Regulation of β-adrenoceptor function:a study of the three subtypes.

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by

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Note: The opinions expressed in this thesis are not necessarily representative of the industrial partner.

ABBREVIATIONS

α	alpha subunit of G proteins
αMEM	alpha modified Eagle's medium
ADP	adenosine-5'-diphosphate
Arg	arginine
ATP	adenosine-5'-triphosphate
BAAM	bromoacetyl alprenolol menthane
βARK	beta-adrenergic receptor kinase
β_1	beta-1
β_2	beta-2
β_3	beta-3
β	beta subunit of G proteins
B_{max}	maximum binding sites
$\beta\gamma$	beta-gamma subunit of G proteins
BSA	bovine serum albumin
cAMP	adenosine 3':5'-cyclic monophosphate
cDNA	complementary DNA
CHO	Chinese Hamster Ovary
Ci	Curie
cpm	counts per minute
C-terminus	carboxyl terminus
CYP	cyanopindolol
DHA	dihydroalprenolol
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethyl sulphoxide
dpm	disintegrations per minute

EC_{50}	concentration of agonist producing a half-maximal response
EDTA	ethylenediamine tetra-acetic acid
FBS	foetal bovine serum
γ	gamma subunit
G_i	inhibitory G-protein
G_o	G-protein of unknown function
G_s	stimulatory G-protein
$G_s\alpha$-L	G_s alpha subunit-long isoform
$G_s\alpha$-S	G_s alpha subunit-short isoform
Gly	glycine
Gpp(NH)p	guanylyl 5' imidodiphosphate
G-protein	guanine nucleotide-binding protein
GDP	guanosine 5'-diphosphate
GPCRs	G-protein coupled receptors
GRK	G-protein receptor kinase
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-(3'-O-thiotriphosphate)
HAT	hypoxanthine-aminopterin-thymidine
HCl	hydrochloric acid
HEPES	(N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
IA	intrinsic activity
IBMX	isobutylmethylxanthine
kDa	kiloDalton
K_D	dissociation constant
mA	milliAmpere
mg	milligram
mRNA	messenger ribonucleic acid

M_r	molecular weight
NAD^+	nicotinamide adenine dinucleotide
N-terminus	amino terminus
NHE	sodium hydrogen ion exchange
NIDDM	non-insulin dependent diabetes mellitus
ob	obesity gene
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCA	perchloric acid
pmol	picomoles
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
$t_{1/2}$	half-life (time)
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	transmembrane
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan

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Summary

The β-adrenoceptors are located in a number of different tissues, from vital organs such as the heart (primarily a β_1-adrenoceptor population) and lung (mainly β_2-adrenoceptors) to adipose tissue (largely β_3-adrenoceptors), and are involved in a variety of regulatory processes. They are stimulated by the endogenous agonists adrenaline and noradrenaline, resulting in a range of effects on the body via the sympathetic nervous system; these include smooth muscle relaxation (β_2-adrenoceptors) and alterations in heart rate (β_1-adrenoceptors). The β_3-adrenoceptor effects thermogenesis and lipolysis, thus offering potential opportunities for β_3-agonists to be developed as anti-obesity and anti-diabetic drugs.

In this study, my first aim was to investigate agonist-induced regulation of the β-adrenoceptors, especially at the level of G-protein down-regulation. This included investigations into a number of the desensitization processes, including sequestration, G-protein down-regulation, and G-protein mRNA level changes (Chapter 3). Some areas of receptor desensitization have been researched a great deal, while other areas, such as at the level of the G-protein, have been less thoroughly investigated. In these studies I found that G-protein down-regulation occurred upon agonist occupancy of all three β-adrenoceptor subtypes (after expression in CHO cells). This was surprising in the case of the β_3-adrenoceptor, as the receptor itself has previously been shown to be refractory to down-regulation (Chambers *et al*, 1994, Thomas *et al*, 1992). This, therefore, showed that co-down-regulation of the receptor and associated G-protein, previously thought to occur extensively, is not inextricably linked.

There has been a growing awareness in recent years of the vital importance to account for the effect of receptor density in tissues or cells under investigation,

on the potency and/or efficacy of agonists at receptors. In Chapter 4, I assessed the effects of receptor density of the β_2-adrenoceptor expressed in NCB20 cells, on the potency and efficacy of agonists such as isoprenaline, salbutamol, ephedrine and adrenaline. It was clear from my data that as receptor levels increased in the three cell lines assessed, the efficacy of the individual agonist increased also. It was also possible to observe that on increasing receptor levels, the EC_{50} value for each agonist at that cell line decreased, or that the potency was increased.

In my final results Chapter, I studied the molecular basis of pharmacological differences previously observed between the rat and human β_3-adrenoceptor. Chimeric constructs of the human β_3-adrenoceptor were produced enabling evaluation of each transmembrane region of the receptor separately. Assays employed were two different functional assays, a cAMP accumulation assay and the microphysiometer, which detected minute pH changes in cells attached to a silicon sensor in response to receptor activation. Through the two assays, I was able to produce a thorough assessment of the role of each transmembrane region in the overall pharmacological response to agonist stimulation. The region which appeared to shift the pharmacology of the receptor from that of the human β_3-adrenoceptor to that of the rat β_3-adrenoceptor was transmembrane region 2. Further investigations employing computer modelling of the β_3-adrenoceptor indicated that this region was not involved in agonist binding, but agonist stimulation must somehow activate TM2 to cause alterations in a number of other transmembrane regions, thus producing an overall conformational change in the receptor.

CHAPTER 1.
INTRODUCTION

1.1. SIGNALLING

The interest and research into signal transduction in cells has been developing rapidly over the past couple of decades. A rapid form of communication between cells is essential for responses to environmental stimuli. It is vital for the cell to develop some form of communication for the rapid propagation of intercellular signals. In the case of hormones and other chemical messengers acting on cell-surface receptors, it is essential for the cell to interpret the presence of the agonist. It is then able to trigger another messenger to propagate the signal through the intracellular environment.

At first, it was believed that the cell surface receptors were able to facilitate a response or effect to chemical messengers within a cell, thus producing an effect. Over the last few decades though, intermediary proteins have been discovered, important for their roles in transducing the effect of neurotransmitter or hormone stimulation on receptors through the cell. For example, it was in the 1950 s that it was first observed by Rall and Sutherland (1957) that adenylyl cyclase was allosterically activated by hormones at a subtype of the adrenoceptor family, the β-adrenoceptor. Once the link between the pharmacologically well characterised β-adrenoceptors and the well defined biochemical reaction was demonstrated, an intensive effort was begun to define the molecular components of hormone-dependent adenylyl cyclase and the mode of interaction between receptors and adenylyl cyclase. It was found that an intermediary protein which was guanine-nucleotide dependent, hence its name as a G-protein, was allosterically connected to the receptor. Upon stimulation of the receptor, the α subunit of the G-protein became separated from the receptor and activated adenylyl cyclase into production of cyclic AMP.

This acts as an allosteric effector which in turn activates a protein kinase, protein kinase A, which then produces the desired effect. It is therefore now known that, in the case of the adrenoceptors at least, that production of a response is not a 'one-step' reaction but is a chain of events involving a diverse set of components, receptors, G-proteins (in this case G_s, the stimulatory G-protein), adenylyl cyclase, cyclic AMP and protein kinase A. Each of these distinct proteins and their role in signal transduction will be discussed separately, beginning at the level of the receptor.

There are a number of different classes of cell surface receptors. For example, there are growth factor receptors, with intrinsic tyrosine kinase activity, but by far the largest class are those which interact with and activate G-proteins. In the case of my research, the G-protein linked receptors to be studied in detail are the family of β-adrenoceptors. This family has been sub-classified according to agonist potencies into the β_1-, β_2-, β_3-, and perhaps β_4-adrenoceptor subtypes. My work investigated some of the biochemical mechanisms employed by the first three subtypes of β-adrenoceptors. In this introductory Chapter, I will detail some of the research already performed on this family, mentioning the second messengers involved, and also discuss regulation of these receptors upon stimulation by agonists. It is also vital to relate such work to a human perspective and relate alterations in function of the β-adrenoceptors to disease states in man. This will be discussed towards the end of this Chapter.

This introductory Chapter will outline the important components of the signalling cascade, beginning in descending order from the receptor, G-protein, through to adenylyl cyclase. After these components have been

individually discussed, the important processes of desensitization will be detailed.

1.2. THE β-ADRENOCEPTOR FAMILY

1.2.1. *INTRODUCTION*

β-adrenoceptors effect their responses via the sympathetic nervous system. This system is an important regulator of the activities of organs such as the heart and peripheral vasculature, especially in responses to stress. The ultimate effects of sympathetic stimulation are mediated by release of noradrenaline from nerve terminals which serves to activate the adrenoceptors on postsynaptic sites.

Ahlquist, in 1948, first identified that catecholamines acted via two principal receptors. These receptors were at that stage only termed α and β-adrenoceptors. α-adrenoceptors were classified as those which exhibit the potencies noradrenaline ≥ adrenaline > isoprenaline. β-adrenoceptors have a differing potency series isoprenaline > adrenaline ≥ noradrenaline. Ahlquist's hypothesis was dramatically proved correct by the development of drugs that selectively antagonize β-adrenoceptors but not the α-adrenoceptors. β-adrenoceptors were subsequently divided into β_1- and β_2-adrenoceptors on the basis of the rank order of potencies of catecholamines acting on tissues producing different responses, β_1 (isoprenaline > noradrenaline > adrenaline) and β_2 (isoprenaline > adrenaline > noradrenaline). This was confirmed by the development of selective agonists dobutamine (β_1), salbutamol (β_2) and antagonists CGP20712A (β_1) and ICI118551 (β_2). In recent years a third β-adrenoceptor subtype was found, known for a number of years as the 'atypical' adrenoceptor, but now re-named the β_3-adrenoceptor. This adrenoceptor shows lower affinity for the classical β-agonists with the rank order of potency being noradrenaline > isoprenaline > adrenaline and low affinity for known β-antagonists. No selective antagonists have yet been discovered for

the β_3-adrenoceptor, which causes difficulty in investigations into this adrenoceptor. Adrenoceptors are located at the plasma membrane and exert their effects through an interaction with guanine nucleotide binding proteins (G-proteins). The three β-adrenoceptors are single polypeptide chain glycoproteins embedded in the plasma membrane with their binding domain facing extracellularly. Hydrophobicity profiles for the receptors have shown seven hydrophobic regions, which are membrane spanning. The presence of these seven transmembrane domains typify the G-protein coupled receptors (GPCRs), and are connected by intracellular and extracellular loops of variable lengths. An N-terminus is located extracellularly and contains glycosylation sites while the positioning of the C-terminus is intracellular. The C-termini are of differing lengths in the three different molecularly identified β-adrenoceptors. For example, the β_1-adrenoceptor has a C-terminal tail length of 85 amino acids, the β_2-adrenoceptor a tail length of 73 amino acids, while the β_3-adrenergic receptor has the shortest C-terminus of 40 residues.

1.2.2. *MOLECULAR CLONING OF THE THREE SUBTYPES*

Three distinct mammalian β-adrenoceptor cDNAs β_1, (Frielle *et al*, 1987) β_2, (Dixon *et al*, 1986) and β_3 (Emorine *et al*, 1989) have been isolated in recent years. Two distinct splice variants of the β_3-adrenoceptor have been isolated, varying only by the presence or absence of six C-terminal amino acids. Genomic clones of both the β_2- and β_1-adrenoceptors predict that both arise from intronless genes, thus defining that neither subtype can generate diversity by differential splicing of pre-mRNA. The human β_3-adrenoceptor (Granneman *et al*, 1992a), however, consists of two exons and a single intron and the rat

β_3 adrenoceptor gene has three exons and two introns (Bensaid *et al*, 1993), giving scope for multiple forms of the β_3-adrenoceptor.

1.2.3. *TISSUE LOCALIZATION OF THE β-ADRENOCEPTORS*

The original subclassification and localization of β-adrenoceptors into β_1-adrenoceptors in the heart (where noradrenaline and adrenaline are equally potent) and β_2-adrenoceptors in vascular and bronchial smooth muscles (where adrenaline is about 10- to 30-fold more potent than noradrenaline) presumes a high degree of organ specificity of the β-adrenoceptor subtypes. This hypothesis has now evolved, mainly due to data from radioligand binding studies, into the concept that in a variety of organs, including the heart, both β_1- and β_2-adrenoceptors co-exist, although often one subtype dominates over the other (Carlsson *et al*, 1972, Brodde *et al*, 1991). This ratio can be altered in diseased tissue, as will be mentioned later in this Chapter.

1.2.4. *THE β_1-ADRENOCEPTOR*

The mammalian β_1-adrenoceptor is coupled through the stimulatory G-protein, G_s to the activation of adenylyl cyclase. It is the production of cAMP by adenylyl cyclase activation which is important in the regulation of heart rate and contractility (Brodde *et al*, 1993), lipolysis by adipose tissue (Arner *et al*, 1991), and blood pressure homeostasis (Kopp *et al*, 1983), among other vital functions. The cAMP activates protein kinase A which in turn alters the phosphorylation status within the cell and the activity of key enzymes which control processes such as lipolysis and contractility. Heart rate and force can be increased by noradrenaline and adrenaline through an interaction with both β_1-adrenoceptors and β_2-adrenoceptors (Kaumann *et al*, 1991, Brodde *et al*, 1993). Several ionic currents can flow upon β-adrenoceptor activation: calcium (through either β_1-adrenoceptors or β_2-adrenoceptors), sodium, potassium and chloride ions. Calcium currents can be increased directly by the α_s unit of the GTP binding protein, G_s, or by coupling of G_s to adenylyl cyclase with subsequent formation of cyclic AMP, release of the catalytic unit of cyclic AMP-dependent protein kinase, and phosphorylation of calcium channels and other proteins. Catecholamines reduce the cardiac efficiency and can also cause disturbance of the cardiac rhythm, culminating in ventricular fibrillation. In normal hearts the dose required to cause marked dysrhythmia is greater than that which produces the chronotropic and inotropic effects, but in ischaemic conditions dysrhythmias are produced more readily.

Like many G_s-coupled receptors the β_1-adrenoceptor demonstrates receptor-specific or homologous desensitization (Arner *et al*, 1991); persistent or repetitive stimulation which decreases the receptor's ability

to activate adenylyl cyclase (Freedman *et al*, 1995). Chronic exposure (days or months) but not acute exposure (hours) to a catecholamine down-regulates human heart β_1-adrenoceptors. Acute desensitization partially uncouples human heart β-adrenoceptor from the adenylyl cyclase. Both acute and chronic desensitization reduce positive inotropic responses to catecholamines.

The hypothesis that agonist-induced desensitization of the β_1-adrenoceptor involves phosphorylation of the receptor itself has recently been investigated by Freedman *et al* (1995). The β_2-adrenoceptor is regulated by cAMP-dependent protein kinase (Bouvier *et al*, 1987) and the β-adrenoceptor kinase (βARK1) or other G-protein coupled receptor kinases (GRKs). Freedman *et al* (1995) have shown that both Chinese Hamster Fibroblasts and human embryonic kidney 293 cells transfected to express β_1-adrenoceptors demonstrate receptor-specific desensitization of the β_1-adrenoceptor within 3-5 minutes. These two cell types also express βARK1 and the associated inhibitory proteins β-arrestin-1 and β-arrestin-2. They have been able to quantify the agonist-induced β_1-adrenoceptor phosphorylation, which seems to be derived equally from protein kinase A and GRK activity. Desensitization was reduced by 50% when protein kinase A inhibitors were added to the cells. Consistent with a GRK mechanism, receptor-specific desensitization of the β_1-adrenoceptor was enhanced by overexpression of β-arrestin-1 and -2 in transfected 293 cells. From these studies, it was concluded that, like the β_2-adrenoceptor, the β_1-adrenoceptor appears to bind either β-arrestin-1 or -2 and phosphorylated by βARK1, βARK2, and GRK5.

1.2.5. *THE β_2-ADRENOCEPTOR*

β_2-adrenoceptors cause smooth muscle relaxation in many organs. This can therefore be a very useful therapeutic effect, and so considerable effort has been made to find selective β_2-agonists. These would have the ability to relax smooth muscle without affecting the large population of β_1-adrenoceptors in the heart, as the activation of β-adrenoceptors in the heart leads to increased rate, which is not a desired effect. It is important to realise however, that the selectivity of such drugs is likely to always be relative rather than absolute.

Bronchial smooth muscle is strongly dilated by activation of β_2-adrenoceptors, and selective β_2-agonists are important in the treatment for asthma. Isoprenaline, given sub-lingually, or as an aerosol, produces a rapid effect, but is liable to cause tachycardia or ventricular dysrhythmias due to its β_1-adrenoceptor actions. Salbutamol does not have these drawbacks, and is probably the most widely used anti-asthmatic drug. Polymorphisms of the β_2-adrenoceptor have been found in the last few years and an overrepresentation of these have been found in asthmatic patients when compared to the normal population (Ohe *et al*, 1995, Turki *et al*, 1995). These will be mentioned in greater detail later.

Uterine smooth muscle also responds to activation of β_2-adrenoceptors, and β_2-agonists are used to delay premature labour. Myometrial cells respond to α-adrenoceptor agonists with contraction and to β-adrenoceptor agonists with relaxation. During pregnancy the β_2-adrenoceptors become dominant and sympathetic nerve stimulation produces relaxation (whereas in the non-pregnant uterus it may elicit relaxation or a biphasic response). Many β-agonists are used clinically

as uterine relaxants. Salbutamol is the one used most recently; while fenoterol and orciprenaline are amongst the most potent relaxants. Antagonists of β_2-adrenoceptors may cause increased uterine activity by blocking the relaxant effects of endogenous β-adrenoceptor agonists.

Skeletal muscle is affected by adrenaline, acting on β_2-adrenoceptors, though the effect is far less dramatic than that on the heart. The twitch tension of fast-contracting fibres, white muscle, is increased by adrenaline, particularly if the muscle is fatigued, whereas the twitch of slow, or red muscle is reduced.

The process of receptor desensitization has been particularly well studied in the β_2-adrenoceptor/G_s/adenylyl cyclase system (Bouvier *et al*, 1987, 1988, 1989, Hausdorff *et al*, 1989, Lohse *et al*, 1989). Two types of rapid alteration in the function and disposition of β_2-adrenoceptors are induced by agonists, i.e. functional uncoupling of the receptors from G_s in the membrane and sequestration of the receptors to internal sites in the cells. Evidence suggests that functional uncoupling from G_s is triggered by phosphorylation of the receptors either by protein kinase A or by one of the two isoforms of βARK. Whereas phosphorylation of β_2-adrenoceptors by protein kinase A can directly lead to uncoupling of the receptors from G_s (Sibley *et al*, 1986), phosphorylation by βARK serves to enhance the affinity of the receptors for an inhibitory protein, β-arrestin, which appears to bind to the βARK-phosphorylated receptors and thereby causes uncoupling. Along with functional uncoupling between receptors and G_s (Sibley *et al*, 1986), there also appears to be spatial uncoupling, caused by internalization of the receptors, a process usually termed receptor sequestration (Yu *et al*, 1993, Pippig *et al*, 1995).

1.2.6. *THE β_3-ADRENOCEPTOR*

The primary classification of the β-adrenoceptors into β_1- and β_2-adrenoceptor subtypes was soon proved insufficient to account for the non-β_1- and non-β_2-adrenergic responses of rat brown and white adipose tissues. Studies in the rat adipose tissue first led investigators to believe in the existence of a third β-adrenoceptor (Arch *et al*, 1984, Zaagsma *et al*, 1990, Muzzin *et al*, 1991). This 'atypical' β_3-adrenoceptor was capable of activating lipolysis when activated by β-agonists (Arch *et al* , 1984). This atypical β-adrenoceptor showed striking dissimilarities in its pharmacology to the β_1- and β_2-adrenoceptor subtypes. It showed a strongly reduced binding affinity for classical β_1- and β_2-adrenoceptor antagonists (Arch *et al* , 1993). Secondly, it was activated by certain β_1- and β_2-adrenoceptor antagonists such as CGP12177 and was selectively stimulated by a series of novel agonists, notably BRL37344 (Arch *et al* , 1984).

The rat adipose tissue β-adrenoceptor was able to be selectively stimulated by a series of compounds developed by SmithKlineBeecham Pharmaceuticals (Arch *et al*, 1984). Activation by these compounds led to fat cell lipolysis. Brown adipose tissue is the main effector of cold- and diet-induced thermogenesis in rodents, which can result in major energy expenditure, thus forming an important role in overall energy balance. Because brown adipose tissue has been demonstrated in humans of all ages and is often atrophied and quiescent in obese animals, much interest has been directed towards it. These receptors have therefore gained attention as potential therapeutic targets of specific agonists that might provide anti-diabetic, thermoregulatory, or anti-obesity properties. So far, anti-obesity drugs, which in animals seem to act selectively on adipose tissue β_3-adrenoceptors, are less

efficient in humans, where they display β_1- and β_2-adrenoceptor mediated side-effects.

This work led to the characterization and cloning of a gene encoding an atypical β-adrenoceptor from human (Emorine *et al*, 1989) and mouse (Nahmias *et al*, 1991) genomic libraries, as well as a rat brown adipose tissue cDNA library (Granneman *et al*, 1991, Muzzin *et al*, 1991). A bovine β_3-adrenoceptor has also been investigated pharmacologically and recently cloned (Pietri-Rouxel *et al*, 1995), showing high similarity to the human β_3-adrenoceptor. Mouse and rat β_3-adrenoceptors were found to be expressed to significant levels only in brown and white adipose tissue, suggesting that the β_3-adrenoceptor might be a fat cell specific β-adrenoceptor. Controversy has remained regarding the distribution of β_3-adrenoceptor mRNA and protein in human tissues. Initially, Northern blot analysis by Emorine *et al* (1989) demonstrated the presence of β_3-adrenoceptor mRNA in rat adipocytes, liver, skeletal muscle, and ileum. Ensuing reports using polymerase chain reaction (PCR) technology provided conflicting evidence regarding β_3-adrenoceptor mRNA distribution in human tissues. A recent report by Berkowitz *et al* (1995) has extended the range of human tissues containing β_3-adrenoceptor mRNA. It has now been identified in an extensive range of human tissues: cerebral cortex, cerebellum, liver, gall bladder, pancreas, stomach, small intestine, white and red skeletal muscle, several white fat tissues, left atrium, left ventricle, lung, kidney, prostate, and corpus cavernosa.

In 1994, Zhang *et al* cloned a gene referred to as the obese gene (*ob*), whose mutation is believed to be responsible for the phenotype of the hereditary obese (*ob/ob*) mouse. The *ob* gene codes for a protein

which presumably controls the size of the body fat mass by acting on the ventromedial nucleus of the hypothalamus to inhibit food intake and/or to stimulate energy expenditure via activation of the sympathetic nervous system. To date, the *ob* gene expression has been demonstrated in white adipose tissue, and also detected in brown adipose tissue. Experiments by Moinat *et al* (1995) have shown that fasting (36 h) or semi-starvation (10 days) decreased the *ob* gene mRNA level in both brown and white adipose tissue. Acute administration of the β_3-adrenergic agonist Ro16-8714 decreased the *ob* gene mRNA level in brown and white adipose tissue of lean Zucker rats. This new gene is regulated by activation of the β_3-adrenoceptor and is therefore of interest to investigators in this area. The product of the *ob* gene is a protein called leptin (Greek leptos = thin) It plays an important role in regulating body weight. Addition of this to mice has caused a reduction in weight of up to 30%. It has been postulated that the *ob* protein is secreted in a graded fashion from adipocytes into the circulation, where it acts as a hormone to control adipose tissue mass. Using polyclonal antibodies to recombinant *ob* protein, it is established that the *ob* protein circulates in mouse, rat and human plasma. The circulating form has a similar molecular weight to the 145 amino acid open reading frame predicted by the nucleotide sequence without the signal sequence, suggesting that *in vivo* the protein is not processed after cleavage of the signal sequence. The protein circulates as a monomer in human serum, and as a monomer and disulphide linked dimer in mouse serum. It is hoped to develop leptin as a therapy for obesity in humans, although with a short half-life, leptin will have to be genetically manipulated first to extend this.

Recently, Clement *et al* (1995) have discovered a genetic variation in the β_3-adrenoceptor, which has been suggested to lead to an increased capacity to gain weight in patients with morbid obesity. This condition is defined as a body-mass index (the weight in kilograms divided by the square of the height in metres) which is greater than 40. Morbid obesity is believed to have a particularly strong genetic component. The functional deficiency of the β_3-adrenoceptor in genetically obese mice (Collins *et al*, 1994, Arbeeny *et al*, 1994), and the results of studies in which the gene for the receptor has been disrupted in mice (Susulic *et al*, 1994), led Clement *et al* (1995) to investigate the role of the β_3-adrenoceptor in patients with morbid obesity. Genomic DNA was extracted from leukocytes, and from this DNA a polymorphism resulting in the replacement of tryptophan by arginine at position 64 was detected. The frequency of this 'Trp64Arg' allele was similar in the morbidly obese patients and the normal subjects. However, the patients with morbid obesity who were heterozygous for the Trp64Arg polymorphism had an increased capacity to gain weight. The role of this polymorphism in the pathogenesis of obesity may be conjectural but may be related to a lowering of the resting metabolic rate, which is genetically determined.

Another study released at the same time (Widen *et al* , 1995) showed that this Trp64Arg allele of the β_3-adrenoceptor is associated with abdominal obesity and a resistance to insulin, which may contribute to the early onset of non-insulin-dependent diabetes mellitus (NIDDM). Another group in the United States performed a similar study (Walston *et al*, 1995) on the Pima Indians, a group with a very high prevalence of NIDDM. Pima subjects homozygous for the Trp64Arg mutation had an earlier onset of NIDDM and tended to have a lower resting metabolic

rate. This polymorphism may therefore accelerate the onset of NIDDM by altering the balance of energy metabolism in visceral adipose tissue.

The degree of amino acid sequence identity between human, bovine, mouse and rat β_3-adrenoceptors is 80-90%. This is much higher than that existing between different β-adrenoceptor subtypes (40-50%) and of the same order as that observed for a given receptor subtype across species. Several residues located in the functional domains of the receptor are shared specifically by the human, bovine, murine and rat β_3-adrenoceptor, and are not found in the β_1- and β_2-adrenoceptor sequences. The human, bovine, mouse and rat β_3-adrenoceptor genes have a similar genomic organization, with an intron interrupting the 3' end of the coding sequence (Granneman *et al*, 1992a, Bensaid *et al*, 1993), whereas the other β-adrenoceptor subtypes are intronless. Moreover, the human and mouse genes have been assigned to a chromosomal linkage group that is conserved between species. Together, this structural data strongly suggests that the human, bovine, mouse and rat genes do not encode distinct β-adrenoceptor subtypes, but are species homologues of the β_3-adrenoceptor gene.

Although high sequence homology has been observed between the human, bovine, murine and rodent β_3-adrenoceptor, species differences in their pharmacology have been shown to exist. For example, Liggett *et al* (1992), have noted the increased efficacy of BRL37344 at the rat β_3-adrenoceptor, when compared with the human β_3-adrenoceptor. Liggett *et al* (1992) have also shown the higher efficacy of CGP12177 for the human β_3-adrenoceptor over the rat β_3-adrenoceptor. These species differences between the rat and human β_3-adrenoceptor are used to classically define the two receptors.

Because β_3-adrenoceptor agonists are being developed as potential therapies for obesity and diabetes (Arch *et al*, 1984), regulation of the receptor is an important consideration. It is already well established that acute adrenergic stimulation of β_1- and β_2-adrenoceptors leads to loss of responsiveness or desensitization. As noted earlier, desensitization of the β_2-adrenoceptor has been particularly well characterized and is thought to result from the phosphorylation of multiple serine and threonine residues in the third cytoplasmic loop and carboxyl terminal tail of the receptor by regulatory kinases which impairs the ability of the β_2-adrenoceptor to couple to G_s. In contrast to the β_1- and β_2-adrenoceptors, the β_3-adrenoceptor has fewer potential phosphorylation sites and thus would be expected to be resistant to agonist-induced desensitization. This prediction was first confirmed in isolated rat adipocytes where acute adrenergic stimulation was shown to desensitize β_1-adrenoceptors, but not β_3-adrenoceptors (Granneman *et al*, 1992c). The lack of β_3-adrenoceptor desensitization has since been shown in Chinese Hamster Fibroblasts and murine Ltk$^-$ cells that have been transfected to express the recombinant human β_3-adrenoceptor (Nantel *et al*, 1993, Liggett *et al*, 1993). Resistance to desensitization, however, may not be an intrinsic property of the β_3-adrenoceptor.

There is also strong evidence that the mRNA encoding β-adrenoceptor subtypes are differentially regulated by agonist exposure. Thus, sympathetic denervation of rat brown fat up-regulates β_3-adrenoceptor mRNA whereas cold exposure, increasing neural stimulation, dramatically down-regulates β_3-adrenoceptor transcripts (Granneman *et al*, 1992b). Treatment with exogenous β-adrenoceptor agonists *in vivo* also down-regulates the β_3-adrenoceptor mRNA.

1.2.7. *THE β_4-ADRENOCEPTOR*

Evidence for a fourth β-adrenoceptor, designated the β_4-adrenoceptor, has emerged from work looking at another 'atypical' adrenoceptor in turkey erythrocytes, showing different properties to the other three β-adrenoceptors (Rooney *et al*, 1991, Vaziri *et al*, 1992). It was initially classified as the turkey homologue of the mammalian β_1-adrenoceptor based on its approximately equal affinities for adrenaline and noradrenaline. The gene for the β_4-adrenoceptor has been cloned from a foetal turkey blood cDNA library, and its deduced amino acid sequence is between 38-59% homologous to the sequences of the mammalian β-adrenoceptor subtypes (Chen *et al*, 1994). The β_4-adrenoceptor displays highest homology (59%) to the mammalian β_1-adrenoceptor, but apparently does not represent an avian homologue of the β_1-adrenoceptor. The β_4-adrenoceptor has been noted to interact with a G-protein other than G_s. It has also been observed that the β_4-adrenoceptor does not internalize upon agonist stimulation and displays only a low affinity agonist binding state insensitive to guanine nucleotides (Hertel *et al*, 1990, Minneman *et al*, 1980). Agonist occupation of this receptor results in stimulation of a phosphoinositidase C in addition to activation of adenylyl cyclase. This turkey adrenoceptor may perhaps play an important role in avian physiology. It was not, however, studied in my investigations, and I will refer only to the three major adrenoceptor subtypes.

1.3. G-PROTEINS

1.3.1. *INTRODUCTION*

Heterotrimeric guanine-nucleotide-binding proteins (G-proteins) function as molecular switches in a diverse set of pathways by coupling seven-transmembrane-helix receptors to specific intracellular effectors. In order to do so they associate with the cytoplasmic face of the plasma membrane. This association may be partly mediated by the βγ subunits, but the N-terminus of the α-subunits also seems to be involved. The transduction of signals depends on the ability of the α-subunits to cycle between the resting (GDP-bound) conformation primed for interaction with agonist-stimulated receptors and an active (GTP-bound) conformation capable of activating or inhibiting a variety of downstream effectors including enzymes as well as ion channels.

1.3.2. *G-PROTEIN SUBUNITS*

All of the G-proteins identified so far contain three subunits (α, β, and γ). In mammals, G-protein α, β and γ subunits are encoded by at least 16, 4, and 7 genes, respectively. The α-subunits are the largest in size (M_r = 39 kDa - 52 kDa), contain the binding site for guanine nucleotides and are the most diverse. The α-subunits also contain the sites for modification by bacterial toxins. The β-subunits are less diverse (M_r = 36 kDa and 35 kDa) and are believed to be common to the different G-proteins. While there are multiple γ-subunits, they are the least investigated subunit. The βγ component forms a single functional unit, as native β and γ do not dissociate. βγ subunit complexes, alone or in co-operation with α subunits, regulate effectors such as adenylyl cyclase, phospholipase C and ion channels. These subunits can vary in hydrophobicity from very hydrophobic to hydrophilic.

G-protein activation is a multi-stage, magnesium dependent process. The inactive form of the G-protein contains bound GDP. Exchange of this GDP for GTP results in activation of the G-protein. The α subunits bind and hydrolyze this GTP. When GTP enters the empty nucleotide binding site of G_α, the α-GTP complex takes on a new active conformation, which causes it to rapidly dissociate from the receptor and from the $\beta\gamma$ unit. α-GTP has a high affinity for binding to and activating the appropriate effectors, such as adenylyl cyclase and phosphoinositidase C. Hydrolysis of the bound GTP terminates regulation of the effector, returning G_α to its inactive GDP-bound state.

In the past year the crystal structure of the G-protein heterotrimer ($G_{\alpha\beta\gamma}$) was finally achieved (Lambright *et al*, 1996). From studies on this crystal structure of $G_{\alpha\beta\gamma}$ (Lambright *et al*, 1996) and mutagenesis, it would seem that the whole signalling complex is not a set of bimolecular reactions, as originally believed and described above, but that it is a 'nanomachine', in that the receptor is acting as a lever activating a switch (G_α) and a propeller, ($G_{\beta\gamma}$). Lambright *et al* (1996) have identified two major contact sites between the G_α and G_β subunit. The first is a tethered prow of G_α, which is the myristoylated or palmitoylated amino terminus. The larger and more important site is the interface between the G_α switch regions and one of the G_β subunit's electronegative faces. The apposition of the two subunits in this region shields this vital domain, protecting the G_α face until the cationic lever of the receptor 'flips' the switch domain and triggers the conformational change in G_α. After the receptor catalyses the magnesium dependent replacement of GDP by GTP in the G_α helical/G_α GTPase domain cleft, the G_α switch region springs shut. The now active, compacted and presumably free G_α thus exposes itself and the freed, but

unchanged $G_{\beta\gamma}$, to interactions with effector molecules (Clapham, 1996).

1.3.3. MEMBRANE ASSOCIATION OF G-PROTEINS

Membrane association of the G-protein α subunit is achieved via lipid modification with either myristate and/or palmitate (Wedegaertner *et al*, 1994, Milligan *et al*, 1995). Membrane association is critical for ensuring high surface concentrations of signal-transduction components and proper relative orientation. Palmitoylation has been suggested to provide a membrane anchorage point. The α-subunits of some, but not all, G-proteins have been shown to contain co-translationally attached myristic acid (Jones *et al*, 1990, Mumby *et al*, 1990). For example, while the G_o polypeptide is myristoylated, G_s is not. Agonist regulation of the palmitoylation status of the α-subunit of G_s is likely to alter the interactions between the subunits of the G-protein heterotrimer and may result in a physical release of the α-subunit from the plasma membrane.

Other investigators (Neubig, 1994) have not only been interested in how the G-protein is held in the plasma membrane, but also whether the G-protein is freely mobile or restricted in the plasma membrane. Neubig (1994) looked at the evidence known showing receptors, G-proteins and effectors to be less mobile in the plasma membrane than previously thought. He believes that signal transduction events in intact cells are actually more complex than in reconstituted systems. The specificity of signalling by G-proteins and receptors in intact cells (Kleuss *et al*, 1992) appears to be greater than that seen in reconstitution systems (Rubenstein *et al*, 1991). Recently, direct demonstrations of G-protein-cytoskeleton interactions and limited mobility of receptors and G-proteins are becoming common. Laboratories have gained data

showing association of G_α or $G_{\beta\gamma}$ subunits with tubulin or other cytoskeletal proteins (Rasenick *et al*, 1988, 1990, Wang *et al*, 1991). Other possible methods of limiting G-protein movement in membranes have been unearthed by the discovery of several types of G-protein in Triton X-100 insoluble membranes with characteristics of noncoated pits or caveolae (Sargiacomo *et al*, 1993). Caveolae ('small caves'), which contain the protein caveolin, are flask-shaped invaginations of the plasma membrane, and are a prominent feature of many mammalian cells (Severs, 1988). β-adrenergic receptors have been shown to accumulate in such noncoated pits (Raposo *et al*, 1989) and to co-purify with caveolin (Dupree *et al*, 1993) when cross-linked with antibodies. Caveolae may therefore represent sites of assembly of a signal transducing complex that could include receptors, G-proteins, effectors, and even the intracellular targets of the second messengers generated.

1.3.4. G-PROTEIN STOICHIOMETRY

Some groups have studied the quantitative stoichiometry of the G-protein and its receptor (Kim *et al*, 1994a). While a large amount of information is available on receptor number due to the availability of radiolabelled drugs, the same is not true for G-proteins and effectors. However, Kim *et al* (1994a) found that in NG108-15 cells it was possible to estimate the copies per cell of each member of the signalling cascade, such as $G_s\alpha$ and adenylyl cyclase. Kim *et al* (1994a) found that while there were as many as 100,000 copies per cell of the IP prostanoid receptor, there were even more copies per cell of $G_s\alpha$ (1,250,000), but only 17,500 copies/cell of the effector adenylyl cyclase. These results suggest that quantitatively, it is the adenylyl cyclase member of the cascade which is the least highly expressed

component and implies that a lot of the cellular $G_s\alpha$ may not have access to adenylyl cyclase.

1.3.5. *THE STIMULATORY G-PROTEIN G_S*

G_s is the G-protein that activates adenylyl cyclase; its α subunit is predominantly responsible for this effect, although $\beta\gamma$ appears to act synergistically with certain forms of adenylyl cyclase. G_s can be covalently modified by a cholera toxin produced by the bacterium *Vibrio cholera*. The toxin is an enzyme which transfers ADP-ribose from NAD^+ to a specific arginine residue of $G_s\alpha$.

Most cells contain two forms of the α subunit of G_s; their apparent molecular weights are 45 and 42 kDa on SDS-polyacrylamide gels (Northup *et al*, 1980). Although most tissues contain both forms of the polypeptide, their relative amounts vary. In heart, for example, the 42 kDa protein predominates, while in brain and adrenal medulla the 45 kDa form is the major species (Mumby *et al*, 1986). The two distinct $G_s\alpha$ subunits represent differentially spliced forms of $G_s\alpha$. The isolation and characterization of the human $G_s\alpha$ chromosomal gene was completed by Kosaza *et al* in 1988. They found the gene to be a split gene, having 13 exons and 12 introns spanning about 20 kilobases of genomic DNA.

