Ser207 (tm5), Phe290 (tm6), Asn293 (tm6) and Asn312 (tm7) (Green *et al.*, 1993; Strader *et al.*, 1987; 1989; Suryanarayana & Kobilka, 1993; Wieland *et al.*, 1996). Several residues are conserved in the β_3-AR that appear to be important in ligand-binding interactions. The aa and regions involved in binding of ligands were identified by site-directed mutagenesis of the β_3-AR and the use of chimeric β_3-ARs. The aa residues in the human β_3-AR that have been shown to be involved in ligand interactions are equivalent to those illustrated with the human β_2-AR (although sequence numbers differ, this is due to the different number of aa in the N-terminal tail and the third intracellular loop as seen in Figure 1.1). Asp117 (tm3) is essential for ligand binding in several bioamine receptors including the β_3-AR (Gros *et al.*, 1998) as its acidic side chain has been postulated to form a salt bridge with NA. Gly53 (tm1) was proposed to be involved in β_2-/β_3-AR subtype selectivity and the sequence Val-Leu-Ala (tm1) was proposed to be involved in species specificity although mutation/deletion of these residues showed an unaltered pharmacological profile, dismissing this notion (Gros *et al.*, 1998). Residues Ser209 and Ser212 (tm5) that are located in the binding pocket (Strosberg & Pietri-Rouxel, 1996) are thought to form hydrogen bonds with the hydroxyl groups of the catechol side chain of NA, and are implicated in high affinity binding to BRL37344 (Guan *et al.*, 1995). Asn312 (tm6) is present in all three β-ARs and is essential for binding of β-AR antagonists such as propranolol (Strosberg & Pietri-Rouxel, 1996) but for the β_3-AR, mutation of this residue affects only signal transduction and not binding (Gros *et al.*, 1998). The β_3-AR ligand-binding site has been based on that for the β_2-AR and visualised by computer modelling (Strosberg & Pietri-Rouxel, 1996). In addition to Asp117, Ser209 and Ser212, Ser169 (tm4) and Phe309 (tm6) are proposed to be in the β_3-AR ligand-binding pocket. Ser169 is thought to form hydrogen bonds with the hydroxyl

Figure 1.6: Topological model for the mouse β_{3a}-AR. The sequences are represented in the one-letter aa code.


– (NH₂)
S-S
S-S
COOH –

group of the ethanolamine side chain of NA, while Phe309 is postulated to have a hydrophobic interaction with the aromatic ring of NA (Strosberg & Pietri-Rouxel, 1996). Two other residues located in the binding pocket, Asp83 (tm2) and Tyr336 (tm7) are thought to be involved in Gαs activation. It must be stressed that residues implicated in β_3-AR ligand binding are mostly based on experiments performed for the β_2-AR. There are almost certainly differences in ligand interactions with aa residues that are still undetermined.

The structures of selective β_3-AR agonists are based on the structure of catecholamines, such as NA. Catecholamines are small molecules with a 10-carbon backbone, containing a catechol group (which is a potential hydrogen bond donor) and a positively charged β-hydroxylalkylamine chain that ends in apolar alkyl substitutions. Structural features of β_3-AR ligands include an 18-20-carbon backbone, an aromatic ring, and a hydroxylalkylamine chain ending in an indol, phenyl hydroxyl, ether or acid function, that increases steric bulk and lipophilicity of the ligand (Blin *et al.*, 1993). These long and bulky amine substituents were proposed to occupy the whole space in the β_3-AR ligand-binding site (the binding pocket is larger in the β_3-AR), that included residues in tm regions 1,2, and 7, as well as those in tm 3,4, and 5 that are occupied by β_1- and β_2-AR ligands (Blin *et al.*, 1993). It should be noted that this was inferred from computer modelling only. Replacing β_1-AR tm7 with tm7 from the β_3-AR conferred on β_3-AR ligands an ability to bind and activate β_1-ARs. Conversely, replacing β_3-AR tm7 with that from β_1-ARs reduced the affinity and potency of β_3-AR ligands at the chimeric β_1-/β_3-AR, illustrating that this seventh tm region is important in conferring subtype specificity, with tm7 needed for receptor activation in β_3-ARs (Granneman *et al.*, 1998). Several β_3-AR agonists may act as antagonists at β_1- or β_2-ARs, since these agonists can adopt stacked conformations in the restricted space at β_1- or β_2-ARs, while still having agonistic actions at β_3-ARs in their extended form (Blin *et al.*, 1993).

1.3 Signalling pathways of GPCRs

Binding of an agonist to a GPCR results in an interaction of the receptor with a G-protein, to cause a diverse range of downstream signalling events. G-proteins are heterotrimeric molecules composed of three subunits (Gα, Gβ and Gγ). After receptor-induced exchange of guanosine 5'-diphosphate (GDP) for guanosine 5'-triphosphate (GTP) on the Gα subunit, the Gα and Gβγ subunits dissociate. The GTP-bound Gα subunit continues to interact with its respective second messenger until GTP is hydrolysed to GDP by the GTPase activity of the subunit. The GDP associated Gα subunit then reassociates with the Gβγ subunits. Currently, at least 20 different Gα subunits are known based on primary sequence, but are divided into four main groups based on their structure and function (Gαs, Gαi, Gαq and Gα12). Classically, Gαs activates AC to increase intracellular cAMP levels, Gαi inhibits AC, Gαq activates phospholipase C (PLC) to increase intracellular levels of inositol trisphosphate (IP_3) and diacylglycerol (DAG), while the role of Gα12 is still unclear. At least 6 different Gβ and 12 different Gγ subunits are known based on their primary sequence.

1.3.1 Adenylate cyclase

AC is a membrane bound protein that is responsible for the formation of cAMP from adenosine 5'-triphosphate (ATP). At present, at least 6 different isoforms of AC have been cloned from mammalian systems. AC is activated by Gαs (as well by the plant diterpene forskolin (Seamon & Daly, 1981) and inhibited by Gαi. Two toxins are able to interfere with AC signalling. Cholera toxin (from *Vibrio cholerae*) catalyses the transfer of adenosine 5'-diphosphate (ADP)-ribose from nicotinamide adenine dinucleotide (NAD) to Gαs, thereby blocking its GTPase activity, rendering it permanently activated. Pertussis toxin (PTX) (from *Bordetella pertussis*) catalyses the ADP-ribosylation of Gαi, preventing GDP displacement by GTP, thereby blocking inhibition of AC by Gαi.

In the β_2-AR, the structural determinants for G-protein coupling reside in the second and third intracellular loops and C-terminal tail (Cheung *et al.*, 1989; Liggett *et al.*,

1989; O'Dowd *et al.*, 1988). These regions are well conserved between all three β-AR subtypes, suggesting that all subtypes share a common second messenger pathway by coupling to Gαs. A series of mutant β_3-ARs that lacked a small segment from the amino- or carboxyl-terminal of the third intracellular loop were less effective at stimulating AC than the wild type human β_3-AR (Guan *et al.*, 1995).

The third intracellular loop of the human β_1- and β_3-AR contain 14 and 12 proline residues respectively that are absent in the β_2-AR. This proline rich region has been shown to impair functional Gαs coupling and agonist-promoted sequestration. Removal of this region from the β_1-AR improves AC coupling and increases sequestration of the receptor towards a more β_2-AR phenotype while insertion of this region into the β_2-AR impairs AC coupling and decreases receptor sequestration towards a β_1-AR phenotype (Green & Liggett, 1994).

β_3-ARs couple to Gαs to activate AC, increasing intracellular cAMP levels and activation of protein kinase A (PKA). The first report of β_3-AR coupling to Gαi was in rat adipocytes, where Gαi proteins could limit β_3-AR but not β_1-AR stimulated AC activity (Chaudhry *et al.*, 1994). In 3T3-F442A adipocytes endogenously expressing β_3-ARs, PTX treatment enhanced cAMP accumulation in response to CL316243 (Soeder *et al.*, 1999). Brown adipocytes in primary culture show a 50% increase in BRL37344-stimulated cAMP production in the presence of PTX (Lindquist *et al.*, 2000). In rat ileum, PTX treatment increased responses to β_3-AR activation (Roberts *et al.*, 1999).

1.3.2 Phosphorylation by PKA and βARK

Unlike the β_1- or β_2-AR (Bouvier *et al.*, 1988; Freedman *et al.*, 1995), the distal cytoplasmic tail and third intracellular loop of the mouse, rat and human β_3-AR contain fewer Ser/Thr residues and no consensus sites (Arg/Lys-Arg-(X)-Ser/Thr) for phosphorylation by PKA (Emorine *et al.*, 1989; Granneman *et al.*, 1991; Muzzin *et al.*, 1991). Phosphorylation of these residues by PKA in the β_2-AR results in rapid uncoupling of the receptor from Gαs (Bouvier *et al.*, 1989) since phosphorylation

sites in the third intracellular loop adjoin regions of the β_2-AR that interact with Gαs (O'Dowd *et al.*, 1988; Strader *et al.*, 1987).

G-protein coupled receptor kinase (GRK) phosphorylation of Ser/Thr residues in the C-terminal tail of the β_2-AR results in rapid agonist promoted homologous desensitisation of the receptor (Bouvier *et al.*, 1988) that requires the presence of β-arrestin which appears to interfere with Gαs activation of AC (Lohse *et al.*, 1990). Replacement or substitution of the Ser/Thr residues in the C-terminal tail resulted in delays in the onset of desensitisation in the β_2-AR (Bouvier *et al.*, 1988). Comparison of the molecular structure of the three β-AR subtypes shows that in contrast to the 11 Ser/Thr residues in the distal cytoplasmic tail in the β_2-AR, there are only 3 Ser residues in the same region of the β_3-AR (Emorine *et al.*, 1989; Frielle *et al.*, 1987; Kobilka *et al.*, 1987a). Thus it was thought that the β_3-AR was less liable to agonist promoted desensitisation compared to the β_2-AR as it lacked the structural requirements involved in β_1- and β_2-AR desensitisation. In addition, the Ser residues that are present are not in close proximity to acidic residues which have been shown to be important in β-adrenergic receptor kinase (βARK) phosphorylation (Onorato *et al.*, 1991). As these sites are not present in the C-terminal tail or third intracellular loop of the β_3-AR, this may contribute to the lack of agonist promoted desensitisation of the β_3-AR, which is supported by studies with chimeric β_2-/β_3-ARs (Liggett *et al.*, 1993; Nantel *et al.*, 1993), although determinants in areas other than the C-terminus may be involved.

1.3.3 Phosphorylation by tyrosine kinase

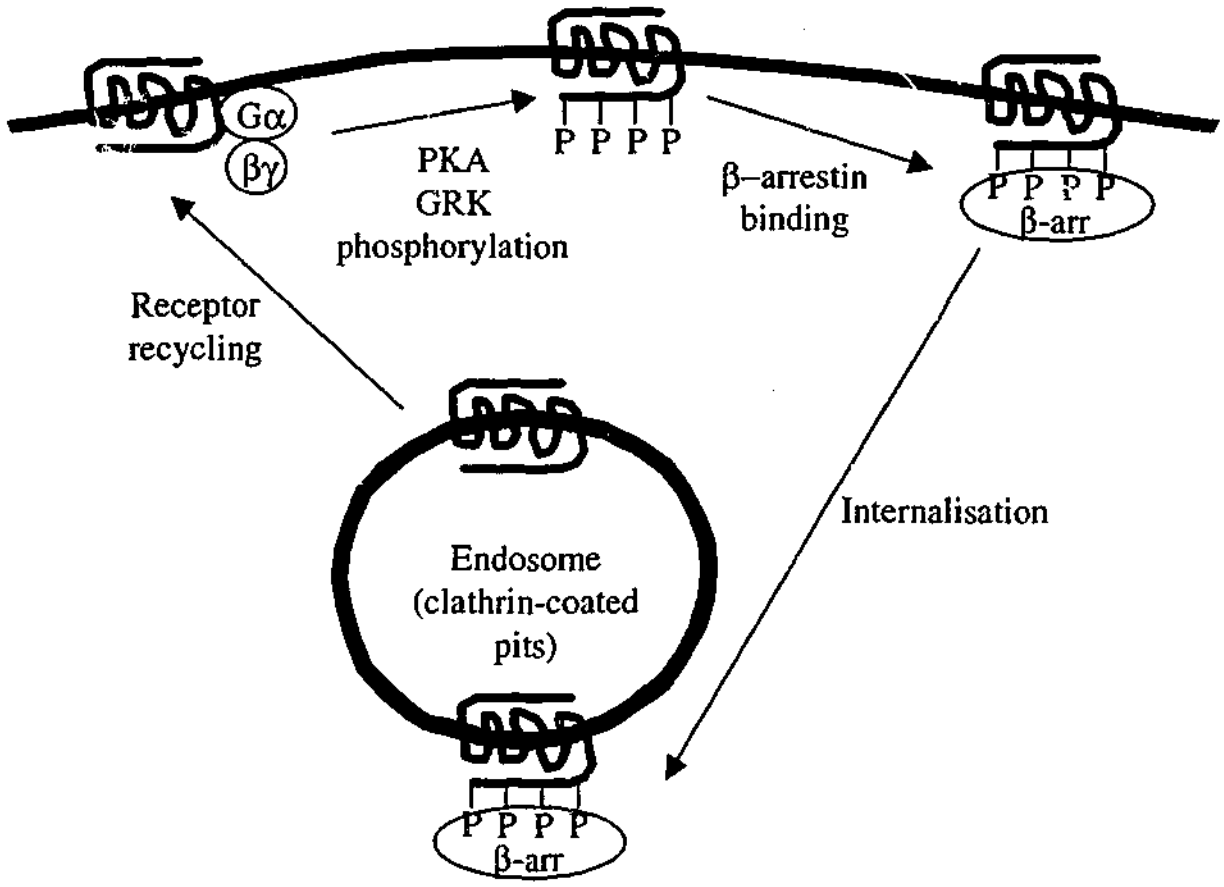
Tyrosine residues (Tyr350, Tyr354) present in the C-terminal tail of the β_2-AR are target sites for tyrosine kinase (TK) that has been shown to be important in agonist promoted down-regulation of the β_2-AR (Valiquette *et al.*, 1990). Replacement of these residues with alanine resulted in 50% less down-regulation of the receptor as compared to the wild type (Valiquette *et al.*, 1990). The β_3-AR contains none of these tyrosine residues and therefore would not be expected to be susceptible to phosphorylation by TK.

1.3.4 Receptor internalisation/endocytosis/sequestration and desensitisation

Endocytosis is defined as the movement of receptor-ligand from the cell surface to an internal compartment, occurring within minutes of agonist application. This process occurs in the following manner: binding of β-arrestin to the phosphorylated receptor causes two effects, it uncouples the receptor from its G-protein leading to a decreased receptor signalling, and initiates the process of receptor internalisation (also termed sequestration) to endosomes. The β-arrestin-bound, desensitised GPCRs undergo β-arrestin-mediated targeting to clathrin-coated pits where they are sequestered, internalised and either recycled to the cell surface or targeted for degradation (see Figure 1.7). GPCRs are only endocytosed in response to agonist stimulation, thought to be due to exposure of an endocytosis motif in the receptor after agonist activation. Endocytosis motifs exist for receptors for low density lipoprotein (LDL) and transferrin. Mutational analysis has revealed that the cytoplasmic domains YXXZ and NPXY (Z is a hydrophobic residue) act as recognition sequences for endocytosis (Trowbridge *et al.*, 1993). Similar motifs exist in the β_2-AR (NPLIY) and many other GPCRs contain the motif $NPX_{2,3}Y$ in their tm7 region but their functional relevance to endocytosis is unclear (Barak *et al.*, 1994; 1995; Laporte *et al.*, 1996; Slice *et al.*, 1994).

The β_3-AR lacks the 10 aa sequence in the C-terminal tail (SSNGNTGEQS) which has been implicated in β_2-AR sequestration (Hausdorff *et al.*, 1989; 1991). Phosphorylation of the β_2-AR leads to sequestration, a process whereby the phosphorylated receptor is internalised to an intracellular compartment, dephosphorylated, and then returned to the plasma membrane (Sibley *et al.*, 1986). The β_2-AR, which contains the highest number of phosphorylation sites, is also the most prone to receptor sequestration, whereas the β_1-AR which contains fewer phosphorylation sites undergoes agonist promoted sequestration to a lesser extent (Frielle *et al.*, 1987). The β_3-AR does not undergo agonist induced sequestration (Jockers *et al.*, 1996; Liggett *et al.*, 1993; Nantel *et al.*, 1993) and also contains the fewest number of phosphorylation sites of the three β-AR subtypes. Studies with chimeric β_2-/β_3-ARs indicated that regions in the second and third intracellular loop

Figure 1.7: Schematic drawing of homologous desensitisation (endocytosis) for the β_2-AR. The activated receptor is phosphorylated by PKA and GRK on specific residues on its intracellular domain, specifically on the third intracellular loop and C-terminal tail. The phosphorylated receptor dissociates from its respective G-protein (Gα). This allows β-arrestin to bind to these phosphorylated residues, which promotes internalisation of the receptor to intracellular endosomes (clathrin-coated pits). Here, the receptor is dephosphorylated and recycled back to the plasma membrane. Alternatively, the receptor may be degraded if not recycled.


Gα
βγ
PKA
GRK
phosphorylation
P P P P
β–arrestin
binding
P P P P
β-arr
Receptor
recycling
Internalisation
Endosome
(clathrin-coated
pits)
P P P P
β-arr

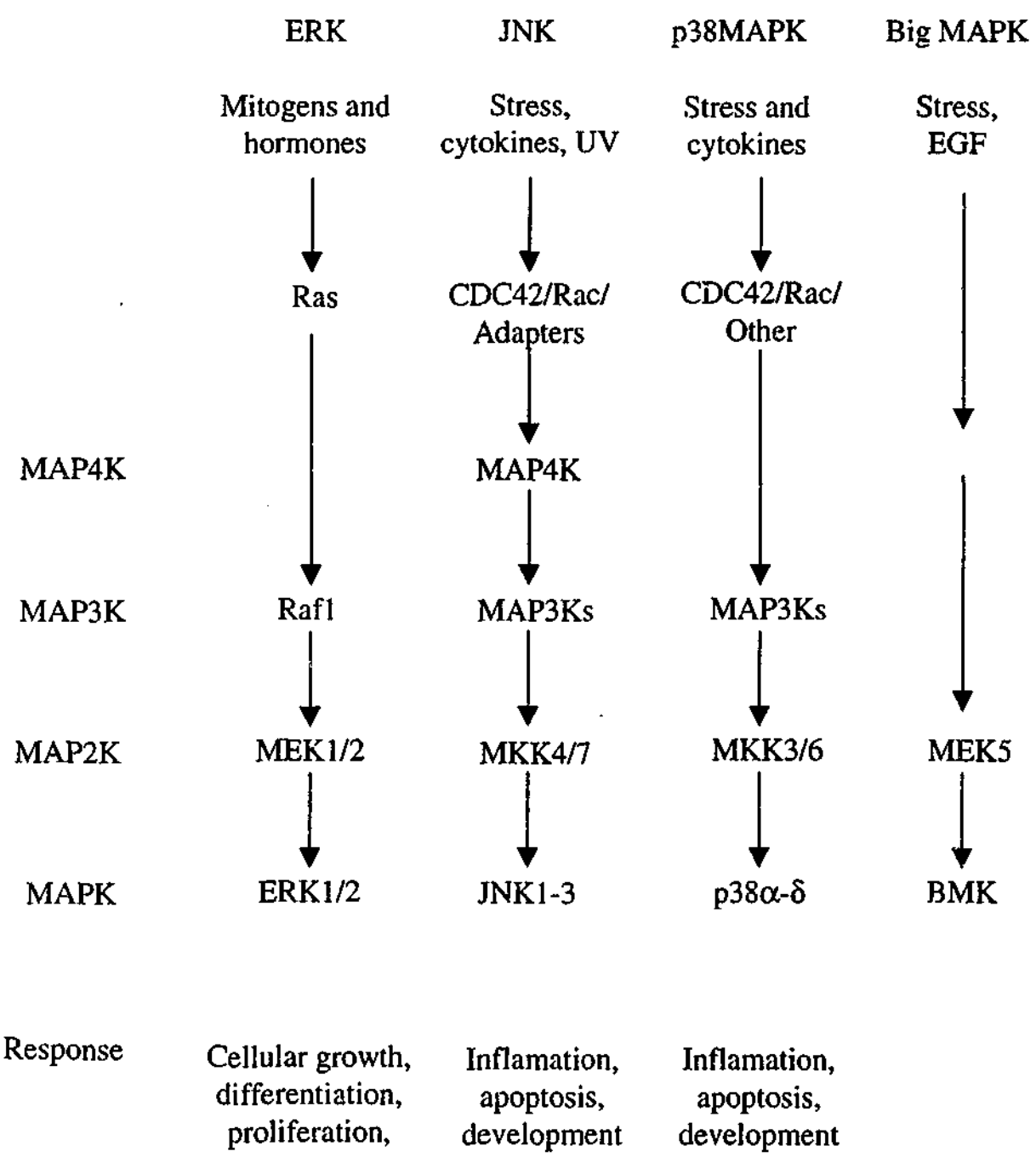
and C-terminal tail of the β_2-AR are important for agonist promoted sequestration (Jockers *et al.*, 1996; Liggett *et al.*, 1993; Nantel *et al.*, 1993).

The β_3-AR, while not undergoing endocytosis or phosphorylated by kinases such as βARK, can be down-regulated. Acute exposure of β_3-ARs to selective and non-selective agonists (or elevation of sympathetic nervous system activity by cold exposure) results in down-regulation of β_3-AR gene expression and/or β_3-AR mediated responses in adipose tissue, adipocytes, gastrointestinal smooth muscle and various cell lines transfected with β_3-ARs that contain the 5' upstream promoter of the gene (Bengtsson *et al.*, 1996; Feve *et al.*, 1992; 1994; Granneman & Lahners, 1992; 1994; 1995; Hutchinson *et al.*, 2000; Klaus *et al.*, 1995; McLaughlin & MacDonald, 1990; Nantel *et al.*, 1994; Revelli *et al.*, 1992). In adipose tissue, down-regulation of β_3-AR function is associated with down-regulation of β_3-AR mRNA levels, but this is not the case in gastrointestinal smooth muscle where agonist exposure does not alter β_3-AR mRNA levels but does cause desensitisation (Hutchinson *et al.*, 2000).

1.3.5 The MAP kinase cascade

Mitogen activated protein kinase (MAPK) cascades participate in a wide variety of cellular responses, most notably cellular differentiation, proliferation, movement, and apoptosis. These cascades are organised in a three-tier scheme, consisting of a MAPK kinase activator (MAP3K), a MAPK activator (MAP2K) and a MAPK, which sequentially activate each other by phosphorylation. Activation of the cascade occurs by small binding or adaptor proteins signalling either directly or indirectly to MAP3K, where the signal is then relayed down the cascade. In mammalian systems, at least four MAPK cascades have been identified, which include the extracellular signal-regulated kinase 1/2 (Erk1/2), c-Jun N-terminal kinase (JNK), p38MAPK and big MAPK cascade. Each cascade appears to mediate different responses. While activation of Erk1/2 regulates cellular growth and differentiation, activation of JNK or p38MAPK is implicated in stress responses such as inflammation and apoptosis (Figure 1.8 for scheme of several MAPK cascades).

Figure 1.8: MAPK cascades.


ERK
JNK
p38MAPK
Big MAPK
Mitogens and hormones
Stress, cytokines, UV
Stress and cytokines
Stress, EGF
Ras
CDC42/Rac/ Adapters
CDC42/Rac/ Other
MAP4K
MAP4K
MAP3K
Raf1
MAP3Ks
MAP3Ks
MAP2K
MEK1/2
MKK4/7
MKK3/6
MEK5
MAPK
ERK1/2
JNK1-3
p38α-δ
BMK
Response
Cellular growth, differentiation, proliferation, development
Inflamation, apoptosis, development
Inflamation, apoptosis, development

1.3.5.1 Components of Erk1/2 cascade

1.3.5.1.1 Raf

Raf proteins are Ser/Thr specific kinases that phosphorylate and thereby activate MAPK/Erk1/2 kinase (MEK). Three forms of Raf have been identified: A-Raf, B-raf and Raf-1 (C-Raf). Raf activation involves several events that include its interaction with small GTP-binding proteins such as Ras and Rap-1, and phosphorylation of multiple residues. Ras activation of Raf occurs by translocation of Raf to the membrane and mediation of its activation (Sun *et al.*, 2000), although Ras can also activate other pathways such as activation of the phosphoinositide 3-kinase (PI3K) pathway.

1.3.5.1.2 MEK

Several upstream regulators including Raf activate MEK by phosphorylating its Ser/Thr residues. The interaction between Raf and MEK occurs through proline-rich domains unique to MEK in the cytosol or plasma membrane since the interaction between Ras and Raf occurs at the plasma membrane. MEK, once activated, can in turn activate Erk1/2 by phosphorylating its tyrosine and threonine residues. MEK can be inhibited by PD98059 (Dudley *et al.*, 1995) by binding to MEK1, thereby preventing activation by Raf through allosteric mechanisms.

1.3.5.1.3 ERK1/2

Activation of Erk1/2 requires phosphorylation of Thr and Tyr residues (Boulton *et al.*, 1991). Phosphorylated Erk1/2 can bind to and phosphorylate cytoplasmic and nuclear targets, its substrate recognition sequence being Pro-Leu-(Ser/Thr)-Pro or (Ser/Thr)-Pro (Seger & Krebs, 1995). Hence Erk1/2 can potentially affect many different proteins. Erk1/2 can affect transcriptional factors such as the Ets family which include Elk-1, Sap-1 and Sap-2 (Yang *et al.*, 1998), which can then regulate p53, c-fos, c-jun and c-myc genes (Wasylyk *et al.*, 1998). Erk1/2 can affect cytoskeletal proteins and upstream protein kinases, including tyrosine kinase

receptors (TKRs) such as the epidermal growth factor receptor (EGFR) (Northwood *et al.*, 1991), suggesting that Erk1/2 may be able to negatively regulate its own activity.

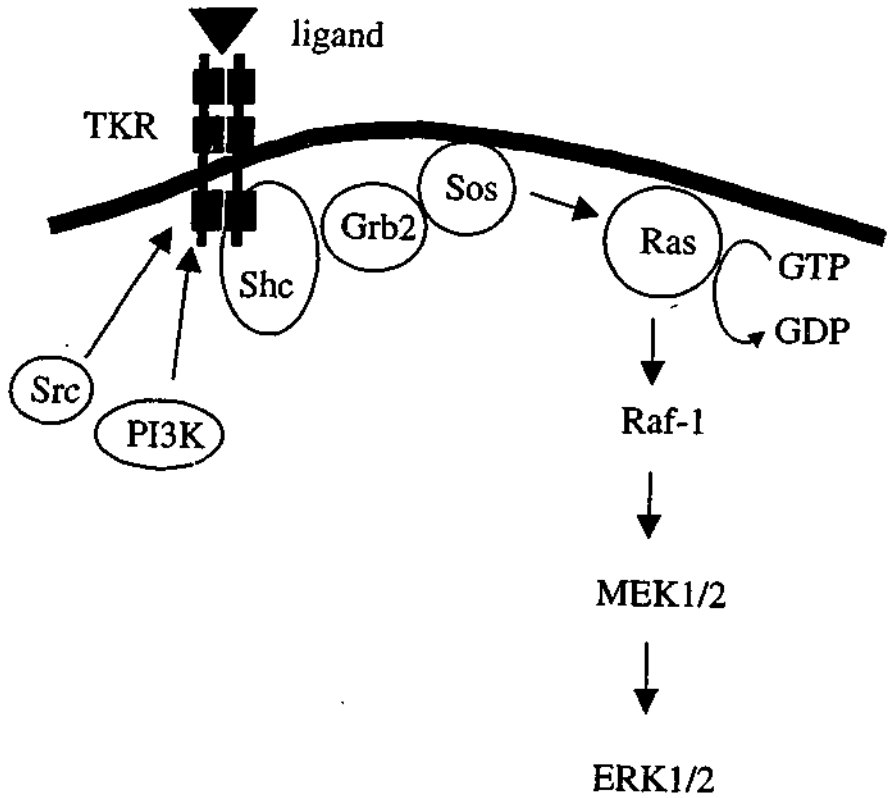
1.3.5.2 Activation of ERK1/2 by TKRs

Classically, activation of Erk1/2 occurs through stimulation of various TKR, such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor. Following activation of the receptor, dimerisation, and autophosphorylation of its intracellular Tyr residues, a series of interactions with intracellular proteins occurs before activation of the Erk1/2 pathway. The intracellular domains of TKRs have a Src homology 2 (SH2) domain which binds an array of proteins with SH2 binding motifs, including enzymes activated by Tyr phosphorylation (ie. Src and PI3K) or adaptor/docking proteins (eg. Shc and growth factor-receptor-bound protein-2 (Grb2). These adaptor/docking proteins recruit guanine nucleotide exchange factors (ie. Sos) to the receptor complex, to then allow further activation of small GTPases like Ras or Rap, and hence activation of the Erk1/2 pathway. This scheme of Erk1/2 activation by TKRs has been simplified and is shown diagrammatically in Figure 1.9.

1.3.5.3 Activation of ERK1/2 by GPCRs

GPCRs usually signal rapidly through intracellular second messengers such as cAMP, DAG, IP_3 and calcium. But some GPCRs can also signal through TK signalling pathways that are activated by TKRs to modulate long-term cellular responses. The MAPK/Erk1/2 pathway is known to be involved in the proliferation and differentiation of several cell types, including adipocytes (Sale *et al.*, 1995). β_3-AR agonist treatment can increase rodent and canine BAT (Champigny *et al.*, 1991; Ghorbani *et al.*, 1997) and β_3-AR activation can stimulate adipocyte differentiation in cultured mouse brown adipocytes (Bronnikov *et al.*, 1992) leading to suggestions that Erk1/2 may play a critical role in cell survival in brown adipocytes (Lindquist & Rehnmark, 1998). β_3-ARs may therefore couple to pathways linked to cellular growth and differentiation.

Figure 1.9: Model of Erk1/2 activation by TKRs. Ligand binding to TKRs causes dimerisation of the receptor and autophosphorylation of intracellular Tyr residues. The components of the cascade vary greatly depending on the receptor and stimuli but usually include adaptor/docking proteins (Shc, Grb2, etc) which recruit guanine nucleotide exchange factors (Sos, etc), which then transduce the signal to small GTPases (Ras, Rap) that can activate the MAPK cascade. Enzymes such as Src and PI3K are able to bind to intracellular domains on the activated receptor.


ligand
TKR
Sos
Grb2
Ras
GTP
GDP
Shc
Src
PI3K
Raf-1
MEK1/2
ERK1/2

β_3-ARs have recently been shown to activate Erk1/2 in a wide variety of cell lines transfected with a recombinant receptor or in cells endogenously expressing the receptor. The mechanism of Erk1/2 activation is wide and in some cases complex. The discussion below illustrates potential mechanisms that may be involved in Erk1/2 activation by GPCRs. In general, these receptors can couple to MAP kinase via pertussis toxin-sensitive (Gαi or Gαo) and pertussis toxin-insensitive (Gαs or Gαq) mediated pathways.

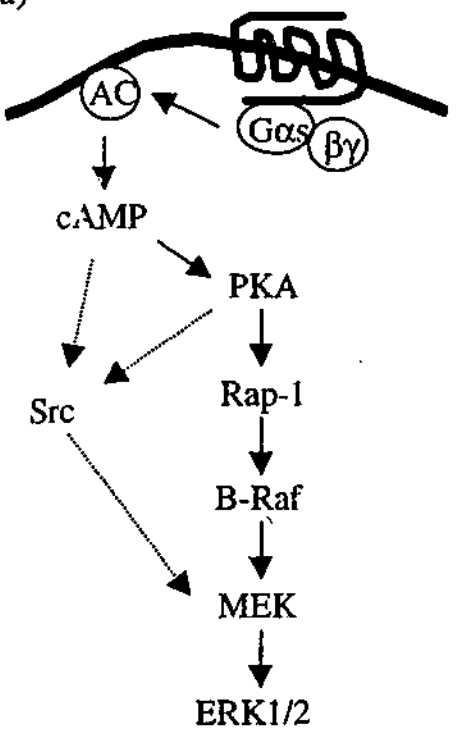
Studies investigating Erk1/2 activation by β_3-ARs have been performed in several cell models including the adipocyte-like cell line 3T3-F442A (Soeder *et al.*, 1999) and the white adipocyte-like cell line C3H10T1/2 (Cao *et al.*, 2000) both of which endogenously express the β_3-AR, and in cells transfected with human (Gerhardt *et al.*, 1999) or mouse (Cao *et al.*, 2000) β_3-AR. In all of these cells, the β_3-AR mediates Erk1/2 activation via a PTX-sensitive Gαi/o protein and is independent of β_3-AR activation of AC (Figure 1.10). However other studies using mouse primary brown adipocytes (Lindquist *et al.*, 2000) or 3T3-L1 adipocytes (Mizuno *et al.*, 1999; 2000) showed that this Gαi/o pathway is not universal for β_3-AR activation of Erk1/2, since in these studies Erk1/2 activation was mediated via the classical cAMP/PKA pathway (Figure 1.10).

1.3.5.3.1 Gα proteins

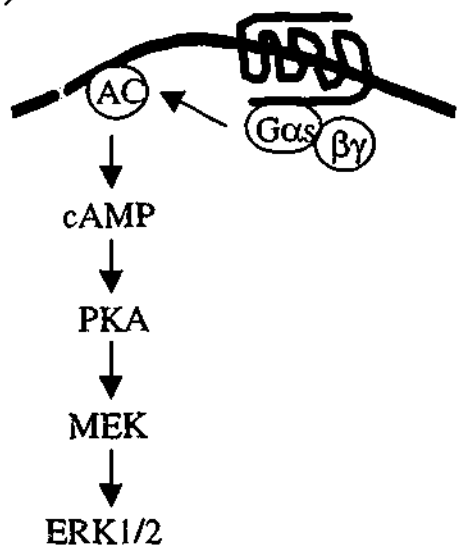
Gαs activation stimulates AC to increase intracellular levels of cAMP, which can then activate either PKA or the cAMP-responsive Ras-guanine nucleotide exchange factor (Ras-GEF) Epac by a PKA-independent mechanism (Beebe, 1994; De Rooij *et al.*, 1998). Both of these factors can then activate Rap and precipitate the MAPK cascade. The action on MAPK can be either inhibitory or stimulatory and appears to be cell specific. In nerve and endocrine cells, such as pheochromocytoma-12 (PC12) and thyroid cells, that express B-Raf, this action is stimulatory. In fibroblast and haematopoietic cells that express mainly Rap-1, the action on the MAPK cascade is inhibitory. This however is a very generalised concept.

Figure 1.10: Proposed models for β_3-AR activation of Erk1/2 MAP kinase in (a) primary brown adipocytes (Lindquist *et al.*, 2000), (b) 3T3-L1 adipocytes (Mizuno *et al.*, 1999; 2000), (c) CHO-K1 cells transfected with the human β_3-AR (Gerhardt *et al.*, 1999), or (d) HEK293 cells transfected with the human β_3-AR (Soeder *et al.*, 1999).

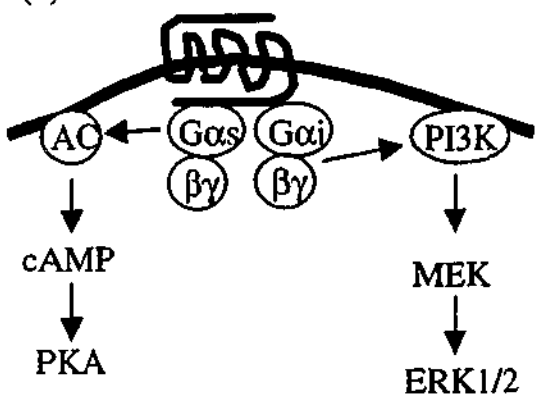
(a)


AC
Gαs
βγ
cAMP
PKA
Src
Rap-1
B-Raf
MEK
ERK1/2

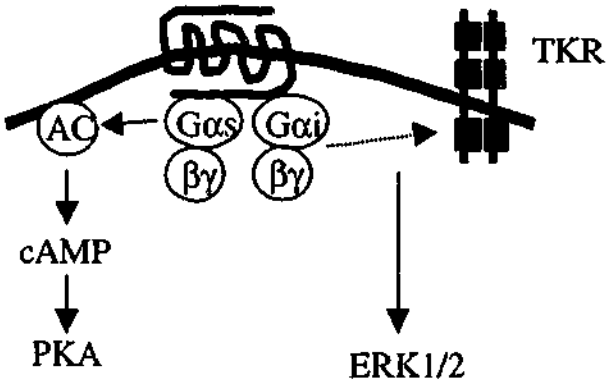
(b)


AC
Gαs
βγ
cAMP
PKA
MEK
ERK1/2

(c)


AC
Gαs
Gαi
βγ
βγ
PI3K
cAMP
PKA
MEK
ERK1/2

(d)


TKR
AC
Gαs
Gαi
βγ
βγ
cAMP
PKA
ERK1/2

Activation of endogenous β_2-ARs in human embryonic kidney293 (HEK293) cells activates Erk1/2 in a manner requiring PKA, suggesting the involvement of Gαs and cAMP. This effect was mimicked by forskolin (Schmitt & Stork, 2000). The involvement of Gαs and cAMP was also observed in primary brown adipocytes expressing the β_3-AR (Lindquist *et al.*, 2000) and in 3T3-L1 adipocytes (Mizuno *et al.*, 1999; Mizuno *et al.*, 2000). In both systems, Erk1/2 activation was insensitive to PTX and in one study sensitive to cholera toxin (Mizuno *et al.*, 2000). This is supported by studies showing cAMP activation of Erk1/2 in other cell models and by transfection of constitutively activated Gαs (as well as Gαq and Gα12/13) to increase Erk1/2 levels (Faure *et al.*, 1994; Frodin *et al.*, 1994; Voyno-Yasenetskaya *et al.*, 1994). However an earlier report showed that isoprenaline and forskolin actually inhibited Erk1/2 activation by insulin or EGF in rat adipocytes (Sevetson *et al.*, 1993).

The action of Gαi on MAPK appears to result from the free Gβγ subunits released following Gαi activation by receptor mediation and not from the Gαi subunit itself (explained in greater detail below) since transfection of cells with constitutively activated Gαi is ineffective in activating Erk1/2 (Faure *et al.*, 1994). However, several reports show direct interaction of Gαi/o with Rap-Gap (Jordan *et al.*, 1999; Mochizuki *et al.*, 1999) to affect Erk1/2 activity by either stimulation or inactivation of Rap-1 to inhibit or stimulate Erk1/2 activity respectively.

Gerhardt *et al.* (1999) and Soeder *et al.* (1999) postulated that the human β_3-AR expressed in either CHO-K1 or HEK293 cells signals through a non-Gαs coupled mechanism to increase Erk1/2 phosphorylation. CGP12177A increased Erk1/2 phosphorylation (and cAMP levels), while forskolin, while increasing cAMP levels, had no effect on Erk1/2 phosphorylation. This elevation of Erk1/2 by CGP12177A was insensitive to H89 (thereby not PKA dependent) but was sensitive to PTX (suggesting a Gαi mediated pathway, independent of effects on cAMP) (Gerhardt *et al.*, 1999). Similarly, CL316243 increased Erk1/2 phosphorylation 4 fold, and this effect was inhibited by PTX but insensitive to H89. Dibutyryl cAMP had no effect on Erk1/2 phosphorylation (Soeder *et al.*, 1999). These studies suggested that cAMP was not required for activation of Erk1/2 and that another pathway must exist.

However the pathway for β_3-AR activation of Erk1/2 independent of the cAMP pathway may be cell specific. β_3-AR activation of Erk1/2 in primary mouse brown adipocytes is sensitive to H89 and insensitive to PTX, with forskolin able to increase Erk1/2 phosphorylation in a PKA-dependent manner, suggesting a Gαs and not a Gαi/o pathway (Lindquist *et al.*, 2000). Similarly, Mizuno *et al.* (1999; 2000) demonstrated that β_3-AR activation of 3T3-L1 adipocytes was sensitive to both cholera toxin and H89 but not to PTX suggesting a Gαs and not Gαi/o mediated pathway for Erk1/2 activation.

Studies using H89 (PKA inhibitor (Engh *et al.*, 1996)) should be examined closely, especially those involving β_2-ARs, since H89 is a potent antagonist at β_1-/β_2-ARs (Penn *et al.*, 1999) but not at β_3-ARs (Fredriksson *et al.*, 2001).

1.3.5.3.2 Gβγsubunits

There are suggestions that Gβγ subunits are responsible for GPCR mediation of Erk1/2. Gβγ signalling from Gαs coupled receptors has not been reported in detail. However, Gβγ release from Gαi and Gαq is known to activate a number of intracellular kinases including PI3K (Hawes *et al.*, 1996; Lopez-Ilasaca *et al.*, 1997), Src (Luttrell *et al.*, 1996) and Erk1/2 (Florio *et al.*, 1999; Luttrell *et al.*, 1995). Gβγ subunits are also able to activate the protein Ser/Thr kinase suppressor of Ras (KSR-1) to inhibit Erk1/2 activation (Bell *et al.*, 1999) and cause phosphorylation-dependent activation of Ras-GRK leading to Ras and Erk1/2 activation (Mattingly & Macara, 1996). Other studies show that transactivation of TKRs by GPCRs (section 1.3.5.3.5) is mediated by Gβγ subunits, where Gβγ over-expression can increase TKR expression and sustained activation of Erk1/2 (Crespo *et al.*, 1994; Hawes *et al.*, 1995; Luttrell *et al.*, 1997b). Most evidence for Gβγ activation of Erk1/2 comes from studies where PTX inhibits, and the over-expression of Gβγ activates Erk1/2 activation (Crespo *et al.*, 1994; Faure *et al.*, 1994; Koch *et al.*, 1994). Additionally, sequestration of Gβγ by binding to transfected α subunit of retinal transducin or to PH domain of over-expressed βARKCT inhibits Erk1/2 activation by Gαi linked

GPCRs (Crespo *et al.*, 1994; Faure *et al.*, 1994; Koch *et al.*, 1994; Luttrell *et al.*, 1995).

β_2-AR activation of Erk1/2 had been suggested to be mediated by G$\beta\gamma$ subunits released from Gαs (Crespo *et al.*, 1995) but it was later hypothesised that phosphorylation of β_2-ARs by PKA switches coupling of the receptor from Gαs to Gαi, and it is the G$\beta\gamma$ subunits released from Gαi that are responsible for Erk1/2 activation (Daaka *et al.*, 1997). This mechanism may not be universal in β_2-AR stimulation of Erk1/2 since a role for Gαs in the signal transduction from β_2-AR activation to Erk1/2 is still observed in Gαs and PKA-deficient S49 mouse lymphoma cells (Wan & Huang, 1998) and in HEK293 cells endogenously expressing β_2-ARs (Schmitt & Stork, 2000). Similarly in primary human endothelial cells, isoprenaline induced Erk1/2 activation is insensitive to PTX but down-regulation of Gαs by cholera toxin is effective (Sexl *et al.*, 1997).

While most evidence suggests that G$\beta\gamma$ subunits from Gαi can couple to MAPK pathways, G$\beta\gamma$ subunits from Gαs have been shown to couple to other pathways including inhibition of reduced nicotinamide adenine dinucleotide (NADPH) dependent H_2O_2 generation by isoprenaline in human adipocyte plasma membranes (Krieger-Brauer *et al.*, 2000).

1.3.5.3.3 Src

Src is a non-receptor protein TK and is attached to the plasma membrane by its N-terminal myrsistate modification. Src consists of 4 well-characterised protein domains: a TK domain, and SH2, SH3 and SH4 domains. It is essential for the mitogenic action of both TKRs and GPCRs. When activated, Src phosphorylates a number of cytoskeletal associated proteins and probably contributes to the activation of both Raf-1, PI3K and Shc (Erpel & Courtneidge, 1995; Ptasznik *et al.*, 1995). Src, and its related family members, may interact with activated TKRs since phosphorylation sites on the platelet-derived growth factor receptor (PDGFR) may act as docking sites for the SH2 domain on Src (Mori *et al.*, 1993), and may phosphorylate the EGFR (Maa *et al.*, 1995) although this is not involved in

activating the Erk1/2 cascade (Biscardi *et al.*, 1999; Tice *et al.*, 1999). Src, and Hck (a Src family member), could also be direct effectors of G-proteins (mainly Gαs and Gαi but not Gαq, Gα12 or Gβγ) to stimulate TK activity (Ma *et al.*, 2000). This could explain PKA-independent actions of Gαs on Erk1/2 activity (Gu *et al.*, 2000; Wolfgang *et al.*, 1996).

c-Src is required for Erk1/2 activation by the β_2-AR, and is recruited to the activated receptor, following binding of β-arrestin, by binding of the Src SH3 domain to proline-rich regions of β-arrestin (Barak *et al.*, 1997; Lin *et al.*, 1997; Luttrell *et al.*, 1997b; Miller *et al.*, 2000). A recent report indicated that c-Src associates to a conditional SH2 binding domain of the β_2-AR which is created by phosphorylation of Tyr350, thereby activating c-Src. This in turn leads to GRK2 phosphorylation followed by recruitment of β-arrestin, initiating clathrin-mediated endocytosis (Fan *et al.*, 2001).

Although the intracellular regions of the β_2-AR that are phosphorylated and bound by β-arrestin are absent from the β_3-AR, Erk1/2 activation by β_3-ARs can be inhibited by the c-Src inhibitor PP2 (Cao *et al.*, 2000) and is independent of β-arrestin and involves a novel mechanism. The β_3-AR can directly interact with the SH3 domains of Src through proline-rich motifs (PXXP) located in the third intracellular loop and carboxyl terminus (Cao *et al.*, 2000). In primary brown adipocytes, Erk1/2 activation by both β_3-AR and α_1-AR activation is inhibited by PP2, although the role of Src in this system is still unclear (Src was postulated not to be a target of PKA since PKA is only a Ser/Thr kinase and other steps still unknown must be present) (Lindquist *et al.*, 2000). Src itself was activated following NA, but not EGF, stimulation in this system, suggesting that the EGFR pathway does not involve Src (PP2 was ineffective in inhibiting EGFR Erk1/2 activation) whereas the NA pathway does (Lindquist, personal communication).

1.3.5.3.4 PI3K

PI3K is a lipid kinase with a 85k Dalton (Da) regulatory (containing a SH3 and a SH2 domain) and a 110kDa catalytic subunit, with three subtypes (I-III) described

which are structurally and functionally related to each other. Through the SH2 domain, PI3K is able to bind to phosphorylated tyrosine residues, such as activated TKRs to become activated, as well as being activated by GPCRs. Once activated, PI3K generates phosphatidylinositol-(4,5)-bisphosphate (PIP2) and phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) from inositol lipids. PI3Ks are involved in receptor-regulated signal transduction processes as well as protein trafficking and mitogenesis.

The PI3K subtype involved in Erk1/2 signalling is the PI3Kγ subtype. PI3Kγ can be activated by Gβγ complex (Leopoldt *et al.*, 1998; Zhang *et al.*, 1995) and plays a critical role in linking Gαi and Gαq coupled receptors and Gβγ to the MAPK pathway (Hu *et al.*, 1996; Lopez-Ilasaca *et al.*, 1997), where PI3K acts downstream from Gβγ and upstream of Src-like kinases. Src may be able to bind to the SH3 domain present in PI3K. It is postulated that Gβγ subunits locate PI3Ks to the plasma membrane to allow access to lipid substrates, enhancing the activity of Src-like kinases, leading to the activation of the Shc-Grb2-Sos-Ras pathway and thereby increasing MAPK activity (Lopez-Ilasaca *et al.*, 1997). However, the relationship between PI3K and the MAPK pathway is still debatable and may involve protein kinase C (PKC), protein kinase B (PKB, also referred to as Akt), p21-activated kinase or intrinsic Ser/Thr kinase activity (reviewed in Toker (2000).

Evidence to suggest a role for PI3K in Erk1/2 signalling comes from studies using the inhibitors of PI3K, wortmannin and LY294002. Inhibitors of PI3K (as well as the dominant-inhibitory mutant of Gβγ subunit regulated by PI3Kγ) block Erk1/2 activation by Gαi coupled receptors or Gβγ subunits in COS-7 and CHO-K1 cells (Hawes *et al.*, 1996; Lopez-Ilasaca *et al.*, 1997). However, wortmannin is ineffective in preventing GPCR EGFR transactivation, suggesting that PI3K acts downstream of TKR activation (Daub *et al.*, 1997). Gerhardt *et al.* (1999) showed that β_3-AR stimulation of Erk1/2 was dependent upon PI3K, and that one of the main targets of PI3K, PKB (Franke *et al.*, 1997), is activated by β_3-AR stimulation through Gαi/o-mediated PI3K activation. However, in primary brown adipocytes where β_3-ARs act via a cAMP/PKA dependent pathway to activate Erk1/2, no PI3K involvement is

observed (Lindquist *et al.*, 2000). This could suggest that PI3K involvement in Erk1/2 signalling requires Gβγ.

1.3.5.3.5 Transactivation with TKRs

GPCR induced Erk1/2 activation has been suggested to proceed via the transactivation of TKRs which then precipitates the cascade to increase Erk1/2. The first indirect indication that TKs might mediate the activation of Erk1/2 by GPCRs came from the inhibitory action of genistein, a non-specific TK inhibitor. This TK could either be the TK c-Src or Src-like kinases (discussed above), or TKRs such as PDGFR (inhibited by AG1295), EGFR (inhibited by AG1478) or the insulin-like growth factor receptor (IGFR). At least three TKRs (the PDGFR, EGFR or IGFR) become tyrosine phosphorylated following GPCR activation (Daub *et al.*, 1996; Linseman *et al.*, 1995; Rao *et al.*, 1995). GPCR activation can induce a rapid increase in tyrosine phosphorylation of several other adaptor proteins such as Shc and Grb2-associated binder 1 (Gab1), and association of Shc and Grb2 (Cazaubon *et al.*, 1994; Chen *et al.*, 1996; Daub *et al.*, 1997; Ohmichi *et al.*, 1994; Sadoshima & Izumo, 1996; Vanbiesen *et al.*, 1995) following transactivation of TKRs. Other evidence indicated that Erk1/2 itself can phosphorylate the EGFR, suggesting that transactivation of the EGFR by GPCRs may be an artefact of Erk1/2 phosphorylation of target proteins and not a primary step in activating Erk1/2.

Evidence for transactivation has come from several Gαq and Gαi coupled receptors (Daub *et al.*, 1997; Daub *et al.*, 1996; Grosse *et al.*, 2000a; Maudsley *et al.*, 2000a; 2000b; Pierce *et al.*, 2000). For example, in rat-1 and COS-7 cells, activation of lysophosphatidic acid (LPA), endothelin-1, thrombin or β_2-AR receptors results in tyrosine phosphorylation of the EGFR, and both GPCR induced tyrosine phosphorylation and Erk1/2 activation can be blocked by dominant-negative mutants of the EGFR and/or inhibition of the EGFR by AG1478 (Daub *et al.*, 1996; 1997; Maudsley *et al.*, 2000a). In cells that lack endogenous EGFRs, such as L cells or CHO-K1 cells, LPA or β_2-AR stimulation can transactivate another TKR, the PDGFR to increase Erk1/2, which is sensitive to AG1295 (Herrlich *et al.*, 1998; Maudsley *et al.*, 2000b). NA activation of Erk1/2 through β_1-ARs expressed in PC12

cells acted synergistically with the nerve growth factor receptor (NGFR) to promote cellular differentiation (Williams *et al.*, 1998).

Several other authors have failed to show transactivation of GPCRs with TKRs (Schaeffer & Weber, 1999; Zhong & Minneman, 1999; Lindquist, personal communication), where NA activation of α_1-ARs in PC12 cells, NA activation of β_3-ARs in mouse primary brown adipocytes, or isoprenaline activation of β_2-ARs in HEK293 cells activate Erk1/2 in a manner independent of EGFR transactivation, despite the presence of EGFRs in these cells. Andreev *et al.* (2001) showed through studies using Src and EGF receptor knock-out mice that both Src and the EGF receptor are not essential for GPCR-induced activation of MAPK, although GPCRs may interact with other TKs and other intracellular proteins not investigated in their study.

There is recent evidence of a novel pathway of GPCR-activation of Erk1/2, which utilises heparin-binding EGF (HB-EGF). HB-EGF is a single transmembrane-spanning protein that is proteolytically cleaved by metalloproteases, leading to the "shedding" of a soluble EGFR ligand that is capable of activating the EGFR (Raab & Klagsbrun, 1997). Activation of GPCRs can activate these metalloproteases to release HB-EGF (Prenzel *et al.*, 1999), which can activate EGFRs, which can then activate Erk1/2 (Pierce *et al.*, 2001).

GPCRs signalling to Erk1/2 can also occur through focal adhesion complexes (Luttrell *et al.*, 1999a).

1.3.5.3.6 Endocytosis

A novel mechanism of Erk1/2 activation by GPCRs (and also TKRs) has been proposed where endocytosis of the receptor is needed before signalling to Erk1/2 can occur. These studies have arisen primarily from use of endocytosis inhibitors. EGFR and IGFR activation of Erk1/2 is decreased following inhibition of clathrin-mediated endocytosis (Chow *et al.*, 1998; Vieira *et al.*, 1996) and LPA receptor activation of Erk1/2 is inhibited by concanavalin A, hypertonic medium and potassium depletion

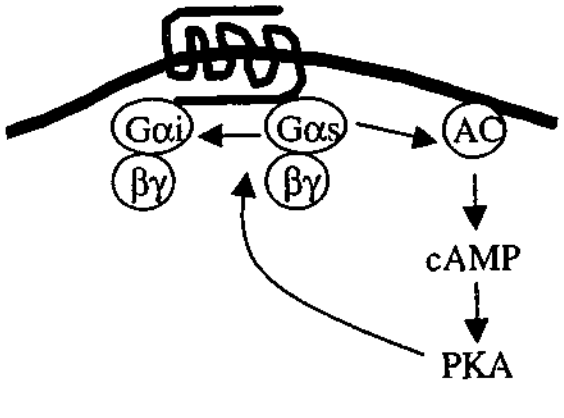
(all inhibitors of GPCR endocytosis) in rat-1 fibroblasts (Luttrell *et al.*, 1997a). Additionally, dominant-negative mutants of β-arrestin-1 or dynamin (GPCR internalisation inhibitors) impair β_2-AR or LPA receptor activation of Erk1/2 in several cell models (Daaka *et al.*, 1998; Ferguson *et al.*, 1996; Luttrell *et al.*, 1999b; Maudsley *et al.*, 2000b; Pierce *et al.*, 2000). Src-mediated tyrosine phosphorylation of dynamin, involved in fission of the budding clathrin-coated vesicle from the plasma membrane, is also involved in β_2-AR internalisation and Erk1/2 activation (Ahn *et al.*, 1999).

These results indicate, as shown in Figure 1.11, that agonist activation of the β_2-AR, following phosphorylation by GRK2, results in β-arrestin-dependent formation of a signalling complex consisting of the receptor, β-arrestin and Src, which then internalises through targeting of this complex to clathrin-coated pits, to activate the Erk1/2 pathway (Ahn *et al.*, 1999; Luttrell *et al.*, 1999b; Maudsley *et al.*, 2000a). This process of internalisation of the β_2-AR to signal to Erk1/2 may involve transactivation of the EGFR to form a more intricate complex (Maudsley *et al.*, 2000a), and Erk1/2 may negatively regulate this process by inactivating GRK2 (Pitcher *et al.*, 1999). Recently, Naga Prasad *et al.* (2001) showed that following β_2-AR activation, β-ARK1 and PI3K can form a cytosolic complex, leading to β-ARK1 mediated translocation of PI3K to the membrane, leading to sequestration of the receptor. However it is unclear if this interaction is involved in β_2-AR signalling to Erk1/2.

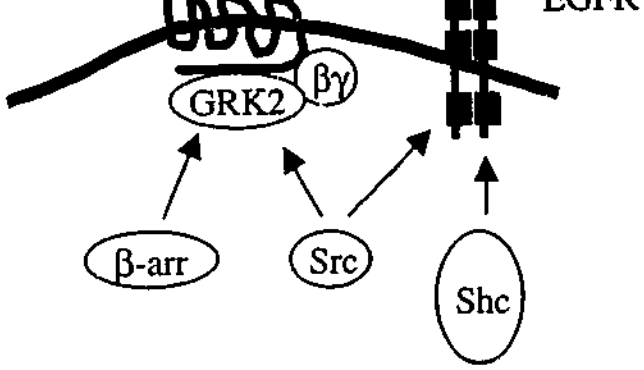
However internalisation to activate Erk1/2 is not always necessary since α_2-AR and opioid receptors can activate Erk1/2 under conditions that would preclude internalisation (De Graff *et al.*, 1999; Li *et al.*, 1999; Schramm & Limbird, 1999; Whistler & von Zastrow, 1999). Additionally, mutant receptors that are unable to undergo endocytosis activate Erk1/2 similar to their wild type counterparts (Blaukat *et al.*, 1999; Budd *et al.*, 1999). Endocytosis appears not to be involved in Erk1/2 activation for β_3-ARs in mouse primary brown adipocytes (Lindquist, personal communication).

Figure 1.11: One proposed model of human β_2-AR activation of Erk1/2. This model has been simplified and other proteins may be involved in this scheme. The steps indicated in this model are not necessarily correct as differing results have been obtained on the order of events by different groups. Step1: Coupling of the β_2-AR to Gαs activates AC to increase cAMP levels. Subsequent activation of PKA phosphorylates the receptor which mediates a switch in receptor coupling from Gαs to Gαi, since Gβγ subunits derived from Gαi appear to be important in Erk1/2 activation by the β_2-AR. Step 2: GRK phosphorylation of the receptor promotes the binding of β-arrestin. Subsequent EGFR transactivation occurs with the β_2-AR, with other proteins such as c-Src and Shc suggested to form part of this complex (one study however suggests that c-Src may bind to the receptor prior to GRK) Step 3: This complex then undergoes internalisation to clathrin-coated pits (endosomes) and hence Erk1/2 activation occurs through the EGFR. This model is based from several studies (Ahn *et al.*, 1999; Daaka *et al.*, 1997; 1998; Ferguson *et al.*, 1996; Luttrell *et al.*, 1999b; Maudsley *et al.*, 2000a; 2000b; Pierce *et al.*, 2000).

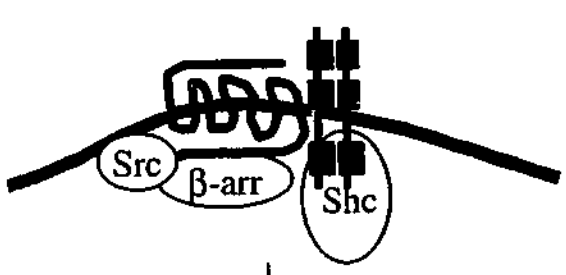
Step 1



Step 2



Step 3



Internalisation

ERK1/2

Further information on GPCR signalling to MAPKs, components of the MAPK cascade and MAPK signalling can be found in several reviews (Brunet & Poiyssegur, 1997; Gudermann *et al.*, 2000; Gutkind, 1998; Lopez-Ilasaca, 1998; Luttrell *et al.*, 1999a; Naor *et al.*, 2000; Schaeffer & Weber, 1999; Sugden & Clerk, 1997; Toker, 2000).

1.4 Functional roles of the β_3-AR

In rodents, β_3-ARs are expressed in adipose tissue (both white and brown adipocytes) where they mediate lipolysis and thermogenesis, and in gastrointestinal tissues (including ileum and colon) where they mediate smooth muscle relaxation. This section will focus on studies using β_3-AR knock-out (KO) mice since several reviews have already extensively illustrated the roles of β_3-ARs in these two tissues (Arch & Kaumann, 1993; Manara *et al.*, 1995).

1.4.1 β_3-AR knock-out mouse models

Two groups have successfully targeted the inactivation of the mouse β_3-AR gene (Revelli *et al.*, 1997; Susulic *et al.*, 1995). Susulic *et al.* (1995) used a novel approach, targeting the β_3-AR in embryonic stem (ES) cells to yield a high targeting frequency and subsequent direct microinjection of the targeting vector in mouse fertilised cells (FVB strain) to obtain two independent targeted mouse lines. Revelli *et al.* (1997) used a more traditional approach of homologous recombination in ES cells and subsequent microinjection into blastocysts (mixed 129 Sv and C57Bl/6 strain background). These animals are susceptible to slowly developing mild increases in fat content, although the compensatory mechanisms in these two models differ. In the Susulic *et al.* (1995) model, there is an increase in β_1-ARs, while in the Revelli *et al.* (1997) model, there is a slight decrease in β_1-ARs. For several early reviews on the physiological and pharmacological consequences of disruption of several adrenergic receptors, and a brief summary of gene modification techniques, the reader is referred to Lowell (1998), Rohrer (1998) and Rohrer & Kobilka (1998).

1.4.2 Adipose tissue

The most convincing evidence for a role of β_3-ARs in lipolysis and thermogenesis comes from studies using the β_3-AR KO model (Grujic *et al.*, 1997; Konkar *et al.*, 2000a; Preitner *et al.*, 1998; Revelli *et al.*, 1997; Susulic *et al.*, 1995). β_3-AR KO mice demonstrate a modest increase in body fat (in females only) in comparison to other genetic models of obesity (Collins *et al.*, 1994; 1999; Evans *et al.*, 1998; Gettys *et al.*, 1997; Hollenga *et al.*, 1991a), which was not associated with increases in food consumption, but rather decreased energy expenditure (Revelli *et al.*, 1997; Susulic *et al.*, 1995). Several explanations for the relative lack of obesity in these animals were given: either the β_3-AR is only marginally involved in thermogenesis and energy balance, or mechanisms develop that compensate for the lack of β_3-ARs, as occurs in other gene KO models (Rudnicki *et al.*, 1992; 1993). In all β_3-AR KO mice there was an abolition of metabolic responses to CL316243 (Grujic *et al.*, 1997; Revelli *et al.*, 1997; Susulic *et al.*, 1995), supporting a role for β_3-ARs in mediating thermogenic, lipolytic and metabolic responses to β_3-AR agonists and re-expression of β_3-ARs in WAT and BAT in these mice completely restores CL316243 mediated effects (Grujic *et al.*, 1997).

In rodent white adipocytes, all three β-AR subtypes can be identified by radioligand binding and functional studies, as well as by detection of mRNA or protein (Bahouth & Malbon, 1988; Bojanic & Nahorski, 1983; Carpene *et al.*, 1994; Collins *et al.*, 1994; 1999; Germack *et al.*, 1997; Granneman, 1992; Hollenga & Zaagsma, 1989; Langin *et al.*, 1991; Ohsaka *et al.*, 1998; Van Liefde *et al.*, 1993). The general consensus from these studies is that there is a major role for β_3-ARs, a minor to important role for β_1-ARs, and a minor to non-existent role for β_2-ARs. In rodent brown adipocytes, β_1- and β_3-ARs were found by radioligand binding, functional and molecular studies, while β_2-ARs were hard to detect (Bengtsson *et al.*, 2000; Bronnikov *et al.*, 1999; Collins *et al.*, 1999; D'Allaire *et al.*, 1995; Muzzin *et al.*, 1992). In bovine perirenal adipose tissue, β_3-AR levels increased dramatically in the third trimester before birth and then virtually disappear after the conversion of brown to white adipose tissue following birth (Casteilla *et al.*, 1994).

Since adipose tissue expresses all three β-ARs, the most likely candidate to compensate for the β_3-AR was the β_1-AR. In the Susulic *et al.* (1995) model, there was up-regulation of β_1-AR mRNA. In contrast, in the Revelli *et al.* (1997) model, the absence of β_3-ARs was not compensated for by an increase in either β_1-/β_2-AR mRNA, and even a decrease in β_1-AR mRNA was observed in BAT of these animals. The differing regulation of β_1-ARs obtained in these two studies (Revelli *et al.*, 1997; Susulic *et al.*, 1995) may be due to the genetic background of the mice and similar differences have been shown for the mouse EGFR KO model (Threadgill *et al.*, 1995).