It was not known until recently if these long and short splice variants of $G_s\alpha$ interacted differently with either receptors or with adenylyl cyclase. Findings by Yagami (1995) show that a β-adrenoceptor agonist/receptor complex catalyzes the exchange from GDP to GTP on $G_s\alpha$-L but not on $G_s\alpha$-S. Yagami (1995) was studying the glucagon receptors and β-adrenoceptors in the liver, using partially hepatectomized male rat

livers to understand whether glucagon receptors shared the common $G_{s\text{-}L}$ with the β-adrenoceptors or have their own independent pathway through $G_{s\text{-}S}$. Using a newly developed tryptic digestion method, Yagami (1995) proved that glucagon receptors do share $G_{s\text{-}L}$ with the β-adrenoceptors but are also coupled to $G_{s\text{-}S}$.

1.4. ADENYLYL CYCLASE

1.4.1. *INTRODUCTION*

Adenylyl cyclase converts MgATP into 3',5'-cyclic AMP (cAMP) and pyrophosphate. The cAMP then acts as an intracellular messenger to activate the cAMP-dependent protein kinase A. Current findings show that the adenylyl cyclase enzymes comprise a heterogeneous multigene family. Members of the adenylyl cyclase family are variously regulated by the α and $\beta\gamma$ subunits of G-proteins. There are now eight known isoforms of adenylyl cyclase. Adenylyl cyclase activity has been localized to the plasma membranes of most mammalian tissues where it produces intracellular cAMP in response to agonist occupancy of receptor for numerous hormones, neurotransmitters, and autocrine and paracrine factors. The cAMP then activates protein kinase A, which phosphorylates cellular substrates such as enzymes or channels which then directly produce or set in motion the cascade that eventually leads to the production of the desired biochemical or physiological response to the hormonal signal.

1.4.2. *STRUCTURE*

The adenylyl cyclase structure bears a remarkable resemblance to certain membrane transporters. They have a common double motif, each with six membrane-spanning domains, and two large cytoplasmic segments. The loop between the first and second transmembrane domains is some 350 amino acids in length, while there is a 250-300 amino acid tail following the second membrane domain. Both the N- and the C-termini are predicted to be cytoplasmic, while the membrane-spanning domains are not highly conserved in the isoforms. The two cytosolic segments are the most highly conserved regions in adenylyl cyclase structure (up to 92% similarity) and are likely to represent the catalytic and regulatory

domains. Based on sequence similarities, the isoforms have been classified into three subfamilies: the type I-like group (I, III and VIII); the type II-like group (types II, IV and VII); and the type V-like group (types V and VI). The overall similarity between the various mammalian adenylyl cyclases is about 50%. Functional properties are shared within these sub-families, although individual isoforms display unique responses.

As well as the complex structure of the adenylyl cyclase family, they are all multiply regulated. Each adenylyl cyclase can be regulated by α subunits of G-proteins, although type I, in particular, is quite refractory to stimulation by $G_s\alpha$ and the type-II-like group is refractory to inhibition by $G_i\alpha$. However, apart from idiosyncratic responses to $G_s\alpha$ subunits, individual isoforms show a diverse range of responses to factors such as calcium, protein kinase C and $\beta\gamma$ subunits of G-proteins.

Types I and VIII adenylyl cyclase appear to be brain-specific species, while type II is detected in both brain and lung. mRNA for type IV is widely distributed in a variety of tissues, while type VII is found in S49 lymphoma cells and rat brain and heart. Type III enzyme has only been found in significant levels in the olfactory tissue and bulb while both type V and VI mRNAs are found at high levels in brain, heart, and lower levels in several other tissues, including kidney, liver, lung and testes.

1.4.3. *ACTIVATION & REGULATION OF ADENYLYL CYCLASE*

Most mammalian adenylyl cyclases are stimulated by forskolin, a diterpine isolated from *Coleus* roots (Seamon *et al*, 1981). This stimulation results from the direct interaction of forskolin with adenylyl cyclase. The construction of forskolin-agarose represented a breakthrough that led to the isolation and purification of mammalian adenylyl cyclases (Pfeuffer and Metzger, 1982). Although forskolin stimulation of adenylyl cyclase does not require G_s, it appears that only G_s-stimulated adenylyl cyclases are regulated by forskolin. Forskolin greatly synergizes G_s stimulation of the enzyme (Clark *et al*, 1982), and G_s increases the binding affinity of forskolin on adenylyl cyclase (Nelson and Seamon, 1986).

1.5. DESENSITIZATION

1.5.1. *INTRODUCTION*

The phenomenon by which cells and tissues develop reduced sensitivity over time to the maintained presence of a stimulus of constant intensity is termed desensitization (Lefkowitz *et al*, 1980). This can be sub-divided into homologous and heterologous effects, where homologous desensitization is when agonist treatment leads to reduced sensitivity to the agonist, but not to a reduction in sensitivity to other signals that work through distinct receptors. Often a more general desensitization occurs, such that reduced sensitivity to several distinct agonists develops. This is termed heterologous desensitization.

The rapid desensitization mechanisms affect receptor function. Phosphorylation of the receptors is a critical step in these mechanisms (Strasser *et al*, 1986). The most rapid and quantitatively most important mechanism is triggered by phosphorylation of the receptors by the group of kinases known as the β-adrenoceptor kinases, βARK (Lohse *et al*, 1990, Benovic *et al*, 1988). These kinases phosphorylate only agonist-occupied, active receptors.

1.5.2. *G-PROTEIN COUPLED RECEPTOR KINASES & β-ARRESTIN*

βARK is a member of a growing family of G-protein coupled receptor kinases (GRKs). βARK activation occurs following interaction of the kinase with the agonist-occupied form of the receptor and G-protein βγ subunits. A 50-70% loss of receptor function is observed in intact cells due to phosphorylation by βARK. The binding of βARK to βγ subunits is shown to be mediated by a stretch of amino acids near the C-terminal of the kinase, including sequences in and extending beyond the

most C-terminal region of the pleckstrin homology domain (Touhara *et al*, 1994). βARK phosphorylates the agonist-occupied form of the receptor, enabling the binding of the co-factor β-arrestin to the receptor, resulting in the uncoupling of receptor from G-proteins and hence effector second messenger systems.

Two possible mechanisms could explain the enhanced phosphorylation of the activated form of the receptor by kinases of the GRK family. First, receptor occupancy may induce a conformational change exposing potential phosphorylation sites previously sequestered from the kinase. Alternatively, interaction of the kinase with the agonist-bound form of the receptor could result in enhanced catalytic activity of the kinase.

To date, six mammalian members of the G-protein-coupled receptor kinase family have been cloned. They have been named GRK1-6 due to the apparently unique functional association of such kinases with this receptor family. Two isoforms of βARK are known so far. They are now re-named thus: rhodopsin kinase corresponds to GRK1, βARK1 to GRK2, and βARK2 to GRK3. The βARK1 gene spans approximately 23 kilobases and is composed of 21 exons interrupted by 20 introns. The common structural features of G-protein-coupled receptor kinases include a centrally localized catalytic domain of approximately 240 amino acids that shares significant identity (46-95%), an N-terminal domain of 184-188 amino acids (except GRK-2) and a variable length C-terminal domain of 100-263 amino acids (Inglese *et al*, 1993).

While βARK modulates receptor-mediated production of second messengers, the likely existence of a feedback loop by which second

messengers and/or their downstream components impinge on βARK to modulate its activity remains an intriguing question. Results have shown that βARK activity can be increased through phosphorylation by protein kinase C, thus indicating that βARK can be pre-conditioned to modulate the subsequent cellular responsiveness to receptor activation, providing the cell with a mechanism by which homologous desensitization can be regulated heterologously.

Two isoforms also exist for β-arrestin. In addition, there appear to exist splice variants of the β-arrestins, containing or not a stretch of eight amino acids which correspond to exon 13 in retinal arrestin (Yamaki *et al*, 1990). Among the two isoforms of the kinases, βARK1 is about twice as active at β_2-adrenoceptors than is βARK2, and the two isoforms of β-arrestin have identical effects on β_2-adrenoceptors.

The phosphorylation sites of the β_2-adrenoceptor have been shown to reside in the C-terminus (Thompson *et al*, 1984). So far, an accurate mapping of the individual phosphorylated residues has not been accomplished. The presence of acidic amino acids located 2 or 3 positions N-terminal to a serine or threonine seems to be required for phosphorylation by βARK, but there is no strict consensus sequence (Onorato *et al*, 1991). The most likely reason for this lack of a small consensus sequence is that βARK recognizes the active conformation of the receptor and, probably, therefore, has several attachment points. The hypothesis of such attachment sites is supported by the finding that a peptide corresponding to the first intracellular loop of the β_2-adrenoceptor is an inhibitor of βARK and can impair βARK-dependent receptor desensitization (Lohse *et al*, 1989).

It has been suggested that the ability of protein kinase A to desensitize the β_2-adrenoceptor does not depend on receptor occupancy by agonist and therefore mediates heterologous desensitization (Hausdorff *et al*, 1990). In contrast, phosphorylation of the β_2-adrenoceptor by βARK depends on receptor occupancy and occurs only in the presence of β-adrenoceptor agonists (Strasser *et al*, 1986). Thus, in contrast to protein kinase A, βARK-mediated phosphorylation is generally thought to be critical for homologous desensitization of the β_2-adrenoceptor. Recent studies using mutations have also demonstrated a role for βARK-mediated phosphorylation in facilitating β_2-adrenoceptor sequestration (Ferguson *et al*, 1995), a desensitization process to be described later in this Chapter.

1.5.3. *EFFECTOR KINASES*

In addition to phosphorylation by their specific kinases, β-adrenoceptors are also phosphorylated by their effector kinases, such as protein kinase A and protein kinase C (Lohse, 1993). This provides a direct negative feedback loop, whereby the effector enzyme shuts off its own stimulation. At the same time, this pathway allows a generalized, non receptor-specific form of desensitization, termed heterologous desensitization. Direct phosphorylation of the β_2-adrenoceptor by protein kinase A has been shown in a reconstituted system (Benovic *et al*, 1985). There are two consensus sites for protein kinase A-mediated phosphorylation in the β_2-adrenoceptor sequence (Bouvier *et al*, 1989), one in the third intracellular loop, in a region essential for coupling to G_s, the other at the N-terminal region of the C-terminus, which also appears to play a role in receptor-G_s coupling (O'Dowd *et al*, 1988).

Protein kinase C can also phosphorylate the β_2-adrenoceptor, probably at the same sites utilized by protein kinase A, again with a clear preference for the site in the C-terminus of the third intracellular loop (Bouvier *et al*, 1987). The effects of protein kinase C-mediated receptor phosphorylation are, therefore, similar to those described for the protein kinase A-mediated process (Bouvier *et al*, 1991).

Under optimal conditions the extent of protein kinase A-mediated desensitization of β_2-adrenoceptors appears comparable in extent to that mediated by the βARK/β-arrestin mechanism, i.e., a loss of 40-50% of receptor function compared to 50-70% for the βARK-mediated effect. There are two important differences however: agonist-induced protein kinase A-mediated desensitization is considerably slower than the βARK-mediated process ($t_{1/2}$ of 2 minutes vs. 15 seconds), but is much more sensitive to agonist concentrations, probably because of the amplification of signalling from receptor occupancy to protein kinase A activation.

1.5.4. *SEQUESTRATION*

Apart from functional uncoupling of β-adrenoceptors from G_s, agonists can also cause rapid physical uncoupling, which is effected by translocation of the receptors to intracellular sites such that they can no longer interact either with their hydrophilic agonists or with G_s.

The exact localization of the sequestered receptors is still not clear. Some researchers believe that receptors are internalized into intracellular endosomes (Perkins *et al*, 1984). This hypothesis is based on the following observations: that sequestered receptors are inaccessible to hydrophilic ligands, but remain accessible to hydrophobic ligands

(Wang *et al*, 1989); sequestered receptors have been found in a membrane fraction that is lighter than the plasma membrane fraction (Harden *et al*, 1980); and agents that disrupt internalization into clathrin-coated vesicles also disrupt sequestration of β_2-adrenoceptors (Hertel *et al*, 1986). The best evidence to date for a true intracellular location of the sequestered receptors was obtained by immunofluorescence confocal microscopy (Von Zastrow *et al*, 1992), which showed β-adrenoceptor immunoreactivity associated with intracellular vesicles and co-localized with transferrin receptors. In this study, the intracellular receptors were detected not only with antibodies against the receptors themselves, but also with antibodies that specifically recognized an epitope that had been added to recombinant expressed receptors. It was not certain whether G-proteins co-localize with the adrenoceptor intracellularly. Balch (1989) showed, however, that 'low molecular weight' G-proteins (i.e. $M_r < 20{,}000$ kDa) are essential components in the transport, at least, of receptors between secretory compartments.

Sequestration was originally thought to be a mechanism of receptor desensitization, as it was observed that the light membrane fraction containing the sequestered receptors did not contain G_s, so that coupling could not occur (Waldo *et al*, 1983). Since then, several findings have disagreed with this role: firstly, in most systems receptor sequestration is slow compared to the rapid phosphorylation and uncoupling processes described above, and, hence, sequestered receptors will already have an impaired function (Roth *et al*, 1991). Second, in most, but not all cell systems the extent of receptor sequestration is too small to account for the observed extent of desensitization (Lohse *et al*, 1990). An alternative hypothesis is that sequestration may be a mechanism for receptor resensitization (Pippig *et al*, 1995). It was thought that upon

removal of agonist, receptors were recycled to the cell surface in a fully functional state (Hertel *et al*, 1983). It has also been observed, however, that following agonist-induced stimulation, sequestered β_2-adrenoceptors are less phosphorylated than those still residing in the plasma membrane (Sibley *et al*, 1986). Furthermore, inhibition of β_2-adrenoceptor sequestration by various means prevents the resensitization of the receptors after removal of the agonist (Yu *et al*, 1993). Sequestration may serve to enable dephosphorylation of receptors and subsequent recycling to the cell surface, where the receptors become reintegrated and fully active once more.

Other groups (Barak *et al*, 1994, 1995, Strader *et al*, 1987, Cheung *et al*, 1989) have produced mutants of the β_2-adrenoceptor to localize the critical residues for sequestration. Barak *et al* (1994) found that a highly conserved tyrosine residue was required for agonist-mediated β_2-adrenoceptor sequestration. Two other groups (Strader *et al*, 1987, Cheung *et al*, 1989) have also investigated regions of the β_2-adrenoceptor by production of mutants. They postulated a causal relationship between receptor-G-protein coupling and sequestration, suggesting that the regions of the β_2-adrenoceptor responsible for functional coupling with G_s are identical to those mediating receptor sequestration. Receptor subtypes within a family may show different sequestration behaviour. For example, the β_2-adrenoceptor shows marked sequestration, while the β_1- and β_3-adrenoceptors do not (Nantel *et al*, 1993, Suzuki *et al*, 1992).

1.5.5. *DOWN-REGULATION*

Another set of mechanisms which can lead to desensitization via a decrease in the number of receptors is often termed down-regulation.

This is a much slower process compared to the alteration of receptor function mentioned above: it usually occurs over hours, and can take up to 24 hours to reach maximal effect from continuous agonist exposure (Lohse *et al*, 1993). It is effected by two sets of mechanisms: enhanced degradation of the receptors, or decreased synthesis (Bouvier *et al*, 1989, Collins *et al*, 1989). As the basal turnover of G-protein-coupled receptors is generally very slow, half-lives in the region of many days have been reported. Decreases in receptor synthesis probably take even longer than 24 hours to affect receptor numbers.

Down-regulation has been studied extensively for the β_2-adrenoceptor (Bouvier *et al*, 1989, Collins *et al*, 1989, Kobilka *et al*, 1987). Degradation of this receptor may have two components: one which can only be initiated by agonists, and another which is protein kinase A-mediated (Bouvier *et al*, 1989). The agonist-dependent, protein kinase A-independent component is documented by the fact that cells defective in post-receptor components G_s, adenylyl cyclase (Shear *et al*, 1976) or protein kinase A, can still show agonist-induced receptor down-regulation. However, receptor-G_s coupling may still be required since defects of this coupling have been found to result in impaired receptor down-regulation (Shear *et al*, 1976, Hadcock *et al*, 1989). These findings suggest that either a G_s-mediated but protein kinase A-independent signal is required for degradation, or that receptor-G_s complexes are preferentially degraded. Protein kinase A-dependent phosphorylation of β_2-adrenergic receptors appears to enhance their degradation, as receptor mutants lacking the phosphorylation sites are down-regulated slower than wild-type receptors (Bouvier *et al*, 1989). βARK-dependent receptor phosphorylation, does not however, seem to play a role in this respect, as several studies have shown that receptor

mutants lacking phosphorylation sites for βARK down-regulate normally (Cheung *et al*, 1989). Down-regulation of β_2-adrenoceptors can be also mediated via reduction of the receptor mRNA (Hadcock *et al*, 1988, 1989). This is due to destabilization of the mRNA. It is not clear whether this is agonist-specific, or protein kinase A-mediated.

The three β-adrenoceptor subtypes show different degrees of down-regulation: β_2-adrenoceptors show marked, β_1- modest and β_3-adrenoceptors little or no down-regulation (Nantel *et al*, 1993, Suzuki *et al*, 1992, Thomas *et al*, 1992).

1.5.6. *REGULATION OF G-PROTEIN FUNCTION & EXPRESSION*

While regulation at the receptor level appears to be the predominant site of desensitization of G-protein coupled receptors, recent data indicate that such regulation may exist at post-receptor levels, most notably at the level of G-proteins. It is a complex set of events, involving alterations in receptor function, intracellular localization and expression, as well as alterations in the function and expression of G-protein α subunits. G-protein elimination has been observed to occur in response to agonists at receptors which regulate a variety of transmembrane signalling cascades including stimulation and inhibition of adenylyl cyclase and stimulation of phosphoinositidase C activity.

Agonist-induced loss of $G_s\alpha$ has been demonstrated to be independent of regulation of levels of $G_s\alpha$ mRNA and to be unaffected by the presence of the protein synthesis inhibitor cycloheximide. These observations indicate that a substantial role for either transcriptional or translational control is unlikely. It was thought at first that

down-regulation of the G-protein always occurred at the same time as the receptor. This has proved not to be the case with the β_3-adrenoceptor, as the receptor is refractory to down-regulation but G_s is not. It does remain possible, however, that there is an initial co-sequestration of the two polypeptides followed by separate sorting processes.

1.6. DISEASES CAUSED BY ALTERATION IN β-ADRENOCEPTOR EXPRESSION, REGULATION AND FUNCTION

1.6.1 *INTRODUCTION*

The β-adrenoceptors play an important role in modulating a variety of target cell responses to catecholamines. There has therefore been much speculation as to whether alterations in these receptors contribute to disease states characterized by alterations in catecholamine response. Disease states with possible alterations in β-adrenoceptors are numerous, including atopic disorders, cardiac disorders, hypertension, diabetes mellitus, thyroid disorders, and neurologic and psychiatric disorders. These major diseases and the effects of the β-adrenoceptor group on their development will be described below.

1.6.2. *ATOPIC DISORDERS*

It is thought that a defective β-adrenoceptor system or an imbalance between α- and β-adrenoceptors could be responsible for asthma and other so-called atopic ("allergic") disorders. This concept is based primarily on clinical observations. The hypothesis implies that individuals with atopic disorders develop (or are genetically susceptible) with target cells whose β-adrenoceptors are unable to maintain normal function of the tissues responsible for expressing atopic systems (e.g. respiratory tract, nasal mucosa, etc). β-adrenoceptor agonists are assumed to induce airway smooth muscle relaxation through the cAMP-protein kinase A phosphorylation cascade. For over thirty years, β-adrenoceptor agonists have been used routinely in the treatment of bronchial asthma. The therapeutic use of these compounds is derived from their ability to prevent and reverse bronchoconstriction. A major theoretical advantage of the β-adrenoceptor agonists is that they act as

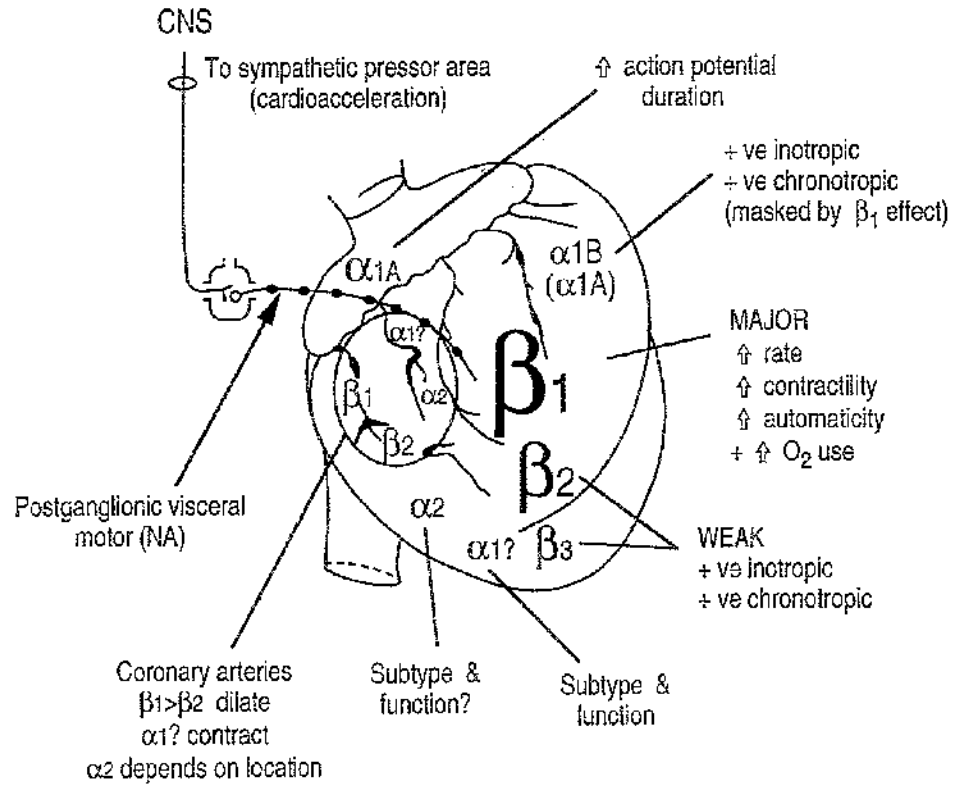
functional antagonists to produce bronchorelaxation regardless of the contractile agonist used to induce tone. This universal activity is important since it is likely that the bronchoconstriction associated with asthma is produced by the concerted actions of several mediators acting through distinct classes of receptors. Recently, a number of groups have investigated a possible genetic basis for reported differences in β_2-adrenoceptor expression in atopic subjects (Ohe *et al*, 1995, Turki *et al*, 1995). A two allele polymorphism of this receptor gene has been identified in Caucasian people. Ohe *et al* (1995) found that a restriction fragment length polymorphism of the β_2-adrenoceptor gene had some association with the responses of airways to β_2-agonists, and the incidence of bronchial asthma. Similarly, Turki *et al* (1995) studied nocturnal asthma, where the β_2-adrenoceptors down-regulate at night. They found an overrepresentation of a Gly16 allele of the β_2-adrenoceptor in these patients. A glycine at position 16 imparted an accelerated agonist-promoted downregulation of the β_2-adrenoceptor as compared to arginine at this position. It was therefore concluded from this study that the Gly16 polymorphism may be an important genetic factor in the expression of this asthmatic phenotype.

1.6.3. *CARDIAC DISORDERS*

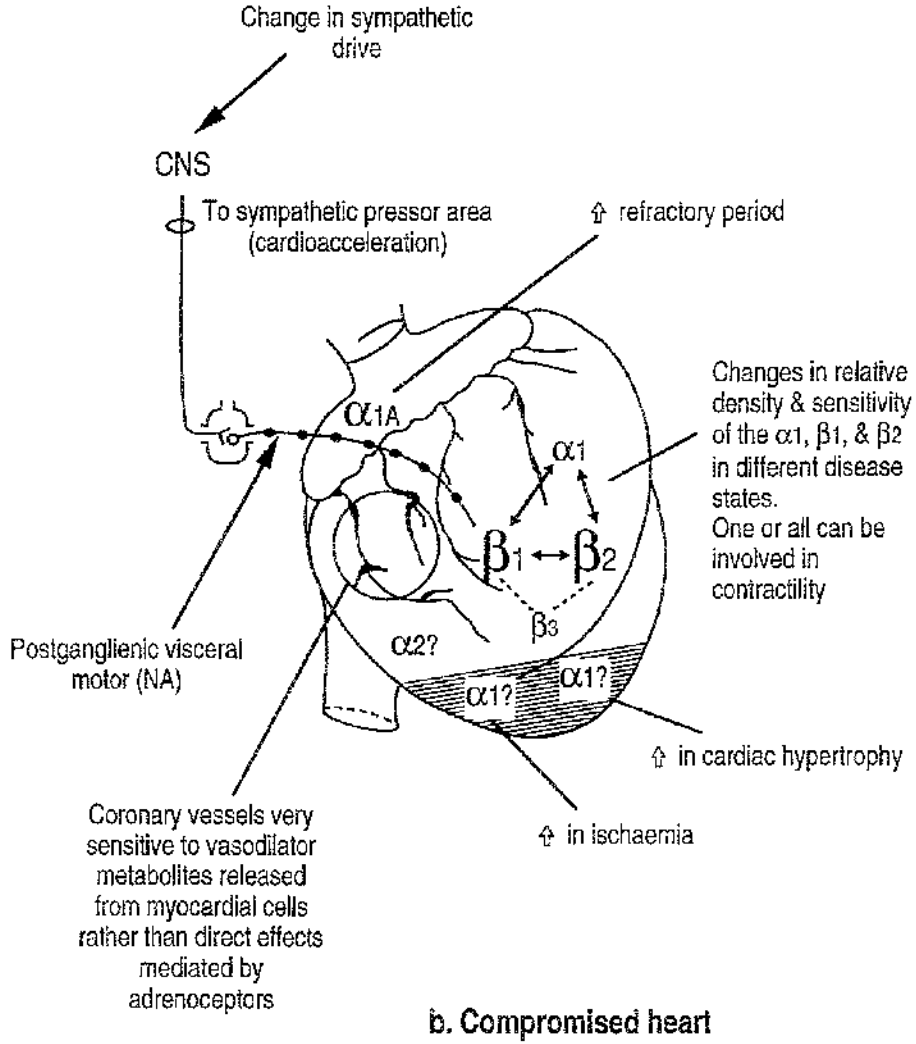
β-adrenoceptors are widely recognised as playing an important role in enhancement of cardiac function- in particular of rate (chronotropy) and force (inotropy) of cardiac contraction and in contributing to the genesis of cardiac arrhythmias. Although adrenergic agonists are sometimes employed to elicit such effects, more commonly β-adrenoceptor antagonists are used to block one or more of these actions in patients with cardiac disorders. β-adrenoceptors, aside from their usefulness as therapeutic targets, are involved in contributing to the pathophysiology

of cardiac disease. Figure 1.1. shows the β-adrenoceptor distribution in the normal and compromised hearts.

Figure 1.1. β-adrenoceptor distribution in the normal and compromised human heart.



a. Normal heart



b. Compromised heart

1.6.4. *HYPERTENSION*

Adrenergic neurotransmitters are important regulators of blood pressure. Whereas α-adrenoceptor stimulation increases blood vessel contraction and peripheral resistance, β-adrenoceptor stimulation causes vessel relaxation and decreased resistance. Considerable evidence has suggested that alterations in the sympathetic nervous system contribute to the development and/or maintenance of systemic hypertension. In addition, sympatholytic drugs, in particular β-adrenoceptor blocking drugs, are important in the management of hypertensive patients. The precise mechanism by which β-blockers lower blood pressure has not yet been resolved, but β-adrenoceptors are known to contribute to the regulation of blood pressure through effects at several target sites, including the central nervous system, adrenergic nerve teminals, blood vessels, heart and kidney.

1.6.5. *DIABETES MELLITUS*

Changes in β-adrenoceptors can be associated with the absolute or relative deficiency of insulin that characterizes the diabetic state. An agonist of the β_3-adrenoceptor would have potential as an anti-type II diabetic therapy. Type II diabetes, or insulin independent diabetes, is caused by the inability of the insulin receptor to be activated by insulin, or loss of the receptor from the cell surface, which leads to reduced uptake of glucose by cells. Glucose levels in the blood therefore rise until an equilibrium is reached between its production and uptake. This leads to very high circulating glucose levels of up to 1200 mg/dl. At this concentration of glucose, the reabsorption process in the kidney is overloaded and as a result glucose is excreted. Due to osmotic effects, water and salts are also excreted, and the frequent urination that occurs can lead to dehydration. At high glucose concentration, non-enzymatic

glycosylation of proteins, including haemoglobin and collagen may occur, which can lead to long term tissue damage in the kidney, retina, nerves and cardiovascular system. Glucose can also be converted to other sugars, e.g.sorbitol which causes the lens of the eye to swell, leading to blurred vision and cataracts. An agonist to β_3-adrenoceptors would be of use for the fact that it stimulates the uptake of glucose by cells.

1.6.6. *NEUROLOGICAL DISORDERS*

Neurological disorders that are characterized by dysfunction of the autonomic nervous system may be associated with alterations in β-adrenoceptors. These disorders include primary neurological diseases as well as diseases, such as diabetes mellitus,mentioned above, that may secondarily manifest abnormalities in autonomic nervous system activity.

1.6.7. *PSYCHIATRIC DISORDERS*

Noradrenaline in the brain has been implicated as an important regulator of several functions, including mood, memory, neuroendocrine control, and stimulation of the autonomic nervous system. Several of these functional responses are thought to be controlled by β-adrenoceptors in the central nervous system.

CHAPTER 2.

MATERIALS AND METHODS

2.1. MATERIALS

All reagents employed were of the highest quality available and were obtained from the following suppliers:

2.1.1. GENERAL REAGENTS

B.R.L., Paisley, U.K.

Pre-stained molecular weight markers.

Boehringer (U.K.) Ltd. Lewes, East Sussex, U.K.

GTPγS, Gpp(NH)p, GDP, creatine phosphate, creatine kinase

Fisons Scientific Equipment, Loughborough, U.K.

Acrylamide, EDTA, glucose, glycine, HEPES, magnesium chloride, sodium hydroxide, di-sodium hydrogen orthophosphate, SDS.

RBI, Natick, MA, U.S.A.

Bromoacetyl alprenolol menthane

Sigma Chemical Co., Poole, Dorset, U.K.

Agarose, N,N'-methylenebisacrylamide, cholera toxin, bovine serum albumin, gelatin, TEMED, trypsin, o-dianisidine hydrochloride, ATP disodium salt, cAMP sodium salt, thimerisol, Dowex AG50W-X4, alumina, imidazole, forskolin, sodium acetate, deoxycholic acid, Ponceau S, nonidet-P40.

Speywood Ltd, Berkshire, U.K.

Pertussis toxin.

Whatman International Ltd. Maidstone,U.K

GF/C Glassfibre filters.

All other reagents were obtained from Merck Ltd, Poole, Dorset, U.K.

2.1.2. TISSUE CULTURE PLASTICWARE

Costar, 205 Broadway, Cambridge, MA., U.S.A.

Biofreeze vials, 12-chamber microphysiometer plates, nitrocellulose.

Molecular Devices, Sunnyvale, CA , USA

Microphysiometer disposables: plungers, capsule cups, blue spacers, sterilant.

Nunc, Denmark.

75 cm^2 tissue culture flasks, 25 cm^2 tissue culture flasks, tissue culture plates, 96-well plates, 24-well plates.

2.1.3. CELL CULTURE MEDIA

Gibco Life Technologies, Paisley, U.K.

Dulbecco's modification of Eagle's medium (10 x), glutamine (200mM), sodium bicarbonate, 50 x HAT (hypoxanthine, aminopterin, thymidine), penicillin (100 I.U./ml) and 100 x streptomycin (100 mg/ml), nutrient mixture Ham's F12, Alpha modified Eagle's medium (without ribonucleosides, deoxyribonucleosides), foetal bovine serum, newborn bovine serum, phosphate buffered saline, versene.

2.1.4. RADIOCHEMICALS

Amersham plc, Amersham, Buckinghamshire

[8-^{3}H]adenosine 3', 5', cyclic phosphate, ammonium salt.

Specific activity =24 Ci/mmol

1-[propyl-2,3-^{3}H]dihydroalprenolol.

Specific activity =64 Ci/mmol

[^{125}I]-low specific activity cyanopindolol.

Specific activity =300 Ci/mmol

Adenosine 5'-[α-^{32}P]triphosphate, triethylammonium salt.

Specific activity =400 Ci/mmol

NEN DuPont, Boston, MA

[12-^{3}H]forskolin. Specific activity=31 Ci/mmol

[^{125}I]cAMP flashplate assay containing cAMP [^{125}I]Tracer (Succinyl cAMP Tyrosine methyl ester [^{125}I]

2.2. METHODS

2.2.1. CELL CULTURE

a) *CELL GROWTH*

Chinese Hamster Ovary (CHO) cells transfected with the human β-adrenoceptors (β_1, β_2, and β_3-adrenoceptors) were grown in 75 cm^2 tissue culture flasks in 0.04% (w/v) sodium bicarbonate buffered α-modified Eagle's medium (αMEM), containing 10% (v/v) foetal bovine serum (FBS) which had been heat inactivated at 56°C for 90 minutes. The medium was supplemented with glutamine, penicillin and streptomycin at final concentrations of 2 mM, 1000 units/ml and 100 μg/ml, respectively.

Parental, non-transfected CHO cells were grown in Ham's F12 nutrient medium containing 10% FBS (heat inactivated as above). CHO cells transfected with the rat β_3-adrenoceptor were grown in Ham's F12 nutrient medium with 10% FBS and G418 (geneticin) at a concentration of 50 μg/ml.

Murine neuroblastoma x embryonic Chinese hamster brain NCB20 cells were grown in DMEM with 0.04% (w/v) sodium bicarbonate and 10% FCS. The medium was supplemented with glutamine, penicillin and streptomycin at final concentrations of 2 mM, 1000 units/ml and 100 μg/ml, respectively. They were maintained in the presence of geneticin sulphate (800 μg/ml).

All cells were grown in a humidified atmosphere of 5% CO_2/95% air.

b) *CELL SUBCULTURE*

Confluent cells were passaged using a sterile trypsin solution containing 0.1% (w/v) trypsin, 0.67 mM EDTA and 10 mM glucose in PBS (0.27 mM potassium chloride, 14 mM sodium chloride, 0.15 mM potassium dihydrogen orthophosphate, 0.8 mM disodium hydrogen orthophosphate, heptahydrate, pH 7.4.). Growth media was removed from the cells and 2 ml of trypsin solution added. When the cells were removed from the surface of the flask, trypsinization was stopped by the addition of growth medium. This cell suspension was centrifuged at 800 x *g* in a MSE centaur centrifuge for five minutes to pellet the cells. The cell pellet was resuspended in growth medium and split to fresh flasks as required.

Cells to be placed on the microphysiometer from transient transfections were lifted gently with versene (0.2 g/l isotonic buffered saline), then spun down and the pellet seeded into capsules for use on the microphysiometer the following day.

c) *CELL MAINTENANCE*

Confluent cells were removed from the surface of the flask by trypsinization as described above and the cells resuspended in freezing medium, which consisted of 70% FBS, 20% growth medium and 10% dimethyl sulphoxide for both CHO cells and NCB20 cells. The suspension was aliquoted into 0.5 ml volumes in Biofreeze vials, frozen overnight packed in cotton wool at -80°C, and then transferred the following day to liquid nitrogen for storage. Cells to be brought up from liquid nitrogen storage were thawed rapidly at 37°C, resuspended in appropriate growth medium and centrifuged at 800 x *g* in a MSE Centaur centrifuge for five minutes to pellet the cells. The cell pellet

was resuspended in growth medium and plated out in a final volume of 10 ml per 75 cm^2 flask.

d) *CELL HARVESTING*

When confluent, growth medium was removed from the cell culture flask and 10 ml of ice-cold PBS (0.27 mM potassium chloride, 14 mM sodium chloride, 0.15 mM potassium dihydrogen orthophosphate, 0.8 mM disodium hydrogen orthophosphate, heptahydrate, pH 7.4) added. Cells were gently washed from the surface of the flask, collected in a 50 ml conical tube and centrifuged at 800 x *g* in a Beckmann TJ6 centrifuge for 10 minutes. The resulting cell pellet was washed with ice-cold PBS and re-centrifuged. The final pellet was stored at -80°C until use.

2.2.2. PRODUCTION OF CRUDE PLASMA MEMBRANE FRACTIONS

Membranes were produced according to Koski and Klee (1981). Frozen cell pellets were thawed and suspended in 5 volumes of ice-cold 10 mM Tris-HCl, 0.1 mM EDTA pH 7.5, then homogenised with 20-25 strokes of a ground glass on teflon homogeniser. The homogenate was centrifuged at 500 x *g* for 10 minutes in a Beckmann L5-50B centrifuge with a Ti50 rotor, to remove unbroken cells and nuclei. Plasma membranes were collected by further centrifugation of the supernatant at 48,000 x *g* for 10 minutes, washed in 10 volumes of the same buffer and after a second centrifugation at the same speed, were resuspended in the same buffer to a final protein concentration of between 0.5-3 mg/ml. These samples were aliquoted and stored at -80°C until required.

2.2.3. PROTEIN DETERMINATION

The method used is based on that of Lowry *et al*, 1951. Stock solutions to be used were 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide, 1% (w/v) copper sulphate, 2% (w/v) sodium potassium tartrate. Just prior to use, these stock solutions were mixed in the following ratio: one hundred parts sodium carbonate in sodium hydroxide to one part copper sulphate plus one part sodium potassium tartrate. Protein standards were prepared using 1 mg/ml BSA, and a standard curve constructed with a maximum of 30 μg of protein per sample. Unknown samples were usually assayed in 1, 3 and 5 μl volumes in duplicate. Samples were made up to a constant volume with Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5). 1 ml of the mixed solution was added to each sample, mixed and left to stand for 15 minutes. 100 μl of Folin and Ciocalteau's phenol reagent diluted 1:1 with water was added to each sample, mixed and allowed to stand for a further 20 minutes. The absorbance of light by each sample was assessed spectrophotometrically at 750 nm in a Shimadzu model of spectrophotometer.

2.2.4. ANTISERUM PRODUCTION

All antisera used were generated against synthetic peptides, essentially as described by Goldsmith and colleagues (Goldsmith *et al*, 1988). This section is for information only, as I did not perform the techniques myself. 3 mg of the appropriate peptide and 10 mg of keyhole limpet haemocyanin were dissolved slowly in 1 ml of 0.1 M phosphate buffer pH 7.0. 0.5 ml of 21 mM glutaraldehyde (also in 0.1 M phosphate buffer, pH 7.0) was then added dropwise with stirring and the combined 1.5 ml incubated overnight at room temperature. The 1.5 ml solution was mixed with an equal volume of complete Freund's

adjuvant and briefly sonicated with a Branson model 'soniprobe'. 1 ml aliquots of the resulting emulsion were injected in multiple subcutaneous sites in New Zealand white rabbits. Four weeks later each animal received a booster immunization with material identically prepared, except one half as much keyhole limpet haemocyanin and peptide were injected in incomplete Freund's adjuvant. Bleeds were performed monthly with approximately 15 ml taken from the ear artery and collected into a glass universal. Blood was left to clot overnight at 4°C and the plasma removed and centrifuged at 1000 x *g* in a Beckman TJ 6 centrifuge for 10 minutes to pellet any remains of the clot. The supernatants thus produced were aliquoted into 250 μl volumes and stored at -80°C until use. The antibody named CS which I used to identify G_s was constructed from the C-terminal peptide sequence RMHLRQYELL of $G_s\alpha$ at positions 385-394. Antiserum SG was produced in a similar fashion against the C-terminal decapeptide of the α subunit of rod transducin (KENLKDCGLF) This antiserum identifies both $G_i1\alpha$ and $G_i2\alpha$. Antiserum CQ was generated against a synthetic peptide (QLNLKEYNLV) which represents the C-terminal decapeptide which is conserved between $G_q\alpha$ and $G_{11}\alpha$.

2.2.5. GEL ELECTROPHORESIS

a) *SAMPLE PREPARATION*

Samples were prepared for gel electrophoresis by sodium deoxycholate/ trichloroacetic acid precipitation; 10 μl of 2% (w/v) sodium deoxycholate was added to each sample, followed by 750 μl of water, then 250 μl of 24% (w/v) trichloroacetic acid. Samples were placed in a microfuge for 20 minutes at 12,000 x *g* , after which the supernatants were removed. The pellets were then dissolved in 20 μl of 1M Tris base followed by 20 μl of Laemmli buffer, which consisted of 5 M

urea, 0.17 M SDS, 0.4 M dithiothreitol, 50 mM Tris-HCl (pH 8.0), and 0.01% bromophenol blue.

b) *LOWER RESOLVING GEL:10% (w/v) SDS-PAGE*

Gel electrophoresis was carried out according to the discontinuous system described by Laemmli (1970). The gel plates were 180 mm by 160 mm with spacers of 1.5 mm. The slabs were run as a part of a Bio-Rad Protean I electrophoresis apparatus (Bio-Rad Laboratories Ltd, Watford, Herts.). Separating gels contained 10% (w/v) acrylamide and 0.27% (w/v) bisacrylamide with 0.375 M Tris (pH 8.8), 0.1% (w/v) SDS, 0.033% (v/v) glycerol, 0.0003% (v/v) TEMED and 0.0004% (w/v) ammonium persulphate. The final volume was 24 ml with the 1.5 mm spacers and 36 ml with the 2.5 mm spacers. The solution was immediately mixed and poured into a Biorad protean I gel casting apparatus. The gel was layered with 0.1% (w/v) SDS to exclude air, and left to set at room temperature for approximately 2 hours.

c) *UPPER STACKING GEL*

The upper stacking gels contained 3% (w/v) acrylamide and 0.08% (w/v) bisacrylamide with 0.125 M Tris (pH 6.8), 0.1% (w/v) SDS, 0.0005% (v/v) TEMED and 0.001% (w/v) ammonium persulphate. These were left to set around a 15 well teflon comb for at least 30 minutes. The solution was mixed, layered on top of the resolving gel and allowed to polymerize.

d) *ELECTROPHORESIS*

The running buffer contained 25 mM Tris (pH 8.5), 0.192 M glycine and 0.1% (w/v) SDS. Samples were loaded onto the gels described above using a Hamilton syringe (Hamilton Co., Reno, Nevada).

Molecular mass determinations were based on prestained SDS molecular weight markers (Sigma). Electrophoresis was performed overnight at 50 V (Bio-Rad system). Electrophoresis was towards the anode at 25 mA per slab until the bromophenol blue dye front was 0.5 cm from the bottom.

e) *GEL PROTEIN STAINING*

After electrophoresis, the gel was placed in a tray and covered in stain solution of Ponceau S for five minutes or until proteins were apparent on the gel as discrete bands. The Ponceau S was completely removed with PBS (0.27 mM potassium chloride, 14 mM sodium chloride, 0.15 mM potassium dihydrogen orthophosphate, 0.8 mM disodium hydrogen orthophosphate, heptahydrate, pH 7.4.) .

2.2.6. WESTERN BLOTTING

Proteins were separated under appropriate resolving conditions on SDS-polyacrylamide gels overnight at 50 V, 25 mA. The proteins were transferred to a nitrocellulose sheet for 2 hours at 1.5 mA in an LKB transblot apparatus. Blotting buffer consisted of 0.192 M glycine, 25 mM Tris and 20% (w/v) methanol. After immunoblotting, the nitrocellulose was placed in a tray and covered in stain solution of Ponceau S for five minutes or until proteins were apparent on the gel as discrete bands. Once protein loading was confirmed as being accurate, the Ponceau S was completely removed with PBS. The sheet was then "blocked" for 2 hours at 37°C in 5% (w/v) gelatin in PBS, containing 0.0004% (w/v) thimerisol as an anti-bacterial agent. After this time gelatin was washed off with large amounts of distilled water, and the nitrocellulose sheet incubated overnight at 37°C with the appropriate dilution of antiserum in 1% (w/v) gelatin in PBS. This antiserum was

the specific anti-G-protein antiserum. On the following day, the antiserum was removed and the nitrocellulose sheet subjected to a series of washes in water to remove all the unbound antiserum, then PBS plus 0.2% (v/v) NP-40 for 10 minutes each, finally followed by a series of PBS washes. The blot was then incubated with a secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG) for 2 hours at 37°C. The second antibody was then removed and the nitrocellulose sheet subjected to the same series of washes as before. The blot was then developed in 50 ml PBS plus 1% o-dianisidine as substrate. Development of the blot was begun by addition of 10 μl of hydrogen peroxide, and stopped by immersion of the blot in 1% (w/v) sodium azide. The blot was finally removed to water, before being dried on blotting paper. Both first and second antibodies could be reused up to four times, and were stored at 4°C containing 0.004% (w/v) thimerisol. Immunoblots were quantified by densitometric scanning using a Bio-Rad imaging densitometer which was linked up to a software package called 'Molecular Analyst' on a Macintosh Quadra 800 computer.

2.2.7. RADIOLIGAND BINDING ASSAYS

Binding assays were performed by the rapid filtration method as described by Pert and Snyder (1973) using a binding buffer (unless otherwise stated) of ice-cold 10 mM Tris HCl, 50 mM sucrose, 20 mM magnesium chloride pH 7.5. 10-50 μg of membrane protein was used plus the appropriate radiolabelled ligand to a final volume of 250 μl. Non-specific binding was assessed in parallel tubes containing the appropriate drug at saturating concentrations. Blank values were determined by replacement of membrane protein with buffer.

The assay was initiated by transferral of tubes to the appropriate incubation temperature. For example, for the dihydroalprenolol binding, tubes were incubated at 30°C for 30 minutes. After this period of time the tubes were rapidly filtered through Whatman glassfibre (GF/C) filters which had been presoaked in binding buffer, followed by three 5 ml washes of the filter with ice-cold binding buffer. Filters were soaked overnight in Ecoscint scintillation fluid prior to being counted in a Rackbeta scintillation counter.

a) [^{3}H]dihydroalprenolol (DHA) binding

25 μl [^{3}H] DHA (64 Ci/mmol) was added at a final concentration of 2 nM to 50 μl membranes (5 μg per assay), with or without the presence of 100 μM propranolol to define non-specific binding. The tubes were made up to a final volume of 250 μl with binding buffer. Tubes were incubated at 30°C for 30 minutes.

b) [^{3}H]forskolin binding

50 μl of [^{3}H]forskolin (31 Ci/mmol) was added at a final concentration of 10 nM to assays with or without 50 μl of appropriate agonist, 300 μl of whole cells (1-2 x10^6 /ml) in DMEM/HEPES (DMEM + 20 mM HEPES pH 7.4 at 4°C) and in the presence or absence of 50 μl 100 μM forskolin to define nonspecific binding. Samples were made up to a final volume of 500 μl with DMEM/HEPES and were incubated at 4°C for 90 minutes.

c) [^{125}I]cyanopindolol (ICYP) binding

Low specific activity (300 Ci/mmol) ICYP was added at a range of concentrations for the determination of B_{max} values of the β_3-adrenoceptors. A volume of 25 μl [^{125}I]-CYP was added to the 50

μl homogenate (20 μg membrane protein), with or without the addition of 100 μM (S)- propranolol to define non-specific binding. This was made up to a total volume of 125 μl with an assay buffer consisting of 50 mM Tris pH 7.7, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4 at 37°C. Assays were incubated for 60 minutes at 37°C.

2.2.8. ADENYLYL CYCLASE ASSAY

This is essentially the method of Salomon (1979) except that the amount of $\alpha[^{32}P]$ATP was reduced to 1 μCi per sample.This assay monitors the production of $[^{32}P]$cAMP from the substrate $\alpha[^{32}P]$ATP. The cAMP thus produced is separated from unreacted $\alpha[^{32}P]$ATP by a two-step column method.

a) SAMPLE PREPARATION

Reaction mixtures of 50 μl were prepared containing: 5 mM creatine phosphate, 100 mM sodium chloride, 100 U/ml creatine phosphokinase, 25 mM Tris acetate pH 7.0, 0.5 mM ATP pH 7.0, 0.05 mM cAMP,10 mM GTP pH 7.0, 10 mM $MgCl_2$, $\alpha[^{32}P]$ATP (1×10^6 c.p.m.). These mixtures also contained between 10 to 20 μg of membrane protein, together with the ligand(s) of interest. Reaction tubes were kept on ice at all times and the reaction started by removal to a 30°C water bath. After 15 minutes the reaction was stopped by removal to ice and the addition of 100 μl of stopper solution containing 2% (w/v) SDS, 45 mM ATP, 1.3 mM 3'5'cAMP. Prior to boiling for 15 minutes, 50 μl of [8-^{3}H]3'-5'cAMP (approximately 10,000 c.p.m.) was added to each tube. To each sample 750 μl of water was added and the $[^3H]$cAMP and $[^{32}P]$cAMP content of each tube determined.

b) PREPARATION OF DOWEX AND ALUMINA COLUMNS

The method used to quantitate the amount of cAMP produced by each sample was identical to that of Salomon (1979) and involves the separation of cAMP from other nucleotides by Dowex and then alumina chromatography. Dowex H^+ 50 x 4 (200-400) was washed in twice its packed volume with 1 M HCl and then with the same volume of water four times. The Dowex was mixed with water to a slurry (1:1 w/v ratio), 3 ml removed and added to glass wool stoppered columns. The water was allowed to drain out the columns and washed with 2 ml of 1 M HCl . Prior to use, the columns were washed with 1 ml of 1 M HCl followed by 20 ml of water. The alumina columns were prepared by the addition of 0.6 g of dry neutral alumina to glass wool stoppered columns and the columns washed with 15 ml of 0.1 M imidazole. On the day of use, each column was washed with 8 ml of 0.1 M imidazole, pH 7.3.

c) DETERMINATION OF COLUMN ELUTION PROFILES

Prior to sample chromatography, the nucleotide elution profiles for each column were determined. This was performed by applying a mixture of [^{3}H]cAMP and [^{32}P]ATP to the column and determining the elution volume. Stock [^{3}H]cAMP was diluted with water to give approximately 10,000 c.p.m. in 50 μl. [^{32}P]ATP was diluted from stock of 1 mCi/ml to give approximately 2,000 c.p.m. in 50 μl. 50 μl of each of the cAMP and ATP solutions were added to 900 μl of water and the mixture applied to a Dowex column. The ATP and cAMP were eluted from the column by successive washes of the column with 0.5 ml of water. Fractions were collected in a vial with 15 ml of Ecoscint and radioactivity determined by scintillation counting using a dual label programme. The elution volumes required to elute the cAMP from the

Dowex columns were then determined graphically. Typical recovery from the Dowex columns was greater than 80%. The elution volume required to elute the cAMP from the alumina columns was determined as for the Dowex columns except that only [^{3}H]cAMP was used, and the eluting buffer was 0.1 M imidazole. Recoveries were similar to that obtained for the Dowex columns.

d) DETERMINATION OF cAMP PRODUCED BY MEMBRANE FRACTIONS

Samples (total volume 950 μl) were added to prepared Dowex columns and the ATP eluted with 1.8 ml of water. 3.5 ml of water was then added to the Dowex columns and this eluate allowed to run directly onto the alumina columns. The cAMP fraction was eluted into vials containing 15 ml of Ecoscint, with 6 ml of 0.1 M imidazole, pH 7.3. The recovery of cAMP from the columns was routinely greater than 80% and when the column recovery fell below 60%, columns were discarded and fresh columns prepared. This gave a column life of approximately 6 months. The cAMP fractions obtained were counted on a dual label scintillation counting programme which automatically corrected for 'spillover' from each channel. The amount of cAMP produced by each sample was calculated by taking into account the recovery from each column, based on the recovery of the [^{3}H]cAMP internal standard. Data was thus calculable in pmoles of cAMP produced per minute per milligram of membrane protein (pmol/min/mg), and the assay was sensitive to approximately 5 pmoles/min/mg.

2.2.9. cAMP FLASHPLATE ASSAY

The basic principle of this method to assess cAMP production is the competition between a radioactive and a non-radioactive antigen (sample) for a fixed number of antibody binding sites. In the [^{125}I]cAMP flashplate, the antibody is fixed to scintillant-coated microplate wells and the counting of the bound fraction is dependent upon the distance of the material to the walls of the wells. Separation of the bound from the free antigen is not necessary to quantitate the bound tracer.

a) *TREATMENT OF 96-WELL PLATES*

Cells were seeded into 96-well plates and grown until confluency in 100 μl of growth medium per well. Plates were then treated at room temperature, with 10 μl 0.5 mM isobutyl methylxanthine (IBMX) per well for 10 minutes, before treatment with agonists for 30 minutes. The reaction was stopped with 10 μl 5% perchloric acid (PCA), mixed, then placed at 4°C overnight. The following day the plates were neutralised with 100 μl of 50 mM potassium hydroxide in 250 mM sodium acetate per well. Wells were then ready to be assayed for cAMP production.

b) FLASHPLATE PROCEDURE

From a total reaction volume after neutralisation of 200 μl, 30 μl of sample was added to the flashplate, along with 70 μl of cAMP assay buffer. The remaining amount was stored at 4°C in case the assay needed to be repeated. For comparison a standard curve was set up in parallel, preferably on the same plate, with a range of cAMP levels from 1 to 50 pmol/ml. To all samples 100 μl of working tracer is added (cAMP[^{125}I]tracer) and the plates left overnight at 4°C before being

counted on a 'TopPlate' counter (Packard). Unknown levels of cAMP from samples could be calculated directly from the standard curve.

2.2.10. MICROPHYSIOMETER EXPERIMENTAL PROCEDURE

The Cytosensor® Microphysiometer (Molecular Devices) detects cellular responses to a wide variety of specific and nonspecific stimuli by measuring the rate of excretion of acidic metabolites with a silicon sensor that functions as an extremely rapid and sensitive extracellular pH meter.

Cells were sandwiched between two porous polycarbonate membranes inside disposable cell capsules, on which they were seeded at 300,000 cells per capsule the day prior to placing on the microphysiometer. On the day of the experiment capsules were placed on the microphysiometer into the silicon sensor chambers. A plunger created a microvolume cavity, 100 μm deep and 6 mm in diameter, to contain the cells. The cells were supplied with a controlled flow of nutrients, with or without the effector of interest. This supply of nutrients consisted of a pot of powdered DMEM (without salts) dissolved in 1 l of deionised water, 6.6 ml of 4.0 M NaCl at pH 7.4. This medium lacked bicarbonate so that it has a low buffering capacity. 1 mg/ml bovine serum albumin (BSA) was added to 800 ml of this, which was fed through the tubes to the silicon chambers. These chambers containing the cells were rinsed at the start of each experiment with medium lacking BSA. BSA was included to prevent drugs sticking to the tubes of the microphysiometer, although is probably not vital in the case of such ligands as isoprenaline.

Acidification rates were determined in microvolts per second during periods of flow cessation (1 μV/sec =1 millipH/min) called 'off-rates'.

Flow was interrupted for an interval of 30 s, during which the rate of acidification was measured and recorded. The flow was then resumed and the next cycle begun. The rates were recorded and plotted as a function of time. Data was exported into appropriate database and graphic programs for further processing, such as the computation of the percentage change in acidification rates.

After experiments were completed, the microphysiometer was washed through with a sterilant solution obtained from Molecular Devices to avoid the formation and build-up of *Mycoplasmae* between use. Pumps were continuously kept running when the microphysiometer was not in use.

2.2.11. MOLECULAR BIOLOGY TECHNIQUES

2.2.11.1. CONSTRUCTION OF CHIMERIC β_3-ADRENOCEPTORS

Eight human/rat β_3-adrenoceptor chimeras were constructed using oligonucleotide-directed *in vitro* mutagenesis. Chimeras were generated by a method based on the work of Kunkel *et al* (1985), using a Muta-Gene® phagemid *in vitro* mutagenesis kit (Bio-Rad). The native human β_3-adrenoceptor gene, was subcloned from the in-house CNOD vector (SB Pharmaceuticals) into the pRc/CMV vector (Invitrogen). This vector contained the f1 origin required for generating single stranded uracil containing DNA to which the mutagenic oligonucleotide anneals prior to acting as a primer for synthesis of the complementary strand by T7 DNA polymerase. *E.coli* MV1190 was transformed with the resulting dsDNA, whereupon replication of the plasmid, the uracil-containing, non-mutant strand was selected against. The frequency of mutants was therefore increased allowing identification directly by DNA sequencing.

Oligonucleotides were designed to independently mutate each transmembrane domain from the human to the rat amino acid sequence. There is no TM3 chimera as transmembrane 3 is identical in both human and rat β_3-adrenoceptors and additionally, a double TM mutant consisting of both the TM1 and TM7 mutations was constructed. The oligonucleotides, ranging in length from 35 to 82 nucleotides, were synthesised by R&D Systems Europe Ltd. A single mutagenic oligonucleotide was designed for each transmembrane domain introducing up to five mutations simultaneously.

2.2.11.2. EXPRESSION OF THE β_3-ADRENOCEPTOR IN CHO CELLS

a) *TRANSIENT TRANSFECTIONS*

Day 1: CHO cells were seeded in an 80 cm^2 plate such that cells will be 70-90% confluent the following day. Cell density on the day of transfection is a crucial factor for successful transfection.

Day 2: For each flask of cells to be transfected, 36 μg of plasmid DNA was made up in 1.8 ml of serum free growth medium (SFGM) and 108 μl of lipofectAMINE in 1.8 ml of SFGM. The lipofectAMINE and plasmid solutions were thoroughly mixed in a 20 ml universal tube and allowed to stand for 45 minutes. Meanwhile the cells were washed gently four times with 10 ml of warm SFGM to remove serum. They were then left in the incubator for about 30 minutes before washing twice more. After standing for 45 minutes, the transfection reaction volume per flask was brought to 18 ml with SFGM (i.e.14.4 ml SFGM +3.6 ml of reaction mixture). The SFGM was aspirated from the cells and 18 ml of the diluted reaction mixture added to each flask. These were incubated at 37°C/5% CO_2 for five hours. After five hours 18 ml

of growth medium containing twice the normal strength of serum was added to each flask. This quenched the transfection.

Day 3: The medium was gently aspirated and carefully replaced with 36 ml of fresh medium containing normal levels of serum.

Day 4: Medium was aspirated from the cells and replaced with 5 ml of warm EDTA (200 mg/l). This was left for 1-2 minutes then removed and added to a universal tube. A further 5 ml of EDTA was added to the cells and the step repeated. This step was repeated once more and the EDTA left on the flasks until all cells had been lifted off. The cells were collected in the universal tube and spun down. The resulting cell pellet was resuspended and either plated onto microphysiometer capsules or onto 96-well plates for use in the Flashplate cAMP accumulation assay the following day.

b) *MASS CULTURE PRODUCTION*

For the production of mass cultures the transient transfection steps above were followed but instead of lifting cells with EDTA, cells were left to grow in normal medium until confluency, then split to new flasks and left to settle down for a number of weeks.

2.2.11.3. RNA ISOLATION BY THE RNAZOLE METHOD

a) *HOMOGENIZATION*

Cells were scraped from the flask wall into the growth medium to obtain a cell suspension. 750 μl of RNAzole B was added to the flask and cells lysed by mixing with a Pasteur pipette. RNA was solubilised by passsing the lysate several times through the Pasteur pipette. Cells were then placed on ice for 30 minutes.

b) *EXTRACTION*

After 30 minutes on ice 100 μl of chloroform was added per 2 ml of homogenate formed. The tube was covered tightly and shaken vigorously for 15 seconds, after which it was placed on ice for 5 minutes. The suspension was then centrifuged at 12,000 x *g* for 15 minutes at 4°C. After centrifugation, the upper aqueous phase containing RNA was obtained by carefully avoiding the lower phase of phenol-chloroform containing the DNA and protein.

c) *PRECIPITATION*

To this tube an equal volume of isopropanol was added and placed on ice for 15 minutes. This sample was then spun at 12,000 x *g* for 15 minutes to produce a small white pellet of RNA at the base of the tube.

d) *WASHING*

The supernatant was removed and the pellet washed with 75% ethanol by vortexing and mixing. This was then spun at 7,500 x *g* for 8 minutes, followed by drying. The RNA was then dissolved in 0.5% SDS and the RNA concentration measured by taking 5 μl RNA diluted to 1 ml with sterile water and recording the optical density at 260 and 280 nm.

2.2.11.4. REVERSE TRANSCRIPTION (RT)

5 μg obtained from the RNAzole method above was added to a sterile tube and diluted with diethylpyrocarbonate (DEPC) treated water to 20 μl. The RNA solution was then heated to 65°C for 10 minutes, chilled on ice and spun down for 5 seconds. From a Pharmacia kit, a cDNA reaction mix was gently pipetted to obtain a uniform suspension. 11 μl of this mix was added to a tube , plus 1 μl DTT, 1 μl oligo dT and the heat denatured RNA. This was mixed thoroughly and incubated at 37°C for 1 hour. The completed first strand cDNA reaction product was now

ready for immediate second strand synthesis, PCR amplification or could be frozen down.

2.2.11.5. POLYMERASE CHAIN REACTION (PCR)

The completed first strand reaction was heated for 5 minutes at 90°C, then chilled on ice. This denatured the RNA-cDNA duplex and inactivated the reverse transcriptase. A PCR mix for 5 tubes was made up consisting of 25 μl 10 x PCR buffer, 15 ul of 15 mM $MgCl_2$, 5 μl of 25 mM dNTPs and 2.5 μl of Taq polymerase. Tubes containing 5 μl of the cDNA reaction, 9.5 μl PCR mix, 1 μl of oligo A, 1 μl oligo B were made up to 50 μl with water. For the prevention of sample evaporation 50 μl of mineral oil was placed over the sample. Positive and negative controls were included, and samples placed into the thermocycler for appproximately 30 cycles. One cycle consisted of 42 s of denaturation at 95°C, then 1 minute of primer annealing at 54°C, followed by 5 minutes of polymerization at 72°C. At the end of the PCR reaction, tubes were removed and the DNA extracted carefully from below the mineral oil layer.

2.2.11.6. AGAROSE GELS

An agarose gel of 1.25% was used, onto which the negative and positive controls were loaded alongside 5 μl of sample. The electrophoresis buffer used was prepared as a 50 x stock solution of 40 mM Tris-acetate, 1 mM EDTA buffer, pH 8, and then diluted on the day of use with H_20 to give a 1 x stock. The gel was stained with ethidium bromide for 25 minutes, rinsed twice in water and visualized under ultraviolet light. Verification of the desired PCR product was obtained by comparison of the sample size with markers and the positive control.

2.2.12. STATISTICAL ANALYSIS

The data were analysed by the use of Student's *t* test. Probability values (P) < 0.05 determined the validity or otherwise of the hypothesis.

CHAPTER 3.

PROCESSES OF DESENSITIZATION OF THE β-ADRENOCEPTORS AND THE ROLE OF THE G-PROTEIN G_s

3.1. METHODS OF DESENSITIZATION

INTRODUCTION

During continuous agonist exposure, it has been observed that a number of G-protein coupled receptors, including the β-adrenoceptors, undergo certain regulatory processes to effect a reduction of their function despite the continued presence of ligand (Liggett *et al*, 1993, Dohlman *et al*, 1987, Hausdorff *et al*, 1990). This waning of the cellular response, known as desensitization, has been particularly well studied for the β-adrenoceptors. In the case of the β-adrenoceptors, desensitization may serve to limit cellular responsiveness during continuous neural firing or where there are persistent levels of catecholamines in circulation. This may be the condition in many types of heart disease, where there are raised levels of catecholamines. Desensitization may also contribute to limiting the clinical effectiveness of therapeutic agents. It is therefore important to build up a clear picture of the underlying processes and how they are controlled.

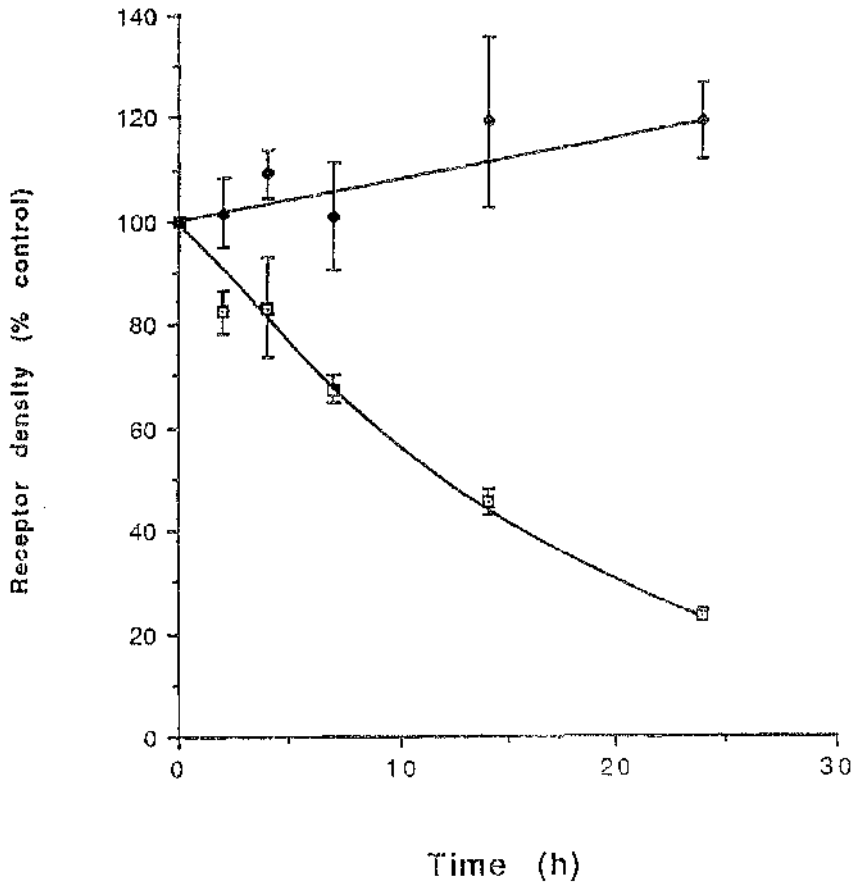
The three subtypes of β-adrenoceptors desensitize via a number of different processes. These include such varied processes as downregulation, sequestration, phosphorylation, and G-protein downregulation. The degree of involvement of each of these different methods in the overall process of desensitization varies between the three different subtypes, depending on such properties as their molecular structure and physiological importance, tissue localization and function in the body.

To investigate the desensitization of the three β-adrenoceptor subtypes, a number of groups have gathered large amounts of evidence by the

production of chimeric constructs (Strader *et al* 1987, Nantel *et al*, 1993). Nantel *et al* (1993) investigated the rapid desensitization of the β_2-adrenoceptor compared with the resistance to rapid desensitization of the β_3-adrenoceptor. While a thirty minute exposure to isoprenaline led to a significant desensitization of β_2-adrenoceptor-stimulated adenylyl cyclase activity, it only produced a marginal effect on β_3-adrenoceptor responsiveness. However, following addition of β_2-adrenoceptor phosphorylation sites to the sequence of the β_3-adrenoceptor, by substitution of the third cytoplasmic loop and the carboxyl-terminal tail of the β_3-adrenoceptor with the corresponding regions of the β_2-adrenoceptor, desensitization of the chimera was partially, but not completely restored. This data implies that other molecular determinants are also involved in the development of agonist-promoted desensitization, but confirms the importance of the cytoplasmic tail of receptors, rich in serine and threonine residues.

From receptor binding studies, groups have assessed the levels of β-adrenoceptor desensitization upon agonist stimulation (Thomas *et al*, 1992, Chambers *et al*, 1994). For example, Chambers *et al* (1994) studied the rate of desensitization of the β_2- and β_3-adrenoceptors transfected into CHO cells after treatment with isoprenaline (100 μM) for varying times (Figure 3.1.1). While a steady reduction in levels of the β_2-adrenoceptor population in membranes derived from β_2-adrenoceptor transfected CHO cells was noted, reaching some 80% following exposure to the agonist for 24 hours, no reduction in levels of the β_3-adrenoceptor was recorded, as assessed by specific binding of [^{125}I]CYP. In fact, a slight increase in β_3-adrenoceptor levels occurred over 24 hours treatment. This was in agreement with observations by Thomas *et al* (1992).

Figure 3.1.1. Time course of β-adrenoceptor down-regulation in β_2- and β_3-adrenoceptor expressing CHO cells. β_2- and β_3-adrenoceptor expressing CHO cells were exposed to isoprenaline (100 μM) for varying times and membranes prepared. Levels of either the β_2- (open squares) or β_3-(closed circles) adrenoceptor were then measured using the specific binding of [^{125}I]CYP. The graph is taken from Chambers *et al* (1994).


Receptor density (% control)
140
120
100
80
60
40
20
0
0
10
20
30
Time (h)

In this Chapter I set out to investigate the possible methods of G-protein down-regulation and its effect on β-adrenoceptor function. I also attempted to clarify the methods of sequestration and recovery of G_s in response to activation of different β-adrenoceptors.

It had already been confirmed that the β_1 and β_2-adrenoceptors down-regulate extensively following sustained exposure to agonists, but whether their G-proteins co-down-regulate was unknown. While it was known that the β_3-adrenoceptor was particularly resistant to down-regulation, and in fact on some occasions up-regulates upon agonist stimulation (Thomas *et al*, 1993, Chambers *et al*, 1994), it could be a possibility that the G-protein down-regulates alone. Although a great deal of work has been performed by different groups on the three β-adrenoceptors, very little is known of their regulation at the G-protein level.

My first set of investigations was to assess how the β-adrenoceptors regulate their activity upon agonist activation. A number of agonists are available for the study of the β-adrenoceptors, such as the physiological agonist adrenaline, but for my studies I selected the agonist isoprenaline.

For the investigation of β-adrenoceptor desensitization and down-regulation of the G-protein, G_s, associated with it, a number of different experiments were performed. The functional assay used for estimation of the level of β-adrenoceptor desensitization was the already well-established Johnson and Salomon (1991) adenylyl cyclase assay. Separation of radiolabelled cyclic AMP and ATP was achieved using the double column method described by Johnson and Salomon (1991).

In parallel with these adenylyl cyclase assays, SDS-PAGE and western blotting was performed for identification of alterations in levels of G_s. An antiserum produced to the C-terminal of G_s (named CS) was used for G_s identification, following which blots were scanned on a densitometer linked to a Macintosh Quadra 800 using a 'Molecular Analyst' software package. The specificity of antiserum CS for $G_s\alpha$ had previously been demonstrated (Milligan and Unson, 1989). Antiserum SG was produced against the C-terminal decapeptide of the α subunit of rod transducin (KENLKDCGLF), and was used here to identify $G_i2\alpha$. Antiserum CQ was generated against a synthetic peptide (QLNLKEYNLV) which represents the C-terminal decapeptide which is conserved between $G_q\alpha$ and $G_{11}\alpha$.

Assays were also performed to investigate the loss of G_s from the plasma membrane into the cytosol, and also whether recovery occured with the transferral of G_s back to the plasma membrane. These were also performed by the use of SDS-PAGE (10% acrylamide) and western blotting techniques.

RT-PCR was performed on mRNA extracted from the β_1-adrenoceptor expressing CHO cells, (chosen because it showed the associated G_s polypeptide to down-regulate most rapidly in response to agonist occupancy of the three β-adrenoceptors) in order to assess the role of alterations in G_s mRNA levels in the overall desensitization process.

Receptor levels of the three different subtypes investigated in these experiments are shown in Table 3.1.1.below, where the D43 and C15 cell lines represent β_3-adrenoceptor expressing CHO cell lines of different receptor densities. These receptor densities were calculated

from [^{3}H]DHA binding performed by the Department of Biotechnology at SmithKline Beecham.

Table 3.1.1. β-adrenoceptor expression in the different CHO cell lines.

CHO cell lines	receptor levels (fmol/mg membrane protein)
β_1-adrenoceptor	7130 ± 258
β_2-adrenoceptor	2300 ± 120
β_3-adrenoceptor (D43)	3000 ± 400
β_3-adrenoceptor (C15)	390 ± 83

3.2. RESULTS

INVESTIGATION OF MECHANISMS OF AGONIST-MEDIATED G_S DOWN-REGULATION BY β_1-ADRENOCEPTOR EXPRESSING CHO CELLS.

SDS-PAGE (10% acrylamide) and western blotting were performed on membranes produced from the harvesting of β_1-adrenoceptor transfected CHO cells. These cells had previously been treated for 16 hours with concentrations of isoprenaline ranging from 1 μM to 0.01 nM. Figure 3.2.1.b shows the curve produced from values obtained after a densitometer scan of the nitrocellulose blot (Figure 3.2.1.a). Western blotting techniques detect mainly the 45 kDa form of $G_s\alpha$, previously identified as $G_s\alpha$-long. Half-maximal reduction in G_s was produced by 2.66 ± 0.88 nM isoprenaline, while the maximal effect was achieved at concentrations greater than 10 nM. After 16 hours of treatment with 1 μM isoprenaline, G_s levels were very low, suggesting almost complete down-regulation of the G-protein. This experiment was repeated at least three times and the results produced were similar.

For an investigation into the time course of down-regulation of G_s upon β_1-adrenoceptor stimulation, β_1-adrenoceptor expressing CHO cells were treated with a maximal concentration of isoprenaline (100 μM), for time periods ranging from 60 minutes to 4 hours, at which time points the cells were then harvested (Figure 3.2.2.a & b). Figure 3.2.2.b. shows the quantitative analysis of immunologically detectable levels of G_s, and after 60 ± 4 minutes, loss of $G_s\alpha$ (L) was 50% of original levels. Maximal down-regulation occured after approximately 200 minutes. Equivalent immunoblots to detect $G_q\alpha$ and $G_i2\alpha$,

demonstrated that there were no significant differences in these polypeptides between untreated and isoprenaline-treated β_1-adrenoceptor transfected CHO cells (Figure 3.2.2 c).

Cholera toxin-catalysed [^{32}P]ADP-ribosylation of β_1-adrenoceptor expressing CHO cell membranes following treatment with 100 μM isoprenaline for different time points produced similar time courses to the immunoblots (Figure 3.2.3.). Half-maximal down-regulation was once again achieved in approximately 60 minutes. Cholera toxin catalyses the [^{32}P]ADP-ribosylation of the α subunit of G_s, confirming once again the disappearance of the G-protein from the plasma membrane.

Figure 3.2.1.a. $G_s\alpha$ down-regulation in β_1-adrenoceptor transfected CHO cells: a typical immunoblot. Concentration-response to isoprenaline in β_1-adrenoceptor expressing CHO cells treated with agonist for 16 hours. Membranes from CHO cells were loaded onto a 10% polyacrylamide gel and $G_s\alpha$ detected using the CS antiserum, previously described. The concentrations of isoprenaline treatment of β_1-adrenoceptor expressing CHO cells were as follows: Control (Lane 1), 0.01 nM isoprenaline (Lane 2), 0.1 nM isoprenaline (Lane 3), 0.5 nM isoprenaline (Lane 4), 1 nM isoprenaline (Lane 5), 50 nM isoprenaline (Lane 6), 100 nM isoprenaline (Lane 7), 1 μM isoprenaline (Lane 8).

Figure 3.2.1 b. Quantitative analysis of a typical immunoblot showing $G_s\alpha$ down-regulation: concentration-response to isoprenaline. Concentration response to isoprenaline in β_1-adrenoceptor expressing CHO cells for 16 hours.