In human adipose tissue most studies show low or no lipolytic or thermogenic responses to β_3-AR activation, and instead lipolytic and thermogenic responses are mediated primarily by β_1- and β_2-ARs (Bousquet-Melou *et al.*, 1995; Carpene *et al.*, 1994; 1999; Hoffstedt *et al.*, 1996; Hollenga *et al.*, 1990; 1991a; Langin *et al.*, 1991; Rosenbaum *et al.*, 1993; Tavernier *et al.*, 1996). However there are reports that indicate a functional β_3-AR in human adipocytes and the human brown adipocyte cell line PAZ6 (Enocksson *et al.*, 1995; Lonnqvist *et al.*, 1993; Zilberfarb *et al.*, 1997). Several studies indicating functional β_3-ARs in human adipocytes have used CGP12177A and caution must be used in interpretation of these results since this drug also acts at the putative β_4-AR (see section 1.4.7.1). The development of new β_3-AR agonists with higher affinities and efficacy at the cloned human β_3-AR may clarify this point (Fisher *et al.*, 1998; Sennitt *et al.*, 1998). Despite this, molecular studies demonstrate the presence of β_3-AR mRNA in human adipocytes, although levels are lower than those of β_1- and β_2-ARs (Berkowitz *et al.*, 1995; Granneman & Lahners, 1994; Granneman *et al.*, 1992; Krief *et al.*, 1993; Lonnqvist *et al.*, 1993; Revelli *et al.*, 1993; Tavernier *et al.*, 1996). β_3-AR mRNA expression in BAT (Krief *et al.*, 1993) and the presence of BAT morphology (Garruti & Ricquier, 1992; Zancanaro *et al.*, 1995) occurs in humans in infancy/pre-term babies and in certain conditions such as pheochromocytoma (a condition of brown fat activation).

1.4.3 Gastrointestinal tissue

β_3-ARs mediate relaxation in a wide variety of gastrointestinal tissues from various species as reviewed elsewhere (Manara *et al.*, 1995), and the presence of β_3-ARs is supported by radioligand binding and molecular studies (Evans *et al.*, 1996; Hutchinson *et al.*, 2000; Roberts *et al.*, 1995; 1999). In addition to the well characterised role of β_3-ARs in these tissues, β_1-ARs may also mediate relaxation in gastrointestinal tissues. β_2-AR mediated relaxation of gastrointestinal tissues is virtually non-existent, although radioligand binding and molecular studies show the presence of both β_1- and β_2-ARs (De Ponti *et al.*, 1995; Ek & Nahorski, 1986; MacDonald & Lamont, 1993; Oostendorp *et al.*, 2000; Roberts *et al.*, 1997; 1999). In some cases β_2-AR agonists (zinterol, ritodrine, clenbuterol, fenoterol, salbutamol) cause relaxation but act at β_3-ARs (Bianchetti & Manara, 1990; Horinouchi & Koike, 2001b; Roberts *et al.*, 1999), and the β_1-AR agonist Ro363 is a partial agonist at the cloned β_3-AR (Molenaar *et al.*, 1997a). It is likely that β_2-ARs (and to some degree, β_1-ARs) have roles other than relaxation, and are known to increase the secretion of several peptides in the gut ie. glucagon-like peptide-1 and peptide YY (Brechet *et al.*, 2001; Claustre *et al.*, 1999; Dumoulin *et al.*, 1995). In human colon preparations, β_1- and β_3-ARs are the predominant subtypes present as determined primarily with selective β_1-, β_2-AR or β_3-AR antagonists against non-selective β-AR agonist-mediated relaxation, with all three β-AR mRNAs present in human colon (Bardou *et al.*, 1998; De Ponti *et al.*, 1996; 1999; MacDonald *et al.*, 1996; Manara *et al.*, 2000; Roberts *et al.*, 1997). However, no studies to date have utilised β_3-AR selective human agonists in human gastrointestinal preparations.

Several studies have investigated gastrointestinal function in β_3-AR KO mice (Cohen *et al.*, 2000; Fletcher *et al.*, 1998; Oostendorp *et al.*, 2000). Investigation of the relaxant effects of β-ARs in colon suggested that β_1-ARs compensate for β_3-ARs in β_3-AR KO mice, not by up-regulation of β_1-AR mRNA, but possibly by more efficient coupling of β_1-ARs to pathways leading to relaxation (Oostendorp *et al.*, 2000). Responses to the selective β_3-AR agonist CL316243 were abolished in tissues from β_3-AR KO mice (Cohen *et al.*, 2000; Oostendorp *et al.*, 2000). β_3-ARs also

mediate gastrointestinal transit time since β_3-AR agonists increase gastrointestinal transit time in wild type mice but not β_3-AR KO mice (Fletcher *et al.*, 1998).

1.4.4 Genito-urinary system

It was originally proposed that β_1- and β_2-ARs mediate relaxation of urinary tract smooth muscle (Elmer, 1974; Larsen, 1979; Li *et al.*, 1992; Nergardh *et al.*, 1977). However, recent studies demonstrate β_3-AR mediated relaxation of bladder detrusor muscle in a wide variety of species. In rat, both β_2- and β_3-ARs mediate detrusor smooth muscle relaxation (Fujimura *et al.*, 1999; Oshita *et al.*, 1997; Yamazaki *et al.*, 1998). *In vivo* experiments in rats indicate that β_3-ARs may be a useful target for the treatment of pollakiuria (frequency of urination) since β_3-AR agonists decrease bladder pressure and increase bladder capacity with minimal cardiovascular effects (Takeda *et al.*, 2000b). Similar results were obtained in ferrets (Takeda *et al.*, 2000a). β_3-ARs increase cAMP levels in rat detrusor smooth muscle (Fujimura *et al.*, 1999) and although all three β-AR subtype mRNAs are present, no functional roles for β_1-AR have been proposed (Fujimura *et al.*, 1999; Seguchi *et al.*, 1998). There are species differences in β-AR mediated relaxation of detrusor smooth muscle. The predominant β-AR subtype present in rabbit are β_2-ARs, whereas in dogs, β_3-ARs predominate (Oshita *et al.*, 1997; Yamazaki *et al.*, 1998).

In humans, mRNA for β_2- and β_3-ARs are present in detrusor muscle although there are conflicting results as to the presence of β_1-AR mRNA (Fujimura *et al.*, 1999; Igawa *et al.*, 1999; Takeda *et al.*, 1999). Isoprenaline mediated relaxation is antagonised by β_3-AR antagonists but to a negligible extent by β_1-/β_2-AR antagonists (Igawa *et al.*, 1999; Takeda *et al.*, 1999). β_3-AR agonists such as BRL37344 and CL316243 relax human detrusor smooth muscle but only at high concentrations (Igawa *et al.*, 1999). The development of new β_3-AR agonists with higher affinity and efficacy at the cloned human β_3-AR may be useful for establishing the role of human β_3-ARs in this tissue.

1.4.5 Oesophagus

In rodent oesophageal smooth muscle, β_3-ARs are the predominant β-AR subtype responsible for smooth muscle relaxation (De Boer *et al.*, 1993; Lezama *et al.*, 1996; Oostendorp *et al.*, 2000), with β_1-ARs also contributing (Oostendorp *et al.*, 2000). A role for β_2-ARs is not clear as two studies have conflicting results, with studies indicating a role in rat (De Boer *et al.*, 1993), but not in mouse (Oostendorp *et al.*, 2000), possibly reflecting a species difference.

1.4.6 Brain

In the rat CNS, β_3-AR mRNA is present in several regions including the hippocampus, hypothalamus and cerebral cortex (Summers *et al.*, 1995). In human brain β_3-AR mRNA is located in the brain stem, cortex, insula and substantia nigra (Rodriguez *et al.*, 1995). Only a few studies have postulated a functional role for β_3-ARs in brain. Intracerebroventricular injection of BRL37344 causes a dose dependent decrease in food intake in obese and lean rats, suggesting a role for central β_3-ARs in controlling food intake (Tsujii & Bray, 1992). This is supported by a recent study showing that intracerebroventricular injection of CL316243 caused c-fos protein expression in discrete regions of the hypothalamus (including the lateral, ventromedial and paraventricular hypothalamic regions) in a dose dependent manner and that the effect was blocked by SR59230A (Castillo-Melendez *et al.*, 2000). Since these regions regulate food intake and energy balance, this raises the possibility that β_3-ARs have a direct role in the central regulation of food intake and energy expenditure.

1.4.7 Cardiovascular

1.4.7.1 Heart

Distinct from the putative β_4-AR in cardiac tissues (section 1.4.7.1), a functional β_3-AR in human heart has been postulated. Several β_3-AR agonists cause dose dependent negative inotropic effects in human ventricular myocardium (Gauthier *et*

al., 1996). This is the only study that illustrates β_3-AR-mediated effects in heart. Although they demonstrate the presence of β_3-AR mRNA, this was demonstrated by polymerase chain reaction (PCR) performed on poly$^+$ RNA (20-40 fold purification of mRNA) with the use of 40 cycles. Other studies show conflicting evidence for β_3-AR mediated roles in producing positive inotropic effects (Kaumann & Molenaar, 1997; Skeberdis *et al.*, 1999). Positive chronotropic effects in humans with β_3-AR agonists may be due to interactions at β_1-/β_2-ARs result from baroreflex effects (Wheeldon *et al.*, 1993; 1994).

In other species, the cardiac effects of β_3-AR agonists are variable. In rat atria, β_3-AR agonists caused no change in chronotropic or inotropic responses (Cohen *et al.*, 1999; Kaumann & Molenaar, 1996), whereas Gauthier *et al.* (1999) reported small decreases in peak tension following β_3-AR stimulation in both rat and guinea-pig ventricular tissues. In dog ventricular tissue, β_3-AR agonists produced negative inotropic effects that were less than those observed in human ventricular tissues (Gauthier *et al.*, 1999). In dog ventricular myocytes, there were decreases in the amplification of contraction and in the peak systolic $[Ca^{2+}]i$ transient (Cheng *et al.*, 1998). Several studies indicate that the effects of β_3-AR agonists in producing positive chronotropic effects in both dogs and rats may not be due to direct interaction with cardiac β_3-ARs, rather they may result from β_3-ARs on blood vessels or by actions at β_1-/β_2-ARs (Shen *et al.*, 1994; 1996; Tavernier *et al.*, 1992).

1.4.7.2 Blood vessels

In vascular smooth muscle, the predominant β-AR subtype mediating vasodilation and vasorelaxation is the β_2-AR although this can depend on the vascular tissue since β_1-ARs can predominate in some vessels such as coronary arteries (for review, see Guimaraes & Moura (2001)). Recently β_3-ARs have been postulated to have roles in vasodilation and vasorelaxation.

One of the first indications of β_3-AR mediated vasodilation was in rat BAT (Takahashi *et al.*, 1992), and this was supported by other studies showing that

vasodilation of peripheral blood vessels by β_3-ARs in dog skin and adipose tissue differed from the pattern of vasodilation observed following stimulation of β_1-/β_2-ARs (Berlan *et al.*, 1994; Shen *et al.*, 1994). β_3-AR stimulation can also increase pancreatic islet blood flow and insulin secretion in rats (Atef *et al.*, 1996) and mediate vasodilation and vasorelaxation in a number of other vascular tissues (Oriowo, 1994; 1995; Rohrer *et al.*, 1999; Shen *et al.*, 1996; Trochu *et al.*, 1999). A recent study showed that BRL37344 and CGP12177A caused vasorelaxation in rat isolated aorta, but responses to CGP12177A and isoprenaline were not antagonised by β_3-AR antagonists (Brawley *et al.*, 2000b). This suggests that the responses were mediated by other β-ARs. The role of β_3-ARs in blood vessels has been examined using β_1-/β_2-AR double KO mice (on a combined 129SvJ and FVB/N mouse background) (Rohrer *et al.*, 1999). In control animals, β_3-AR stimulation caused vasorelaxation, but this was increased substantially in β_1-/β_2-AR KO mice. It was suggested that this was due to compensatory up-regulation of β_3-ARs or their signalling pathways in β_1-/β_2-AR KO mice. While β_3-AR mediated vasodilation and vasorelaxation has been observed in rat, dog and mice, β_3-ARs do not appear to mediate vasodilation in primates (Shen *et al.*, 1996).

It has been postulated that β_3-ARs are located on endothelial cells and act in combination with β_1-/β_2-ARs to mediate vascular relaxation through activation of a nitric oxide synthase pathway and increases in cyclic GMP (cGMP) (Trochu *et al.*, 1999). However, Brawley *et al.* (2000a) showed that while the endothelium/nitric oxide pathway is involved in classical β-AR mediated vasorelaxation, its role in CGP12177A mediated vasorelaxation was less convincing.

1.4.8 Other putative β-ARs

1.4.8.1 The putative β_4-AR

CGP12177A is a high affinity hydrophilic β_1-/β_2-AR antagonist (Staehelin & Hertel, 1983) and a partial agonist at β_3-ARs in both adipose and gastrointestinal tissues (Feve *et al.*, 1991; Granneman & Whitty, 1991; Kaumann & Molenaar, 1996; Koike

et al., 1995a; 1996; Langin *et al.*, 1991; Molenaar *et al.*, 1997a; Sennitt *et al.*, 1998; Zhao *et al.*, 1998). Evidence for a fourth β-AR was based on the cardiostimulant effects of several non-conventional β-AR partial agonists related to pindolol, including CGP12177A (Kaumann, 1989). The putative β_4-AR resembles the β_3-AR in that it is stimulated by non-conventional partial agonists and resistant to antagonism by propranolol but differs in that it is not stimulated by β_3-AR agonists and is resistant to blockade by β_3-AR antagonists. The pharmacology and characterisation of the 'β_4-AR' has been described in cardiac, gastrointestinal and adipose tissues, and even more recently in oesophagus and ureter (Galitzky *et al.*, 1997; Kaumann, 1996; Kaumann & Molenaar, 1996; 1997; Kaumann *et al.*, 1998; Molenaar *et al.*, 1997b; Oostendorp *et al.*, 2000).

Support for the hypothesis suggesting the β_4-AR came from studies using β_3-AR KO mice. Responses to CGP12177A in adipose, cardiac and gastrointestinal tissues remained intact in β_3-AR KO mice (Cohen *et al.*, 2000; Kaumann *et al.*, 1998; Konkar *et al.*, 2000a; Oostendorp *et al.*, 2000; Preitner *et al.*, 1998). These studies illustrated that the β_3-AR could not account for the β_4-AR pharmacology displayed in these tissues, which apart from cardiac tissues, all express β_3-AR (section 1.2.2).

However, it was also clear that responses to CGP12177A via the 'putative β_4-AR' only existed in tissues with high levels of β_1-ARs, such as adipose and cardiac tissue. In addition, CGP12177A was known to act as an agonist at β_1-ARs in recombinant systems (Pak & Fishman, 1996). The notion that β_1-ARs were responsible for the observed β_4-AR pharmacology was discounted since it was thought that β_1-ARs needed to be highly expressed and thus physiologically irrelevant (Pak & Fishman, 1996). Recent studies call this into question and show that CGP12177A has agonistic actions in cells expressing physiologically relevant levels of β_1-ARs (Konkar *et al.*, 2000a; 2000b). Use of β_1-AR KO or β_1-/β_2-AR KO mice provide the most convincing data that the putative β_4-AR is a state or form of the β_1-AR since responses to CGP12177A in cardiac and adipose tissue were abolished in these mice (Kaumann *et al.*, 2001; Konkar *et al.*, 2000a). CGP12177A may interact with an altered conformation of the β_1-AR to produce its agonistic actions: these could

include active/inactive conformations of the β_1-AR, interactions with a β_1-AR dimer complex, or interactions with an allosteric site on the β_1-AR.

1.4.8.2 Atypical β-AR in skeletal muscle

β-ARs affect a number of metabolic and physiological processes in skeletal muscle, including glucose uptake and metabolism, non-shivering thermogenesis and contractility. Insulin increases glucose transport by causing translocation of glucose transporter (GLUT4) to the cell surface. However several reports show that NA can decrease glucose uptake in muscle (Huang *et al.*, 1997), either by inhibition of insulin stimulated glucose transport (Laurent *et al.*, 1998) or through a mechanism independent of insulin-stimulated glucose uptake (Lee *et al.*, 1997). Several β_3-AR agonists are potent and selective stimulants of non-shivering thermogenesis *in vivo* and *in vitro*, and are able to increase glucose uptake and tissue glucose utilisation in adipose tissue and skeletal muscle (Abe *et al.*, 1993; Board *et al.*, 2000; Liu *et al.*, 1996; Liu & Stock, 1995). This effect is independent of the action of insulin (Abe *et al.*, 1993). The effect of BRL37344 on glucose uptake in rat skeletal muscle is biphasic, with low concentrations increasing glucose uptake (possibly through β_3-ARs) and at high concentrations decreasing glucose uptake (Liu *et al.*, 1996). This decrease in glucose uptake is mediated by β_2-ARs which inhibit insulin stimulated glycogen synthesis (Liu *et al.*, 1996). This could explain why studies with NA decrease glucose uptake if higher concentrations are used. In addition, cAMP accumulation in rat soleus muscle is mediated by β_2-ARs and not by β_3-ARs (Roberts & Summers, 1998). High affinity and low affinity [^{125}I]-cyanopindolol (ICYP) sites (with characteristics similar to β_3-ARs ie. propranolol resistance) are present in rat skeletal and soleus muscle (Molenaar *et al.*, 1991; Roberts *et al.*, 1993; Sillence & Matthews, 1994; Sillence *et al.*, 1993). However, molecular studies have failed to detect the presence of β_3-ARs in soleus muscle (Evans *et al.*, 1996).

L6 cells (a rat skeletal muscle cell line) contain lower levels of glycogen than skeletal muscle (Elsner *et al.*, 1998; Nakatani *et al.*, 1997), which may explain why β-AR stimulation increases glucose uptake in these cells, although the receptor mediating these effects is disputed (Nevzorova *et al.*, 2000; Tanishita *et al.*, 1997). A

recent study (Nevzorova *et al.*, 2000) investigating β-AR stimulation of glucose uptake in L6 cells clearly show that the β_2-AR is the sole β-AR mediating glucose uptake, even with BRL37344, since this drug was shown to act solely through β_2-ARs and not β_3-ARs. This work was supported with molecular studies showing the presence of β_2-AR mRNA, but not β_1-or β_3-AR mRNA. While β_2-ARs can explain β-AR mediated glucose uptake in L6 cells, this mechanism does not explain the effect of β_3-AR agonists and the results of radioligand binding studies performed in skeletal muscle.

1.5 Pharmacological characterisation of the β_3-AR

β_1- and β_2-ARs can be pharmacologically characterised by:

(i) blockade by non-selective (propranolol, alprenolol) and selective antagonists (CGP20712A (β_1-AR); ICI118551 (β_2-AR))

(ii) activation by selective agonists (Ro363 (β_1-AR); zinterol, salbutamol (β_2-AR))

(iii) high level of stereoselectivity for β-AR agonist and antagonist enantiomers

(iv) activation by the catecholamines NA, AD and isoprenaline, with rank orders of potency isoprenaline > NA > AD for β_1-AR and isoprenaline > AD > NA for β_2-AR

The pharmacological criteria to define the β_3-AR include:

(i) low affinities and potencies for conventional β-AR antagonists and agonists

(ii) low stereoselectivity of agonist and antagonist stereoisomers compared with β_1- or β_2-ARs

(iii) partial agonist activities of several β_1- and β_2-AR antagonists related to pindolol

(iv) high affinity and potency for several selective activators of lipolysis, thermogenesis and smooth muscle relaxation (CL316243 and BRL37344)

(v) antagonism by selective antagonists (SR59230A)

Examination of the chemical structure of a number of β-AR agonists and antagonists show that most compounds active at the β_3-AR share a common structural feature, the phenylethanolamine side chain linked to a catechol ring (Figure 1.12 and 1.13). The affinities and potencies of a range of compounds at the cloned human, mouse and rat β_3-AR are given in Table 1.1. For data in native tissues, refer to (Arch & Kaumann, 1993; Manara *et al.*, 1995) where it has been extensively reported. It should be noted that the references cited below are a summary of the whole body of research performed to support the pharmacology of each drug, and not a comprehensive list.

1.5.1 Agonists

1.5.1.1 Non-selective β-AR agonists

The non-selective β-AR agonists comprise the endogenous catecholamines NA and AD, as well as the synthetic catecholamine isoprenaline. All of these agonists act at β_1-, β_2- and β_3-ARs, albeit with differing rank orders of potency. The main evidence for an atypical β-AR came from studies in adipose and gastrointestinal tissues which showed resistance to antagonism by several non-selective and β_1-/β_2-AR selective antagonists when either NA, AD or isoprenaline was utilised as the agonist (for reviews refer to Arch & Kaumann (1993) and Manara *et al.* (1995). These non-selective agonists are still employed since they easily allow elucidation of β-AR subtypes involved in a given tissue when used together with selective antagonists. This section will focus primarily on selective β_3-AR agonists.

1.5.1.2 β_3-AR selective agonists

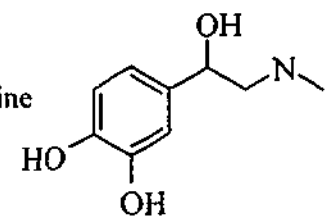
1.5.1.2.1 BRL compounds

The BRL series of compounds, synthesised by Beecham Pharmaceuticals, were the first selective β_3-AR agonists described. They were shown to selectively stimulate brown and white adipocyte β_3-ARs with higher potency than isoprenaline (Arch *et*

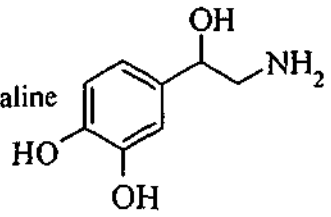
Figure1.12: Structures of β-AR agonists used in the identification and classification of β-ARs.

Non-selective β-AR agonists

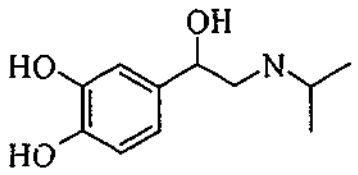
Adrenaline



Noradrenaline

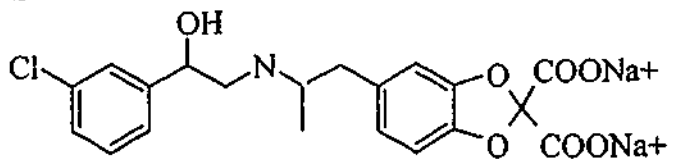


Isoprenaline

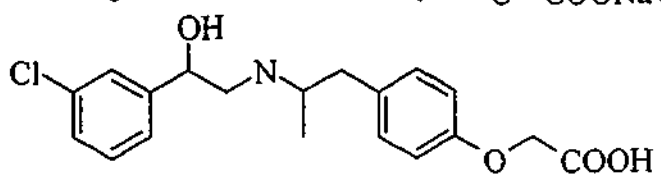


Selective β_3-AR agonists- rodent

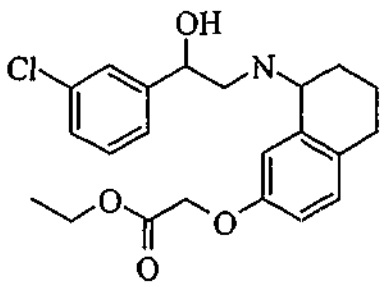
CL 316243



BRL 37344

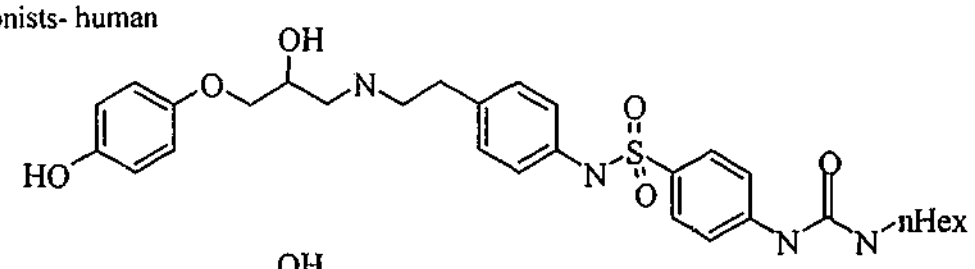


SR 58611A

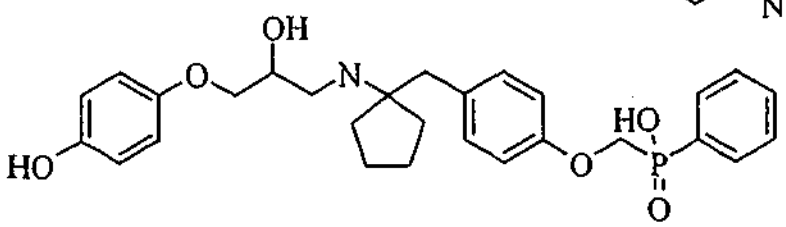


Selective β_3-AR agonists- human

L755507



SB251023



β_3-AR agonist / β_1-/β_2-AR antagonist

CGP 12177A

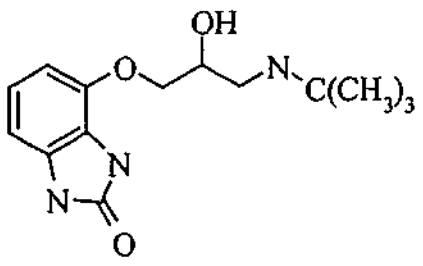
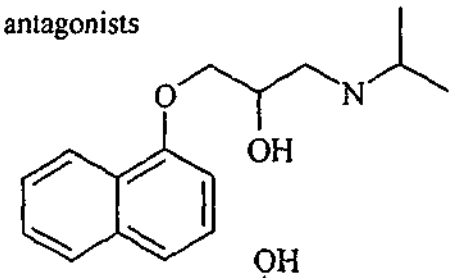


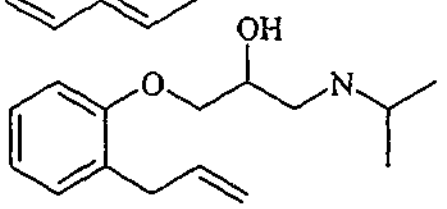
Figure 1.13: Structures of β-AR antagonists used in the identification and classification of β-ARs.

Non-selective β-AR antagonists

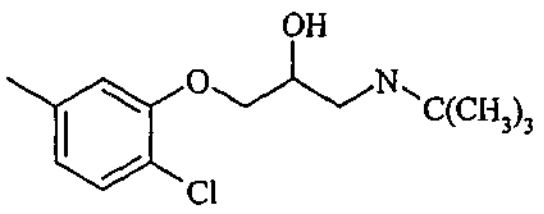
Propranolol



Alprenolol

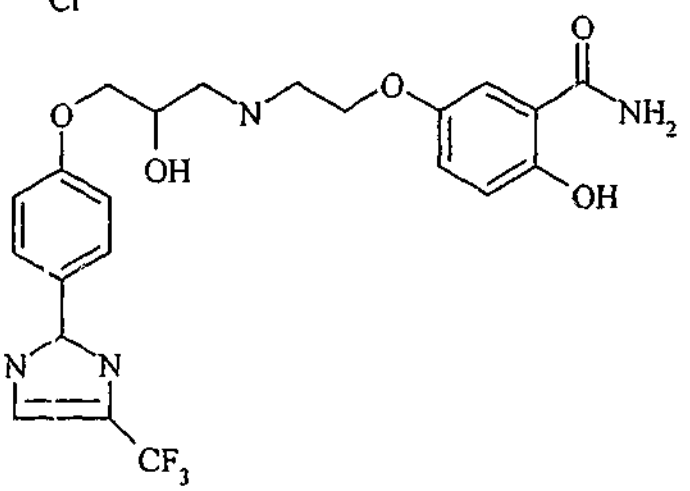


Bupranolol



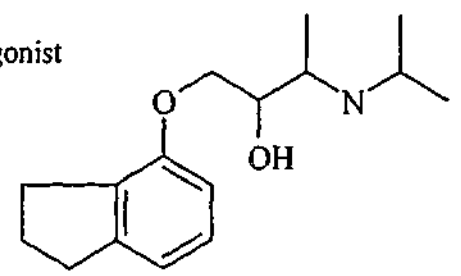
Selective β_1-AR antagonist

CGP 20712A



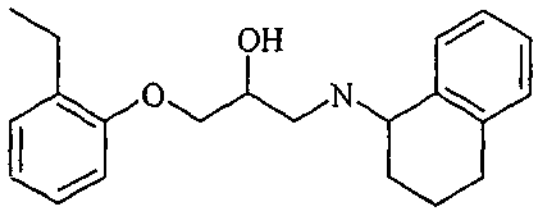
Selective β_2-AR antagonist

ICI 118551



Selective β_3-AR antagonist

SR 59230A



al., 1984; Wilson *et al.*, 1984). Two effective compounds were the methyl esters BRL26830A and BRL35135, which are metabolised *in vivo* to their parent acids BRL28410 and BRL37344 respectively. The most potent compound, BRL37344, was 400 and 20 times more selective for the adipose tissue β_3-AR as compared to β_1-AR (heart) or β_2-AR (trachea) mediated responses respectively (Arch *et al.*, 1984). BRL37344 is also a selective β_3-AR agonist in gastrointestinal tissues (reviewed in Arch & Kaumann (1993) and Manara *et al.* (1995), with potency values that are generally similar to those of isoprenaline depending on tissue and species (Manara *et al.*, 1995). BRL37344, and its related compounds, fit the pharmacological criteria for actions at β_3-ARs since non-selective β-AR antagonists, such as propranolol, and β_1- and β_2-AR selective antagonists all show low affinity in antagonising responses to BRL37344 in both adipose and gastrointestinal tissues (Arch *et al.*, 1984; Bond & Clarke, 1988; Carpene *et al.*, 1994; Granneman, 1990; Hollenga *et al.*, 1991b; Hollenga & Zaagsma, 1989; McLaughlin & MacDonald, 1990; 1991; Wilson *et al.*, 1984).

One of the main disadvantages of BRL37344 is its ability to act as an agonist at β_2-ARs in a wide variety of tissues, such as bronchi (Ten Berge *et al.*, 1995) and L6 myocytes (Nevzorova *et al.*, 2000). In rat ileum, BRL37344 causes a biphasic relaxation response. Whereas the high affinity component was due to actions on the β_3-AR, no mechanism for the lower affinity component has been elucidated (Roberts *et al.*, 1999), although it appears unlikely that BRL37344 interacts with the novel ICYP binding protein in rat colon (Sugasawa *et al.*, 1997).

1.5.1.2.2 CL316243

One of the main side effects of the BRL series of compounds was muscle tremor and hypokalaemia (Connacher *et al.*, 1988; 1990; Mitchell *et al.*, 1989), effects most likely due to interactions at receptors other than β_2-ARs. CL316243, developed by American Cyanamid Company, was slightly more potent than BRL37344 or isoprenaline in adipose and gastrointestinal tissues (Cohen *et al.*, 1995; Dolan *et al.*, 1994), but showed markedly lower affinities at β_1- and β_2-ARs (Dolan *et al.*, 1994). CL316243 is 10,000 times more selective for the β_3-AR compared to the β_1- or β_2-

ARs (Bloom *et al.*, 1992; Dolan *et al.*, 1994), in contrast to BRL37344 which is only 667 and 23 times more selective for the β_3-AR than the β_1- or β_2-AR respectively (Dolan *et al.*, 1994). In β_3-AR KO mice, all responses to CL316243 in both adipose and gastrointestinal tissues are abolished (Cohen *et al.*, 2000; Grujic *et al.*, 1997; Oostendorp *et al.*, 2000; Revelli *et al.*, 1997; Susulic *et al.*, 1995), illustrating the specific action of CL316243. CL316243 fits the pharmacological profile for interactions with β_3-ARs since responses are resistant to antagonism by β_1-/β_2-AR antagonists and non-selective β-AR antagonists such as propranolol in adipose and gastrointestinal tissues (Ohsaka *et al.*, 1998; Roberts *et al.*, 1999)

1.5.1.2.3 Other β_3-AR agonists

Several other β_3-AR agonists have been developed and reported.

The phenylethanolaminotetralins developed by Sanofi Pharmaceuticals, which include SR58611A were originally described as gut specific β_3-AR agonists (Bianchetti & Manara, 1990; Croci *et al.*, 1988), with more selectivity for the β_3-AR in rat colon and insensitive to antagonism by β_1-/β_2-AR antagonists and only weakly blocked by non-selective antagonists (Bianchetti & Manara, 1990). Similar results were obtained in adipose tissue (Nisoli *et al.*, 1994), although SR58611A is approximately 23 times more potent in gastrointestinal tissues than in adipose tissue, due to its conversion to a more active metabolite in gastrointestinal tissues (Landi *et al.*, 1993). Recently two other related compounds, SR59119A and SR59104A, (Bardou *et al.*, 1998) have been described as having β_3-AR agonistic properties in human gastrointestinal tissues.

The Zeneca compound ZD7114 (formerly ICID7114) and its acid metabolite ICI215001 are potent and selective β_3-AR agonists that stimulate whole body oxygen consumption with minimal cardiovascular side effects, (Holloway *et al.*, 1991), with potency values similar to those of isoprenaline in BAT. In rodent WAT, it acts as a partial agonist (Mayers *et al.*, 1996) and in rodent gastrointestinal tissues as a competitive antagonist of isoprenaline-mediated relaxation (Growcott *et al.*, 1993; MacDonald & Lamont, 1993; Roberts *et al.*, 1999).

The main problem with the majority of β_3-AR agonists available are their limited effects in humans and primates. The first generation β_3-AR agonists, such as BRL37344 and CL316243, while active at rodent β_3-ARs, are mostly ineffective at the human receptor (although can have some effect at highly expressed cloned human β_3-AR) (Bousquet-Melou *et al.*, 1995; Carpene *et al.*, 1999; Connacher *et al.*, 1992; Hoffstedt *et al.*, 1996; Langin *et al.*, 1991; Liggett, 1992; Tavernier *et al.*, 1996; Wheeldon *et al.*, 1994). A number of new human-selective, or at least human-potent, β_3-AR agonists have been described, with most development being performed in transfected cell lines expressing the human β_3-AR to determine potency and affinity. One of these compounds, L755507, is active at both the human and rhesus monkey β_3-AR expressed in CHO-K1 cells, and is more than 1,000 times selective for the β_3-AR compared to the β_1- or β_2-AR in both species (Fisher *et al.*, 1998; Parmee *et al.*, 1998). *In vivo* and *in vitro* studies in rhesus monkey showed L755507 to have potential anti-obesity effects with only minor cardiovascular side effects (Fisher *et al.*, 1998). A group of new β_3-AR agonists synthesised by Smith Kline Beecham have also been reported to have β_3-AR agonist actions at the cloned human β_3-AR with varying degrees of activity on β_1-/β_2-ARs (Sennitt *et al.*, 1998).

1.5.2 β_3-AR agonists/ β_1-/β_2-AR antagonists

CGP12177A was originally developed as a non-selective β-AR antagonist (Staehelin & Hertel, 1983) but studies in adipose tissues found that it was an agonist comparable to NA for stimulation of respiration and thermogenesis (Mohell & Dicker, 1989). Further characterisation showed that it could act at two sites in adipose tissue: at the β_1-AR as a high affinity antagonist, and at the β_3-AR as a low affinity partial agonist (Granneman & Whitty, 1991) which could also be shown in gastrointestinal tissues (Koike *et al.*, 1996). Numerous other studies have shown agonistic actions of CGP12177A in both adipose (Feve *et al.*, 1991; Germack *et al.*, 1997; Langin *et al.*, 1991; Van Liefde *et al.*, 1993; Zhao *et al.*, 1998) and gastrointestinal (Kaumann & Molenaar, 1996; Koike *et al.*, 1995; Koike *et al.*, 1996; Molenaar *et al.*, 1997a; Sennitt *et al.*, 1998) tissues.

Other β_1-/β_2-AR antagonists that display β_3-AR agonist properties include alprenolol and cyanopindolol (Blue *et al.*, 1990; Hoey *et al.*, 1996; Kaumann & Molenaar, 1996), despite evidence showing that cyanopindolol, alprenolol and CGP12177A can also act as antagonists (Kaumann & Molenaar, 1996; Kelly & Houston, 1996; Kirkham & Kelly, 1992; Landi *et al.*, 1993; MacDonald *et al.*, 1994; MacDonald & Watt, 1999; McLaughlin & MacDonald, 1990; 1991; Roberts *et al.*, 1999).

In β_3-AR KO mice, the responses to CGP12177A in both adipose and gastrointestinal tissues remain (detailed previously in section 1.4). This agonist effect of CGP12177A is probably mediated by interactions at the β_1-AR (Konkar *et al.*, 2000b; Pak & Fishman, 1996).

1.5.3 Antagonists

1.5.3.1 Non-selective β-AR antagonists, β_1- and β_2-AR selective antagonists

The low affinity of propranolol (a classical non-selective β-AR antagonist) and other β_1- and β_2-AR selective antagonists in blocking both adipocyte and gastrointestinal responses to both non-selective β-AR and β_3-AR agonists was one of the earliest pieces of evidence supporting the involvement of a non-β_1-/β_2-AR subtype mediating effects in these tissues (Arch *et al.*, 1984; Bianchetti & Manara, 1990; Bond *et al.*, 1986; Bond & Clarke, 1987; 1988; Carpene *et al.*, 1994; D'Allaire *et al.*, 1995; Germack *et al.*, 1997; Granneman, 1990; Harms *et al.*, 1974; 1977; Hollenga *et al.*, 1991b; Hollenga & Zaagsma, 1989; McLaughlin & MacDonald, 1990;, 1991; Nisoli *et al.*, 1994; Ohsaka *et al.*, 1998; Roberts *et al.*, 1999; Van Liefde *et al.*, 1993; Wilson *et al.*, 1984).

1.5.3.2 SR59230A, the β_3-AR selective antagonist

The first β_3-AR selective antagonists, the aryloxypropanolaminotetralins (APATs), such as SR59230A designed by Sanofi Midy, were first tested in rat colon and adipose tissue. SR59230A inhibited β_3-AR mediated responses to a greater extent in rat colon or adipose tissue than β_1-AR or β_2-AR mediated responses in atria or

trachea respectively (Manara *et al.*, 1996; Nisoli *et al.*, 1996). Further studies have supported the potency of SR59230A as a β_3-AR antagonist (Galitzky *et al.*, 1997; Kaumann & Molenaar, 1996; MacDonald & Watt, 1999; Oostendorp *et al.*, 2000; Roberts *et al.*, 1999), although one report indicating poor antagonism of CGP12177A mediated responses postulated that this indicated the presence of a β_4-AR (Galitzky *et al.*, 1997).

A recent report has indicated that in cloned human β_3-AR, SR59230A displays little selectivity in binding for the β_3-AR over the cloned β_1- or β_2-AR, which may suggest that SR59230A is not as selective as first reported, although it was more potent at β_3-ARs than at β_1-/β_2-ARs (Candelore *et al.*, 1999). This same paper also reported several other β_3-AR antagonists synthesised by Merck that were potent and selective for the human β_3-AR. No literature has yet been cited on the actions of these new antagonists at rodent β_3-ARs.

1.5.4 Radioligands

Several radioligands have been used to identify β_1- and β_2-ARs in receptor binding techniques with homogenates and by autoradiography. These ligands include ICYP (Engel *et al.*, 1981) and ^{3}H-CGP12177 (Levin & Sullivan, 1986; Mohell & Dicker, 1989; Staehelin & Hertel, 1983). Early studies were unsuccessful in identifying atypical/β_3-ARs due to the low concentrations of radioligand used since ICYP and ^{3}H-CGP12177 display markedly lower affinity for β_3-ARs than for β_1-/β_2-ARs (10-100 times lower affinity, refer to Table 1.1 for data on ICYP acting at β_3-ARs). Higher levels of non-specific binding were reported when using higher concentrations of radioligand which suggested that these radioligands were unsuitable for labelling of β_3-ARs (Landi *et al.*, 1992; Langin *et al.*, 1991; Muzzin *et al.*, 1988). Despite these earlier studies showing difficulties when using these radioligands, successful binding studies have been performed with both radioligands in a number of tissues including adipose and gastrointestinal tissues, and in cells endogenously expressing or transfected with the β_3-AR (Blin *et al.*, 1994; D'Allaire *et al.*, 1995; Emorine *et al.*, 1989; Feve *et al.*, 1991; Germack *et al.*, 1997; Hutchinson *et al.*, 2000; Koike *et al.*, 1996; Muzzin *et al.*, 1991; 1992; Nahmias *et*

al., 1991; Roberts *et al.*, 1995; Sillence *et al.*, 1993; Tate *et al.*, 1991). ICYP may also bind to receptors other than β-ARs, including 5-HT_1 and 5-HT_2 receptors (Hoyer *et al.*, 1985a; 1985b), dopamine receptors (Lew & Summers, 1987) and a novel site in rat colon smooth muscle that may mediate relaxation and which is not a β-AR, α-AR or 5-HT (Sugasawa *et al.*, 1997). This SM-11044 binding protein has recently been cloned (Sugasawa *et al.*, 2001) and does not display any structural features of β-ARs.

The ICYP binding profile at the cloned human, mouse and rat β_3-AR expressed in CHO-K1 cells is shown in Table 1.1. The characteristic radioligand binding properties for interactions at β_3-ARs include low radioligand affinity for ICYP, low affinity (pK_i values) for non-selective β-AR agonists with higher affinity for β_3-AR agonists, and a profile for antagonists as follows: β_3-AR antagonists > non-selective β-AR antagonists >> β_1-/β_2-AR antagonists. The lower affinity values for β_3-AR agonists over antagonists may reflect the lack of ability of agonists to compete for antagonist binding under certain conditions that favour low affinity agonist states (McPherson *et al.*, 1984).

1.6 Aims of the present study

Splice variants of GPCR that differ only in their C-terminus may exhibit differences in second messenger coupling and regulation. The primary aim of this thesis was to characterise the splice variant of the mouse β_3-AR, the β_{3b}-AR, in relation to the known receptor, the β_{3a}-AR. Secondly, the β-AR subtypes involved in mouse ileal smooth muscle relaxation were examined in mice lacking the β_3-AR.

Table1.1: Pharmacological characterisation of the mouse (Blin *et al.*, 1994; Nahmias *et al.*, 1991), rat (Blin *et al.*, 1994; Candelore *et al.*, 1999; Cohen *et al.*, 1995; Dolan *et al.*, 1994; Granneman *et al.*, 1991; 1998; Mejean *et al.*, 1995; Muzzin *et al.*, 1991) and human (Blin *et al.*, 1993; Candelore *et al.*, 1999; Cohen *et al.*, 1999; Dolan *et al.*, 1994; Emorine *et al.*, 1989; Feve *et al.*, 1991; Fisher *et al.*, 1998; Granneman *et al.*, 1993; Gros *et al.*, 1998; Mejean *et al.*, 1995; Molenaar *et al.*, 1997a; Sennitt *et al.*, 1998; Tate *et al.*, 1991) β_3-AR expressed in CHO-K1 cells and in 3T3-F442A mouse adipocytes (El Hadri *et al.*, 1996; Feve *et al.*, 1991; 1992; 1994; 1995; Krief *et al.*, 1994) which endogenously express the β_3-AR. Equilibrium dissociation constant (K_D) and negative logarithm of the affinity of a ligand derived from competition binding studies (pK_i) values are from radioligand binding studies using ICYP and negative logarithm of the activation constant (pK_{act}) values are from cAMP accumulation or AC stimulation assays.

	Mouse β_3-AR		Rat β_3-AR		Human β_3-AR		β_3-AR mouse 3T3-F442A adipocytes	
	pK_{act}	pK_i	pK_{act}	pK_i	pK_{act}	pK_i	pK_{act}	pK_i
<u>β-AR agonists</u>								
(-)-Isoprenaline	8.3	5.6	8.3	5.6	8.4	6.2		
(+)-Isoprenaline	7			3	7	4.7		
Isoprenaline	8.3		6.2, 8.4, 7.9, 8.7, 5.5	4.2, 4.9	5.3, 8.4, 5.9, 7.4, 5.9, 7.8, 6.3	6.2, 5.2, 5.4, 5.2	5.3, 5.7, 5.9	5.2
NA	7.9	5.7	5.2, 7.0, 7.4, 4.7, 7.9	3.3, 4.3, 5.7	4.3, 8.2, 5.9	> 5, 6.3	4.7, 5.4	3.9
AD	7.6	5.3	5.2, 6.7, 4.2, 7.6	3.8, 5.3	4.3, 7.3, 5.9	4.7	5.1	3.6
<u>β_2-AR agonists</u>								
BRL37344	9.4	6.5	9.4, 7.1, 8.9, 9.7, 9.0	6.5, 6.6, 7.0, 6.8	5.1, 7.8, 8.3, 8.2, 7.5, 6.0, 7.1	6.5, 6.7, 7.0, 6.2	7.0, 6.8, 6.6, 6.7	6.9, 6.5, 6.7, 6.9-6.6
SR59611A	7.7	5.9			7.6	5.2		
CGP12177A	7.4	6.8	7.4, 6.3, 6.7	6.8	6.3, 6.9, 6.0, 5.2, 5.9, 6.5	7.7, 7.1, 5.7, 6.1	6.3	9.2
CL316243			9.8, 9.1, 7.2	6.6, 6.0	7.2, >5.5	7.9		
CL314514					6	5.7		
L755507					9.4	7.9		
carazolol			7.6	7.7	8	8.7		
<u>β-AR antagonists</u>								
propranolol	antagonist	6.8	antagonist	5.8, 6.8	antagonist	7.8, 6.8		
Alprenolol	antagonist	7	antagonist	6.4, 7.0	antagonist	7		
nadolol					antagonist	5.6, 6.2, 6.3		
tertatolol						8		
Pindolol	antagonist	6.5	antagonist	6.5	antagonist	8	5	
cyanopindolol					antagonist			
Bupranolol	antagonist	7.4	antagonist	7.4	antagonist	7.3, 6.8		
<u>β_1-AR antagonists</u>								
CGP20712A	antagonist	4.9	antagonist	4.9	antagonist	5.6, 5.7	antagonist	5.1, 5.3, 5.6-5.3
<u>β_2-AR antagonists</u>								
ICI118551	antagonist	5.7	antagonist	5.3, 5.7	antagonist	6.6	antagonist	5.1, 5.3, 5.4-5.2
<u>β_3-AR antagonists</u>								
SR59230A			antagonist	8.4	antagonist	8.4		
ICYP K_D (pM)	880		1300		920, 4170, 500, 230, 610, 1890		1500, 1900, 3500, 3100, 2580, 2820	

Chapter 2

General Methods

2.1 *Cell Culture*

CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). Basic cell culture techniques are not explained here. For more information, refer to Freshney (1994).

2.1.1 *Maintenance of cells*

CHO-K1 cells were grown as monolayers in 50:50 Dulbecco's modified Eagle's Medium (DMEM): Ham's F-12 media containing heat inactivated foetal bovine serum (FBS, 10% v v^{-1}), glutamine (2mM), penicillin (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}). Transfected CHO-K1 cells were grown in the above media with the addition of G418 (400 µg ml^{-1}) and pH readjusted to 7.4 by the addition of sterile NaOH. In some experiments, cells were grown in the above media containing 0.5% v v^{-1} heat inactivated FBS (as indicated in the relevant sections). FBS was heat inactivated by incubation at 50°C for 30 min.

Cells were passaged every 3-4 days using trypsin/ ethylenediaminetetraacetic acid (EDTA) (0.1% trypsin w v^{-1}, 0.02% EDTA w v^{-1}). Briefly, cells were grown up to 90% confluence, media discarded and cells washed once with 10-20ml sterile Hepes-buffered saline (HBS) (composition (mM): Hepes 10, NaCl 150, pH 7.4 room temperature). To a 150cm^2 flask, at least 3ml trypsin/EDTA mix was added to the flask (for a 75cm^2 flask, at least 2ml) and the flask incubated for 5-10 min at 37°C. After 10 min, the cells detach from the bottom of the flask and the cell suspension is pipetted up and down to disaggregate the cells into a single cell suspension. Approximately one-tenth of the solution was transferred to a new flask and media added to the flask. Cells are incubated at 37°C, 5% CO_2, 85% humidity in a CO_2 water-jacketed incubator (Forma Scientific, Marietta, OH, U.S.A.).

2.1.2 *Freezing and thawing cells*

Cells were frozen in cryovials at early passage numbers. Cells were not used for experiments if passage numbers were high (typically cells over passage number 16

were discarded), if cells became too confluent, or if infection of cells occurred (cells were discarded and new cells set up). To freeze cells, they were grown to 70-90% confluence in 75cm^2 flasks and harvested as described in section 2.1.1. Cells were transferred to sterile centrifuge tubes and centrifuged at 800 x g at room temperature for 5 min. The supernatant was discarded and the pellet resuspended in 1ml heat inactivated FBS. In a separate tube, a 1ml mixture of 20% dimethyl sulfoxide (DMSO; cell culture grade) in FBS was prepared and the mixture added slowly to the cell mixture. The mixture was transferred to two 1ml cryovials, labelled and placed in a polystyrene container at –70°C for at least 2 days. Vials were then placed in liquid nitrogen for long term storage.

To thaw cell stocks, cryovials containing cells were removed from liquid nitrogen and quickly placed in a beaker containing warm water (~ 37°C) until the contents had thawed. The vial was sprayed with 70% ethanol, and contents quickly transferred to flasks containing pre-warmed media using sterile Pasteur pipettes. Flasks were placed in the incubator for several hours until the cells adhered to the flask then media was replaced with new media (to ensure removal of DMSO).

2.1.3 Counting cells

Cells were harvested as detailed in section 2.1.1 and the cell suspension transferred to centrifuge tubes and centrifuged at 800 x g for 5 min at room temperature. The supernatant was discarded and the cell pellet resuspended in appropriate media (approximately 5ml for cells harvested from one 75cm^2 flask, 90% confluence). With a cover slip in place, a Pasteur pipette was used to transfer a small amount of the cell suspension to a chamber on a Neubauer bright-line haemocytometer (Sigma Chemical Company, St Louis, MO, U.S.A.). This was done by carefully touching the edge of the cover slip with the pipette tip and allowing the chamber to fill by capillary action, making sure the chamber was not overfilled or underfilled. Counting was performed as per established cell counting protocols (refer to Freshney (1994) for more details).

2.1.4 Mouse ileum cell culture

Mice were anaesthetised with 80% CO_2/20% O_2 and decapitated. A 10-15 cm segment of the small intestine finishing 2-3 cm above the ileocaecal junction was removed and its contents flushed out with Krebs-Henseleit buffer (composition (mM): NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaCl_2$ 2.5, pH 7.4) containing ascorbic acid (0.1mM) and EDTA (0.04mM), and then with Hanks buffer containing 200mM L-glutamine. For preparation of smooth muscle strips, tissues were pinned out on a Petri dish containing Hanks buffer and the mesenteric fat carefully dissected away. The tissues were then cut open along the mesenteric fat line and pinned out as a flat tissue. The mucosa was gently scraped off with a sterile microscope slide. Tissues were then cut into segments of approximately 1 cm in length, washed in DMEM media and cultured in 12-well plates containing DMEM (with 200mM L-glutamine). Tissues were incubated at 37°C 5% CO_2, 85% humidity in a CO_2 water-jacketed incubator.

2.2 General molecular biology methods

2.2.1 RNA extraction

Male C57BL/6J mice (8 weeks old) were obtained from Monash University Central Animal Facilities (Monash University, VIC, Australia). FVB mice (female, ~ 4 months old) were obtained from the Animal Resources Centre, Canning Vale, Western Australia. β_3-AR KO mice (male, ~ 1 year old) were the offspring of a previously described strain (Susulic *et al.*, 1995).

Mice were anaesthetised with 80% CO_2/20% O_2 and decapitated. Interscapular BAT was removed from C57BL/6J mice and snap frozen in liquid nitrogen and stored at -70°C. Ileal tissue was obtained from β_3-AR KO and FVB mice. A 10-15 cm segment of the small intestine finishing 2-3 cm above the ileocaecal junction was removed and its contents flushed out with cold Krebs-Henseleit solution containing ascorbic acid (0.1mM) and EDTA (0.04mM). Tissues were pinned out on a petri dish and the mesenteric fat carefully dissected away. The tissues were then cut open along the

mesenteric fat line and pinned out as a flat tissue. The mucosa was scraped away using sterile microscope slides and washed in saline before being snap frozen in liquid nitrogen and stored at -70°C.

Frozen tissue was crushed in 10ml polypropylene tubes using a stainless steel pestle and mortar pre-cooled in liquid nitrogen. Total ribonucleic acid (RNA) was extracted by homogenising crushed tissue in Trizol reagent (1ml) using a PRO200 homogeniser (ProScientific Inc., Monroe, CT, U.S.A.) as per manufacturer's instructions.

Briefly, after addition of Trizol reagent, samples were incubated at room temperature for 5 min, solution transferred to microcentrifuge tubes, and chloroform (200µl) added. Tubes were shaken vigorously by hand for 15 sec, incubated (room temperature, 3 min), and centrifuged (12,000 x g, 15 min, 4°C). Following centrifugation, the mixture separates into a lower red phenol-chloroform phase (containing DNA and protein), an interphase, and a clear upper aqueous phase (containing RNA). The upper aqueous phase was transferred to a microcentrifuge tube, making sure not to contaminate it with the interphase. RNA was precipitated by the addition of isopropyl alcohol (500µl, 10 min) and centrifuged (12,000 x g, 10 min, 4°C). Supernatant was removed and the RNA pellet washed with 75% ethanol (200µl). Samples were mixed by vortexing, centrifuged (7,500 x g, 5 min, 4°C) and supernatant discarded. The RNA pellet was air dried for 10 min and RNA dissolved in sterile water. Samples could then be kept at -70°C for several months. The yield and quality of RNA was assessed by measuring absorbance at 260 and 280nm (Ultrospec 2000, PhamaciaBiotech, Uppsala, Sweden) and by electrophoresis on 1.3% agarose gels stained with ethidium bromide.

Total RNA was treated with DNase to remove any contaminating DNA. Briefly, 20µg RNA was incubated with reaction mix (100mM sodium acetate pH 7.0, 5mM $MgSO_4$, 5mM dithiothreitol, 36 U RNasin, 10 U DNase I) in a total volume of 40µl at 37°C for 30 min. The reaction was diluted to 400µl with sterile water and extracted with an equal volume of phenol:chloroform (1:1). The RNA was precipitated with ethanol (1ml) and 2M sodium acetate (40µl). The yield and quality

of DNase-treated RNA was determined by measuring absorbance at 260 and 280nm and by electrophoresis on 1.2% agarose gels. DNase treatment of total RNA was only performed on samples obtained from BAT from C57BL/6J mice which were used for cloning of the β_{3a}- and β_{3b}-AR (section 2.3).

2.2.2 Reverse transcription/Polymerase chain reaction

cDNA was synthesised by reverse transcription (RT) of 1µg of each total RNA using oligo $(dT)_{15}$ as a primer. RNA in a volume of 7.5µl was heated (70°C, 5 min) and cooled on ice for 2 min. Reaction mix (12.5µl) containing 1 x RT buffer, 1mM dNTPs, 18 U RNAsin, 5mM $MgCl_2$, 50 µg ml^{-1} oligo $(dT)_{15}$ and 20 U AMV reverse transcriptase was added to samples (final concentrations in 20µl). Samples were mixed briefly, centrifuged, and incubated at 42°C for 45 min, then 95°C for 3 min before cooling on ice for 2 min. Samples were briefly centrifuged and 1mM EDTA (20µl) added. The cDNA product was stored at -70°C and used for polymerase chain reaction (PCR) without further treatment. A negative control for each RNA sample was produced by setting up the RT reaction as normal but omitting the reverse transcriptase. RT reactions performed from total RNA prepared from ileum was modified slightly. RNA was prepared in a volume of 7.7µl and reaction mixture was in a volume of 12.3µl and contained 20 U RNAsin instead of 18 U RNAsin.

PCR amplification was carried out on cDNA equivalent to 100ng of starting RNA using oligonucleotide primers specific for the mouse β_1-, β_2- or β_3-AR or the internal standard β-actin (see Table 2.1 for sequence of primers and probes used). Reactions were performed in a FTS-1S capillary thermal sequencer (Corbett Research, Lindcombe, NSW, Australia). For β_2-, β_3-AR or β-actin PCR, PCR reaction mixes contained cDNA, 1 U Taq polymerase, 1 x PCR buffer, 200µM dNTPs, 2mM Mg-acetate, forward primer (2.8pmol β_2-, β_3-AR or 1.5pmol β-actin) and reverse primer (2.8pmol β_2-, β_3-AR or 1.5pmol β-actin) in a total volume of 10µl. For β_1-AR PCR, PCR reaction mixes contained cDNA, 0.5 U Platinum Pfx DNA polymerase, 1 x AMP buffer, 1 x enhancer solution, 130µM dNTPs, 1.5mM $MgSO_4$, and 5.8pmol reverse and forward primer. Reverse primer for β-actin was end-labelled with

Table 2.1: Oligonucleotides used as primers and hybridisation probes.

	Strand	Length	Sequence (5' -> 3')	T_m (^oC)[a]
Primers for mRNA analysis				
β_1-AR	for	22	CCG CTG CTA CAA CGA CCC CAA G	71
β_1-AR	rev	26	AGC CAG TTG AAG AAG AGC AAG AGG CG	71
β_2-AR	for	26	GGT TAT CGT CCT GGC CAT CGT GTT TG	71
β_2-AR	rev	29	TGG TTC GTG AAG AAG TCA CAG CAA GTC TC	70
β_3-AR	for	26	TCT AGT TCC CAG CGG AGT TTT CAT CG	76
β_3-AR	rev	25	CGC GCA CCT TCA TAG CCA TCA AAC C	77
β-actin	for	22	ATC CTG CGT CTG GAC CTG GCT G	71
β-actin	rev	25	CCT GCT TGC TGA TCC ACA TCT GCT G	71
Primers for genotyping animals				
neo	for	25	CGC ATC GCC TTC TAT CGC CTT CTT G	77
wt	for	24	GTT GCG AAC TGT GGA CGT CAG TGG	77
neo/wt	rev	22	AAT GCC GTT GGC GCT TAG CCA C	75
Primers for sequencing				
	for	20	TAA TAC GAC TCA CTA TAG GG	48
	rev	18	TAG AAG GCA CAG TCG AGG	50
Primers for PCR cloning				
β_3-AR	for	20	GGA AGC TTC CCA CCC CAG GC	69
β_{3a}-AR	rev	30	GAA TCT AGA TTC CTT GCT GGA TCT TCA CGG	70
β_{3b}-AR	rev	26	CCT TCT AGA GAG AGC GGG ACT GAG GC	70
Probes for hybridisation				
β_1-AR		22	AAG AAG ATC GAC AGC TGC GAG C	68
β_2-AR		20	GCC AGC ATC GAG ACC CTG TG	70
β_3-AR		25	TGC CAA CTC CGC CTT CAA CCC CCT C	81

[a] T_m determined by PRIMER (version 0.5, Whitehead Institute)

[γ-^{33}P]ATP prior to PCR. Briefly, 40pmol reverse primer was end-labelled in a 25µl reaction mix containing 1 x One-Phor-All Plus buffer, 14 U T4 polynucleotide kinase and 50µCi [γ-^{33}P]ATP, incubated at 37°C for 30 min, then at 95°C for 3 min (labelled primer was stored at -20°C). For each set of primers, a single PCR reaction mix was made containing all components apart from the cDNA and then added in aliquots to minimise variation between the samples. Each PCR experiment contained a negative control consisting of a cDNA reaction carried out with the negative RT reaction. Following heating at 95°C for 2 min, amplification cycles were 30 sec at 95°C, 30 sec at 64°C, 30 sec extension at 72°C, for a specified number of cycles (see below), and a final extension at 72°C for 4 min. Annealing temperature for all PCR reactions was 64°C except for β_1-AR PCR where it was 60°C. For each set of primers the log (PCR product) *versus* cycle number was plotted and a cycle number chosen within the linear portion of the graph (data not shown). Cycle numbers were 16 for β-actin, 26 for β_1- and β_2-AR, and 30 for β_3-AR.

Following amplification, PCR products, along with a 100bp molecular weight marker, were electrophoresed on 1.3% agarose gels stained with ethidium bromide, and transferred onto Hybond N^+ membranes by Southern blotting. Briefly, gels were soaked in 0.4M NaOH, 1M NaCl for 30 min and transferred in 0.4M NaOH/1M NaCl overnight (for more detail, refer to Sambrook (1989)). Membranes were rinsed for 5 min in 0.5M Tris pH 7.5/1M NaCl, then 2 x SSC (1 x SSC: 0.15M NaCl, 15mM sodium citrate, pH 7.0) for 5 min. Filters were then allowed to dry for 30 min and stored in cling-film.

For detection of β_1-, β_2- and β_3-AR products, membranes were hybridised with a probe specific for each expected product (Table 2.1). Probes were end-labelled with [γ-^{33}P]ATP. Briefly, 6pmol probe was labelled in a 20µl reaction mix containing 1 x One-Phor-All buffer, 9.5 U T4 polynucleotide kinase and 50µCi [γ-^{33}P]ATP before incubation at 37°C for 30 min and then 95°C for 5 min. Reactions were diluted with 40µl sterile water. Labelled probe was stored at -20°C. Hybridisation of probe to the membranes was carried out. Firstly, membranes were exposed to UV light for 2 min and pre-hybridised in a hybridisation incubator (Bartelt Instruments, Heidelberg West, VIC, Australia) for at least 4 h at 42°C in buffer (5 x Denhardts solution (0.1%

w v^{-1} ficoll type 400, 0.1% w v^{-1} polyvinylpyrrolidone, 0.1% w v^{-1} bovine serum albumin (BSA)), 5 x SSC, 0.5% w v^{-1} sodium dodecyl sulfate (SDS), 100 µg ml^{-1} salmon sperm DNA (heated 5min at 95°C before addition), 0.1mM ATP). An aliquot of the labelled probe (no less than 20µl) was added and the blot hybridised overnight at 42°C. Membranes were rinsed then washed in 2 x SSC/0.1% w v^{-1} SDS for 30 min at 30°C, then rinsed with gentle agitation in the same buffer for 5 min at 37°C. Membranes were dried for 30 min and stored in cling-film.

The PCR protocol was modified for samples used for cloning of the mouse β_{3a}- or β_{3b}-AR. PCR amplification on was carried out on cDNA equivalent to 100ng of starting RNA using intron spanning primers as indicated in Table 2.1. PCR reaction mixes contained cDNA, 2.5pmol each of the forward and reverse primers, 1.5mM Mg-acetate, 1 x Expand PCR buffer, 130µM dNTPs and 2.5 U Taq Expand in a total volume of 20µl (final concentrations in 20µl). Following heating at 95°C for 2 min, amplification cycles were 30 sec at 95°C, 30 sec at 64°C, 30 sec extension at 72°C, with 30 cycles and a final extension at 72°C for 4 min. PCR products were electrophoresed on 1% low melting temperature agarose gels.

2.2.3 Analysis of mRNA products

Radioactivity was detected with a Molecular Dynamics SI phosphoimager (Sunnyvale, CA, U.S.A.), and bands quantified using ImageQuantNT software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). β-AR levels were normalised for β-actin levels (β-AR/β-actin) and expressed as a percentage of the mean ± standard error of the mean (s.e.mean) value of *n* animals from FVB tissue.

2.3 Molecular cloning of the mouse β_{3a}- and β_{3b}-AR

We generated inserts carrying the coding region of the β_{3a}- or β_{3b}-AR by RT-PCR using high-fidelity polymerase (Expand High Fidelity PCR System). The primers included Hind III or Xba I sites for subcloning fragments into the mammalian expression vector pcDNA3.1^+. The complete inserts and junctions with pcDNA3.1^+

were checked by DNA sequencing on both strands (Micromon, Monash University, Australia). Plasmids were linearised with Sca I prior to transfection.

RNA was obtained from BAT from male C57BL/6J mice and DNase treated to remove any contaminating DNA (section 2.2.1). RT and PCR were performed as described in section 2.2.2.

2.3.1 Purification of bands

Bands were excised from gels using a scalpel blade. The agarose was transferred to microcentrifuge tubes and incubated at 70°C until melted. Bands were further purified with the use of the Wizard PCR Prep DNA purification system with provided reagents. Wizard resin (1ml) was added and mixed by inversion for 20 sec. PCR product purification was performed using a 3ml syringe. For each PCR product, one Wizard minicolumn was prepared according to manufacturer's instructions. The resin/DNA solution was pipetted into the syringe barrel and gently pushed into the minicolumn attached to the syringe. The column was washed with 80% isopropanol (2ml) and the minicolumn placed into a microcentrifuge tube and centrifuged (12,000 x g, 20 sec) to dry the resin. The minicolumn was transferred to a new microcentrifuge tube, 50µl water added to the column, incubated for 1 min, and centrifuged (12,000 x g, 30 sec) to elute the bound DNA fragment. The purified DNA can be stored at -20°C without further treatment.

Purified DNA products are run on 1% agarose gels along with 200ng pUC19 DNA digested with Hpa II to quantitate DNA concentration. Figure 2.1 shows gels of β_{3a}- and β_{3b}-AR PCR products before and after purification.

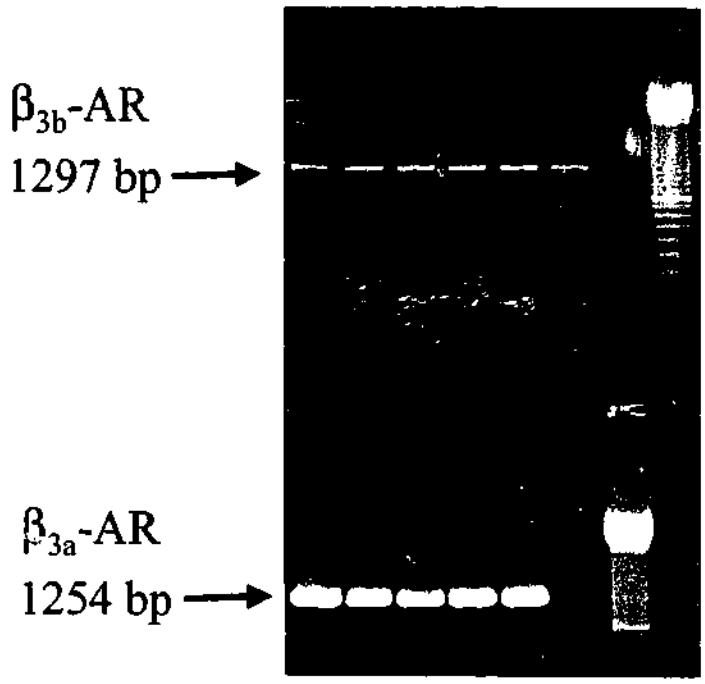
2.3.2 Digestion of purified DNA products and vector

The vector used was pcDNA3.1$^+$. Digestion reaction mixes consisted of pcDNA3.1$^+$ (2µg), 1 x One-Phor-All buffer, 0.01% w v^{-1} BSA, 15 U Hind III and 15 U Xba I in a total volume of 20µl. Reactions were incubated at 37°C for 1 h, briefly centrifuged, and incubated at 60°C for 20 min. 2µl of the digested vector was run on a 1.5%

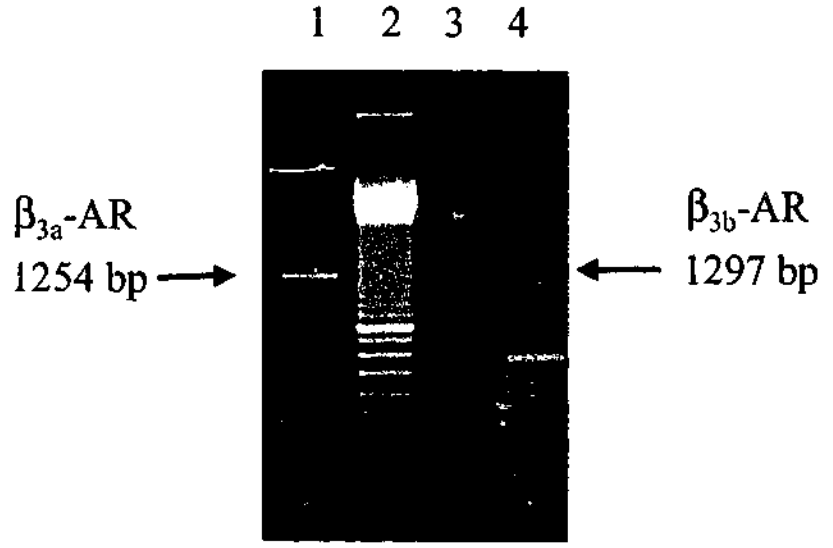
Figure 2.1a: PCR product (16µl) from PCR performed on RT samples using primers specific for the β_{3a}- or β_{3b}-AR and run on 1% low melting point agarose gels. Bands were isolated and purified.

Figure 2.1b: Purified β_{3a}- or β_{3b}-AR were run on 1% agarose gels (lane 1 β_{3a}-AR fragment, lane 3 β_{3b}-AR fragment) along with 100bp ladder (lane 2) and pUC19 DNA digested with Hpa II (lane 4).

(a)


β_{3b}-AR
1297 bp
β_{3a}-AR
1254 bp

(b)


1 2 3 4
β_{3a}-AR
1254 bp
β_{3b}-AR
1297 bp

agarose gel to verify that the vector had been digested. The remaining portion of the digested vector was run on a 1% low melting temperature agarose gel and bands purified as described in section 2.3.1. Figure 2.2 shows a gel of digested pcDNA3.1$^+$ and a vector map of pcDNA3.1$^+$.

Purified β_{3a}- or β_{3b}-AR DNA was digested in the same manner except 40ng DNA was used.

2.3.3 Ligation of β_{3a}- or β_{3b}-AR DNA into pcDNA3.1$^+$

Digested PCR fragments were ligated into digested pcDNA3.1$^+$. Briefly, digested β_{3a}- or β_{3b}-AR PCR fragment (20µl) was incubated with 100ng of digested pcDNA3.1$^+$, 1 x One-Phor-All buffer, 1mM ATP and 5 U T4 DNA ligase in 50µl volume. Samples were incubated at room temperature for 2 h and the ligated DNA was precipitated. A mixture consisting of 100% ethanol (150µl), 1 M NaCl (50µl) and transfer RNA (tRNA) (10µg) was added to a 50µl ligation reaction and samples centrifuged (14,000 x g, 5 min). The supernatant was removed and the pellet washed with ice-cold 70% ethanol and centrifuged (14,000 x g, 2 min). The supernatant was discarded and the pellet resuspended in 10µl of sterile water. Samples could then be stored at -20°C.

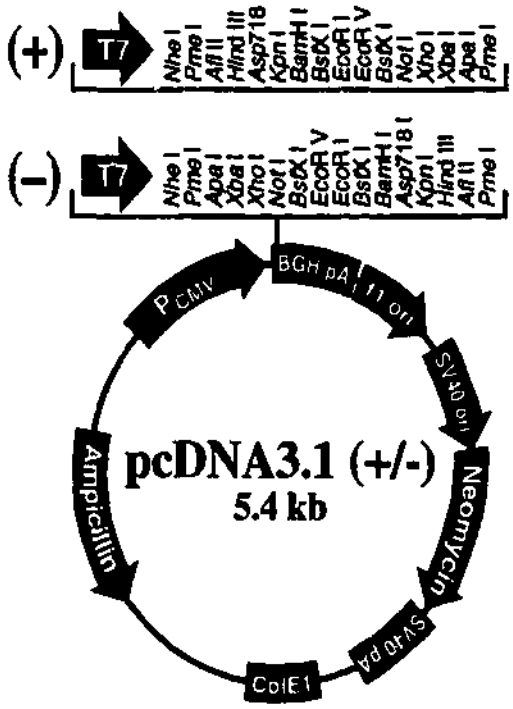
2.3.4 Transformation of ligated pcDNA3.1$^+$ vector into DH$_{5\alpha}$ cells

Ligation mixtures are transformed into competent DH$_{5\alpha}$ cells. Competent DH$_{5\alpha}$ cells were prepared as previously described (Sharma & Schimke, 1996). Competent DH$_{5\alpha}$ cells (50µl) and 3µl ligation mixture were incubated on ice for 5 min. The mixture was transferred to a BioRad Gene Pulser/*E. coli* Pulser cuvette (0.2cm electrode gap) and electroporation performed at 2500V, 25µF capacitance, 200 Ohms (τ= 5-5.3msec) in a Gene Pulser II electroporation system (BioRad Laboratories, Hercules, CA, U.S.A.). SOC media (1ml) (SOB media composition: 20 g l^{-1} casein hydrolysate, 5 g l^{-1} yeast extract, 0.5 g l^{-1} NaCl, 2.5mM KCl, pH 7, 10mM $MgCl_2$; SOC media: SOB media containing 20mM glucose) was quickly added to the cuvette, mixed briefly, and contents transferred to a microcentrifuge tube and placed

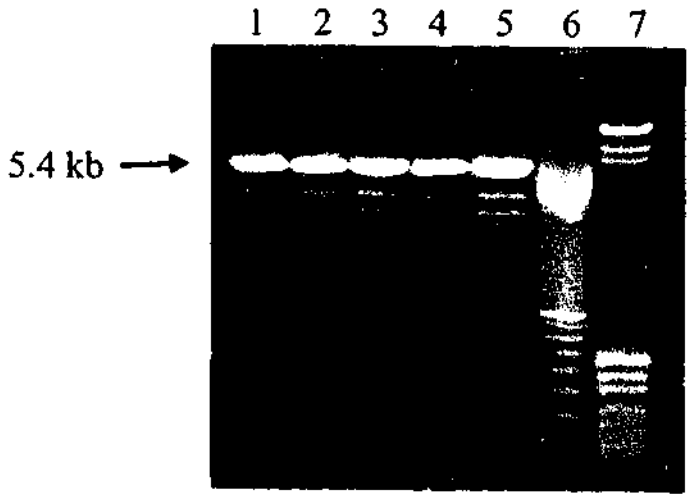
Figure 2.2a: pcDNA3.1+ vector illustrating cytomegalovirus (CMV) enhancer-promoter for high-level expression, large multiple cloning site, bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability, f1 origin for single-stranded rescue of sense strand, SV40 origin for episomal replication, ampicillin resistance gene and ColE1 origin for selection and maintenance in *E. coli*, and neomycin resistance gene for selection and maintenance in mammalian cells.

Figure 2.2b: Purification of digested pcDNA3.1$^+$. Digested products were run on 1% low melting point agarose gels. Order of gel: lanes1-5 2µl of pcDNA3.1$^+$ cut with Hind III and Xba I, lane 6 100bp ladder (5µl), lane 7 λ cut with Hind III (10µl). The 5.4 kb fragment was then purified. Additional fragments below the 5.4kb band probably result from star activity of the Hind III (Nasri & Thomas, 1986). The 80bp fragment released from pcDNA3.1+ by Hind III/Xba I digestion can be seen at the bottom of the gel.

(a)


(+) T7
Nhe I
Pme I
Afl II
Hind III
Asp718 I
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
Pme I
(–) T7
Nhe I
Pme I
Apa I
Xba I
Xho I
Not I
BstX I
EcoR V
EcoR I
BstX I
BamH I
Asp718 I
Kpn I
Hind III
Afl II
Pme I
P CMV
BGH pA
f1 ori
SV40 ori
Neomycin
SV40 pA
ColE1
Ampicillin
pcDNA3.1 (+/-)
5.4 kb

(b)


1 2 3 4 5 6 7
5.4 kb

in an orbital shaker for 60 min. Aliquots of transformed $DH_{5\alpha}$ cells were plated onto agar LB plates (LB broth composition: 20 g l^{-1} casein hydrolysate, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, pH 7, 75 mg l^{-1} ampicillin; agar LB plates: LB broth, 15 g l^{-1} agar) and incubated overnight at 37°C.

2.3.5 Colony selection

LB plates incubated overnight contained colonies of cells. Approximately 20 colonies were selected from all plates and minipreps made using a commercial kit (Wizard Plus Miniprep DNA purification system with provided cell suspension, lysis, neutralisation and wash solutions). Briefly, colonies were picked from plates using sterile toothpicks and placed in 15ml round bottom tubes with capped lids containing 5ml of LB broth and incubated in an orbital shaker overnight at 37°C. Bacterial cells were pelleted by centrifugation (10,000 x g, 1-2 min), supernatant discarded and cell pellet resuspended in 200µl cell suspension solution. Cell lysis solution (200µl) was added and mixed by inversion. Neutralisation solution (200µl) was added, tubes mixed by inversion and centrifuged (10,000 x g, 5 min). Plasmid purification was performed without a vacuum manifold using a Wizard minicolumn according to manufacturer's instructions. Resuspended resin (1ml) and the lysate from each miniprep was transferred to a syringe barrel attached to a minicolumn and solutions gently pushed into the minicolumn. The minicolumn was washed with column wash solution (2ml). The minicolumn was placed into a microcentrifuge tube and centrifuged (10,000 x g, 2 min) to dry the resin. The minicolumn is transferred to a new microcentrifuge tube, 50µl water added to the column, incubated for 1 min, and centrifuged (10,000 x g, 30 sec) to elute the DNA. The DNA can be stored at -20°C without further treatment. Glycerol stocks of bacterial cells were also made (cells stored in 50% glycerol at -70°C) for long term storage.

Purified plasmids were checked for correct inserts. The concentration of DNA was calculated by measuring absorbance at 260 and 280nm and samples (250ng) digested with a reaction mixture (1 x One-Phor-All buffer, 0.01% BSA, 6 U Hind III, 6 U Xba I), in a total volume of 10µl. Reactions were incubated at 37°C for 2 h, then 65°C for 20 h. An aliquot (4µl) of the digested product was run on a 1% agarose gel

for visualisation of correct fragment sizes. Clones with correct restriction enzyme profiles were precipitated.

To precipitate, 2.5 x volume 100% ethanol and 1/4 volume 1M NaCl was added to 2µg of each purified plasmid, samples vortexed and centrifuged (14,000 x g, 15 min). The supernatant was discarded and pellet washed with ice-cold 70% ethanol (100µl) and centrifuged (14,000 x g, 5 min). The supernatant was discarded, and the pellet allowed to air dry for 10 min. The pellet was resuspended in 6µl sterile water. Samples could then be stored at -20°C.

2.3.6 Sequencing of purified plasmids containing DNA inserts

Sequencing was performed on both strands of DNA. Reactions were performed in a FTS-1S capillary thermal sequencer on 400ng of double stranded DNA in a mixture containing either 4pmol reverse or forward primer (see Table 2.1 for primer sequences) and 6µl of ABI Prism Big Dye Terminator reagent version 2 (provided by Micromon, Monash University, Australia) in a total volume of 15µl. Samples were heated to 96°C for 10 sec, 50°C for 5 sec, and then 60°C for 4 min for 25 cycles. Reactions were stopped by incubation at 4°C for 4 min.

Products were purified by the addition of 1/10 volume 3M Na-acetate pH 5.2 and 2.5 volumes 95% ethanol, vortexed and incubated on ice for 10 min. Samples were centrifuged (14,000 x g, 25 min), supernatant removed and pellet washed with 70% ethanol (250µl). Samples were centrifuged (14,000 x g, 2 min), supernatant removed and pellet air dried. Samples were then sequenced at Micromon.

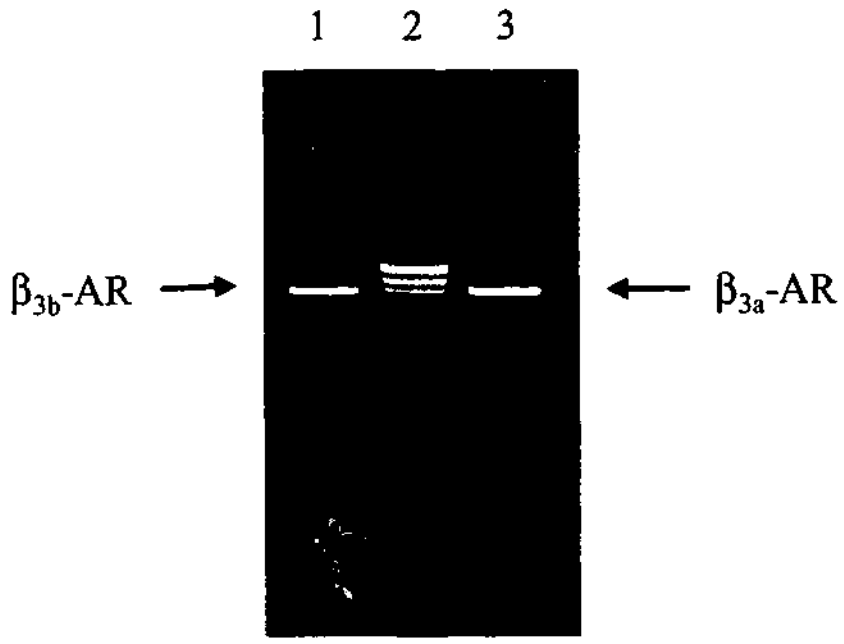
2.3.7 Linarisation of plasmid containing DNA inserts

Large-scale preparation of constructs was performed using a Wizard Plus Midiprep DNA purification system (using solutions provided). Briefly, 100µl of cell glycerol stock was incubated in 100ml of SOC media overnight at 37°C in an orbital shaker. Cells were pelleted by centrifugation (4,000 x g, 10 min, 4°C), supernatant discarded and cell pellet resuspended in 3ml cell suspension solution. Cell lysis solution (3ml)

was added and samples mixed by inversion. Neutralisation solution (3ml) was added, tubes mixed by inversion and centrifuged (14,000 x g, 15 min, 4°C). Plasmid purification was performed with a vacuum manifold using a Wizard midicolumn according to manufacturer's instructions. 10ml of resuspended resin was added to a syringe barrel, and the lysate from each midiprep transferred to the syringe barrel containing the resin and the solution gently pushed into the midicolumn by use of a vacuum. Wash solution (15ml) was added three times to the syringe to wash the column. The midicolumn was placed into a tube and centrifuged (10,000 x g, 2 min) to dry the resin. The midicolumn was transferred to a new tube, 300µl water added to the column, incubated for 1 min, and centrifuged (10,000 x g, 20 sec) to elute the DNA. The eluted DNA was centrifuged for 5 min to pellet resin fines and the supernatant transferred to a new tube. The DNA can be stored at -20°C without further treatment. Glycerol stocks were also made (stored with 50% glycerol at -70°C) for long term storage.

To linarise DNA, digestion reactions were performed on 60µg of purified plasmid containing the DNA of the β_{3a}- or β_{3b}-AR using 2 x One-Phor-All buffer and 150 U Sca I in a total volume of 400µl. Reactions were performed overnight at 37°C. An aliquot of plasmid was run on a 1% agarose gel to check for complete digestion. The remaining Sca I digest was phenol-chloroform extracted. Briefly, 200µl of phenol was added to the mixture, vortexed and centrifuged (12,000 x g, 2 min). Chloroform (200µl) was added to the top aqueous phase, vortexed and centrifuged (12,000 x g, 5 min). To the top aqueous phase, 3 x volume 100% ethanol and 1/10 volume Na-acetate pH 5.2 was added and samples incubated at -20°C for 30 min. Samples were centrifuged (12,000 x g, 10 min), supernatant removed, and the resulting pellet resuspended in 30µl TE buffer (10mM Tris pH 7.4, 1mM EDTA pH 8). Linarised plasmid DNA (100ng) and pUC19 DNA digested with Hpa II (200ng) were run on a 1% agarose gel to quantitate DNA concentration. Figure 2.3 shows the linarised constructs before transfection into CHO-K1 cells.

Figure 2.3: Linarised pcDNA3.1$^+$ vector containing DNA of either the β_{3a}- or β_{3b}-AR (approximately 100ng), which had been linarised with Sca I. Lane 1: β_{3a}-AR containing vector; Lane 2: pUC19 DNA digested with Hpa II (200ng); Lane 3: β_{3b}-AR containing vector.


1
2
3
β_{3b}-AR
β_{3a}-AR

2.3.8 *Cell transfection*

CHO-K1 cells were transfected with linarised plasmid DNA (section 2.3.7) by electroporation with a Gene Pulser II electroporation system. CHO-K1 cells were grown to ~70% confluence and harvested as detailed in section 2.1.1 (except cells were washed with phosphate buffered saline (PBS) twice (composition (mM): NaCl 140, KCl 2.7, Na_2HPO_4 10, KH_2PO_4 1.8, pH 7.4). CHO-K1 cells ($5x10^6$) were resuspended in PBS (700µl) in a BioRad Gene Pulser cuvette (0.4 cm electrode gap) containing 15µg linearised plasmid DNA (no DNA for negative control) in 15-20µl TE buffer (10mM Tris pH 7.4, 1mM EDTA pH 8.0) and incubated on ice for 5 min. Electroporation was performed at 270V, 950µF capacitance (τ= 15-25msec) and then left on ice for 5 min. Cells were transferred to flasks with a Pasteur pipette and incubated for 16-24 h. Media was changed once after this period, and media changed to that containing G418 (800 µg ml^{-1}) 48 h after electroporation.

2.3.9 *Dilution cloning*

Single cell colonies were obtained by the method of dilution cloning. This method relies on seeding cells at low density, incubating until colonies form, and propagation into a clonal cell line. Briefly, cells were harvested (section 2.1.1) after transfected CHO-K1 cells were selected with G418. Cells were counted (section 2.1.3) and serial dilutions performed to obtain a concentration of 5-8 cells per ml media. Aliquots (100µl) of cell suspensions were transferred to 96-microtiter plates. Plates were placed in the incubator for ~2 weeks before being examined for cellular growth. Colonies were isolated by standard trypsinisation (section 2.1.1) and expanded. Approximately 25% of wells contained cells (assumed to arise from single cell colonies) after this period.

Colonies (once expanded) were evaluated for receptor expression using a radioligand binding screen (use of one concentration of ICYP (800pM; section 2.5). Suitable clones were used for further study.

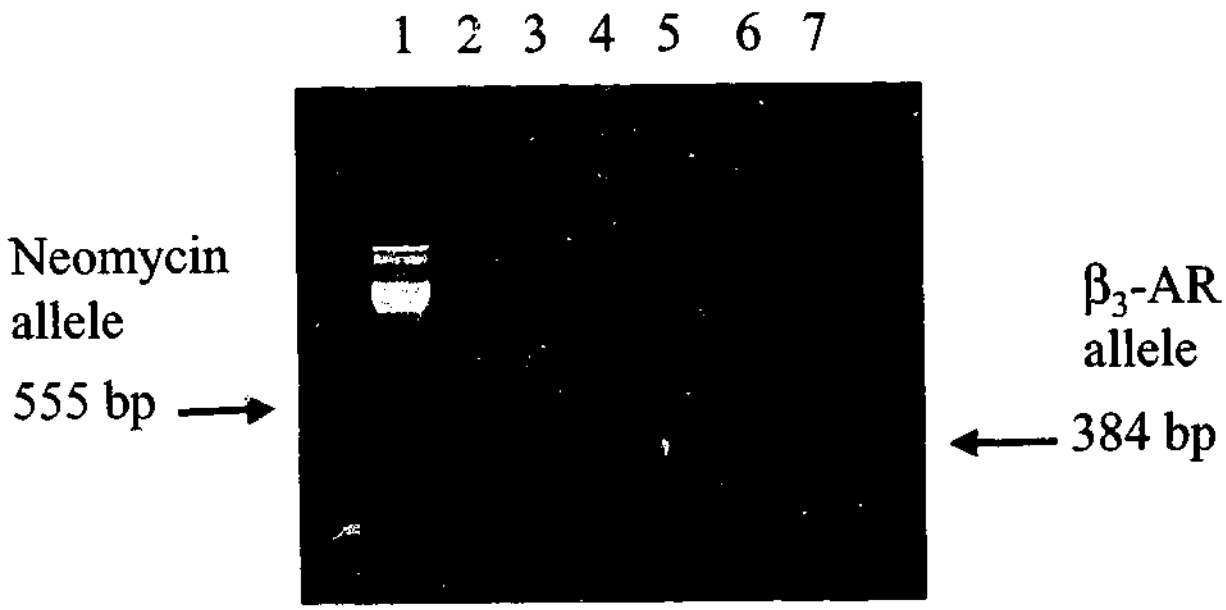
2.4 *Genotyping of animals*

FVB mice (female, ~ 4 months old) were obtained from the Animal Resources Centre, Canning Vale, Western Australia. β_3-AR KO mice (male, ~ 1 year old) were the offspring of a previously described strain (Susulic *et al.*, 1995).

Genomic DNA analysis was conducted on mouse tails to determine the genotype of all mice used for breeding and experimental studies. Genomic DNA was isolated by proteinase K digestion overnight followed by phenol-chloroform extraction. Mouse tail (approximately 0.1g tissue) was incubated with 400µl proteinase K digestion mixture (10mM Tris HCl pH 8, 10mM NaCl, 10mM EDTA, 0.5% w v^{-1} SDS, 80 µg ml^{-1} proteinase K) overnight at 62°C in an air shaker in microcentrifuge tubes. Tubes were centrifuged (14,000 x g, 2 min), supernatant transferred to a fresh microcentrifuge tubes, a half volume of phenol (buffered with Tris pH 8) added and tubes mixed by inversion. Chloroform (200µl) was added, tubes mixed by inversion, and spun at 14,000 x g for 5 min. The supernatant was transferred to a new tube, 1/10 volume of 3M Na-acetate pH 5.2 added followed by addition of 2.5-3 volumes of ethanol, and tubes mixed by inversion and spun at 14,000 x g for 5 min. Supernatant was discarded and the pellet washed with 70% ethanol (200µl), vortexed and spun (14,000 x g, 5 min). Supernatant was removed and the pellet resuspended in sterile water and vortexed. DNA samples were then stored without any other treatment at -20°C.

PCR was performed on 0.5µg DNA using primers designed to indicate the presence/absence of neomycin disruption to the β_3-AR allele (Table 2.1). PCR reaction mixes contained DNA, 1 U Taq polymerase, 1 x PCR buffer, 200µM dNTPs, 2mM Mg-acetate, and 2.5pmol each of forward and reverse primer in a total volume of 10µl. PCR was carried out in a FTS-1S capillary thermal sequencer. Samples were denatured (95°C, 2 min) and each amplification stage consisted of sequential denaturation (95°C, 30 sec), annealing (62°C, 30 sec), extension (72°C, 30 sec) and final extension (72°C, 4 min) for a total of 32 cycles. Products were run on a 1.3% agarose gel stained with ethidium bromide to evaluate genotype of mice (Figure 2.4).

Figure 2.4: Gel illustrating expected PCR products from genotyping of mice. Lane 1: 100bp molecular weight marker; Lane 2-4: PCR to indicate presence/absence of neomycin disruption to β_3-AR allele; Lane 5-7: PCR to indicate presence/absence of wild type β_3-AR allele. Wild type mice (+/+) (lane 2, 5) display only the β_3-AR allele. Heterozygote mice (+/-) (lane 3, 6) display both neomycin and β_3-AR allele. β_3-AR KO (-/-) mice (lane 4, 7) display only neomycin disruption to β_3-AR allele.


1 2 3 4 5 6 7
Neomycin allele
555 bp
β_3-AR allele
384 bp

2.5 *Radioligand binding experiments*

2.5.1 *Membrane preparation*

2.5.1.1 *Cell membranes*

Cells were grown to 95% confluence as a monolayer before membranes were harvested for radioligand binding studies. Cells were washed twice with HBS, and gently scraped from flasks into lysis buffer (25mM Tris pH 7.5 room temperature, 1mM EDTA, 10 mg ml^{-1} bacitracin, 10 mg ml^{-1} leupeptin, 10 mg ml^{-1} pepstatin A, 0.5mg ml^{-1} aprotinin) using a cell scraper. Cells were homogenised with a Dounce tissue grinder (Wheaton, Millville, NJ, U.S.A., approximately 10 strokes per pestle), and centrifuged at low speed (800 x g, 10 min) to separate membranes and nuclear fractions. Supernatant was kept and the resulting pellet re-homogenised in lysis buffer and centrifuged again. Supernatants were pooled and centrifuged (39,000 x g, 15 min, 4°C). The resulting pellet was homogenised in binding buffer (50mM Tris pH 7.4 room temperature, 5mM $MgCl_2$, 1mM EDTA, 10 mg ml^{-1} bacitracin, 10 mg ml^{-1} leupeptin, 10 mg ml^{-1} pepstatin A, 0.5mg ml^{-1} aprotinin) and placed on ice until use on the same day and the unused portion frozen (-20°C) for determination of protein levels (section 2.10).

2.5.1.2 *Mouse ileum membranes*

For ileum membrane preparation, tissue was dissected as described in section 2.2.1 and homogenised in 20-25ml of ice cold buffer (10mM Tris, pH 7.4 4°C, containing 10μM phenylmethylsulphonyfluoride (PMSF), 250mM sucrose) using a PRO200 homogeniser. The homogenate was centrifuged (39 x g, 10 min, 4°C), the supernatant kept and centrifuged (39,000 x g, 15 min, 4°C). The pellet was resuspended in binding buffer and stored on ice until use on the same day and the unused portion frozen (-20°C) for determination of protein levels (section 2.10).

2.5.2 *Kinetic studies*

The association of ICYP (500pM) to specific binding sites in 50µl aliquots of cell membranes was determined at various times up to 90 min. Non-specific binding was determined with (-)-alprenolol (1mM). The dissociation of ICYP was determined after 60 min incubation with ICYP (500pM) at intervals up to 90 min after the addition of (-)-alprenolol (1mM). All kinetic studies were performed in duplicate in a total volume of 100µl at room temperature in 96 well microtiter plates. Reactions were terminated by rapid filtration through pre-wetted GF/C Uni filter plates (Packard Instruments, Meriden, CT, U.S.A.) pre-soaked for 30 min in 0.5% polyethylenimine using a Packard FilterMate (Packard Instruments, Meriden, CT, U.S.A.). Polyethylenimine is a cationic polymer that reduces the non-specific binding of the radioligand to the glass fibre filters (Bruns *et al.*, 1983). Filters were washed four times with wash buffer (50mM Tris pH 7.4 4°C), dried, 30µl Microscint-O (Packard Instruments, Meriden, CT, U.S.A.) added and radioactivity measured using a Packard TopCount (Packard Instruments, Meriden, CT, U.S.A.).

2.5.3 *Saturation binding*

Saturation of ICYP binding at β_3-ARs in CHO-K1 cells or ileum membranes was determined by incubating aliquots of membranes (50µl) with ICYP (100-2000pM) at room temperature for 60 min in a final volume of 100µl in 96 well microtiter plates. Non-specific binding was defined by (-)-alprenolol (1mM). All experiments were performed in duplicate. Reactions were terminated by rapid filtration through pre-wetted GF/C Uni filter plates pre-soaked for 30 min in 0.5% polyethylenimine using a Packard FilterMate. Filters were washed four times with wash buffer (50mM Tris pH 7.4 4°C), dried, 30µl Microscint-O added and radioactivity measured using a Packard TopCount.

To study the saturation of ICYP binding at β_1-/β_2-ARs in mouse ileum membranes, aliquots of membrane (50µl) were incubated with ICYP (5-100pM) at room temperature for 60 min in a final volume of 100µl in microcentrifuge tubes. Non-specific binding was defined by (-)-propranolol (1µM). After incubation, tubes were

centrifuged briefly (14,000 x g, 2 min), supernatants discarded and the pellet resuspended in 100µl binding buffer and transferred to 96 well microtiter plates for 30 min to minimise any low affinity binding. All experiments were performed in duplicate. Reactions were terminated by rapid filtration through GF/B Uni filter plates (Packard) pre-soaked in wash buffer (50mM Tris pH 7.4 room temperature) using a Packard FilterMate. Filters were washed 4 times with wash buffer, dried, 30µl Microscint-O added and radioactivity measured using a Packard Top Count.

2.5.4 Competition binding

Aliquots of cell membranes (50µl) were incubated with ICYP (500pM) and a range of concentrations of competing agents, as indicated in the relevant chapters, in a total volume of 100µl for 60 min at room temperature in 96-microtiter plates. Non-specific binding was defined by (-)-alprenolol (1mM). Experiments were performed in duplicate. All reactions were terminated by rapid filtration through GF/C filters pre-soaked for 30 min in 0.5% polyethylenimine using a Packard Cell Harvester. Filters were washed 4 times with wash buffer (50mM Tris pH 7.4 4°C), dried, 30µl Microscint-O added and radioactivity measured using a Packard Top Count.

2.5.5 Analysis of radioligand binding experiments

2.5.5.1 Kinetic data

Kinetic constants were obtained using GraphPad PRISM to obtain the observed association rate constant (K_{obs}) and dissociation rate constant (K_{-1}) using one phase exponential association or decay analysis respectively. The association rate constant (K_1) was then determined by

$$K_1 = K_{obs} - K_{-1} / [\text{radioligand}] \; M^{-1} \; min^{-1}$$

That allowed the calculation of the equilibrium dissociation constant (K_D) from

$$K_D = K_{-1} / K_1$$

2.5.5.2 Saturation data

Saturation binding data was analysed using GraphPad PRISM using a non-linear regression one site binding site model to obtain K_D and maximum density of binding sites (B_{max}) values. B_{max} is expressed as fmol mg protein^{-1} and K_D is expressed in pM for the data analysed. Data was plotted as the specific binding (expressed as fmol mg protein^{-1}) against the concentration of radioligand used.

2.5.5.3 Competition data

Competition data was analysed with GraphPad PRISM using a one-site competitive non-linear curve fitting program to obtain the concentration of compound which inhibits 50% of the binding of a radioligand (IC_{50}), which was then converted to the negative logarithm of the affinity of a ligand (pK_I) by the program using the Cheng & Prusoff (1973) equation

$$IC_{50}=K_i(1+[L]/K_D)$$

Where IC_{50} is the concentration of unlabelled drug which inhibits 50% of the binding, K_i is the equilibrium dissociation constant of the unlabelled compound, [L] is the concentration of the radioligand used, and K_D is the equilibrium dissociation constant of the radioligand as determined from saturation studies.

Competition binding curves were plotted as a percentage of the radioligand bound against the log concentration of the competing drug.

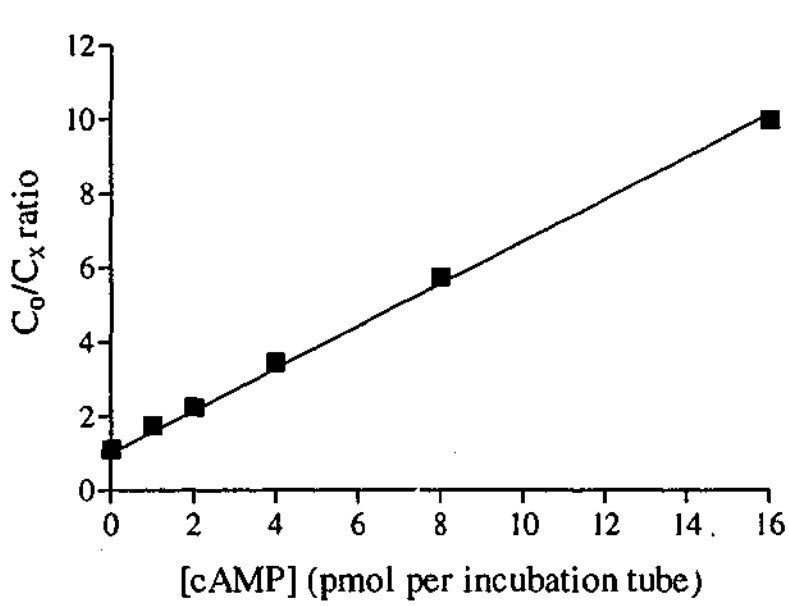
2.6 cAMP accumulation studies

The assay used to measure cAMP (Amersham cyclic AMP (^{3}H) assay system, Uppsala, Sweden), is based on the competition between unlabelled cAMP (unknown or standard) and a fixed amount of [^{3}H]cAMP for a binding protein purified from bovine muscle which has high specificity and affinity for cAMP (Figure 2.5; refer to manufacturers instructions for more detail).

Figure 2.5a: Basis of cAMP accumulation assay.

Figure 2.5b: A typical cAMP standard curve.

Free cAMP		**Bound cAMP**
[^{3}H]cAMP		[^{3}H]cAMP-binding protein
	+ binding protein $\rightleftharpoons$	
cAMP		cAMP-binding protein



2.6.1 Sample preparation

Cells were harvested as described in section 2.1.1 and seeded into 12-well plates at $1x10^5$ cells per well in media containing FBS (0.5% v v^{-1}) for 2 days. On the day of the experiment, media was replaced to contain 3-isobutyl-1-methylxanthine (IBMX, 1mM) and ascorbic acid (1mM) for 2 h. IBMX is a non-specific phosphodiesterase inhibitor (Wells & Miller, 1988) and ascorbic acid was added to prevent the degradation of catecholamines used. Cells were exposed to agonists for times as specified in the relevant chapters. In experiments where cells were pretreated, drugs were added prior to agonist addition for specified times as indicated with the data. Experiments were performed in duplicate.

Segments of mouse ileum were prepared as previously described (section 2.1.4). Following an initial equilibration period of 1-2 h, media was replaced to contain IBMX (1mM) and ascorbic acid (1mM) for 1 h. Tissues were exposed to drugs for 30 min.

2.6.2 Extraction and determination of cAMP by radiobinding assay

Following cell treatments, the reactions were stopped by aspiration of the media and addition of 0.8ml ice cold 75% ethanol containing EDTA (1mM). Cells were scraped off with a cell scraper and the suspension transferred to microcentrifuge tubes before being dried in a Speedvac centrifuge (Savant, Holbrook, NY, U.S.A.). The dried samples were dissolved in 50mM Tris pH 7.4 4°C (containing 1mM EDTA) and samples sonicated (Sonifier cell disrupter, Branson Sonic Power Company) for a few seconds. Samples could then be stored at -20°C.

Following treatment of ileum segments, tissues were blotted dry and snap frozen in liquid nitrogen and stored at -70°C. cAMP was extracted by homogenisation of tissues in 1ml 75% ethanol containing EDTA (1mM) using a PRO200 homogeniser. Samples were centrifuged (2,500 x g, 2 min) and the supernatant transferred to a new tube. The pellet was washed with 100µl 75% ethanol (containing EDTA (1mM)) and the supernatant combined. The pellet was kept for determination of protein levels as

described in section 2.10. The supernatant was dried in a Speedvac centrifuge and the dried samples dissolved in 50mM Tris pH 7.4 4°C (containing 1mM EDTA) and samples sonicated for a few seconds. Samples were used immediately for determination of cAMP levels.

The cAMP in the samples was measured using a commercial kit according to manufacturer's instructions. Briefly, aliquots of unknown samples containing cAMP were incubated with a specific amount of [^{3}H]cAMP and binding protein for 2-3 h in microcentrifuge tubes, in an ice bath kept at 4°C. Charcoal was added to samples (to separate protein bound cAMP from the unbound cAMP by adsorption of the free cAMP to the charcoal), vortexed immediately and centrifuged to sediment the charcoal. A sample of the supernatant was removed and placed in scintillation vials containing 3ml scintillant (EcoLite, ICN, Costa Mesa, CA, U.S.A.) for counting on a β-counter (Packard Minaxi Tri-Carb 400 series, Packard Instruments, Meriden, CT, U.S.A.). cAMP was used as the standard (1-16pmol per incubation tube) and samples treated the same as the set of unknowns.

2.6.3 Analysis of cAMP data

For each standard and unknown replicate, the amount of [^{3}H]cAMP in the absence of unlabelled cAMP (C_0) over the amount of [^{3}H]cAMP bound in the presence of standard or unknown unlabelled cAMP (C_x) was expressed (C_0/C_x) against pmoles of inactive cAMP standard (measurements measured as counts per minute (cpm)). A linear standard curve was produced (see Figure 2.5 for a representative standard curve) using linear-regression with GraphPad PRISM and interpolation of unknowns was based on the standard curve for each experiment. Data was analysed using non-linear regression using a sigmoid dose response (variable slope) curve fitting program (GraphPad) to construct concentration-response (c-r) curves to the data and the negative logarithm of the EC_{50} (pEC_{50}) values obtained. C-r curves are plotted as mean ± s.e.mean of *n* individual experiments. In experiments where cells were treated, paired cell samples were used where cells were obtained from the same flask of cells before plating out into 12 well plates. In some experiments, data is presented as a bar chart.

2.7 *Western blotting/Immunoblotting*

For information on standard Western blotting/immunoblotting techniques, refer to Harlow & Lane (1988).

2.7.1 *Sample preparation*

Cells ($1x10^5$ cells per well) were grown in 12 well plates in media containing 0.5% v v^{-1} FBS for 2 days and media replaced 2 h before experiments commenced. Some cells were pretreated before being exposed to agonist and times of treatments are indicated in the relevant chapters. All experiments were performed in duplicate. After treatment, media was aspirated and cells immediately lysed by the addition of 40µl 2 x SDS sample buffer (composition: 62.5mM Tris pH 6.8, 2% SDS, 10% glycerol, 50mM dithiothreitol, 0.1% bromophenol blue). 2 x SDS buffer was heated to 65°C before addition. Cells were scraped, transferred to microcentrifuge tubes on ice, sonicated briefly and boiled for 5 min. Samples could then be stored at -20°C. Aliquots (4µl) of samples were separated on 12% polyacrylamide gels.

Segments of mouse ileum were prepared as previously described (section 2.1.4). Following an initial equilibration period of 1-2 h, media was replaced and tissues equilibrated for a further 1 h before tissues were exposed to agonist (times of treatment is indicated in the relevant chapter). After treatment, tissues were blotted dry and snap frozen in liquid nitrogen and stored at -70°C. Tissues were homogenised with a Dounce homogeniser in 100-300µl ice-cold PBS buffer (composition (mM): dibasic sodium phosphate 9.1, monobasic sodium phosphate 1.7, NaCl 150mM, pH 7.4) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF (dissolved in isopropanol), 30µl ml^{-1} aprotinin and 1mM sodium orthovanadate. PMSF (10µl of 10 mg ml-^{1} stock) was added to samples and incubated on ice for 30 min. The insoluble protein was removed by centrifugation (10,000 x g, 10 min, 4°C), the supernatant retained and protein measured (section 2.10). Protein (~50µg) was mixed with an equal volume of 2 x SDS sample buffer (without addition of dithiothreitol) and boiled for 3 min. The unused portion of the

sample could be stored at -20°C. Samples were separated on 12% polyacrylamide gels.

2.7.2 Gel conditions and antibody conditions

Samples were separated on 12% polyacrylamide gels along with a standard marker (BioRad Kaleidoscope prestained standards) at 100V (constant) until the bromophenol blue front reached the bottom of the gel (2-3 h). Following electrophoresis, gels were soaked in transfer buffer number 2 (0.025M Tris, 20% methanol, pH 10.4) for 5-10 min with gentle shaking before being electrotransfered to Hybond-C Extra nitrocellulose membranes (pore size 0.45μm) with a semi-dry blotter (CBS Scientific, Del Mar, CA, U.S.A.) for 30 min at 2-2.5 mA cm^{-2} of membrane. On the bottom of the semi-dry blotter (positive electrode), assembly of the gel, nitrocellulose membrane and Whatman 3MM paper was in the following order: positive electrode, 6 sheets of 3MM paper soaked in transfer buffer number 1 (300mM Tris, 20% methanol, pH 10.4), 3 sheets of 3MM paper presoaked in transfer buffer number 2, nitrocellulose membrane presoaked in distilled water, polyacrylamide gel, 6 sheets of 3MM paper presoaked in transfer buffer number 3 (38mM Tris, 10mM β-alanine, 20% methanol, pH 9.4), negative electrode. Following transfer, the membranes were washed in Tris buffered saline (TBS; 20mM Tris, 140mM NaCl, pH 7.6) for 5 min followed by quenching of non-specific binding in blocking buffer (5% nonfat dry milk, 0.1% Tween-20 in TBS) for 1 h at room temperature followed by three 5 min washes with TBS-Tween-20 (TBST; TBS, 0.1% Tween-20). Membranes were incubated at 4°C overnight with gentle shaking with primary antibody (phospho-p44/42 MAP kinase (Thr-202/Tyr-204) and at 1:1000 dilution factor) in primary antibody dilution buffer (TBST containing 5% w v^{-1} Fraction V BSA). Membranes were washed times 3 times for 5 min in TBST. Primary antibody was detected using a secondary antibody (HRP linked anti-rabbit) at 1:2000 dilution factor in blocking buffer, membranes washed three times for 5 min in TBST and detected with enhanced chemiluminescence (ECL) according to manufacturer's instructions. Blots were exposed to Hyperfilm ECL film. Membranes could then be stripped (30 min, 50°C in 10M urea, 50mM sodium phosphate, 100mM β-mercaptoethanol), washed in TBST until almost no β-mercaptoethanol

smell can be detected on the membrane and reprobed with another primary antibody (p44/42 MAP kinase (Thr-202/Tyr-204)) and detected as above.

Polyacrylamide gels were assembled with the Mini Protean 3 electrophoresis module (BioRad Laboratories, Hercules, CA, U.S.A.) and run in running buffer (25mM Tris, 200mM glycin, 0.1% SDS). Separation gels were composed of the following: 30% acrylamide (4.8ml), Tris pH 8.8/SDS buffer (1.5M Tris pH 8.8, 0.4% SDS; 6ml), 5% glycerol (0.8ml), ammonium persulphate (100 mg ml^{-1}, 60µl), TEMED (5µl). Stacking gels were composed of: 30% acrylamide (0.5ml), Tris pH 6.8/SDS buffer (0.5M Tris pH 6.8, 0.4% SDS; 2.5ml), water (2ml), bromophenol blue solution (4mg bromophenol blue in 0.5M Tris pH 6.8; 20µl), ammonium persulphate (100 mg ml^{-1}, 25µl), TEMED (5µl).

2.7.3 Analysis of data

Radioautographs were visualised on a Molecular Dynamics densitometer and bands quantified using ImageQuantNT Software (Molecular Dynamics). Results are expressed as the ratio between phosphorylated and total Erk1/2 protein (α-p-Erk/α-Erk), with the ratio normalised in each experiment to that of controls, with *n* referring to the number of independent experiments performed in duplicate.

2.8 Cytosensor microphysiometer studies

The cytosensor microphysiometer (Molecular Devices Corp., California, U.S.A.) is a silicon light addressable sensor-based device that can be used to measure the cellular activity of isolated cells (McConnell *et al.*, 1992). Cells are placed between two porous polycarbonate membranes and placed in a sensor chamber where a plunger creates a microvolume cavity and cells are superfused with a controlled flow of medium. Effector agents can then be introduced to the cell chamber and incubated for defined periods of time. The basis of measurement involves the interaction of a range of effector agents (including ligands for cell surface receptors such as cytokines, mitogens, hormones and pharmacological agents) with their respective receptor/target that leads to a functional response requiring energy. Energy

metabolism in living cells is tightly coupled to cellular ATP usage so that events which alter cellular ATP levels such as receptor activation and subsequent initiation of signal transduction pathways, will alter acid excretion in cells (Figure 2.6). The rate of acid secretion (acidification rate) is detected as a change in potential across a silicon light-addressable potentiometric sensor (LAPS) during periods of cessation of flow of medium. Alterations in the metabolic activity of cells induced by effector agents will result in changes in the rate of excretion of acidic products, detected to very low levels by the LAPS within the cytosensor unit. This variation in acidification rate as compared to basal acidification rates is reported by the Cytosensor system. Figure 2.7 shows a schematic diagram of the cytosensor microphysiometer set up.

The cytosensor microphysiometer has been used for determination of the activity and mode of action of various compounds/ligands that interact with specific cell surface receptors, including muscarinic (Baxter *et al.*, 1994), dopamine (Coldwell *et al.*, 1999), α-AR (MacLennan *et al.*, 2000; Pihlavisto & Scheinin, 1999), somatostatin (Chen & Tashjian, 1999) and 5-HT (Dunlop *et al.*, 1998) receptors to name a few. Measurements of compounds tested (such as EC_{50} (concentration of compound which produces 50% of maximum effect)) in the cytosensor microphysiometer generally demonstrate good correlation with EC_{50} values determined using more traditional approaches (Hirst & Pitchford, 1993). Hence the cytosensor microphysiometer has the potential for use in a wide variety of applications in conjunction with traditional pharmacological methods.

The cytosensor microphysiometer used for experiments described in this thesis consisted of two separate four-chamber machines connected together to produce an eight channel system. Chambers were maintained at 37°C.

2.8.1 Cytosensor consumables

Items required to operation and maintenance of the cytosensor microphysiometer are listed below. All consumables were obtained from Molecular Devices unless stated otherwise.

Figure 2.6: Schematic diagram illustrating the cell biology of extracellular acidification. Not all possible pathways are shown in this figure. Diagram annotated from McConnell *et al.* (1992).

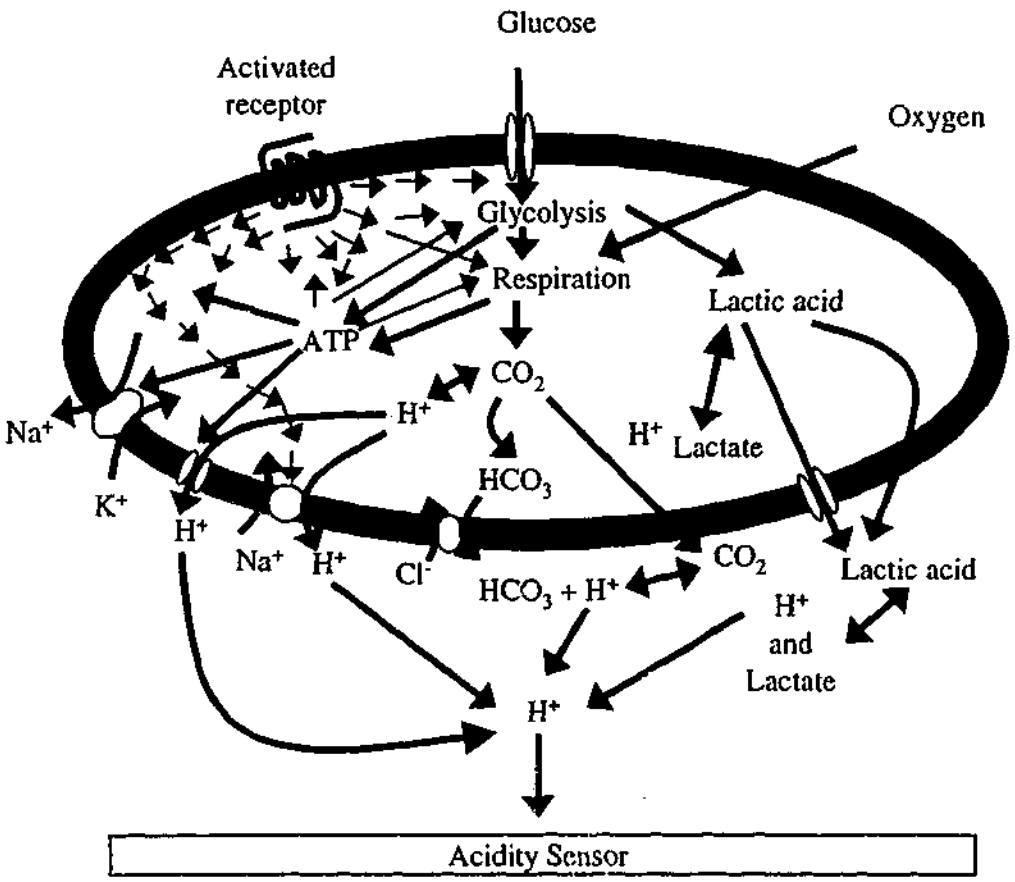
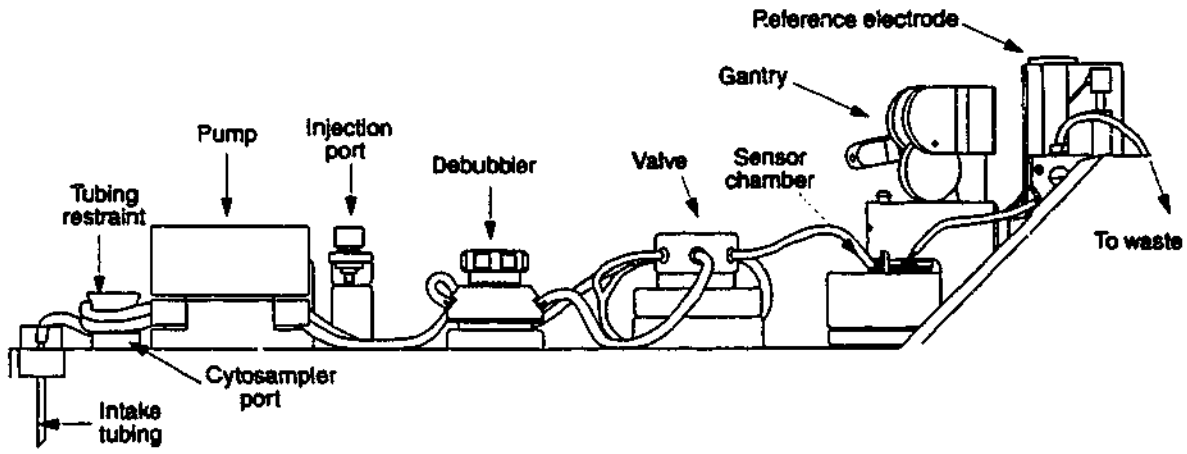
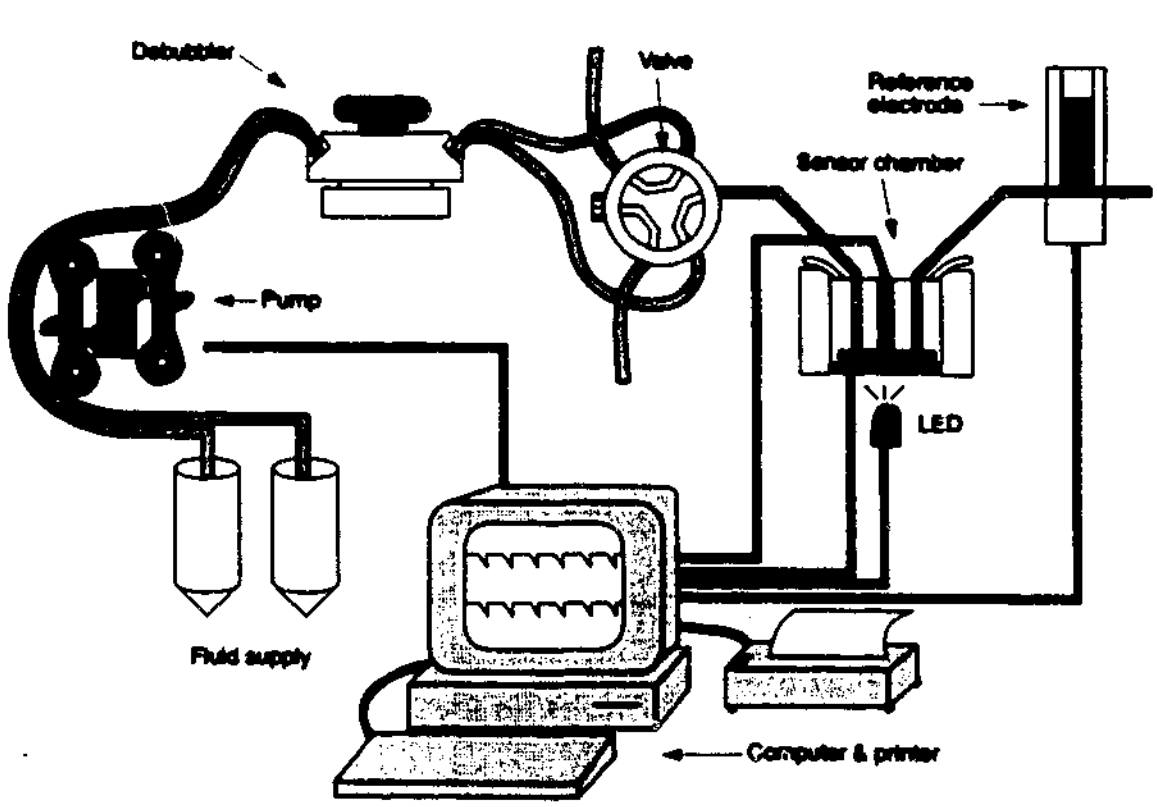

Glucose
Activated receptor
Oxygen
Glycolysis
Respiration
Lactic acid
ATP
CO_2
Na^+
H^+
H^+ Lactate
HCO_3
K^+
H^+
Na^+
H^+
Cl^-
$HCO_3 + H^+$
CO_2
Lactic acid
H^+ and Lactate
H^+
Acidity Sensor

Figure 2.7a: A diagram of side view of a microphysiometer fluid path (from cytosensor microphysiometer system user's manual, Molecular Devices, 1995).

Figure 2.7b: Schematic representation of the Cytosensor microphysiometer system (from cytosensor microphysiometer system user's manual, Molecular Devices, 1995).


Reference electrode
Gantry
Pump
Injection port
Debubbler
Valve
Sensor chamber
Tubing restraint
To waste
Cytosampler port
Intake tubing


Debubbler
Valve
Reference electrode
Sensor chamber
Pump
LED
Fluid supply
Computer & printer

For cell assembly and operation of cytosensor

12mm transwell inserts (capsule cups) (Costar, Corning, NY, U.S.A.): sterile, disposable cell capsule with polycarbonate membrane (3µm pore size) used for growing cells directly on to the membrane.

Spacers: sterile, blue plastic spacer used to define the height and diameter of compartment containing cells and protect cells from being squashed by insert.

Inserts: sterile, plastic cup with polycarbonate membrane used to protect cells from media flow and prevent washout of cells from capsule cup.

15 and 50 ml plastic centrifuge tubes (Life Technologies): sterile, used for media and drug solutions and attached directly on to the cytosensor.

12-well plates (Life Technologies): sterile, used to contain cells growing on capsule cups.

RPMI160 media: powdered low buffering RPMI1640 media (bicarbonates, phosphates and other buffers omitted) specifically designed for use with the cytosensor was stored at 4°C. Media was reconstituted with MilliQ filtered deionised water (Millipore Corp., Marlborough, MA, U.S.A.), pH adjusted to 7.2-7.4, and sterilised by filtration through sterile disposable 0.2µm pore size filter units (Nalgene Nunc, Rochester, NY, U.S.A.) and stored in sterile 1L bottles at 4°C for up to 2 weeks. On the day of the experiment, media was pre-warmed to room temperature before use.

For maintenance of cytosensor

Detergent A and bleach B solutions: liquid concentrates that are diluted and combined prior to use to sterilise and remove pyrogens from fluid pathways.

Tubing: tubing of various types for fluid paths. Tubing was replaced at least every six months or more if needed.

Debubbler membranes: used in the removal of bubbles from fluid paths. Replaced at the end of every experiment.

Electrode solution and membranes: nitrocellulose membranes used in reference electrodes were replaced every six weeks or more frequently if damaged. Electrode solution was replaced when membranes replaced.

Sterile water: Used to constantly hydrate fluid paths and silicon sensor chambers when not in use.

2.8.2 Preparation of cells for cytosensor microphysiometer

Cells were harvested as described in section 2.1.1. Cells were seeded into capsule cups at $5x10^5$ cells per cup in media devoid of FBS (capsule cups were surrounded by 2ml media) and left to adhere overnight at 37°C. Capsules were placed in sensor chambers in the cytosensor and maintained by a flow (100µl/min) of low-buffered modified RPMI1640. Each cell chamber was served by fluid from either of two reservoirs, which could be altered using a software command. The flow was stopped for 40 sec at the end of each 2 min pump cycle and the rate of acidification (µvolts sec^{-1}) measured for 30 sec during that period. Cells were superfused with media for a period of 2 h to stabilise baseline extracellular acidification rates. Following stabilisation of baseline rates, the fluid path sources to the cell chambers was switched using a software command to make sure that altering which of the two reservoirs media came from did not influence results obtained.

2.8.3 Incubations and concentration-response curves

Cumulative c-r curves to specified agonists were constructed as detailed in the relevant chapters. Each concentration of agonist was exposed to cells starting at the lowest concentration used and moving sequentially through the concentrations to the highest concentration used, with no washout time between doses. The time periods for agonist exposure were long enough to produce a stable state with each concentration of agonist used before exposure to the following concentration. Stock solutions of drugs were thawed on the day of experiment and were diluted in modified media. All unused media solutions were discarded at the end of the experiment.

For experiments utilising antagonists, antagonists were incubated for 1h following the 2 h equilibration time period as detailed in the relevant chapters. Paired samples

were used to investigate the effect of antagonists where cells seeded from the same flask were used, either in the presence/absence of antagonist.

In experiments where cells were pretreated, see relevant chapters for more detail.

2.8.4 Analysis of cytosensor microphysiometer data

Acidification rates were measured by periodically interrupting the flow of modified media to the cells, allowing the accumulation of excreted acid metabolites. The pumps were automatically switched off and flow stopped for 40 sec at the end of each 2 min pump cycle and the rate of acidification (μvolts sec^{-1}) measured for 30 sec during that period. During this time, the accumulation of acidic products resulted in a drop in pH of the media. Flow was then resumed and the acidic metabolites were removed and the pH returned to basal levels.

There are two types of data output generated by the microphysiometer:

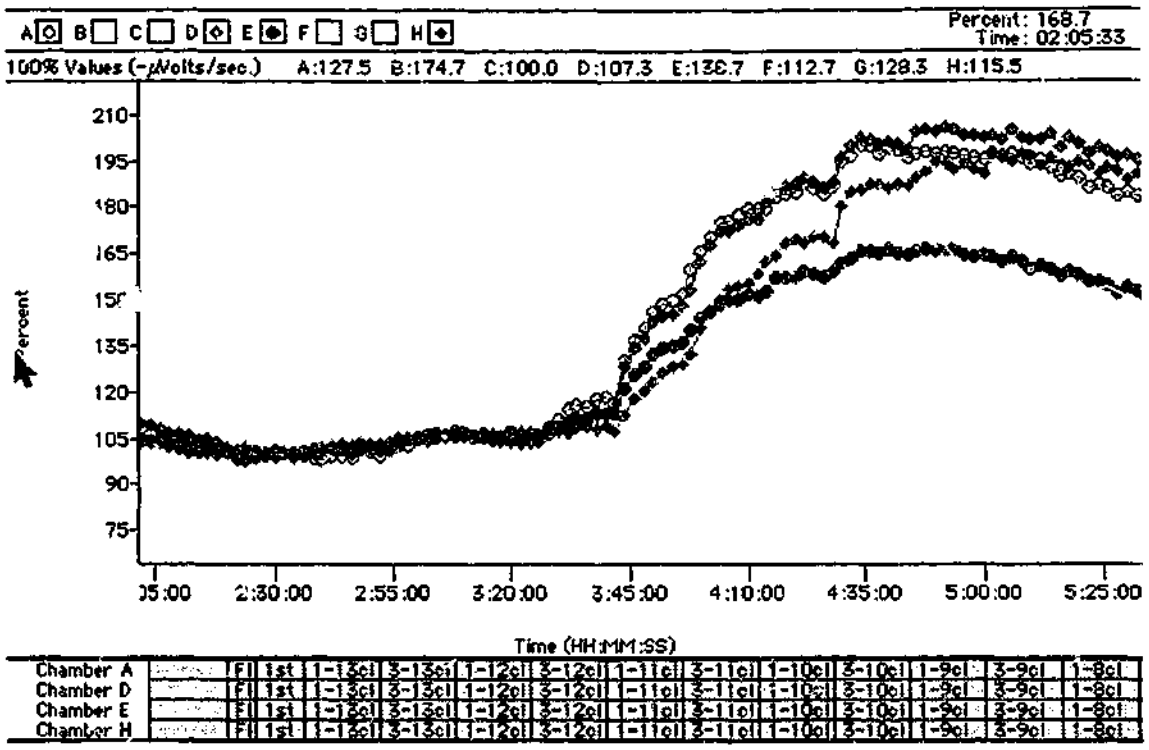
(a) raw data graph showing the pH of the chamber containing cells (expressed in millivolts where 60mV = 1 pH unit) against time (Figure 2.8 shows an example of the raw data trace obtained from an experiment).