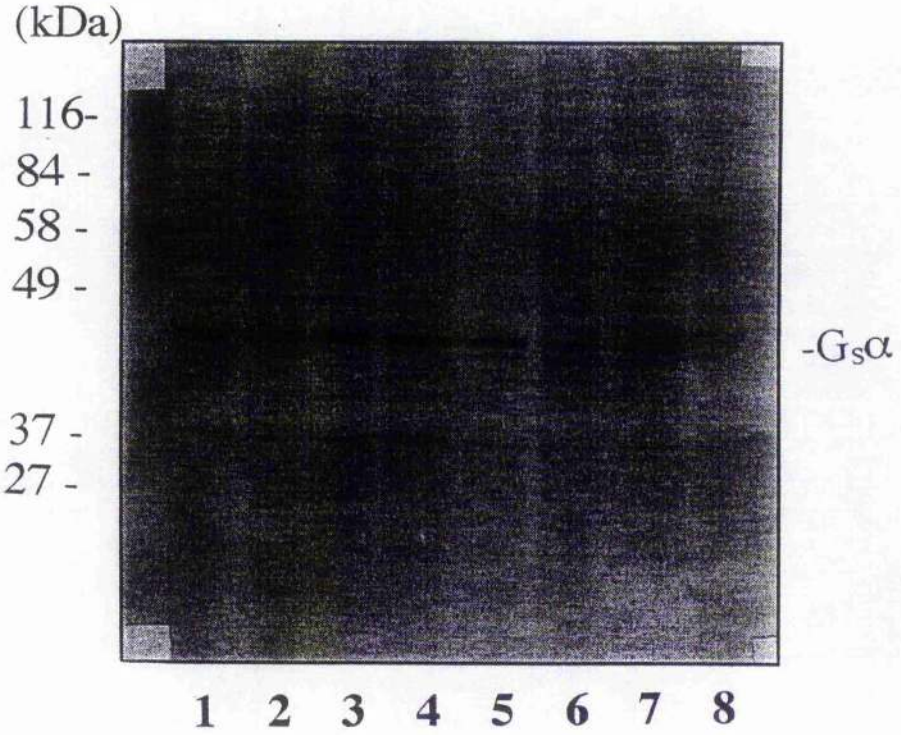

(kDa)
116-
84 -
58 -
49 -
37 -
27 -
-$G_S\alpha$
1 2 3 4 5 6 7 8

Figure 3.2.1.b.

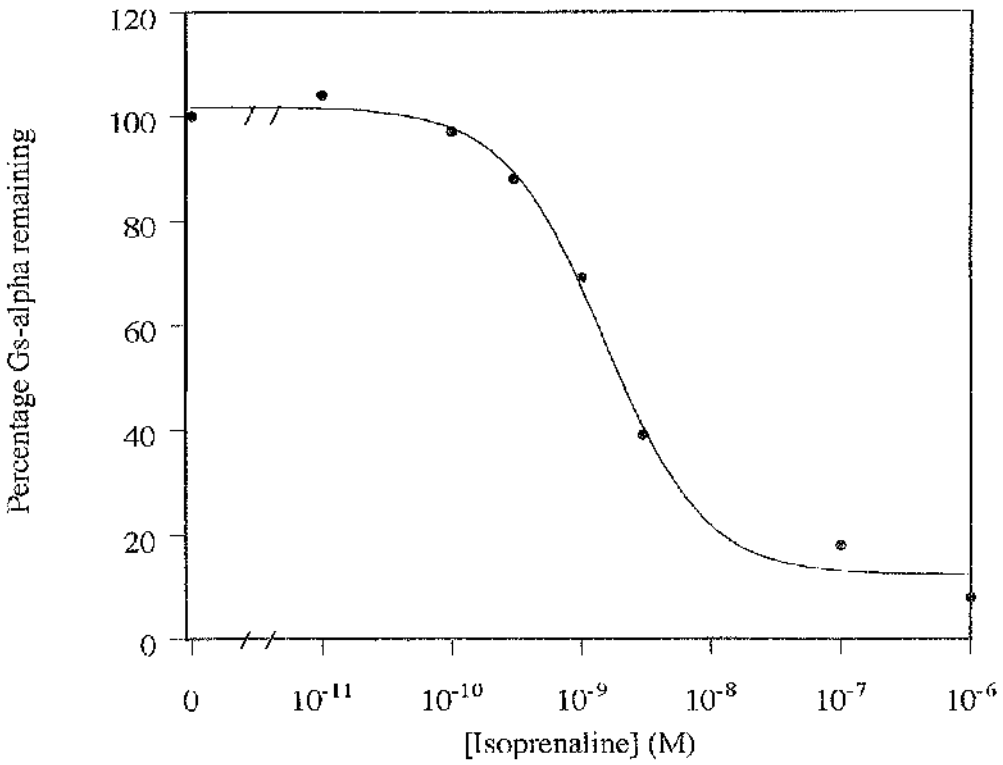

Percentage Gs-alpha remaining
120
100
80
60
40
20
0
0
10-11
10-10
10-9
10-8
10-7
10-6
[Isoprenaline] (M)

Figure 3.2.2.a. Time course of $G_s\alpha$ down-regulation in β_1-adrenoceptor expressing CHO cells: a typical immunoblot. Lanes show membranes of β_1-adrenoceptor expressing CHO cells treated for the following lengths of time with 100 μM isoprenaline: Control (Lane 1), 15 minutes (Lane 2), 30 minutes (Lane 3), 45 minutes (Lane 4), 60 minutes (Lane 5), 75 minutes (Lane 6), 90 minutes (Lane 7), 120 minutes (Lane 8), 180 minutes (Lane 9), 240 minutes (Lane 10).

Figure 3.2.2.b. Quantitative analysis of a typical immunoblot showing $G_s\alpha$ down-regulation during a time course to isoprenaline in β_1-adrenoceptor expressing CHO cells.

Figure 3.2.2.c. Immunoblots of $G_s\alpha$, $G_i2\alpha$ and $G_q\alpha$ down-regulation in β_1- and β_2-adrenoceptor expressing CHO cells treated for 16 hours with isoprenaline (100 μM). Part i shows levels of $G_s\alpha$ in the β_1-adrenoceptor expressing CHO cells (Lanes 1 and 2) and β_2-adrenoceptor expressing CHO cells (Lanes 3 and 4), with (Lanes 2 and 4) and without (Lanes 1 and 3) isoprenaline treatment. Part ii shows levels of $G_q\alpha$ in the β_1-adrenoceptor expr essing CHO cells (Lanes 5 and 6) and β_2-adrenoceptor expressing CHO cells (Lanes 7 and 8), with (Lanes 6 and 8) and without (Lanes 5 and 7) isoprenaline treatment. Part iii shows levels of $G_i2\alpha$ in the β_1-adrenoceptor expr essing CHO cells (Lanes 9 and 10) and β_2-adrenoceptor expressing CHO cells (Lanes 11 and 12), with (Lanes 10 and 12) and without (Lanes 9 and 11) isoprenaline treatment.

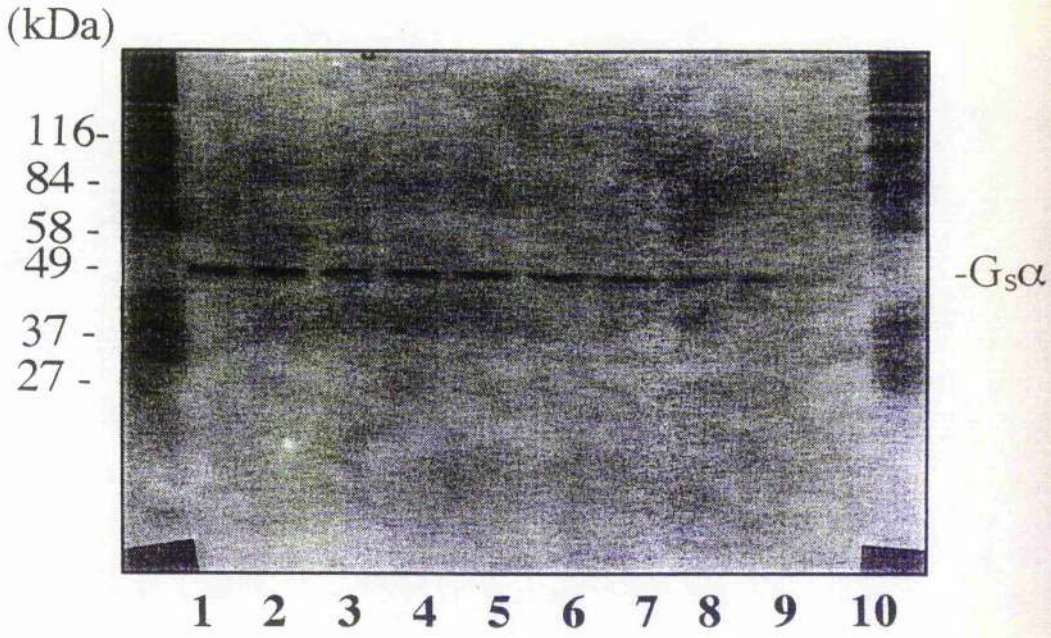
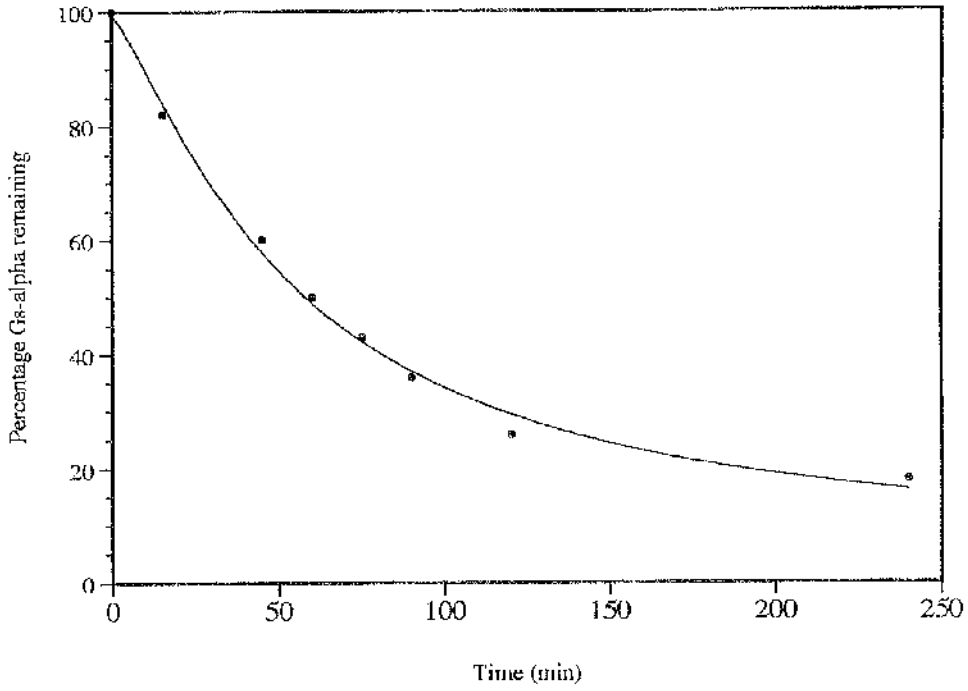

(kDa)
116-
84 -
58 -
49 -
37 -
27 -
-$G_s\alpha$
1 2 3 4 5 6 7 8 9 10

Figure 3.2.2.b.


Percentage Gs-alpha remaining
100
80
60
40
20
0
0
50
100
150
200
250
Time (min)

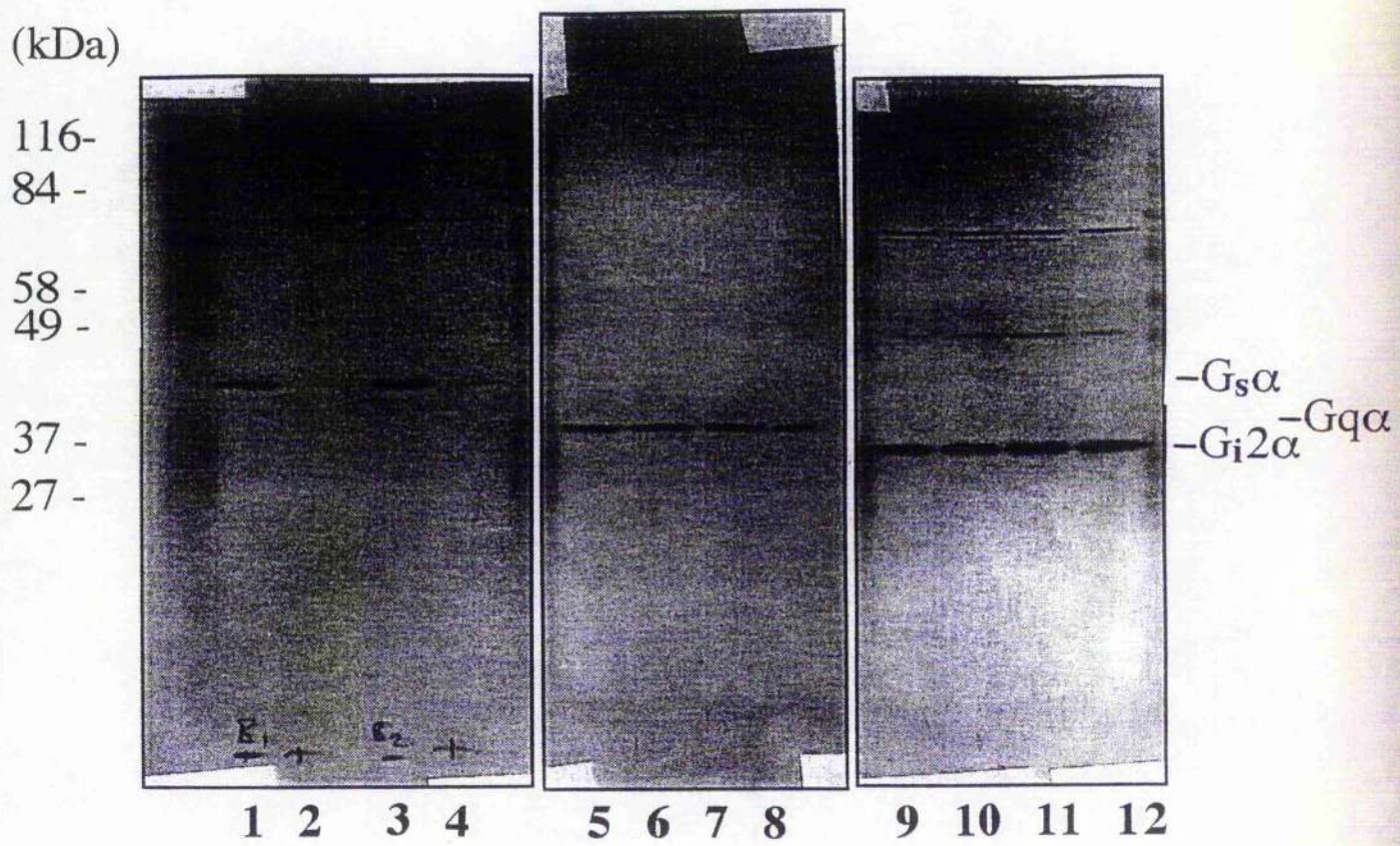
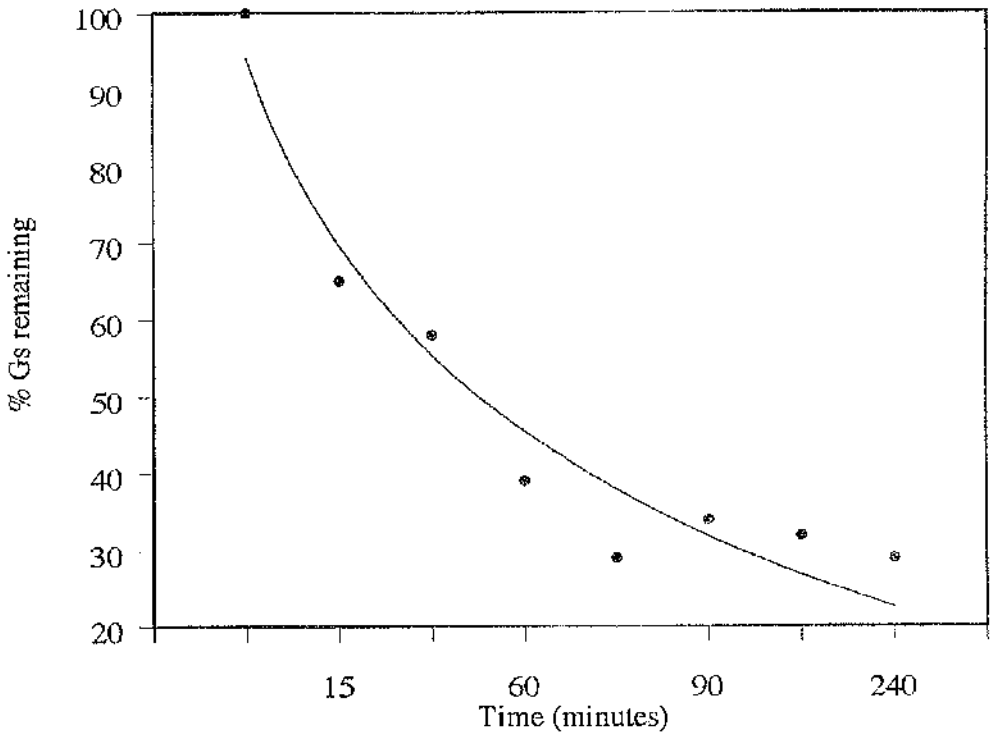

i.
ii.
iii.
(kDa)
116-
84 -
58 -
49 -
37 -
27 -
$G_s\alpha$
$G_i2\alpha$
$Gq\alpha$
1 2 3 4
5 6 7 8
9 10 11 12

Figure 3.2.3. Cholera toxin-catalysed [^{32}P] ADP ribosylation of membranes from β_1-adrenoceptor expressing CHO cells following exposure to isoprenaline: time course.

Figure 3.2.3.


100
90
80
70
60
50
40
30
20
% Gs remaining
15
60
90
240
Time (minutes)

Adenylyl cyclase assays (Johnson and Salomon, 1991) were carried out concurrently with western blots on the same membranes from treated β_1-adrenoceptor expressing CHO cells. Concentration-responses to isoprenaline treatment for 16 hours were performed and results are shown in Figure 3.2.4. Samples were stimulated during the assay with 100 μM isoprenaline. Cyclase activity was measured in units of pmol/min/mg protein. An EC_{50} value for isoprenaline of 1.3 ± 0.9 nM was estimated from concentration-effect curves. This value, an indication of the desensitization of the functional response to agonist, was similar to the EC_{50} value of $G_s\alpha$ down-regulation (2.66 ± 0.88 nM) obtained from western blots, and suggests that $G_s\alpha$ down-regulation is an important component of the overall desensitization process of the β_1-adrenoceptor.

Adenylyl cyclase assays were also performed on membranes from β_1-adrenoceptor expressing CHO cells treated for different times with 100 μM isoprenaline. These samples were also treated in the assay with a maximal concentration (100 μM) of isoprenaline. These produced half-lives for receptor desensitization by isoprenaline of 42.5 ± 3.2 minutes. This value, showing the rate of desensitization of the β_1-adrenoceptor, was similar to the values obtained (60 ± 4 minutes) using the western blotting technique, indicating once again that loss of $G_s\alpha$ may be indicative of receptor desensitization. Figure 3.2.5. shows a typical cyclase assay concentration-response for β_1-adrenoceptor expressing CHO cell membranes treated with 100 μM isoprenaline for varying lengths of time. Experiments were performed more than three times.

Figure 3.2.4. Desensitization of β_1-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with isoprenaline. β_1-adrenoceptor expressing CHO cells.were treated with isoprenaline at varying concentrations for 16 hours. Samples were also stimulated again during the assay with 100 μM isoprenaline. Two further experiments produced similar results.

Figure 3.2.4.

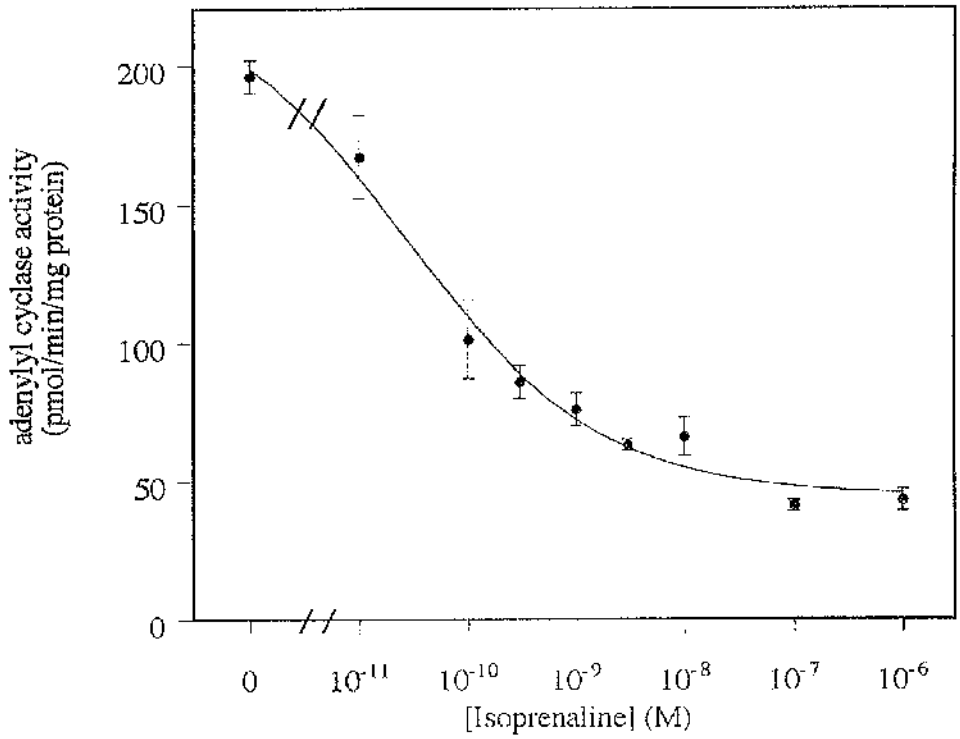
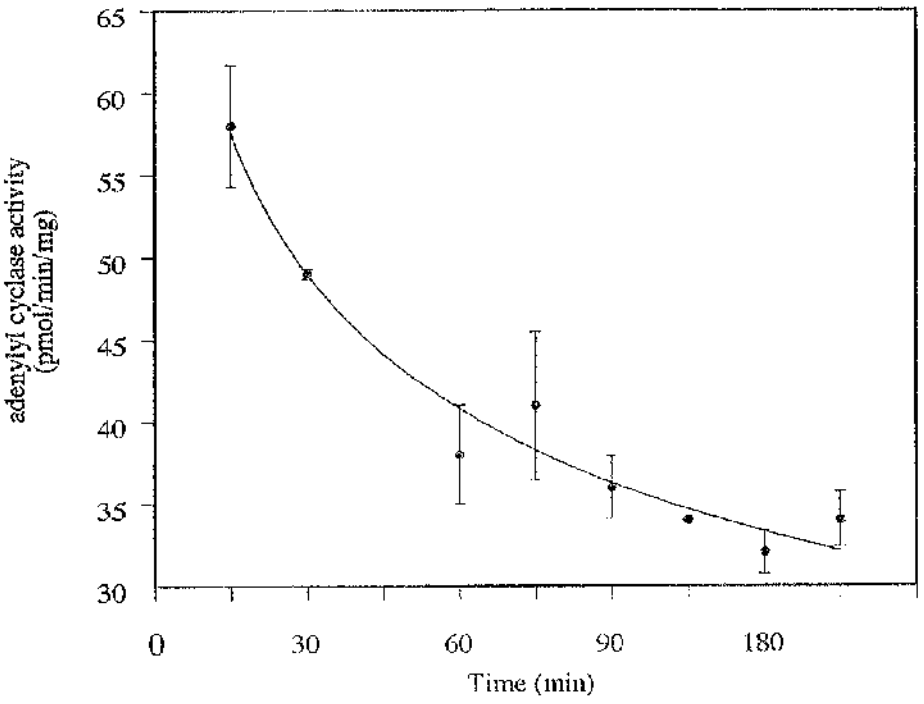

adenylyl cyclase activity
(pmol/min/mg protein)
200
150
100
50
0
0
10-11
10-10
10-9
10-8
10-7
10-6
[Isoprenaline] (M)

Figure 3.2.5. Desensitization of β_1-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with isoprenaline (100 μM) for different times. Samples were also stimulated again during the assay with 100 μM isoprenaline. Two further experiments produced similar results.

Figure 3.2.5.


adenylyl cyclase activity
(pmol/min/mg)
65
60
55
50
45
40
35
30
0
30
60
90
180
Time (min)

3.3. RESULTS

INVESTIGATIONS OF MECHANISMS OF AGONIST-MEDIATED G_S DOWN-REGULATION IN RESPONSE TO ACTIVATION OF β_2-ADRENOCEPTOR EXPRESSING CHO CELLS.

For direct comparison of EC_{50} values and $t_{1/2}$ for the three β-adrenoceptors in CHO cells, SDS-PAGE (10% acrylamide) and western blotting techniques were then performed on membranes obtained from β_2-adrenoceptor expressing CHO cells. These CHO cells were pre-treated for 16 hours with a range of concentrations of isoprenaline (1 mM to 0.1 nM). Figure 3.3.1.b shows the curve which was constructed from values obtained from a densitometer scan (Figure 3.3.1.a) of the nitrocellulose blot and comparison to the control, untreated membranes where basal levels of $G_s\alpha$ in the CHO cells are present. A half-maximal reduction in $G_s\alpha$ (L) levels (representing the EC_{50} value) in the β_2-adrenoceptor CHO cells was achieved by treatment with 39 ± 25 nM isoprenaline. This EC_{50} value, when compared with the 2.66 ± 0.88 nM value for the β_1-adrenoceptor is quite surprising, as most groups have found isoprenaline to be more potent at the β_2-adrenoceptor (Green *et al*, 1992). Green *et al* (1992) found a 4-fold greater potency for the β_2-adrenoceptor than the β_1-adrenoceptor. Suzuki *et al* (1992), however, found no differences in potency of isoprenaline between the β_1- and β_2-adrenoceptor, using adenylyl cyclase assays. Results of my investigations, however, could be affected by the differences in receptor density of the three cell lines (β_1-adrenoceptor expressing CHO cells containing much more β_1-adrenoceptors than there are β_2-adrenoceptors in the β_2-adrenoceptor expressing CHO cells). This phenomenon is discussed in greater detail in Chapter 4.

After chronic treatment (16 h) with a high concentration of isoprenaline (10 μM), $G_s\alpha$ levels were very low, implying near complete down-regulation of the polypeptide. This experiment was repeated on different sets of treated CHO cells at least three times, each producing similar results.

These results were quite similar to those reported by Chambers *et al* (1994), with an EC_{50} value for isoprenaline-induced $G_s\alpha$ down-regulation in β_2-adrenoceptor expressing CHO cells of 9 ± 4 nM. The curve from the Chambers *et al* (1994) study of $G_s\alpha$ down-regulation is shown in Figure 3.3.1.c. alongside the curve of $G_s\alpha$ down-regulation for the β_3-adrenoceptor. To assess the rapidity of the down-regulation of G_s upon β_2-adrenoceptor stimulation, β_2-adrenoceptor expressing CHO cells were treated with a maximal concentration of isoprenaline (100 μM), for times ranging from 60 minutes to 24 hours (Figure 3.3.2). Figure 3.3.2. shows a pattern of $G_s\alpha$(L) down-regulation much slower than the β_1-adrenoceptor expressing CHO cells. Only after approximately 6 hours (247 ± 35 minutes) was half-maximal loss of G_s achieved in the β_2-adrenoceptor expressing CHO cells. Maximal G_s down-regulation, leaving less than 20% of original levels, only occured after approximately 20 hours. This difference between the half-maximal loss of $G_s\alpha$ in the β_1- and β_2-adrenoceptor expressing CHO cells, approximately 1 hour compared with 6 hours, can probably be explained by the differences in receptor expression between the β_1-adrenoceptor (7130 ± 258 fmol/mg membrane protein) and β_2-adrenoceptor (2300 ± 120 fmol/mg membrane protein). This has been shown to result in less rapid down-regulation, depending on the levels of receptor present. This phenomenon is shown with the comparison of

two β_3-adrenoceptor CHO cell lines containing different receptor levels (Figure 3.4.5).

Equivalent immunoblots for detection of $G_q\alpha$ and $G_i2\alpha$ on the same membranes demonstrated that there were no detectable differences in levels of these polypeptides between untreated and isoprenaline treated β_2-adrenoceptor transfected CHO cells (Figure 3.2.2.c).This is to be expected as it is believed that the β-adrenoceptor signalling process only operates through the G_s-adenylyl cyclase system.

Figure 3.3.1.a. A typical immunoblot of $G_s\alpha$ down-regulation after the treatment of β_2-adrenoceptor expressing CHO cells with varying concentrations of isoprenaline for 16 hours. Membranes from β_2-adrenoceptor expressing CHO cells treated with isoprenaline are shown, with concentrations of isoprenaline treatments as follows: 1 mM isoprenaline (Lane 1), 0.3 mM isoprenaline (Lane 2), 0.1 mM isoprenaline (Lane 3), 30 μM isoprenaline (Lane 4), 10 μM isoprenaline (Lane 5), 1 μM isoprenaline (Lane 6), 0.1 μM isoprenaline (Lane 7), 10 nM isoprenaline (Lane 8), 1 nM isoprenaline (Lane 9), 0.1 nM isoprenaline (Lane 10), untreated (Lane 11).

Figure 3.3.1.b. Quantitative analysis of the typical immunoblot of $G_s\alpha$ down-regulation after the treatment of β_2-adrenoceptor expressing CHO cells with varying concentrations of isoprenaline for 16 hours.

Figure 3.3.1.c. $G_s\alpha$ down-regulation in β_2-adrenoceptor (open circles) and β_3-adrenoceptor (closed squares) expressing CHO cells exposed to varying concentrations of isoprenaline. Taken from Chambers *et al* (1994). β_2- and β_3-adrenoceptor expressing CHO cells were exposed to isoprenaline (0-100 μM) for 7 hours. Membranes were prepared and immunoblotted to detect the presence of $G_s\alpha$45. This polypeptide was down-regulated by exposure to isoprenaline in both the β_2- and β_3-adrenoceptor expressing CHO cells to a similar maximal extent. However, the EC_{50} for isoprenaline-induced $G_s\alpha$45 down-regulation in β_3-adrenoceptor expressing CHO cells was considerably greater (approximately 300 nM) than in the β_2-adrenoceptor expressing CHO cells (approximately 10 nM).

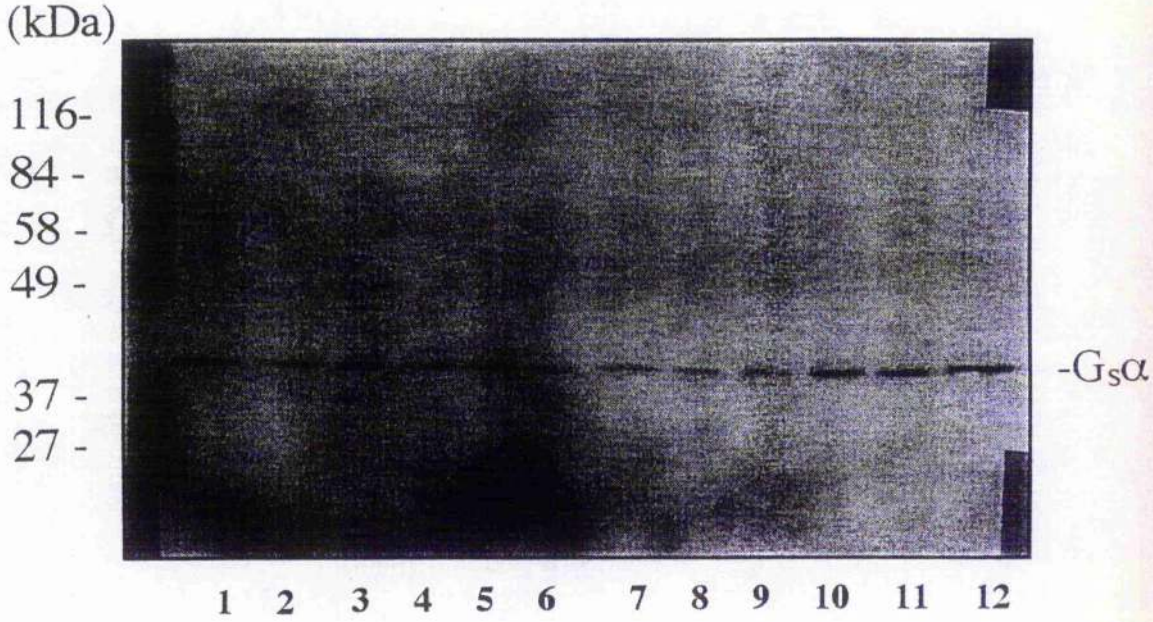
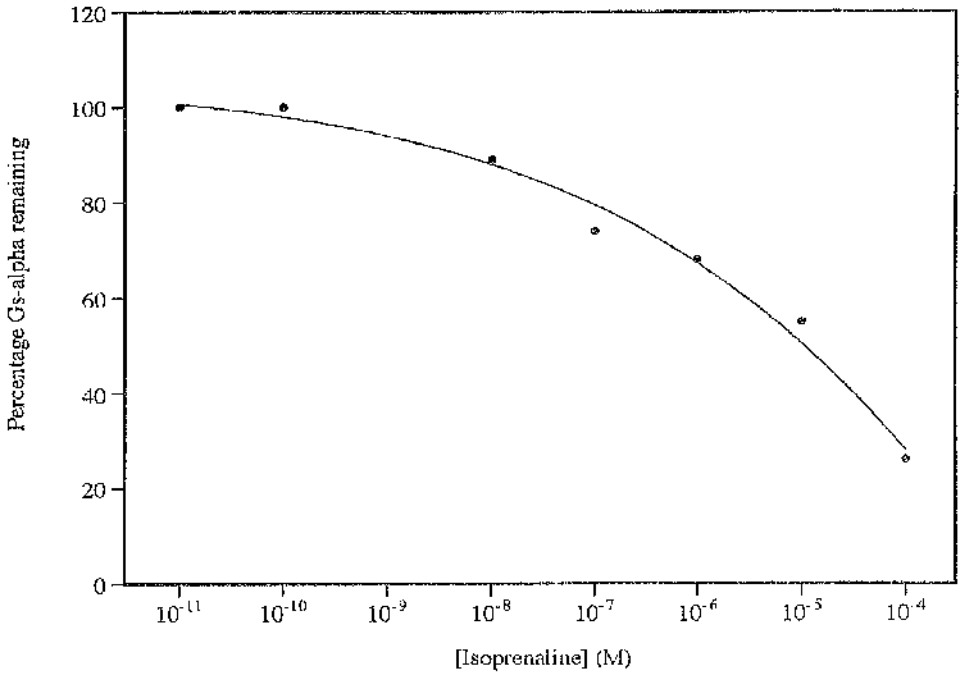

(kDa)
116-
84 -
58 -
49 -
37 -
27 -
-Gsα
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.3.1.b.


120
100
80
60
40
20
0
Percentage Gs-alpha remaining
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[Isoprenaline] (M)

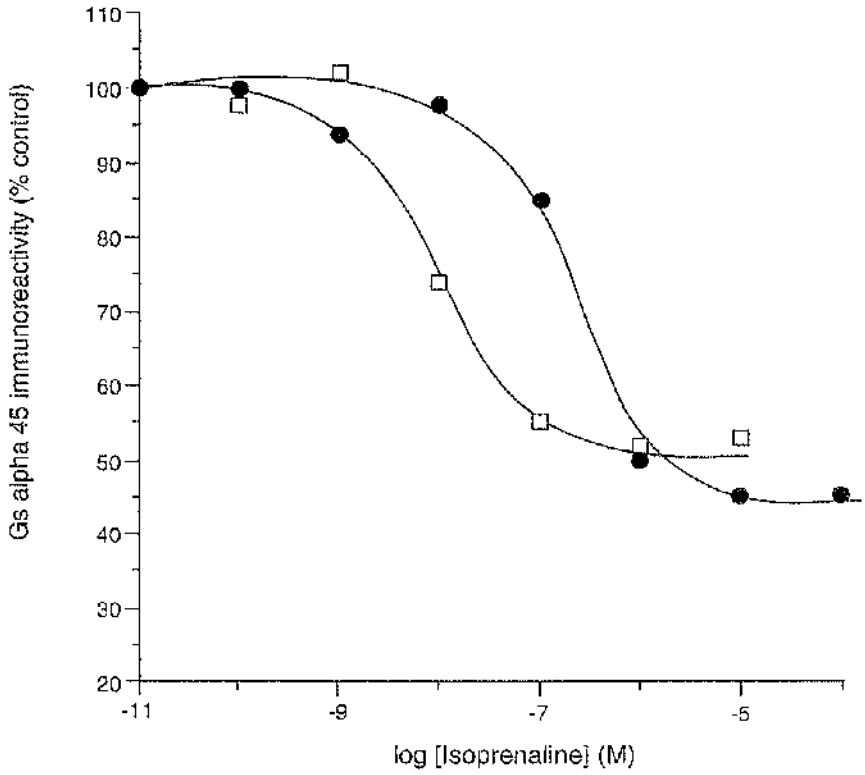
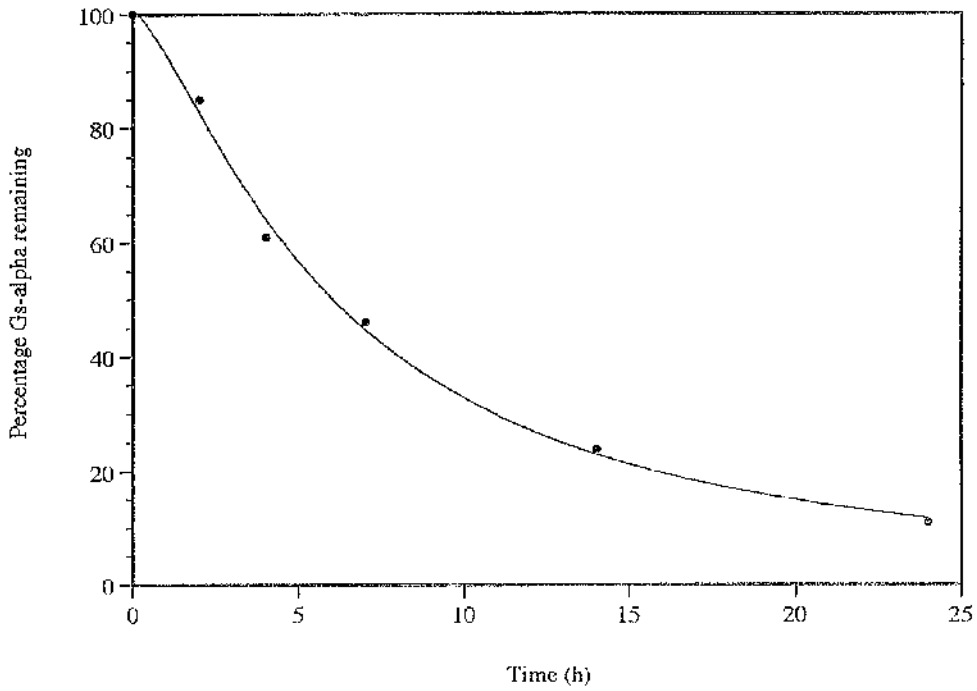

Gs alpha 45 immunoreactivity (% control)
110
100
90
80
70
60
50
40
30
20
-11
-9
-7
-5
log [Isoprenaline] (M)

Figure 3.3.2. Down-regulation of levels of $G_s\alpha$ in β_2-adrenoceptor expressing CHO cells after treatments with 100 μM isoprenaline for different times. Quantitative analysis of a typical immunoblot.

Figure 3.3.2.


Percentage Gs-alpha remaining
100
80
60
40
20
0
0
5
10
15
20
25
Time (h)

Adenylyl cyclase assays were performed on the β_2-adrenoceptor expressing CHO cell membranes produced and used in the above experiments for western blotting. Concentration-responses to isoprenaline treatment are shown in Figure 3.3.3 (data taken from Chambers *et al*, 1994). β_2-adrenoceptor expressing CHO cells were pre-treated with isoprenaline or without for 24 hours before being harvested and membranes produced. Samples were then treated at varying concentrations of isoprenaline during the adenylyl cyclase assay. Results are presented as mean ± S.E.M. of data derived from a single experiment. Values of adenylyl cyclase activity in these β_2-adrenoceptor expressing CHO cells are expressed in units of pmol/min/mg protein. Half-maximal reduction in isoprenaline-stimulated adenylyl cyclase activity was approximately 30 nM, very similar to that observed in the western blot studies of Figure 3.3.1.b.(39 ± 25 nM). This shows a sensitivity of the adenylyl cyclase functional response to isoprenaline, and is proof that the β_2-adrenoceptor desensitization process is occurring. The fact that results are similar to western blotting results, is consistent with the idea that $G_s\alpha$ down-regulation is an important factor in the desensitization of the β_2-adrenoceptor.

To investigate the rate of this β_2-adrenoceptor desensitization process, adenylyl cyclase assays were also performed on membranes from β_2-adrenoceptor expressing CHO cells treated for different times with isoprenaline (100 μM). Once more these produced values for half-maximal reduction in isoprenaline-stimulated adenylyl cyclase of 6-7 hours. These results were indicative of a parallel link with the direct observation of $G_s\alpha$ down-regulation upon isoprenaline stimulation obtained from western blots. This link between the functional assay and

protein detection systems suggests that the loss of $G_s\alpha$ levels is a key element in the process of β_2-adrenoceptor desensitization. Figure 3.3.4. shows a sample adenylyl cyclase assay result for β_2-adrenoceptor expressing CHO cell membranes treated with 100 μM isoprenaline for varying lengths of time.

Figure 3.3.3. Desensitization of β_2-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with varying concentrations of isoprenaline. β_2-adrenoceptor expressing CHO cells.were pre-treated with isoprenaline (open circles) or without (closed circles) for 24 hours before being harvested and membranes produced. Samples were then treated at varying concentrations during the adenylyl cyclase assay. Results are presented as mean ± S.E.M. of data derived from a single experiment. Two further experiments produced similar results.

Figure 3.3.3.

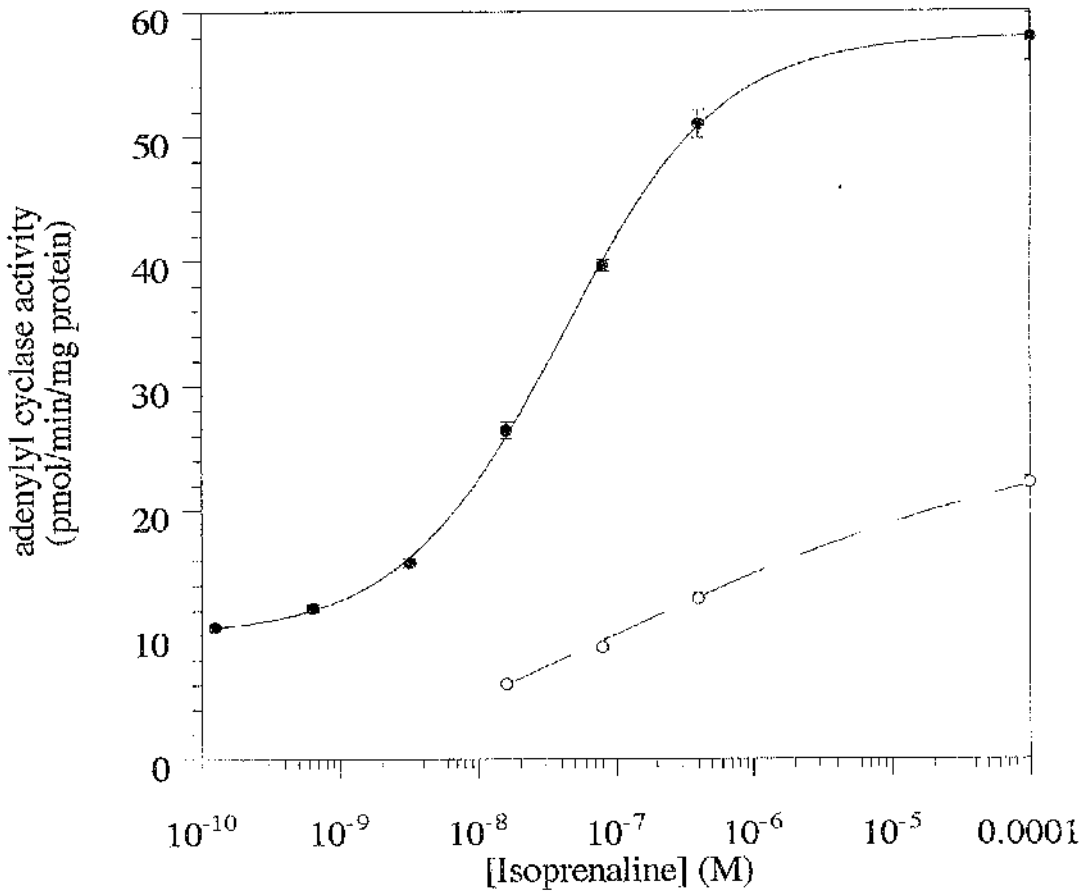
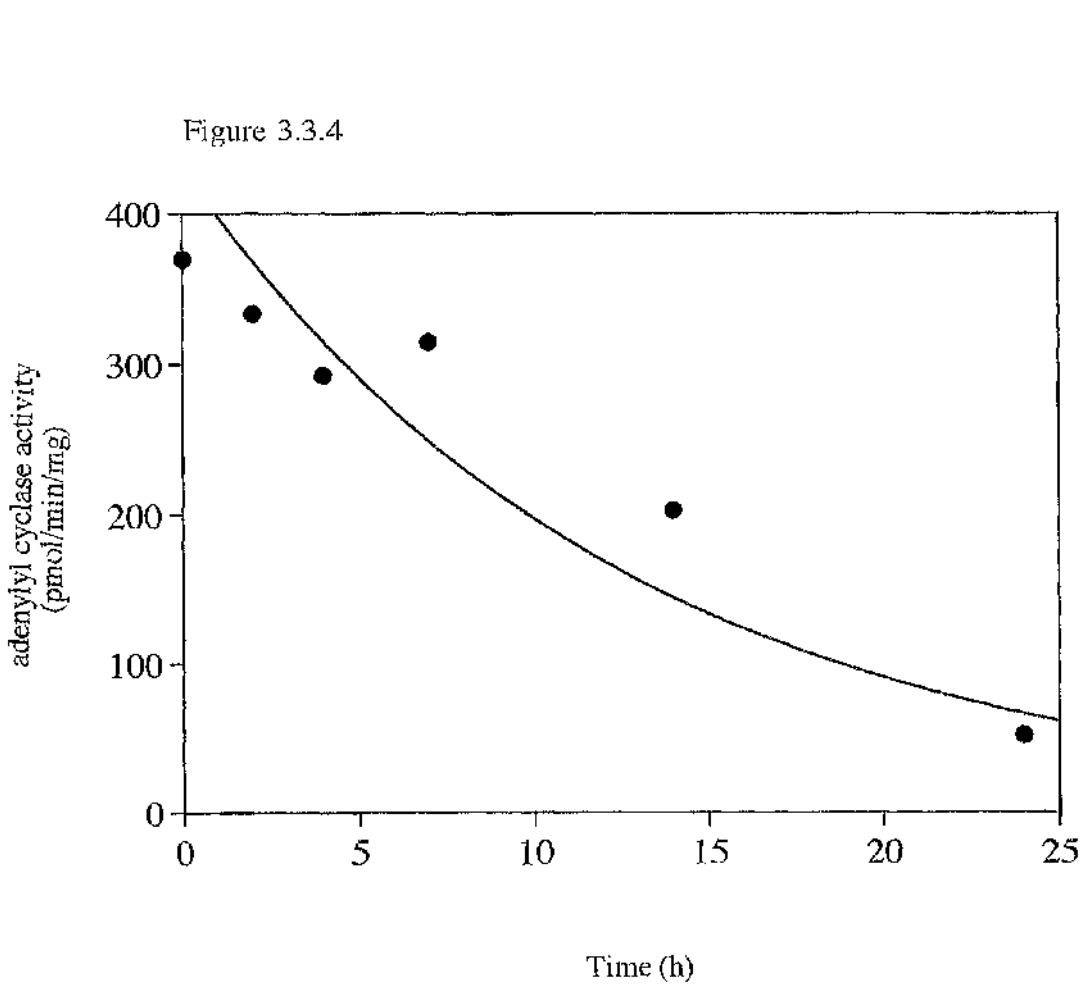

adenylyl cyclase activity
(pmol/min/mg protein)
60
50
40
30
20
10
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
0.0001
[Isoprenaline] (M)

Figure 3.3.4. Desensitization of β_2-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with isoprenaline (100 μM): time course. β_2-adrenoceptor expressing CHO cells.were treated with isoprenaline for varying times. Samples were also stimulated during the adenylyl cyclase assay with 100 μM isoprenaline. Two further experiments produced similar results.

Figure 3.3.4


400
300
200
100
0
adenylyl cyclase activity
(pmol/min/mg)
0
5
10
15
20
25
Time (h)

3.4. RESULTS

INVESTIGATIONS OF MECHANISMS OF AGONIST-MEDIATED G_S DOWN-REGULATION IN THE β_3 ADRENOCEPTOR EXPRESSING CHO CELLS.

To compare the β_1-adrenoceptor and β_2-adrenoceptor processes of desensitization with the lack of desensitization in the β_3-adrenoceptor, SDS-PAGE (10% acrylamide) and western blotting were also performed on membranes taken from treated β_3-adrenoceptor expressing CHO cells. Cells were chronically treated for 16 hours with concentrations of isoprenaline which ranged from 1 mM to 0.01 nM. Figure 3.4.1. shows the curve constructed from results of a densitometer scan on the nitrocellulose blot. This was compared to control, untreated membranes of the β_3-adrenoceptor expressing CHO cells where basal levels of $G_s\alpha$ were shown. The half-maximal reduction in G_s levels by treatment of the β_3-adrenoceptor expressing CHO cells was approximately 200 nM. This was a substantially higher concentration than was necessary to reduce G_s levels by half in the β_1- and β_2-adrenoceptor expressing CHO cells. This observation was consistent with the lower potency of isoprenaline at the β_3-adrenoceptor compared with the potency at the β_2- and β_1-adrenoceptor. Maximal reduction of G_s (only 5-10% of original levels remaining) occurred at a concentration of 10 μM isoprenaline. This experiment was repeated at least three times, each time producing similar results. Equivalent immunoblots of detection for $G_q\alpha$, $G_{11}\alpha$ and $G_i2\alpha$ demonstrated that there were no differences in these polypeptides between untreated and isoprenaline-treated β_3-adrenoceptor transfected CHO cells. This, as mentioned before, is to be expected, in that the β-adrenoceptors only transduce their signalling of agonist responses through the G_s-adenylyl cyclase system.

EC_{50} values for isoprenaline-induced $G_s\alpha$ down-regulation in the β_3-adrenoceptor expressing CHO cells were similar to those found by Chambers *et al* (1994). While my investigations found the EC_{50} to be 177 ± 113 nM, Chambers *et al* (1994) found it to be 214 ± 43 nM. A comparison of the $G_s\alpha$ down-regulation in the β_2-adrenoceptor and β_3-adrenoceptor expressing CHO cells is shown in Figure 3.3.1 c. This shows quite clearly how Chambers *et al* (1994) and my data both show differing EC_{50} values for isoprenaline at the β_2-adrenoceptors and β_3-adrenoceptors, which can be directly related to the lower potency of isoprenaline at the β_3-adrenoceptor.

Figure 3.4.1. Quantitative analysis of a typical immunoblot showing $G_s\alpha$ down-regulation after isoprenaline treatment at varying concentrations in β_3-adrenoceptor transfected CHO cells. Experiments were repeated two more times with similar results.

Figure 3.4.1.

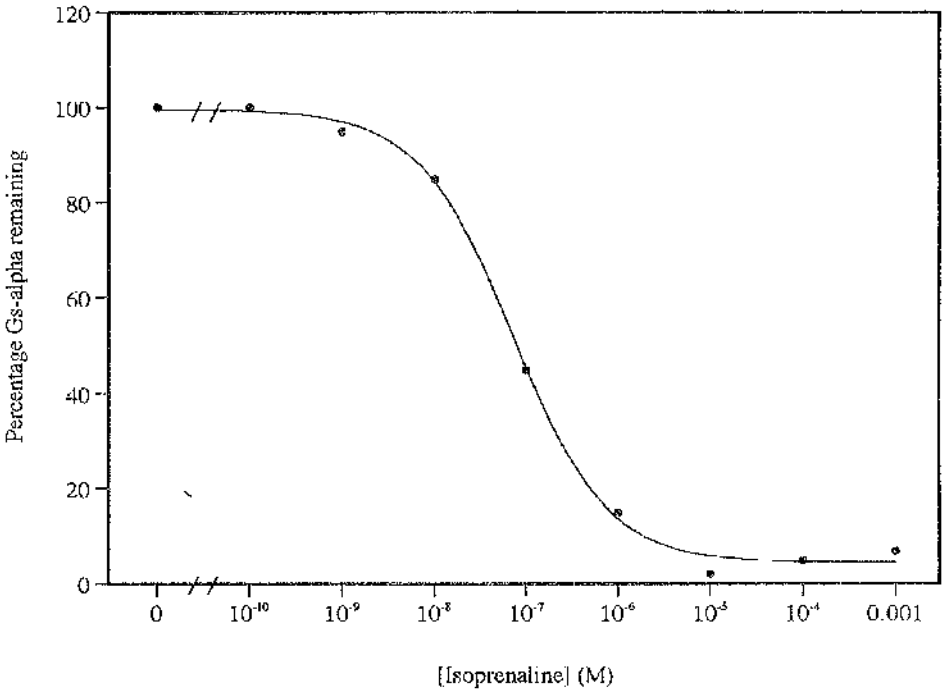
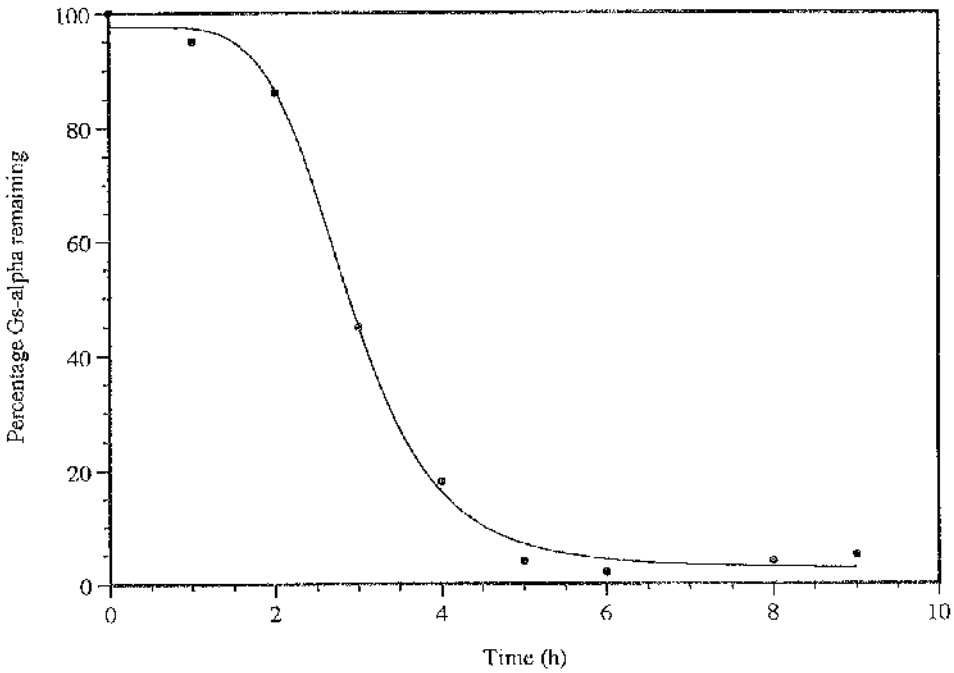

120
100
80
60
40
20
0
Percentage Gs-alpha remaining
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
0.001
[Isoprenaline] (M)

Figure 3.4.2. Quantitative analysis of a typical immunoblot showing $G_s\alpha$ down-regulation after isoprenaline (100 μM) treatment for various times in β_3-adrenoceptor transfected CHO cells. Experiments were performed two more times with similar results.

Figure 3.4.2.


Percentage Gs-alpha remaining
100
80
60
40
20
0
0
2
4
6
8
10
Time (h)

To examine the rate of G_s down-regulation upon β_3-adrenoceptor stimulation with isoprenaline, β_3-adrenoceptor expressing CHO cells were treated with a maximally effective concentration of isoprenaline (100 μM), for time periods ranging from 1-9 hours (Figure 3.4.2). The $G_s\alpha$ (L) polypeptide in these cells was lost at a much slower rate than the $G_s\alpha$ (L) of the β_1-adrenoceptor expressing CHO cells, but followed a similar time course to the loss of G_s in β_2-adrenoceptor expressing CHO cells. Only after approximately 3-4 hours (220 ± 72 minutes) of treatment with isoprenaline was half-maximal loss of $G_s\alpha$ achieved. This is, as highlighted above, due probably to the levels of β_3-adrenoceptor expression (3000 ± 400 fmol/mg membrane protein) when compared with that of β_1-adrenoceptor expression in the CHO cell line (7130 ± 258 fmol/mg membrane protein). The β_2-adrenoceptor expressing CHO cell line used in this study had levels similar to the β_3-adrenoceptor levels, of 2300 ± 120 fmol/mg membrane protein. A study performed to compare the effect on $G_s\alpha$ down-regulation of different receptor levels is shown at the end of Section 3.4. Two β_3-adrenoceptor expressing CHO cell lines were compared with different receptor levels of the β_3-adrenoceptor.

Adenylyl cyclase activity was calculated from assays performed on the β_3-adrenoceptor expressing CHO cell membranes which had previously been chronically treated. Concentration-responses to isoprenaline treatment for 16 hours were performed and results of adenylyl cyclase assays are shown in Figure 3.4.3. Cyclase activity was expressed in units of pmol/min/mg of protein. Half-maximal reduction in isoprenaline-stimulated adenylyl cyclase activity was approximately 150 nM. This value, an indication of agonist-induced receptor desensitization, was very similar to values obtained from the western

blot analysis of $G_s\alpha$ levels (117 ± 113 nM for my studies and 214 ± 43 nM in the study by Chambers *et al* (1994)), indicating that the observed loss of $G_s\alpha$ is a key element in β_3-adrenoceptor desensitization.

Cyclase assays were performed on membranes from β_3-adrenoceptor expressing CHO cells treated for different times with 100 μM isoprenaline. Once more these produced half-lives for rate of agonist-induced β_3-adrenoceptor desensitization by isoprenaline of approximately 6-7 hours, which were in close agreement with values obtained for $G_s\alpha$ down-regulation from western blotting techniques. Figure 3.4.4 shows a typical cyclase assay time course for β_3-adrenoceptor expressing CHO cell membranes treated with 100 μM isoprenaline for varying lengths of time. As rates of desensitization of the β_3-adrenoceptor measured as a loss of adenylyl cyclase activity in β_3-adrenoceptor expressing CHO cells were shown to be following a similar temporal pattern to loss of $G_s\alpha$ levels in the β_3-adrenoceptor expressing CHO cells, this would suggest that loss of $G_s\alpha$ is an important element in the process of β_3-adrenoceptor desensitization.

Figure 3.4.3. Desensitization of β_3-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with isoprenaline: concentration-response. β_3-adrenoceptor expressing CHO cells.were treated with isoprenaline at varying concentrations for 16 hours, followed by a stimulation with 100 μM isoprenaline during the course of the assay. Two further experiments produced similar results.

Figure 3.4.3.

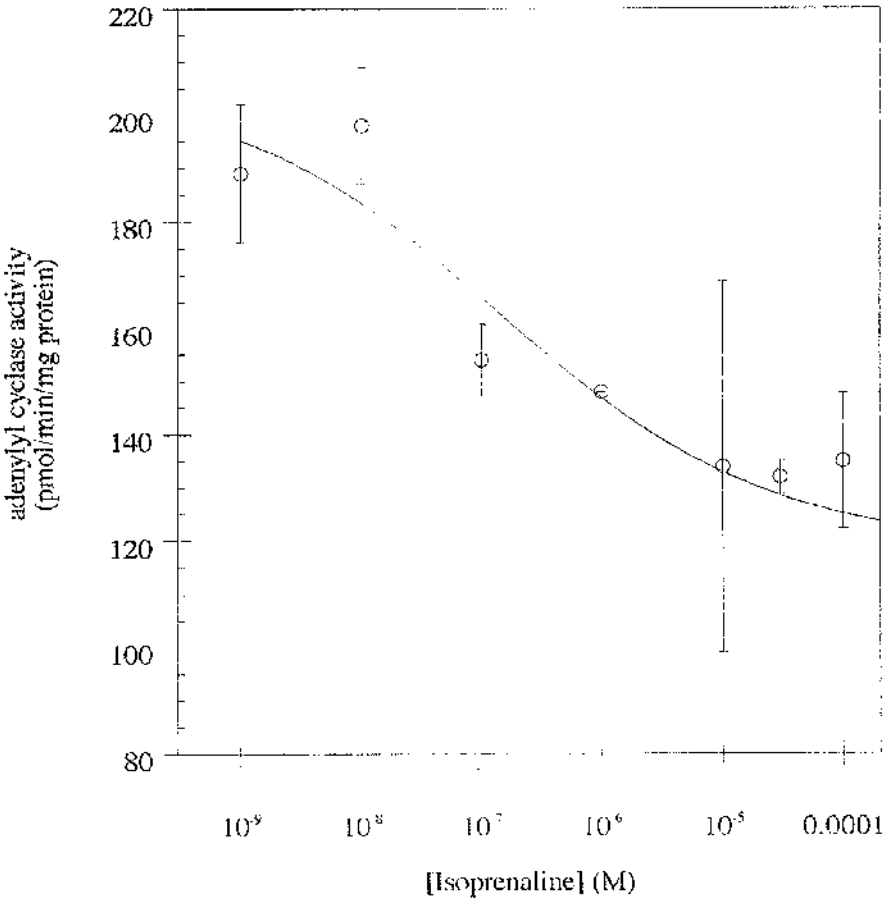
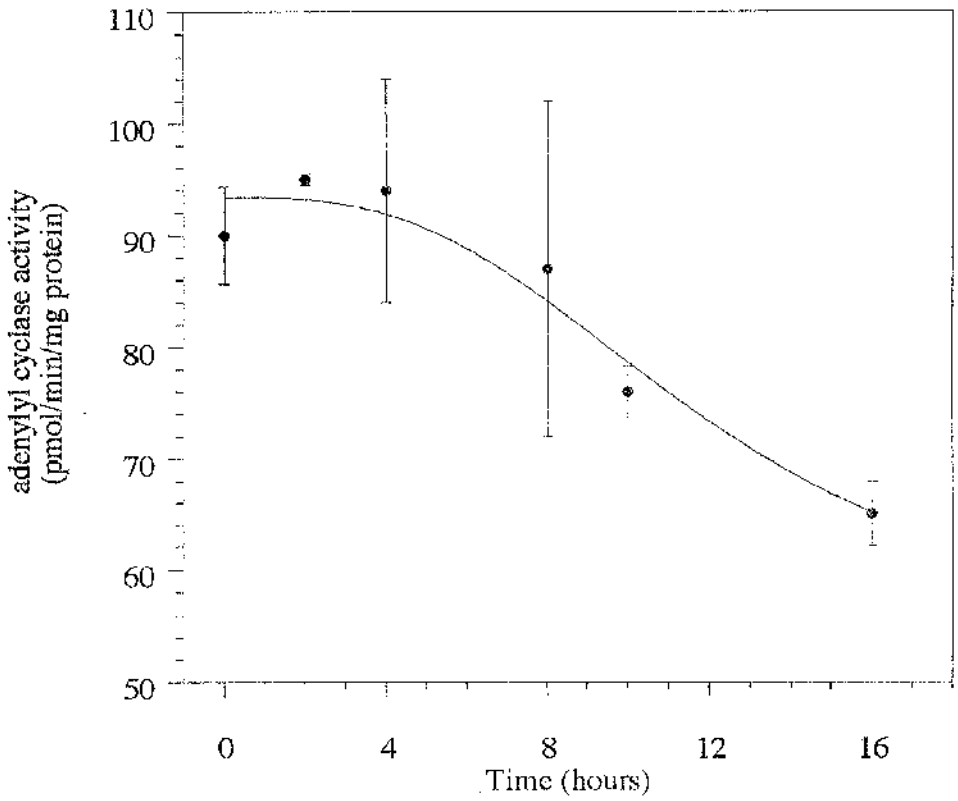

220
200
180
160
140
120
100
80
adenylyl cyclase activity
(pmol/min/mg protein)
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
0.0001
[Isoprenaline] (M)

Figure 3.4.4. Desensitization of β_3-adrenoceptor-mediated stimulation of adenylyl cyclase activity following treatment with isoprenaline (100 μM) for varying times. Samples were also stimulated again during the course of the adenylyl cyclase assay with 100 μM isoprenaline. Two further experiments produced similar results.

Figure 3.4.4.


adenylyl cyclase activity
(pmol/min/mg protein)
110
100
90
80
70
60
50
0
4
8
12
16
Time (hours)

The immunoblot results for all three β-adrenoceptor subtype-mediated $G_s\alpha$ down-regulation are shown in Table 3.4.1. Adenylyl cyclase activity values for agonist-mediated receptor desnsitization were not tabulated but results followed a similar pattern to western blotting results through the three β-adrenoceptor subtypes.

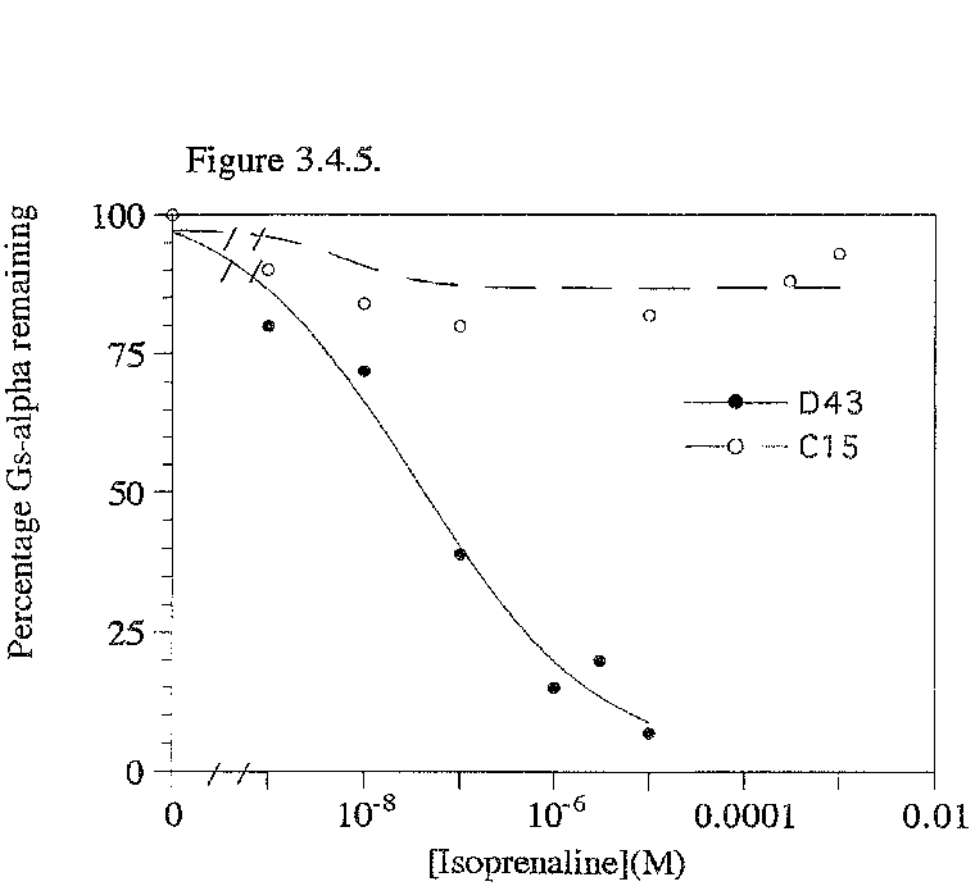
Table 3.4.1. Quantitation of data for $G_s\alpha$ down-regulation following analysis of immunoblots for all three β-adrenoceptor subtypes. Results are from three or more experiments ± S.E.M.

CHO cells	concentration for half-maximal down-regulation of G_s (nM)	time for half-maximal down-regulation of G_s (min)
β_1-adrenoceptor	2.7 ± 0.9	60 ± 4
β_2-adrenoceptor	39 ± 25	247 ± 35
β_3-adrenoceptor	177 ± 133	220 ± 72

From this Table, it can be clearly observed that the three β-adrenoceptors show differences in their respective rates of $G_s\alpha$ down-regulation. This, as discussed in the separate sections above, is likely to be due to different levels of receptor expression of each β-adrenoceptor subtype in the CHO cell lines. Further investigations into this hypothesis took place when I was able to compare two stable CHO cell lines previously transfected to express different levels of the β_3-adrenoceptor. With these two cell lines, it was possible to confirm the phenomenon of the effect of receptor density on rate of down-regulation. These two CHO cell lines differed by approximately 10-fold receptor levels of the β_3-adrenoceptor. The C15 CHO line contained 390 ± 83 fmol/mg membrane protein of β_3-adrenoceptors, while the D43 CHO cell line, used previously in the experiments above, contained 10-fold as many β_3-adrenoceptors with 3000 ± 400 fmol/mg membrane protein. Treatment of these two cell lines with varying concentrations of isoprenaline resulted in down-regulation of $G_s\alpha$ only in the D43 cells: no down-regulation occurred in the lower β_3-adrenoceptor expressing CHO cells (Figure 3.4.5.). This confirmed the belief that receptor density affects the rate and/or extent of down-regulation of $G_s\alpha$, and therefore probably plays a key role in overall receptor desensitization. Figure 3.4.5. shows the graph produced after isoprenaline treatment on D43 and C15 β_3-adrenoceptor expressing CHO cells.

Figure.3.4.5. Quantitation of scans of immunoblots showing $G_s\alpha$ down-regulation in C15 and D43 cell lines following treatment with varying concentrations of isoprenaline. Cells were treated for 16 hours with different concentrations of isoprenaline. Isoprenaline-treated C15 cell lines are shown as open circles, D43 cell lines as closed circles.

Figure 3.4.5.


Percentage Gs-alpha remaining
100
75
50
25
0
0
10-8
10-6
0.0001
0.01
[Isoprenaline](M)
D43
C15

3.5. *INVESTIGATIONS INTO MOVEMENT OF G_S FROM THE PLASMA MEMBRANE INTO THE CYTOSOL.*

As the G_s polypeptide was observed to be down-regulated most rapidly in the β_1-adrenoceptor expressing CHO cells, this cell line was chosen for a study to investigate the movement of G_s away from the cell membrane into the cytosol. Experiments were carried out to assess whether the G_s was broken down in the process of down-regulation or transferred to the cytosol, then perhaps processed into vesicles before being recycled back to the plasma membrane.

β_1-adrenoceptor expressing CHO cells were treated for four hours with a maximally effective concentration of isoprenaline (100 μM), then harvested concurrently with untreated cells. The harvested pellets were homogenised, then centrifuged in an airfuge at 30 psi (178,000 x *g*) for 10 minutes. The pellet produced was assumed to be the membrane fraction and the supernatant the cytosolic fraction. Samples were loaded onto 10% acrylamide gels and western blotting performed using an antiserum produced to the C-terminal of G_s, named CS (Milligan & Unson, 1984). Results are shown in Figure 3.5.1.

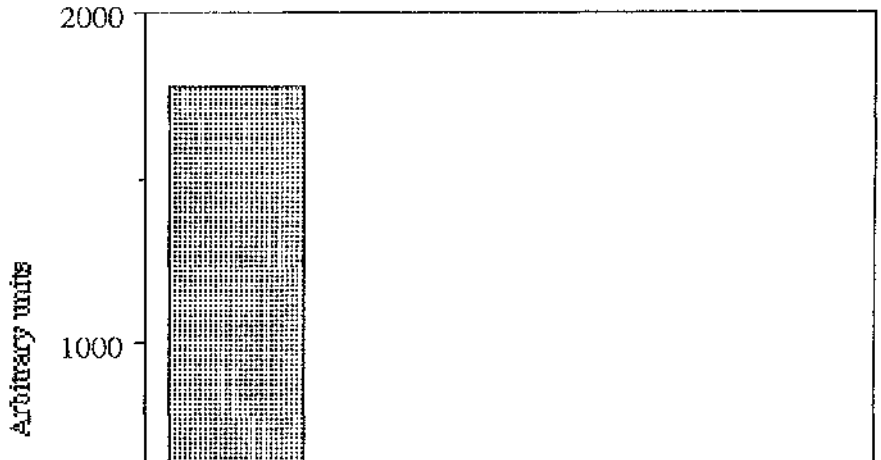
The results with the untreated β_1-adrenoceptor expressing CHO cells proved that all the $G_s\alpha$ was detected in the membrane fraction (including vesicles), while undetectable levels were found in the cytosolic contents of the CHO cells. After the treatment of cells for four hours, relatively small amounts of $G_s\alpha$ remained in the membrane (approximately 5% of original membrane fraction levels), yet still very little was found in the cytosol (approximately 11% of original membrane fraction levels). There was no proof of complete transferral of $G_s\alpha$ from the plasma

membrane to the cytosol, rather, close to complete removal of $G_s\alpha$ from both regions of the cell.

These results showed that the $G_s\alpha$ polypeptide is not present in the cytosolic fraction after agonist-mediated down-regulation, indicating that the polypeptide is not stored for long times, is broken down and *de novo* synthesis is necessary for re-activation to the plasma membrane. This agrees with other groups when investigating receptor loss, (Perkins *et al*, 1991, Von Zastrow *et al*, 1992, Saffitz *et al*, 1992), who suggest that along with the receptor, the G-protein may firstly be packaged into vesicles and later degraded. While I was unable to directly study movement of Gs into vesicles, it was clear from my studies that the G_s was not present in the membrane fraction or cytosolic fraction and therefore had most likely been degraded.

3.5.1. Quantitative analysis of blot showing down-regulation of $G_s\alpha$ levels. Column graph represents movement of G_s from membrane to cytosol. P represents 'pellet' and S represents 'supernatant'. The columns are shown as arbitrary units. This column graph is representative of one immunoblot, but the experiment was performed three times with similar results.

Figure 3.5.1.


2000
1000
0
Arbitrary units
Control(P)
Control(S)
Treated (P)
Treated(S)

3.6. *ALTERATIONS IN mRNA LEVELS OF G_S IN β_1-ADRENOCEPTOR CHO CELLS*

To investigate the effect of agonist stimulation on levels of mRNA of G_s, and whether this could form a major part of the G-protein down-regulation process and the overall receptor desensitization, β_1-adrenoceptor expressing CHO cells were treated with a maximally effective concentration of isoprenaline (100 μM) for either 30 minutes or 4 hours. RNA was then extracted by the RNAzol method outlined in Chapter 2. Oligonucleotides specific to $G_s\alpha$ were produced for the detection by RT-PCR of G_s mRNA levels in the β_1-adrenoceptor expressing CHO cells. Oligonucleotides for the housekeeping gene β-actin were added in parallel as an internal control, as alterations in actin levels would not be expected to occur in response to isoprenaline stimulation of the cells.

As Figure 3.6.1.a. shows, while β-actin mRNA levels did not change upon agonist stimulation, $G_s\alpha$ mRNA levels did not alter significantly either. This experiment was repeated three times and the alterations in G_s mRNA levels shown in the column graph of Figure 3.6.1b. These results suggests that an alteration in mRNA levels does not play a major role in regulation of G-proteins upon agonist stimulation.

Figure 3.6.1.a. Agarose gel showing levels of $G_s\alpha$ mRNA from β_1-adrenoceptor expressing CHO cells, β-actin mRNA and molecular weight markers. β-actin mRNA is shown in Lanes 1, 2, and 3, while $G_s\alpha$ mRNA is shown in Lanes 4, 5, and 6. mRNA extracted from β_1-adrenoceptor expressing CHO cells is shown on the photograph after the following time treatments with isoprenaline (100 μM): no treatment (Lanes 1 and 4), 30 minutes treatment (Lanes 2 and 5), 4 hours of treatment (Lanes 3 and 6).

Figure 3.6.1.b. Column graph produced from quantitative analysis of mRNA levels on agarose gel. The procedure was performed three times.