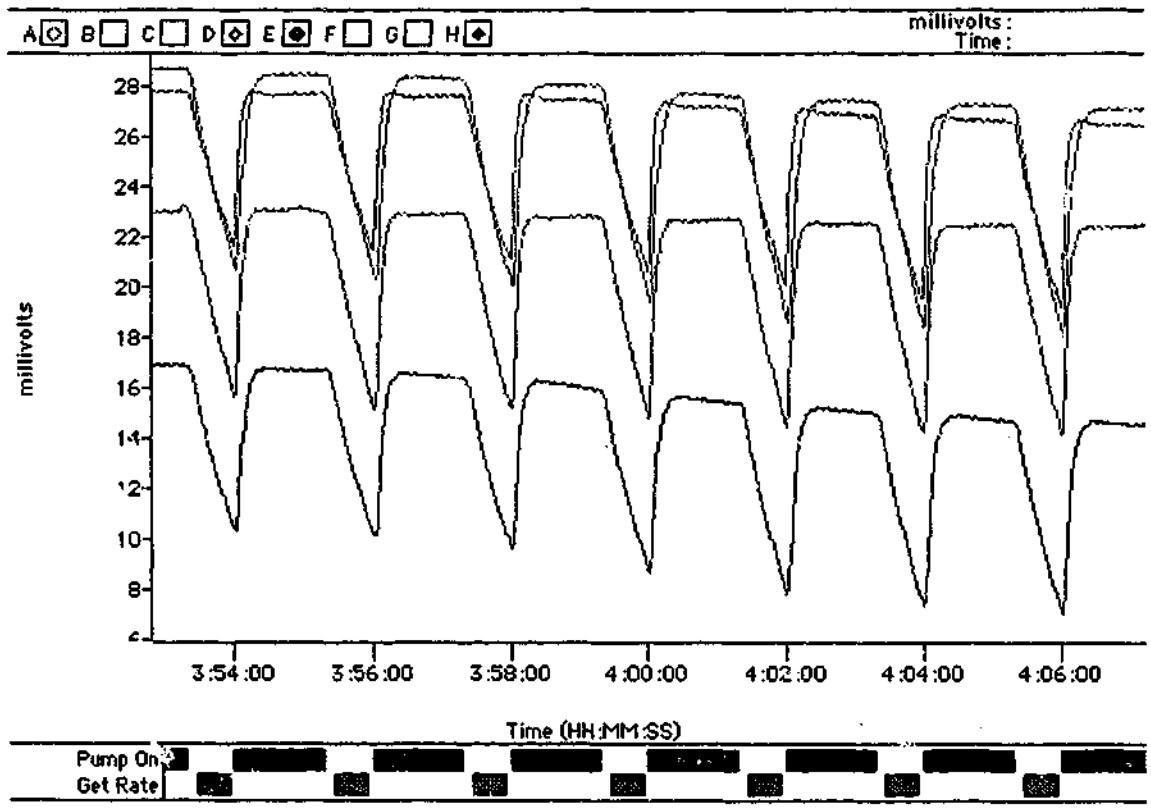
(b) rate data graph which is the gradient of the raw data graph when the pump is off as determined by the cytosensor software (the rate at which the pH in the chamber drops, expressed as -μV sec^{-1}) against time (Figure 2.8 shows an example of the rate data trace obtained from an exp .iment).

To analyse cytosensor microphysiometer data, stable baseline acidification rates were first normalised to 100% in a period just prior to the first addition of drug using the cytosensor software to allow comparison of data obtained from all eight chambers. Changes due to agonist exposure were calculated as a percentage increase over the normalised baseline response. The effect of each concentration of agonist was defined as the response produced with the last acidification measurement made at that concentration. In all experiments, the normalised data was used for further analysis since raw data values (-μV sec^{-1}) were variable and dependent upon the number of cells within each chamber. Variations in cell numbers were due to either

Figure 2.8a: Rate data output from a typical experiment constructing a c-r curve to CL316243 in CHO-K1 cells expressing either the β_{3a}-AR (channel A, D) or the β_{3b}-AR (channel E, H) at high expression levels (note: cells in channel D and H have been pretreated with PTX (100ng ml^{-1}, 16 h)) by the Cytosensor microphysiometer software program, Cytosoft version 2.01 for Macintosh.

Figure 2.8b: Raw data output from a typical experiment (same experiment as in above figure) produced by the Cytosensor microphysiometer software program, Cytosoft version 2.01 for Macintosh.


A B C D E F G H
Percent: 168.7
Time: 02:05:33
100% Values (-µVolts/sec.) A:127.5 B:174.7 C:100.0 D:107.3 E:136.7 F:112.7 G:128.3 H:115.5
210
195
180
165
150
135
120
105
90
75
Percent
05:00
2:30:00
2:55:00
3:20:00
3:45:00
4:10:00
4:35:00
5:00:00
5:25:00
Time (HH:MM:SS)
Chamber A
Chamber D
Chamber E
Chamber H
Fl 1st 1-13cl 3-13cl 1-12cl 3-12cl 1-11cl 3-11cl 1-10cl 3-10cl 1-9cl 3-9cl 1-8cl


A B C D E F G H
millivolts:
Time:
28
26
24
22
20
18
16
14
12
10
8
millivolts
3:54:00
3:56:00
3:58:00
4:00:00
4:02:00
4:04:00
4:06:00
Time (HH:MM:SS)
Pump On
Get Rate

inaccuracies inherent in counting cells or uneven distribution of cells over the capsule cup. This effect of cell numbers was greatest when experiments were performed on different days. As a result, responses were then normalised further to allow comparison of data obtained on different days to limit the influence of differences in cell number, with 0% defined as basal levels (i.e. before addition of drug) and 100% as the response produced at the maximal concentration of agonist used.

The cytosensor software was used for all data collection and analysis was Cytosoft (version 2.0.1 for Macintosh). Data was analysed using non-linear regression using a sigmoid dose response (variable slope) curve-fitting program (GraphPad) to construct c-r curves to the data and pEC_{50} values obtained. C-r curves are plotted as mean ± s.e.mean of *n* individual experiments where each *n* value represents cells grown in different flasks before plating in transwell inserts, apart from experiments where cells were treated or paired for antagonist studies, where paired and treated cells were obtained from the same flask of cells.

2.9 Organ bath studies

2.9.1 Tissue preparation

Mice (8-14 weeks old) were anaesthetised with 80% CO_2/20% O_2 and decapitated. Approximately 10 cm of ileum was removed 2 cm above the ileocaecal junction. The surrounding mesenteric fibres were gently cut away from the muscle with fine scissors in a Petri dish containing ice-cold Krebs-Henseleit solution. Segments of approximately 2 cm were cut and suture needles used to pass silicone treated silk (5-0 size, Deknatel, Fall River, MA, U.S.A.) through a single wall on each end of the ileum.

2.9.2 Incubations and concentration-response curves

Tissue segments were mounted on tissue hooks and suspended in 10ml jacketed organ baths containing 6ml Krebs-Henseleit solution maintained at 37°C and

bubbled with 95% O_2/5% CO_2 (pH 7.4) under 4mN force. Responses were measured with a isotonic transducer (UgoBasile, Italy, model 7006) connected to a MacLab (AD Instruments, Castle Hill, NSW, Australia) system and an Apple IIci computer to measure isotonic changes in the length of the tissues. Tissues were allowed to equilibrate with or without antagonists (as specified) for 30 min.

Following equilibration, tissues were contracted with carbachol (1μM) (approximately 80% maximal response) until responses maintained plateau (10-15 min). Carbachol stimulates muscarinic receptors causing contraction of gastrointestinal smooth muscle (Honda *et al.*, 1993). Control experiments were conducted showing that in the majority of experiments the carbachol-evoked contraction was stable for the whole experiment and tissues where this was not so in the equilibration phase were not used (data not shown). Cumulative c-r curves to specified agonists (as indicated in the relevant chapters) were constructed using increments of 0.5 log units until a stable state was observed. At the end of each c-r curve, tissues were maximally relaxed with papaverine (10μM) and all responses were expressed as a percentage of this papaverine response. Papaverine is structurally but not pharmacologically related to morphine. Its main action is to relax smooth muscle by a mechanism that is poorly understood but thought to involve inhibition of phosphodiesterases, blockade of calcium channels and possibly inhibition of mitochondrial respiration (Berndt *et al.*, 1976; Iguchi *et al.*, 1992; Kaneda *et al.*, 1998).

Where the effect of antagonists was investigated, the protocol used paired tissues. Two pieces of ileum from the same animal were mounted in different organ baths at the same time, and c-r curves to the specified agonist were performed in identical manner, with one tissue being incubated in the presence of the specified antagonist. Tissues were equilibrated in the presence/absence of antagonist for 1 h prior to carbachol contraction. Antagonists employed in these studies are indicated in the relevant chapters.

2.9.3 Analysis of organ bath studies

Non-linear regression using a sigmoid dose-response (variable slope) curve fitting program (GraphPad PRISM) was used to construct dose-response curves to the data and determine pEC_{50} values. Antagonist activity was measured by determining the shift of the c-r curve in the presence of the antagonist and a dose-ratio (dr) calculated for each set of paired tissues. The negative logarithm of the concentration of antagonist to produce a 2-fold shift in the c-r curve for an agonist (pK_B) values were obtained for each single concentration of antagonist with the equation

$$pK_B = \log(dr-1) - \log[\text{antagonist}]$$

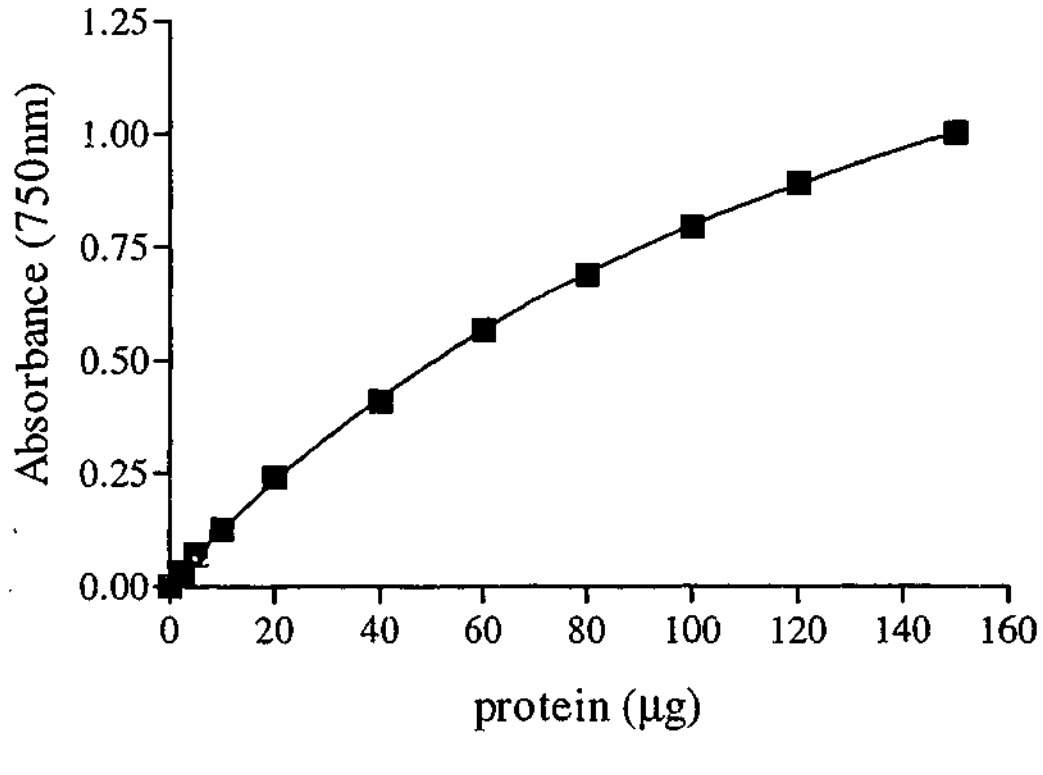
according to the method of Furchgott (1972)

C-r curves are plotted as mean ± s.e.mean of *n* individual experiments with each *n* value referring to the number of individual animals used. All pK_B values are expressed as mean ± s.e.mean of *n* individual experiments.

2.10 Protein determination

Protein measurement was determined by the method of Lowry *et al.* (1951). Briefly, aliquots of membranes or standards (volume adjusted to 150µl with water) containing protein were digested in 2M NaOH (50µl) for 30 min at room temperature followed by the addition (1ml) of an alkaline copper solution (0.001% $CuSO_4.5H_2O$, 0.002% NaK tartrate) made in 2% Na_2CO_3 for 10 min at room temperature. Folin and Ciocalteu's Phenol Reagent was diluted 1:2 with distilled water and 100µl added to the copper treated protein, vortexed immediately and protein colour allowed to develop in the dark at room temperature for 30 min. Absorbances were measured with a Novaspec II spectrophotometer (Pharmacia LKB Biochrom, England) at a wavelength of 750nm. BSA was used as the standard (2.5-150µg). All samples (both standards and unknowns) were performed in duplicate. A representative standard curve is shown in Figure 2.9. A standard curve was produced

Figure 2.9: A typical Lowry standard curve.


1.25
1.00
0.75
0.50
0.25
0.00
Absorbance (750nm)
0
20
40
60
80
100
120
140
160
protein (µg)

with GraphPad PRISM using non-linear regression. Unknown sample absorbance readings were read off the graph to obtain protein values.

2.11 Statistics

A number of statistical tests were performed dependent on the data being analysed. Students t-tests were performed using GraphPad PRISM to determine significance of differences between two sets of data. Two-way analysis of variance (ANOVA) tests were used to determine significance of variations between curves. Probability values (p) less than or equal to 0.05 were considered significant.

2.12 Drugs and reagents

The following drugs were gifts: carvedilol (Dr R.R. Ruffolo III, SB Pharmaceuticals), CL316243 (Dr T. Nash, Wyeth-Ayerst), GR265162X (Dr C. Cowan, Glaxo Wellcome), labetolol (Prof B. Jarrott, Department of Pharmacology, Monash University), L755507 (Dr M. Fisher, Merck), SB251023 (Dr J. Arch, Smith Kline Beecham), SR59230A (Dr L. Manara, Sanofi-Midy). $DH_{5\alpha}$ cells were a gift from Dr D. Bulach (formerly from Department of Microbiology, Monash University).

Drugs and reagents were purchased as follows: EDTA (AJAX Chemicals, Melbourne, VIC, Australia); [^{3}H]cAMP assay system, DNase I, dNTPs, ECL kit, Hyperfilm ECL film, Hind III, Hybond C-extra and Hybond N^+ membranes, 10 x One-Phor-All buffer, Sca I, T4 PNK, Xba I, 100 bp ladder (Amersham Pharmacia Biotech, Uppsala, Sweden); D-glucose, Nonidet P-40, phenol, sodium deoxycholate (Amresco, Solon, OH, U.S.A.); 30% acrylamide, ammonium persulfate, Kaleidoscope marker, non-fat dry milk powder, TEMED (Bio-Rad Laboratories, Hercules, CA, U.S.A.); puC19 DNA restricted with Hpa II (Bresatec, Adelaide, SA, Australia); ATP, Fraction V BSA, proteinase K, tRNA (Boehringer Manneheim, Germany); G418, H89, PP2, Ro-31-8220 (CalBiochem Corp., La Jolla, CA, U.S.A.); CGP20712A (Ciba-Geigy AG, Australia); 2000 Ci $mmol^{-1}$ [γ-^{33}P]ATP (Geneworks, Adelaide, SA, Australia); 10 x AMP buffer, cholera toxin, 10 x enhancer solution,

leupeptin, oligo(dT)$_{15}$, oligonucleotides (primers and probes), 10 x PCR buffer, pepstatin A, platinum Pfx DNA polymerase, pertussis toxin, salmon sperm DNA, SDS, Taq polymerase, Trizol reagent, yeast extract (Gibco BRL, Life Technologies, Gaithersburg, MD, U.S.A.); aprotinin, leupeptin, pepstatin A (ICN, Costa Mesa, CA, U.S.A.); ICI118551 (Imperial Chemical Industries, Wilmslow, Cheshire, U.K.); pcDNA3.1$^+$ (Invitrogen, Groningen, Netherlands); polyvinylpyrrolidone (Koch-Light Laboratories, Coinbrook, Bucks, U.K.); L(+)-ascorbic acid (Merck, Frankfurt, Germany); 2200 Ci mmol^{-1} [^{125}I]-(-)-cyanopindolol (NEN Life Science Products, Boston MA, U.S.A.); α-p-Erk primary antibody, α- Erk primary antibody, secondary HRP-linked anti-rabbit antibody (New England Biolabs, Beverly, MA, U.S.A.); agar bacteriological (number 1), casein hydrolysate (Oxoid Limited, Basingstoke, Hampshire, U.K.); AMV reverse transcriptase, RNasin, 10 x RT buffer, Wizard PCR preps DNA purification system, Wizard Plus Midiprep DNA purification system, Wizard Plus Miniprep DNA purification system (Promega, Madison, WI, U.S.A.); CGP12177A (Research Biochemicals Inc, MA, U.S.A.); agarose LE, ampicillin, Expand high fidelity PCR system (containing 10 x Expand PCR buffer and Taq Expand) (Roche Diagnostics, Indianapolis, IN, U.S.A.); bupranolol (Schwartz Pharma AG, Mannheim, Germany); (-)-tertatolol, (+)-tertatolol (Servier Laboratories, Neuilli Sur Seine, France); (-)-alprenolol, aprotinin, bacitracin, carbachol (carbamylcholine chloride), concanavalin A, cytochalasin D, DMSO (cell culture grade), dithiothreitol, ficoll type 4000, Folin and Ciocalteu's phenol reagent, forskolin, fraction V BSA, genistein, IBMX, (-)-isoprenaline, (+)-isoprenaline, LY294002, MDC, β-mercaptoethanol, (-)-noradrenaline, (+)-noradrenaline, papaverine HCl, PD98059, PMSF, polyethylenimine, (-)-propranolol, (+)-propranolol, sodium orthovanadate, Tween-20 (Sigma Chemical Company, St. Louis, MO, U.S.A.); DMEM, DMEM/Hams F12 media, FBS, Hanks buffer, L-glutamine. penicillin/streptomycin, trypsin/EDTA (Trace Biosciences, Castle Hill, NSW, Australia); BRL37344 (Tocris Cookson Ltd, Bristol, U.K.); low melting point agarose, T4 DNA ligase (USB, Cleveland, OH, U.S.A.); 3MM paper (Whatman International, Maidstone, U.K.).

Cytosensor consumables were obtained from suppliers as provided in section 2.8.1. All cell culture consumables were obtained from Life Technologies (Gaithersburg, MD, U.S.A.) unless stated otherwise.

All other drugs and reagents were of analytical grade.

Drug stocks were prepared in distilled water with the following exceptions. G418 was prepared in sterile PBS. Cytochalasin D, forskolin, genistein, IBMX, LY294002, PD98059, pepstatin A and PP2 were prepared in DMSO. (-)-alprenolol, bupranolol, CGP12177A, CGP20712A, GR265162X, ICI118551, (-)- and (+)-isoprenaline, labetolol, (-)- and (+)-noradrenaline, (-)- and (+)-propranolol, tertatolol were prepared in 10mM HCl. SR59230A was prepared in a mixture of 50% water, 25% ethanol and 25% DMSO. L755507, PMSF and SB251023 were prepared in 100% ethanol.

Chapter 3

Pharmacological characterisation of the mouse β_{3a}- and β_{3b}-AR expressed in CHO-K1 cells

3.1 Introduction

β_3-ARs are pharmacologically characterised by a set of criteria (Arch & Kaumann, 1993; Emorine *et al.*, 1994; Strosberg & Pietri-Rouxel, 1996) that include; (a) low affinity and potency for conventional β-AR antagonists and agonists, including radioligands; (b) low stereoselectivity of agonist and antagonist stereoisomers relative to those at typical β-ARs; (c) partial agonist activity of several β_1-/β_2-AR antagonists; (d) high affinity and potency of selective agonists such as CL316243 and BRL37344; and (e) antagonism by the β_3-AR antagonist SR59230A. β-ARs exhibiting a pharmacological profile consistent with that of the β_3-AR have been cloned from various species, including mouse, rat and human (Emorine *et al.*, 1989; Granneman *et al.*, 1991; Muzzin *et al.*, 1991; Nahmias *et al.*, 1991).

The mouse β_3-AR gene contains two introns, and can undergo alternate splicing (Evans *et al.*, 1999; Granneman & Lahners, 1995; Van Spronsen *et al.*, 1993). Splicing of the first intron 100bp upstream of the previously characterised start of exon 2 results in the production of a mRNA that encodes a β_3-AR variant, termed the β_{3b}-AR, which possesses a unique C-terminal tail with 17 amino acids that differ from the 13 in the known receptor (Evans *et al.*, 1999). Alternate splicing of GPCRs has the potential to diversify the number of receptor subtypes encoded by a single gene. Numerous GPCRs have isoforms with differing C-terminals (Bach *et al.*, 2001; Chang *et al.*, 1998; Gerald *et al.*, 1995; Hasegawa *et al.*, 1996; Heidmann *et al.*, 1997; Hirasawa *et al.*, 1995; Irie *et al.*, 1993; Jin *et al.*, 1997; Krobert *et al.*, 2001; Mialet *et al.*, 2000; Namba *et al.*, 1993; Nambi *et al.*, 2000; Reisine *et al.*, 1993; Schindler *et al.*, 1998; Suzuki *et al.*, 2000; Vanetti *et al.*, 1993). A common feature of these splice variants is differential expression in various tissues. Although most of the splice variants share similar pharmacological profiles, differences in functional responses of splice variants have been identified (Cole & Schindler, 2000; Irie *et al.*, 1993; Mialet *et al.*, 2000; Namba *et al.*, 1993; Negishi *et al.*, 1996; Suzuki *et al.*, 2000).

The aims of the present study are to characterise the pharmacological properties of the mouse β_{3a}-AR and the splice variant, the β_{3b}-AR, expressed in CHO-K1 cells.

The entire coding region of each receptor was cloned, transfected into CHO-K1 cells and stable cell lines generated. Three different clonal cell lines with different expression levels for each receptor were then used to characterise the pharmacology of each receptor in radioligand binding, cAMP accumulation and cytosensor microphysiometer studies.

3.2 Methods

3.2.1 Expression of the mouse β_{3a}- or β_{3b}-AR in CHO-K1 cells

The entire coding region of the mouse β_{3a}- or β_{3b}-AR was cloned and transfected into CHO-K1 cells as described previously (section 2.3).

3.2.2 Cell culture

Cell culture was performed as described in section 2.1.

3.2.3 Radioligand binding studies

3.2.3.1 Membrane preparation

Membranes for radioligand binding experiments were prepared as described in section 2.5.1.1. Membranes were kept on ice and used for studies on the same day. In some studies, a unused portion of membranes was frozen for measurement of protein levels (section 2.10).

3.2.3.2 Kinetic studies

The rate of association of ICYP (500pM) to specific binding sites was determined for up to 90 min at room temperature. Non-specific binding was determined with (-)-alprenolol (1mM). The dissociation of ICYP was determined after a 60 min incubation with ICYP (500pM) at room temperature at intervals up to 90 min after the addition of (-)-alprenolol (1mM) (see section 2.5.2 for more detail).

3.2.3.3 Saturation and competition binding studies

The characteristics of ICYP binding to cell membranes were determined in saturation and competition studies as described in section 2.5.3 with ICYP (100-2000 pM) at room temperature for 60 min. In competition studies, aliquots of membrane suspensions were incubated with ICYP (500pM) and a range of competing agents ((-)-isoprenaline, (+)-isoprenaline, (-)-NA, (+)-NA, BRL37344, CL316243, CGP12177A, (-)-propranolol, (+)-propranolol, (-)-tertatolol, (+)-tertatolol, bupranolol, labetolol, (-)-alprenolol, CGP20712A, ICI118551 or SR59230A) with a concentration range as indicated with the data. Non-specific binding for saturation and competition studies was defined by (-)-alprenolol (1mM). Reactions were incubated, filtered, washed and counted as described in section 2.5.3 and 2.5.4. All experiments were performed in duplicate.

3.2.4 Cytosensor microphysiometer studies

The cytosensor microphysiometer (Molecular Devices Corp., California, U.S.A.) was used to measure extracellular acidification rates of cells following drug administration. Cells were seeded into 12mm transwell plates at 5 x 10^5 cells per well overnight in media devoid of FBS prior to use in the cytosensor the following day as detailed in section 2.8.2. Following a 2 h equilibration period, cumulative c-r curves to several β-AR ligands ((-)-isoprenaline, (+)-isoprenaline, CL316243, BRL37344, CGP12177A, GR265162X, SB251023 or L755507) were constructed as described in section 2.8.3, with cells exposed to each concentration of agonist for 14 min (except (-)- and (+)-isoprenaline where 8 min exposure time was used). Responses were measured as described in section 2.8.4.

To examine the effect of the β_3-AR antagonist SR59230A on CL316243-mediated responses in the cytosensor, agonist c-r curves were performed in paired sister cells in the absence and presence of antagonist. Where possible, pK_B values were calculated. In some experiments, c-r curves to SR59230A were produced in parallel

with CL316243 c-r curves using exposure times of 14 min per dose for both CL316243 and SR59230A. Analysis of results is detailed in section 2.8.4.

To examine the effect of CL316243 and SR59230A in untransfected CHO-K1 cells, cells were exposed to drugs for 30 min in parallel with cells expressing the β_{3a}-AR (high expression levels).

3.2.5 cAMP accumulation studies

Cells were plated in 12-well plates at 1 x 10^5 cells per well, and cultured for 2 days as described in section 2.6.1, then treated with the β_3-AR agonist CL316243 or the β_3-AR antagonist SR59230A for 30 min. To examine the effect of the β_3-AR antagonist SR59230A on CL316243-mediated responses, cells were incubated with a range of concentrations of SR59230A for 10 min before the addition of a single submaximal concentration of CL316243 for 20 min. Concentrations used are shown in the results. cAMP samples were extracted and the determination of levels of cAMP were performed as described in section 2.6.2. Responses are expressed as pmol of cAMP produced per well.

3.2.6 Analysis

All results are expressed as mean ± s.e.mean of *n* experiments. Binding results were analysed and binding parameters calculated as described in section 2.5.5. Cytosensor microphysiometer results were analysed as described in section 2.8.4 and cAMP results were analysed as described in section 2.6.3. Student's t-test or 2-way ANOVA statistical analysis using GraphPad PRISM was utilised and p values less than or equal to 0.05 were considered significant.

3.2.7 Drugs and reagents

Drugs and reagents used and preparation of stock solutions is described in section 2.12. All drugs were diluted either in binding buffer (radioligand binding studies),

modified RPMI-1640 media (cytosensor microphysiometer studies) or water (cAMP accumulation studies) prior to use.

3.3 Results

3.3.1 Examination of the radioligand binding characteristics of ICYP in CHO-K1 cells expressing the β_{3a}- or β_{3b}-AR

3.3.1.1 Kinetics of ICYP binding

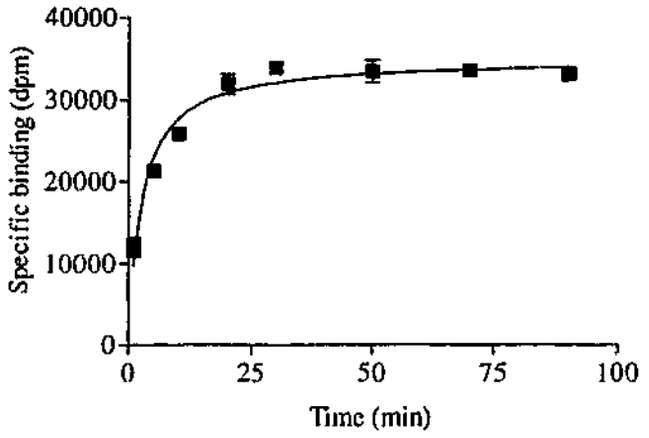
The kinetics of ICYP binding were examined in high expressing clones at room temperature, and showed that association of ICYP to either the β_{3a}- or β_{3b}-AR reached equilibrium in less than 60 min with association rate constants (K_1) of 1.67 ± 0.21 x 10^8 min^{-1} and 3.73 ± 0.72 x 10^8 min^{-1} respectively (n=3) (Figure 3.1). Dissociation kinetics were determined after 60 min preincubation and showed that binding was reversible following the addition of 1mM (-)-alprenolol. Dissociation was monophasic (Figure 3.1) with a dissociation constant (K_{-1}) of 0.143 min^{-1} at the β_{3a}-AR and 0.155 min^{-1} at the β_{3b}-AR. A kinetic pK_D of 9.01 (β_{3a}-AR) and 8.87 (β_{3b}-AR) was determined (n=3).

3.3.1.2 Saturation binding

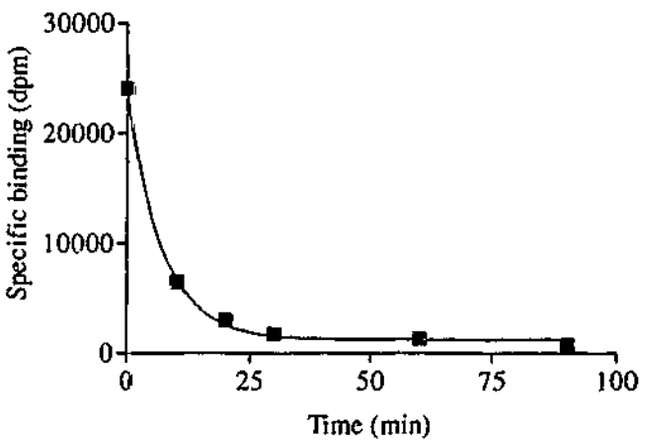
ICYP binding occurred in a saturable manner to a single ICYP site in CHO-K1 cells expressing either the β_{3a}- or β_{3b}-AR. Stably transfected cells were chosen with three levels of expression as determined by saturation binding studies: high (~1200 fmol mg $protein^{-1}$), medium (~500 fmol mg $protein^{-1}$) and low (~100 fmol mg $protein^{-1}$) levels of expression (Figure 3.2 for β_{3a}-AR clones; Figure 3.3 for β_{3b}-AR clones; Table 3.1). Non-specific binding represented ~8%, ~10% or ~40% of the total binding measured in high, medium and low expressing clones respectively. No ICYP binding sites could be detected in untransfected CHO-K1 cells (data not shown).

Figure 3.1: Association and dissociation kinetics of specific ICYP (500pM) binding to membranes from cells transfected either with the β_{3a}- (a, b) or β_{3b}-AR (c, d) at room temperature. (a, c) shows that the association of specific ICYP binding reached equilibrium within 60 min at either the β_{3a}- (a) or β_{3b}-AR (c). (b, d) shows the dissociation of specific ICYP binding at various times after the addition of (-)-alprenolol (1mM) to β_{3a}- (b) or β_{3b}-AR (d) membranes incubated with ICYP for 60 min. Points show mean ± s.e.mean of 3 experiments performed in duplicate.

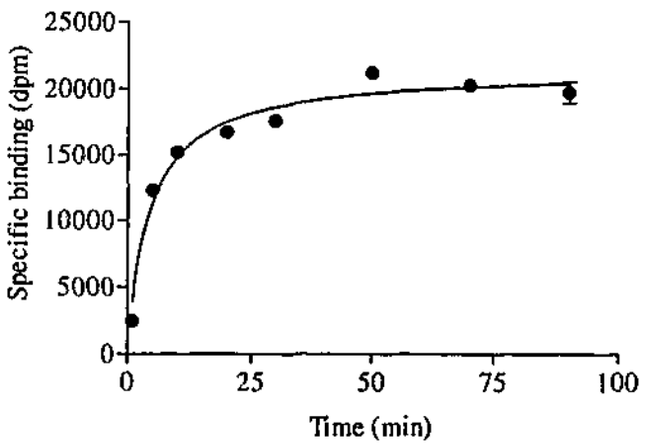
(a)


Specific binding (dpm)
40000
30000
20000
10000
0
0
25
50
75
100
Time (min)

(b)


Specific binding (dpm)
30000
20000
10000
0
0
25
50
75
100
Time (min)

(c)


Specific binding (dpm)
25000
20000
15000
10000
5000
0
0
25
50
75
100
Time (min)

(d)

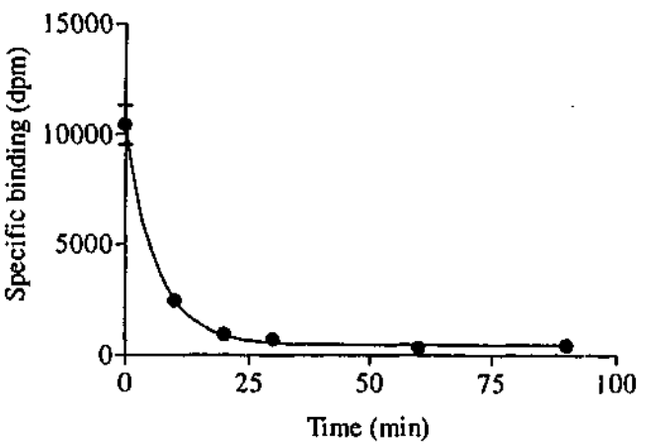
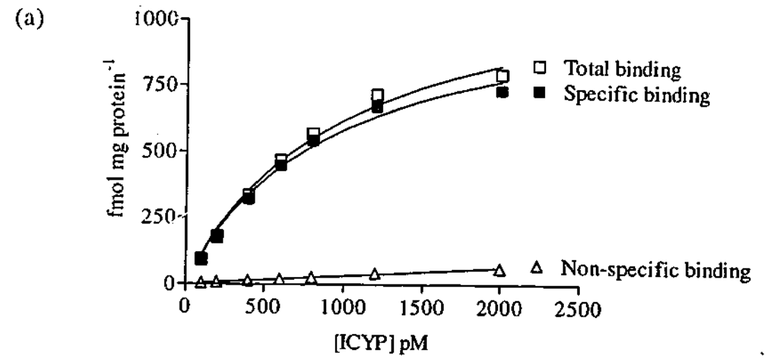
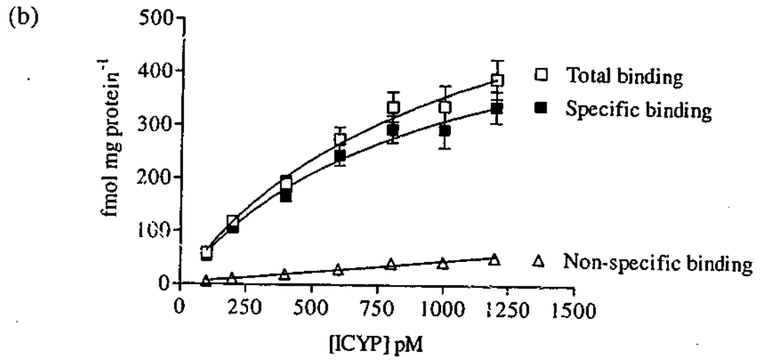

Specific binding (dpm)
15000
10000
5000
0
0
25
50
75
100
Time (min)

Figure 3.2: Saturation binding of ICYP to membranes prepared from CHO-K1 cells transfected with the β_{3a}-AR. (a) data from cells expressing ~1100 fmol mg protein^{-1}, (b) from cells expressing ~600 fmol mg protein^{-1}, and (c) from cells expressing ~100 fmol mg protein^{-1}. Incubations were performed for 60 min and non-specific binding was defined with (-)-alprenolol (1mM). Points show mean ± s.e.mean for 3 experiments performed in duplicate.





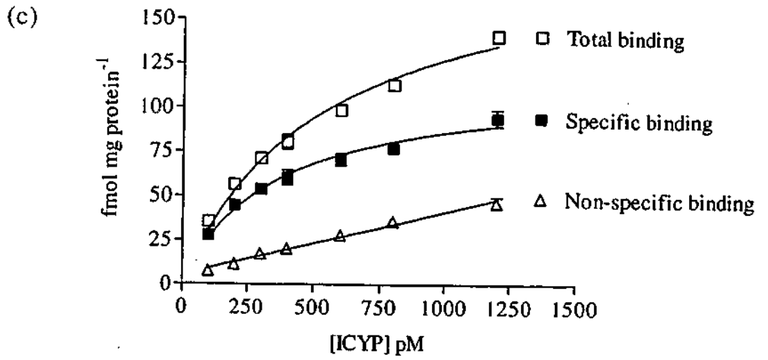


Figure 3.3: Saturation binding of ICYP to membranes prepared from CHO-K1 cells transfected with the β_{3b}-AR. (a) data from cells expressing ~1200 fmol mg protein^{-1}, (b) from cells expressing ~500 fmol mg protein^{-1}, and (c) from cells expressing ~100 fmol mg protein^{-1}. Incubations were performed for 60 min and non-specific binding was defined with (-)-alprenolol (1mM). Points show mean ± s.e.mean for 3 experiments performed in duplicate.

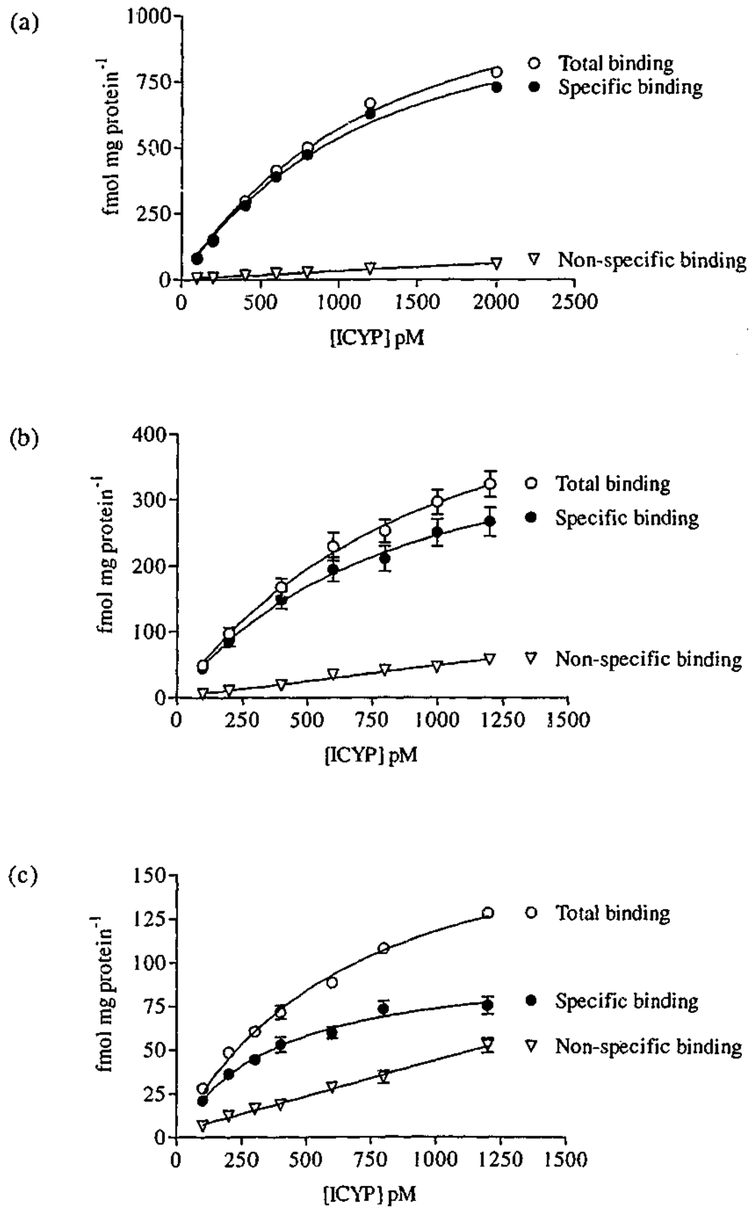


Table 3.1: Summary of affinity (pK_D) and density of binding sites (B_{max}) in membranes prepared from CHO-K1 cells expressing either the β_{3a}- or the β_{3b}-AR at three different levels. Values represent mean ± s.e.mean from 3 experiments performed in duplicate.

	B_{max} (fmol mg protein^{-1})	pK_D	*n*
β_{3a}-AR			
High	1118 ± 48	9.04 ± 0.09	*3*
Medium	587 ± 24***	9.05 ± 0.04	*3*
Low	115 ± 6	9.46 ± 0.14	*3*
β_{3b}-AR			
High	1243 ± 53	8.88 ± 0.08	*3*
Medium	457 ± 67***	9.07 ± 0.03	*3*
Low	101 ± 5	9.43 ± 0.13	*3*

*** indicates $p<0.001$ determined by 2-way ANOVA for comparison of saturation binding curves between the β_{3a}- and β_{3b}-AR

3.3.1.3 Competition binding

Competition studies were performed to determine the affinities (pK_i) of several β-AR ligands at either the β_{3a}- or β_{3b}-AR. All competition curves fitted a one-site model. Studies using high expressing cells showed no significant difference in the affinity for all β-AR ligands between either receptor (see Table 3.2 for pK_i values). Competition curves for a given ligand were not significantly different when performed with β_{3a}- or β_{3b}-AR membranes expressed at similar expression levels (2-way ANOVA ns).

3.3.1.3.1 Agonists

The β_3-AR agonists CL316243, BRL37344 and CGP12177A competed at the ICYP binding site in both the β_{3a}- and β_{3b}-AR (Figure 3.4) with the rank order of affinity CGP12177A > BRL37344 > CL316243 for both receptors.

Competition between ICYP and NA or isoprenaline occurred (Figure 3.5, 3.6 respectively), with similar rank orders of affinity for either the β_{3a}- or β_{3b}-AR. Stereoselective competition for ICYP binding was demonstrated for (-)-NA (pK_i 4.89 ± 0.40 β_{3a}-AR, 4.71 ± 0.09 β_{3b}-AR) > (+)-NA (pK_i < 3.0 β_{3a}- and β_{3b}-AR) and (-)-isoprenaline (pK_i 4.67 ± 0.17 β_{3a}-AR, 4.65 ± 0.09 β_{3b}-AR) > (+)-isoprenaline (pK_i < 3.0 β_{3a}- and β_{3b}-AR) at both receptors (n=3). All isomers produced curves that were significantly different from each other (2-way ANOVA *** $p<0.001$), with the (-)-isomers showing a 44-77 fold higher affinity than the (+)-isomers. Competition curves for the (+)-isomers could not be fully constructed.

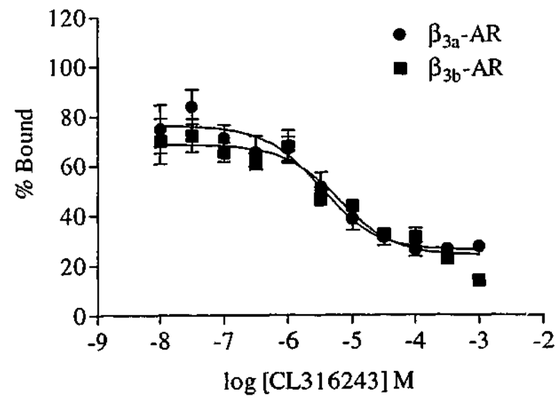
The rank order of affinity for all β-AR agonists investigated at the β_{3a}- or β_{3b}-AR was CGP12177A > BRL37344 > CL316243 > (-)-noradrenaline, (-)-isoprenaline > (+)-noradrenaline, (+)-isoprenaline.

Table 3.2: Comparison of binding affinities (pK_i) for β-AR ligands at the β_{3a}- or β_{3b}-AR. Values represent mean ± s.e.mean from 3-6 experiments performed in duplicate. All values were determined in high expressing cells unless marked otherwise (* denotes medium and $^\#$ denotes low expressing cells; pK_i values compared with SR59230A, (-)-propranolol, CGP20712A or ICI118551 at different levels).

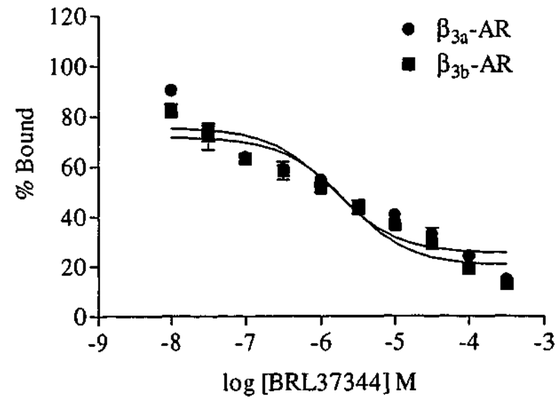
Competitor	pK_i β_{3a}-AR	pK_i β_{3b}-AR
β_3-AR selective agonists		
BRL37344	6.02 ± 0.03 *(3)*	5.83 ± 0.22 *(3)*
CL316243	5.87 ± 0.39 *(3)*	5.42 ± 0.12 *(3)*
CGP12177A	6.54 ± 0.05 *(3)*	6.45 ± 0.07 *(3)*
β_3-AR selective antagonists		
SR59230A	7.32 ± 0.06 *(5)*	7.20 ± 0.08 *(5)*
	6.88 ± 0.08 *(3)**	6.87 ± 0.10 *(3)**
	6.57 ± 0.27 *(3)*$^\#$	6.51 ± 0.27 *(3)*$^\#$
β-AR nonselective agonists		
(-)-Noradrenaline	4.89 ± 0.40 *(3)*	4.71 ± 0.09 *(3)*
(+)-Noradrenaline	< 3.0 *(3)*	< 3.0 *(3)*
(-)-Isoprenaline	4.67 ± 0.17 *(3)*	4.65 ± 0.09 *(3)*
(+)-Isoprenaline	< 3.0 *(3)*	< 3.0 *(3)*
β-AR nonselective antagonists		
(-)-Propranolol	6.29 ± 0.08 *(6)*	5.98 ± 0.14 *(6)*
	6.16 ± 0.11 *(3)**	6.42 ± 0.08 *(3)**
	6.72 ± 0.17 *(3)*$^\#$	6.63 ± 0.16 *(3)*$^\#$
(+)-Propranolol	4.40 ± 0.06 *(4)*	4.30 ± 0.04 *(6)*
(-)-Tertatolol	6.62 ± 0.15 *(3)*	6.66 ± 0.13 *(3)*
(+)-Tertatolol	5.13 ± 0.14 *(3)*	5.07 ± 0.07 *(3)*
Bupranolol	6.53 ± 0.13 *(3)*	6.48 ± 0.09 *(3)*
Labetalol	5.25 ± 0.05 *(3)*	5.13 ± 0.06 *(3)*
(-)-Alprenolol	6.54 ± 0.03 *(4)*	6.47 ± 0.05 *(4)*
β_1-AR selective antagonist		
CGP20712A	4.91 ± 0.04 *(4)*	4.90 ± 0.06 *(4)*
	4.83 ± 0.17 *(3)**	5.31 ± 0.12 *(3)**
	5.14 ± 0.25 *(3)*$^\#$	5.00 ± 0.13 *(3)*$^\#$
β_2-AR selective antagonist		
ICI118551	4.69 ± 0.12 *(3)*	4.68 ± 0.14 *(3)*
	5.18 ± 0.03 *(3)**	5.29 ± 0.06 *(3)**
	5.51 ± 0.19 *(3)*$^\#$	5.34 ± 0.30 *(3)*$^\#$

Figure 3.4: Competition between ICYP (500pM) and (a) CL316243, (b) BRL37344 or (c) CGP12177A for binding sites in homogenates of CHO-K1 cells expressing either the β_{3a}- or β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3).

(a)



(b)



(c)

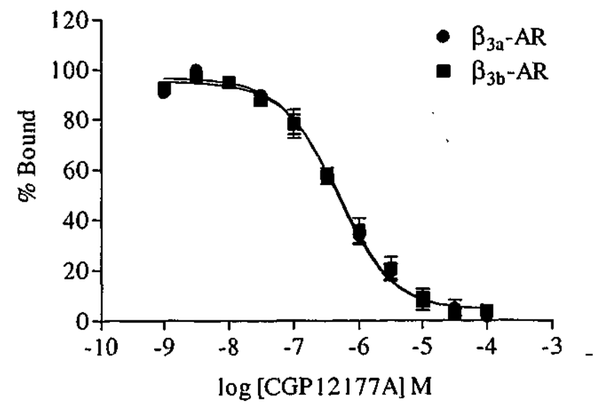
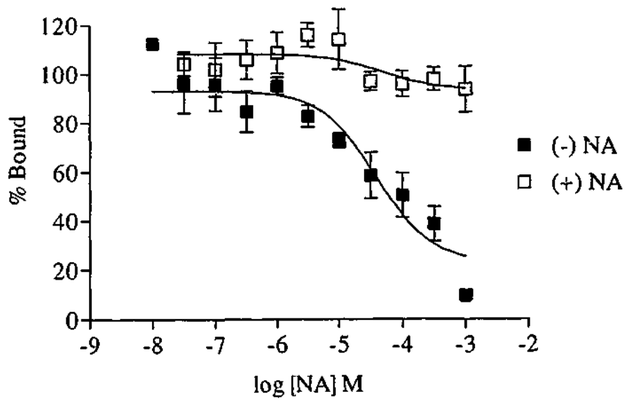


Figure 3.5: Competition between ICYP binding (500pM) and the stereoisomers of NA in membranes prepared from CHO-K1 cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3).

(a)



(b)

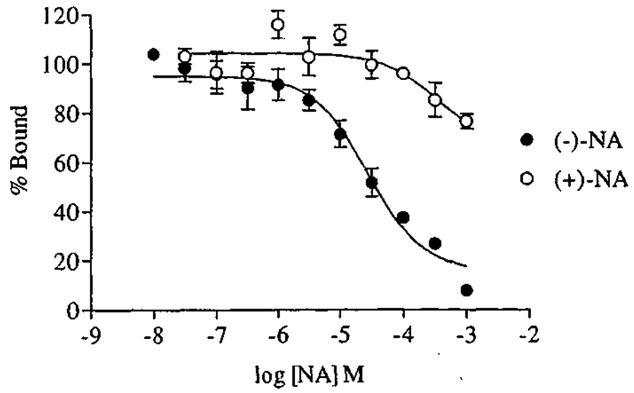
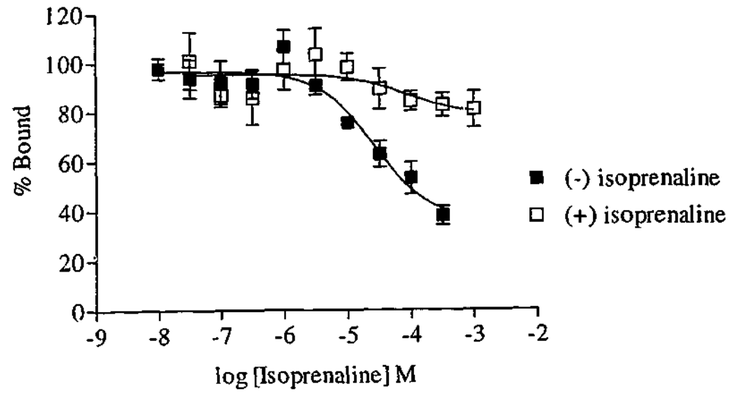
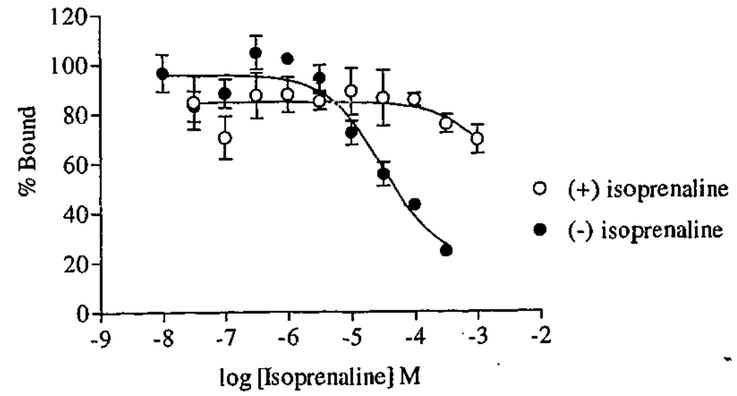


Figure 3.6: Competition between ICYP binding (500pM) and the stereoisomers of isoprenaline in membranes prepared from CHO-K1 cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3).

(a)



(b)



3.3.1.3.2 Antagonists

The β-AR selective subtype antagonists CGP20712A (β_1-AR) (Figure 3.7), ICI118551 (β_2-AR) (Figure 3.8), SR59230A (β_3-AR) (Figure 3.9) and the non-selective antagonists (-)-propranolol (Figure 3.10, 3.11), (-)-tertatolol (Figure 3.12), (-)-alprenolol (Figure 3.13), bupranolol (Figure 3.13) and labetolol (Figure 3.13) competed for ICYP binding to the β_{3a}- and β_{3b}-AR with the following rank order of affinity: SR59230A > (-)-alprenolol, (-)-tertatolol, bupranolol, (-)-propranolol > labetolol > CGP20712A, ICI118551.

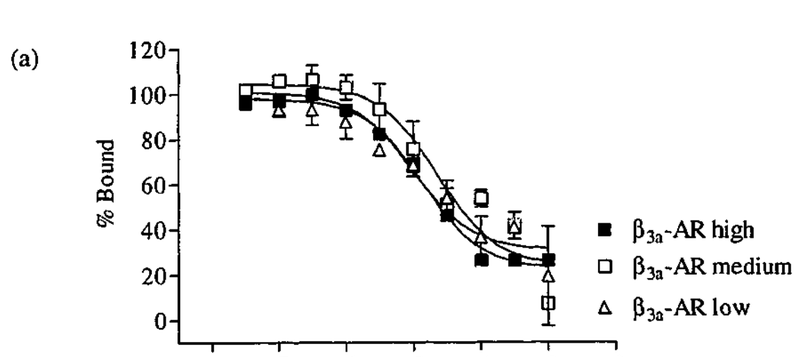
Stereoselectivity was also examined using the stereoisomers of propranolol and tertatolol. Competition for ICYP binding was demonstrated for (-)-propranolol (pK_i 6.29 ± 0.08 β_{3a}-AR, 5.98 ± 0.14 β_{3b}-AR; n=6) > (+)-propranolol (pK_i 4.40 ± 0.06 β_{3a}-AR, 4.30 ± 0.04 β_{3b}-AR; n=4-6) and (-)-tertatolol (pK_i 6.62 ± 0.15 β_{3a}-AR, 6.66 ± 0.13 β_{3b}-AR; n=3) > (+)-tertatolol (pK_i 5.13 ± 0.14 β_{3a}-AR, 5.07 ± 0.07 β_{3b}-AR; n=3) (Figure 3.11 and 3.12 respectively). The curves for the isomers were significantly different in position (2-way ANOVA ***$p<0.001$) and the (-)-isomer showed 31-78 fold higher affinity than (+)-isomers.

There was no effect of receptor density on affinity values for CGP20712A or (-)-propranolol (Figure 3.7 and 3.10 respectively) for either receptor, with similar pK_i values obtained (Table 3.2). The affinity values for ICI118551 decreased as expression levels increased (Figure 3.8, Table 3.2), whereas affinity values for SR59230A increased as receptor expression levels increased (Figure 3.9, Table 3.2) in cells expressing either the β_{3a}- or β_{3b}-AR.

3.3.2 Examination of functional responses mediated by either the β_{3a}- or β_{3b}-AR expressed in CHO-K1 cells using the cytosensor microphysiometer

The β_3-AR agonist CL316243 had no effect on increases in extracellular acidification rates in untransfected CHO-K1 cells (Figure 3.14).

Figure 3.7: The effect of differing levels of receptor expression on the competition for ICYP binding (500pM) by the β_1-AR antagonist CGP20712A in cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3-4).



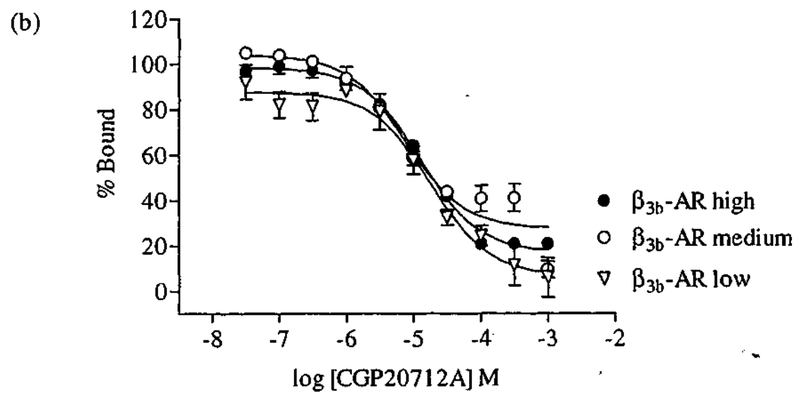
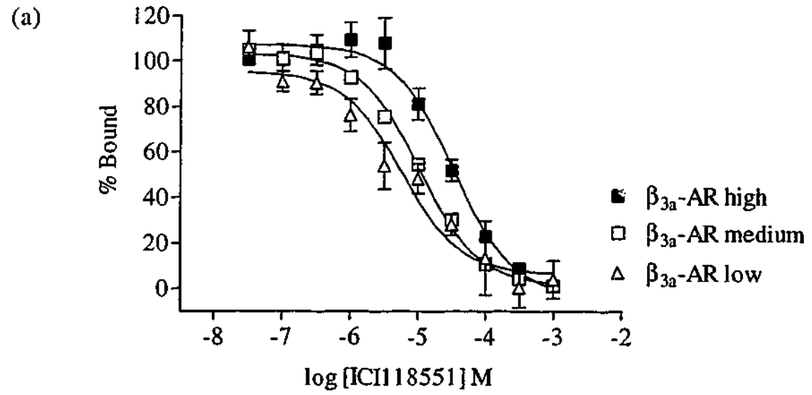


Figure 3.8: The effect of differing levels of receptor expression on the competition for ICYP binding (500pM) by the β_2-AR antagonist ICI118551 in cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3).



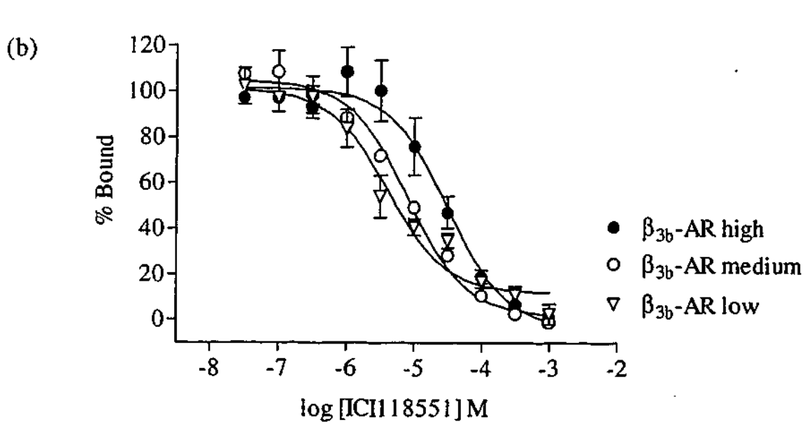
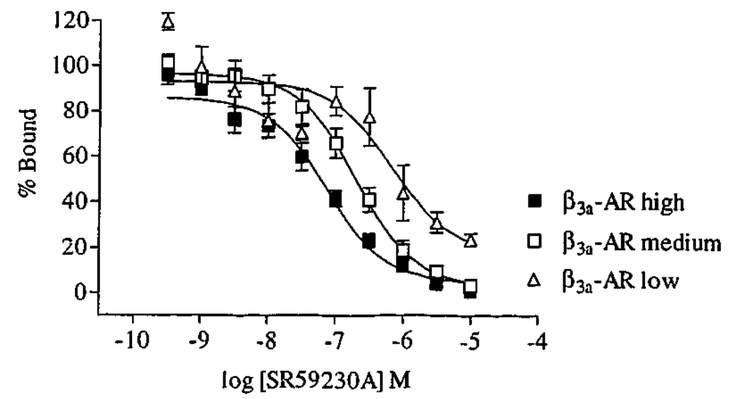


Figure 3.9: The effect of differing levels of receptor expression on the competition for ICYP binding (500pM) by the β_3-AR antagonist SR59230A in cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3-5).

(a)



(b)

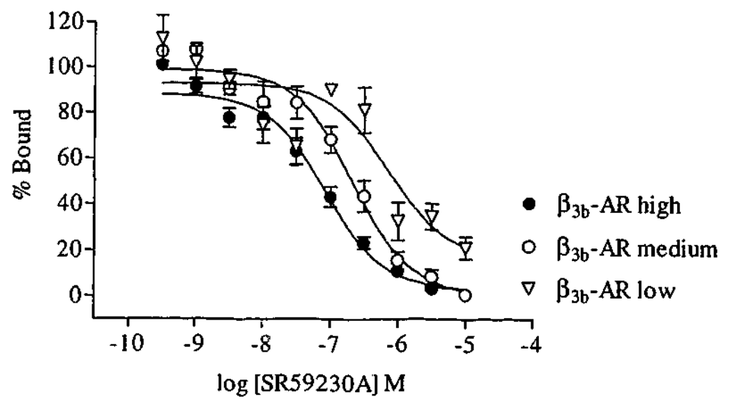
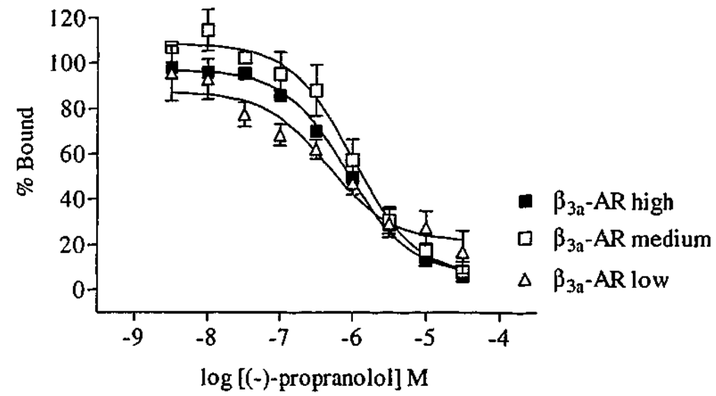


Figure 3.10: The effect of differing levels of receptor expression on the competition for ICYP binding (500pM) by the non-selective β-AR antagonist (-)-propranolol in cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific was binding defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3-6).

(a)



(b)

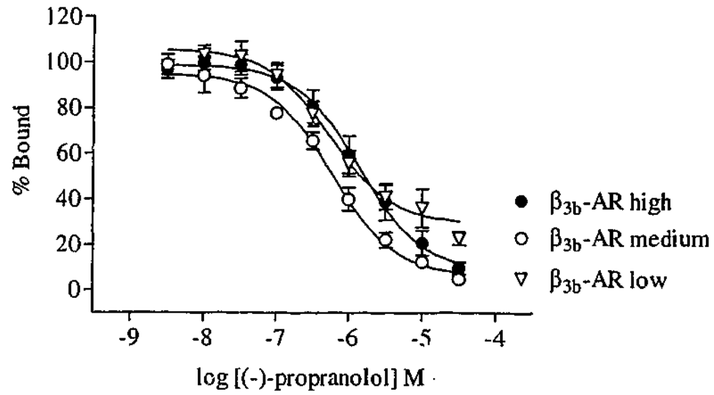
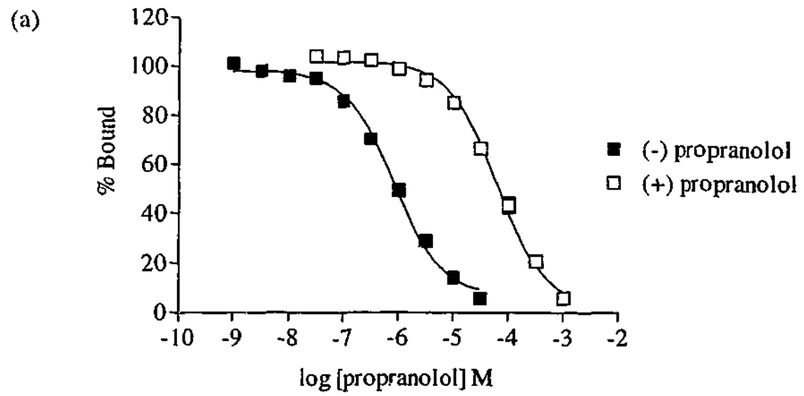


Figure 3.11: Competition between ICYP binding (500pM) and the stereoisomers of propranolol in membranes prepared from CHO-K1 cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=4-6).



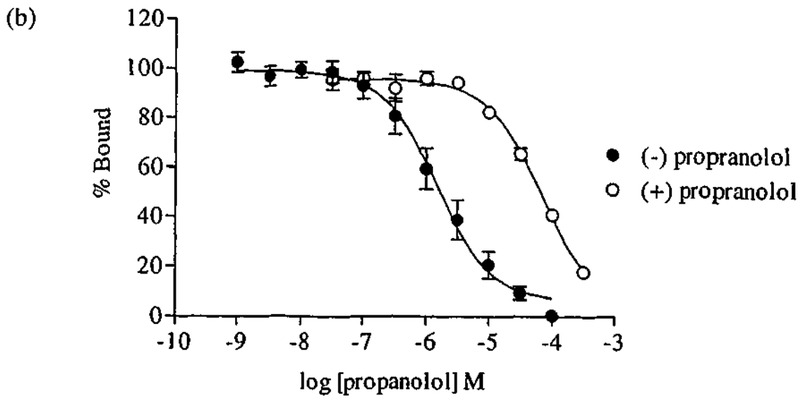
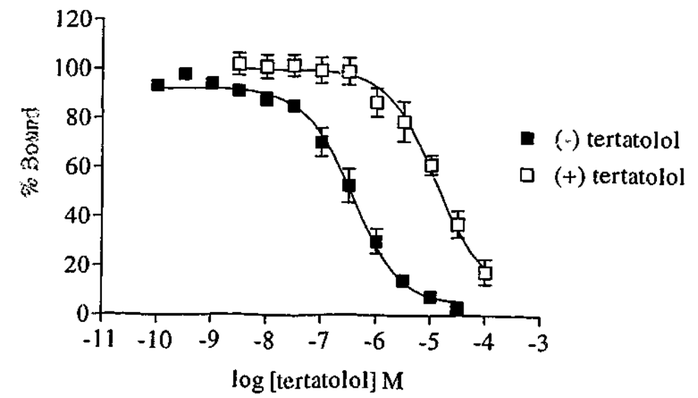


Figure 3.12: Competition between ICYP binding (500pM) and the stereoisomers of tertatolol in membranes prepared from CHO-K1 cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3).

(a)



(b)

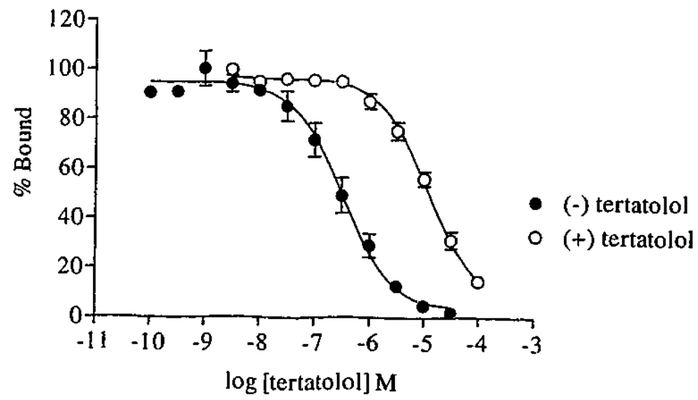
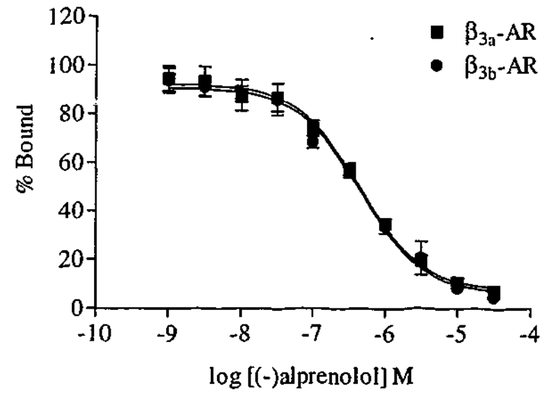


Figure 3.13: Competition between ICYP (500pM) and (a) (-)-alprenolol, (b) bupranolol or (c) labetalol for binding sites in homogenates of CHO-K1 cells expressing either the β_{3a}- or β_{3b}-AR. Incubations were for 60 min at room temperature and non-specific binding defined by (-)-alprenolol (1mM). The results are expressed as a % of the maximum specific binding for ICYP in each individual experiment. Corresponding affinity values are shown in Table 3.2. Points show mean ± s.e.mean (n=3-4).

(a)



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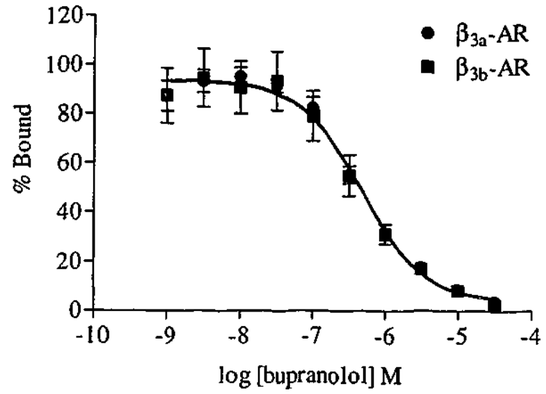

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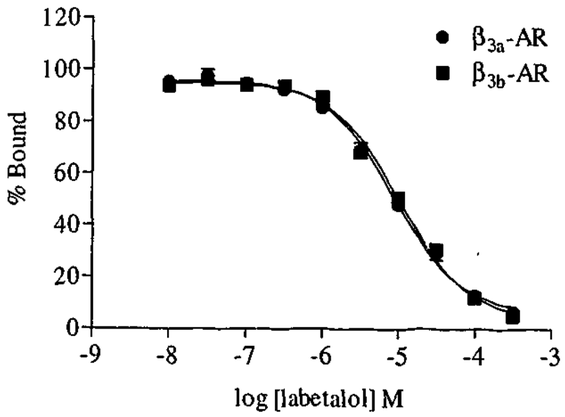
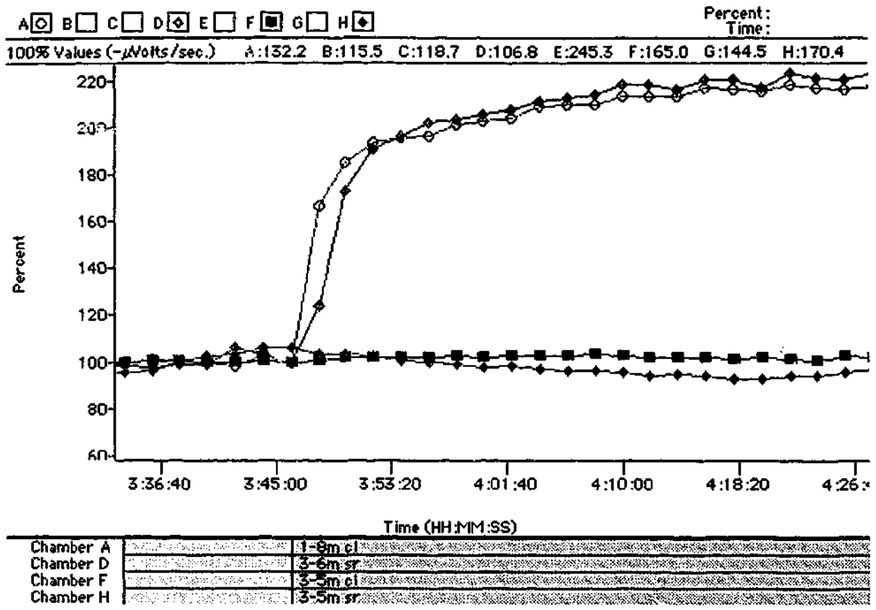


Figure 3.14: Rate data from cytosensor microphysiometer experiment where non-transfected CHO-K1 cells (chambers F and H) were exposed either to 30μM CL316243 (chamber F) or 30μM SR59230A (chamber H). Cells expressing the β_{3a}-AR at high levels (chambers A and D) were exposed to either 10nM CL316243 (chamber A) or 3μM SR59230A (chamber D) in parallel. All cells were exposed to drugs for 30 min. Note the lack of effect of either CL316243 or SR59230A on extracellular acidification responses in non-transfected cells.



3.3.2.1 Agonist profile

Exposure of CHO-K1 cells expressing high levels of β_{3a}- or β_{3b}-AR to all β-AR ligands examined resulted in a concentration-dependent increase in the rate of extracellular acidification relative to baseline acidification rates. Concentration-response curves to the β_3-AR agonists CL316243 (Figure 3.15), BRL37344 (Figure 3.15) (both rodent selective), the β_1-/β_2-AR antagonist/β_3-AR agonist CGP12177A (Figure 3.15), and GR265162X, SB251023 and L755507 (all human selective) (Figure 3.16) were measured. In all experiments performed, pEC_{50} values were similar for each individual agonist at both receptors when expressed at high levels (Table 3.3).

Stereoselective activation of the β_{3a}- and β_{3b}-AR was observed with isoprenaline: (-)-isoprenaline (pEC_{50} 7.88 ± 0.25 β_{3a}-AR, 7.85 ± 0.43 β_{3b}-AR) > (+)-isoprenaline (pEC_{50} 5.73 ± 0.09 β_{3a}-AR, 5.51 ± 0.12 β_{3b}-AR) (Figure 3.17, Table 3.3). These isomers produced c-r curves that were significantly different in position (2-way ANOVA ***$p<0.001$) with the (-)-isomers showing a 120-218 fold higher potency than (+)-isomers (n=4).

Maximal responses produced at the β_{3b}-AR were significantly less than those at the β_{3a}-AR for every agonist examined as shown in Table 3.3 (2-way ANOVA *** $p < 0.001$). The rank order of potency at both receptors was: BRL37344, CL316243, GR265162X > CGP12177A, L755507 > SB251023, (-)-isoprenaline > (+)-isoprenaline.

The effect of receptor expression levels in either β_{3a}- or β_{3b}-AR cells was examined on CL316243-mediated increases in extracellular acidification rates. CL316243 c-r curves were shifted to the right as receptor expression levels decreased in cells expressing either the β_{3a}-AR (high: pEC_{50} 10.62 ± 0.05; medium: pEC_{50} 9.74 ± 0.15; low: 8.86 ± 0.05; n=5-12) or the β_{3b}-AR (high: pEC_{50} 10.50 ± 0.04; medium: pEC_{50} 9.50 ± 0.12; low: 8.23 ± 0.06; n=3-11) (Figure 3.18). This was also accompanied by a decrease in the maximal response (data not shown).

Figure 3.15: The effects of β_3-AR agonists on extracellular acidification rates in the cytosensor microphysiometer. Mean concentration-response curves for (a) BRL37344, (b) CGP12177A and (c) CL316243 to increase extracellular acidification rates in cells expressing either the β_{3a}- or the β_{3b}-AR at high expression levels. The results are expressed as a % of the maximum response produced to the same agonist in cells expressing the β_{3a}-AR. Each point shows the mean ± s.e.mean (n=4-6).

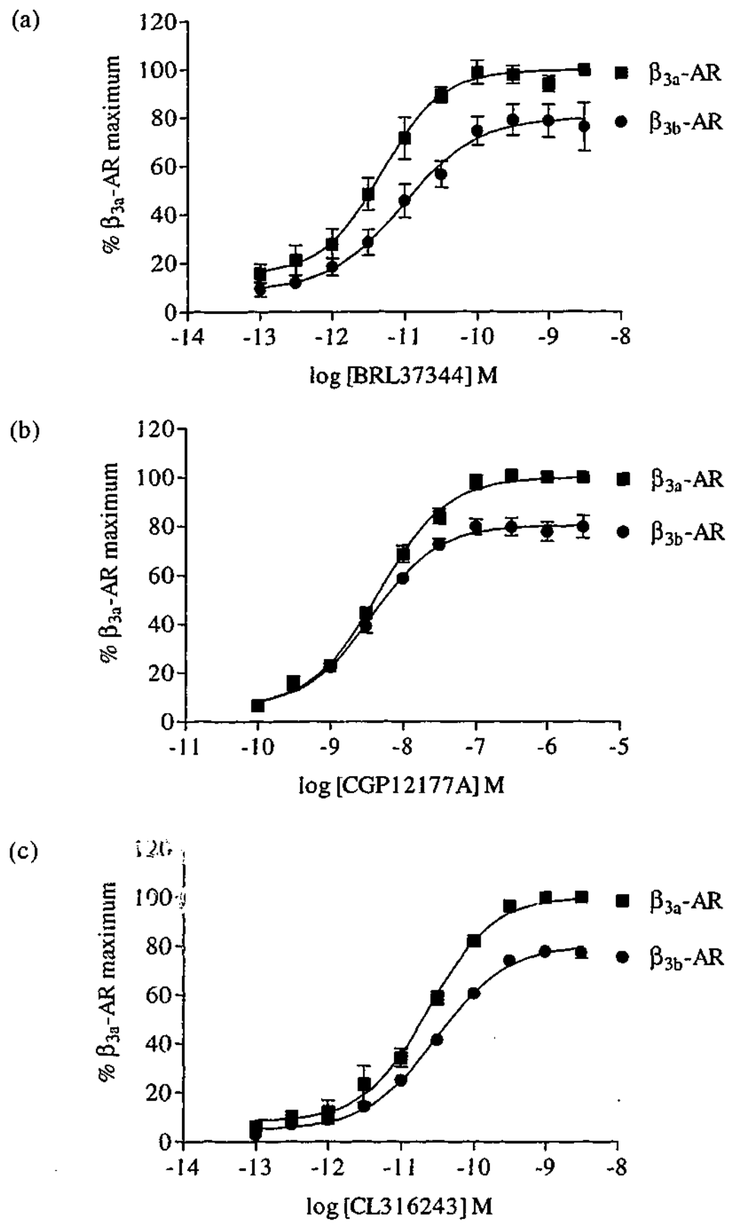
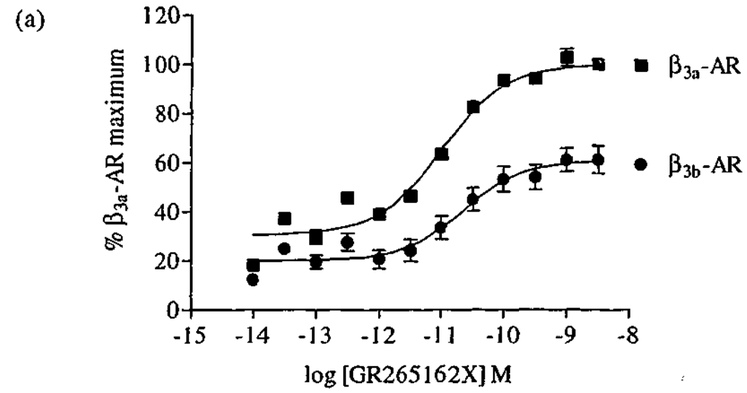
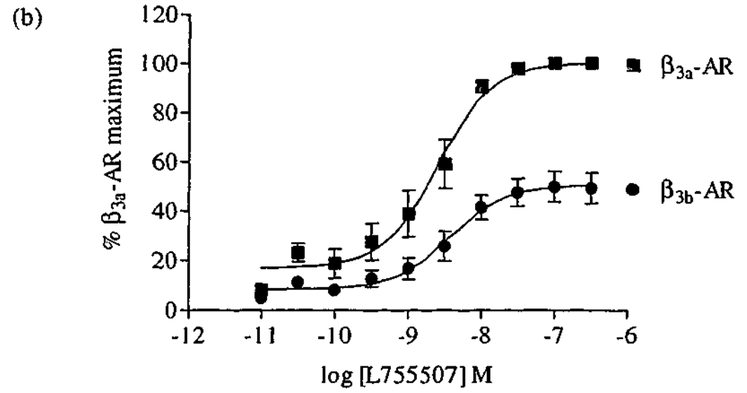


Figure 3.16: The effects of "human selective" β_3-AR agonists on extracellular acidification rates in the cytosensor microphysiometer. Mean concentration-response curves for (a) GR265162X, (b) L755507 and (c) SB251023 to increase extracellular acidification rates in cells expressing either the β_{3a}- or the β_{3b}-AR at high expression levels. The results are expressed as a % of the maximum response produced to the same agonist in cells expressing the β_{3a}-AR. Each point shows mean ± s.e.mean (n=3-4).





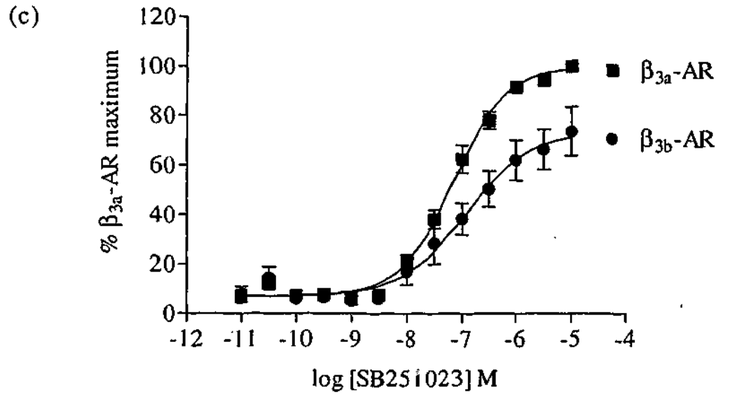


Table 3.3: Summary of agonist potency (pEC_{50}) values at the mouse β_{3a}- or β_{3b}-AR expressed at high levels in CHO-K1 cells assessed using the cytosensor microphysiometer. Agonist potency values are mean ± s.e.mean for *n* experiments. Maximal responses in CHO-K1 cells expressing the β_{3b}-AR are expressed as a % of the maximal response produced in CHO-K1 cells expressing the β_{3a}-AR, defined as 100% (mean ± s.e.mean).

Agonist	pEC_{50} β_{3a}-AR	pEC_{50} β_{3b}-AR	β_{3b}-AR max response % β_{3a}-AR max	*n*
CL316243	10.62 ± 0.05	10.50 ± 0.04	80.5 ± 1.7***	*5-6*
BRL37344	11.30 ± 0.09	11.01 ± 0.15	80.0 ± 4.2***	*4*
CGP12177A	8.32 ± 0.05	8.42 ± 0.07	80.3 ± 1.7***	*6*
GR265162X	11.00 ± 0.08	10.65 ± 0.18	61.1 ± 3.6***	*4*
SB251023	7.14 ± 0.04	6.91 ± 0.20	74.0 ± 6.8***	*4*
L755507	8.60 ± 0.08	8.43 ± 0.15	50.6 ± 3.4***	*3-4*
(-)-Isoprenaline	7.88 ± 0.25	7.85 ± 0.43	60.9 ± 7.3***	*4*
(+)-Isoprenaline	5.73 ± 0.09	5.51 ± 0.12	69.2 ± 5.8***	*4*

*** indicates $p<0.001$ determined by 2-way ANOVA of c-r curves produced in β_{3a}- or β_{3b}-AR cells

Figure 3.17: The effect of the stereoisomers of isoprenaline on increases in extracellular acidification rates in the cytosensor microphysiometer in cells expressing either the β_{3a}- or β_{3b}-AR at high expression levels. The results are expressed as a % of the maximum response produced to 3μM (-)-isoprenaline or 30μM (+)-isoprenaline in cells expressing the β_{3a}-AR. Each point shows mean ± s.e.mean (n=4).

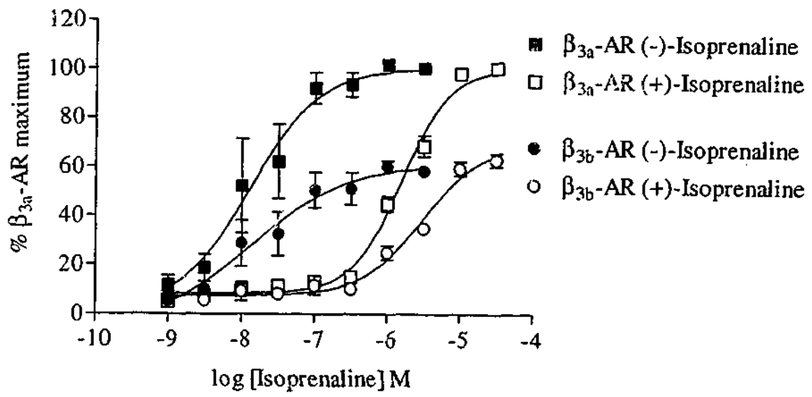
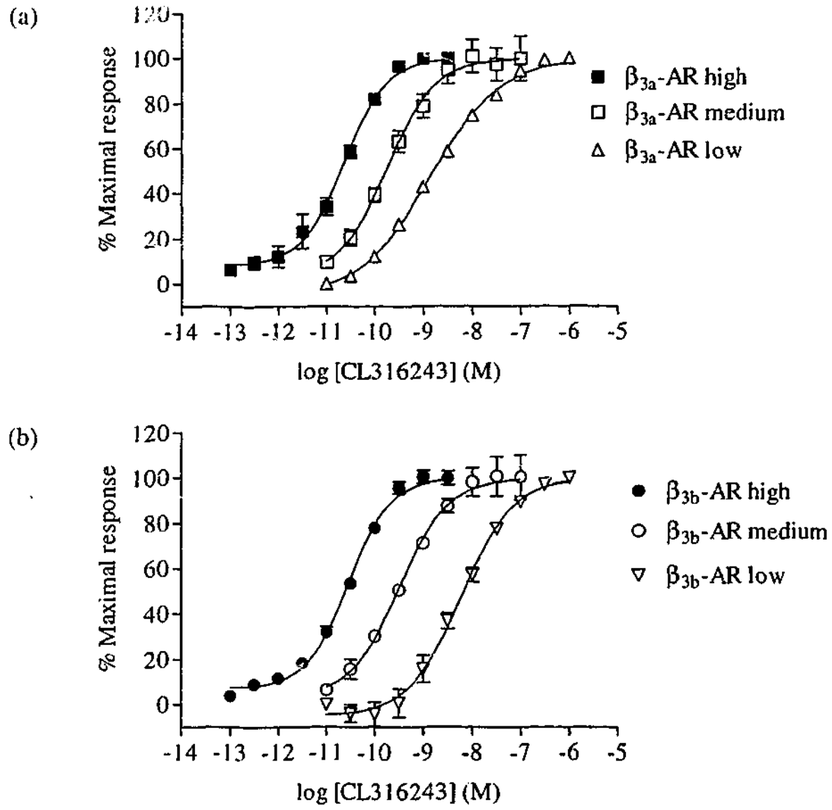


Figure 3.18: The effect of differing levels of receptor expression on CL316243-mediated increases in extracellular acidification rates as measured in the cytosensor microphysiometer in cells expressing either the (a) β_{3a}- or (b) β_{3b}-AR. The results are expressed as a % of the maximum response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=3-12).

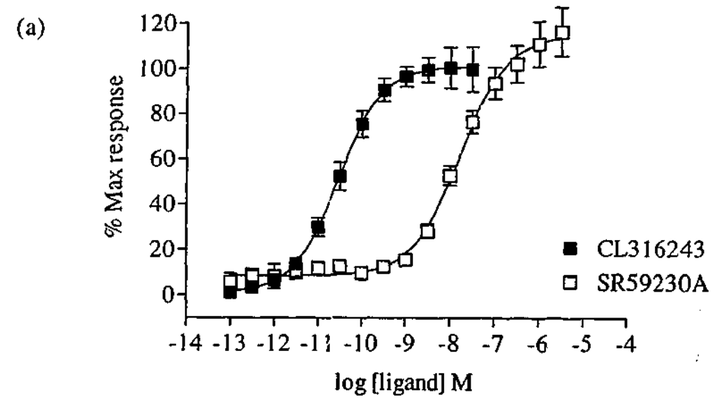


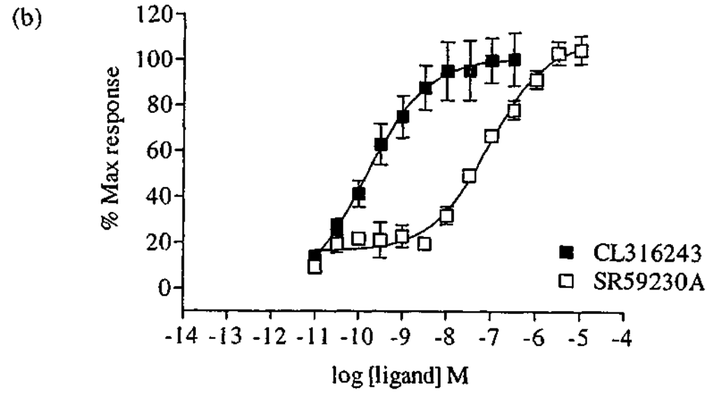
3.3.2.2 SR59230A

The β_3-AR antagonist produced dose-dependent increases in extracellular acidification rates in all three β_{3a}-AR clones examined, with an intrinsic activity equal to that of CL316243 c-r curves that were constructed in sister cells studied in parallel (Figure 3.19, 3.20; Table 3.4). The c-r curve to SR59230A was shifted to the right as expression levels were lowered, consistent with results obtained with CL316243 in the cytosensor microphysiometer. This was also accompanied by a decrease in the maximal response (data not shown). In untransfected CHO-K1 cells, SR59230A failed to increase extracellular acidification rates at a concentration of 10μM (Figure 3.14).

To examine the effect of SR59230A on CL316243-mediated increases in extracellular acidification rates, cells expressing low or medium levels of receptor were exposed to SR59230A (300nM) for 1 h prior to construction of CL316243 c-r curves. This concentration of SR59230A was chosen since it is appropriate for antagonism of β_3-AR-mediated responses and produces a submaximal agonist response in comparison to CL316243 responses in the cytosensor microphysiometer (Figure 3.19). In medium and low expressing cells, SR59230A (300nM) caused an initial increase in extracellular acidification rates, but subsequent CL316243 c-r curves were shifted to the right in comparison to CL316243 c-r curves constructed in the absence of SR59230A, with a pK_B value of 8.23 ± 0.11 (n=8) and 7.95 ± 0.15 (n=4) in medium and low expressing cells respectively (Figure 3.21; Table 3.5). In cells expressing high receptor levels, three different concentrations of SR59230A were used (1, 3 and 10nM). SR59230A (300nM) could not be used since this concentration of SR59230A gives a maximal agonist response in comparison to CL316243-mediated responses in the cytosensor microphysiometer (Figure 3.19). The concentrations of SR59230A used caused an initial increase in extracellular acidification response and subsequent CL316243 c-r curves were not shifted in comparison to CL316243 c-r curves produced in the absence of SR59230A (Figure 3.21). Maximum responses to CL316243 were not altered by SR59230A (Figure 3.21).

Figure 3.19: Comparison of the agonistic effects of SR59230A in the cytosensor microphysiometer to increase extracellular acidification rates performed in parallel with CL316243-mediated increases in extracellular acidification rates in cells expressing the β_{3a}-AR at (a) high, (b) medium and (c) low receptor levels. The results are expressed as a % of the maximum response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=4-8).





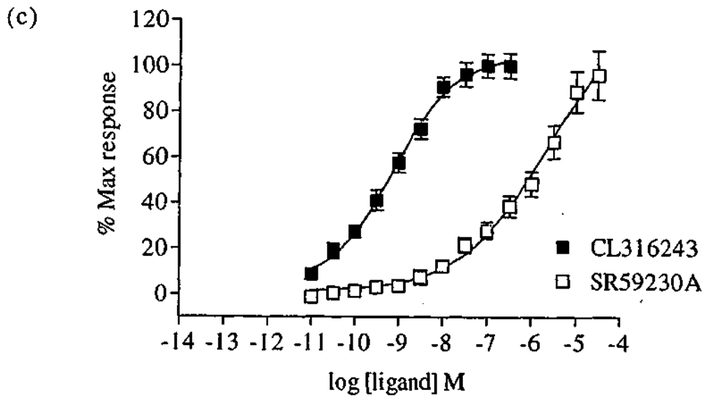


Figure 3.20: Comparison of the mean agonist concentration-response curves to SR59230A (Figure 3.19) in β_{3a}-AR cells expressing differing levels of receptor to illustrate the differences in potency (pEC_{50}) observed at different expression levels. The results are expressed as a % of the maximum response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=4-8).

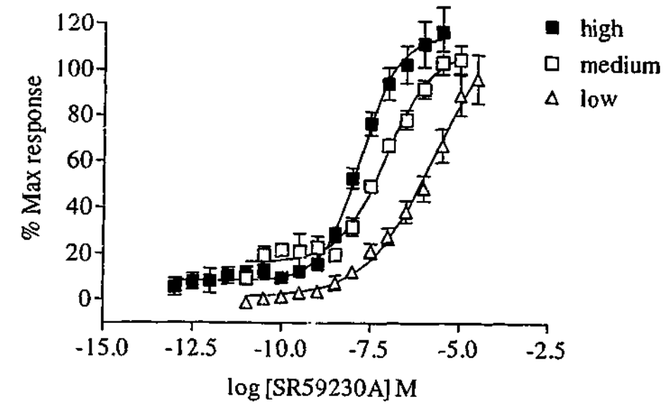


Table 3.4: Summary of agonist potency (pEC_{50}) values for CL316243 and SR59230A at the mouse β_{3a}-AR expressed at different receptor levels assessed using the cytosensor microphysiometer. Agonist potency values are mean ± s.e.mean (n=4-8). Intrinsic activity values are the mean value of the calculated maximum for SR59230A tested in each experiment expressed as a % of the maximum response to CL316243 in that experiment.

Expression level	pEC_{50} CL316243	Intrinsic activity	pEC_{50} SR59230A	Intrinsic activity[#]	*n*
High	10.53 ± 0.07	1.00 ± 0.04	7.77 ± 0.10	1.15 ± 0.05	*8*
Medium	9.83 ± 0.34	1.00 ± 0.07	7.05 ± 0.12	1.08 ± 0.05	*4*
Low	9.11 ± 0.13	1.00 ± 0.04	5.29 ± 0.24	0.96 ± 0.10[##]	*8*

[#]Intrinsic activity relative to maximal CL316243 response, defined as 1.00

[##]Based on 30μM SR59230A since c-r curve could not be fully constructed

Figure 3.21: Interactions between CL316243 and SR59230A in cells expressing the β_{3a}-AR at (a) high, (b) medium and (c) low receptor levels to increase extracellular acidification rates in the cytosensor microphysiometer. The results are expressed as a % of the maximum response produced to the highest concentration of CL316243 in each experiment. Each point shows mean ± s.e.mean (n=4-8).

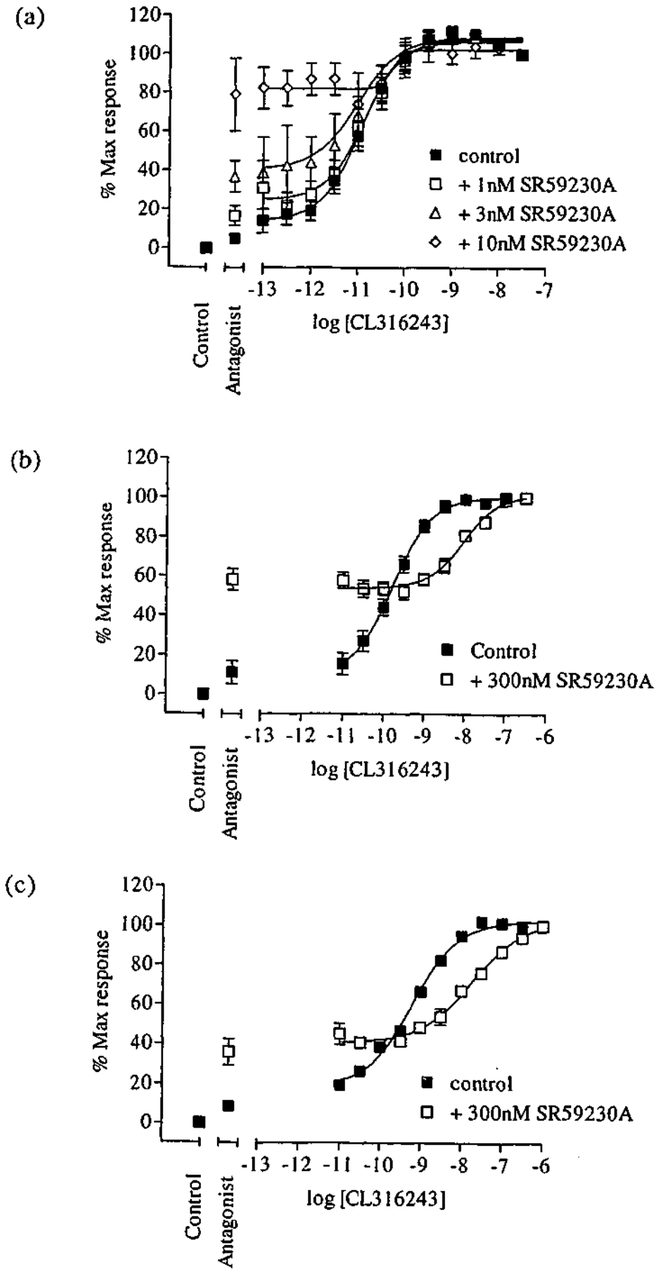


Table 3.5: Summary of agonist potency (pEC_{50}) values for CL316243 in the presence or absence of SR59230A at the mouse β_{3a}-AR expressed at different receptor levels assessed using the cytosensor microphysiometer. % Maximum response values given are the increase over control initial basal extracellular acidification rates following the 1 h incubation of cells in the absence (control) or presence of various concentrations of SR59230A. Affinity values are represented as pK_B values of *n* experiments.

Expression level		% Max response[#]	pEC_{50} CL316243	pK_B	*n*
High	Control	4.50 ± 1.94	10.94 ± 0.08		*5*
	+ 1nM SR59230A	16.70 ± 5.08	10.86 ± 0.11		*4*
	+ 3nM SR59230A	40.57 ± 10.00	11.00 ± 0.23		*5*
	+ 10nM SR59230A	79.18 ± 18.78	10.27 ± 0.33		*5*
Medium	Control	11.14 ± 5.85	9.76 ± 0.10		*8*
	+ 300nM SR59230A	58.33 ± 5.50	8.04 ± 0.12	8.23 ± 0.11	*8*
Low	Control	8.31 ± 1.73	9.18 ± 0.05		*4*
	+ 300nM SR59230A	35.67 ± 6.37	7.72 ± 0.12	7.95 ± 0.15	*4*

[#] Percentage of maximal response after 1 h equilibration period with antagonist or media alone (0% defined as extracellular rate before addition of SR59230A or media)

Experiments were performed in cells expressing the β_{3a}-AR only. Preliminary results indicate similar observations for cells expressing the β_{3b}-AR (data not shown).

3.3.3 Examination of cAMP accumulation responses mediated by either the β_{3a}- or β_{3b}-AR expressed in CHO-K1 cells

3.3.3.1 CL316243

The effect of receptor expression levels in β_{3a}- or β_{3b}-AR cells on CL316243-mediated increases of cAMP accumulation was examined. CL316243 c-r curves were shifted to the right as receptor expression levels were lowered (Figure 3.22 (β_{3a}-AR), 3.23 (β_{3b}-AR); Table 3.6), accompanied by a decrease in the maximum response. The pEC_{50} values obtained in cAMP accumulation studies were ~ 0.5 - 0.75 log units lower than those obtained from cytosensor microphysiometer studies. This may indicate that signalling pathways apart from those mediating increases in cAMP levels are involved in cytosensor microphysiometer responses.

CL316243 did not increase intracellular cAMP accumulation in untransfected CHO-K1 cells (Figure 3.24; Table 3.7).

3.3.3.2 SR59230A

Preliminary data indicated that the β_3-AR antagonist SR59230A increased cAMP levels in cells expressing the β_{3a}-AR (Figure 3.24). Further studies showed that SR59230A increased cAMP accumulation in a dose-dependent manner in cells expressing the β_{3a}-AR at all levels of expression investigated, but with an intrinsic activity significantly lower than that of CL316243 (Figure 3.25; Table 3.8). C-r curves to SR59230A were shifted to the right as expression levels were lowered, an effect that was accompanied by a decrease in the maximum response. In untransfected CHO-K1 cells, SR59230A failed to increase intracellular cAMP levels (Figure 3.24; Table 3.7).

Figure 3.22: The effect of differing levels of receptor expression on CL316243-mediated increases in cAMP accumulation in cells expressing the β_{3a}-AR. The results are expressed as the amount of cAMP produced per well. Each point shows mean ± s.e.mean (n=4-5). The lower graph has an expanded scale to illustrate more clearly the effect of CL316243 on cAMP accumulation in low expressing cells.

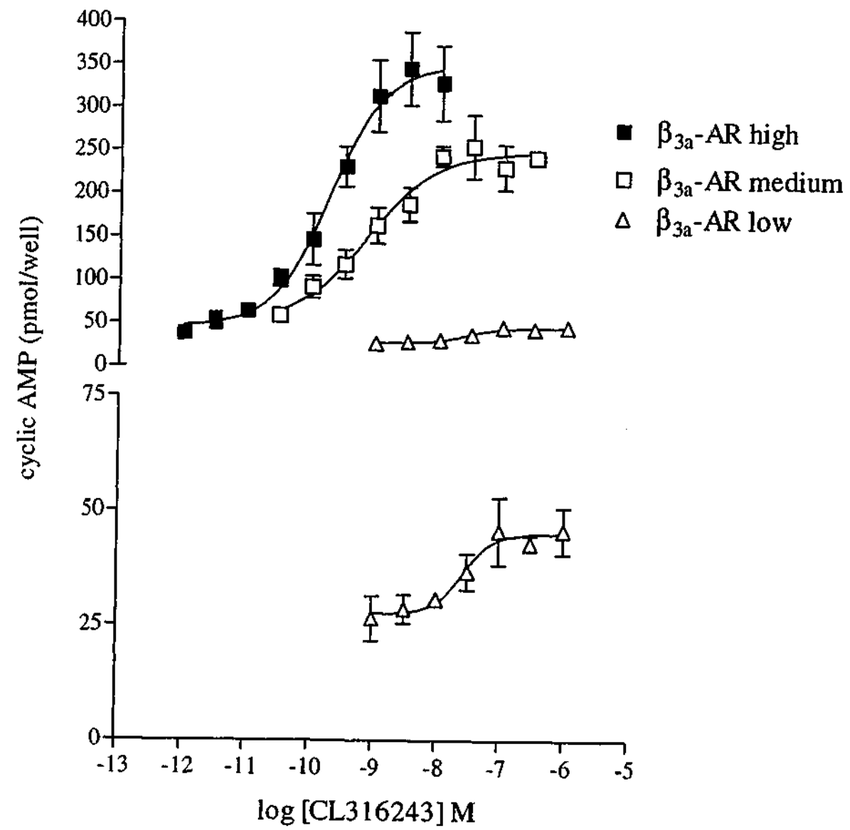


Figure 3.23: The effect of differing levels of receptor expression on CL316243-mediated increases in cAMP accumulation in cells expressing the β_{3b}-AR. The results are expressed as the amount of cAMP produced per well. Each point shows mean ± s.e.mean (n=4-5). The lower graph has an expanded scale to illustrate more clearly the effect of CL316243 on cAMP accumulation in low expressing cells.

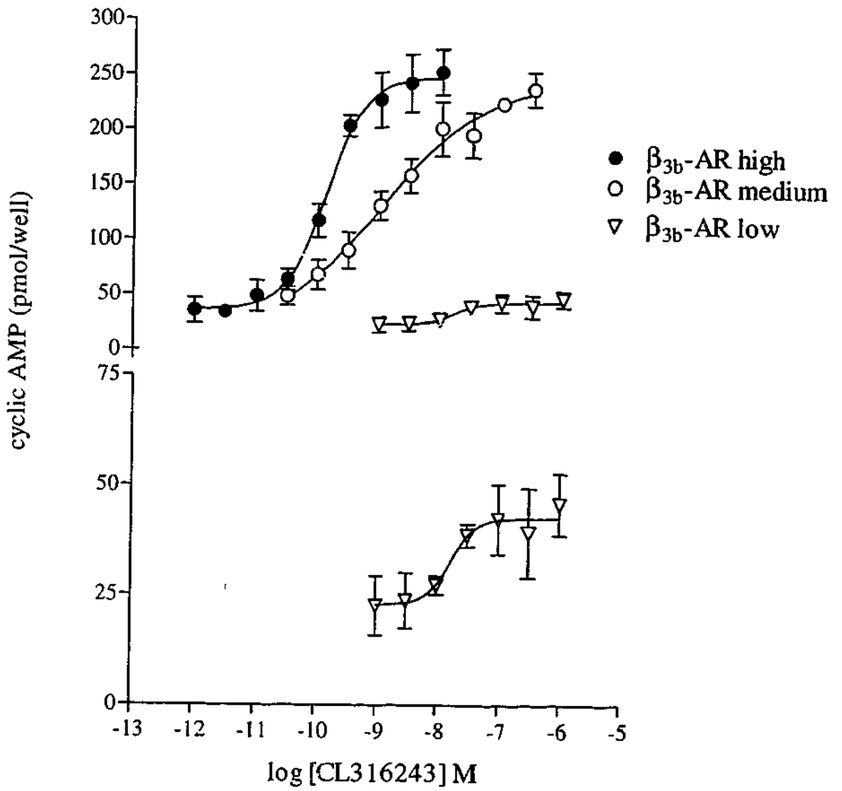


Table 3.6: Summary of agonist potency (pEC_{50}) values for CL316243 at the mouse β_{3a}- or β_{3b}-AR expressed at different receptor levels in CHO-K1 cells to increase cAMP accumulation levels. Agonist potency values are mean ± s.e.mean for *n* experiments. Maximum responses produced are expressed as the amount of cAMP produced per well.

	pEC_{50} CL316243	Maximum (pmol/well)	*n*
β_{3a}-AR			
high	9.74 ± 0.15	347 ± 27	*5*
medium	9.16 ± 0.27	248 ± 15	*4*
low	7.57 ± 0.24	45 ± 3	*4*
β_{3b}-AR			
high	9.87 ± 0.10	247 ± 12	*5*
medium	8.97 ± 0.39	244 ± 27	*4*
low	7.76 ± 0.33	43 ± 4	*4*

Figure 3.24: Comparison of the effect of CL316243 (0.3nM high; 3nM medium; 30nM low; 10μM CHO-K1) and SR59230A (100nM high; 1μM medium; 10μM low and CHO-K1) on cAMP accumulation in untransfected CHO-K1 cells or the β_{3a}-AR expressed at low, medium or high receptor levels. Bars show mean ± s.e.mean of 3 experiments performed in duplicate. The lower graph has an expanded scale to illustrate more clearly the effect of CL316243 and SR59230A on cAMP accumulation in untransfected CHO-K1 cells or the β_{3a}-AR expressed at low receptor levels.

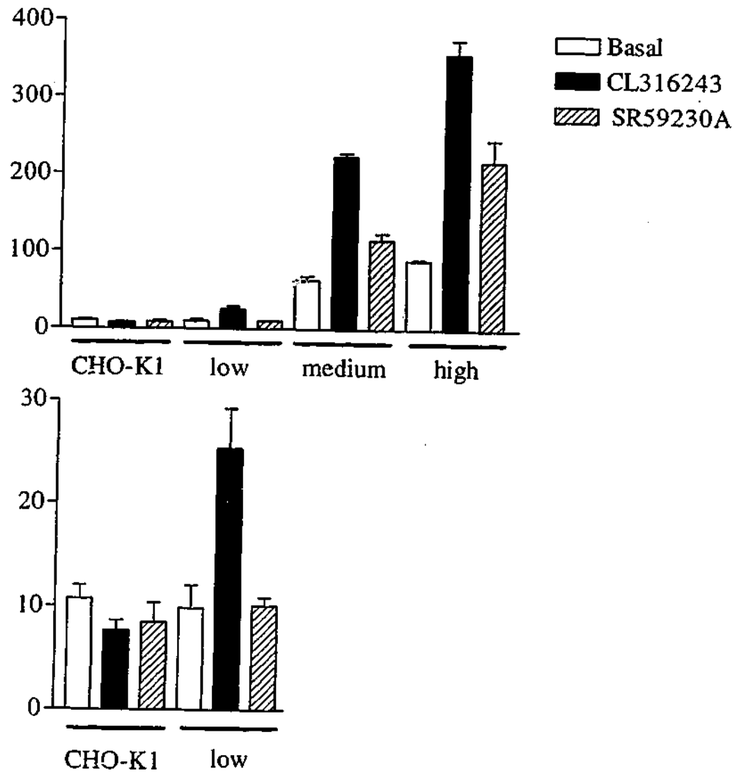


Table 3.7: Summary of the individual effects of CL316243 and SR59230A on cAMP accumulation in untransfected CHO-K1 cells or cells expressing the β_{3a}-AR at differing receptor expression levels. cAMP levels are mean ± s.e.mean for *n* experiments performed in duplicate.

Expression level		cAMP (pmol per well)	*n*
High	Basal	88.79 ± 1.50	*3*
	+ 0.3nM CL316243	356.57 ± 18.45	*3*
	+ 100nM SR59230A	216.97 ± 27.96	*3*
Medium	Basal	62.69 ± 5.91	*3*
	+ 3nM CL316243	222.71 ± 4.71	*3*
	+ 1μM SR59230A	114.48 ± 8.87	*3*
Low	Basal	9.86 ± 2.22	*3*
	+ 30nM CL316243	25.34 ± 3.87	*3*
	+ 10μM SR59230A	10.10 ± .082	*3*
CHO-K1	Basal	10.76 ± 1.3	*3*
	+ 10μM CL316243	7.68 ± 0.98	*3*
	+ 10μM SR59230A	8.48 ± 1.91	*3*

Figure 3.25: The effect of the β_3-AR "antagonist" SR59230A on cAMP accumulation in cells expressing the β_{3a}-AR at (a) high, (b) medium and (c) low receptor levels. The results are expressed as the amount of cAMP produced (pmol) per well. Basal and CL316243 stimulated cAMP accumulation is also shown. Each point shows mean ± s.e.mean (n=3 in duplicate).

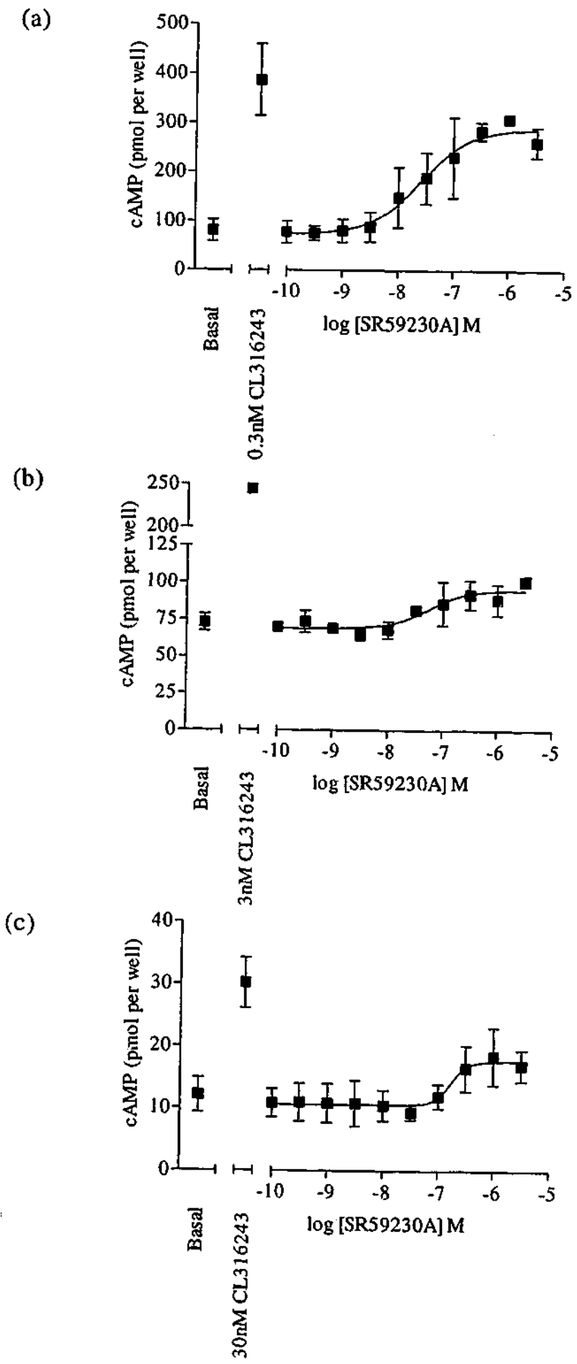


Table 3.8: Summary of agonist potency (pEC_{50}) values for SR59230A to increase cAMP accumulation in cells expressing at the mouse β_{3a}-AR expressed at different receptor levels. Agonist potency values are mean ± s.e.mean (n=3 in duplicate). Intrinsic activity values are the mean value of the calculated maximum for SR59230A tested in each experiment expressed as a % of the submaximal response to CL316243 in that experiment.

Expression level		cAMP (pmol per well)	pEC_{50}	Intrinsic activity[#]	*n*
High	Basal	81.06 ± 22.05			*3*
	CL316243	387.49 ± 72.52		1.00 ± 0.19	*3*
	SR59230A	290.3 ± 30.68	7.60 ± 0.29	0.75 ± 0.08	*3*
Medium	Basal	72.91 ± 5.95			*3*
	CL316243	244.57 ± 4.59		1.00 ± 0.02	*3*
	SR59230A	94.82 ± 5.54	7.28 ± 0.36	0.39 ± 0.02	*3*
Low	Basal	12.12 ± 2.80			*3*
	CL316243	30.16 ± 4.03		1.00 ± 0.13	*3*
	SR59230A	17.68 ± 1.96	6.77 ± 0.35	0.59 ± 0.06	*3*

[#] Intrinsic activity defined as response to CL316243 (1.00)

SR59230A inhibited cAMP accumulation in CHO-K1 cells expressing the β_{3a}-AR in response to a submaximal concentration of CL316243 in medium (3nM) and low (30nM) expressing cells with pEC_{50} values of 7.52 ± 0.33 (n=4) and 7.24 ± 0.55 (n=4) respectively (Figure 3.26). When CHO-K1 cells expressing the β_{3a}-AR at high receptor levels were stimulated with a submaximal concentration of CL316243 (0.3nM), SR59230A initially behaved as an antagonist, but at higher concentrations stimulated cAMP accumulation levels (Figure 3.26).

Experiments were performed in cells expressing only the β_{3a}-AR but preliminary results indicate similar observations for cells expressing the β_{3b}-AR (data not shown).

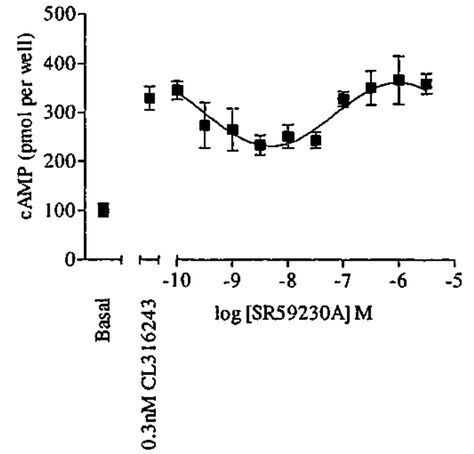
3.4 Discussion

Alternate splicing of genes encoding GPCRs results in isoforms differing at their C-terminus that share similar pharmacological properties. Here, the pharmacology of the mouse β_3-AR splice variants (Evans *et al.*, 1999) expressed in CHO-K1 cells was examined. Several stable cell lines of CHO-K1 cells expressing either the β_{3a}- or β_{3b}-AR at different levels were established by transfection of CHO-K1 cells, selection for neomycin resistance, and subsequent generation of clones.

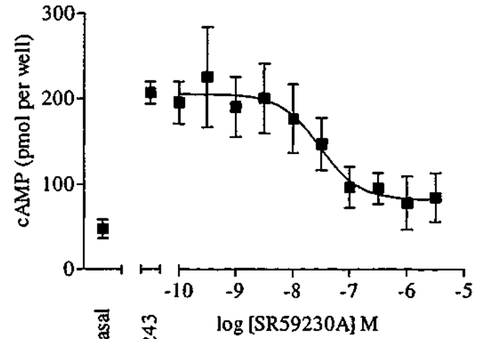
Three levels of receptor expression were chosen: low (~100 fmol mg $protein^{-1}$), medium (~500 fmol mg $protein^{-1}$), and high (~1200 fmol mg $protein^{-1}$). The K_D values for ICYP binding were consistent with that of the mouse β_3-AR expressed in CHO-K1 cells (880pM, Nahmias *et al.*, 1991), the endogenous mouse β_3-AR in 3T3-F442A adipocytes (1500-3500pM, El Hadri *et al.*, 1996; Feve *et al.*, 1991; 1992; 1994; 1995; Krief *et al.*, 1994) or endogenously expressed in mouse ileum (594-782pM, Hutchinson *et al.*, 2000; 887pM, Chapter 5) or mouse adipose tissue (2100pM, Carpene *et al.*, 1994). However, as receptor levels increased, the affinity for ICYP as determined by saturation binding decreased. This was not observed for other receptor systems investigating the effect of receptor number on K_D parameters (Cordeaux *et al.*, 2000; Hirst *et al.*, 1997; Liang *et al.*, 2000; Tsuda *et al.*, 1997; Vilardaga *et al.*, 1994; Wilson *et al.*, 1996) and an explanation for this cannot be

Figure 3.26: Interaction between SR59230A and CL316243 on cAMP accumulation in cells expressing the β_{3a}-AR at (a) high, (b) medium and (c) low receptor levels. Cells were pretreated with SR59230A before stimulation with the appropriate concentration of CL316243. The results are expressed as the amount of cAMP produced (pmol) per well. Basal and CL316243 stimulated cAMP accumulation is also shown. Each point shows mean ± s.e.mean (n=4 in duplicate).

(a)



(b)



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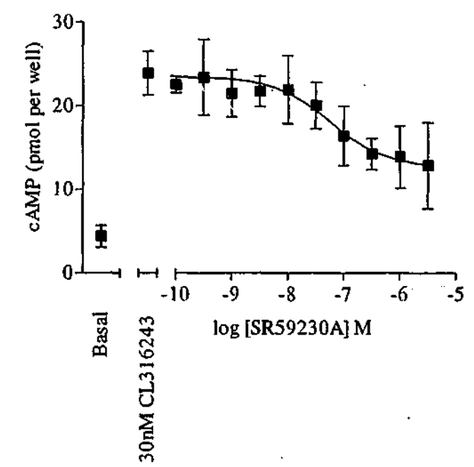


given. If ICYP was an agonist radioligand, one might expect that as one increases expression level a lower K_D (i.e. higher affinity) would be observed.

There was no difference in the affinity of β-AR ligands between the β_{3a}- and β_{3b}-AR expressed at high levels, consistent with the isoforms differing at the C-terminus and not in the transmembrane regions that form the β_3-AR ligand-binding pocket (Granneman *et al.*, 1998; Gros *et al.*, 1998; Guan *et al.*, 1995). This is also consistent with splice variants of other GPCRs showing no difference in affinity values for several ligands investigated (Bach *et al.*, 2001; Mialet *et al.*, 2000; Suzuki *et al.*, 2000). The rank order of β-AR ligands at either the β_{3a}- or β_{3b}-AR was identical: SR59230A > (-)-alprenolol, bupranolol, CGP12177A, (-)-tertatolol > BRL37344, (-)-propranolol > CL316243 > labetolol, (+)-tertatolol > CGP20712A, ICI118551, (-)-isoprenaline, (-)-NA, (+)-propranolol >> (+)-isoprenaline, (+)-NA. Affinity values for these ligands are in good agreement with those obtained at other cloned β_3-ARs (Chapter 1, Table 1.1).

Receptor expression level did not yield differing affinity values for (-)-propranolol or CGP20712A, consistent with other studies examining the effect of receptor expression on affinity of ligands for receptors (Cordeaux *et al.*, 2000; Tsuda *et al.*, 1997). However, affinity values increased for SR59230A, and decreased for ICI118551 as receptor expression levels increased. One possible explanation for ICI118551 may include interactions of ICYP and/or ICI118551 with endogenous β_2-ARs present in CHO-K1 cells. β_2-AR mRNA was detected in untransfected CHO-K1 cells (but not β_1- or β_3-AR mRNA) and the level of β_2-AR mRNA was reduced in transfected CHO-K1 cells (own unpublished observations). Untransfected CHO-K1 cells are believed to express the β_2-AR at approximately 30 fmol mg protein^{-1}. This suggests that at low levels of β_3-AR expression, there is proportionately greater interaction of either ICYP and/or ICI118551 with endogenous β_2-ARs, but as β_3-AR receptor levels are increased, the ratio of β_3-AR to β_2-AR increases, effectively "swamping out" the β_2-AR component in these cells. The influence of receptor density on the affinity for SR59230A may be that increased receptor expression induces a conformation of the receptor that allows SR59230A to have stronger

interactions at the binding pocket, although no precedence for this occurring at the β_3-AR or other GPCRs could be found in the literature.

The potency and efficacy of the selective β_3-AR agonist CL316243 was correlated with levels of expression for both the β_{3a}- and β_{3b}-AR, as assessed by cAMP accumulation and cytosensor microphysiometer studies. This observation is consistent with standard theoretical drug-receptor interaction models where increases in signal amplification result from increased receptor expression (Black & Leff, 1983; Clarke & Bond, 1998; Furchgott, 1966; Kenakin, 1995a; Kenakin & Morgan, 1989). This suggests that amplification occurs between CL316243-receptor binding and activation of AC. The use of clonal cell lines expressing different levels of recombinant receptors can allow the investigation of the relationship between receptor expression and response. In cells variously expressing the β_2-AR or β_3-AR, the potency and maximum responses of several agonists increased as receptor density increased (Bouvier *et al.*, 1988; MacEwan *et al.*, 1995; McDonnell *et al.*, 1998; Samama *et al.*, 1993; Whaley *et al.*, 1994; Wilson *et al.*, 1996). This has also been observed for adenosine, serotonin, secretin, opioid or muscarinic receptors (Bach *et al.*, 2001; Boddeke *et al.*, 1992; Cordeaux *et al.*, 2000; Hirst *et al.*, 1997; Mei *et al.*, 1989; Vilardaga *et al.*, 1994), although this is not a universal observation since several reports indicate that at some receptors intrinsic activities may increase with no apparent changes in pEC_{50} values (or vice versa) with increased receptor number (Tsuda *et al.*, 1997; Varrault *et al.*, 1992; Vogel *et al.*, 1995).

It is also notable that the basal level of cAMP accumulation in CHO-K1 cells increased with expression level, suggesting that high receptor levels may induce a degree of constitutive activity as seen in other systems with over-expressed human β_2-AR (Chidiac *et al.*, 1994; MacEwan *et al.*, 1995; McDonnell *et al.*, 1998; Samama *et al.*, 1993). Chidiac *et al.* (1994) suggested that certain β_2-AR ligands behave like "protean drugs", in that they can act as an agonist in one setting but as an inverse agonist in another. No inverse agonism has been reported for the β_3-AR, and SR59230A showed no inverse agonism in cAMP accumulation studies.

Studies examining CL316243-mediated cAMP accumulation and extracellular acidification responses of the β_{3a}- or β_{3b}-AR revealed that c-r curves to CL316243 in the cytosensor microphysiometer were typically 0.5-0.75 log units shifted to the left compared to those obtained in cAMP accumulation, at all three levels of expression examined. The difference in agonist potency values may be due to the fact that the cytosensor microphysiometer measures the entire consequence of cell stimulation by CL316243, and not simply the activation of Gαs as assessed by cAMP accumulation. One possible interpretation is that the β_3-AR couples to intracellular signalling pathways additional to Gαs (i.e. a cell's ability to activate one pathway may exceed what is needed for activation of another pathway). Alternatively, since the cytosensor microphysiometer measures very small changes in extracellular acidification rate, it may be that it has far greater sensitivity than cAMP assays (McConnell *et al.*, 1992). This discrepancy between the cytosensor microphysiometer and cAMP assay potency values has been reported for the $5HT_{1A}$ receptor (Dunlop *et al.*, 1998) and corticotropin-releasing factor receptor (Smart *et al.*, 1999).

Atypical β-AR (β_3-ARs) are characterised by low stereoselectivity ratios for antagonist enantiomers (Emorine *et al.*, 1994; Zaagsma & Nahorski, 1990) compared to β_1-/β_2-ARs which display high degrees of stereoselectivity (for review see Ruffolo (1991)). Generally, in radioligand binding experiments, β_3-ARs show little stereoselectivity between isomers of isoprenaline, alprenolol and tertatolol (Emorine *et al.*, 1989; Muzzin *et al.*, 1991; Roberts *et al.*, 1995). In functional experiments, there is conflicting data. Most studies show a modest preference for the (-)-isomer of several β_3-AR ligands, including isoprenaline (Carpene *et al.*, 1994; Emorine *et al.*, 1989; Horinouchi & Koike, 2001b; Nahmias *et al.*, 1991; Van der Vliet *et al.*, 1990), NA (Horinouchi & Koike, 2001b), cyanopindolol (Langin *et al.*, 1991), alprenolol (Harms, 1976; Harms *et al.*, 1977; Roberts *et al.*, 1999), tertatolol (Harms *et al.*, 1977; Roberts *et al.*, 1999) and propranolol (Bojanic *et al.*, 1985; Harms, 1976; Harms *et al.*, 1977; Roberts *et al.*, 1999), all ligands that have one chiral centre.

However, there are a few reports that indicate high stereoselectivity of the ligands TMQ7 and BRL37344 at the β_3-AR. TMQ7 is a non-selective β-AR agonist and is

structurally distinct from catecholamines. Its isomers exhibit high stereoselective activation for all three β-ARs expressed in CHO-K1 cells. Its degree of stereoselectivity at β_3-ARs is much greater in functional assays (776 fold selectivity for (-)-isomer compared to (+)-isomer) as opposed to radioligand binding assays (only 5 fold degree of selectivity) (Konkar *et al.*, 1999). At β_1-/β_2-ARs, the degree of stereoselectivity was equal in both functional and radioligand assays. TMQ7 displayed similar functional results in rat tissues and at the cloned rat β_3-AR (Fraundorfer *et al.*, 1994; Konkar *et al.*, 1996). The discrepancy may be due to the structure of the β_3-AR binding pocket which is less sterically hindered than that of the β_1-/β_2-AR since there are fewer bulky amino acid side chains that protrude into the binding pocket (Blin *et al.*, 1993; Granneman *et al.*, 1998).

Most β_3-AR agonists have two chiral centres, with four possible stereoisomers being produced (RR, RS, SR and SS conformations). Only a limited number of studies have investigated the role of these stereoisomers on β_3-AR function. Oriowo *et al.* (1996) investigated the individual stereoisomers of BRL37344 (50:50 mixture of the RR and SS isomers). The RR isomer was found to be 1000-3000 fold more selective for mediation of β_3-AR effects in rat adipose and gastrointestinal tissues (Oriowo *et al.*, 1996). The majority of β_3-AR agonists have bulky alkylamine side chains, that fit into the β_3-AR binding pocket. Bulky side chains are not favoured for interactions with β_1-/β_2-ARs, which have smaller binding pockets. The significance of stereoselectivity of BRL37344 and other β_3-AR agonists has not been further investigated and may be valuable in terms of selectivity since most β_3-AR agonists have low potency at other β-ARs. A high degree of stereoselectivity is also observed with the β_3-AR antagonist SR59230A, where the SS isomer is over 10,000 times more potent than its RR isomer (SR59483A) in antagonising β_3-AR-mediated increases in cAMP in rat BAT membranes and β_3-AR-mediated relaxation in rat proximal colon (Manara *et al.*, 1996; Nisoli *et al.*, 1996).

Stereoselectivity was observed with isoprenaline at both the β_{3a}- and β_{3b}-AR in the cytosensor microphysiometer (120-218 fold preference for (-)-isomer), with the same relative degree of stereoselectivity observed for both receptors. This degree of

stereoselectivity is much greater than described in previous reports (28 fold and 22 fold at the cloned human and mouse β_3-AR in CHO-K1 cells (Emorine *et al.*, 1989; Nahmias *et al.*, 1991) and in native tissues (25-36 fold stereoselectivity, Carpene *et al.*, 1994; Horinouchi & Koike, 2001b; Van der Vliet *et al.*, 1990). In radioligand binding experiments, degrees of stereoselectivity for propranolol (48-78 fold difference) and tertatolol (31-34 fold difference) were again higher than those previously reported (1.2-3 fold preference for (-)-isomer (Roberts *et al.*, 1995)). This did not correlate with functional data, with a higher degree of stereoselectivity being observed against β_3-AR (BRL37344) mediated responses (27 and 32 fold difference for (-)-isomer of tertatolol and alprenolol respectively (Roberts *et al.*, 1999). The degree of stereoselectivity for the (-)-isomers of NA and isoprenaline could not be fully quantitated in radioligand binding experiments at the β_{3a}- or β_{3b}-AR due to the low affinity of the (+)-isomers, but there is at least 47-77 fold selectivity for the (-)-isomer (previous reports show only 16 or 31 fold selectivity for (-)-isoprenaline at the cloned rat or human β_3-AR expressed in CHO-K1 cells (Emorine *et al.*, 1989; Muzzin *et al.*, 1991)). The reasons for the discrepancies between the work presented here and a limited number of previous studies remain unclear. Several explanations can be put forward. One possible explanation for the relative differences may be the degree of purity of isomers; only a minute contamination of the (+)-isomer with the (-)-isomer may influence the results substantially. Alternatively, since the results obtained with the cloned mouse splice variants show more marked stereoselectivity in functional than in radioligand binding experiments (albeit only one agonist was employed in functional experiments and no experiments were performed on stereoisomers of BRL37344 or other β_3-AR ligands with more than one chiral centre due to unavailability of these compounds), this discrepancy may suggest that stereoselectivity is of less importance for binding of the compounds than functional consequences. Experiments using a wider range of stereoisomers at several β_3-ARs would provide more insight into the importance of steric interactions of ligands with the β_3-AR.