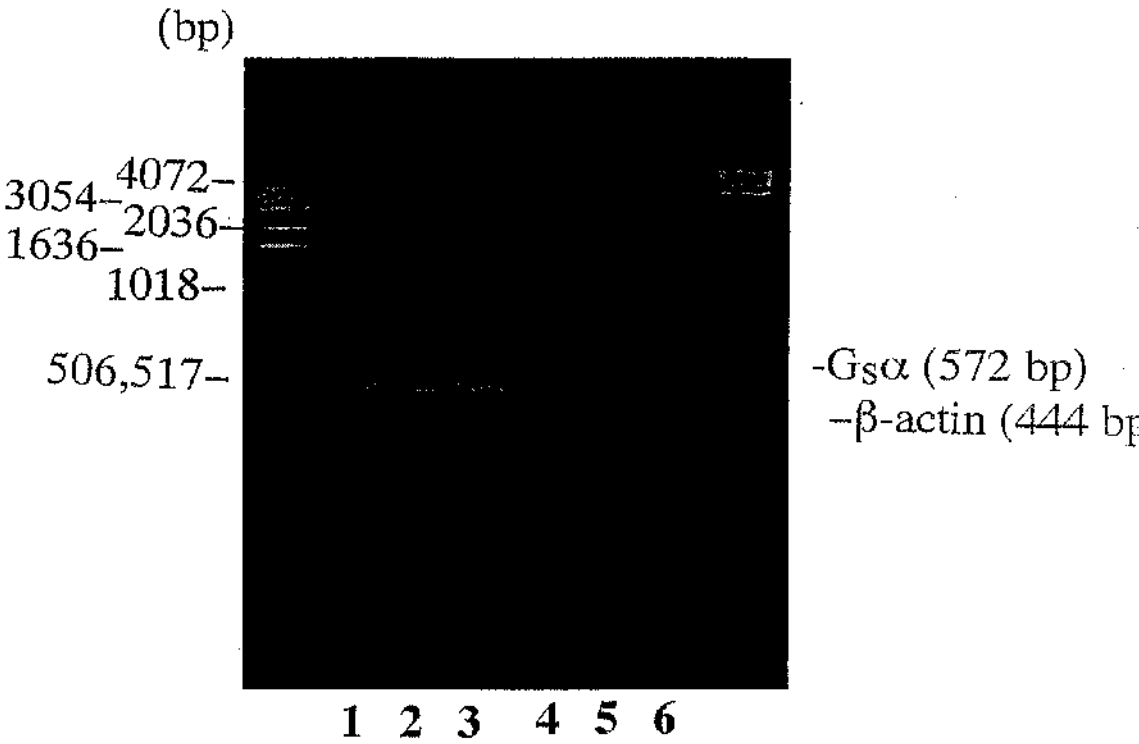
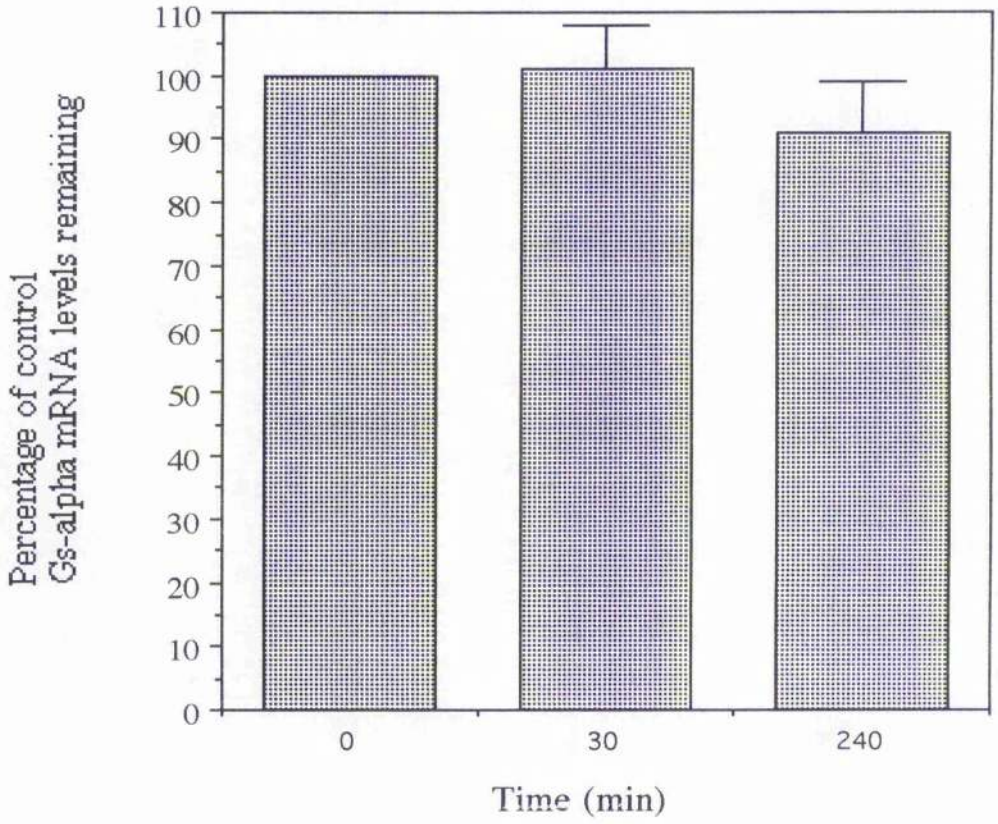

(bp)
4072–
3054–
2036–
1636–
1018–
506,517–
-Gsα (572 bp)
–β-actin (444 bp
1 2 3 4 5 6

Figure 3.6.1.b.


Percentage of control
Gs-alpha mRNA levels remaining
110
100
90
80
70
60
50
40
30
20
10
0
0
30
240
Time (min)

3.7. DISCUSSION

The actual loss of cellular receptors is a prominent mechanism during long-term agonist desensitization of the β_1- and β_2-adrenoceptors (Liggett, 1991, Hausdorff *et al*, 1990, Dohlman *et al*, 1991, Collins *et al*, 1991). During this down-regulation process, a recorded loss of some 50-70% of the receptors after 24 hours of agonist exposure is not uncommon. The β_3-adrenoceptor, however, has been shown to be resistant to down-regulation, with surface receptors staying the same or even increasing upon agonist exposure (Thomas *et al*, 1992, Emorine *et al*, 1989, Liggett *et al*, 1993, Chambers *et al*, 1994). For example, Thomas *et al* (1992) noted that during prolonged exposure to agonist, no down-regulation of β_3-adrenoceptor expression in 3T3-F442A cells occurred. Indeed, β_3-adrenoceptor expression increased during agonist exposure to approximately 165% of basal levels. Similarly, a lack of β_3-adrenoceptor desensitization has been demonstrated in non-recombinant cells which express native β_3-adrenoceptors, such as isolated rat adipocytes (Granneman, 1992c).

Whereas the majority of G-protein-linked receptors are down-regulated by maintained exposure to their agonists, the β_3-adrenoceptor is not the only example of a receptor reported to be resistant to such a process. In studies comparing the desensitization characteristics of the α_2C10, α_2C4 and α_2C2-adrenoceptors following transfection into CHO cells, it was noted that the α2C4-adrenoceptor was not down-regulated but, as with the β_3-adrenoceptor as noted here, the G-protein (G_i) linked to the α_2-adrenoceptors was down-regulated (Eason and Liggett, 1992).

As a number of groups had already investigated β-adrenoceptor down-regulation, I was interested in assessing effects of agonist treatment on elements downstream in the signalling pathway, that is, to assess the levels of G_s down-regulation in these cells expressing the β-adrenoceptors, and whether G_s could be co-down-regulated with the receptor, or was down-regulated separately. It had previously been assumed that down-regulation of the receptor occurred simultaneously with its respective G-protein. Evidence for such co-internalization is numerous. Time course profiles of receptor down-regulation and G-protein loss have often been shown to be similar (Milligan, 1993).

Results with the β_1-adrenoceptor and β_2-adrenoceptor expressing CHO cells showed that G_s is down-regulated in a concentration-dependent manner in response to isoprenaline. This was shown directly by G_s detection by immunoblotting, and indicated by adenylyl cyclase assays, as these showed similar EC_{50} values to the western blots of G_s, thus suggesting that G_s down-regulation is a key element in β_1-adrenoceptor and β_2-adrenoceptor desensitization.

The down-regulation of G_s in a time-dependent manner also occurred in the β_1-adrenoceptor and β_2-adrenoceptor expressing CHO cells. The time for half-maximal loss of G_s in β_1-adrenoceptor expressing CHO cells was remarkably fast, 60 ± 4 minutes, compared to 247 ± 35 minutes in the β_2-adrenoceptor expressing CHO cells and a similar rate of 220 ± 72 minutes in the β_3-adrenoceptor expressing CHO cells. This was assumed to be due to larger receptor levels in these cells (7130 ± 258 fmol/mg membrane protein for the β_1-adrenoceptors and 2300 ± 120 fmol/mg membrane protein for the β_2-adrenoceptors) when compared to the β_2-adrenoceptor expressing CHO cells. While other

groups had studied the down-regulation of the β_1-adrenoceptor and β_2-adrenoceptor with binding studies (Thomas *et al*, 1992, Chambers *et al*, 1994), I was mainly interested in G_s down-regulation, and therefore did not have time courses or concentration-responses for the β-adrenoceptor down-regulation to directly compare results with. However, groups (Chambers *et al*, 1994) working on the same CHO cells transfected with β_2-adrenoceptors, showed a similar time course of receptor down-regulation to my studies of G_s level down-regulation. For example, Chambers *et al*, (1994) observed a < 75% reduction in receptor levels following exposure for 24 hours to isoprenaline. This was in agreement with previous published reports that β_2-adrenoceptor levels in CHO cells are reduced to < 20% of their original levels over the same time period (Suzuki *et al*, 1992). I found that approximately 15% of original levels of G_s remained at the plasma membrane of the β_2-adrenoceptor expressing CHO cells after 24 hours of treatment with maximally effective levels of isoprenaline (100 μM). This suggested that in the case of the β_2-adrenoceptor, at least, G_s and this receptor are down-regulated concurrently. The molecular basis of this pronounced down-regulation is thought to involve, at least partially, the presence of two tyrosine residues (Tyr-350 and Tyr-354) located in the intracellular C-terminal tail of the β_2-adrenoceptor, as replacement of these with alanines by site-directed mutagenesis dramatically reduces the ability of the β_2-adrenoceptor to undergo agonist-induced down-regulation (Valiquette *et al*, 1990).

Thomas *et al* (1992) also studied the down-regulation of the β_1-adrenoceptor which had been transfected into 3T3-F442A cells. Although receptor density of the β_1-adrenoceptor was much lower in these cells (14.3 ± 3 fmol/mg membrane protein) than was present in

my CHO cells (7130 ± 258 fmol/mg membrane protein), a similar time course of down-regulation was observed, which could be compared to the time course of G_s down-regulation in my study. For example, receptor levels after treatment for 5 hours with isoprenaline (100 μM) were approximately 20% of original levels in the study by Thomas *et al* (1992), while G_s levels in β_1-adrenoceptor expressing CHO cells in my studies also reached lowest levels (maximal down-regulation) after this short time. Half-maximal down-regulation of the receptor in the study by Thomas *et al* (1992) occurred at approximately 2.5 hours, while G_s half-maximal down-regulation in my CHO cell line occurred at 60 ± 4 minutes. This, as mentioned previously, and proved by work on two β_3-adrenoceptor expressing CHO cell lines, was probably due to much larger receptor levels. The experiments comparing down-regulation of D43 and C15 β_3-adrenoceptor expressing CHO cells in Section 3.4., confirmed that this was the case. The higher the receptor density, the greater the rate of G_s down-regulation. This, once again, is in agreement with the pattern of down-regulation observed in the β_2-adrenoceptor, in that the receptor and G-protein down-regulate concurrently. The temporal co-incidence of loss of the receptor with the G-protein raised the possibility that down-regulation of the two polypeptides is inextricably linked.

A number of previous studies have also shown that agonist treatment results in a concurrent down-regulation of both receptor and G-protein (Adie *et al*, 1992, Milligan, 1993). Two groups have, however, been able to show that the β_3-adrenoceptor, in contrast to the β_1- and β_2-adrenoceptor, at least when expressed in CHO cells, does not undergo substantial down-regulation (Chambers *et al* 1994, Nantel *et al*, 1994). These groups' results were in agreement with a previous study by

Liggett *et al*, (1993). Clearly, the results of my studies showing G_s to down-regulate extensively in the β_3-adrenoceptor expressing CHO cells upon agonist stimulation, imply that, in some receptor/G-protein signalling systems, the down-regulation of the G-protein occurs independently of down-regulation of the receptor, and co-down-regulation is not required. It could be possible, perhaps, that there is an initial co-sequestration of the two polypeptides followed by separate sorting processes (Milligan *et al*, 1995). It may also be a possibility that the occurrence of two mechanisms (receptor up-regulation and G-protein down-regulation) operating to regulate the β_3-adrenoceptor response, at least *in vivo*, results in a neutral net effect on β_3-adrenoceptor mediated responses (i.e. no long term tolerance to the effect of β_3-agonists).

The mixed and differing results of the previous studies leave many questions to be answered concerning sequestration of the receptor and its respective G-protein. During agonist exposure, receptor sequestration occurs along a similar time frame to that of phosphorylation, again beginning within seconds to minutes. It has previously been suggested that rapid internalization of the receptor away from the plasma membrane G_s occurs (Hausdorff *et al*, 1990).

A classic hallmark of acute sequestration of β_2-adrenoceptors, for example, has been the loss of hydrophilic ligand binding sites, with no change in the total number of sites, as assessed by hydrophobic ligands. Thus receptors are no longer accessible to classical β-adrenoceptor hydrophilic ligands such as isoprenaline and CGP12177. Upon the removal of agonist, both desensitization and sequestration are readily reversed with similar kinetics. That the loss of these sites is due to the

accumulation of receptors within an intracellular compartment has been shown by various methods, including differential centrifugation when a light vesicle fraction of β_2-adrenoceptors can be isolated after agonist exposure (Perkins *et al*, 1991) due to the fact that the membrane compartment has a lower density than plasma membranes, immunofluorescent light microscopy (Von Zastrow *et al*, 1992) and electron microscopy of ligand binding sites (Saffitz *et al*, 1992). This light vesicle fraction hypothesis represents an attractive and simple mechanism for an explanation of loss of receptor activity, but now new lines of evidence suggest that it is not an important mechanism underlying rapid desensitization. Several groups, for example Hausdorff *et al* (1990), have effectively blocked sequestration by pre-treatment of cells with inhibitors such as phenylarsene oxide or concanavalin A, without seeing any effect on rapid desensitization (Waldo *et al*, 1983). Also, the onset of agonist-induced desensitization has been detected before that of β-adrenoceptor sequestration (Waldo *et al*, 1983). It has also been proposed that receptor sequestration is a pre-requisite stage for down-regulation. However, recent experimental manipulations that have impaired or abolished receptor desensitization have had no effect on the sequestration process (Bouvier *et al*, 1988, Hausdorff *et al*, 1989, Lohse *et al*, 1990). Whether sequestration plays any role in the regulation of β-adrenoceptor function has remained controversial. The latest proposals by Wu *et al* (1995) for the role of sequestration are that upon stimulation, β-adrenoceptors are uncoupled from G_s, and sequestered away from the cell surface into phosphatase-enriched vesicle compartments. In these compartments the phosphorylated receptors are presumably dephosphorylated and thus reactivated. Receptors are then recycled back to the plasma membrane where they can recouple to G_s. Mutations produced by Barak *et al*

(1994, 1995) have been used to determine the importance of residues on transmembrane domain seven for sequestration. It was demonstrated that mutation of tyrosine residue 326 to an alanine abolished agonist sequestration of this mutant without affecting its ability to maximally stimulate adenylyl cyclase in membranes. Studies ongoing from this discovery are now focusing on the NPLIY amino acid sequence. The amino acid sequence $NP(XX)_{2,3}Y$ recurs in the seventh transmembrane region throughout the superfamily of G-protein-coupled receptors. Barak *et al* (1994) tested the hypothesis, for the β_2-adrenoceptor, that this sequence behaves as a functional motif. Point mutations were produced of the most conserved amino acids, N, P, and Y. Mutation of asparagine 322 to an alanine resulted in complete uncoupling of the receptor, loss of high-affinity agonist binding, and abolition of receptor sequestration, downregulation, and phosphorylation. In contrast, a mutation of this residue to an aspartic acid resulted in an improvement of G-protein coupling without adversely affecting other receptor properties. Substitution of proline residue 323 with alanine resulted in a receptor with mild deficits in sequestration and coupling, a reduced agonist-mediated phosphorylation, and no change in downregulation. A mutant receptor with tyrosine residue 326 changed to a phenylalanine sequestered at 25% the rate of wild type receptor and was also phosphorylated less well than the wild type receptor in response to agonist. In contrast the alanine mutant of tyrosine 326 does not sequester and is weakly phosphorylated in response to agonist, and it activates adenylyl cyclase less well than wild type receptor. These data suggest that the NPLIY sequence of the β_2-adrenoceptor functions as a motif that may represent a critical determinant for maintaining the normal conformation of the receptor but does not function as a specific sequestration recognition motif.

While most previous sequestration studies have focused on the receptor alone being packaged into vesicles, my studies investigated loss of G_s from the plasma membrane and looked at whether it was transferred into the cytosol, or just rapidly degraded. Previous workers have found conflicting results on whether $G_s\alpha$ remains in the cytosol after down-regulation, whether it is packaged into vesicles, or whether it is rapidly degraded. Ransnas *et al* (1989) reported receptor-mediated down-regulation of $G_s\alpha$ in wild-type S49 lymphoma cells. This resulted in a redistribution of $G_s\alpha$ from the membrane fraction to the cytosol. Their studies were, however, limited by the poor specificity of the antiserum. It did show two interesting findings in that the $G_s\alpha$ was detected in the cytosol, and also that this cytosolic $G_s\alpha$ behaved as if it were persistently activated. Kvapil *et al* (1994) found that the long and short forms of $G_s\alpha$ were unequally distributed after isoprenaline stimulation of β-adrenoceptors in S49 wild-type lymphoma cells. Two cellular pools of membranes, light density membranes and plasma membranes differed in their content of $G_s\alpha$ splice forms. Kvapil and co-workers (1994) had, however, identified the presence of G_s in light vesicle fractions, encouraging the suggestions that G-proteins are firstly packaged into vesicles, before degradation processes.

My investigations showed that, upon agonist stimulation, there was loss of $G_s\alpha$ from the plasma membrane, but this polypeptide was not immunologically detected in the cytosolic fraction after four hours of treatment with 100 μM isoprenaline. $G_s\alpha$ immunoreactivity was observed in the cytoplasmic fractions of both the control cells and treated cells, but only less than 10% of the original membrane fraction of $G_s\alpha$. It may perhaps have been found in the cytosol in a short space of time after treatment, but from my studies I can conclude that $G_s\alpha$ is

rapidly degraded and not stored (for any length of time anyhow) in the cytosol. I cannot make assumptions however, on whether the G_s polypeptide is packaged into vesicles first. My results were in agreement with other studies (Milligan *et al*, 1989, Chang and Bourne, 1989) where they were also unable to detect $G_s\alpha$ in the cytoplasm of cholera-toxin treated cells, believing too that the G-protein must be rapidly degraded. Milligan *et al* (1989) noted that a reduction in $G_s\alpha$ of the membrane fraction upon prostaglandin treatment of NG108-15 cells was not transferred to the cytoplasm, as there was no increase in $G_s\alpha$ levels in that fraction.

Other studies looking at other G-proteins have found movement of the polypeptide from the plasma membrane to the cytosol (Svoboda and Milligan, 1994). In a clone of a CHO cell line expressing high levels of the human muscarinic M1 acetylcholine receptor (Hm1), they observed substantial agonist-specific down-regulation of both Hm1 receptors and the alpha subunits of G_q and G_{11}. The G_q and G_{11} polypeptides were found to be transferred from plasma membranes to distinct light vesicular membranes. These observations suggested that the Hm1 receptor and associated G-proteins was down-regulated by two sequential steps. Firstly, there is a transfer of signal-transducing polypeptides from the plasma membrane to a non-plasma membrane light vesicle fraction. Secondly, agonist-specific down-regulation occurs. A more recent study by Svoboda *et al* (1996) investigating the thyrotropin-releasing hormone (TRH) in human embryonic kidney 293 cells, noted that the two transfected species variants of $G_{11}\alpha$ (human and murine) had identical cellular distribution. Sustained exposure of the cells to TRH resulted in transferral of both species forms of $G_{11}\alpha$ to low density membranes and cytosol fractions from the plasma

membrane. This demonstrated agonist-induced subcellular distribution and down-regulation of $G_{11}\alpha$, suggesting that the G_{11} member of the G-protein family, at least, is tranferred to a light vesicle fraction from the plasma membrane.

Previous studies have investigated the regulation of β-adrenoceptors by alteration in mRNA levels (Hadcock *et al*, 1988, 1989, Nishikawa *et al*, 1993, Bouvier *et al*, 1989). Hadcock *et al* (1988, 1989), demonstrated that homologous and heterologous down-regulation can occur at the level of receptor mRNA. Chronic stimulation by β-adrenoceptor agonists resulted in homologous down-regulation of receptor mRNA, while cholera toxin and forskolin also stimulated cAMP accumulation and heterologous down-regulation of receptor mRNA. Work demonstrated that agonist-promoted down-regulation of receptor mRNA required receptor-G_s coupling and protein kinase A activity, as well as G_s regulation of adenylate cyclase and some other effector. Nishikawa *et al* (1993) observed in rat lung tissue that administration of isoprenaline resulted in a significant decrease in β_1-adrenoceptor mRNA after 2 hours, while the β_2-adrenoceptor mRNA was not altered after 2 hours, but was significantly decreased after 1 day of isoprenaline treatment.

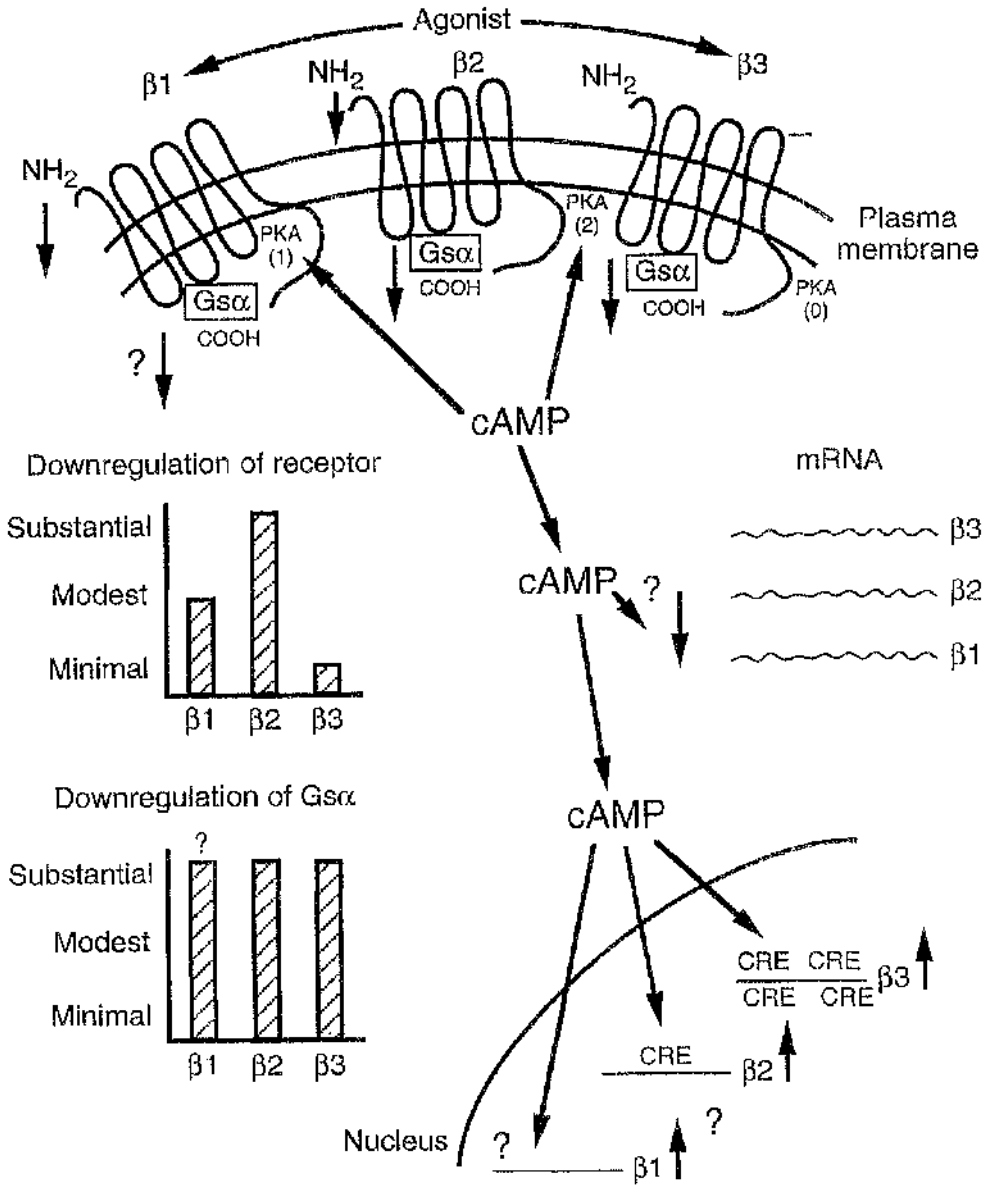
The discovery of cAMP response elements (CRE s) on regions of the β-adrenoceptors indicates the possibility of regulation of expression of the receptor. Transcriptional regulation by cAMP is accomplished by means of cAMP response element binding proteins (CREB), which bind to DNA sequences termed cAMP response elements and alter the rate of transcription (Yamamoto *et al*, 1988). The classic CRE motif has a sequence TGACGTCA, and DNA sequences that are imperfect by as

much as two nucleotides are still effective in binding CREB and regulating transcription (Habener, 1990). Analysis of the 5' flanking region of the β_3-adrenoceptor indicates that it contains potential cAMP response elements. In 3T3-F442A cells which have differentiated towards an adipocyte phenotype by addition of insulin, the β_3-adrenoceptor is expressed although it is not in the undifferentiated fibroblast phenotype (Feve *et al*, 1991). These cells also express the β_1-adrenoceptor at low levels in the fibroblast form, and levels of mRNA encoding this receptor are also increased with differentiation. When differentiated cells were exposed to isoprenaline, levels of β_3-adrenoceptor mRNA were found to be elevated within 4 hours and this was then maintained for at least 30 hours. In addition to this, levels of the β_3-adrenoceptor also increased with a similar temporal pattern. However, in parallel, the levels of the β_1-adrenoceptor declined by some 70% (Thomas *et al*, 1992). mRNA encoding the β_2-adrenoceptor has also been detected in differentiated 3T3-F442A cells (Feve *et al*, 1991). A cAMP-responsive element has also been noted in the promoter region of the β_2-adrenoceptor (Collins et al, 1990). The influence of the cAMP-regulated transcriptional mechanism on β_2-adrenoceptor mRNA levels appears to predominate in the short term. Three putative CRE's, which are imperfect by two nucleotides, are present in the 5' flanking region of the rat β_1-adrenoceptor gene. Very little is known so far of their involvement in regulating reporter gene expression in response to cAMP. Figure 3.8.1. illustrates the presence or absence of CRE's in the three β-adrenoceptors.

Although many groups have investigated the regulation of receptor mRNA (Feve *et al*, 1991, Thomas *et al*, 1992), very few groups have targeted whether alterations occur in G_s mRNA levels upon agonist

stimulation (McKenzie and Milligan, 1990, Shah and Milligan, 1994, Mullaney *et al*, 1995). Alterations in G_s mRNA have previously been studied in NG108-15 cells, however, after iloprost-treatment (McKenzie and Milligan, 1990). Agonist-induced loss of $G_s\alpha$ in this system was found to be independent of regulation of levels of $G_s\alpha$ mRNA, and unaffected by the presence of the *de novo* protein synthesis inhibitor cycloheximide. Mullaney *et al* (1995) also studied regulation of $G_s\alpha$ mRNA levels in two NCB20 neuroblastoma clones. They found that stimulation of cells with isoprenaline produced no alteration in mRNA levels of $G_s\alpha$. My observations in this Chapter were in agreement with these studies, in that after agonist treatment of the β_1-adrenoceptor transfected CHO cells with 100 μM isoprenaline, no alterations in $G_s\alpha$ mRNA levels were observed. It is therefore unlikely that either transcriptional or translational control of $G_s\alpha$ play a major role in desensitization (McKenzie and Milligan, 1990, Shah and Milligan, 1994) but that agonist-induced modulations in G-protein α subunit levels involve changes in the turnover rate of activated G-proteins (Levis and Bourne, 1992, Milligan, 1993, Mitchell *et al*, 1993).

Figure 3.7.1. Illustration of effect of agonist on the β-adrenoceptors: receptor mRNA changes, presence of CRE's, down-regulation of both receptor and G_s levels.


Agonist
β1
β2
β3
NH2
NH2
NH2
Plasma membrane
PKA (1)
PKA (2)
PKA (0)
Gsα
COOH
?
cAMP
Downregulation of receptor
Substantial
Modest
Minimal
β1 β2 β3
mRNA
cAMP ?
Downregulation of Gsα
cAMP
CRE CRE
CRE CRE
CRE
Nucleus
?

CHAPTER 4.

EFFECT OF β-ADRENOCEPTOR DENSITY ON G-PROTEIN DOWN-REGULATION AND THE EFFICACY AND POTENCY OF AGONISTS

4.1. INTRODUCTION

The effect of changes in receptor density on agonist activity and receptor/G-protein regulation is now well-established (Milligan, 1993). However, in many systems little or no regulation is detected. For example, in neuroblastoma x glioma hybrid NG108-15 cells, agonists at each of the endogenously expressed IP prostanoid, adenosine A2 and secretin receptors cause stimulation of adenylyl cyclase but only those at the IP prostanoid receptor can cause down-regulation of $G_s\alpha$ (Kelly *et al*, 1990, McKenzie and Milligan, 1990). This effect was not mimicked by analogues of cAMP (McKenzie and Milligan, 1990) and it was therefore believed that G-protein down-regulation might reflect the levels of expression of the individual receptors (Milligan and Green, 1991). The importance of receptor levels in agonist-mediated down-regulation has been studied traditionally by the use of irreversible receptor blockade (Kim *et al*, 1994b, MacEwan *et al*, 1995), where agonist access to the receptor population is limited. Irreversible blockers have been employed to 'knock out' receptor levels, leaving receptors inaccessible to agonist activation and regulation. The advantages of this method over others is that just one cell line is treated, with no complications resulting from slightly differing proportions of the other signalling components. It is also possible to investigate effects on efficacy and agonist potency via cell lines expressing differing levels of the receptor in the study. While results from such a study provide evidence for the concept that efficacy and potency alterations are likely to reflect levels of expression of a receptor, it may suffer from the fact that they had to be performed on different individual clonal isolates, leading to the possibility of slightly different levels of the signalling components between clones.

Data from other groups demonstrates that the degree of G-protein down-regulation observed is related to the number of receptors available for the agonist ligand to occupy and not the ability of this receptor occupancy to result in the activation of adenylyl cyclase (Kim and Milligan, 1994). It should therefore be anticipated, as previous studies have shown, that such effects are restricted to the G-protein activated by the receptor, and that the effect is a reflection of enhanced degradation of the G-protein without significant transcriptional or translational control (Milligan *et al*, 1993, Mitchell *et al*, 1993). It is not mimicked by treatment with analogues of cAMP (McKenzie *et al*, 1990) and mutationally activated G-protein α subunits are known to have reduced half-lives compared with the wild-type proteins (Milligan *et al*, 1993, Levis *et al*, 1992). Thus, if greater levels of available receptors are able to activate more copies of the G-protein, then greater down-regulation of this polypeptide should be anticipated. For example, investigations have been carried out previously into the concept that agonist-mediated G-protein down-regulation is likely to reflect levels of expression of a receptor (Adie *et al*, 1994a). Adie and co-workers (1994a) generated clonal cell lines of NG108-15 cells with genomic DNA encoding the human β_2-adrenoceptor. Two clones were studied, one containing some 4,000 fmol/mg membrane protein and another with some 300 fmol/mg membrane protein. The clone with high receptor levels responded to challenge with isoprenaline by down-regulating levels of $G_s\alpha$ whereas little effect was observed in the clone expressing much lower levels. While these results provide strong supporting evidence for the concept that agonist-mediated G-protein down-regulation is likely to reflect levels of expression of a receptor, as previously mentioned, they suffer from the fact that they had to be performed on different individual clonal isolates. To counteract this concern, my

second study in this Chapter involved only a single clone of the β_1-adrenoceptor expressing CHO cells (β_1-adrenoceptor levels of 7130 ± 258 fmol/mg membrane protein). I firstly took isoprenaline and stimulated β_1-adrenoceptors, then effected a way of allowing only limited access of the agonist to the receptors by pre-treating cells with an irreversible blocker, bromoacetyl alprenolol menthane (BAAM). Altering receptor levels in one just one cell line by this method provided a clear, reliable assessment of the principles that receptor density affects G-protein down-regulation, without alteration in amounts of the signalling components, which could occur using different cell lines. Although my investigations with BAAM were only preliminary, other investigators have generated similar results to mine (Kim *et al*, 1994b, MacEwan *et al*, 1995). The irreversible antagonist used in this study, bromoacetyl alprenolol menthane (BAAM) is an alkylating β-blocker. The pharmacophore of BAAM is derived from alprenolol, a drug binding with high affinity and specificity to β-adrenoceptors. BAAM also contains a bromoacetamido group which reacts with an amino acid residue of the β-adrenoceptor. BAAM undergoes covalent attachment to the receptor protein through bromoalkylation of sulfhydryl groups and thus irreversible occupancy of the receptor binding site. It can therefore irreversibly decrease the density of β_1-adrenoceptor binding sites at relatively low concentrations. These properties of BAAM were highly useful for my investigation into the effect of receptor level differences on G-protein down-regulation, by performing immunoblotting on crude membrane fractions taken from β_1-adrenoceptor expressing CHO cells. I also used binding assays, with the ligand [^{3}H]DHA, to assess alterations in the β_1-adrenoceptor density, caused by BAAM treatment.

My first study in this Chapter, however, assess effect of altered receptor levels on agonist potency and efficacy, in my case assessing action of ligands on the β_2-adrenoceptor expressed in murine neuroblastoma x Chinese hamster brain NCB20 cells. After selecting three cell lines with a range of β_2-adrenoceptor levels, it was then possible to perform assays of two kinds ([^{3}H]forskolin binding and adenylyl cyclase assays) to assess the intrinsic activities and potencies of four β-adrenoceptor specific agonists, isoprenaline, salbutamol, ephedrine and the physiological agonist adrenaline. While isoprenaline and adrenaline are classically defined as full agonists, both salbutamol and ephedrine are defined as partial agonists. Whether this classification stands, or whether it is in fact dependent on receptor levels, will be assessed in this Chapter.

4.2. INVESTIGATIONS INTO THE EFFECT OF RECEPTOR DENSITY ON AGONIST POTENCY AND EFFICACY.

RESULTS

Three different cell lines of NCB20 neuroblastoma cells previously transfected with the β_2-adrenoceptor (Mullaney *et al*, 1995) at receptor densities shown in Table 4.2.1. were employed in this study. It had previously been confirmed that transfection of these cells had no effect on the cellular morphology or gross polypeptide composition of membranes from wild-type NCB20 cells, as assessed by Coomassie Brilliant Blue staining of SDS-PAGE (Mullaney *et al*, 1995). The L9 cell line expressed the lowest β_2-adrenoceptor levels, while the D4 cell line expressed the highest levels.

Table 4.2.1. Approximate receptor densities for the three cell lines of NCB20 neuroblastoma cells: L9, D1, and D4.

NCB20 cells	**Approximate receptor density** (pmol/mg membrane protein)
L9	0.6
D1	4.7
D4	12.9

Two assays were employed in parallel for this investigation, a well-established assay and a relatively new assay. The well-established

double column method of Johnson and Salomon (1991) was chosen to estimate adenylyl cyclase activity, measured in pmol/min/mg protein, while a more recently established [^{3}H]forskolin assay was also used. This provided a direct comparison of two diverse assays, one being a functional assay and the other a binding assay. It also involved comparison of results taken from a whole cell preparation ([^{3}H]forskolin binding assay) with results using a membrane fraction preparation (adenylyl cyclase assay). Methods for these two assays are as detailed in Chapter 2 (Materials and Methods).

Previous workers (Kim *et al*, 1995) had already found that the optimal temperature for the incubation in the [^{3}H]forskolin binding assay to be 4°C, rather than the more common 30°C or 37°C for a binding assay. I did, however, perform a time course to estimate the optimum incubation time for maximal binding of the radiolabel in NCB20 cells to occur. This time course is shown in Figure 4.2.1.a. It was also vital to use enough cells to produce good levels of specific binding in this binding assay. I therefore performed an assay where varying levels of cells were added and specific binding calculated for each. This is shown in Figure 4.2.1.b.

Figure 4.2.1.a. Time course for binding of [^{3}H]forskolin. Results, showing the mean ± S.E.M.from triplicate samples for each point, were expressed in units of dpm, representing specific binding. The assay was performed at 4°C for 90 minutes, using a final concentration of 10 nM [^{3}H]forskolin. This was a representative experiment of which two others were performed with similar results. Error bars represent the errors of three different binding points.

Figure 4.2.1.b. Assessment of cell number versus specific binding of [^{3}H]forskolin. Error bars shown are from three different assay points. Results, showing the mean ± S.E.M.from triplicate samples for each point, were expressed in units of dpm, representing specific binding. The assay was performed at 4°C for 90 minutes, using a final concentration of 10 nM [^{3}H]forskolin. From this assessment of cell numbers necessary for good, reproducible levels of specific binding, further [3H]forskolin binding assays used cell numbers between 0.75-1.25 x 10^6 cells per assay.

Figure 4.2.1.a.

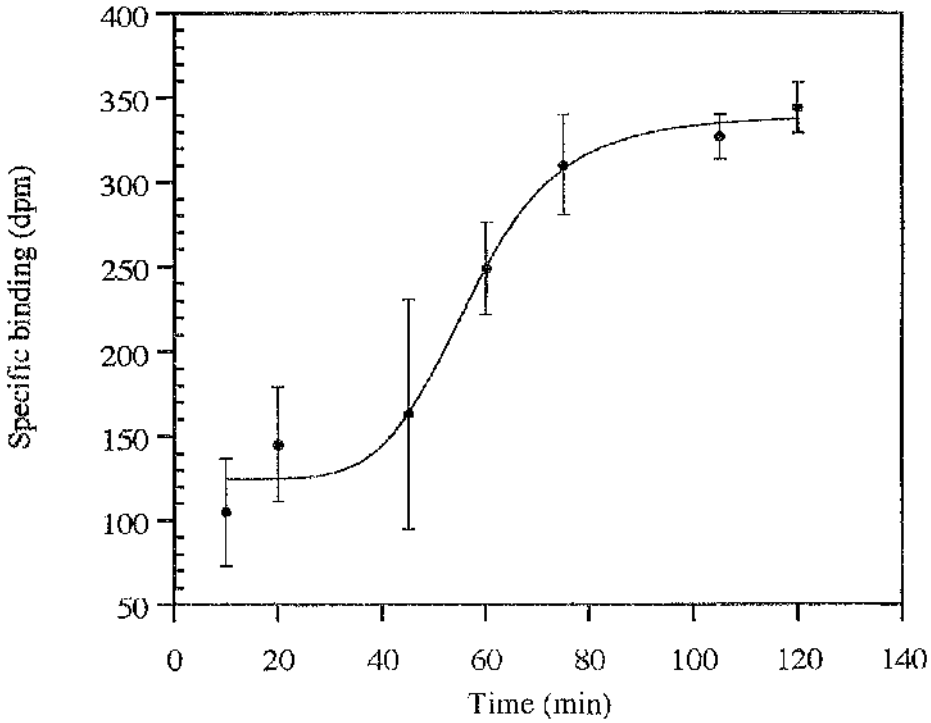
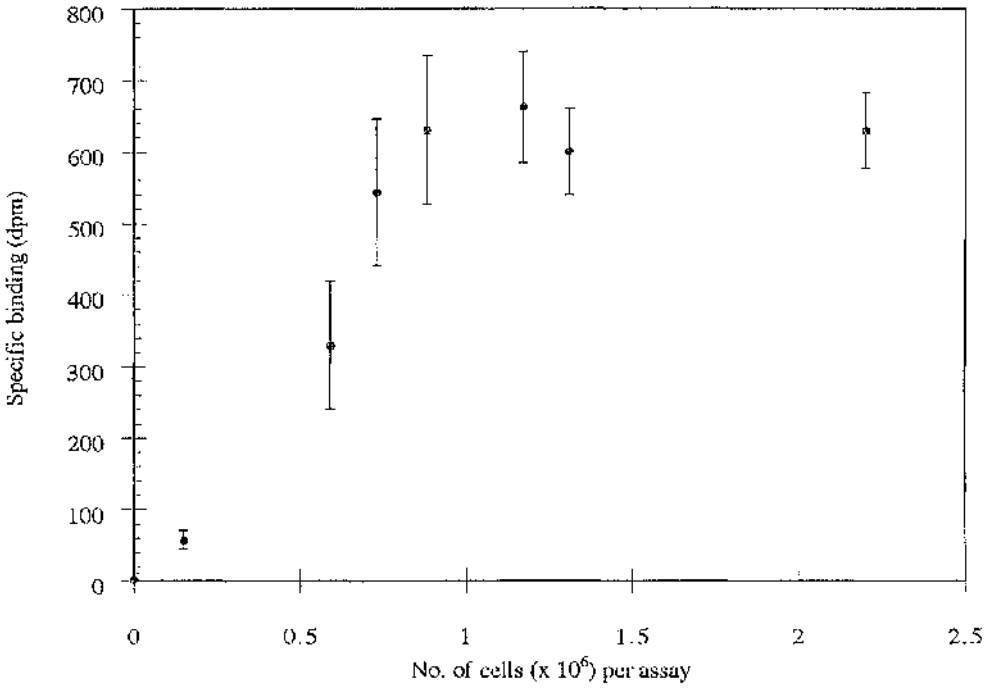

Specific binding (dpm)
400
350
300
250
200
150
100
50
0
20
40
60
80
100
120
140
Time (min)

Figure 4.2.1.b.


800
700
600
500
400
300
200
100
0
Specific binding (dpm)
0
0.5
1
1.5
2
2.5
No. of cells (x 10^6) per assay

After optimal conditions for the specific [^{3}H]forskolin binding assay had been established, concentration-response curves were produced for a number of β_2-adrenoceptor agonists in both this and the adenylyl cyclase assay. The agonists represented a range of efficacies. A partial agonist is defined as an agonist which produces a response, but is unable to achieve a maximal reponse which full agonists can achieve. Therefore, while a full agonist has an intrinsic activity (IA) of 1.00, the partial agonist will have intrinsic activity less than 1.00.

Examples of concentration-response curves are shown in Figures 4.2.2. a &b, 4.2.3. a &b, and 4.2.4. a &b.

4.2.2.a. Concentration-response curve to isoprenaline in L9 cells ([^{3}H]forskolin). Results from [^{3}H]forskolin binding assays are expressed as specific binding, units of which are dpm. Error bars represent S.E.M. and are for mean values determined from triplicate samples at each concentration of isoprenaline. This was a representative experiment of which two others were performed with similar results. The assay was performed at 4°C for 90 minutes, using a final concentration of 10 nM [^{3}H]forskolin. Cell numbers were between 0.75-1.25 x 10^6 per assay.

4.2.2.b. Concentration-response curve to isoprenaline in L9 cells (adenylyl cyclase assay). Results, showing the mean ± S.E.M. are determined from triplicate samples per point and are expressed in pmol/min/mg protein. This was a representative experiment of which two others were performed with similar results.

Figure 4.2.2.a.

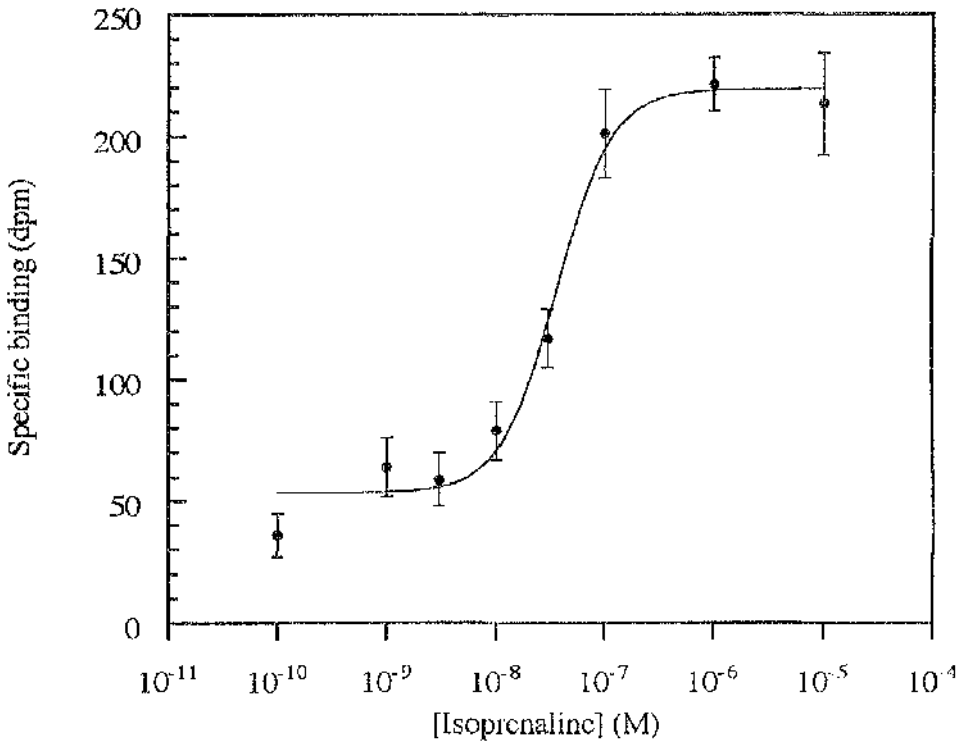
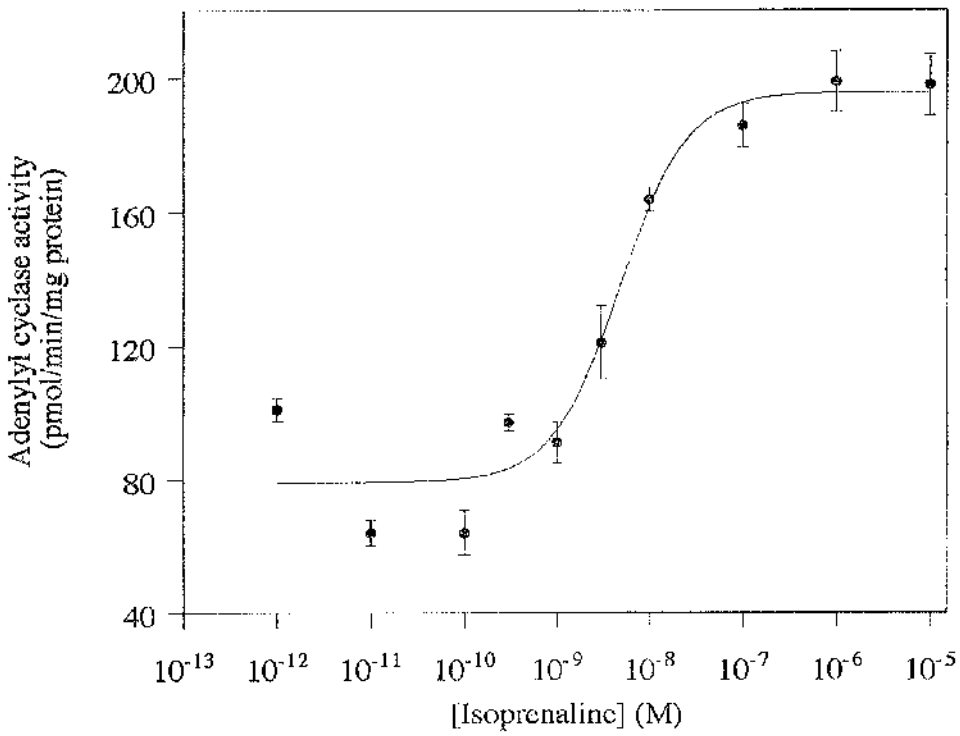

250
200
150
100
50
0
Specific binding (dpm)
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[Isoprenaline] (M)

Figure 4.2.2.b


Adenylyl cyclase activity
(pmol/min/mg protein)
200
160
120
80
40
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[Isoprenaline] (M)

4.2.3.a. Concentration-response curve to isoprenaline and salbutamol in D1 cells ([^{3}H]forskolin). Results from [^{3}H]forskolin binding assays are expressed as specific binding, units of which are dpm. Error bars represent S.E.M. and are for mean values determined from triplicate samples at each concentration of isoprenaline. This was a representative experiment of which two others were performed with similar results. The assay was performed at 4°C for 90 minutes, using a final concentration of 10 nM [^{3}H]forskolin. Cell numbers were between 0.75-1.25 x 10^6 per assay. This was a representative experiment of which two others were performed with similar results.

4.2.3.b. Concentration-response curve to isoprenaline in D1 cells (adenylyl cyclase assay). Results of adenylyl cyclase activity are expressed as pmol/min/mg protein. Mean values ± S.E.M. are determined from triplicate samples per data point. This was a representative experiment of which two others were performed with similar results.

Figure 4.2.3.a.

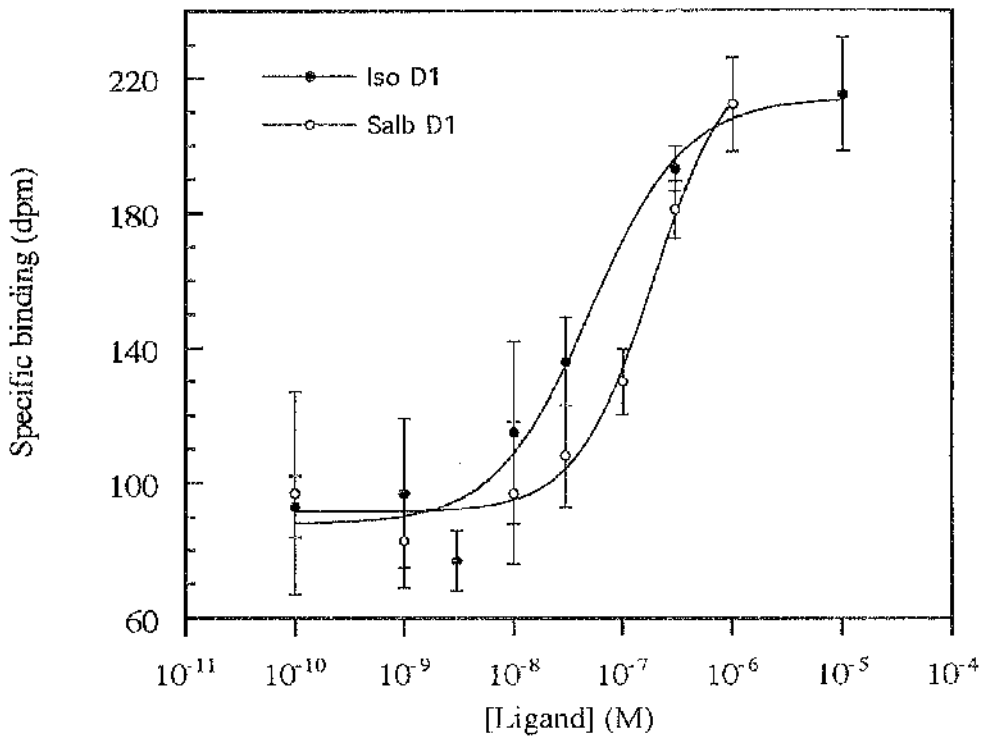
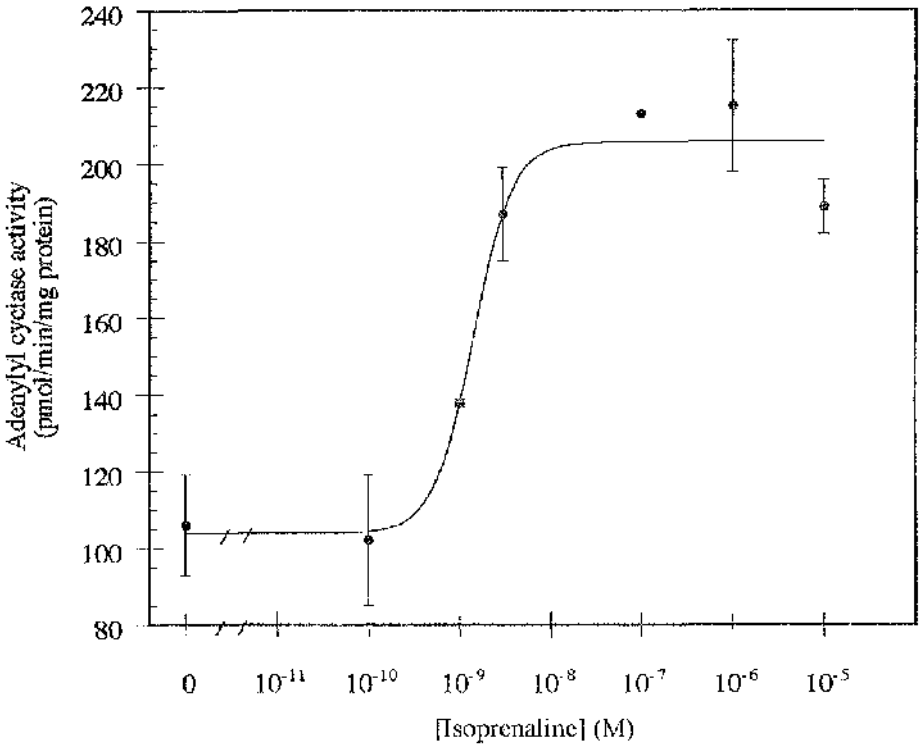

Iso D1
Salb D1
Specific binding (dpm)
220
180
140
100
60
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[Ligand] (M)

Figure 4.2.3.b.


240
220
200
180
160
140
120
100
80
Adenylyl cyclase activity
(pmol/min/mg protein)
0
10-11
10-10
10-9
10-8
10-7
10-6
10-5
[Isoprenaline] (M)

4.2.4.a. Concentration response curve to isoprenaline in D4 cells ([^{3}H]forskolin). Results from [^{3}H]forskolin binding assays are expressed as specific binding, units of which are dpm. Error bars represent S.E.M. and are for mean values determined from triplicate samples at each concentration of isoprenaline. The assay was performed at 4°C for 90 minutes, using a final concentration of 10 nM [^{3}H]forskolin. Cell numbers were between 0.75-1.25 x 10^6 per assay. This was a representative experiment of which two others were performed with similar results.

4.2.4.b. Concentration response curve to isoprenaline in D4 cells (adenylyl cyclase assay). Adenylyl cyclase activity is expressed in pmol/min/mg protein. Mean values ± S.E.M. are determined from triplicate samples per data point. This was a representative experiment of which two others were performed with similar results.

Figure 4.2.4.a.

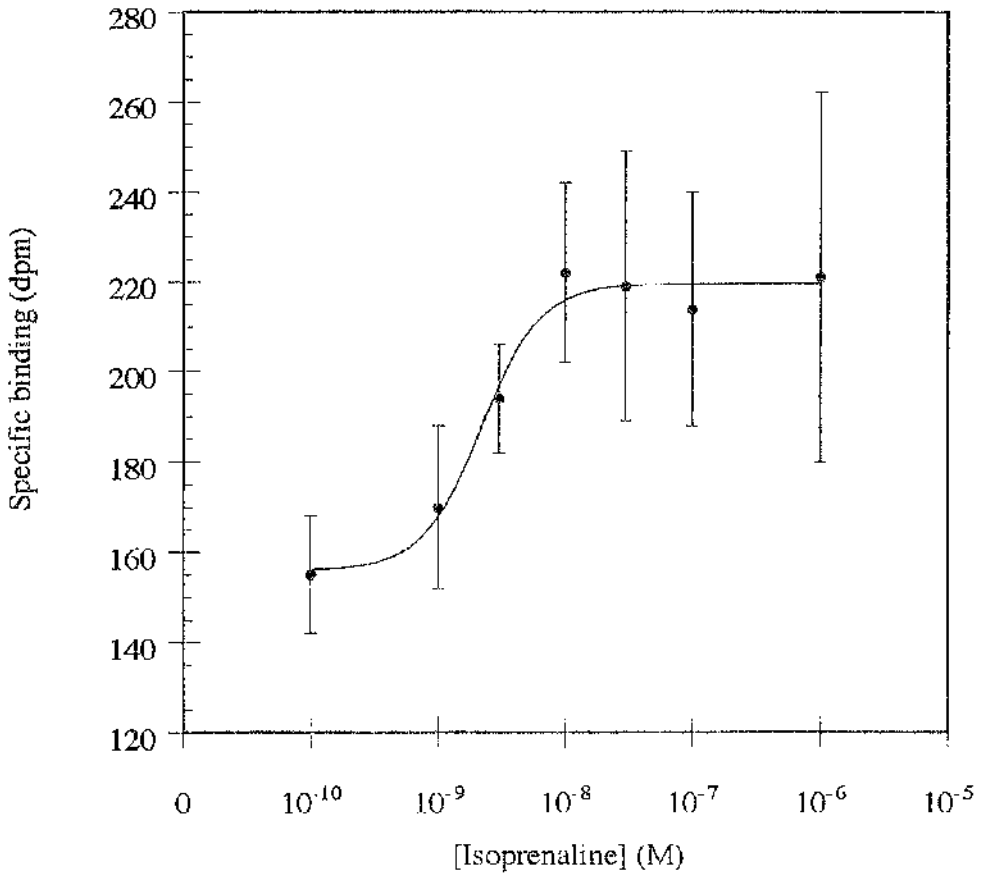
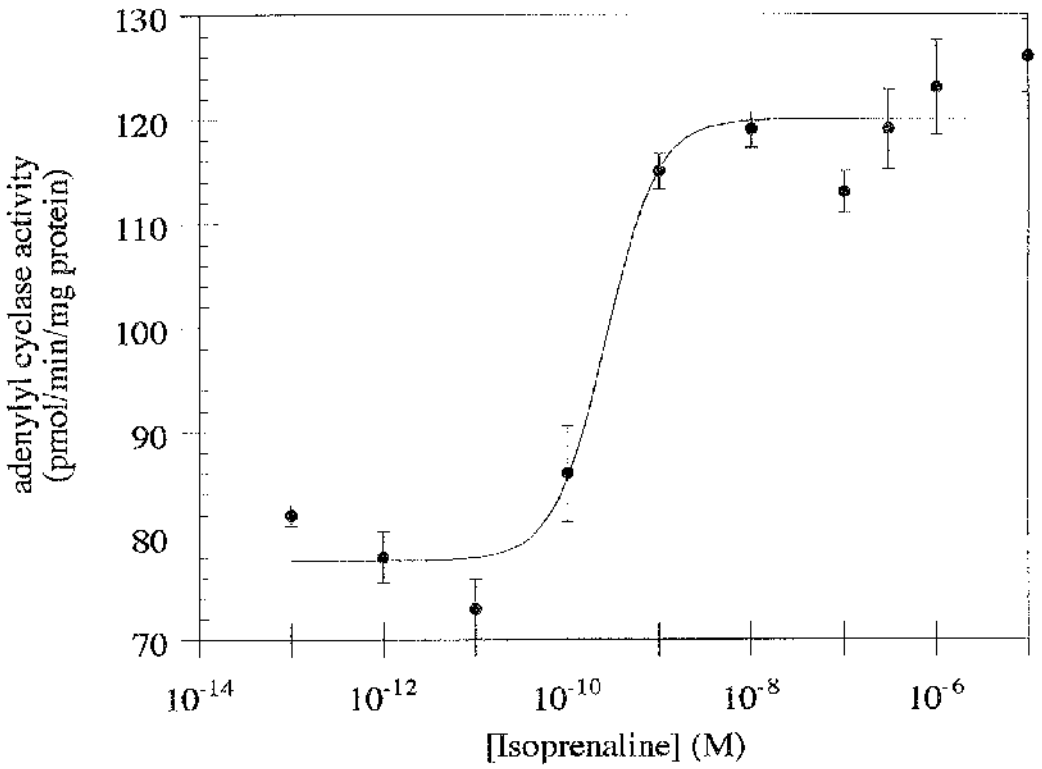

Specific binding (dpm)
280
260
240
220
200
180
160
140
120
0
10-10
10-9
10-8
10-7
10-6
10-5
[Isoprenaline] (M)

Figure 4.2.4.b.


130
120
110
100
90
80
70
adenylyl cyclase activity
(pmol/min/mg protein)
10-14
10-12
10-10
10-8
10-6
[Isoprenaline] (M)

After concentration responses were performed more than three times for each cell line of NCB20 cells, it was then possible to produce, for salbutamol at least, concentration-responses, showing responses as a percentage of the maximal isoprenaline (full agonist) response. These are shown in Figure 4.2.5. a &b, with results of both the adenylyl cyclase assays and [^{3}H]forskolin binding assays shown. It is clear from these concentration-reponse curves that the cyclase and binding results are comparable, with salbutamol shown as a full agonist at the D1 and D4 cell lines, while only a partial agonist at the L9 cell line. The L9 cell line had the lowest β_2-adrenoceptor levels and is therefore clear proof that the efficacy (intrinsic activity) of a ligand is dependent on receptor levels.

Figure 4.2.5. a. Comparison of the [^{3}H]forskolin assay results of the three cell lines, L9, D1 and D4, with different doses of salbutamol. Results are expressed as a percentage of the maximal isoprenaline response. Error bars represent S.E.M. and are for mean values determined from all salbutamol concentration-response curves. The [^{3}H]forskolin binding assay was performed at 4°C for 90 minutes, using a final concentration of 10 nM [^{3}H]forskolin. Cell numbers were between 0.75-1.25 x 10^6 per assay.

Figure 4.2.5.b. Comparison of the adenylyl cyclase assay results of the three cell lines, L9, D1 and D4, with different doses of salbutamol. Results are expressed as a percentage of the maximal isoprenaline response. Error bars represent S.E.M. and are for mean values determined from all salbutamol concentration-response curves.

Figure 4.2.5.a.

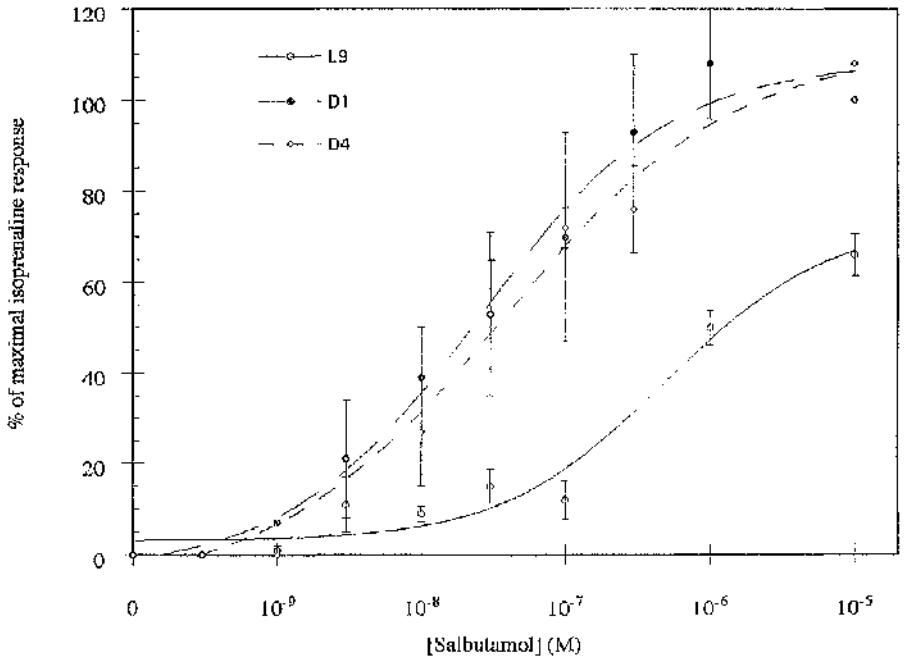
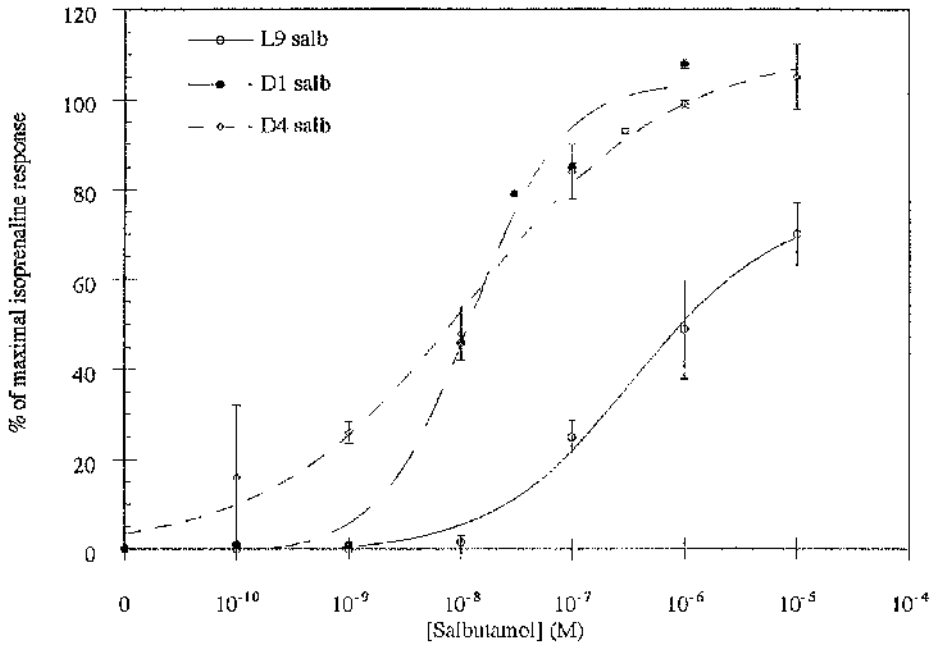

120
100
80
60
40
20
0
% of maximal isoprenaline response
L9
D1
D4
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[Salbutamol] (M)

Figure 4.2.5.b.


L9 salb
D1 salb
D4 salb
% of maximal isoprenaline response
120
100
80
60
40
20
0
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
[Salbutamol] (M)

It was also possible to note alterations in basal signalling in the three cell lines. This has previously been explored by other investigators studying NG108-15 cells (Adie and Milligan, 1994b), and NCB20 cells (Mullaney *et al,* 1995). Mullaney *et al* (1995) observed that basal adenylyl cyclase activity was substantially higher in clone D1 NCB20 cells than either the parental NCB20 cells or clone L9 NCB20 cells. This phenomenon is believed to represent agonist-independent receptor activity, that is, empty receptor activation of the signalling cascade (Adie and Milligan, 1994b). Thus, the higher receptor levels in a cell, the more agonist-independent receptor activity occuring, and a higher basal value recorded. These alterations in basal levels are shown in Figure 4.2.6. a &b, for the [^{3}H]forksolin binding assay and the adenylyl cyclase assay, respectively. Results from the [^{3}H]forskolin binding assay were significantly different ($p< 0.013$ for D1 compared to L9, $p< 0.00001$ for D4 compared to L9, and $p< 0.021$ for D4 compared to D1), but it is not so clear, and only significantly different for the D4 line compared to the L9 line ($p< 0.005$), in results from adenylyl cyclase assays.

From results for all concentration-effect curves at each cell line, it was possible to calculate EC_{50} values and intrinsic activity (IA) values. These are shown in Table 4.2.2. and Table 4.2.3. with comparisons of the [^{3}H]forskolin binding values and adenylyl cyclase assay values. It is clear from Table 4.2.2. that EC_{50} values (defining potency) of each agonist at the β_2-adrenoceptor, are affected by receptor density. For example, in the adenylyl cyclase assay, there was some 2-fold difference in potency of isoprenaline between that of the lowest β_2-adrenoceptor expressing cell line L9, and that of D1 (17 ± 3.5 nM and 8 ± 0.5 nM, respectively). There was also a 2-fold difference in

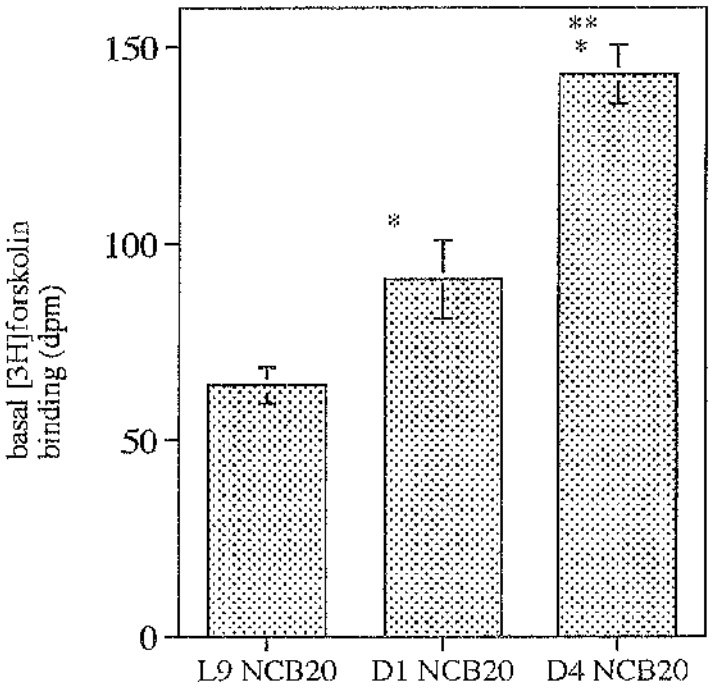
potency between the D1 cell line and the highest β_2-adrenoceptor expressing cell line D4 (8 ± 0.5 nM and 4 ± 0.9 nM, respectively). This suggests that as β-adrenoceptor levels increase, EC_{50} values are decreased, therefore potency of agonist increases with receptor density. These 2-fold differences in potency between cell lines is not a particularly large difference, as other groups have noted much larger differences (Mullaney *et al*, 1995).

In Table 4.2.3., a trend can clearly be seen that as receptor level increases, so does the intrinsic activity of an agonist. For example, while salbutamol has an IA value of 0.7 at the L9 cell line, and therefore classed as a partial agonist, salbutamol has an IA value of 1.0 at the D1 and D4 cell lines, which have higher β_2-adrenoceptor levels. It can therefore be classed as a full agonist at the D1 and D4 NCB20 cell lines, but only a partial agonist at the lower β_2-adrenoceptor expressing L9 NCB20 cells.

Figure 4.2.6.a. Basal level changes in the three NCB20 cell lines, L9, D1 and D4, from results of [^{3}H]forskolin binding assays, measured in levels of specific binding. Results from [^{3}H]forskolin binding assays are expressed as basal binding, units of which are dpm. Error bars represent S.E.M. and are for mean values determined from all basal levels obtained during assays on each cell line. The assay was performed at 4°C for 90 minutes, using a final concentration of 10 nM [^{3}H]forskolin. Cell numbers were between 0.75-1.25 x 10^6 per assay. There was a statistically significant difference in basal levels between cell lines ($p< 0.013$ for D1 compared to D4, $p< 0.00001$ for D4 compared to L9, and $p< 0.021$ for D4 compared to D1).

Figure 4.2.6.b. Basal level changes in the three NCB20 cell lines, L9, D1 and D4, from results of adenylyl cyclase assays, measured in pmol/min/mg protein. Results are obtained from assays performed on each particular cell line, from which mean ± S.E.M. of basal levels are calculated. Results were found to be significantly different between the L9 and D4 lines ($p< 0.005$).

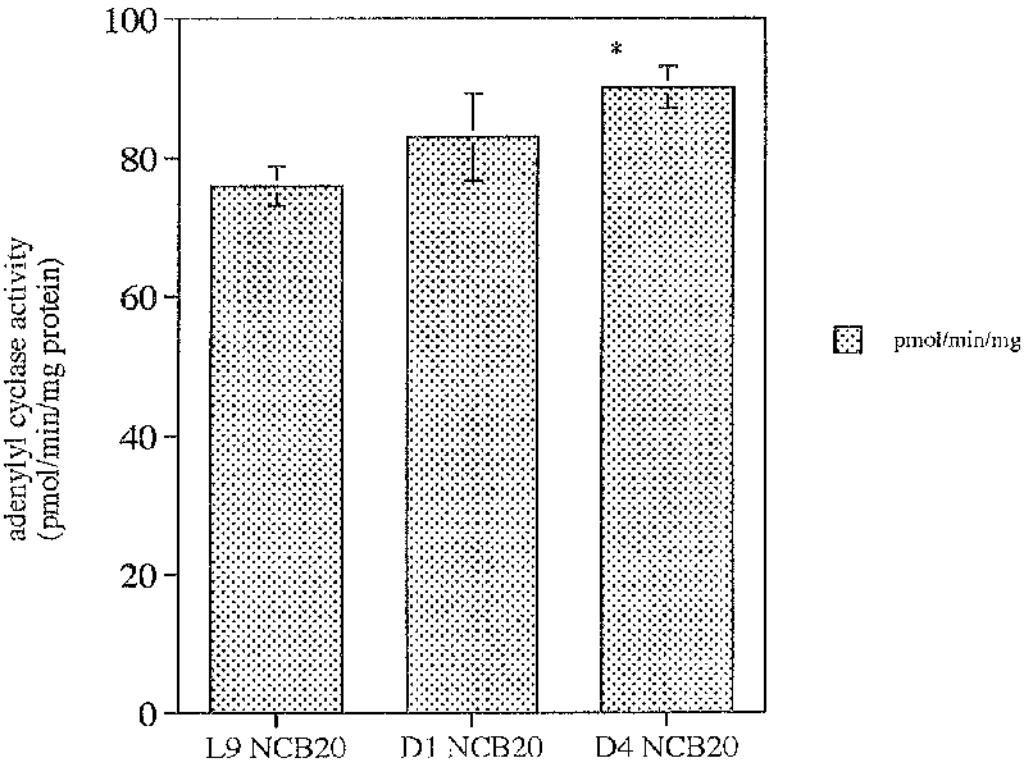
Figure 4.2.6.a



* where p< 0.013 for D1 compared to L9
where p< 0.00001 for D4 compared to L9

** where p< 0.021 for D4 compared to D1

Figure 4.2.6.b.



* where $p < 0.005$ for D4 compared to L9

Table 4.2.2. Potency (EC_{50}) values for ligands at NCB20 cells:a comparison of the two assays, [^{3}H]forskolin binding and adenylyl cyclase. Results are means of three or more experiments (except numbers in brackets) ± S.E.M. N.D=not determined. Iso=isoprenaline, sal=salbutamol, eph=ephedrine, and adren=adrenaline.

	EC_{50} (nM)			
cells	*iso*	*sal*	*eph*	*adren*
L9	37 ±	360 ±	300 ±	200
([^{3}H]forskolin)	8.1	138	50	(1)
L9	17 ±	93 ±	N.D.	24
(adenylyl	3.5	2.5		(2)
cyclase)				
D1	38 ±	18 ±	100	N.D.
([^{3}H]forskolin)	5.9	8.2	(1)	
D1	8 ±	18 ±	340 ±	N.D.
(adenylyl	0.5	5.9	49	
cyclase)				
D4	1 (2)	44 ±	N.D.	N.D.
([^{3}H]forskolin)		4.9		
D4	4 ±	44	140	6 (1)
(adenylyl	0.9	(2)	(1)	
cyclase)				

Table 4.2.3. Intrinsic activity (IA) values for ligands compared to isoprenaline. Comparison of the two assays, [^{3}H]forskolin binding and adenylyl cyclase. Results are means of three experiments ± S.E.M. except where stated in brackets. N.D.=not determined. Iso=isoprenaline, sal=salbutamol, eph=ephedrine, and adren=adrenaline.