SR59230A was the first selective β_3-AR antagonist described (Manara *et al.*, 1996) and has been shown to competitively antagonise β_3-AR mediated-responses in a wide variety of tissues with greater preference for β_3-ARs compared to β_1-/β_2-ARs

(Manara *et al.*, 1996; Nisoli *et al.*, 1996). However recent reports indicate that SR59230A may not be as selective for β_3-ARs as previously reported (Boorman *et al.*, 2001; Brahmadevara *et al.*, 2001; Candelore *et al.*, 1999; Louis *et al.*, 2000) and agonistic actions of SR59230A have been reported. There is no literature on the agonistic actions of SR59230A at cloned rodent β_3-ARs but at the cloned human β_3-AR, SR59230A appears to be a partial agonist with an intrinsic activity of 0.46 compared to isoprenaline and a K_{act} of 18 nM on cAMP production (Strosberg & Pietri-Rouxel, 1997), although the receptor expression level was not indicated. In CHO-K1 cells expressing the human β_3-AR, SR59230A showed negligible agonistic actions on cAMP levels in cells with expression levels of 40-60 fmol mg protein^{-1} (Candelore *et al.*, 1999). However, when the receptor is expressed at 10 times these levels, SR59230A is a partial agonist (intrinsic activity 0.63 compared with isoprenaline) with an EC_{50} value of 71 nM (Candelore *et al.*, 1999). It was suggested that SR59230A had low efficacy, enabling it to act as an antagonist in tissues but as a partial agonist in cells expressing high receptor levels (Arch, 2000) since in cells expressing lower amounts of receptor, SR59230A produced only 0.5% of the cAMP accumulation seen with isoprenaline (Candelore *et al.*, 1999). However, in guinea-pig duodenum and gastric fundus, it behaves as an agonist with potency nearly equal to isoprenaline (pD_2 6.5 and 7.1 respectively) (Horinouchi & Koike, 2001a). At high concentrations SR59230A can produce concentration-dependent relaxation of hypoxic pulmonary vasoconstriction in rats (Dumas *et al.*, 1998), mediate a slow relaxation of rat aortic rings (Brahmadevara *et al.*, 2001) and mediate ileum smooth muscle relaxation (Chapter 5). Other β-AR antagonists reported to have some agonistic actions at β_3-AR include tertatolol (Bond & Vanhoutte, 1992), alprenolol (Blin *et al.*, 1993; Blue *et al.*, 1990; Carpene *et al.*, 1994; Granneman *et al.*, 1991), propranolol (Blin *et al.*, 1993; 1994; Gerhardt *et al.*, 1999; Pietri-Rouxel *et al.*, 1995), oxprenolol (Blin *et al.*, 1993; Nahmias *et al.*, 1991), pindolol (Carpene *et al.*, 1994; Granneman *et al.*, 1991; Horinouchi & Koike, 2001c; Nahmias *et al.*, 1991), cyanopindolol (Langin *et al.*, 1991; Wilson *et al.*, 1996), nadolol (Carpene *et al.*, 1994) and carteolol (Horinouchi & Koike, 2000; Zhao *et al.*, 1998).

Agonistic actions of SR59230A at the cloned mouse β_3-AR were thought to resemble those of CGP12177A at the cloned human and rat β_1-AR, where at low

expression levels it is a partial agonist but at high expression levels it acts as a full agonist (Konkar *et al.*, 2000; Pak & Fishman, 1996). These authors suggested that the simplest explanation for their findings was that CGP12177A is intrinsically a weak agonist at the cloned β_1-ARs, but that as receptor levels increase, the agonistic effect becomes more apparent. This also occurs to a certain degree for the $5HT_{4a}$ and $5HT_{4b}$ receptors, where two partial agonists and an antagonist at low receptor density (400 fmol mg protein^{-1}) become full agonists and a partial agonist respectively at high receptor expression levels (3000-5000 fmol mg protein^{-1}) (Bach *et al.*, 2001). This is similar to β_3-AR responses mediated by SR59230A in cAMP accumulation experiments, where as receptor expression levels increased, the agonist effect of SR59230A becomes more apparent and the antagonist effect becomes less apparent. However, this is not true for cytosensor microphysiometer studies using SR59230A. SR59230A is a full agonist at all levels of receptor expression levels (albeit with lower potency values than CL316243), and its antagonist actions are only evident in low and medium expressing cells. At high receptor levels, SR59230A is unable to antagonise CL316243-mediated increases in extracellular acidification rates, since the agonistic actions of SR59230A occur at concentrations that are much lower than its appropriate concentration for antagonism of β_3-AR responses.

Some hypotheses to consider for the anomalous behaviour of SR59230A include:

1. Binding of SR59230A to the β_3-AR may induce a conformational change in the receptor enabling it to act as an agonist for one effector, and an antagonist of CL316243-mediated responses. Several recent reports indicate that receptors may exist in multiple active states that may correspond to different conformations with specific pharmacological and functional properties and that different ligands and/or G-proteins affect these states in different manners (Ghanouni *et al.*, 2001; Kenakin, 1995b; Palanche *et al.*, 2001; Roettger *et al.*, 1997; Samama *et al.*, 1993; Thomas *et al.*, 2000; Whistler & von Zastrow, 1999). To a certain degree this can be observed with the human β_3-AR. Gerhardt *et al.* (1999) suggest that at least three different receptor states may exist for the human β_3-AR transfected in CHO-K1 cells. One state may favour β_3-AR signalling

towards AC, another favour Erk1/2, and additional receptor states may not discriminate between the two signalling pathways.

2. Since AC is a principal effector of β_3-AR mediated effects, it can be postulated that a different atypical pathway or interactions with other cell surface molecules may mediate some of the agonistic actions of SR59230A. This would seem likely since SR59230A behaves as a full agonist in the cytosensor microphysiometer at all levels of expression examined, but it is a weak partial agonist with respect to CL316243-mediated increases in cAMP accumulation. This type of receptor promiscuity is enhanced when receptors are over-expressed (Cordeaux *et al.*, 2000; Eason *et al.*, 1992; Kenakin, 1995a; 1995b) so influencing the potency of agonist actions and their effects (Cordeaux *et al.*, 2000; 2001; Raymond *et al.*, 1999; Yang & Lanier, 1999). This is unlikely to have influenced the results obtained from the medium and low expressing cells since receptor levels are within the physiological ranges reported for the mouse β_3-AR. It is more likely that the response observed in the cytosensor microphysiometer may result from as yet unknown multiple effector(s).

In conclusion, this study demonstrates several important findings for the mouse β_3-AR. Firstly, it is not possible to discriminate between the mouse β_3-AR splice variants based on pharmacology, and therefore not possible to study the consequence of the coexpression of the receptors in native tissues due to the lack of differences in pharmacological profiles. The use of recombinant receptor expression systems is therefore a useful technique for examination of the properties of the β_3-AR splice variants. Secondly, the mouse β_3-AR displays greater stereoselectivity for the (-)-isomers of several β-AR ligands than previously described. And thirdly, the β_3-AR antagonist SR59230A exhibits agonistic actions at the cloned mouse β_3-AR.

Chapter 4

4

Signal transduction pathways utilised by the mouse β_{3a}- and β_{3b}-AR expressed in CHO-K1 cells

4.1 Introduction

Although it does not always occur, alternate splicing of transcripts encoding GPCRs has the potential to diversify the number of receptor subtypes beyond those encoded by distinct genes. Many GPCRs have isoforms which differ in the C-terminus region. While most of these splice variants share similar pharmacology, some show marked differences in signaling properties. One example is the mouse prostaglandin EP_3 receptor where there are four splice variants (Namba *et al.*, 1993), termed the EP_{3A}, EP_{3B}, EP_{3C} and EP_{3D} receptor. While the receptor that was first cloned (EP_{3A}) was shown to couple to Gαi/o, the EP_{3B} and EP_{3C} receptors coupled solely to Gαs and the EP_{3D} receptor to Gαi/o, Gαs and Gαp[1] (Namba *et al.*, 1993). Other mouse EP_3 receptor splice variants (with differing C-terminal sequences to those reported by Namba *et al.* (1993)) show marked differences in G-protein coupling. While all three splice variants ($EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$) coupled to Gαi/o, the $EP_{3\gamma}$ splice variant was also shown to couple to Gαs (Irie *et al.*, 1993; Negishi *et al.*, 1996). Differences in signaling pathways have also been observed for the rat somatostatin receptor (SSTR) splice variants. The rat SSTR splice variants show only small differences in their ability to couple to AC (Schindler *et al.*, 1998) but they have opposing effects on cellular proliferation (Alderton *et al.*, 1998). Hence differences in the C-terminal tail can play important roles in signalling for receptor splice variants.

Alternate splicing of the mouse β_3-AR generates two isoforms of the receptor, the previously cloned receptor (termed the β_{3a}-AR), and the splice variant, termed the β_{3b}-AR (Evans *et al.*, 1999), which differ only in their C-terminal tail. While both receptors are differentially expressed in mouse tissues (Evans *et al.*, 1999), they have identical pharmacological properties when expressed in CHO-K1 cells (Chapter 3).

All three β-ARs couple to Gαs to increase intracellular cAMP levels which leads to subsequent activation of PKA and other intracellular targets. Recently, β_3-ARs have been shown to couple to Gαi, which limits β_3-AR stimulated AC activity by inhibition of AC, in rat adipocytes (Chaudhry *et al.*, 1994), 3T3-F442A adipocytes (Soeder *et al.*, 1999) and primary mouse brown adipocytes (Lindquist *et al.*, 2000). Coupling of the β_3-AR to Gαi has been suggested to account for β_3-AR mediated

[1] Gαp does not describe any known G-protein but was used by Namba *et al.*, (1993).

activation of the MAPK pathway to increase Erk1/2 phosphorylation (Cao *et al.*, 2000; Gerhardt *et al.*, 1999; Soeder *et al.*, 1999), although this may not be a universal pathway for β_3-AR activation of Erk1/2 since other studies have shown Erk1/2 activation occurring through the classical cAMP/PKA pathway (Lindquist *et al.*, 2000; Mizuno *et al.*, 1999; 2000). Erk1/2 activation by the β_3-AR is complex and differs between different cell systems utilised. Several proteins that may be involved in the signaling pathway cascade include Gαs (Lindquist *et al.*, 2000; Mizuno *et al.*, 2000), Gαi (Cao *et al.*, 2000; Gerhardt *et al.*, 1999; Soeder *et al.*, 1999), PKA (Lindquist *et al.*, 2000; Mizuno *et al.*, 1999), Src (Cao *et al.*, 2000; Lindquist *et al.*, 2000), TKR (Lindquist *et al.*, 2000; Soeder *et al.*, 1999) (including the EGFR (Soeder *et al.*, 1999)) and PI3K (Gerhardt *et al.*, 1999).

The aims of the present study were to examine the signaling pathways of the mouse β_{3a}- and β_{3b}-AR expressed in CHO-K1 cells. Cytosensor microphysiometer and cAMP accumulation studies were performed to examine coupling of the receptors to different signaling pathways (specifically Gαs and Gαi). Additional studies were performed to examine if β_{3a}- or β_{3b}-AR activation increased Erk1/2 phosphorylation and the mechanism of this action.

4.2 Methods

4.2.1 Expression of the mouse β_{3a}- or β_{3b}-AR in CHO-K1 cells

Cloning, transfection and generation of stable single cell populations of cells has been described previously (section 2.3).

4.2.2 Cell culture

Cell culture was performed as previously described (section 2.1).

4.2.3 Cytosensor microphysiometer studies

The cytosensor microphysiometer (Molecular Devices Corp., California, U.S.A.) was used to measure extracellular acidification rates produced in cells following drug administration as detailed in section 2.8.2 and 2.8.3. Cells were seeded into 12mm transwell plates at 5 x 10^5 cells per well overnight in media devoid of FBS prior to use in the cytosensor the following day as detailed in section 2.8.2. Following a 2 h equilibration period, cumulative c-r curves to CL316243 were produced in the absence or presence of PTX pre-treatment (100ng ml^{-1}, 16 h). Control cells were treated with vehicle (0.5mM NaCl, 0.1mM sodium phosphate pH 7.0).

Analysis of cytosensor microphysiometer studies is detailed in section 2.8.4. Responses are expressed as a percentage of the maximal response to CL316243 in vehicle treated cells for each receptor.

4.2.4 cAMP accumulation studies

Cells were plated in 12-well plates at 1 x 10^5 cells per well, and cultured for 2 days as described in section 2.6.1. C-r curves to CL316243 were performed in the presence/absence of PTX pre-treatment (100ng ml^{-1}, 16 h). Control cells were treated with vehicle (0.5mM NaCl, 0.1mM sodium phosphate pH 7.0). Cells were treated with CL316243 for 30 min and concentrations used are indicated with the results. To determine the affect of several other treatments on CL316243-mediated increases of cAMP accumulation levels, cells were pretreated either with a PKA inhibitor (H89, 10μM), PI3K inhibitor (LY294002, 10μM), Src inhibitor (PP2, 10μM), MEK1/2 inhibitor (PD98059, 50 μM), TK inhibitor (genistein, 50 μM), PKC inhibitor (Ro-31-8220, 1 μM), or an endocytosis inhibitor (concanavalin A; 0.25mg ml^{-1}; cytochalasin D, 1 μM; monodansylcadaverine (MDC), 10 μM) for 1 h prior to stimulation with CL316243 (100nM) for 30 min. In other experiments, transfected or untransfected CHO-K1 cells were treated either with forskolin (10μM, 30 min) or cholera toxin (2μg ml^{-1}, 90 min).

Analysis of cAMP accumulation studies is detailed in section 2.6.3. Responses are expressed in pmol of cyclic AMP produced per well.

4.2.5 Erk1/2 phosphorylation studies

Cells were plated in 12-well plates at 1 x 10^5 cells per well, and cultured for 2 days as previously described (section 2.7). Cells were treated either with PTX (100ng ml^{-1} 16 h), H89 (10μM), LY294002 (10μM), PD98059 (50 μM), PP2 (10μM), genistein (50 μM), Ro-31-8220 (1 μM), concanavalin A (0.25mg ml^{-1}), cytochalasin D (1 μM) or MDC (10 μM) for 1 h (apart from PTX treatment) prior to stimulation with CL316243 (10μM, 5 min). In other experiments, transfected or untransfected CHO-K1 cells were treated either with forskolin (10μM, 5 min) or cholera toxin (2μg ml^{-1}, 90 min).

Analysis of data is detailed as previously described (section 2.7.3).

4.2.6 Analysis

All results are expressed as mean ± s.e.mean of *n* experiments (cAMP accumulation and Western blotting experiments were performed in duplicate). Students t-test or 2-way ANOVA statistical analysis using GraphPad PRISM were utilised and p values less than or equal to 0.05 were considered significant.

4.2.7 Drugs and reagents

Drugs and reagents used and preparation of stock solutions is described in section 2.12. All drugs were diluted either in modified RPMI-1640 media (cytosensor microphysiometer studies) or DMEM:Hams F-12 media (cAMP accumulation studies) or water (Erk1/2 studies) prior to use. PTX was resuspended in sterile water to give a concentration of 0.1 mg ml^{-1} in 0.5M NaCl, 0.1M sodium phosphate pH 7.0 as per manufacturers instructions (Life Technologies). Vehicle treated cells for PTX studies were treated with 0.5mM NaCl, 0.1mM sodium phosphate pH 7.0.

4.3 *Results*

4.3.1 *Functional assessment of responses mediated by the β_{3a}- and β_{3b}-AR expressed in CHO-K1 cells using the cytosensor microphysiometer*

CL316243 produced expression related effects on extracellular acidification responses in the cytosensor microphysiometer in cells expressing the β_{3a}- or β_{3b}-AR. Potency values of CL316243 were related to the levels of expression, with pEC_{50} values increasing with increasing expression levels of the β_{3a}- or β_{3b}-AR (Figure 4.1, 4.2, Table 4.1). pEC_{50} values for CL316243 were 10.79 ± 0.07, 9.79 ± 0.05 and 9.09 ± 0.09 for CHO-K1 cells expressing the β_{3a}-AR at high, medium and low expression levels respectively. pEC_{50} values for CL316243 were 10.59 ± 0.05, 9.88 ± 0.11 and 9.12 ± 0.13 for CHO-K1 cells expressing the β_{3b}-AR at high, medium and low expression levels respectively.

PTX (100ng ml^{-1} 16 h) treatment had no effect on extracellular acidification maximal responses to the β_3-AR agonist CL316243 in CHO-K1 cells expressing the β_{3a}-AR at high (vehicle 100%; PTX treated 108 ± 4%), medium (vehicle 100%; PTX treated 111 ± 5%) or low (vehicle 100%; PTX treated 97 ± 8%) expression levels (n=4-6), with no significant changes in pEC_{50} values (2-way ANOVA ns; Figure 4.1; Table 4.1). However, PTX treatment significantly increased extracellular acidification maximal responses in cells expressing the β_{3b}-AR when cells were expressed at either high (vehicle 100%; PTX treated 134 ± 5%), medium (vehicle 100%; PTX treated 139 ± 8%) or low (vehicle 100%; PTX treated 153 ± 11%) expression levels (n=5-6), without changes in pEC_{50} values (***$p<0.001$ 2-way ANOVA; Figure 4.2; Table 4.1).

4.3.2 *Coupling of the β_{3a}- and β_{3b}-AR expressed in CHO-K1 cells to cAMP accumulation*

As seen with the cytosensor microphysiometer, CL316243 produced expression-related effects on cAMP accumulation responses in cells expressing either the β_{3a}- or β_{3b}-AR. The potency of CL316243 (pEC_{50}) and its maximal response produced

Figure 4.1: Lack of effect of PTX (100ng ml^{-1}) treatment on CL316243 extracellular acidification responses measured using the cytosensor microphysiometer in β_{3a}-AR cells expressed at (a) high, (b) medium, and (c) low levels of expression. Values represent mean ± s.e.mean from 4-6 experiments. Data is presented as a percentage of the maximal response to the highest concentration of CL316243 in each experiment in vehicle treated cells.

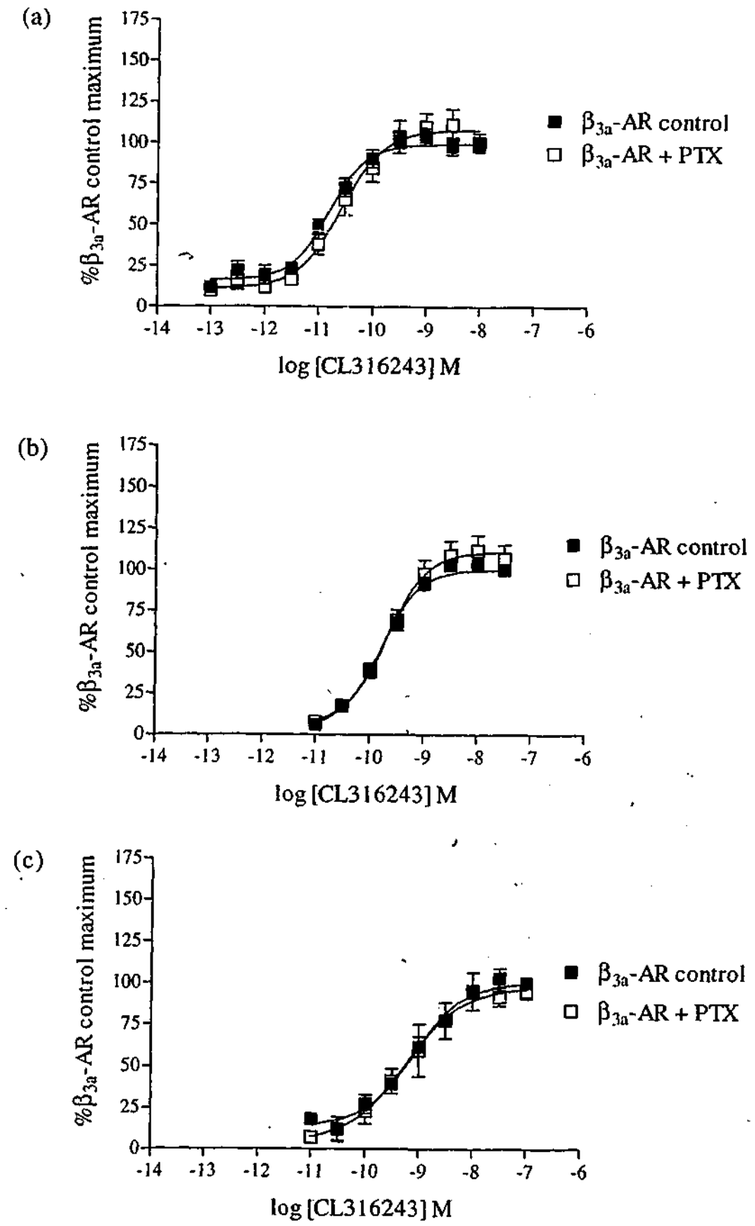


Figure 4.2: PTX (100ng ml^{-1}) treatment increased CL316243 extracellular acidification responses measured using the cytosensor microphysiometer in β_{3b}-AR cells expressed at (a) high, (b) medium, and (c) low levels of expression. Values represent mean ± s.e.mean from 4-6 experiments. Data is presented as a percentage of the maximal response to the highest concentration of CL316243 in each experiment in vehicle treated cells.

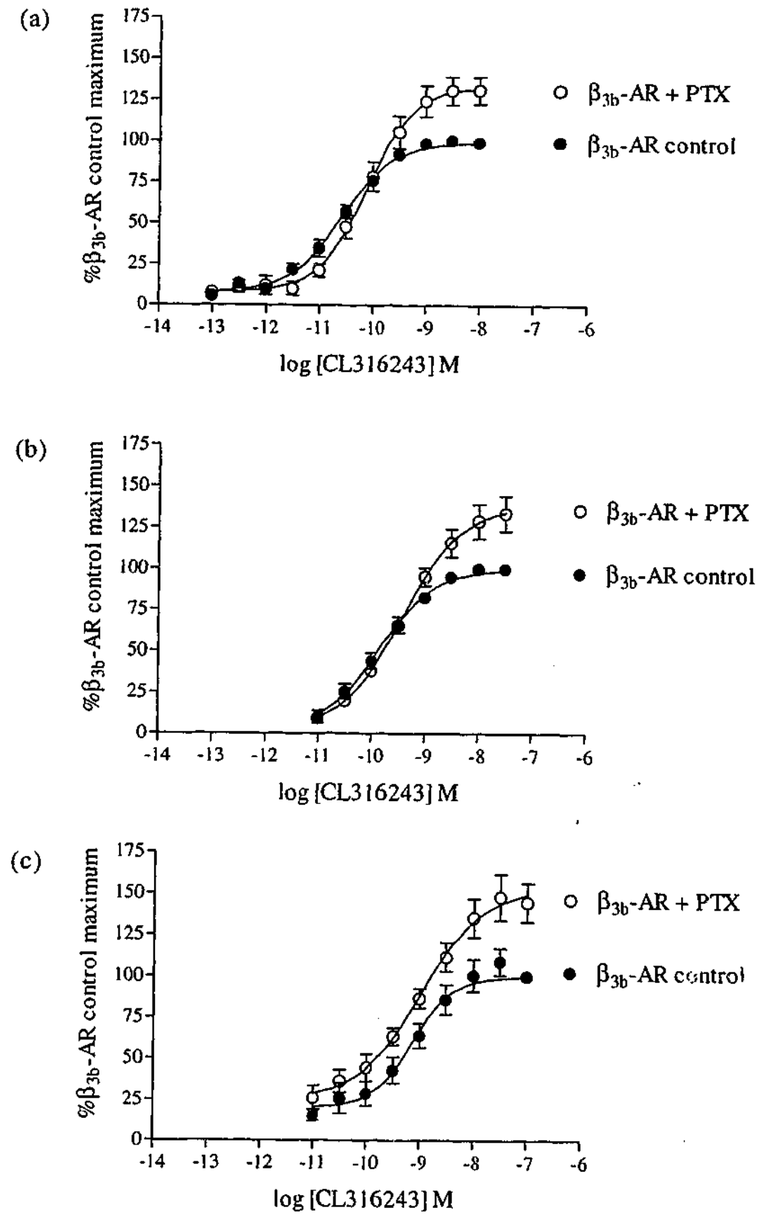


Table 4.1: Effect of PTX (100ng ml^{-1}) on CL316243-mediated increases in extracellular acidification responses as assessed with the cytosensor microphysiometer in cells expressing either the β_{3a}- or β_{3b}-AR at high, medium and low expression levels. Agonist potency values are mean ± s.e.mean for *n* experiments. Maximal responses are expressed as a percentage of the response produced in vehicle treated cells, defined as 100%.

	Control		PTX treated		
	pEC_{50}	Maximum (%)	pEC_{50}	Maximum (%)	*n*
β_{3a}-AR					
high	10.79 ± 0.07	100	10.57 ± 0.10	108 ± 4	*6*
medium	9.79 ± 0.05	100	9.69 ± 0.11	111 ± 5	*6*
low	9.09 ± 0.09	100	9.25 ± 0.20	97 ± 8	*4*
β_{3b}-AR					
high	10.57 ± 0.05	100	10.11 ± 0.09	134 ± 5***	*6*
medium	9.88 ± 0.11	100	9.43 ± 0.13	139 ± 8***	*5*
low	9.12 ± 0.13	100	8.98 ± 0.17	153 ± 11***	*5*

*** indicates $p<0.001$ by 2-way ANOVA of CL316243 concentration-response curves produced in β_{3b}-AR cells in the presence/absence of PTX.

increased with increasing expression of either the β_{3a}- or β_{3b}-AR (Figure 4.3, 4.4, Table 4.2). pEC_{50} values for CL316243 were 9.74 ± 0.15, 9.16 ± 0.27 and 7.57 ± 0.24 for CHO-K1 cells expressing the β_{3a}-AR at high, medium and low expression levels respectively (n=4-5). pEC_{50} values for CL316243 were 9.87 ± 0.10, 8.97 ± 0.39 and 7.76 ± 0.33 for CHO-K1 cells expressing the β_{3b}-AR at high, medium and low expression levels respectively (n=4-5).

PTX (100ng ml^{-1} 16 h) treatment had no effect on maximal cAMP accumulation responses (expressed as pmol of cAMP per well) to the β_3-AR agonist CL316243 in CHO-K1 cells expressing the β_{3a}-AR at high (vehicle 347 ± 27; PTX treated 298 ± 22), medium (vehicle 248 ± 15; PTX treated 249 ± 11) or low (vehicle 45 ± 3; PTX treated 48 ± 6) expression levels (n=4-5), with no significant changes in pEC_{50} values (2-way ANOVA ns; Figure 4.3; Table 4.2). In CHO-K1 cells expressing the β_{3b}-AR, PTX treatment significantly increased cAMP accumulation responses to CL316243 in high (vehicle 247 ± 12; PTX treated 496 ± 72), medium (vehicle 244 ± 27; PTX treated 316 ± 19) or low (vehicle 43 ± 4; PTX treated 61 ± 6) expressing cells (n=4-5), with no significant changes in pEC_{50} values (***$p<0.001$ high and medium expressing cells, *$p<0.05$ low expressing cells; Figure 4.4; Table 4.2).

4.3.3 Coupling of the β_{3a}- and β_{3b}-AR to Erk1/2 phosphorylation

All experiments examining CL316243-mediated increases in Erk1/2 phosphorylation were conducted in cells expressing high levels of β_{3a}- or β_{3b}-ARs. CL316243 caused phosphorylation of Erk1/2 in a concentration-dependent manner (Figure 4.5) with pEC_{50} values of 7.28 ± 0.26 and 7.36 ± 0.24 in cells expressing the β_{3a}- or β_{3b}-AR respectively (n=3), while having no effect in untransfected CHO-K1 cells (Figure 4.5). PTX (100ng ml^{-1} 16 h) treatment had no significant effect on CL316243 (10μM) mediated increases in Erk1/2 phosphorylation in cells expressing β_{3a}- or β_{3b}-AR, yet increased cAMP accumulation levels in β_{3b}-AR cells in a parallel experiment (Figure 4.6). In cells expressing β_{3a}- or β_{3b}-AR, the increase in Erk1/2 phosphorylation levels by CL316243 was not affected by treatment with H89 (Figure 4.7) or Ro-31-8220 (Figure 4.8), but was inhibited by LY294002 (Figure 4.9), PP2

Figure 4.3: Lack of effect of PTX (100ng ml^{-1}) treatment on CL316243-mediated increases in cAMP accumulation in β_{3a}-AR cells expressed at (a) high, (b) medium, and (c) low levels of expression. Values represent mean ± s.e.mean from 4-5 experiments performed in duplicate. Data is presented as the amount of cAMP accumulated (pmol/well) following CL316243 treatment. Note the difference in y-axis scale.

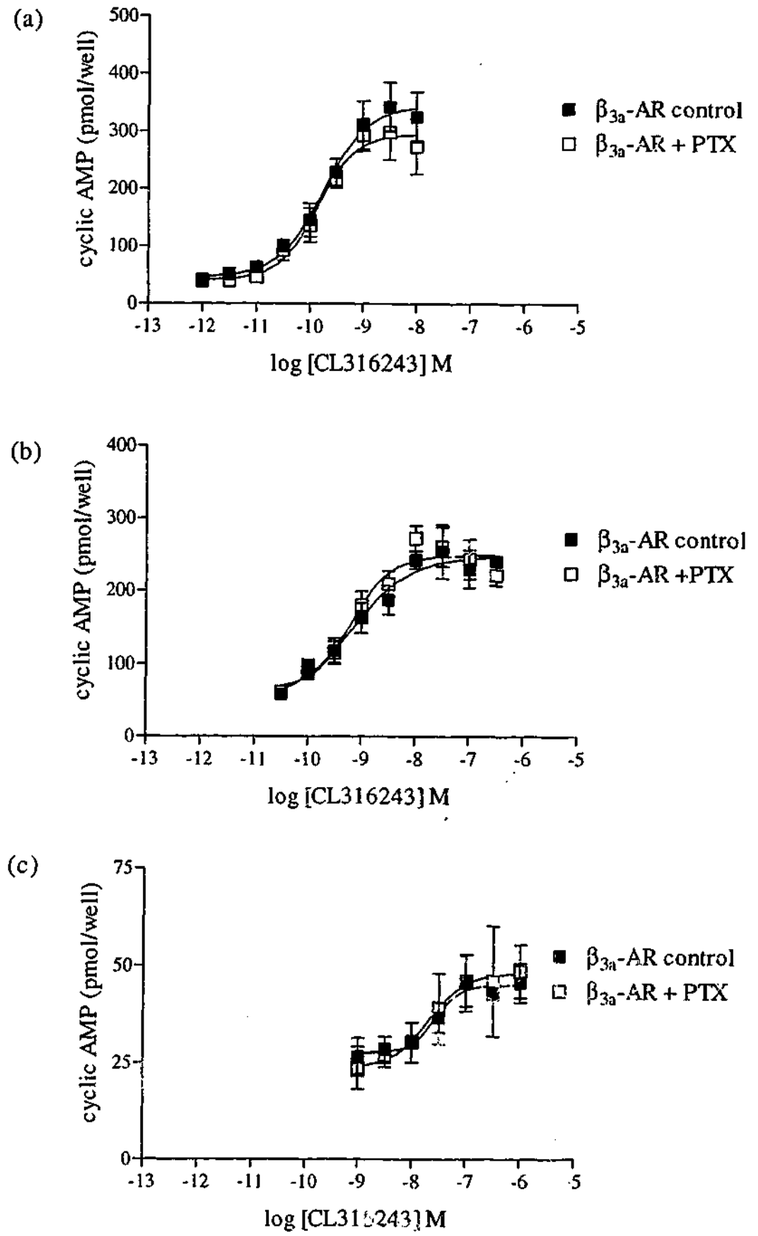


Figure 4.4: PTX (100ng ml^{-1}) treatment increased CL316243-mediated increases in cAMP accumulation in β_{3b}-AR cells expressed at (a) high, (b) medium, and (c) low levels of expression. Values represent mean ± s.e.mean from 4-5 experiments performed in duplicate. Data is presented as the amount of cAMP accumulated (pmol/well) following CL316243 treatment. Note the difference in y-axis scale.

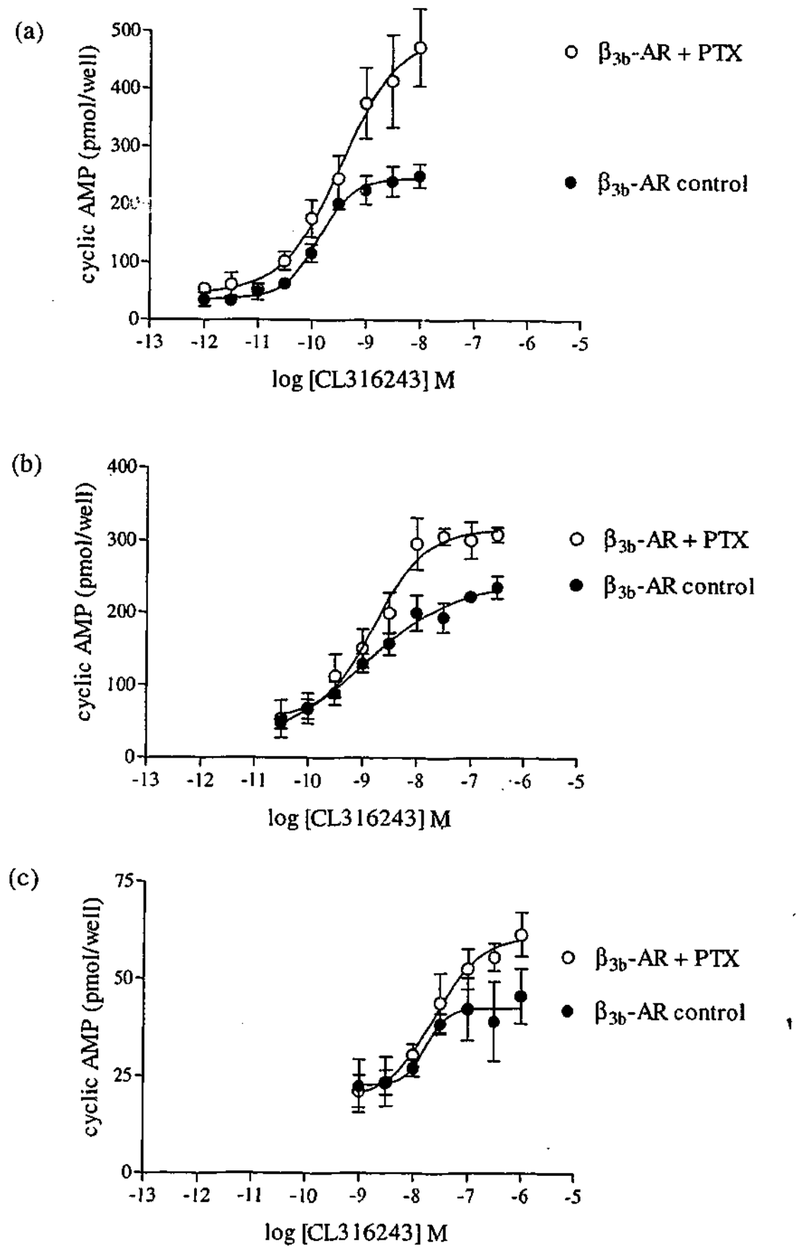


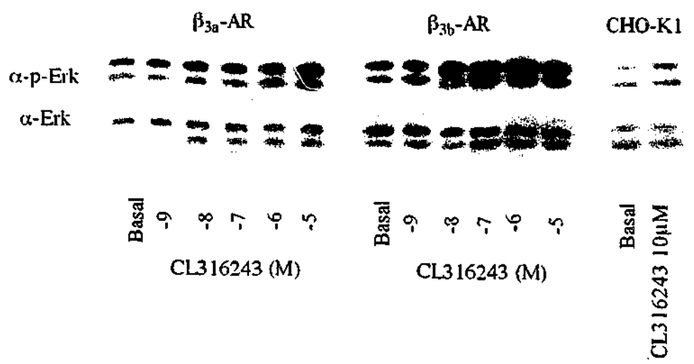
Table 4.2: Effect of PTX (100ng ml^{-1}) on CL316243-mediated increases in cAMP accumulation in cells expressing either the β_{3a}- or β_{3b}-AR at high, medium and low expression levels. Agonist potency values are mean ± s.e.mean for *n* experiments performed in duplicate. Maximal responses are expressed as pmol cAMP produced per well.

	Control		PTX treated		
	pEC_{50}	Maximum (pmol/well)	pEC_{50}	Maximum (pmol/well)	*n*
β_{3a}-AR					
high	9.74 ± 0.15	347 ± 27	9.85 ± 0.15	298 ± 22	*5*
medium	9.16 ± 0.27	248 ± 15	9.17 ± 0.17	249 ± 11	*4*
low	7.57 ± 0.24	45 ± 3	7.68 ± 0.470	48 ± 6	*4*
β_{3b}-AR					
high	9.87 ± 0.10	247 ± 12	9.46 ± 0.25	496 ± 72***	*5*
medium	8.97 ± 0.39	244 ± 27	8.76 ± 0.18	316 ± 19***	*4*
low	7.76 ± 0.33	43 ± 4	7.61 ± 0.33	61 ± 6*	*4*

*** indicates $p<0.001$, * indicates $p<0.05$ determined by 2-way ANOVA of CL316243 concentration-response curves produced in β_{3b}-AR cells in the presence/absence of PTX.

Figure 4.5: C-r curve for CL316243-induced Erk1/2 phosphorylation. (a) shows immunoblot of CL316243-mediated increases in Erk1/2 phosphorylation following 5 min stimulation of cells expressing either the β_{3a}- or β_{3b}-AR with the indicated concentration of CL316243. (b) shows c-r curve for CL316243-mediated increase of Erk1/2 phosphorylation. Values are expressed as mean ± s.e.mean of the ratio between phosphorylated and total Erk1/2 levels and are expressed as a percentage of the ratio to 10μM CL316243. Experiments were performed in duplicate (n=3).

(a)



(b)

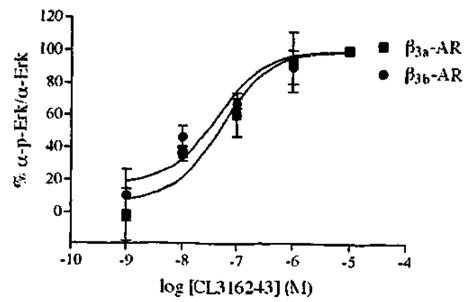


Figure 4.6: Effect of PTX on induced cAMP levels and Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of PTX treatment (100ng ml^{-1}, 16h) in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing the lack of effect of PTX on CL316243-mediated increases in Erk1/2 phosphorylation. Values are mean s.e.mean of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100%. (c) PTX had no effect on CL316243-mediated increases in cAMP accumulation in cells expressing the β_{3a}-AR but significantly increased ($p<0.05$) levels in response to CL316243 in cells expressing the β_{3b}-AR. Data is presented as the amount of cAMP accumulated (pmol/well).

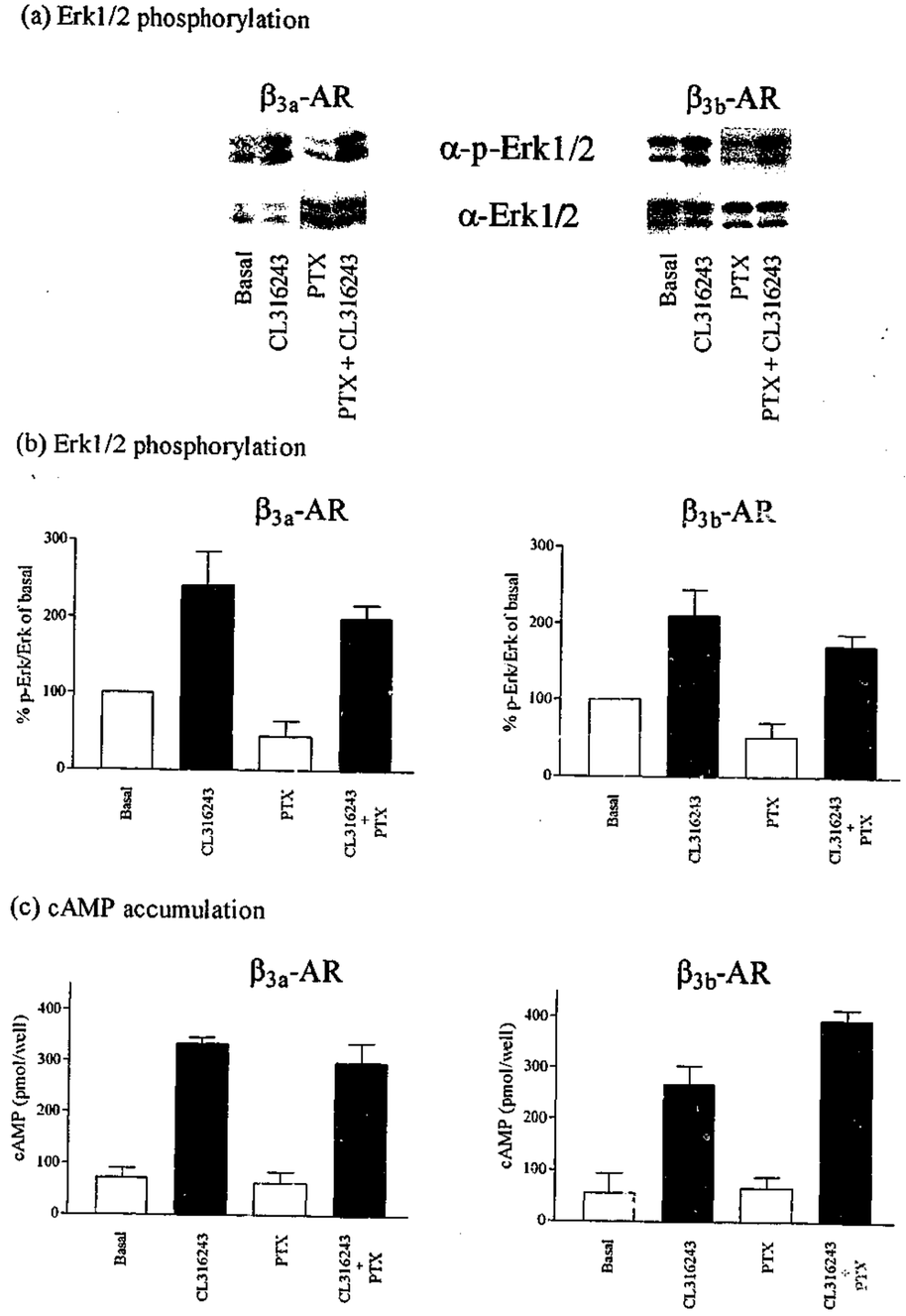
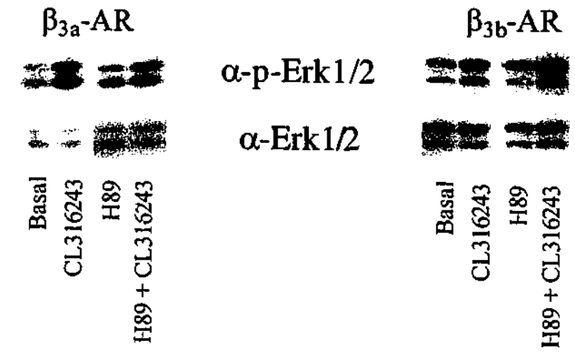
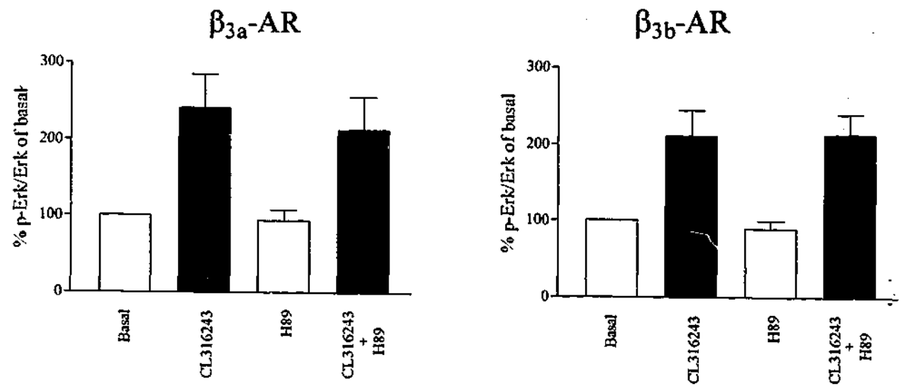


Figure 4.7: Effect of H89 on induced cAMP levels and Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of H89 treatment (10μM, 1h) in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing the lack of effect of H89 on CL316243-mediated increases in Erk1/2 phosphorylation. Values are mean s.e.mean of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100%. (c) H89 had no effect on CL316243-mediated increases in cAMP accumulation in cells expressing either the β_{3a}- or β_{3b}-AR. Data is presented as the amount of cAMP accumulated (pmol/well).

(a) Erk1/2 phosphorylation



(b) Erk1/2 phosphorylation



(c) cAMP accumulation

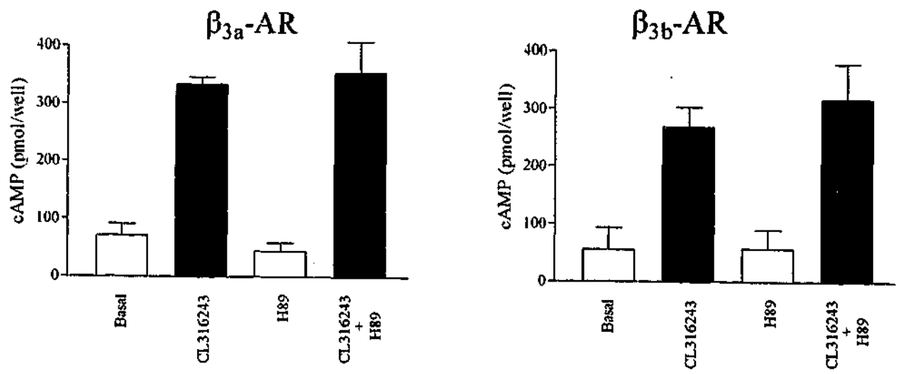


Figure 4.8: Effect of Ro-31-8220 on induced Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of Ro-31-8220 treatment (1μM, 1h) in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing the lack of effect of Ro-31-8220 on CL316243-mediated increases in Erk1/2 phosphorylation. Values are mean s.e.mean of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100%.

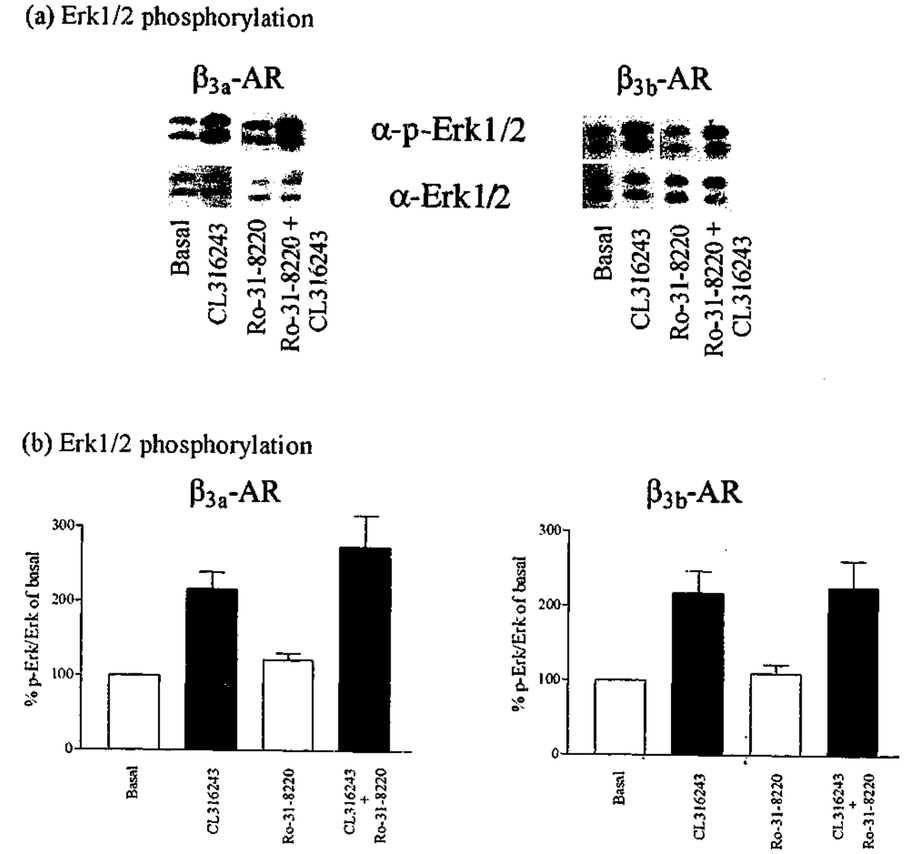
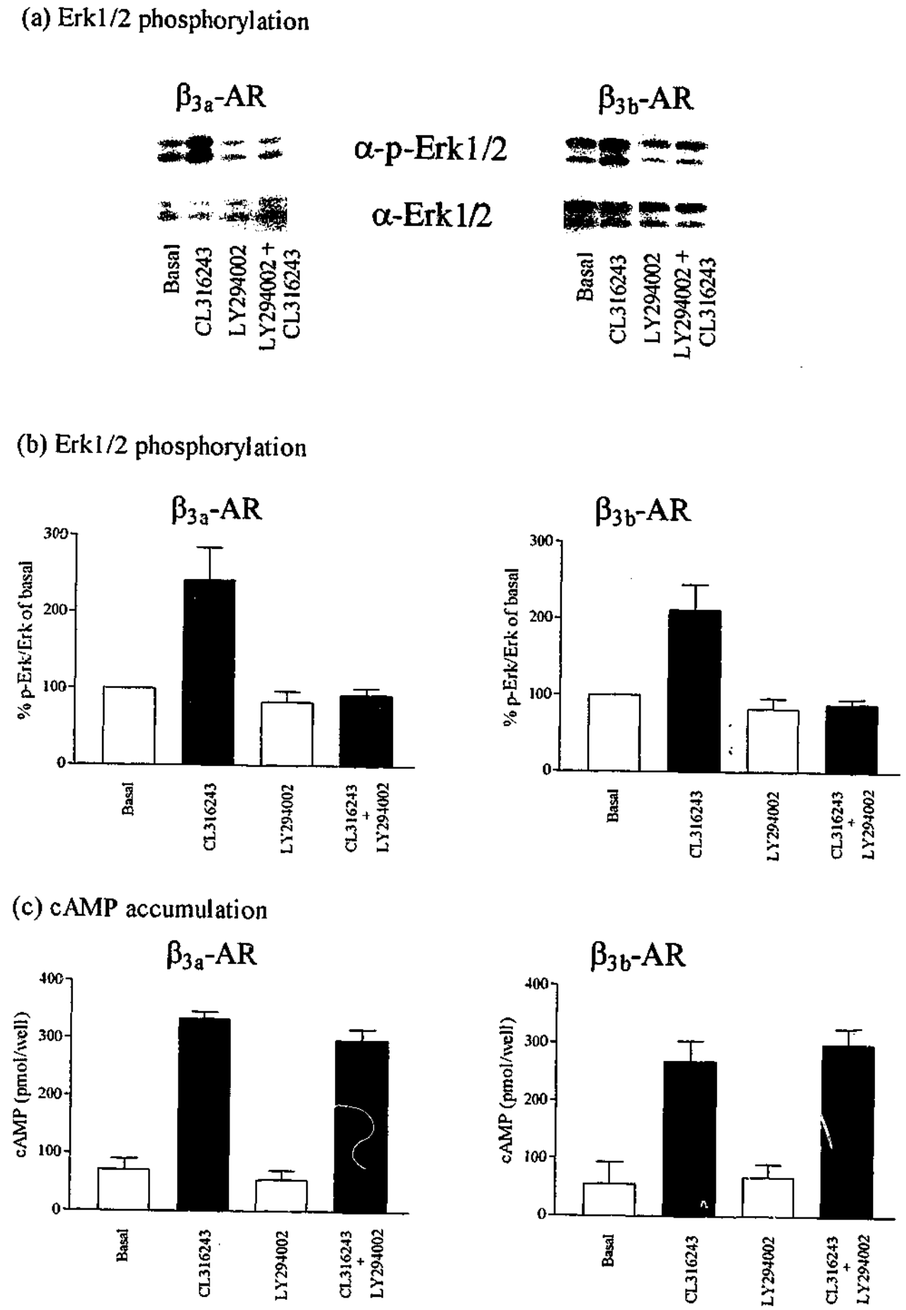


Figure 4.9: Effect of LY294002 on induced cAMP levels and Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of LY294002 treatment (10μM, 1h) in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing LY294002 pretreatment significantly decreased CL316243-mediated increases in Erk1/2 phosphorylation ($p<0.05$). Values are mean s.e.mear of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100%. (c) LY294002 had no effect on CL316243-mediated increases in cAMP accumulation in cells expressing either the β_{3a}- or β_{3b}-AR. Data is presented as the amount of cAMP accumulated (pmol/well).



(Figure 4.10), genistein (Figure 4.11) and PD98059 (Figure 4.12), while having no significant effect on CL316243-mediated increases in cAMP accumulation levels (Figure 4.7-4.12). It should be noted that treatment of cells expressing either the β_{3a}- or β_{3b}-AR, with either PD98059 or PP2 reduced the basal rate of Erk1/2 phosphorylation. Relatively few papers mention the effect of various inhibitors on basal Erk1/2 activity, but PP2 and PD98059 have previously been shown to reduce basal Erk1/2 levels in primary brown adipocytes (Fredriksson *et al.*, 2000). This suggests that Src and MEK1/2 may be involved in basal activation of Erk1/2 in our system under the conditions used here. Preliminary experiments showed that DMSO had no effect on Erk1/2 levels (data not shown), since several inhibitors used were dissolved in DMSO.

In experiments designed to examine whether the changes in Erk1/2 phosphorylation were associated with increases in cAMP, untransfected CHO-K1 cells or β_{3a}- or β_{3b}-AR cells were treated with forskolin or cholera toxin. Forskolin or cholera toxin had no effect on Erk1/2 phosphorylation levels, although they markedly increased cAMP accumulation in these cells (Figure 4.13).

Several reports have suggested that internalisation is critical for β-AR activation of Erk1/2. Treatment of cells with either MDC or cytochalasin A had no effect on the ability of CL316243 to cause increases in Erk1/2 phosphorylation or cAMP accumulation (Figure 4.14), although concanavalin D treatment inhibited CL316243-mediated increases in Erk1/2 phosphorylation without affecting cAMP accumulation responses.

Table 4.3 gives values of Erk1/2 phosphorylation levels (relative to that of basal, defined as 100%) and cAMP accumulation (pmol per well) in response to CL316243 stimulation in the presence and absence of various inhibitors.

Figure 4.10: Effect of PP2 on induced cAMP levels and Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of PP2 treatment (10μM, 1h) in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing PP2 significantly decreased ($p<0.05$) CL316243-mediated increases in Erk1/2 phosphorylation. Values are mean ± s.e.mean of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100% (note that PP2 treatment itself significantly decreased Erk1/2 phosphorylation levels). (c) PP2 had no effect on CL316243-mediated increases in cAMP accumulation in cells expressing either the β_{3a}- or β_{3b}-AR. Data is presented as the amount of cAMP accumulated (pmol/well).

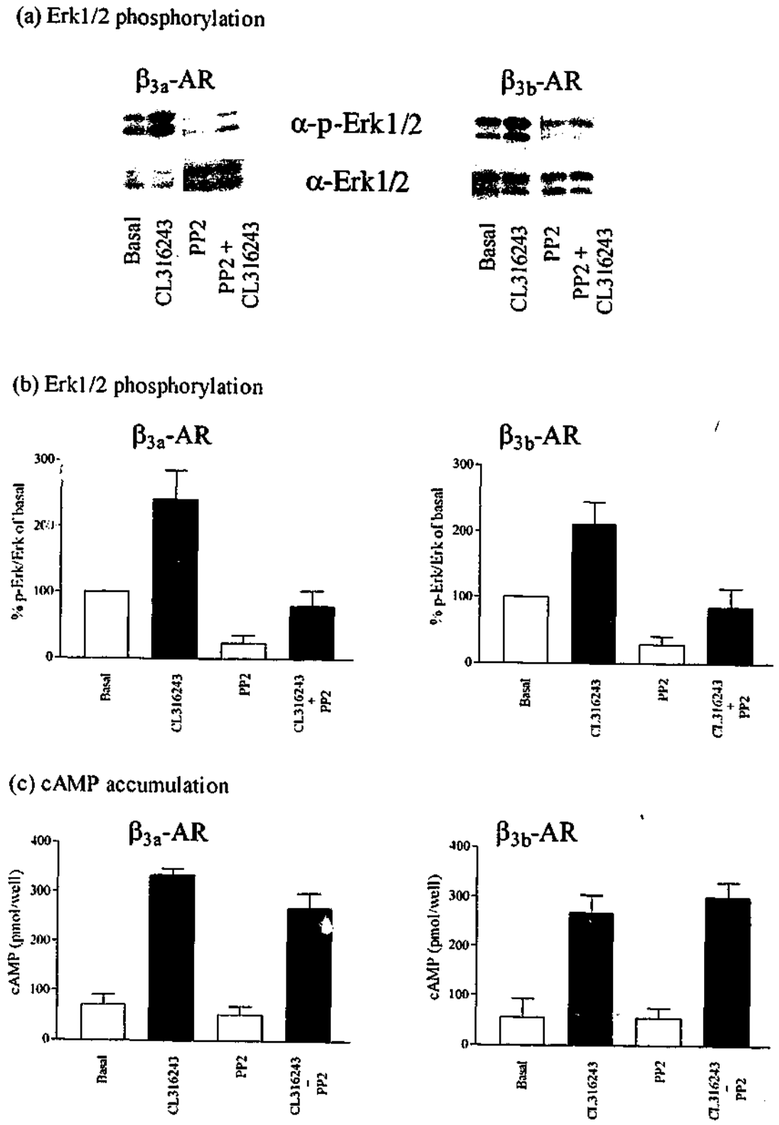


Figure 4.11: Effect of genistein on induced cAMP levels and Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of genistein treatment (50μM, 1h) in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing genistein significantly decreased ($p<0.05$) CL316243-mediated increases in Erk1/2 phosphorylation. Values are mean s.e.mean of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100%. (c) Genistein had no effect on CL316243-mediated increases in cAMP accumulation in cells expressing either the β_{3a}- or β_{3b}-AR. Data is presented as the amount of cAMP accumulated (pmol/well).

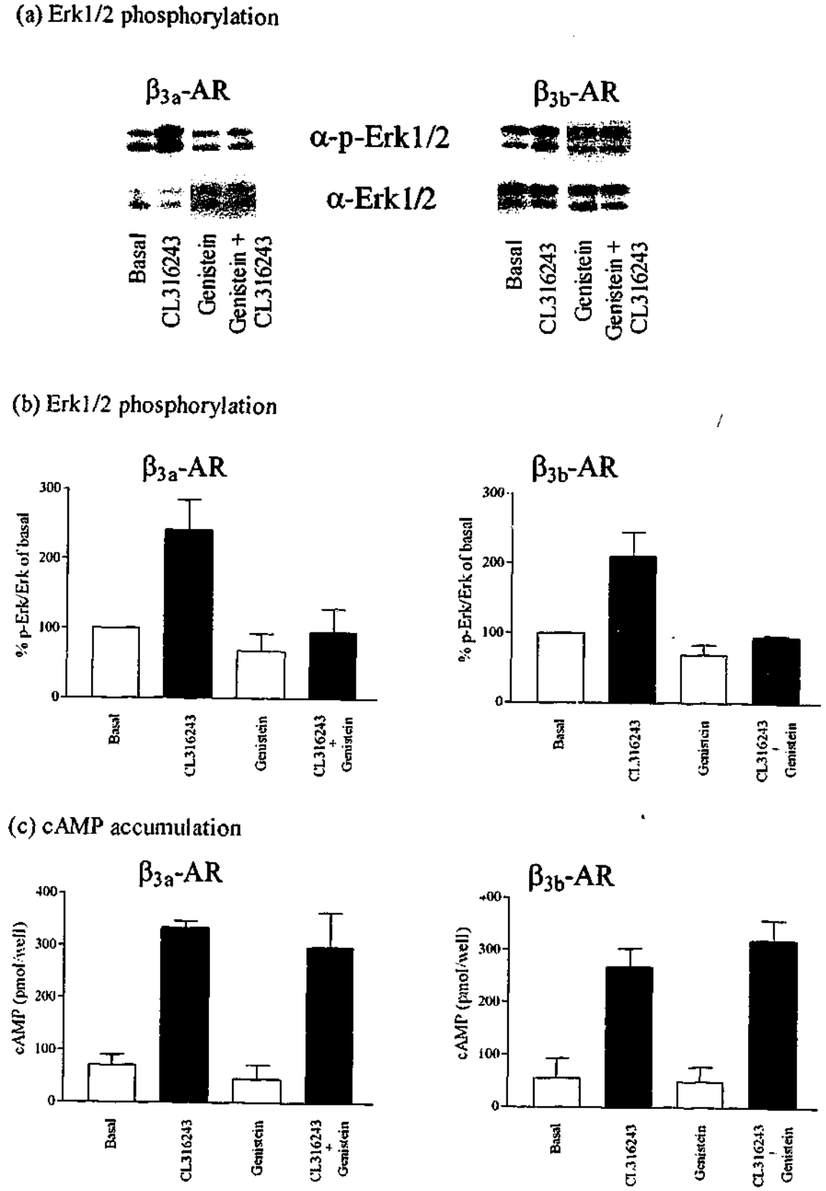
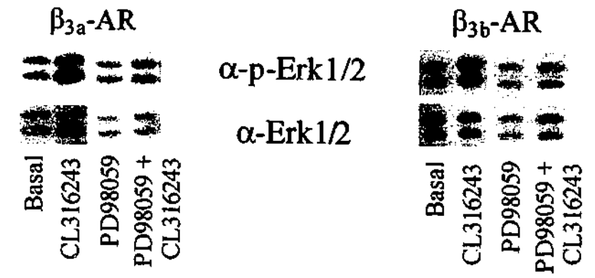


Figure 4.12: Effect of PD98059 on Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of PD98059 treatment (50μM, 1h) in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing PD98059 significantly decreased ($p<0.005$) CL316243-mediated increases in Erk1/2 phosphorylation. Values are mean s.e.mean of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100% (note that PD98059 treatment itself significantly decreased Erk1/2 phosphorylation levels).

(a) Erk1/2 phosphorylation



(b) Erk1/2 phosphorylation

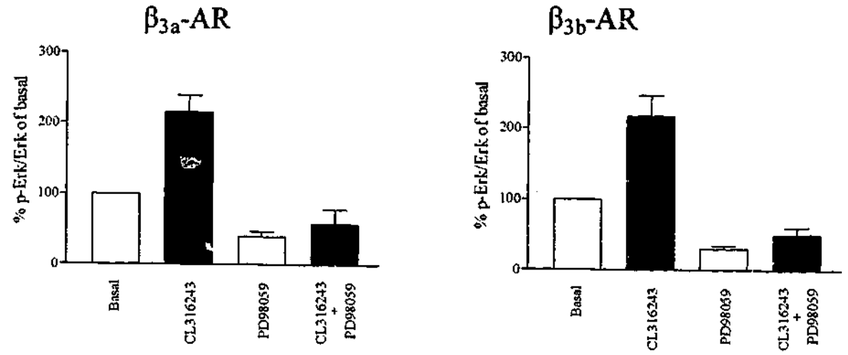
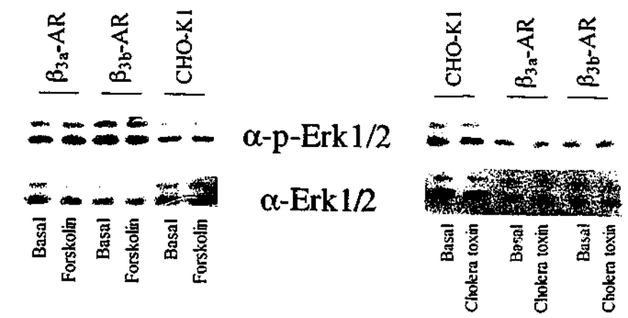


Figure 4.13: Effect of forskolin and cholera toxin on induced cAMP levels and Erk1/2 phosphorylation. (a) shows immunoblot of lack of effect of forskolin (10μM, 5min) or cholera toxin (2μg ml^{-1}, 90min) on Erk1/2 phosphorylation levels in untransfected CHO-K1 cells or cells expressing either the β_{3a}- or β_{3b}-AR, although these treatments were effective in increasing cAMP accumulation in these cells (b) Data is presented as the amount of cAMP accumulated (pmol/well).

(a) Erk1/2 phosphorylation



(b) cAMP accumulation

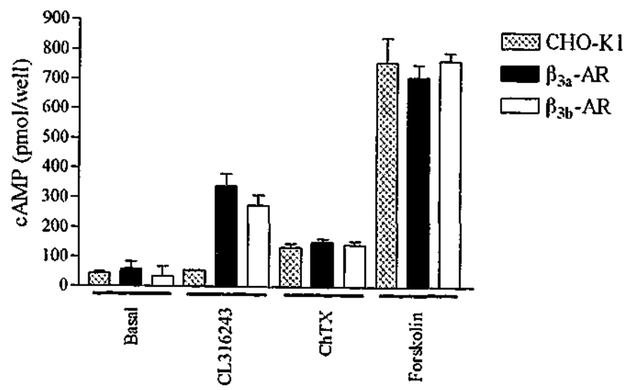


Figure 4.14: Effect of MDC, concanavalin A and cytochalasin D on induced cAMP levels and Erk1/2 phosphorylation. (a) shows immunoblot of CL316243 (5min, 10μM) mediated increases in Erk1/2 phosphorylation in the presence or absence of MDC (10μM, 1h), concanavalin A (0.25mg ml^{-1}, 1h) or cytochalasin D (1μM, 1h) treatment in cells expressing either the β_{3a}- or β_{3b}-AR. (b) Compilation of results from (a) and three other experiments showing concanavalin A significantly decreased ($p<0.05$) CL316243-mediated increases in Erk1/2 phosphorylation. MDC or cytochalasin D treatment had no effect on CL316243-mediated increases of Erk1/2 phosphorylation. Values are mean s.e.mean of 4 experiments performed in duplicate. In each experiment, the basal level was defined as 100%. (c) All treatments had no effect on CL316243-mediated increases in cAMP accumulation in cells expressing either the β_{3a}- or β_{3b}-AR. Data is presented as the amount of cAMP accumulated (pmol/well).

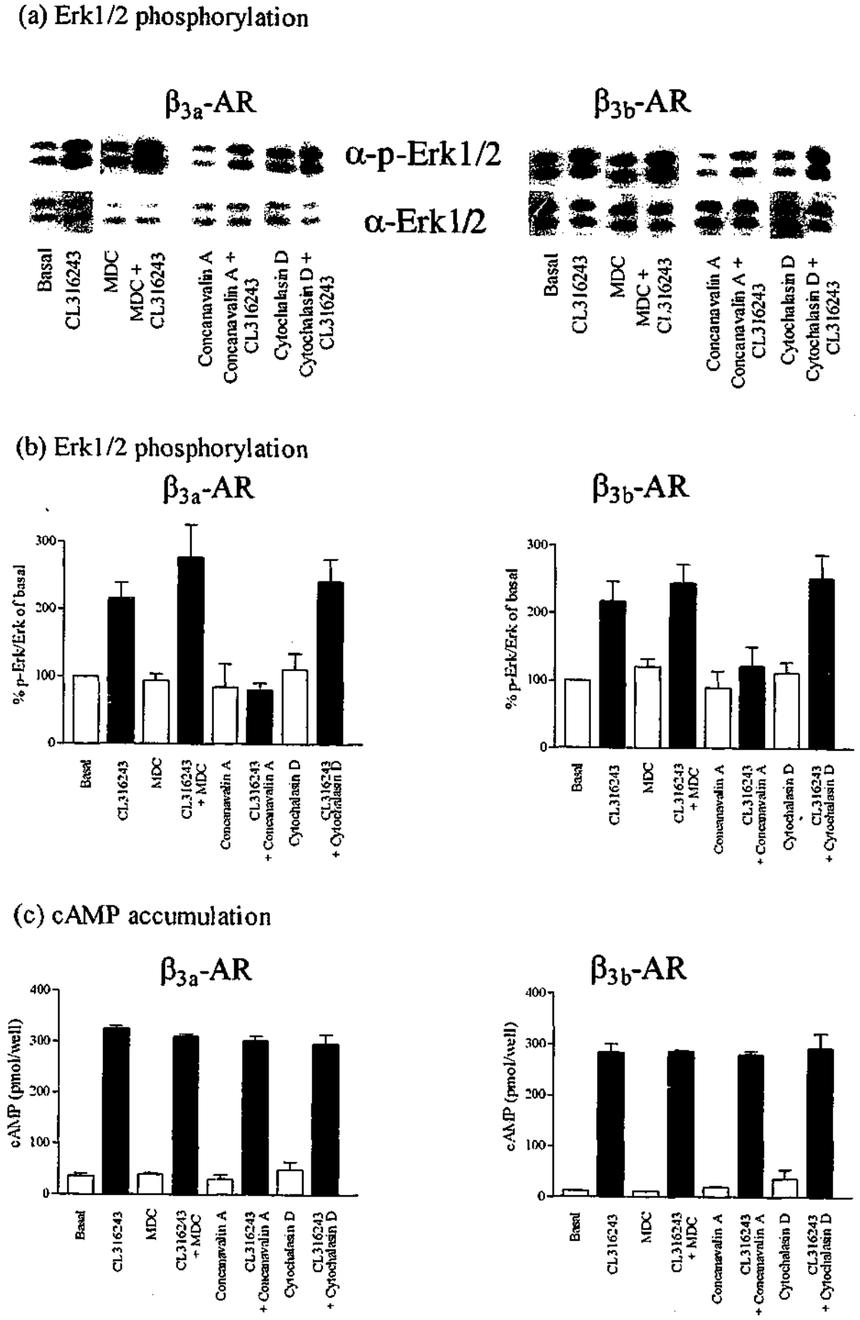


Table 4.3: Effect of various signaling inhibitors on Erk1/2 phosphorylation and cAMP accumulation levels in cells expressing either the β_{3a}- or β_{3b}-AR. Erk1/2 phosphorylation levels are expressed as mean s.e.mean of *n* experiments performed in duplicate. In each experiment, the basal level was defined as 100%. cAMP accumulation levels are expressed as mean s.e.mean of amount of cAMP accumulated (pmol per well).

	Erk1/2 phosphorylation			cAMP accumulation (pmol/well)		
	β_{3a}-AR	β_{3b}-AR	*n*	β_{3a}-AR	β_{3b}-AR	*n*
Basal	100	100	*4*	71.2 ± 19.6	54.7 ± 38.1	*3*
+ CL316243	241.2 ± 43.4	210.5 ± 34.2	*4*	333.4 ± 12.4	267.4 ± 35.7	*3*
+ PTX	43.8 ± 20.3	52.0 ± 19.5	*4*	62.0 ± 21.5	65.7 ± 22.7	*3*
+ PTX, CL316243	199.4 ± 17.4	170.9 ± 16.1	*4*	297.5 ± 39.1	394.1 ± 20.7$^{\#}$	*3*
+ H89	93.2 ± 14.1	89.4 ± 10.4	*4*	43.8 ± 15.4	56.9 ± 33.4	*3*
+ H89, CL316243	212.5 ± 43.4	212.7 ± 27.1	*4*	354.3 ± 54.2	316.4 ± 61.9	*3*
+ LY294002	82.6 ± 14.3	82.1 ± 14.2	*4*	54.8 ± 15.6	67.3 ± 23.0	*3*
+ LY294002, CL316243	92.6 ± 14.3*	88.7 ± 6.6*	*4*	298.0 ± 19.0	298.6 ± 27.8	*3*
+ PP2	23.2 ± 12.0	28.8 ± 12.9	*4*	51.8 ± 15.9	53.7 ± 20.9	*3*
+ PP2, CL316243	79.4 ± 23.9*	85.1 ± 29.4*	*4*	268.8 ± 29.5	300.3 ± 29.0	*3*
+ Genistein	67.8 ± 25.2	69.4 ± 14.0	*4*	45.2 ± 26.9	49.2 ± 28.7	*3*
+ Genistein, CL316243	95.7 ± 33.0*	94.6 ± 2.2*	*4*	297.6 ± 66.2	319.4 ± 38.4	*3*
Basal	100	100	*4*	35.7 ± 5.5	11.7 ± 0.6	*3*
+ CL316243	216.0 ± 23.1	217.1 ± 29.5	*4*	325.5 ± 4.8	283.0 ± 17.2	*3*
+ Ro-31-8220	121.5 ± 9.3	109.9 ± 11.7	*4*			
+ R0-31-8220, CL316243	273.7 ± 43.2	224.3 ± 36.8	*4*			
+ PD98059	39.3 ± 7.3	30.5 ± 3.9	*4*			
+ PD98059, CL316243	58.3 ± 20.7**	50.2 ± 10.6**	*4*			
+ MDC	94.1 ± 9.4	119.9 ± 12.1	*4*	38.9 ± 3.5	10.1 ± 1.0	*3*
+ MDC, CL316243	277.4 ± 47.4	243.9 ± 28.2	*4*	310.8 ± 4.3	285.5 ± 2.4	*3*
+ Concanavalin A	84.7 ± 34.3	89.9 ± 24.2	*4*	30.4 ± 8.5	18.5 ± 2.6	*3*
+ Concanavalin A, CL316243	80.1 ± 10.5**	121.0 ± 19.2*	*4*	301.8 ± 10.3	280.6 ± 6.2	*3*
+ Cytochalasin D	110.5 ± 23.5	111.7 ± 15.4	*4*	49.6 ± 15.7	35.6 ± 17.9	*3*
+ Cytochalasin D, CL316243	241.7 ± 33.4	252.1 ± 34.8	*4*	296.9 ± 18.1	293.0 ± 28.2	*3*

* indicates $p<0.05$, ** indicates $p<0.005$ determined by Student's t-test of CL316243-mediated increases of Erk1/2 phosphorylation levels in the presence or absence of the indicated inhibitor

$^{\#}$ indicates $p<0.05$ by Student's t-test of CL316243 stimulated increases in cAMP accumulation in the presence or absence of PTX at the β_{3b}-AR

4.4 Discussion

As mentioned in Chapter 3, in the cytosensor microphysiometer all β-AR agonists caused a larger maximal response in cells expressing the β_{3a}-AR compared to cells expressing the β_{3b}-AR at high expression levels, although the pEC_{50} values were similar at each receptor for every agonist investigated. Similar results were obtained for CL316243 in cAMP accumulation studies.

The rank order of potency for signalling pathways for the β_{3a}- or β_{3b}-AR was similar: extracellular acidification > cAMP accumulation >> Erk1/2 phosphorylation. The difference between the potency values for responses measured with the cytosensor microphysiometer and cAMP assays has been discussed previously (Chapter 3). Briefly, this may due to increased sensitivity of responses measured in the cytosensor microphysiometer as compared to cAMP assays, or that since responses measured with the cytosensor microphysiometer are a measure of the whole cell, the β_3-AR may couple to other intracellular signalling pathways not reflected in Gαs activity. The discrepancy in pEC_{50} values for Erk1/2 phosphorylation cannot be as easily dismissed as the c-r curve for CL316243 expressing either receptor is shifted over 100-fold to the right compared to cAMP accumulation responses. This indicates that activation of Gαs/AC/cAMP occurs at low concentrations of agonist, but that Erk1/2 phosphorylation becomes significant at high levels of receptor occupancy. Since Erk1/2 activation is not dependent upon cAMP, it may be feasible that the abundance of the signalling components responsible for Erk1/2 phosphorylation is less than that for cAMP, giving rise to such a difference.

β_3-ARs couple to Gαs to activate AC, increasing intracellular cAMP levels and activating PKA, which then presumably activates β_3-AR mediated functions such as lipolysis and thermogenesis. Only one study to date has directly demonstrated that β_3-AR activation results in PKA activation (Fredriksson *et al.*, 2001) since other studies have been based on the ability of cAMP activators to mimic β_3-AR responses, therefore implying that β_3-AR responses are mediated by PKA. Substrates for PKA are diverse and affect multiple cellular functions, including gene transcription, regulation of ion channels, cell growth and death. Substrates for PKA

(due to β_3-AR activation) can be found in the cytoplasm (e.g. hormone sensitive lipase (Anthonsen *et al.*, 1998; Nedergaard & Lindberg, 1982) and p38 MAPK (Cao *et al.*, 2001)) or in the nucleus (e.g. effects on uncoupling protein 1 (Fredriksson *et al.*, 2001), vascular endothelial growth factor (Fredriksson *et al.*, 2000) and CREB (Chaudhry & Granneman, 1999; Thonberg, personal communication) gene transcription).

Several reports show that as well as coupling of the β_3-AR to Gαs, coupling to PTX-sensitive Gαi can occur. In rat adipocytes Gαi limits β_3-AR but not β_1-AR stimulation of AC activity (Chaudhry *et al.*, 1994). In 3T3-F442A adipocytes that endogenously express β_3-ARs, PTX treatment enhanced cAMP accumulation in response to CL316243 (Soeder *et al.*, 1999). In brown adipocytes in primary culture, BRL37344-stimulated cAMP accumulation is enhanced in the presence of PTX (Lindquist *et al.*, 2000). The β_3-AR agonist CL316243 increased intracellular cAMP levels in a concentration-dependent manner in both β_{3a}- or β_{3b}-AR cells, supporting coupling of both receptors to Gαs. However, in the presence of PTX, CL316243-mediated cAMP accumulation or cytosensor microphysiometer responses were increased in β_{3b}-AR cells but not in β_{3a}-AR cells. Thus both receptors couple to Gαs, but only the β_{3b}-AR couples to Gαi. Although promiscuous receptor-second messenger coupling may occur at high receptor expression levels (Cordeaux *et al.*, 2000; Eason *et al.*, 1992; Kenakin, 1995a; 1995b), it is unlikely to have influenced the results obtained from medium (~500 fmol mg^{-1} protein) and low (~100 fmol mg^{-1} protein) expressing cells used here since these receptor levels are similar to physiological levels reported in rat white adipocytes (~400-600 fmol mg^{-1} protein; Germack *et al.*, 1996), rat brown adipocytes (~430 fmol mg^{-1} protein; Sillence *et al.*, 1993), and mouse ileum (~60 and ~150 fmol mg^{-1} protein; Hutchinson *et al.*, 2000 and chapter 5 respectively).

The explanation for the difference in Gαi coupling between the β_{3a}-AR in CHO-K1 cells and the endogenous β_3-AR in 3T3-F442A adipocytes (Soeder *et al.*, 1999) and brown adipocytes (Lindquist *et al.*, 2000) is unclear. Factors that need to be investigated include the influence of receptor density and cell type, and the extent to which responses to agonist treatment in 3T3-F442A adipocytes, or primary mouse

brown adipocytes are mediated by endogenous β_{3b}-ARs. Several studies have illustrated that coupling of a receptor to multiple effector pathways is dependent on the different properties (i.e. cell signaling proteins) of the cell in which the receptor is expressed (Law *et al.*, 1997; Logsdon, 1999; Perez *et al.*, 1993; Zambon *et al.*, 2000), thus results reported in one cell model can not automatically be applied to other cell models. Soeder *et al.* (1999) reported that PTX pretreatment of CHO-K1 cells expressing the mouse β_3-AR also increased CL316243-mediated cAMP accumulation. However no data was presented. They implied that the cells used in the study were the stably transfected line "CHO-Moβ_3" (with 200,000 receptors per cell, or approximately 600 fmol mg protein^{-1}) created by Nahmias *et al.* (1991). The β_3-AR construct reported in this paper contained 15bp of the 5' untranslated region, 1164bp of coding region, and 186 bp of 3' untranslated region. However the coding region was derived from exon 1 of the β_3-AR gene, since the work pre-dated the discovery of an intron interrupting the β_3-AR coding region in human, rat and mouse genes (Bensaid *et al.*, 1993; Granneman *et al.*, 1992; Van Spronsen *et al.*, 1993). The C-terminal tails of the receptors studied are:

β_3-AR (Nahmias *et al.*, 1991) SYGGRGPEEPRAVTFPASPVEARQSPPLNR

β_{3a}-AR (Evans *et al.*, 1999) SYGGRGPEEPRAVTFPASPVEARQSPPLNRFDGYEGARPFPT

β_{3b}-AR (Evans *et al.*, 1999) SYGGRGPEEPRAVTFPASPVEARQSPPLNSSLLREPRHLYTCLGYP

It is possible that the truncated "CHO-Moβ_3" couples to Gαs and Gαi like the β_{3b}-AR but that the β_{3a}-AR couples only to Gαs. It is certain that the data reported by Soeder *et al.* (1999) is not comparable to this study due to differences in receptor sequence.

Treatment of CHO-K1 cells expressing either the β_{3a}- or β_{3b}-AR with CL316243 increases Erk1/2 phosphorylation by 2-fold in a concentration-dependent manner, as reported in other studies using CL316243 (Cao *et al.*, 2000; Gerhardt *et al.*, 1999; Soeder *et al.*, 1999). The absence of CL316243-mediated increases of Erk1/2 phosphorylation in untransfected CHO-K1 cells confirms that the β_3-AR is necessary for activation of Erk1/2 phosphorylation and that CL316243 produces this effect by activation of the β_3-AR. Erk1/2 activation was inhibited by the MEK1/2 inhibitor

PD98059 (Dudley *et al.*, 1995), illustrating that activation of the MAPK cascade is necessary and that CL316243 does not have direct interactions with Erk1/2. The concentration-response curve for Erk1/2 activation to CL316243 in CHO-K1 cells expressing either receptor is shifted significantly to the right compared to cAMP accumulation (over 100-fold shift, compared to 30-fold shift reported by Soeder *et al.* (1999)). This suggests that activation of Gαs/AC/cAMP (occurring at low concentration of agonist) rather than Erk1/2 phosphorylation (which becomes significant at high levels of receptor occupancy) is the dominant signal transduction pathway following β_3-AR stimulation, although the importance of subsequent intracellular targets will determine the importance and contribution of each pathway.

β_3-ARs have been shown to activate Erk1/2 in several cell models including the mouse adipocyte-like cell lines 3T3-F442A (Soeder *et al.*, 1999) and C3H10T1/2 (Cao *et al.*, 2000), both of which endogenously express the β_3-AR, and in cells transfected with the human (Gerhardt *et al.*, 1999) or mouse (Cao *et al.*, 2000) β_3-AR. In these studies, Erk1/2 activation results from coupling of the β_3-AR to PTX-sensitive Gαi proteins and is independent of β_3-AR activation of AC. Several other studies in primary mouse brown adipocytes (Lindquist *et al.*, 2000) or 3T3-L1 adipocytes (Mizuno *et al.*, 1999; 2000) indicate that this pathway is not universal since β_3-AR activation of Erk1/2 occurs through the classical cAMP/PKA pathway. Hence the mechanism of Erk1/2 activation by β_3-ARs is dependent upon cell type. In most cell systems, Erk1/2 phosphorylation is inhibited by PTX (inhibitor of Gαi/o) or β-ARK CT (which sequesters Gβγ subunits). The involvement of Gαi or Gβγ subunits derived from Gαi in Erk1/2 phosphorylation differs between brown and white adipocytes. In mouse primary brown adipocytes, a Gαs/PKA-dependent rap-1/B-raf pathway mediates β_3-AR stimulation of Erk1/2 activation (Lindquist *et al.*, 2000), while in white adipocytes H89 has no effect and there is clear inhibition by PTX, indicating a requirement for Gαi (Soeder *et al.*, 1999). Activation of Erk1/2 by human β_3-ARs expressed in HEK293 (Soeder *et al.*, 1999) or CHO-K1 (Gerhardt *et al.*, 1999) cells is sensitive to PTX but not H89, indicating role a for Gαi but not Gαs.

The β_{3b}-AR appears to couple to Gαi since PTX treatment increased cAMP accumulation and extracellular acidification rates, whereas this does not occur with the β_{3a}-AR. However, PTX had no effect on Erk1/2 phosphorylation in response to CL316243 in either β_{3a}- or β_{3b}-AR expressing cells. This is unlike what occurs in CHO-K1 cells expressing the human β_3-AR where PTX treatment inhibits Erk1/2 phosphorylation following β_3-AR activation (Gerhardt *et al.*, 1999). Additionally, H89 (PKA inhibitor (Engh *et al.*, 1996)) had no effect on Erk1/2 phosphorylation or cAMP responses in response to CL316243 in cells expressing either receptor. This suggests that CL316243-mediated increases in Erk1/2 are not dependent upon coupling of the receptor to Gαi or a PKA-dependent pathway. It is unlikely that the effects of H89 are due to antagonism with the β_3-AR (Fredriksson *et al.*, 2001), although H89 is a potent antagonist at β_2-ARs (Penn *et al.*, 1999). This does not rule out the possibility that intracellular cAMP exerts its effects on Erk1/2 in a PKA-independent pathway. A family of cAMP-binding proteins differentially distributed in mammalian brain and peripheral organs, termed cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF, also referred to as Epac) (De Rooij *et al.*, 1998; Kawasaki *et al.*, 1998), can bind cAMP and activate Rap-1 in a cAMP-dependent but PKA-independent manner (De Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Other targets for PKA-independent cAMP activation include chloride (Shintani & Marunaka, 1996) and sodium channels (Niisato *et al.*, 1999). Isoprenaline can inhibit acetylcholine-mediated contractions in guinea-pig tracheal smooth muscle in an exclusively cAMP-PKA-independent manner (Spicuzza *et al.*, 2001) and cAMP can induce thyroid cell proliferation through a PKA-dependent mechanism but can activate PI3K and PKB through PKA-independent mechanisms (Cass *et al.*, 1999).

In the present study, cells treated with either forskolin (activator of AC (Seamon & Daly, 1981)) or cholera toxin (which irreversibly activates the α-subunit of Gαs (Gill & Meren, 1978)) showed increased cAMP accumulation in both transfected and untransfected CHO-K1 cells but failed to increase Erk1/2 phosphorylation levels. This suggests that Gαs or AC activation is not needed for Erk1/2 phosphorylation in CHO-K1 cells. This finding is somewhat curious since several reports indicate cAMP-mediated Erk1/2 phosphorylation in CHO-K1 cells (Seidel *et al.*, 1999;

Verheijen & Defize, 1997) while others show opposite results (Ai *et al.*, 1999; Gerhardt *et al.*, 1999; Montrose-Rafizadeh *et al.*, 1999).

PKC is proposed to be an intermediate step in Gαq receptor signaling to Erk1/2. PKC activation by phorbol esters can activate Erk1/2 (Burgering & Bos, 1995; Lindquist & Rehnmark, 1998), although the mechanism of this action is presently unclear. PKC can directly phosphorylate and thereby activate Raf-1 (Kolch *et al.*, 1993) but may not be essential for substantial Erk1/2 activation since PKC may serve to recruit Raf to the membrane by an unknown mechanism (Schonwasser *et al.*, 1998). PKC may exert its action on Erk1/2 through binding to Src (Dikic *et al.*, 1996; Levi *et al.*, 1998) or activation of the protein kinase Pyk2, which is an intermediate between PLC and Src/Shc/Sos (Della Rocca *et al.*, 1997). Treatment of β_{3a}- or β_{3b}-AR cells with the PKC inhibitor Ro-31-8220 (Davis *et al.*, 1992) did not affect CL316243-mediated Erk1/2 phosphorylation. Hence PKC is not involved in β_3-AR activation of Erk1/2, consistent with another study (Gerhardt *et al.*, 1999).

Src is a non-receptor PTK that is essential for the mitogenic action of TKRs in a wide variety of cellular systems. One of its well-characterised protein domains, the SH3 domain, is able to bind to proline-rich motifs (PXXP) in the third intracellular loop and C-terminal tail of the mouse β_3-AR (Cao *et al.*, 2000). A specific inhibitor of Src, PP2 (Hanke *et al.*, 1996), can inhibit β_3-AR-mediated activation of Erk1/2 (Cao *et al.*, 2000; Lindquist *et al.*, 2000), consistent with PP2 inhibiting CL316243-mediated increases in Erk1/2 phosphorylation in β_{3a}- or β_{3b}-AR cells, without affecting CL316243-mediated cAMP responses. Treatment with PP2 in the absence of CL316243 significantly lowered the basal levels of Erk1/2 phosphorylation compared to control cells. This implies that there is a "basal" activation of Erk1/2 through Src. Src can be activated by Gβγ release from PTX-sensitive G-proteins (Luttrell *et al.*, 1996). In cells expressing the human β_3-AR, Src may be activated by Gβγ subunits released from PTX-sensitive G-proteins since the human β_3-AR activation of Erk1/2 is PTX sensitive. However, the Erk1/2 activation by CL316243 in β_{3a}- or β_{3b}-AR cells is not sensitive to PTX. The difference in the PTX sensitivity of Erk1/2 phosphorylation may reflect sequence differences between the mouse and human β_3-AR. The mouse has four PXXP motifs for Src binding, whereas the human

β_3-AR has only two. This could raise the possibility of a weaker association of Src with the human β_3-AR and increase its dependence on Gαi and G$\beta\gamma$ to activate the MAPK pathway.

PI3Ks are stimulated by G$\beta\gamma$ subunits (Hawes *et al.*, 1996; Leopoldt *et al.*, 1998; Lopez-Ilasaca *et al.*, 1997; Zhang *et al.*, 1995) and play a major role in PTX-sensitive GPCR coupling to the MAPK pathway through interactions of G$\beta\gamma$ subunits to recruit PI3K to the plasma membrane where it can activate the classical TKR pathway of Erk1/2 activation (Lopez-Ilasaca *et al.*, 1997). Use of PI3K inhibitors such as LY294002 (Vlahos *et al.*, 1994) or wortmannin (Powis *et al.*, 1994) show that the human β_3-AR requires PI3K to activate Erk1/2 and PKB, with Gαi needed for PI3K action on PKB and Erk1/2 (Gerhardt *et al.*, 1999). In contrast, PI3K involvement is not required in β_3-AR activation of Erk1/2 in a cAMP/PKA dependent system (Lindquist *et al.*, 2000). In β_{3a}- or β_{3b}-AR cells, LY294002 abolished the ability of CL316243-mediated increases in Erk1/2, without affecting cAMP responses, illustrating the involvement of PI3K. Since Erk1/2 activation in these cells is not mediated by Gαs/cAMP/PKA or PTX-sensitive G-proteins, one may speculate that PI3K may be activated by G$\beta\gamma$ subunits released following Gαs activation. Several studies have shown that G$\beta\gamma$ release from Gαs coupled receptors can activate a wide variety of other signaling pathways (Krieger-Brauer *et al.*, 2000).

Erk1/2 activation by GPCRs can be mediated through transactivation with TKRs which then signal to Erk1/2. This has been observed for several Gαq and Gαi coupled receptors (Daub *et al.*, 1996; 1997; Grosse *et al.*, 2000b; Maudsley *et al.*, 2000a; 2000b; Pierce *et al.*, 2000). Several studies have illustrated transactivation of the β_2-AR with the EGFR (Daub *et al.*, 1996; 1997; Maudsley *et al.*, 2000a). This does not occur in CHO-K1 cells since they lack EGFRs, but β_2-AR and dopamine D_4 and D_{2L} receptor activation can transactivate another TKR, the PDGFR in CHO-K1 cells (Maudsley *et al.*, 2000b; Oak *et al.*, 2001). CHO-K1 cells endogenously express low levels of PDGFRs (Duckworth & Cantley, 1997) and the PDGFR can act as a scaffold for several other kinases and adaptor proteins including Src, Shc, Grb2/Sos, PI3K and Ras-GAP (for review refer to Heldin *et al.* (1998)). Transactivation is not a universal pathway for GPCR activation of Erk1/2 (Schaeffer

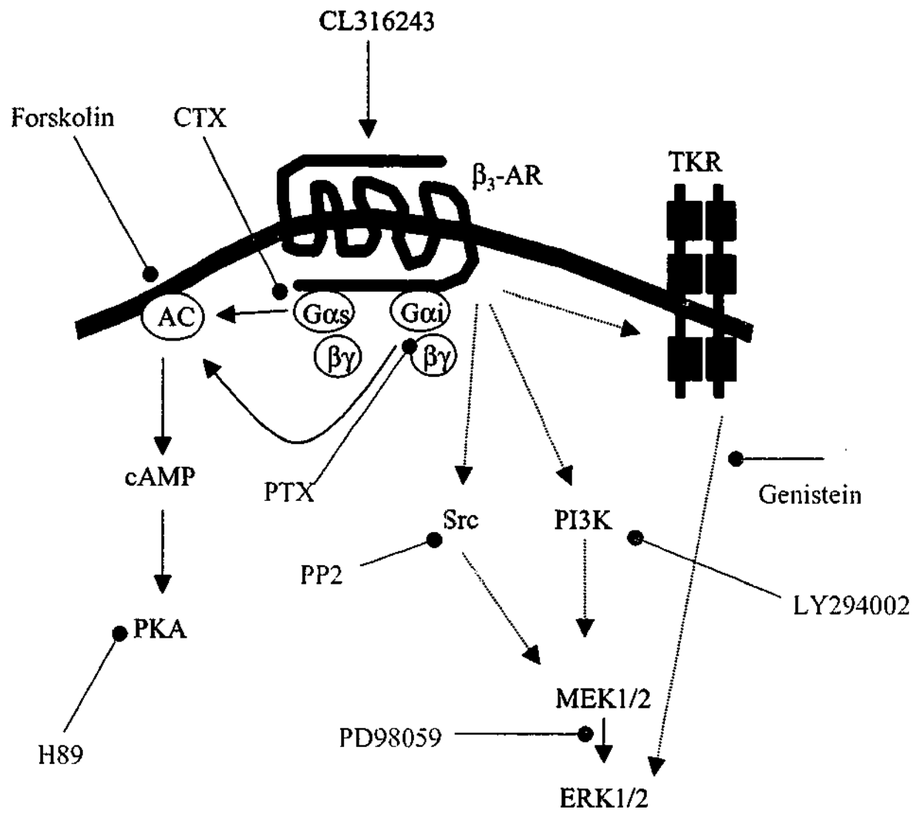
& Weber, 1999; Zhong & Minneman, 1999; Lindquist personal communication). β_{3a}- or β_{3b}-AR activation of Erk1/2 is sensitive to a general tyrosine kinase inhibitor (genistein (Akiyama *et al.*, 1987)), which may indicate cross-talk between these receptors and TKRs, presumably the PDGFR.

Associated studies investigating β_2-AR activation of Erk1/2 show that as well as transactivation with TKRs, endocytosis of the β_2-AR is required for Erk1/2 activation. Receptor endocytosis has been considered as a process that terminates or attenuates receptor signaling. A number of recent studies have demonstrated that TKRs may be internalised to signal to Erk1/2 (Chow *et al.*, 1998; Pol *et al.*, 2000; Vieira *et al.*, 1996). Following β_2-AR phosphorylation by GRK2, subsequent β-arrestin and Src binding, internalisation to clathrin-coated pits occurs which is essential for Erk1/2 activation (Ahn *et al.*, 1999; Daaka *et al.*, 1998; Luttrell *et al.*, 1997a; 1999b; Maudsley *et al.*, 2000a). Since many GPCRs undergo ligand-dependent endocytosis via a clathrin-dynamin-mediated process (Koenig & Edwardson, 1997) and GPCR activation of Erk1/2 occurs predominantly through activation of Raf-1 (Gutkind, 1998), it has been suggested that GPCRs employ endocytosis to signal through to Erk1/2 (Lefkowitz, 1998). However, this mechanism is not universal for all GPCRs, even though these receptors undergo receptor-mediated endocytosis (Blaukat *et al.*, 1999; Budd *et al.*, 1999; De Graff *et al.*, 1999; Kramer & Simon, 2000; Li *et al.*, 1999; Schramm & Limbird, 1999). Several inhibitors of endocytosis are commonly employed to test this hypothesis. MDC is an inhibitor of clathrin-mediated endocytosis that acts by stabilising clathrin cages and inhibiting transglutaminase (Davies *et al.*, 1980; Katoh *et al.*, 1994). The lectin concanavalin A prevents clathrin coat-protein assembly by binding specifically to terminal mannose residues on cell surface glycoproteins and glycolipids to impair their mobility within the membrane bilayer (Lis & Sharon, 1986). Cytochalasin D is an actin-disrupting agent that inhibits endocytosis through clathrin-independent and dependent mechanisms (Fujimoto *et al.*, 2000; Sandvig & Van Deurs, 1990). Treatment of β_{3a}- or β_{3b}-AR cells with MDC or cytochalasin D had no effect on the ability of CL316243 to activate Erk1/2 or increase cAMP accumulation. This is not surprising considering that β_3-ARs are thought not to be susceptible to internalisation since the receptor lacks sites for phosphorylation by GRK and PKA, there is no

phosphorylation of the receptor with agonist stimulation (Liggett *et al.*, 1993) and the receptors are unable to bind β-arrestin (Cao *et al.*, 2000). Instead regulation of the β_3-AR occurs at the level of the gene in adipose tissue (Bengtsson *et al.*, 1996) although this itself is not a mechanism of β_3-AR regulation that occurs in all tissues (Hutchinson *et al.*, 2000). However Erk1/2 phosphorylation was inhibited by concanavalin A, without affecting CL316243-mediated increases in cAMP. No explanation can be given for this finding. Since clathrin-coated vesicles can convey membrane traffic between specific cellular compartments, this may indicate that other proteins involved in the β_3-AR-mediated increase in Erk1/2 are dependent upon a concanavalin A sensitive mechanism that is not sensitive to MDC or cytochalasin D.

The present study demonstrates that the β_{3a}- and β_{3b}-AR couple to Gαs, and that in addition the β_{3b}-AR couples to Gαi. Increases in Erk1/2 phosphorylation following either β_{3a}- or β_{3b}-AR activation are independent of receptor coupling to Gαi or increases in cAMP, and involve a mechanism that includes activation of PI3K and Src. A schematic of the proposed signaling pathway for Erk1/2 activation by the β_{3a}- or β_{3b}-AR is shown in Figure 4.15. Activation of Erk1/2 signaling by β-ARs and other GPCRs is clearly a complex process modulated by multiple signaling proteins that differ between cell and receptor types.

Figure 4.15: A model for Erk1/2 activation in response to CL316243 in CHO-K1 cells transfected with either β_{3a}- or β_{3b}-AR. For more detail, refer to text. Inhibition is denoted by red lines whereas treatments that did not affect CL316243 activation of Erk1/2 is denoted by blue lines. The figure shows receptor coupling to Gαi but this only applies to the β_{3b}-AR.



Chapter 5

β_3-ARs in mouse ileum: roles and signal transduction pathways

5.1 *Introduction*

β_3-ARs are known to mediate relaxation in a wide variety of gastrointestinal tissues from various species including guinea pig, rat, rabbit and man (for review see Manara *et al.* (1995)). A functional role for β_3-ARs is supported by studies demonstrating β_3-AR mRNA in gastrointestinal tissues (Bensaid *et al.*, 1993; Evans *et al.*, 1996; 1998; Granneman *et al.*, 1991; 1993; Hutchinson *et al.*, 2000; Krief *et al.*, 1993; Roberts *et al.*, 1997; 1999) and radioligand binding studies using ICYP that show a site with characteristics resembling β_3-ARs (Hutchinson *et al.*, 2000; Roberts *et al.*, 1995; 1997).

While β_3-ARs predominate in gastrointestinal tissues, β_1- and β_2-ARs may also have roles. The β_1-AR agonist Ro363 relaxes rat ileum, an effect which is antagonised by CGP20712A (Roberts *et al.*, 1999). Ro363 has an intrinsic activity equal to that of isoprenaline in rat colon and guinea pig ileum (Molenaar *et al.*, 1997a), and caused a relaxation in rat ileum amounting to 60-70% of the isoprenaline response (Hoey *et al.*, 1996). Later studies showed responses that were less than 20% of maximum responses with pEC_{50} values (6.2) in rat terminal ileum (Roberts *et al.*, 1999) intermediate between values at β_1- and β_3-ARs in rat colon (8.5 and 5.6 respectively) (Molenaar *et al.*, 1997a). A role for β_2-AR mediated smooth muscle relaxation in gastrointestinal tissues has been suggested (MacDonald & Lamont, 1993; Oostendorp *et al.*, 2000; Thollander *et al.*, 1996; Van der Vliet *et al.*, 1990) although β_2-ARs may also mediate secretory roles (Claustre *et al.*, 1999; Dumoulin *et al.*, 1995).

However one of the problems in interpreting some studies is the use of agonists that also act at other β-ARs. Ro363 is a partial agonist at the cloned human β_3-AR and on intestinal β_3-ARs of the rat and guinea pig (Molenaar *et al.*, 1997a), and also has affinity for β_2-ARs, albeit lower than that at β_1-ARs (McPherson *et al.*, 1984; Molenaar *et al.*, 1997a). Zinterol (β_2-AR agonist) causes relaxation in rat ileum through β_3-ARs since responses were antagonised by β_3-AR and not β_2-AR antagonists (Roberts *et al.*, 1999). Zinterol may also have agonistic actions at cardiac

β_1-ARs (Freyss-Beguin *et al.*, 1983; Juberg *et al.*, 1985; Kuznetsov *et al.*, 1995) and β_1-ARs in Sertoli cells (Hool & Harvey, 1997).

The aims of the present study were to characterise β_3-ARs in mouse ileum and to examine the importance of β_3-ARs in that tissue by comparing responses of ileum from β_3-AR KO and FVB mice. Relaxation of ileal smooth muscle to (-)-isoprenaline was performed to assess the relative roles of all three β-AR subtypes in mediating smooth muscle relaxation. Molecular and radioligand binding studies were employed to characterise the β-AR subtypes present in mouse ileum.

5.2 Methods

5.2.1 Genotyping of animals

Genotyping of FVB and β_3-AR KO mice is described in section 2.4. All mice used for breeding or experimental studies were genotyped to confirm genotype status.

5.2.2 Analysis of β-AR mRNA levels in ileum

Analysis of mRNA levels were performed as previously described (section 2.2).

5.2.3 ICYP binding studies in mouse ileum

Mouse ileum membranes were prepared as previously described (section 2.5.1.2). Saturation and competition radioligand binding assays were performed in binding buffer as previously described (section 2.5.3 and 2.5.4 respectively). Preliminary experiments carried out in mouse ileum to study competition at β_1-/β_2-AR sites were difficult to perform due to the low levels of binding and were not continued. Competition studies at β_3-ARs in FVB ileum were performed using (-)-isoprenaline, CL316243, BRL37344, CGP12177A, (-)-propranolol, SR59230A, CGP20712A or ICI118551, with a concentration range of 30nM to 1μM. ICYP concentration was 500pM and non-specific binding was defined by (-)-alprenolol (1mM). All experiments were performed in duplicate.

Analysis of saturation and competition binding data is detailed in section 2.5.5.

5.2.4 Organ bath studies

Preparation of tissues and c-r curves to agonists (either in the presence/absence of antagonist) were performed as described in section 2.9. Initially, c-r curves were performed to carbachol (1nM-30μM) to determine the concentration that produced 80% maximal contraction of mouse ileum and to assess if contraction of ileum was different between FVB and β_3-AR KO tissue samples.

C-r curves to β-AR agonists were constructed in tissues precontracted with carbachol (1μM). Cumulative c-r curves were constructed to (-)-isoprenaline, CL316243, CGP12177A, zinterol or Ro363as described in section 2.9.2. In some experiments, c-r curves to forskolin or the β_3-AR antagonist SR59203A were performed. Relaxation responses were measured as a percentage of the R_{max} response to papaverine (10μM). Concentration ranges used for agonists are indicated with the data.

The protocol examining antagonist effects on β-AR agonist stimulated relaxation of mouse ileum is described in section 2.9.2. Briefly, agonist c-r curves were performed in paired tissue segments from the same animal in the presence or absence of antagonist. Antagonists (and concentrations) used were: (-)-propranolol (100nM, 1μM), carvedilol (100nM), CGP20712A (100nM), ICI118551 (100nM), SR59230A (100nM) or CGP12177A (100nM). For the investigation of stereoselectivity in the enantiomers of propranolol, ((-)-propranolol (10μM) or (+)-propranolol (100μM)) and tertatolol ((-)-tertatolol (10μM) or (+)-tertatolol (100μM)) were used in FVB ileum under β_1- and β_2-AR blockade (100nM CGP20712A, 100nM ICI118551).

The effect of antagonists on agonist c-r curves were evaluated from calculation of pK_B values as detailed in section 2.9.3.

The effect of PTX treatment on CL316243 c-r curves was assessed by preincubation of mouse ileum for 2 h with 500ng ml^{-1} PTX prior to carbachol contraction.

5.2.5 cAMP accumulation studies

Mouse ileum segments were prepared as previously described (section 2.1.4). Tissues were exposed either to forskolin (100μM), CL316243 (10μM) or (-)-isoprenaline (10μM) for 30 min and cAMP extracted and measured as described previously (section 2.6.1 and 2.6.2). Responses are expressed as pmol of cyclic AMP produced per mg of tissue.

5.2.6 Erk1/2 phosphorylation

Studies were performed as previously described (section 2.7). Tissues were exposed to CL316243 (10μM) for 2, 5, 10, 15, 20 or 30 min. Control tissues were obtained at 0, 10 and 30 min. Analysis of data is detailed as described previously (section 2.7.3), with all values normalised to those of control samples at 0 min (defined as 100%).

5.2.7 Analysis

All results are expressed as mean ± s.e.mean of *n* experiments. mRNA levels were calculated as previously described (section 2.2.2). Radioligand binding results were analysed and binding parameters calculated as described in section 2.5.5 and organ bath experiments were analysed as described in section 2.9.3. Levels of cAMP and Erk1/2 were calculated as previously described (section 2.6.3 and 2.7.3 respectively). Students t-test or 2-way ANOVA statistical analysis using GraphPad PRISM were utilised and p values less than or equal to 0.05 were considered significant.

5.2.8 Drugs and reagents

The drugs and reagents used and the preparation of stock solutions is described in section 2.12. All drugs were diluted either in binding buffer (radioligand binding

experiments), Krebs-Henseleit solution (organ bath experiments) or water (cAMP accumulation and Erk1/2 phosphorylation experiments) prior to use.

5.3 *Results*

5.3.1 *Detection of β_1-, β_2- and β_3-AR mRNA in FVB and β_3-AR KO ileum*

RT-PCR detected all three β-AR subtypes and β-actin mRNA in ileal smooth muscle from FVB mice. Direct comparison of the levels of β-AR mRNA in β_3-AR KO compared to FVB samples showed that β_1-AR mRNA levels were increased more than three fold in β_3-AR KO as compared to FVB ileum (FVB 100 ± 37.8%, KO 350.9 ± 92.5%; n=6; Student's t-test *$p<0.05$), β_2-AR mRNA levels were not significantly altered (FVB 100 ± 12.2%, KO 95.2 ± 9.0%, n=6; Student's t-test ns), and as expected no significant β_3-AR mRNA was detected in β_3-AR KO samples (FVB 100 ± 15.5%, KO 2.4 ± 1.1%, n=6; Student's t-test ***$p<0.001$) (Figure 5.1).

5.3.2 *Characteristics of ICYP binding in FVB and β_3-AR KO ileum*

5.3.2.1 *ICYP saturation binding in FVB and β_3-AR KO ileum*

ICYP bound in a saturable manner to a high affinity site in membranes prepared from FVB ileum (K_D 44.6 ± 30.4 pM; B_{max} 16.7 ± 4.9 fmol mg^{-1} protein; n=5) (Figure 5.2) and also to a low affinity site in FVB ileum (K_D 886.7 ± 62.2 pM; B_{max} 158.7 ± 48.7 fmol mg^{-1} protein; n=3) (Figure 5.3). High affinity ICYP binding was increased in β_3-AR KO ileum (K_D 57.2 ± 25.5 pM; B_{max} 30.6 ± 6.5 fmol mg^{-1} protein; n=6) compared to FVB ileum (2 way ANOVA ***$p=0.0001$) (Figure 5.2).

5.3.2.2 *Competition studies in FVB ileum*

Competition studies were performed to determine the affinities of several β-AR ligands for the low affinity ICYP binding site in FVB ileum (see Table 5.1 for summary). All competition curves fitted to a one site fit. Studies using β-AR

Figure 5.1: β_1, β_2-, and β_3-AR mRNA levels in FVB and β_3-AR KO ileum. Bars show mean ± s.e.mean (n=6).

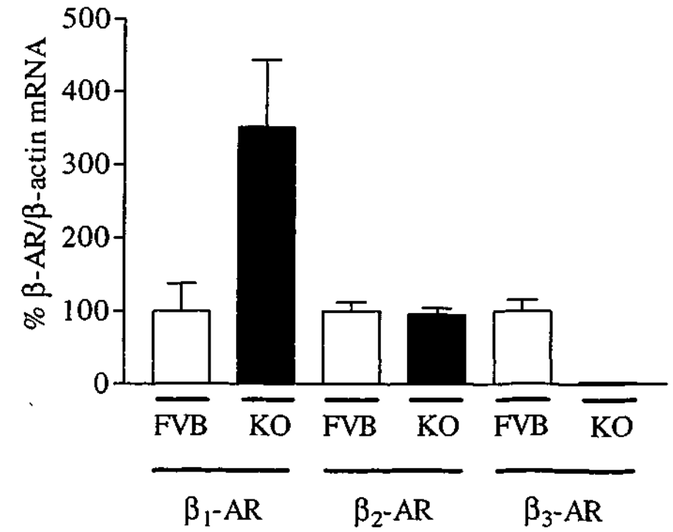


Figure 5.2: Saturation binding curve of ICYP to FVB and β_3-AR KO ileal membrane preparations to a high affinity ICYP site. Incubations were for 1 h at room temperature and non-specific binding was defined by (-)-propranolol (1μM). Points show mean ± s.e.mean (n=5-6).

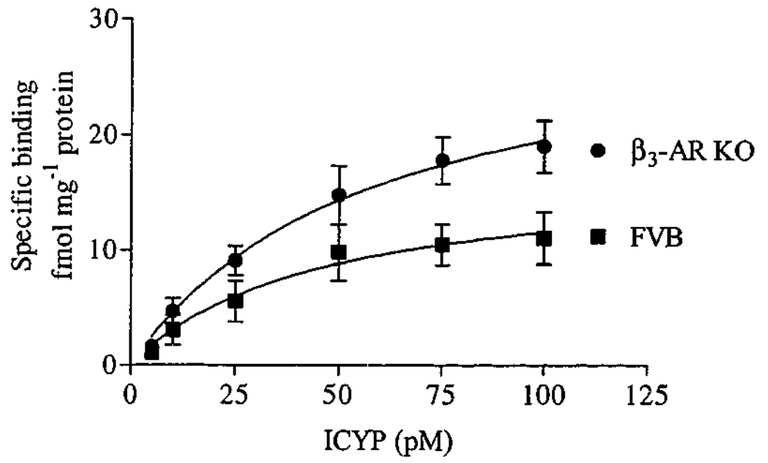


Figure 5.3: Saturation binding curve of ICYP to a low affinity ICYP site in mouse ileal membrane preparations. Incubations were for 1 h at room temperature and non-specific binding was defined by (-)-alprenolol (1mM). Points show mean ± s.e.mean (n=3).

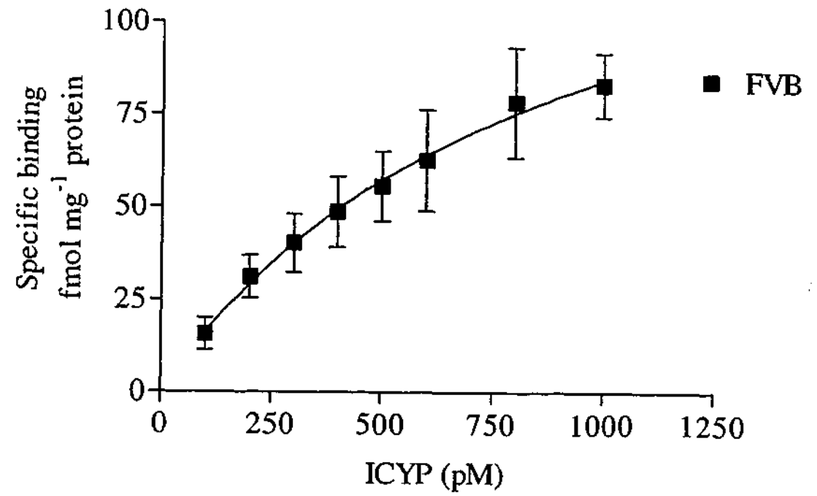


Table 5.1: Summary table of binding affinities (pK_i) of several β-AR ligands at the low affinity ICYP site in FVB mouse ileum.

Competitor	*n*	pKi ± s.e.mean
Agonists		
(-)-isoprenaline	3	3.01 ± 0.11
CGP12177A	3	4.87 ± 0.15
BRL37344	3	ND
CL316243	3	3.16 ± 0.79
Antagonists		
(-)-propranolol	3	5.14 ± 0.10
SR59230A	3	5.54 ± 0.28
ICI118551	3	4.57 ± 0.05
CGP20712A	3	4.28 ± 0.84

ND: non-linear regression was not obtainable

antagonists showed that SR59230A had the highest affinity at this site, and the rank order of affinity (pK_i) for antagonists was SR59230A (5.7) > (-)-propranolol (5.3) > ICI118551 (4.8), CGP20712A (4.6) (Figure 5.4).

The β-AR agonists (-)-isoprenaline, CL316243 and CGP12177A competed at the low affinity ICYP site with the following rank order of affinity (pK_i) CGP12177A (5.1) > CL316243 (3.9), (-)-isoprenaline (3.2), whereas BRL37344 did not compete for this site (Figure 5.5).

5.3.3 Organ bath studies in ileum from FVB and β_3-AR KO mice

Carbachol c-r curves were carried out to determine an optimal concentration to produce approximately 80% contraction of tissues (determined to be approximately 1μM from c-r curve, Figure 5.6). There was no difference in the carbachol c-r curves between FVB (pEC_{50} 7.07 ± 0.10; n=7) and β_3-AR KO (pEC_{50} 6.99 ± 0.07; n=7) ileum (2-way ANOVA ns).

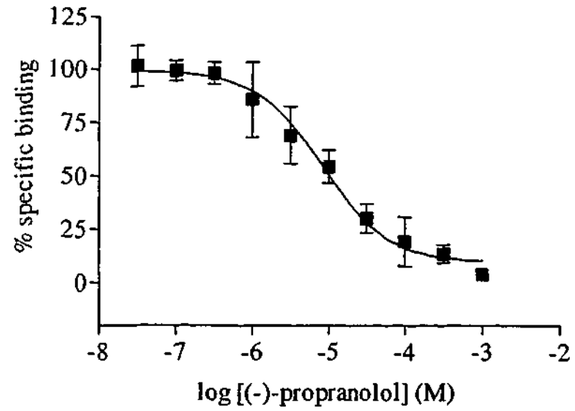
5.3.3.1 Ro363

The β_1-AR agonist Ro363 relaxed smooth muscle in carbachol precontracted FVB (pEC_{50} 8.22 ± 0.47; n=7) and β_3-AR KO (pEC_{50} 7.97 ± 0.25; n=9) ileum smooth muscle in a dose-dependent manner, with no significant difference between curves (2-way ANOVA ns) (Figure 5.7a). Due to a limited amount of drug available, no antagonist studies were performed using Ro363.

5.3.3.2 Zinterol

The β_2-AR agonist zinterol relaxed smooth muscle in carbachol precontracted ileum from FVB (pEC_{50} 7.23 ± 0.41; n=9) and β_3-AR KO (pEC_{50} 6.89 ± 0.54; n=6) (2-way ANOVA ns) (Figure 5.7b).

Figure 5.4: Competition between ICYP (500pM) and (a) (-)-propranolol (b) SR59230A, ICI118551 or CGP20712A in FVB ileal membranes. Incubations were for 1 h at room temperature. Non-specific binding was defined by (-)-alprenolol (1mM). pK_i values are given in Table 5.1. Points show mean ± s.e.mean (n=3).



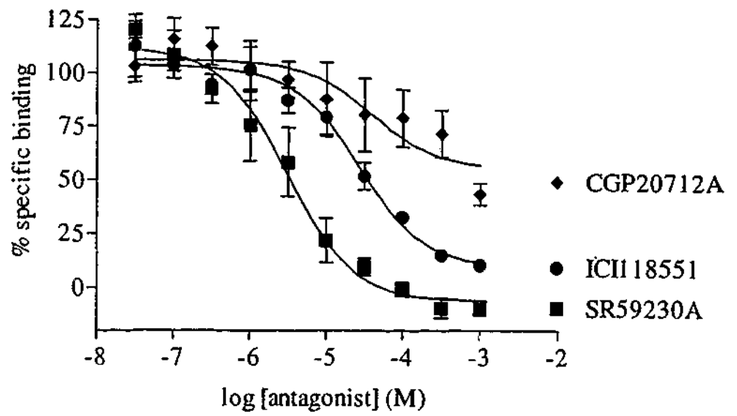
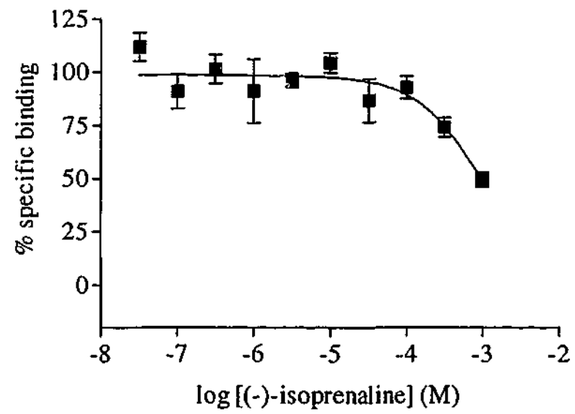


Figure 5.5: Competition between ICYP (500pM) and (a) (-)-isoprenaline (b) BRL37344, CL316243 or CGP12177A in FVB ileal membranes. Incubations were for 1 h at room temperature. Non-specific binding was defined by (-)-alprenolol (1mM). pK_i values are given in Table 5.1. Points show mean ± s.e.mean (n=3).



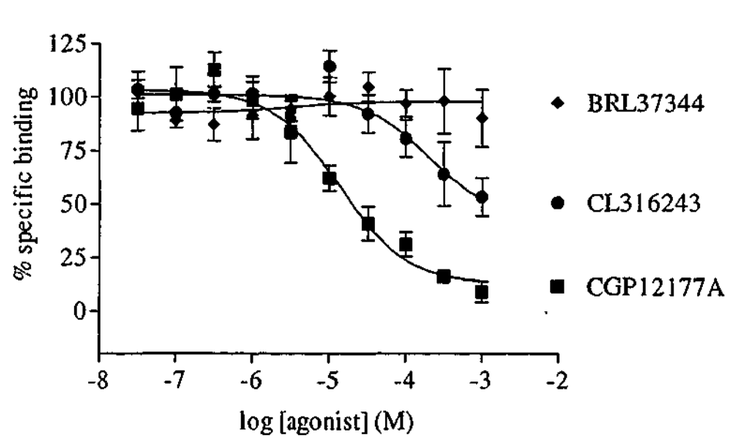


Figure 5.6: Mean concentration-response curves for carbachol contraction of mouse ileum from FVB or β_3-AR KO mice. Results are expressed as a percentage of the response to 30μM carbachol. Points show mean ± s.e.mean (n=7).

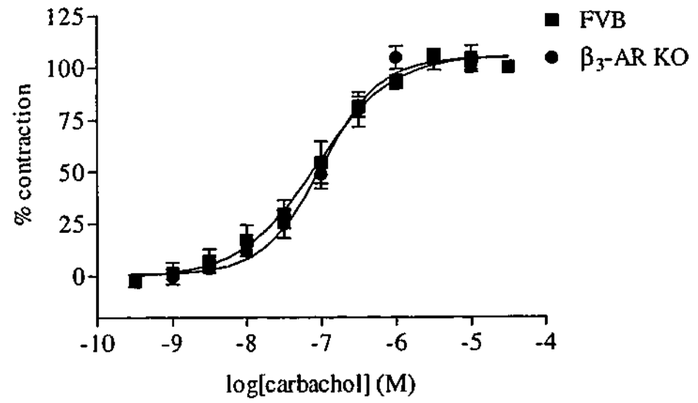
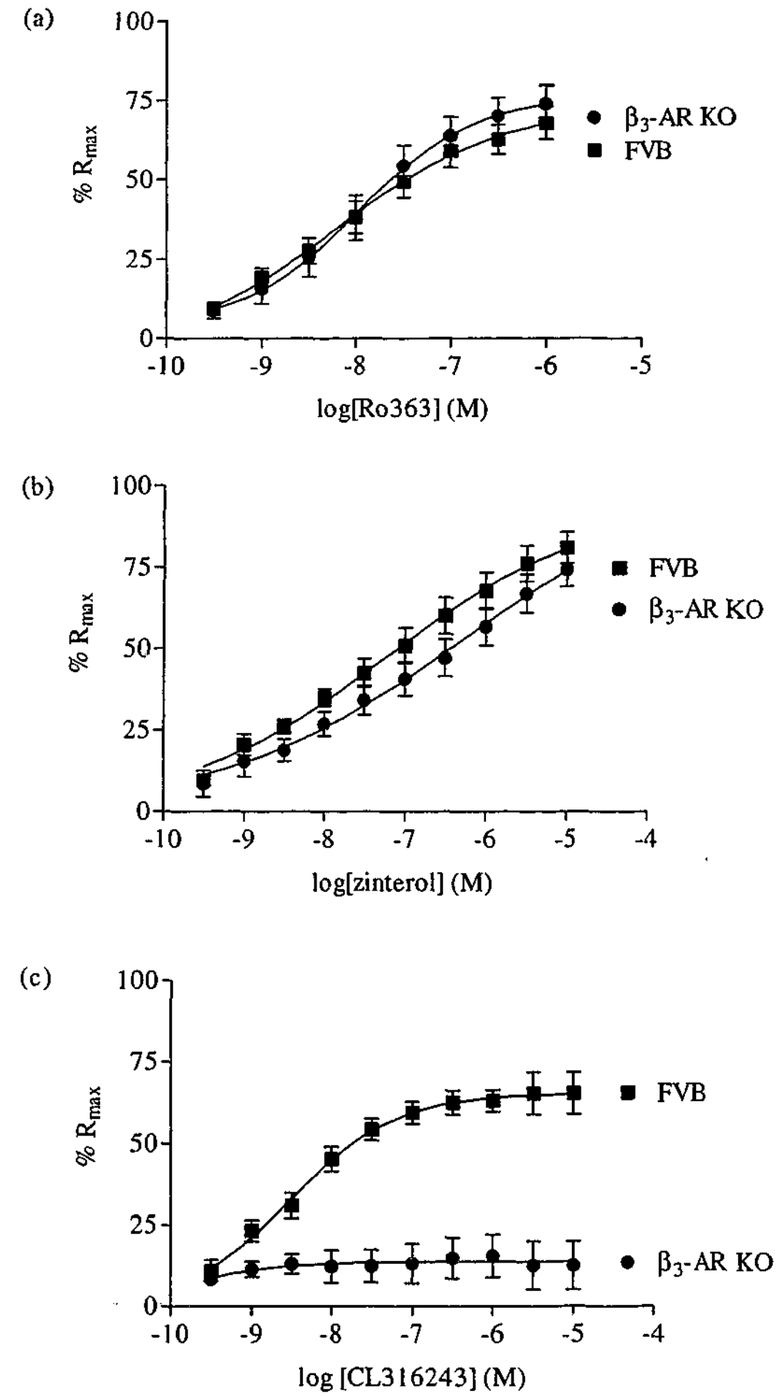


Figure 5.7: Relaxation of mouse ileum by (a) Ro363, (b) zinterol or (c) CL316243 in tissues from FVB or β_3-AR KO mice precontracted with carbachol (1μM). Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=7-12). The slope for Ro363 and zinterol in both types of tissue were significantly less than unity and no response to CL316243 was observed in β_3-AR KO mouse ileum.



5.3.3.3 *CL316243*

The β_3-AR agonist CL316243 relaxed smooth muscle in carbachol precontracted FVB ileum in a dose-dependent manner (pEC_{50} 8.52 ± 0.35; n=7) whereas ileum from β_3-AR KO animals (n=7) was unresponsive to CL316243 (2-way ANOVA ***p<0.001) (Figure 5.7c). Responses to CL316243 in FVB ileum were antagonised by SR59230A (100nM) (control: pEC_{50} 8.77 ± 0.50; SR59230A (100nM): pEC_{50} 7.65 ± 0.15; n=6) with a pK_B value of 8.3 ± 0.2 (Figure 5.8a). Significant antagonism of CL316243 responses by both propranolol (100nM) (control: pEC_{50} 8.80 ± 0.60; propranolol (100nM): pEC_{50} 8.33 ± 0.25; n=6) and carvedilol (100nM) (control: pEC_{50} 8.35 ± 0.37; carvedilol (100nM): pEC_{50} 8.07 ± 0.09; n=3) was not observed (Figure 5.7b,c).

5.3.3.4 *(-)-Isoprenaline*

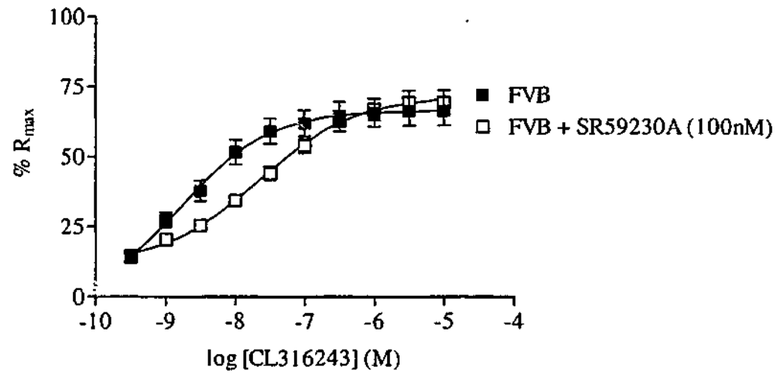
The non-subtype selective β-AR agonist (-)-isoprenaline caused concentration-dependent relaxation of carbachol-precontracted ileum from both FVB (pEC_{50} 7.59 ± 0.06; n=39) and β_3-AR KO (pEC_{50} 7.95 ± 0.07; n=38) mice (Figure 5.9).

The β_1-AR selective antagonist CGP20712A (100nM) (Figure 5.10a) caused a rightward shift in the (-)-isoprenaline c-r curve in β_3-AR KO (control: pEC_{50} 8.20 ± 0.13; CGP20712A (100nM): pEC_{50} 5.67 ± 0.34; n=9) and FVB ileum (control: pEC_{50} 7.82 ± 0.13; CGP20712A (100nM): pEC_{50} 6.60 ± 0.12; n=7), with the larger shift observed in β_3-AR KO rather than FVB (pK_B values 9.4 ± 0.3 and 8.1 ± 0.3 respectively; Student's t-test ***p<0.001).

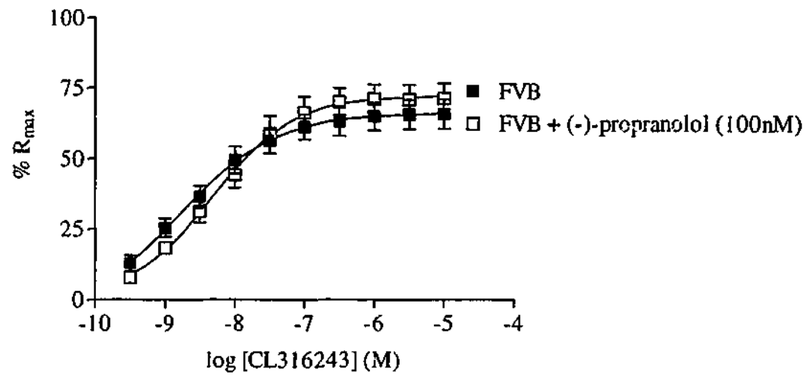
The β_2-AR antagonist ICI118551 (100nM) (Figure 5.10b) was a very weak antagonist in β_3-AR KO (control: pEC_{50} 7.98 ± 0.15; ICI118551 (100nM): pEC_{50} 7.51 ± 0.12; n=8) and FVB (control: pEC_{50} 7.66 ± 0.11; ICI118551 (100nM): pEC_{50} 7.37 ± 0.10; n=6) ileum and produced no significant shift in responses to (-)-isoprenaline.

Figure 5.8: Antagonism of CL316243-mediated relaxation of carbachol (1μM) precontracted ileum from FVB mice by (a) SR59230A, (b) (-)-propranolol or (c) carvedilol. Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=3-6). pK_B values are shown in Table 5.2.

(a)



(b)



(c)

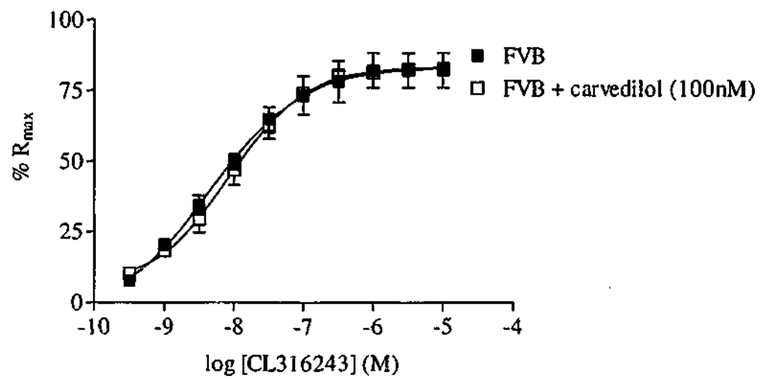


Figure 5.9: Relaxation of carbachol (1μM) precontracted ileum by (-)-isoprenaline in tissues from either FVB or β_3-AR KO mice. Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=38-39).

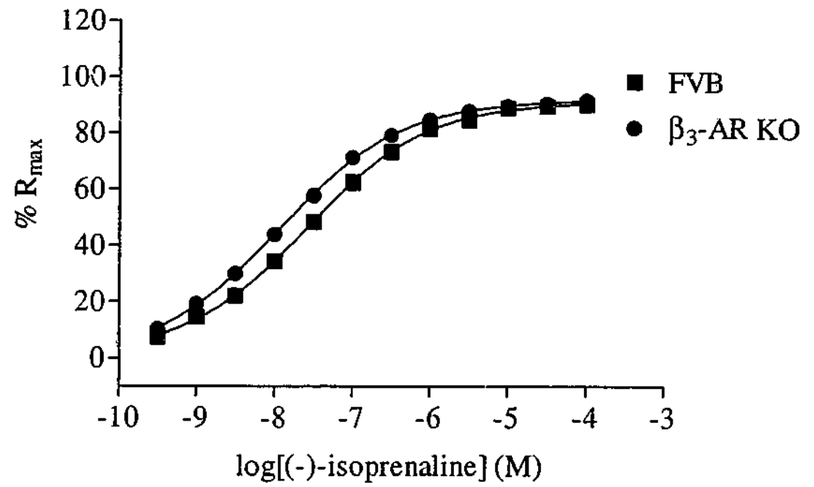
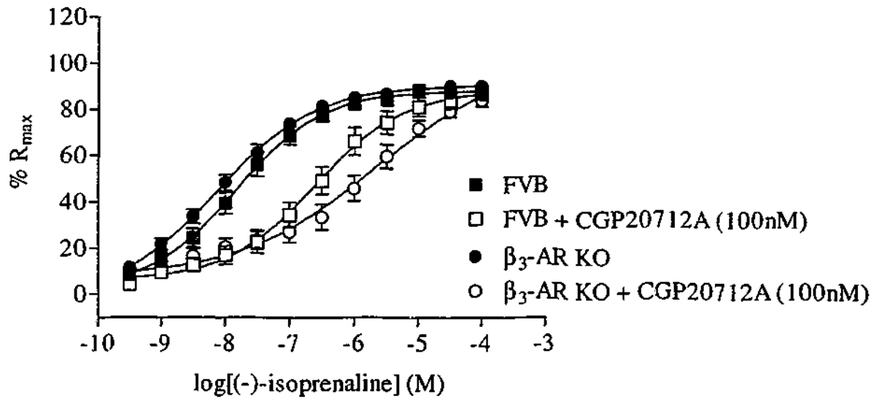
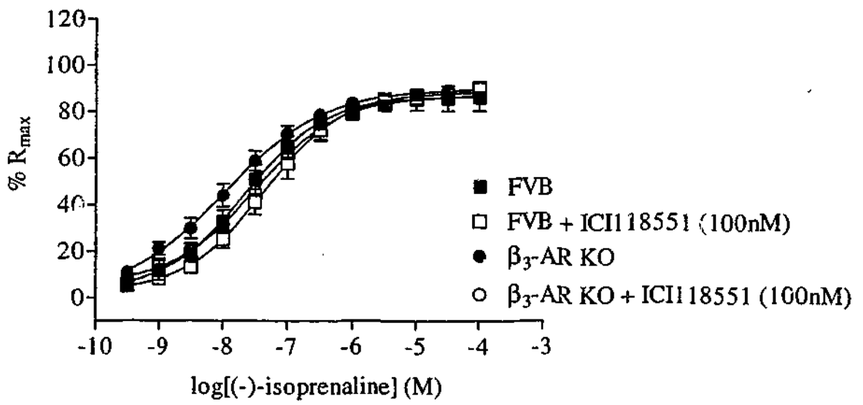


Figure 5.10: Antagonism of (-)-isoprenaline-mediated relaxation of carbachol (1μM) precontracted ileum from FVB or β_3-AR KO mice by (a) CGP20712A, (b) ICI118551 or (c) SR59230A. Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=6-9). pK_B values are shown in Table 5.2

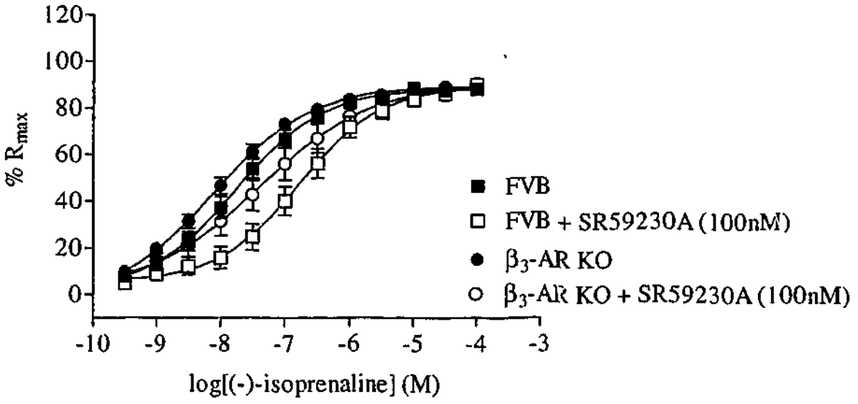
(a)



(b)



(c)



The β_3-AR antagonist SR59230A (100nM) (Figure 5.10c) significantly shifted the (-)-isoprenaline c-r curve to the right in FVB ileum (control: pEC_{50} 7.76 ± 0.12; SR59230A (100nM): pEC_{50} 6.78 ± 0.11; pK_B 8.0 ± 0.2; n=8). (-)-Isoprenaline responses in β_3-AR KO ileum was antagonised to a weaker degree by SR59230A (100nM) (control: pEC_{50} 8.11 ± 0.12; SR59230A (100nM): pEC_{50} 7.39 ± 0.20; pK_B 7.4 ± 0.3; n=9). There was no statistical difference between pK_B values obtained from FVB or β_3-AR KO mice (Student's t-test ns).

The non-selective β-AR antagonist (-)-propranolol (1μM) (Figure 11a) caused a rightward shift of the (-)-isoprenaline c-r curve with pK_B values of 7.7 ± 0.2 and 8.9 ± 0.3 (Student's t-test ***$p<0.001$) in FVB (control: pEC_{50} 7.29 ± 0.14; propranolol (1μM): pEC_{50} 5.63 ± 0.44; n=7) and β_3-AR KO (control: pEC_{50} 7.96 ± 0.27; propranolol (1μM): pEC_{50} 4.57 ± 0.75; n=6) ileum respectively.

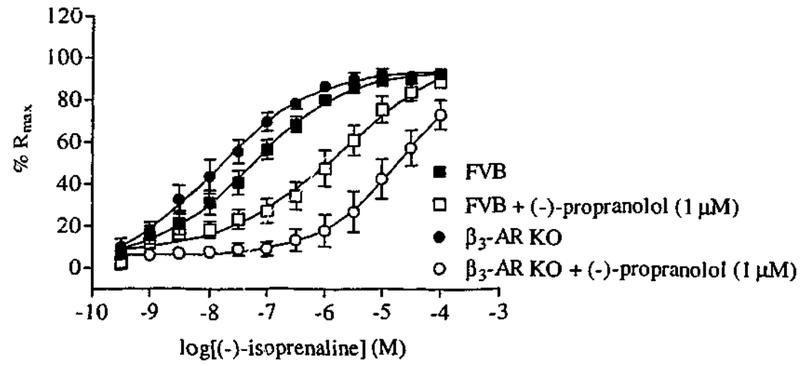
Responses to (-)-isoprenaline were antagonised by carvedilol (100nM) (non-specific β-AR antagonist) (Figure 5.11b) more strongly in ileum from β_3-AR KO (control: pEC_{50} 7.75 ± 0.29; carvedilol (100nM): pEC_{50} 5.12 ± 0.22; n=3) compared to FVB ileum (control: pEC_{50} 7.36 ± 0.14; carvedilol (100nM): pEC_{50} 5.99 ± 0.26; n=5) with pK_B values of 10.1 ± 0.4 and 8.4 ± 0.4 respectively (Student's t-test ***$p<0.001$).

CGP12177A (β_1-/β_2-AR antagonist) (Figure 5.11c) antagonised responses to (-)-isoprenaline in both FVB and β_3-AR KO ileum, with responses more strongly antagonised in ileum from β_3-AR KO (control: pEC_{50} 7.29 ± 0.17; CGP12177A (100nM): pEC_{50} 4.96 ± 0.08 n=3) as compared to FVB ileum (control: pEC_{50} 7.36 ± 0.14; CGP12177A (100nM): pEC_{50} 5.91 ± 0.27; n=5) with pK_B values of 9.6 ± 0.6 and 8.6 ± 0.4 respectively (Student's t-test ***$p<0.001$).

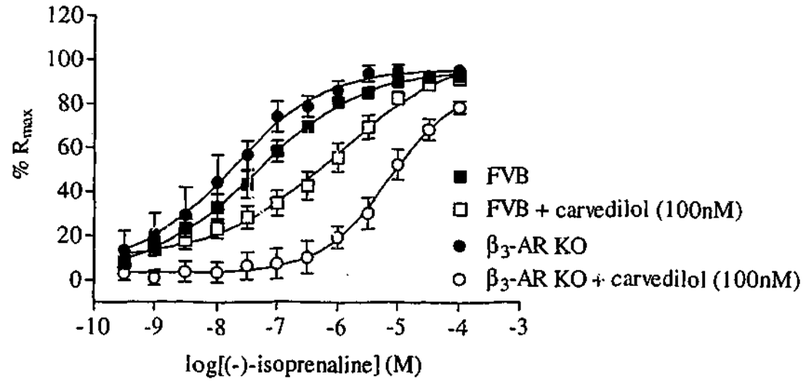
Responses to (-)-isoprenaline were antagonised by the stereoisomers of propranolol under conditions chosen to block β_1- and β_2-ARs (100nM ICI118551 and 100nM CGP20712A) (Figure 5.12a) (control: pEC_{50} 7.61 ± 0.17; ICI118551 (100nM) and CGP20712A (100nM): pEC_{50} 6.67 ± 0.07; ICI118551 (100nM), CGP20712A (100nM) and (-)-propranolol (10μM): pEC_{50} 5.43 ± 0.30; ICI118551 (100nM),

Figure 5.11: Antagonism of (-)-isoprenaline-mediated relaxation of carbachol (1μM) precontracted ileum from FVB or β_3-AR KO mice by (a) (-)-propranolol, (b) carvedilol or (c) CGP12177A. Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=3-7). pK_B values are shown in Table 5.2.

(a)



(b)



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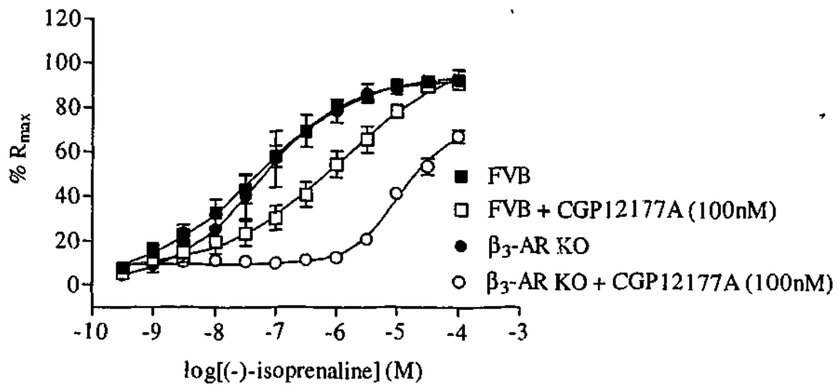
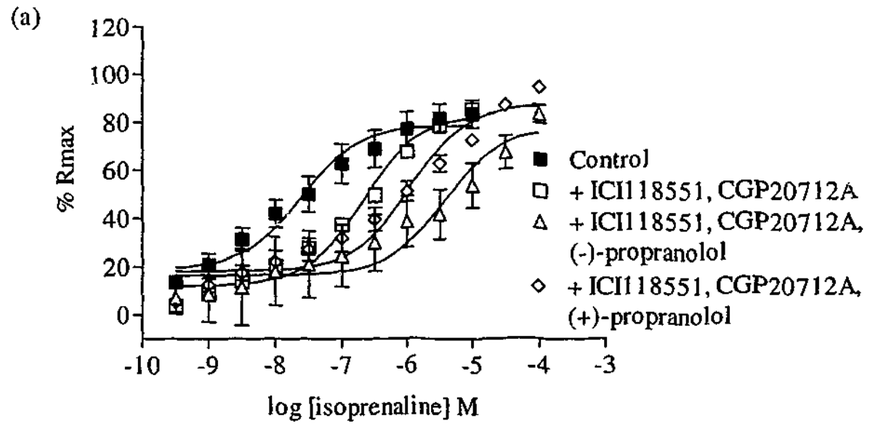


Figure 5.12: Effect of the stereoisomers of (a) propranolol or (b) tertatolol on (-)-isoprenaline-mediated relaxation of FVB ileum precontracted with carbachol (1μM). Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=5-6).



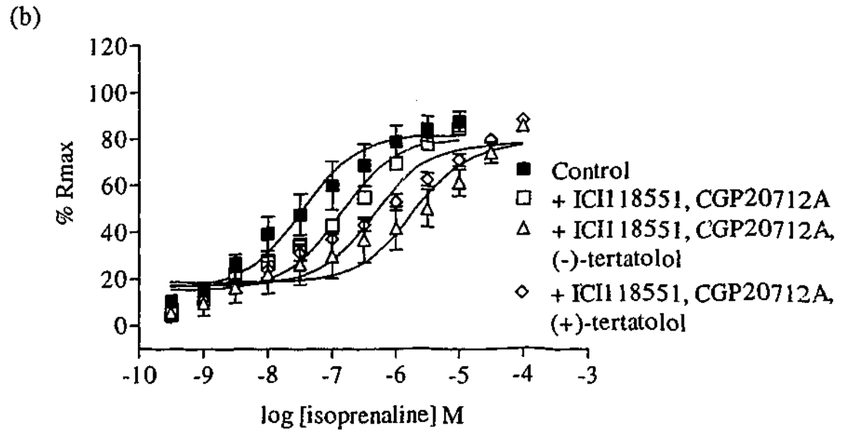


Table 5.2: Summary table of pK_B values for a range of compounds obtained against either CL316243 c-r curves in FVB ileum or against (-)-isoprenaline c-r curves in FVB or β_3-AR KO ileum.

Compound	CL316243	(-)-isoprenaline	
	$pK_B \pm$ s.e.mean (n)	$pK_B \pm$ s.e.mean (n)	$pK_B \pm$ s.e.mean (n)
	FVB	FVB	β_3-AR KO
β_1-AR antagonist			
CGP20712A		8.1 ± 0.3 (7)	9.4 ± 0.3 (9)
β_2-AR antagonist			
ICI118551		No shift (6)	No shift (8)
β_3-AR antagonist			
SR59230A	8.3 ± 0.2 (6)	8.0 ± 0.2 (8)	7.4 ± 0.3 (9)
β-AR antagonist			
(-)-propranolol	No shift (6)	7.7 ± 0.1 (7)	8.9 ± 0.3 (6)
carvedilol	No shift (3)	8.4 ± 0.4 (5)	10.1 ± 0.4 (3)
CGP12177A		8.6 ± 0.4 (5)	9.6 ± 0.6 (3)
β-AR antagonist under β_1- and β_2-AR blockade*			
(-)-propranolol		6.2 ± 0.3 (5)	
(+)-propranolol		4.6 ± 0.2 (6)	
(-)-tertatolol		6.1 ± 0.3 (5)	
(+)-tertatolol		4.4 ± 0.3 (6)	

* CGP20712A (100nM) and ICI118551 (100nM)

CGP20712A (100nM) and (+)-propranolol (100μM): pEC_{50} 5.96 ± 0.10; n=5-6) with pK_B values of 6.15 ± 0.3 and 4.58 ± 0.2 for (-)- and (+)-propranolol respectively (Student's t-test ***p<0.001). Additionally, under conditions to block β_1- and β_2-ARs (100nM ICI118551 and 100nM CGP20712A), responses to (-)-isoprenaline were antagonised by the stereoisomers of tertatolol (Figure 5.12b) (control: pEC_{50} 7.48 ± 0.16; ICI118551 (100nM) and CGP20712A (100nM): pEC_{50} 6.87 ± 0.09; ICI118551 (100nM), CGP20712A (100nM) and (-)-tertatolol (10μM): pEC_{50} 5.72 ± 0.20; ICI118551 (100nM), CGP20712A (100nM) and (+)-tertatolol (100μM): pEC_{50} 6.34 ± 0.10; n=5-6) giving pK_B values of 6.10 ± 0.3 and 4.40 ± 0.3 for (-)- and (+)-tertatolol respectively (Student's t-test ***p<0.001). The degree of blockade with the combination of ICI118551 (100nM) and CGP20712A (100nM) was similar to that with CGP20712A (100nM) alone (Figure 5.10a).

A summary of the pK_B values obtained in FVB or β_3-AR KO mice is shown in Table 5.2.

5.3.3.5 CGP12177A

CGP12177A failed to exert any significant agonistic actions in either β_3-AR KO or FVB ileum, even at concentrations of 10μM (Figure 5.13). Subsequent addition of (-)-isoprenaline (10μM) failed to produce any relaxation of the ileum, suggesting that CGP12177A acted purely as an antagonist at β_1- and β_2-ARs. Subsequent additions of CL316243 (1μM) in FVB mice caused relaxation in the same tissue, suggesting an intact β_3-AR response, whereas in β_3-AR KO mice CL316243 had no effect (Figure 5.13, 5.7c).

5.3.3.6 SR59230A

Although generally regarded as a β_3-AR antagonist, it was found that SR59230A could also act as an agonist and produce relaxation responses in FVB ileum. C-r curves to SR59230A were biphasic in ileum from FVB mice (Figure 5.14). The first phase (pEC_{50} 7.97 ± 0.30, n=6) occurred at concentrations similar to that of (-)-isoprenaline but lower than those for CL316243, and caused a response that was

Figure 5.13: Original traces obtained from (a,b) FVB or (c,d) β_3-AR KO ileal tissues. Tissues were contracted with carbachol (1μM) and then exposed to CL316243 (1μM) followed by CGP12177A (10μM) or, CGP12177A (10μM) followed by CL316243 (1μM) as indicated. (-)-Isoprenaline (10μM) and finally papaverine (10μM) was added. Arrows indicate administration of drug. CL316243 was effective only in FVB ileum while GCP12177A was ineffective in causing ileal relaxation in both FVB and β_3-AR KO mice. Note the lack of effect of (-)-isoprenaline in FVB and β_3-AR KO ileum following CGP12177A administration. Traces are representative of n=4 experiments.

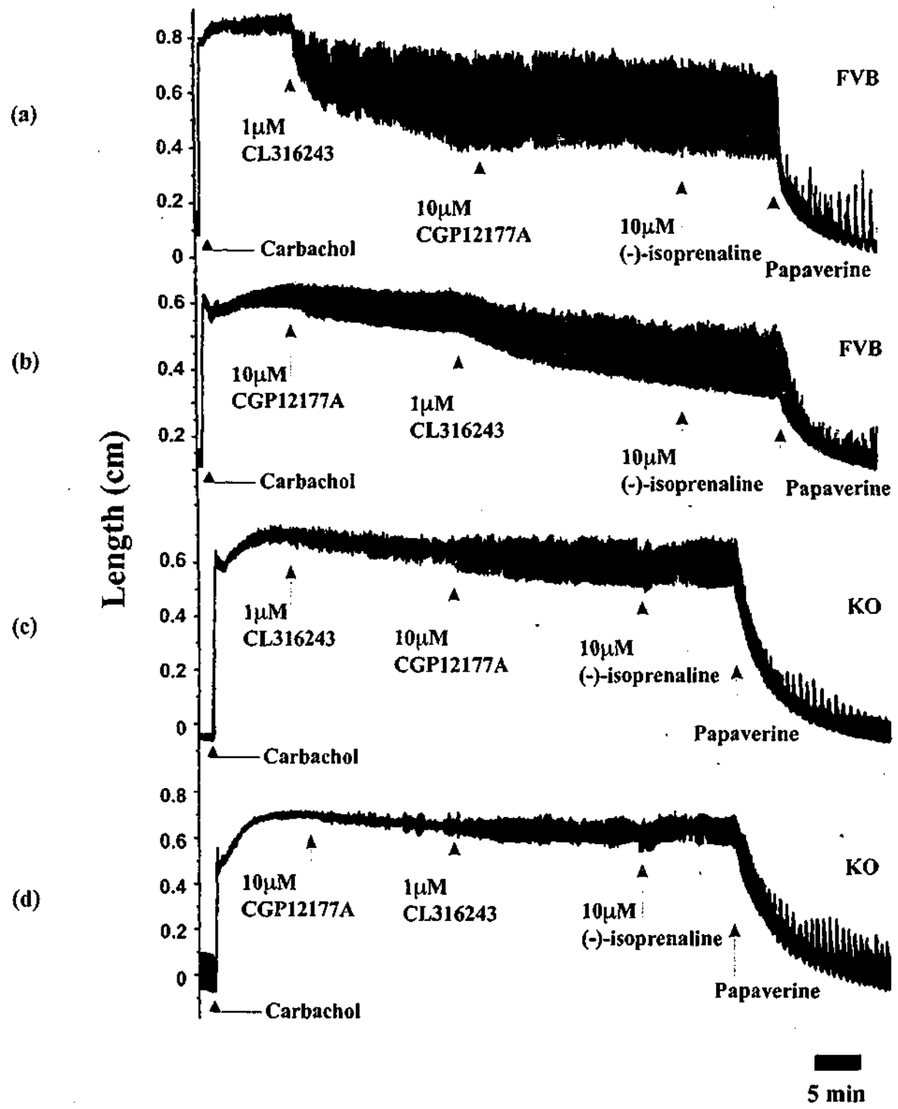
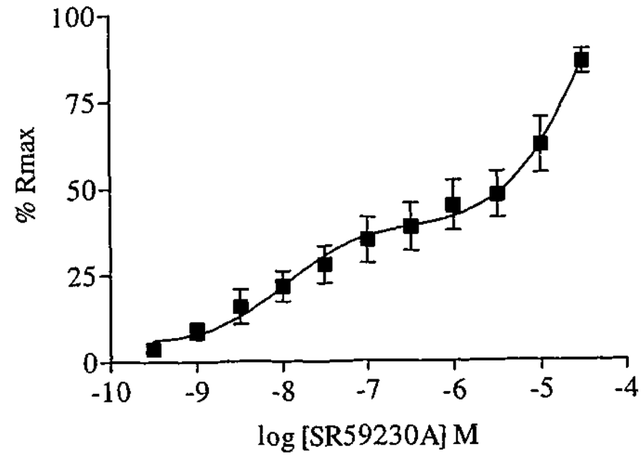


Figure 5.14: Agonist effects of SR59230A on mouse ileum from FVB mice precontracted with carbachol (1μM). Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=6).



approximately 40% of the maximum relaxation to papaverine. The second phase had an estimated pEC_{50} of 4.58 ± 0.42 (n=6) but failed to reach a maximum within the concentration range used.

5.3.3.7 Effect of forskolin and PTX on relaxation of mouse ileum smooth muscle

Forskolin produced dose-dependent relaxations in carbachol pre-contracted ileum from FVB mice (pEC_{50} 6.38 ± 0.09, n=4), with a maximal relaxation response of 91.6 ± 5.7% compared to the papaverine response (Figure 5.15).

Preincubation of ileum from FVB mice with PTX (500ng ml^{-1}, 2h) produced a significant increase (26%) in the maximum relaxation of the smooth muscle to CL316243 without any significant changes in pEC_{50} values (control: pEC_{50} 8.35 ± 0.36, R_{max} 67.9 ± 8.8%; + PTX: pEC_{50} 7.94 ± 0.28, R_{max} 93.4 ± 6.6%; n=4-6; 2-way ANOVA *** $p<0.001$) (Figure 5.16).

5.3.4 cAMP accumulation levels

cAMP accumulation levels in ileum from FVB mice were significantly increased compared to basal levels (28.52 ± 1.51 pmol cAMP mg $protein^{-1}$, n=3) following forskolin (100μM; 45.00 ± 1.67 pmol cAMP mg $protein^{-1}$; **$p<0.01$, n=3), (-)-isoprenaline (10μM; 37.28 ± 1.90 pmol cAMP mg $protein^{-1}$; **$p<0.01$, n=3) or CL316243 (10μM; 37.79 ± 1.32 pmol cAMP mg $protein^{-1}$; *$p<0.05$, n=3) (Figure 5.17). There was no significant difference in the level of cAMP measured following CL316243 or (-)-isoprenaline treatment (Student's t-test ns). However, both these treatments were significantly different (* $p<0.05$ Student's t-test) from the forskolin response, with only approximately half as much cAMP measured following CL316243 or (-)-isoprenaline treatment compared to forskolin treatment.

Figure 5.15: Forskolin-mediated relaxation of ileum from FVB mice precontracted with carbachol (1μM). Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=4).

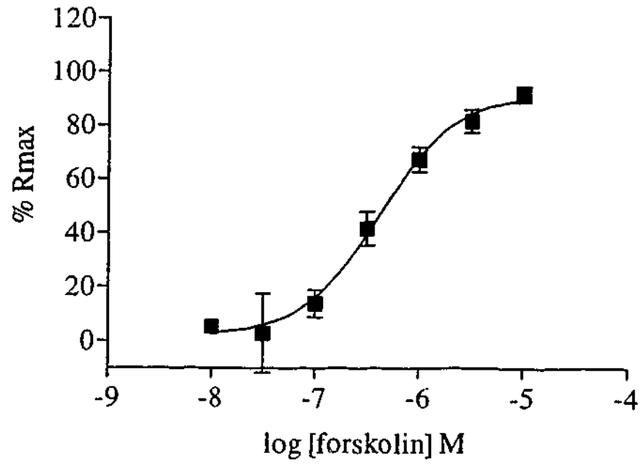


Figure 5.16: PTX treatment (2 h, 500ng ml^{-1}) significantly increased relaxation responses to CL316243 in ileum from FVB mice in tissues precontracted with carbachol (1μM). Points are expressed as a percentage of R_{max} to papaverine (10μM) and show mean ± s.e.mean (n=4-6).

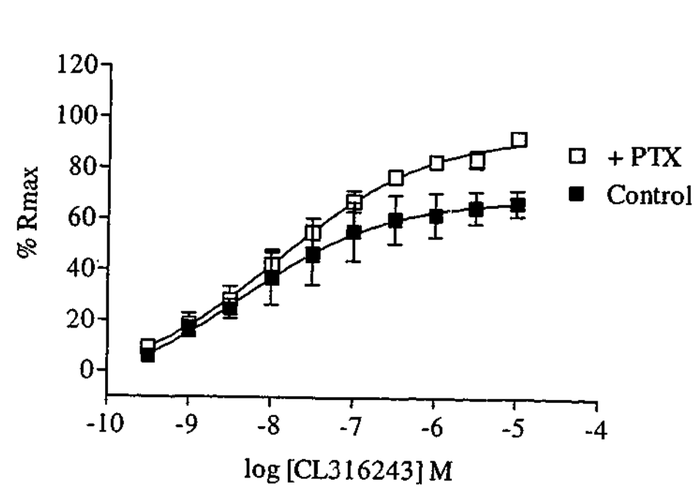
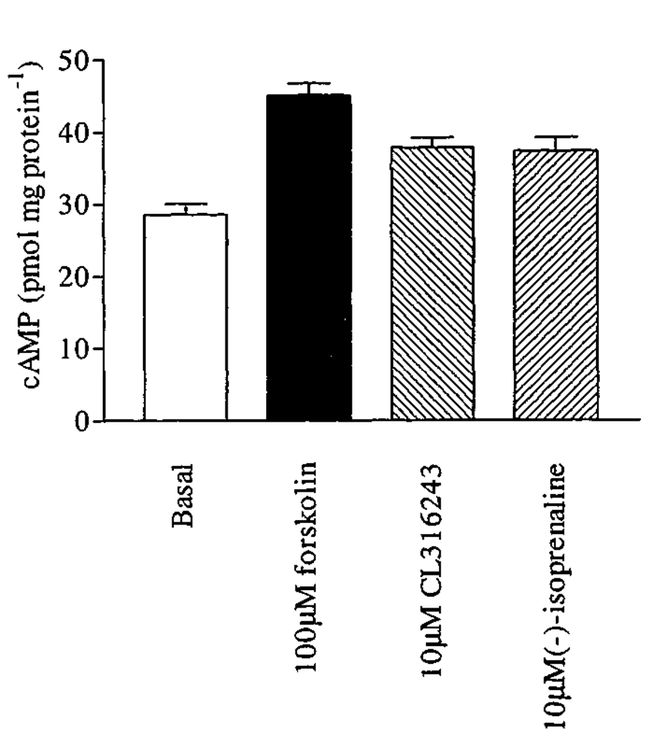


Figure 5.17: Forskolin, CL316243 and (-)-isoprenaline increased cAMP accumulation levels in FVB mouse ileum segments. Data is presented as pmol cAMP accumulated mg protein^{-1}. Bars show mean ± s.e.mean (n=3).



5.3.5 CL316243 mediated effects on basal levels of Erk1/2 in mouse ileum

CL316243 (10μM) treatment of mouse ileum segments resulted in a 2.5 fold increase in Erk1/2 phosphorylation after 5 min treatment which was transient in nature (Figure 5.18, n=9).

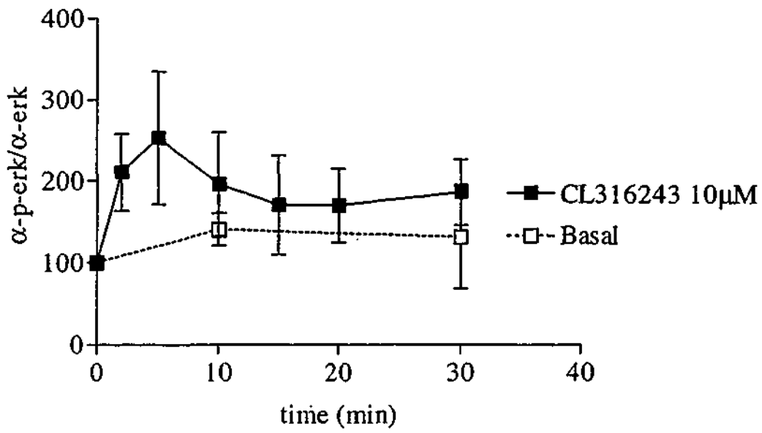
5.4 Discussion

Both β_1- and β_3-ARs mediate relaxation of mouse ileal smooth muscle in FVB mice. The relaxant effects of (-)-isoprenaline are antagonised significantly by the selective β_1- (CGP20712A) and β_3-AR (SR59230A) antagonists but weakly by the β_2-AR antagonist (ICI118551), suggesting that both β_1- and β_3-ARs, but not β_2-ARs, are involved in relaxation to (-)-isoprenaline. Responses to (-)-isoprenaline were antagonised by the β_1-/β_2-AR antagonist propranolol, the non-selective β-AR antagonist carvedilol, and the β_1-/β_2-AR antagonist (and putative β_4-AR agonist) CGP12177A. The degree of blockade caused by CGP20712A and SR59230A was similar, indicating that β_1- and β_3-ARs contribute to a similar extent to (-)-isoprenaline relaxation.

Although responses to Ro363 occurred in ileum from both FVB and β_3-AR KO mice, the slopes of c-r curves were significantly different from unity. Ro363 may exert its action on two or more β-ARs in these tissue preparations, or interact at other atypical states of the β_1-AR that have been proposed to exist (Konkar *et al.*, 2000b). Interactions at β_3-ARs seem unlikely since responses to Ro363 were identical in both FVB and β_3-AR KO ileum.

The pK_B values observed in ileum from FVB mice with propranolol (7.7) were higher than the values found in studies in rat distal colon (6.6; McLaughlin & MacDonald. 1990), ileum (6.8; Roberts *et al.*, 1999) and gastric fundus (6.3; McLaughlin & MacDonald, 1991), but not other studies where higher pK_B values have been reported (8.4, guinea pig taenia caecum (Koike *et al.*, 1995b); 8.2 & 8.5, rat colon (Bianchetti & Manara, 1990; Croci *et al.*, 1988)). In rat colon, there is a

Figure 5.18: Time course for CL316243-induced Erk1/2 phosphorylation. CL316243 (10μM) was added to FVB mouse ileum segments at time 0. Values are expressed as percentage increase over control at time zero. Values are mean ± s.e.mean (n=9).



mixture of β-ARs since in studies where higher pA_2 values for propranolol are reported, Schild plot slopes well below unity were found, suggesting the presence of more than one β-AR (Bianchetti & Manara, 1990; Croci *et al.*, 1988; McLaughlin & MacDonald, 1990). Under β_1-/β_2-AR blockade, a pK_B value of 6.2 for propranolol was found, consistent with its actions on β_3-ARs (McLaughlin & MacDonald, 1990; 1991; Roberts *et al.*, 1999). Other studies of antagonism of (-)-isoprenaline by propranolol show higher pK_B values in guinea pig atria (β_1-AR) (pA_2 8.7-8.9), trachea (β_2-AR) (pA_2 8.3) and diaphragm (β_2-AR) (pA_2 9.2) (Harms *et al.*, 1977), all tissues with predominantly β_1- or β_2-AR mediated responses. Antagonism with CGP20712A also revealed a pK_B value of 8.1 which was lower than that at cardiac β_1-ARs (pA_2 9.0; Molenaar & Summers, 1987) but higher than that at atypical β-ARs (pA_2 4.1-4.6 (Hollenga & Zaagsma, 1989; Van Liefde *et al.*, 1993)). Studies carried out in rat ileum, distal colon and guinea pig colon (De Ponti *et al.*, 1995; MacDonald & Lamont, 1993; Roberts *et al.*, 1999) demonstrated no shift with CGP20712A. Carvedilol antagonised (-)-isoprenaline responses in FVB ileum with a pK_B (8.4) similar to that observed in ferret myocardium (pK_B 8.1; Lowe *et al.*, 1999). This data taken together suggests that a significant β_1-AR component exists in mouse ileum, as evidenced by the higher than expected pK_B values obtained from antagonism of (-)-isoprenaline by propranolol and CGP20712A at β_3-ARs, and the similarity of the pK_B values to those reported in tissues rich in β_1-ARs.

Stereoselectivity at β_3-ARS in FVB ileum for the enantiomers of propranolol and tertatolol against (-)-isoprenaline mediated relaxations were evident in this study, with stereoselectivity indices of 1.3 and 1.4 respectively, similar to other studies (Bojanic *et al.*, 1985; Harms, 1976; Harms *et al.*, 1977; Roberts *et al.*, 1999).

A functional role of the β_3-AR in mouse ileum was also confirmed in this study, as responses were produced to the β_3-AR agonist CL316243 which were antagonised by SR59230A but not by propranolol or carvedilol. Affinity values for antagonism by SR59230A were consistent with previous reports (this study pK_B 8.3, in rat colon pA_2 8.1 (Manara *et al.*, 1996)). Carvedilol was investigated since it was reported to have high affinity at the cloned human β_3-AR (pK_i 9.4; Candelore *et al.*, 1999). However in the present study it had no antagonist action against responses to

CL316243 in mouse ileum. Propranolol resistance of β_3-AR mediated responses has been used in many studies of gastrointestinal smooth muscle to demonstrate the presence of atypical β-ARs (Bianchetti & Manara, 1990; De Ponti *et al.*, 1995; McLaughlin & MacDonald, 1991), and this was confirmed in this study where propranolol failed to antagonise responses to CL316243. In the present study no responses to CL316243 were observed in ileum from KO animals. This is supported by other studies using β_3-AR KO animals where responses to CL316243 were abolished in stomach fundus (Cohen *et al.*, 2000), colon (Oostendorp *et al.*, 2000), adipose tissue (Grujic *et al.*, 1997; Preitner *et al.*, 1998; Susulic *et al.*, 1995) and there was also evidence for decreased gastrointestinal motility (Fletcher *et al.*, 1998).

Radioligand binding studies identified a population of binding sites in FVB ileum with characteristics similar to those displayed by β_3-ARs in other studies. Saturation studies revealed a low affinity ICYP site (K_D 886pM) similar to K_D values for ICYP at the cloned mouse β_3-AR (880pM; Nahmias *et al.*, 1991) and in rat ileum (1.29nM; Roberts *et al.*, 1995). Saturation studies also revealed a high affinity ICYP site in both FVB and β_3-AR KO ileum (K_D 45 and 57pM respectively), which closely correlates with high affinity ICYP binding in rat colon (K_D 75pM; Ek & Nahorski, 1986), guinea-pig atria and uterus (K_D 22-40pM; Engel *et al.*, 1981; McPherson *et al.*, 1984). Competition studies at the low affinity ICYP site in FVB ileum showed a site with characteristics similar to β_3-ARs. Competition studies with β-AR antagonists showed that (-)-propranolol, ICI118551 and CGP20712A competed for binding with affinities lower than expected for β_1-/β_2-ARs, but similar to those reported in rat ileum (Roberts *et al.*, 1995). SR59230A had the highest affinity for the selection of β-AR antagonists examined. However, the affinity of SR59230A (pK_i 5.54) was much lower than would be expected (8.4 at cloned human β_3-AR (Candelore *et al.*, 1999); 7.2-7.3 at cloned mouse β_{3a}- and β_{3b}-AR, Chapter 3), and may reflect binding to other sites such as β_1- or β_2-ARs (pK_i 8.4 and 9.3 respectively at cloned human receptors (Candelore *et al.*, 1999)) or could be due to ICYP binding to the other site in gut described by Sugasawa *et al.* (1997). This site may have very low affinity for the β-AR antagonists and cause a low pK_i value. The β-AR agonists (-)-isoprenaline and CL316243, but not BRL37344, competed weakly with the low affinity ICYP binding site in FVB ileum. The lower affinity displayed by agonists in

receptor binding assays compared to their effect in functional assays is not uncommon since agonist binding prefers high affinity states acquired in functional studies. Receptors in binding studies tend to be predominantly in the low affinity sate (Kent *et al.*, 1980) and can be affected by the assay buffer (McPherson *et al.*, 1985). CGP12177A, a β_1-/β_2-AR antagonist and β_3-AR agonist, had the highest affinity of β-AR agonists examined, but its higher affinity may be due to interactions at other β-ARs or different affinity states of the β_1-AR.

A role for β_1- and β_3-ARs is supported by the presence of mRNA for both receptors in ileum. In contrast, although β_2-AR mRNA was present, relaxation responses to (-)-isoprenaline were not antagonised by the β_2-AR antagonist ICI118551 in FVB mice. Despite other *in vitro* studies showing a minor β_2-AR mediated relaxation in mouse colon (Oostendorp *et al.*, 2000), rat distal colon, and jejunum (MacDonald & Lamont, 1993; Van der Vliet *et al.*, 1990), and *in vivo* studies in rat duodenum and jejunum showing (-)-isoprenaline and ritodrine can disrupt migrating myoelectric complexes, an effect which is blocked by ICI118551 (Thollander *et al.*, 1996), other studies in rat ileum show that despite β_2-AR mRNA being present, no β_2-AR mediated relaxation responses could be found (Roberts *et al.*, 1999). C-r curves to zinterol had slopes different from unity, suggesting interactions with multiple β-AR subtypes. Since zinterol c-r curves were identical in FVB and β_3-AR KO ileum, zinterol may exert its actions at both β_1- and β_2-ARs in these preparations since zinterol can act through β_1-ARs, albeit mainly in cardiac tissues (Freyss-Beguin *et al.*, 1983; Juberg *et al.*, 1985; Kuznetsov *et al.*, 1995). β_2-ARs may play roles other than relaxation in mouse ileum. β-AR agonists (isoprenaline, AD and terbutaline) increase glucagon-like peptide-1 and peptide YY secretions in rat ileum, an effect which is antagonised by propranolol and likely to be mediated by β_2-AR activation (Claustre *et al.*, 1999; Dumoulin *et al.*, 1995).

β_1-ARs appear to compensate for the lack of β_3-ARs in ileum from β_3-AR KO mice. There was increased antagonism of (-)-isoprenaline mediated relaxation by CGP20712A, propranolol and carvedilol in ileum from β_3-AR KO as compared to FVB mice. The increase in pK_B values for these agents suggests a greater

contribution of β_1-ARs to the response. There may also be a slight increase in β_2-AR function in β_3-AR KO mice since while responses to (-)-isoprenaline were not antagonised by ICI118551 in FVB mice (pK_B 7.0), there was a somewhat greater shift in β_3-AR KO mice (pK_B 7.3), despite no increase in β_2-AR mRNA levels. Compensation by β_1-ARs was associated with an increase in receptor density and β_1-AR mRNA levels. Interestingly, this parallels the increase in β_1-AR mRNA levels seen in adipose tissue in this strain of β_3-AR KO mice (Grujic *et al.*, 1997; Susulic *et al.*, 1995) where these animals compensate for the lack of β_3-ARs in adipose tissue by up-regulation of β_1-AR mRNA and function. Studies with another strain of β_3-AR KO mice on a 129Sv+C57Bl/6 mouse background which have lower levels of β_1-AR mRNA in BAT but not WAT (Revelli *et al.*, 1997), show that in mouse colon, while β_1-ARs also compensate for the lack of β_3-ARs functionally, no differences in β_1-AR mRNA levels were observed in 129Sv+C57Bl/6 and β_3-AR KO ileum (Oostendorp *et al.*, 2000). These authors suggested that in the β_3-AR KO animals, existing β_1-ARs may become coupled more strongly to signalling pathways governing gastrointestinal relaxation. This difference may be a result of the different strain backgrounds of the mice used, the knock-out strategy used or other unknown variables. Nevertheless, both studies show several similar findings, including β_3-AR KO animals showing slowly developing mild increases in body fat content, with no associated increase in food intake, and no changes in circulating levels of insulin, glucose or free fatty acids.

Another interesting feature of the (-)-isoprenaline mediated relaxation in β_3-AR KO ileum is that antagonism by SR59230A was still observed, although much weaker than that in FVB mice (although the pK_B values obtained were not statistically different (Student's t-test ns)). This would suggest that SR59230A may not be as specific for the β_3-AR as previously thought (Manara *et al.*, 1996). There was apparent selectivity of SR59230A for β_3-ARs observed in tissues where β_3-ARs are the predominate β-AR subtype, with a pA_2 of 8.8 and a pK_B value of 8.9 in rat colon and brown adipocytes respectively (Manara *et al.*, 1996; Nisoli *et al.*, 1996), similar to values observed here with either CL316243 or (-)-isoprenaline as the agonist (pK_B 8.3 and 8.0 respectively) in FVB mice. However, the potent antagonism of

SR59230A has not been shown in other studies (Boorman *et al.*, 2001; Galitzky *et al.*, 1997; Kaumann & Molenaar, 1996). In β_3-AR KO mice, a pK_B value against (-)-isoprenaline of 7.4 was observed, in comparison with a pA_2 value of 7.3 observed at β_1-ARs in guinea pig atria against (-)-isoprenaline mediated effects (Manara *et al.*, 1996). A recent study has suggested that in cloned human β-ARs, SR59230A displays little selectivity in binding for the β_3-AR compared to β_1- or β_2-ARs (Candelore *et al.*, 1999). This may suggest that SR59230A is not as selective as previously reported and may also interact with β_1-ARs but this needs further investigation.

SR59230A also caused a concentration-dependent relaxation of FVB ileum that was biphasic in nature. Other studies have illustrated agonistic actions of SR59230A in tissues (Brahmadevara *et al.*, 2001; Dumas *et al.*, 1998; Horinouchi & Koike, 2001a) and at the cloned mouse β_3-AR (Chapter 3). The mechanism of SR59230A's agonistic actions has not been further studied here and clearly warrants investigation since SR59230A is typically used to define the presence of functional β_3-ARs.

CGP12177A is a high affinity β_1-/β_2-AR antagonist (Staehelin & Hertel, 1983) which also shows partial agonist activity at β_3-ARs in adipose tissues (Mohell & Dicker, 1989). It has also been used as the prototype partial agonist at another site termed the putative β_4-AR in the heart of several species including man and mouse (Kaumann, 1996; Kaumann & Molenaar, 1997; Molenaar *et al.*, 1997b), as well as in mouse and human adipocytes (Galitzky *et al.*, 1997; Preitner *et al.*, 1998). In cardiac tissues, CGP12177A and other non-conventional partial agonists cause antagonism of responses mediated by β_1- and β_2-ARs but at higher concentrations has agonist effects that are resistant to blockade by propranolol. These effects are not be mediated by β_3-ARs since the heart does not express this subtype (Evans *et al.*, 1996) and CGP12177A still produces these effects in heart from β_3-AR KO mice (Kaumann *et al.*, 1998). In tissues that do express β_3-ARs, such as rat colon or guinea-pig taenia caecum, CGP12177A can act as an agonist (Kaumann & Molenaar, 1996; Koike *et al.*, 1995b; 1996; Molenaar *et al.*, 1997a; Sennitt *et al.*, 1998). Support for the β_4-AR concept came from studies showing that CGP12177A activated brown adipose tissue and cardiac responses in β_3-AR KO mice (Kaumann

et al., 1998; Preitner *et al.*, 1998). In β_3-AR KO mice, CGP12177A has agonist actions in atria (Cohen *et al.*, 2000), adipose tissue (Preitner *et al.*, 1998) and oesophageal and colonic smooth muscle (Oostendorp *et al.*, 2000). However it is clear that CGP12177A effects at the 'putative β_4-AR' only occur in tissues (heart and adipose) that express high levels of β_1-AR, and more tellingly also in cultured cells over expressing the β_1-AR (Pak & Fishman, 1996). It is now clear from a recent study in β_1- or β_3-AR KO mice, that β_1-ARs mediate most, if not all, of the β_3-AR independent effects of CGP12177A on brown adipocyte AC activity (Konkar *et al.*, 2000a). In the present study in mouse ileum, no agonist actions of CGP12177A were observed in either β_3-AR KO or FVB ileum, even at concentrations of 10μM, indicating no agonist actions at either β_3- or β_1-ARs, despite several attempts to show agonist effects with different batches of drug and different stock solutions made up in different solutions (data not shown). Instead, CGP12177A acted as a potent antagonist of (-)-isoprenaline mediated relaxations with pK_B values of 9.6 in β_3-AR KO and 8.6 in FVB ileum. These values are much higher than those reported in rat ileum (7.6; Roberts *et al.*, 1999) and consistent with those in guinea pig taenia caecum (9.3; Koike *et al.*, 1996). The findings are in accord with an action of CGP12177A to block β_1-ARs. The pK_B for CGP12177A would be expected to increase in β_3-AR KO mice even if β_1-ARs were not expressed at higher levels. The pK_B value of 8.6 in FVB mice would suggest antagonism at both β_1- and β_3-ARs, whereas the pK_B of 9.6 in β_3-AR KO mice would be due to blockade at β_1-ARs only.

The signaling pathways activated following β_3-AR stimulation have primarily been assessed in adipose tissue or cells transfected with the β_3-AR. There has been little focus on the pathways activated following β_3-AR stimulation in gastrointestinal tissues, where β_3-ARs mediate smooth muscle relaxation in a wide variety of species (for review, refer to Manara *et al.* (1995)). Relaxation of gastrointestinal smooth muscle is primarily mediated by cAMP or cGMP (predominantly through activation of Gαs). Targets for cAMP and cGMP are proposed to include inhibition of PLC, Raf, myosin light chain kinase, IP_3 receptor and Rho kinase, and the promotion of uptake of calcium to the sarcoplasmic reticulum. Contraction of gastrointestinal smooth muscle is primarily due to agents that increase intracellular calcium

predominantly through Gαi/o and Gαq G-proteins (for reviews on gastrointestinal signaling, refer to Abdel-Latif (2001) and Makhlouf & Murthy (1997)).

Studies to date have shown that β_3-AR activation results in increases of cAMP (Koike *et al.*, 1995a; 1995b) and that the relaxation response to CL316243 in rat ileum is enhanced following PTX treatment, indicating coupling of the β_3-AR to Gαi (Roberts *et al.*, 1999). This was further supported in this study where forskolin and CL316243 both caused relaxation of carbachol contracted mouse ileum in a concentration-dependent manner, which was associated with increases in cAMP accumulation levels. PTX treatment increased relaxation responses to CL316243 in tissues precontracted with carbachol. This suggests that in mouse ileum, the β_3-AR couples to both Gαi and Gαs. The relative contribution of the two mouse splice variants to this cannot be easily assessed since they share similar pharmacological profiles (Chapter 3). While the β_{3a}- and β_{3b}-AR both couple to Gαs, only the β_{3b}-AR appeared to couple to Gαi in transfected CHO-K1 cells (Chapter 4). However no conclusions can be drawn to the relative importance of the two splice variants in conferring Gαi coupling in mouse ileum since several studies have illustrated that coupling of a receptor to multiple effector pathways is dependent on the different properties of the cell in which the receptor is expressed. (Law *et al.*, 1997; Logsdon, 1999; Perez *et al.*, 1993; Zambon *et al.*, 2000) i.e. results reported in one cell model cannot automatically be applied to other cell models.

The effect of PTX on CL316243-mediated relaxations is probably not through any PTX actions on the contraction to carbachol. Radioligand binding and mRNA studies show the presence of a large population of M_2 receptors and a smaller population of M_3 receptors in gastrointestinal smooth muscle (Giraldo *et al.*, 1988; Maeda *et al.*, 1988; Michel & Whiting, 1988a; 1988b; Sales *et al.*, 1997). M_3 receptors are coupled to Gαq to activate PLC and IP_3 production while M_2 receptors are coupled to Gαi/o to inhibit AC activity (reviewed in Eglen (2001)). The action of carbachol is through the M_3 (and not M_2) receptor, to stimulate PI hydrolysis and activation of PLC to contract gastrointestinal smooth muscle (Honda *et al.*, 1993; Konno & Takayanagi, 1989; Matsui *et al.*, 2000). Therefore since carbachol mediates its contractile effects through M_3 receptors, it is unlikely that PTX would affect contractions to carbachol.

cAMP has dual effects on cellular proliferation which are dependent upon cellular background. In various cells (including epithelial, heapocyte, keratinocyte, pancreatic β islet, Schwann and Swiss 3T3 cells), cAMP stimulates cellular proliferation (Dumont *et al.*, 1989). However, in normal fibroblasts, smooth muscle, lymphoid, neuronal and glial cells, cAMP inhibits cellular proliferation (Blomhoff *et al.*, 1988; Dugan *et al.*, 1999; Hollenberg & Cuatrecasas, 1973; Mark & Storm, 1997; Nilsson & Olsson, 1984) through cAMP-dependent inactivation of the Erk1/2 pathway (Dugan *et al.*, 1999; Hordijk *et al.*, 1994; Schmitt & Stork, 2001).

In the present study, β_3-AR stimulation resulted in a time-dependent increase of Erk1/2 phosphorylation levels in mouse ileum. This is the first report of β_3-AR activation of Erk1/2 in gastrointestinal smooth muscle, since all other studies have been conducted in transfected cell line or adipocytes (Chapter 4; Cao *et al.*, 2000; Gerhardt *et al.*, 1999; Lindquist *et al.*, 2000; Mizuno *et al.*, 1999; 2000; Soeder *et al.*, 1999). However, activation of β-ARs in cat iris sphincter or bovine tracheal smooth muscle with isoprenaline inhibits basal Erk1/2 levels and Erk1/2 levels that are stimulated by calcium mobilising agents (Koch *et al.*, 2000). The mechanism of the activation of Erk1/2 in mouse ileum has not been further elucidated and further studies here are needed to determine the exact mechanism of this action.

In conclusion, in mouse ileum, β_3-ARs and β_1-ARs mediate smooth muscle relaxation, with no apparent β_2-AR involvement. In β_3-AR KO mice, β_1-ARs functionally compensate for the lack of β_3-ARs, supported by increased β_1-AR mRNA and levels of binding. CGP12177A acted as an antagonist in this preparation with no agonist actions and SR59230A appears not to be as selective for the β_3-AR as previously reported.

Chapter 6

6.1 General Discussion

The β_3-AR has been cloned from several species, including human (Emorine *et al.*, 1989), rat (Bensaid *et al.*, 1993; Granneman *et al.*, 1991; Muzzin *et al.*, 1991) and mouse (Nahmias *et al.*, 1991). In rodents, β_3-ARs are expressed in adipose tissues (both white and brown adipocytes) where they mediate lipolysis and thermogenesis, and in gastrointestinal tissues (including ileum and colon) where they mediate smooth muscle relaxation. An additional level of complexity was introduced with the discovery of a splice variant of the mouse β_3-AR, termed the β_{3b}-AR (Evans *et al.*, 1999). This receptor has a unique C-terminal tail compared to the known β_{3a}-AR. There is differential expression of the two isoforms in mouse tissues, with the lowest proportion of β_{3b}-AR transcripts in BAT, and the highest in hypothalamus. The work presented in this thesis examines the pharmacological properties and signalling pathways utilised by the mouse β_{3a}- or the β_{3b}-AR expressed in CHO-K1 cells using a range of selective pharmacological tools in binding and functional studies to determine whether the mouse β_3-AR splice variants can be discriminated on a pharmacological basis. The role of the β_3-AR in mouse ileum was further assessed by the use of mice lacking the β_3-AR.

The primary focus of the work presented in this thesis was to compare the properties of the two mouse β_3-AR splice variants expressed in CHO-K1 cells. There was no difference in the affinity of β-AR ligands between the β_{3a}- and β_{3b}-AR expressed in CHO-K1 cells, consistent with the isoforms differing at the C-terminus and not in the transmembrane regions that are believed to form the β_3-AR ligand-binding pocket (Granneman *et al.*, 1998; Gros *et al.*, 1998; Guan *et al.*, 1995). The rank order of potency of β-AR agonists assessed using the cytosensor microphysiometer was identical in cells expressing either receptor at high levels. However, the potency and efficacy of the β_3-AR selective agonist CL316243 was reduced with levels of expression for both receptors as assessed by cAMP accumulation and cytosensor microphysiometer studies, consistent with standard drug-receptor interaction models, both in theoretical (Kenakin, 1995a) and practical applications (Cordeaux *et al.*, 2000; McDonnell *et al.*, 1998; Wilson *et al.*, 1996). This shows that there is no basis for the discrimination between the mouse β_3-AR splice variants based on

pharmacological properties, and therefore it is not possible to directly study the consequence of the presence of both receptors in native tissues due to the lack of differences in pharmacological profiles. This study also highlights the importance of expression levels in determining the efficacy and potency of β-AR ligands at the cloned mouse splice variants. Most studies using recombinant systems generally over-express the receptor to levels well beyond those encountered in native tissues. Hence this study highlights the need to also express recombinant receptors at physiologically relevant levels and examine more than one level of expression.

One of the main criteria that characterise β_3-ARs has been the relatively low level of stereoselectivity for β-AR ligands. Work presented in this thesis indicates that there is greater stereoselectivity for the enantiomers of several β-AR ligands than previously reported in cells expressing either the β_{3a}- or the β_{3b}-AR (Chapter 3). However, the stereoselectivity observed for the enantiomers of propranolol and tertatolol in antagonising isoprenaline-mediated relaxation in mouse ileum (Chapter 5) was not as great as those at the recombinant receptor. Such discrepancies further highlight the importance of examining receptors not only in recombinant systems, but also in tissues that endogenously express the receptor.

Another criterion for the pharmacological characterisation of the β_3-AR is antagonism by the β_3-AR antagonist SR59230A. This drug was originally reported to be selective for antagonism of β_3-AR-mediated effects rather than effects mediated by β_1- or β_2-ARs (Manara *et al.*, 1996; Nisoli *et al.*, 1996). However, the work presented in this thesis highlights the need for close examination of the effects of SR59230A at the mouse β_3-AR. In CHO-K1 cells expressing the β_3-AR, SR59230A exhibited both antagonistic and agonistic properties (Chapter 3). Agonist properties of SR59230A were more evident in cells expressing higher levels of receptor as assessed by both cytosensor microphysiometer and cAMP accumulation. The antagonist activity of SR59230A was weak in high expressing cells in comparison to medium or low expressing cells. The intrinsic activity of SR59230A was equal to that of the β_3-AR agonist CL316243 in the cytosensor microphysiometer, but in cAMP accumulation studies, SR59230A was only a partial agonist relative to responses produced by CL316243. This may suggest that the β_3-AR can couple to

pathways other than AC. This is probably not due to promiscuous coupling of the receptor, which occurs for other receptor systems (Cordeaux *et al.*, 2000; Eason *et al.*, 1992; Kenakin, 1995a; 1995b), since agonist actions of SR59230A have been observed in mouse ileum (Chapter 5) and in other tissues endogenously expressing the β_3-AR (Brahmadevara *et al.*, 2001; Dumas *et al.*, 1998; Horinouchi & Koike, 2001a). Further investigation of the agonist properties of SR59230A is needed before the actions of the drug can be fully elucidated.

It is also worthy of note that the agonistic actions of SR59230A were more evident in the cytosensor microphysiometer, than in cAMP accumulation assays. The cytosensor microphysiometer measures the whole cellular response following receptor activation, and not just one pathway, as in the case of cAMP. Previous studies of β_3-AR antagonists have been primarily performed using cAMP assays. However, the cytosensor revealed anomalous behaviour of SR59230A that was not originally observed with cAMP assays (SR59230A inhibition of CL316243-stimulated cAMP accumulation). Hence the cytosensor microphysiometer has proved to be a valuable tool for the study of drug-receptor interactions.

In addition to the agonistic properties of SR59230A, this drug may not be as selective for the β_3-AR as previously reported. This was shown in several other studies (Boorman *et al.*, 2001; Brahmadevara *et al.*, 2001; Candelore *et al.*, 1999; Louis *et al.*, 2000). In this thesis, pK_B values for antagonism of β_3-AR-mediated effects at the cloned receptor (Chapter 3) and at the endogenous receptor in mouse ileum (Chapter 5) were consistent with previous reports (7.95 and 8.23 in low and medium expressing cells against CL316243-mediated increases in extracellular rates; 7.24 and 7.52 in low and medium expressing cells against CL316243-mediated increases in cAMP levels; 8.0 and 8.3 in FVB mouse ileum against (-)-isoprenaline and CL316243-mediated relaxations respectively). In mice lacking the β_3-AR, SR59230A was still effective in antagonising (-)-isoprenaline mediated relaxation of ileal smooth muscle (pK_B 7.4). This would suggest that SR59230A might not be as specific for the β_3-AR as previously suggested (Manara *et al.*, 1996).

Another major aim of this thesis was to determine the functional roles of the β_3-AR in mouse ileum, using a transgenic mouse model (Chapter 5). In mice lacking the β_3-AR, responses to the β_3-AR agonist CL316243 were abolished, as reported in other studies using β_3-AR KO animals (Cohen *et al.*, 2000; Grujic *et al.*, 1997; Oostendorp *et al.*, 2000; Preitner *et al.*, 1998; Susulic *et al.*, 1995). β_1-ARs appear to compensate for the lack of β_3-ARs in ileum from β_3-AR KO mice and this was supported functionally by the increased antagonism of (-)-isoprenaline mediated relaxation by the β_1-AR antagonist CGP20712A and also by the non-selective β-AR antagonists (-)-propranolol and carvedilol (no functional role for β_2-ARs was evident in FVB or β_3-AR KO mouse ileum). This compensation was associated with an increase in ICYP binding to a β_1-/β_2-AR site and increased mRNA levels for the β_1-AR. β_3-ARs may therefore be functionally relevant for relaxation of mouse ileum since in β_3-AR KO mice, β_1-ARs compensate for their absence.

In CHO-K1 cells transfected with the mouse β_3-AR splice variants, there was a larger maximal response produced at the β_{3a}-AR than at the β_{3b}-AR, even though the receptors were expressed at similar levels (Chapter 3). This indicated that differential coupling of each receptor to intracellular pathways, rather than differences in expression levels, were responsible for the observed difference in maximal response. β_3-ARs are known to couple to both Gαs and Gαi (Chaudhry *et al.*, 1994; Lindquist *et al.*, 2000; Soeder *et al.*, 1999). Work presented in this thesis shows that CL316243-mediated cAMP accumulation or cytosensor microphysiometer responses are increased following PTX treatment only in cells expressing the β_{3b}-AR, but not those expressing the β_{3a}-AR (Chapter 4). Promiscuous second messenger coupling can occur at high receptor expression levels, but this is unlikely to be a factor in cells expressing lower levels of receptor similar to physiological levels. This suggests that, at least in CHO-K1 cells expressing the splice variants, that both receptors couple to Gαs, and only the β_{3b}-AR couples to Gαi. In mouse ileum however, PTX treatment increased responses to CL316243, suggesting that at least some of the β_3-AR in this tissue couple to both Gαs and Gαi (Chapter 5). In mouse ileum, the β_{3a}-AR is the predominant receptor expressed. The results obtained in the recombinant system and that in a native tissue may be explained by (a) the cell type involved can

greatly influence second messenger coupling (Law *et al.*, 1997; Logsdon *et al.*, 1999; Perez *et al.*, 1993; Zambon *et al.*, 2000) or (b) that the β_{3b}-AR is strongly coupled to Gαi in ileum, even though it makes up only approximately 20% of the total β_3-AR mRNA levels (Evans *et al.*, 1999). However, investigation of the turnover and synthesis rates of β_{3a}- and β_{3b}-AR proteins has not been conducted and may result in a different ratio of proteins to mRNA levels.

Recent studies have illustrated that the β_3-AR agonist treatment of cells expressing the β_3-AR results in Erk1/2 activation (Cao *et al.*, 2000; Gerhardt *et al.*, 1999; Lindquist *et al.*, 2000; Mizuno *et al.*, 1999; 2000; Soeder *et al.*, 1999), although the mechanism of this activation varies considerably (Chapter 1). In this thesis, stimulation of CHO-K1 cells expressing either of the mouse splice variants with CL316243 increased Erk1/2 phosphorylation. The activation of Erk1/2 was not dependent on a cAMP-PKA or a PTX-sensitive pathway, but was dependent on interactions with Src, PI3K and possibly a TKR (Chapter 4). This mechanism of Erk1/2 activation differs from that described in other β_3-AR systems (Cao *et al.*, 2000; Gerhardt *et al.*, 1999; Lindquist *et al.*, 2000; Mizuno *et al.*, 1999; 2000; Soeder *et al.*, 1999), and illustrates that activation of Erk1/2 is a complex process modulated by multiple signalling proteins that may differ between cell types.

The studies presented in this thesis have utilised several different methods to pharmacologically characterise the mouse β_{3a}- or β_{3b}-AR expressed in CHO-K1 cells, and to assess the relevance of the β_3-AR in mouse ileum using β_3-AR KO mice. The work described in this thesis has revealed several important findings: (a) the mouse β_3-AR splice variants share similar pharmacological properties and therefore cannot be discriminated pharmacologically in tissues that coexpress both receptors (although there may be different regulatory properties that have yet to be described); (b) both receptors couple to Gαs, but only the β_{3b}-AR appears to couple to Gαi; (c) the mechanism of Erk1/2 activation by the mouse splice variants is identical and independent of the receptor coupling to Gαs or Gαi, but appears to involve a mechanism that includes activation of PI3K and Src; (d) the β_3-AR antagonist SR59230A appears not to be as selective for the β_3-AR as previously thought, and exhibits both agonistic and antagonistic actions at the cloned mouse

receptor and that endogenously expressed in mouse ileum; (e) the β_1-AR appears to compensate for the lack of β_3-ARs in ileum from β_3-AR KO mice.

Chapter 7

7

References

7.1 References

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