	Intrinsic activity (IA)			
cells	*iso*	*sal*	*eph*	*adren*
L9 ([^{3}H]forskolin)	1.0	0.7 ± 0.1	0.5 ± 0.1	N.D.
L9 (adenylyl cyclase)	1.0	0.7 ± 0.1	0.3 (2)	1.2 (1)
D1 ([^{3}H]forskolin)	1.0	1.0 (2)	0.4 ± 0.1	N.D.
D1 (adenylyl cyclase)	1.0	1.0 (2)	0.7 ± 0.2	1.0 (1)
D4 ([^{3}H]forskolin)	1.0	1.0 (2)	N.D.	N.D.
D4 (adenylyl cyclase)	1.0	1.0 (2)	0.9 (1)	N.D.

4.3. INVESTIGATIONS INTO EFFECTS OF ALTERATIONS IN RECEPTOR LEVEL ON DOWN-REGULATION OF G_S.

RESULTS

Crude membrane fractions derived from β_1-adrenoceptor expressing CHO cells were examined for expression of these receptors by measuring the specific binding of the β-adrenoceptor antagonist [^{3}H]dihydroalprenolol (10 nM). Exposure of the β_1-adrenoceptor expressing CHO cells to varying concentrations of BAAM for 4 hours prior to cell harvesting and crude membrane preparation resulted in a decrease in the number of detectable specific binding sites for [^{3}H]DHA (Figure 4.3.1). In the absence of BAAM, some 7,100 fmol/mg membrane protein of binding sites for the β_1-adrenoceptor are available in the passages of CHO cells used here. Half-maximal reduction in the number of available β_1-adrenoceptors was achieved by treatment with 30nM BAAM, and treatment with 1μM BAAM reduced the number of β_1-adrenoceptors to some 15% of original levels.

Figure 4.3.2. shows percentage $G_s\alpha$ levels remaining after concentration-reponses to isoprenaline were performed on β_1-adrenoceptor expressing CHO cells, treated or untreated with 10 μM BAAM for four hours. It clearly shows that BAAM treatment blocks agonist-receptor interactions to such an extent that the process of G-protein down-regulation is decreased, in direct proportion to the fraction of receptors irreversibly blocked by BAAM.

Figure 4.3.1. Effect of different concentrations of BAAM on β_1-adrenoceptor levels. Results are expressed as the percentage of β_1-adrenoceptors remaining after BAAM treatment, obtained from [^{3}H]DHA binding assays. Cells were treated with concentrations of BAAM for four hours prior to harvesting and the production of crude membrane fractions. This Figure was a representative experiment of which two others were performed with similar results.

Figure 4.3.1.

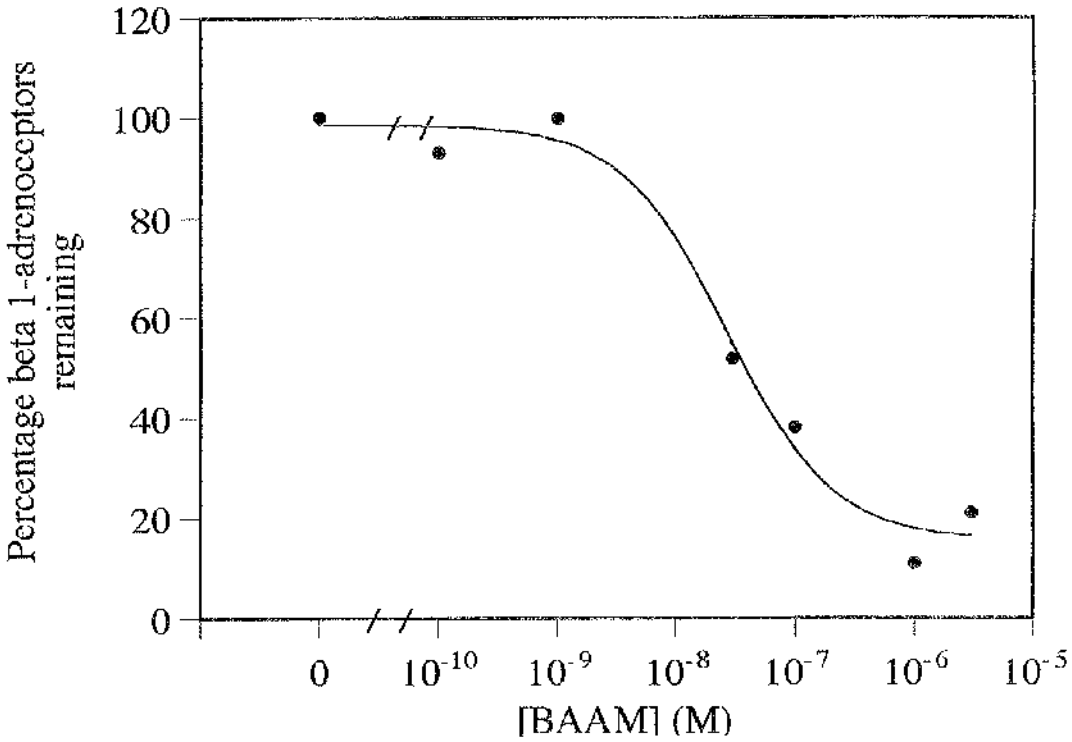
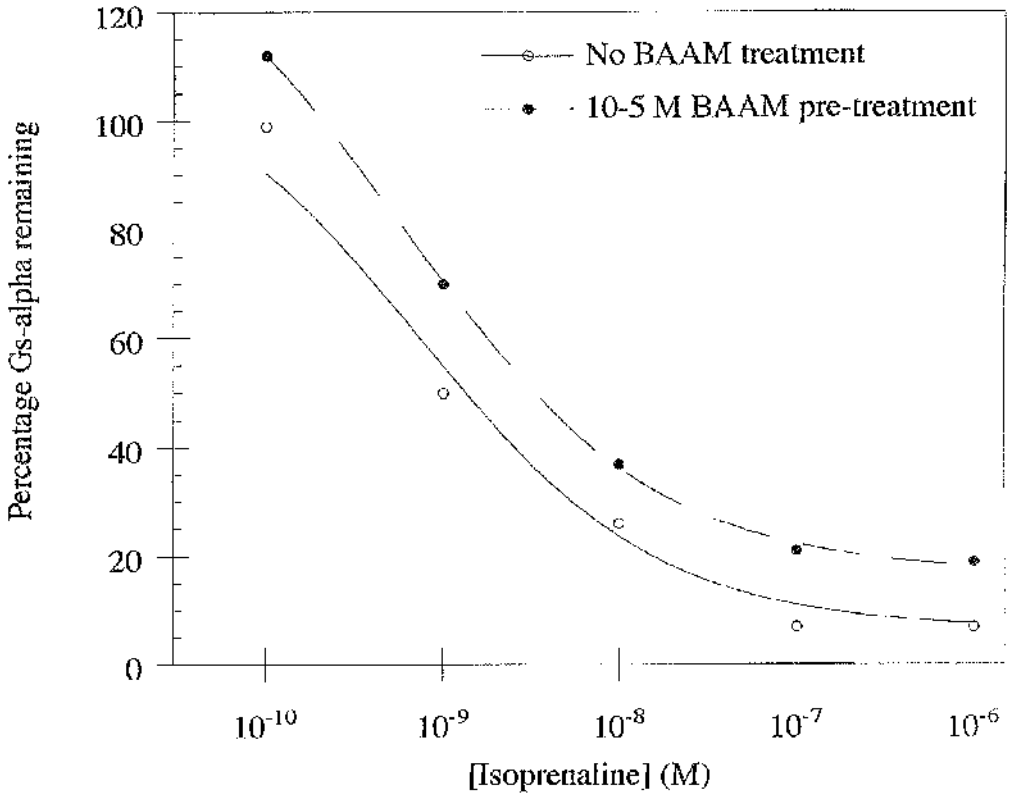

Percentage beta 1-adrenoceptors remaining
120
100
80
60
40
20
0
0
10-10
10-9
10-8
10-7
10-6
10-5
[BAAM] (M)

Figure 4.3.2. Concentration-response to isoprenaline in β_1-adrenoceptor expressing CHO cells with or without BAAM (10 μM) treatment. Cells were treated with different concentrations of isoprenaline, with or without BAAM pre-treatment for four hours. Results were expressed as a percentage of $G_s\alpha$ remaining. This was a representative experiment of which two others were performed with similar results.

Figure 4.3.2.


120
100
80
60
40
20
0
Percentage Gs-alpha remaining
No BAAM treatment
10-5 M BAAM pre-treatment
10-10
10-9
10-8
10-7
10-6
[Isoprenaline] (M)

4.4. DISCUSSION

In recent years, many biochemical studies have utilised wild-type or mutant receptors which have been stably or transiently expressed into model cell systems over wide ranges of receptor densities. In the past decade, however, a large amount of evidence has been accumulated from studies into the effect of receptor density on the kinetic parameters of adenylyl cyclase activation (Johnson *et al*, 1979, Bouvier *et al*, 1988, George *et al*, 1988). No effort has been made so far, to fit the consequences of these varying receptor levels to any currently accepted model for adenylyl cyclase activation. Many studies looking at the β-adrenoceptors have not touched on the effect of different receptor densities on results. Very few groups have previously investigated this effect of receptor density on agonist potency and efficacy (Whaley *et al*, 1994, MacEwan *et al*, 1995). This may lead, in turn, to incorrect conclusions concerning the ability of mutant and wild-type β-adrenoceptors to activate adenylyl cyclase when different receptor levels are present in different studies. I therefore investigated the effects of varying receptor density on signalling processes, such as G-protein down-regulation. I also assessed the effect of alterations in β_2-adrenoceptor density on partial and full agonists.

The main investigations in this Chapter were to obtain data on the importance of receptor levels (the β_2-adrenoceptor in NCB20 cells) in defining the intrinsic activity and potency of agonists. This could be performed without alterations in amounts of the other components of the signaling cascade in three clones of NCB20 cells transfected with different levels of the β_2-adrenoceptor. The concept that different degrees of intrinsic activity of agonists should be observed in cells and

tissues expressing different levels of a G-protein-linked receptor is a classical element of receptor theory (Stephenson *et al*, 1956, Kenakin *et al*, 1989, Hoyer *et al*, 1993). Experimental examination of this has traditionally focused on the observations that many drugs act as full agonists in some tissues but not in others, or by the use of irreversible antagonists to limit access of agonists to receptors. The first approach is limited by the fact that levels of both the G-protein and effector systems are likely to vary widely between the cell type, and the second approach by the possibility that irreversible antagonism of a fraction of the receptors might be anticipated to limit access of the remaining receptors to a proportion of the G-protein population. In cells in which G-protein levels are comparable to those of the receptor this might alter receptor output.

In my first study, alongside changes in efficacy due to changes in receptor levels, I was also able to assess alterations in EC_{50} values of agonists due to alterations in receptor levels. The relationship between EC_{50} values for agonist and the nature and levels of receptor expression has been examined previously (Whaley *et al*, 1994, Samama *et al*, 1994). Whaley *et al* (1994) noted an increased potency (decreased EC_{50}) of the ligand adrenaline with increasing receptor number (from 200 to 0.2 nM as receptor density increased from 5 to 5000 fmol/mg membrane protein). I also observed an increased potency with the full agonist isoprenaline related to an increase in receptor number. This confirmed the results of Whaley *et al* (1994) and results of two previous studies performed with Chinese Hamster fibroblast membranes (Bouvier *et al*, 1988) and Chinese Hamster ovary whole cells (Samama *et al*, 1993). My results did not show this phenomenon so clearly with the partial agonist salbutamol, but the trend was similar. These effects

of receptor density on agonist activity were consistent with the predictions of classical pharmacological models, and emphasise the importance of relating data on agonist potency at cloned receptors to receptor expression levels. Mathematical predictions have been developed by Whaley *et al* (1994), based on the cycle of G-protein activation first proposed by Cassell and Selinger (1977) and the mobile receptor model, to analyse alterations in β-adrenoceptor full agonist EC_{50} values with receptor number. Equations predict the relationship of receptor number to the EC_{50} (potency) and IA (efficacy).

In systems in which the effector species is quantitatively the limiting component of the cascade, it is also often observed that elevation in receptor number will result in a leftward shift in the dose-effect curve, consistent with the notion of a receptor population reserve. Adie *et al*, (1994) noted this phenomenon when studying isoprenaline stimulation of adenylyl cyclase activity in membranes of βN22 and βN17 cells. A number of other studies have reported this phenomenon with cells expressing various levels of the β_2-adrenoceptor (Whaley *et al*, 1994, Bouvier *et al*, 1988). However, one report, using S49 lymphoma cells did not report such an effect (Johnson *et al*, 1979).

The phenomenon that efficacy increased as receptor levels increased was confirmed in my study, very clearly by observations with the agonist salbutamol. At the lowest receptor density NCB20 cell line, it was only a partial agonist (IA of 0.7 ± 0.1 in both [^{3}H] forskolin binding assays and adenylyl cyclase assays) while at the higher expressing β_2-adrenoceptor cell lines of D1 and D4 NCB20 cells, salbutamol was found to be a full agonist. Ephedrine followed a similar trend, although results were only preliminary. For example, using the adenylyl cyclase

assay, at the L9 NCB20 cells, ephedrine was only a partial agonist with an IA value of approximately 0.3. At the higher β_2-adrenoceptor expressing D1 NCB20 cells, the IA value was 0.7 ± 0.2, indicating that although ephedrine was still only a partial agonist at the β_2-adrenoceptor, the efficacy was increasing with increasing receptor levels. Finally, at the D4 NCB20 cells, although the result of just one experiment, the IA value was 0.9, suggesting that the results for ephedrine are consistent with the phenomenon of increasing receptor levels leading to increasing efficacy of agonist.

Further to the previous investigations into efficacy and potency of agonists at the β_2-adrenoceptor, and the effect of changes in receptor levels, I also made preliminary investigations to assess the effect of alterations in receptor levels on down-regulation of the G-protein associated with the β_1-adrenoceptor, G_s. This followed similar investigations by Kim *et al* (1994) and MacEwan *et al* (1995) where they had utilised BAAM to investigate G-protein down-regulation (Kim *et al*, 1994b) and intrinsic activity and potency (MacEwan *et al*, 1995). From assays performed here, using the irreversible antagonist BAAM, from preliminary investigations, one was able to demonstrate that the degree of G-protein down-regulation observed was related to the number of receptors available for the agonist to occupy. This was in agreement with observations by Kim *et al* (1994) while investigating the regulation of the β_2-adrenoceptor, in that receptor availabilty defined the extent of agonist-mediated down-regulation in neuroblastoma x glioma hybrid cells transfected to express the β_2-adrenoceptor.

For example, Figure 4.3.2. shows that loss of receptors results in less down-regulation of G_s, the G-protein associated with the β_1-

adrenoceptor. A slightly larger concentration of isoprenaline (0.56 nM) is necessary to stimulate half-maximal G_s down-regulation after BAAM (10 μM) treatment, when compared to the β_1-adrenoceptor-expressing CHO cells which did not receive BAAM pre-treatment (0.71 nM).

These preliminary results using the antagonist BAAM should be anticipated from previous studies (Kim *et al*, 1994b, MacEwan *et al*, 1995), which have shown that such effects are restricted to the G-protein activated by the receptor, and that the effect is a reflection of enhanced degradation of the G-protein without significant transcriptional or translational control (Milligan *et al*, 1993b, Mitchell *et al*, 1993). Effects are also not mimicked by treatment with cAMP analogues (McKenzie *et al*, 1990), and also as mutationally activated G-protein α subunits are known to have reduced half-lives compared with the wild-type proteins (Milligan *et al*, 1993b, Levis *et al*, 1992). Therefore, if greater levels of available receptors are able to activate more copies of the G-protein then greater down-regulation of this polypeptide is to be expected. However, the observation that reduction of β_2-adrenoceptor availability in clone βN22 cells to some 300 fmol/mg membrane protein still resulted in a detectable down-regulation of $G_s\alpha$ whereas this was not observed in clone βN17 which expresses this level of receptor endogenously, demonstrates that it is unwise to extrapolate results from data obtained in different clonal isolates (Adie *et al*, 1994a).

The implications of my studies are, along with other workers such as Whaley *et al*, (1994), Kim *et al* (1994), and MacEwan *et al* (1995) that investigators cannot ignore the now well-established phenomenon that receptor expression levels in wild-type or transfected cell lines, has

a direct result on efficacies and potencies of agonists, and can also affect the rates of down-regulation of components in the signaling cascade.

CHAPTER 5.

INVESTIGATIONS INTO THE MOLECULAR BASIS OF PHARMACOLOGICAL DIFFERENCES BETWEEN THE RAT AND HUMAN β3-ADRENOCEPTORS

5.1. INTRODUCTION

The β_3-adrenoceptor is a mediator of the lipolytic and thermogenic responses which occur in rodent adipose tissues *in vitro* (Hollenga *et al*, 1991, Arch *et al*, 1993), and atypical β-adrenoceptor agonists that activate these receptors have potent therapeutic effects in *in vivo* rodent models of adult-onset diabetes and obesity. Rodent adipocytes have been shown to exhibit the strongest response to β_3-adrenoceptor agonists while human fat cells are only poorly responsive to β_3-agonists. These species-related differences may be partly explained by lower β_3-adrenoceptor mRNA levels found in human adipocytes compared to rat adipocytes. Poor coupling efficiency of these human adipocyte β_3-adrenoceptors cannot, however, be ruled out. Experiments with rodent cells natively expressing the β_3-adrenoceptor and other cells into which this receptor has been transfected, have suggested a number of differences in the pharmacology of the β_3-adrenoceptor between the different species known to investigators, i.e. the rat, mouse, human and bovine β_3-adrenoceptors.

Given that agonists specific for the β_3-adrenoceptor are in the process of being developed as potential therapeutic agents for such diseases as diabetes and obesity, and that these drugs are primarily tested in rodent models, it is of vital importance to understand the molecular differences in the rat and human β_3-adrenoceptors from which the variation in pharmacology arises.

The sequence homology is very high in the β_3-adrenoceptors, approximately 80-90% identity between the human, bovine, mouse and rat β_3-adrenoceptors. Figure 5.1.1. represents a divergence tree constructed to show these similarites between the human, bovine, rat and mouse β_3-adrenoceptors. The bovine and human β_3-adrenoceptors are similar to each other (>90% homology at amino acid level) and the mouse and rat β_3-adrenoceptors show even closer similarity to each other (>95% homology at amino acid level). Several residues located in

the transmembrane domains of the receptor are shared uniquely by these four species of β_3-adrenoceptors and are not found in the β_1- or the β_2-adrenoceptor of the same species. Even though their sequence homology is so high, cross-species differences in β_3-adrenoceptor pharmacology have been observed. For example, Liggett's group (Liggett *et al*, 1992), with their published data shown in Table 5.1.1, were able to demonstrate that, using cell lines with comparable β_3-adrenoceptor expression levels, while some agonists were only partial agonists at the human β_3-adrenoceptor, they were able to perform as full agonists at the rat β_3-adrenoceptor. In this same system, they also observed a reversal of intrinsic activity between CGP12177 and BRL37344 in the rat and human β_3-adrenoceptors. The SmithKlineBeecham β_3-adrenoceptor agonist BRL37344, a phenylethanolamine, when compared to isoprenaline, is a full agonist at the rat β_3-adrenoceptor but only a partial agonist at the human β_3-adrenoceptor. Due to these published pharmacological differences between species, and work carried out by other groups, including studies at SB Pharmaceuticals, the agonist BRL37344 was never entered for human clinical trials. An understanding of the molecular basis of differences between the rat and human β_3-adrenoceptor may allow future workers to more rationally design agonists with greater efficacy at the human β_3-adrenoceptor.

Deletion and site directed mutagenesis studies have led to a good understanding of the catecholamine binding pocket (Strader *et al*, 1987, 1989), and the molecular basis for pharmacological differences between the catecholamine receptor subtypes has been explored with the use of chimeric receptors (Frielle *et al*, 1989: Kobilka *et al*, 1988). Recently, Guan *et al* (1995) employed a similar strategy to investigate the difference in efficacy of the phenylethanolamine BRL37344 for the β_3-adrenoceptor and other β-adrenoceptors by using a series of β_2/β_3 chimeras. These studies suggested that TM5 (transmembrane region 5) is a key molecular determinant responsible

for the higher affinity binding of BRL37344 at β_3-adrenoceptors when compared to β_2-adrenoceptors.

As recent work on the β_3-adrenoceptor has suggested, it would be of great interest to discover how subtle molecular differences in the structure of the β_3-adrenoceptor produce such well-defined alterations in its pharmacology between the rat and human β_3-adrenoceptors.

Figure 5.1.1. Divergence tree showing differences in percentage terms between different species of β_3-adrenoceptors (bovine, human, mouse and rat).

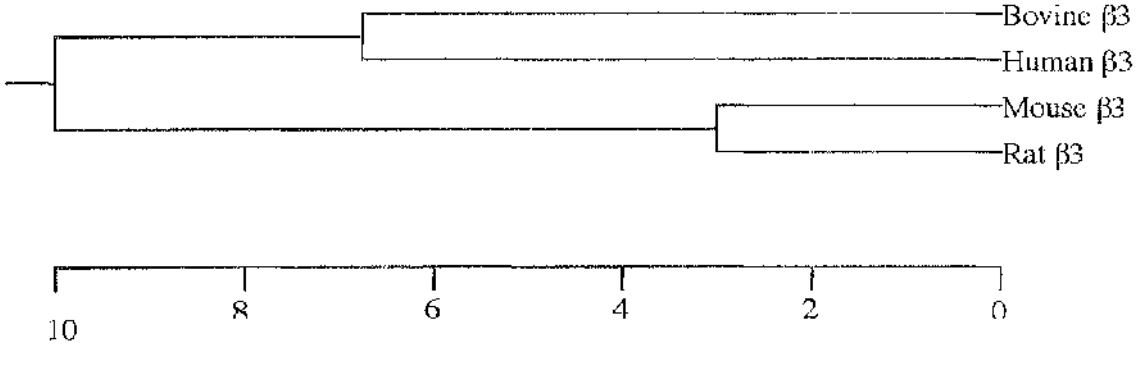


Table 5.1.1. Summary of published data, taken from Liggett *et al* (1992) showing significant differences in intrinsic activity between the rat and human β_3-adrenoceptor. Values are relative to the maximal level of isoprenaline stimulation in each cell line.

	Intrinsic activity (IA)	
agonist	human β_3-adrenoceptor	rat β_3-adrenoceptor
isoprenaline	1.00	1.00
BRL37344	0.60 ± 0.01	1.00 ± 0.01
CGP12177	0.64 ± 0.05	0.31 ± 0.02

The β_3-adrenoceptor and its malfunction have been linked with a number of common diseases. There is evidence that molecular abnormalities in the β_3-adrenoceptor may lead to obesity and the development of non-insulin-dependent diabetes mellitus (NIDDM). Groups have shown that expression of the receptor is markedly decreased in rodent models of obesity (Muzzin *et al*, 1991); mice with knockout (disruption) of the gene for the receptor have marked reductions in lipolysis stimulated by β-selective agonists (Susulic *et al*, 1995), and β_3-specific agonists have potent anti-obesity and anti-diabetic effects in both animals and humans (Himms-Hagen *et al* , 1994, Connacher *et al* , 1992, Mitchell *et al* , 1989). To examine the potential role of inherited defects in this gene, a number of groups have investigated molecular abnormalities in the β_3-adrenoceptor, and its effect on the development of obesity and onset of NIDDM.

A single site polymorphism of the β_3-adrenoceptor was recently discovered by Clement *et al* (1995), and has been suggested to lead to an increased capacity to gain weight in patients with morbid obesity. The frequency of this polymorphism was similar in morbidly obese French patients and normal subjects. However, the patients with morbid obesity who were heterozygous for the Trp64Arg mutation had an increased capacity to gain weight.

This Trp64Arg mutation appears at the beginning of the first intracellular loop of the β_3-adrenoceptor. This loop is thought to be important for the proper movement of the receptor to the cell surface and possibly also for its coupling to G-proteins. Defective expression at the cell surface or impaired signalling may lead to decreased lipolysis and thermogenesis in visceral fat tissue that may contribute to central obesity, insulin resistance, and NIDDM. Morbid obesity, where the body mass index is greater than 40, is believed to have a particularly strong genetic component. The functional deficiency of the β_3-adrenoceptor in

genetically obese mice, and the results of studies in which the gene for the receptor has been disrupted in mice, led Clement *et al* (1995) to investigate the role of the β_3-adrenoceptor in patients with morbid obesity. Genomic DNA was extracted from leukocytes and a polymorphism resulting in the replacement of a tryptophan residue by arginine at position 64 in the β_3-adrenoceptor was detected. The frequency of this Trp64Arg allele was similar in the morbidly obese patients and the normal subjects. However, the patients suffering morbid obesity who were heterozygous for the Trp64Arg mutation had an increased capacity to gain weight. The role of this mutation in the pathogenesis of obesity may be conjectural but may be related to a lowering of the resting metabolic rate, which is genetically determined. Another study released at the same time (Widen *et al*, 1995) showed that this Trp64Arg allele of the β_3-adrenoceptor is associated with abdominal obesity and a resistance to insulin, which may be a causal factor in the early onset of NIDDM. Obesity is a known risk factor for the development of NIDDM. NIDDM is one of the most common inherited diseases. Although most forms of the disease do not have a simple Mendelian pattern of inheritance, the contribution of heredity is well recognized. It is likely that the common forms of NIDDM are complex and heterogeneous and that they may develop when a pool of mutant genes, each contributing in a small and subtle way, interact with one another and with environmental, ageing, and behavioral influences to lead to the expression of the disease.

Another group in the U.S. performed a similar study (Walston *et al*, 1995) on the Pima Indians, a group with a very high prevalence of NIDDM. Pima subjects homozygous for the Trp64Arg polymorphism had an earlier onset of NIDDM and tended to have a lower resting metabolic rate. This mutation may therefore accelerate the onset of NIDDM by altering the balance of energy metabolism in visceral adipose tissue.

To investigate the molecular basis for the species selectivity of compounds such as BRL37344 for the β_3-adrenoceptor a series of human/rat chimeric β_3-adrenoceptors were constructed. Mutants were produced by identification of the changes in the amino acid residues of the transmembrane regions in the rat and human β_3-adrenoceptors.

Single point mutations were not performed but the whole transmembrane regions substituted to produce the chimeric constructs. This was after results from other groups (Guan *et al*, 1995) suggested that single point mutations were not as useful, in the first instance, as whole transmembrane region mutation, as this is more likely to represent the sum effects of both binding and efficacy differences between mutants. Altering the whole transmembrane region from one species to another would hopefully result in more global changes in the receptor structure.

Each chimeric receptor consists largely of the human sequence, except that in each case a single transmembrane (TM) region has been substituted with the corresponding sequence in the rat homologue. Each receptor was named according to the TM region which had been replaced by the rat sequence. Table 5.1.2. shows the specific amino acid changes. Thus the TM1 mutant consists of the human β_3-adrenoceptor, except for the TM1 region which is from the rat β_3-adrenoceptor transmembrane region. An exception to this was the TM1+7 chimera which contains both its TM1 and TM7 regions from the rat β_3-adrenoceptor.

The Trp64Arg mutant was produced with somewhat different intentions, as mentioned previously. This mutation is just a single amino acid change at position 64 from an arginine residue to a tryptophan residue, as it had recently been observed (Walston *et al*, 1995) that in a population where high numbers of

their group developed NIDDM, there was a higher incidence of this human β_3-adrenoceptor polymorphism. It was therefore of interest to examine whether drugs stimulating the normal human β_3-adrenoceptor were just as effective at stimulating this mutated form.

Methods for the construction and expression of the chimeric β_3-adrenoceptors are detailed in Chapter 2 (Materials and Methods). These were kindly constructed by C.Chapman, T.Whitton, and H.Clinkenbeard of the Department of Biotechnology, SB Pharmaceuticals, Harlow, Essex.

Table 5.1.2 Amino acid substitutions for chimera production.

Transmembrane domain	Mutation (human β_3 to rat β_3-AR)
1	V_{48}, L_{49} & A_{50} deletions
	V_{60} -T, W_{64} -R
2	A_{81} -T, M_{86} -V
	V_{91} -M, A_{94} -G
4	T_{156} -A, V_{163} -I,
	A_{167} -T
5	V_{205} -A
6	C_{292} -R, T_{300} -I,
	T_{302} -S
7	G_{325} -S, P_{326} -G,
	A_{327} -V, L_{329} -I
1+7	V_{48}, L_{49} & A_{50} deletions
	V_{60} -T, W_{64} -R, G_{325} -S,
	P_{326} -G, A_{327} -V, L_{329} -I
Trp64Arg	W_{64} -R

The functional assays chosen for the identification of molecular differences between the chimeras and the wild type rat and human β_3-adrenoceptors were the microphysiometer and the cAMP accumulation 'flashplate' assay, a commercially available kit.

The microphysiometer produces a non-invasive, physiological measurement in real time by detecting cellular responses to stimulation. This is performed by measuring the excretion of acidic metabolites with a silicon sensor functioning as an extracellular pH meter. Changes of less than 0.01 pH unit per minute can be monitored. Activation of hormone receptors on intact cells has been shown to increase solution acidity. Cellular metabolism produces protons primarily by generating lactic acid during glycolysis or by generating carbon dioxide during respiration. Lactic acid and carbon dioxide passively diffuse across the cell membrane and they are also actively transported out via acid and anion transport proteins. In addition, protons are excreted via exchangers, channels and pumps on the cell membranes. Energy metabolism is largely regulated by the supply and demand for cellular ATP. When cell surface receptors are activated, the secondary signals they generate can result in an increase or decrease in ATP consumption, which in turn causes changes in the rate of glycolysis and/or respiration. The energy pathway that produces the greatest number of hydrogen ions per ATP consumed is glycolysis, usually the predominating energy generating pathway for cells grown in culture.

Compounds were employed in the study which classically define the β_3-adrenoceptor; agonists isoprenaline, BRL37344 and CGP12177. Isoprenaline is a full agonist at both the rat and human β_3-adrenoceptor, while the phenylethanolamine BRL37344 is an agonist at the rat and human β_3-adrenoceptors but is less efficacious in humans than in rodents. CGP12177 is an aryloxypropanolamine which is a β_1- and β_2-adrenoceptor antagonist, but

at the human and rat β_3-adrenoceptor it is a partial agonist, showing a higher potency for the human β_3-adrenoceptor, previously shown by Liggett's studies (1992) in Table 5.1.1.

Acidification rates are determined as microvolts per second during periods of flow cessation. Rates are recorded and plotted as a function of time. Data may be exported into appropriate database and graphics programs for further processing, such as the computation of the percentage change in acidification rates. Data for up to eight chambers can be displayed simultaneously. These will be displayed on the Figures in this Chapter as A, B, C, D, E, F, G, and H.

The cyclic AMP accumulation 'flashplate' assay, the second type of functional assay to be performed, was used in parallel with the microphysiometer, to confirm and back up data obtained on the microphysiometer. The cAMP accumulation assay records results much further upstream than the microphysiometer, which itself records the final overall response of the whole cells to stimulation. As explained in Chapter 2 (Materials and Methods), the basic principle of the 'flashplate' assay is the competition between a radioactive and non-radioactive antigen for a fixed number of antibody binding sites. Samples in my assay were added to the wells of the kit, 30 μl of unknown cAMP sample per well, then after addition of radioactive tracer, values could be obtained by comparison with a standard curve to cAMP. Scintillant was already present in the wells.

As results emerged, it became obvious that the mutants, transiently expressed in CHO cells or mass cultures produced from transient transfections, did not produce strong enough responses on the microphysiometer to conduct full concentration-response curves to individual compounds, and therefore results of the highly sensitive 'flashplate' assay were vital to this study.

5.2. RESULTS USING THE MICROPHYSIOMETER

Firstly, cumulative concentration-response curves to isoprenaline and BRL37344 at the wild-type human and rat β_3-adrenoceptors were obtained, for comparison with transiently transfected chimera mass cultures. Cumulative concentration-response curves are ideal for β_3-adrenoceptor responses as they are not affected by short-term desensitization, but with most other receptors, cumulative concentration-reponses are not possible. Cells were treated with increasing concentrations of agonist for 12 minutes, after which time the next concentration could be immediately added. Figure 5.2.1.a, b, and c show typical cumulative concentration-responses to isoprenaline, BRL37344 and CGP12177 performed on the human and rat β_3-adrenoceptor expressing CHO cells. The curves produced from one set of cumulative concentration-responses are shown in Figure 5.2.2.a , b, and c. These curves were similar over 3-5 repeat cumulative concentration-response curves and resulted in EC_{50} values being obtained.

Table 5.2.1 shows the potency (EC_{50}) values for the rat and human β_3-adrenoceptor CHO cells upon stimulation with the three β_3-agonists isoprenaline, BRL37344 and CGP12177. Results are the means of 3-5 separate experiments ± S.E.M. values. It can be seen quite clearly from this table that the potency for BRL37344 at the rat β_3-adrenoceptor is much higher, approximately 100-fold greater, than BRL37344 at the human β_3-adrenoceptor. These results are similar to the potency reversal observed by Liggett *et al* (1992), in that BRL37344 had a higher potency for the rat β_3-adrenoceptor than isoprenaline, while at the human β_3-adrenoceptor the potency was reversed.

Figure 5.2.1a. Cumulative concentration-responses to isoprenaline in human and rat β_3-adrenoceptor expressing CHO cells. Chambers contained the following cells:

A= rat β_3-adrenoceptor expressing CHO cells

B= rat β_3-adrenoceptor expressing CHO cells

E= human β_3-adrenoceptor expressing CHO cells

F= human β_3-adrenoceptor expressing CHO cells

Cells were first treated with 0.01 nM isoprenaline for 12 minutes, as shown on the x-axis, then increasing concentrations as shown on the legend, eventually up to a maximal concentration of 1 μM isoprenaline. Responses are expressed on the y-axis as the percentage change in acidification rate.

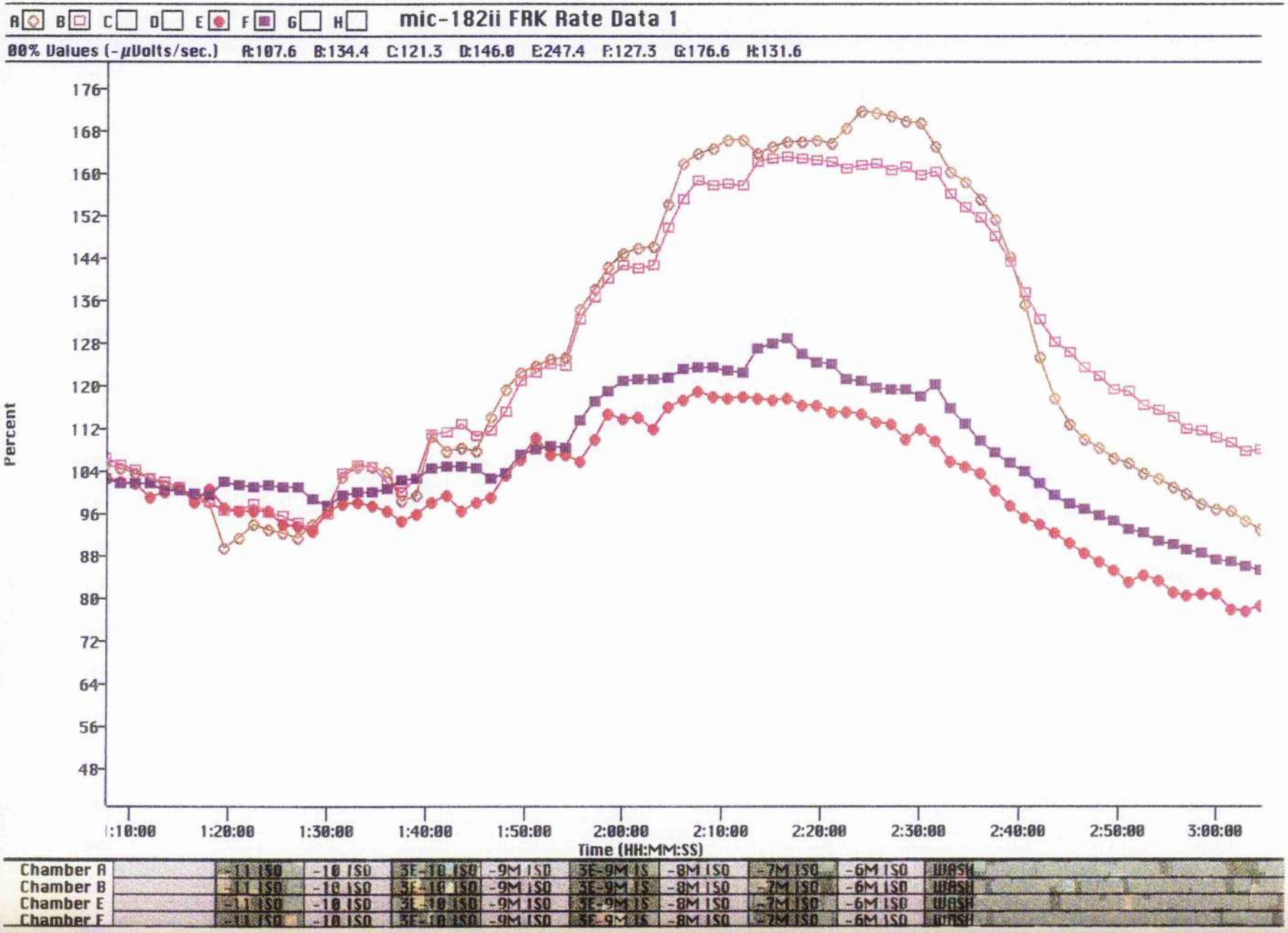

A B C D E F G H
mic-182ii FRK Rate Data 1
00% Values (-µVolts/sec.) A:107.6 B:134.4 C:121.3 D:146.0 E:247.4 F:127.3 G:176.6 H:131.6
Percent
176
168
160
152
144
136
128
120
112
104
96
88
80
72
64
56
48
1:10:00
1:20:00
1:30:00
1:40:00
1:50:00
2:00:00
2:10:00
2:20:00
2:30:00
2:40:00
2:50:00
3:00:00
Time (HH:MM:SS)
Chamber A
Chamber B
Chamber E
Chamber F
-11 ISO
-10 ISO
3E-10 ISO
-9M ISO
3E-9M IS
-8M ISO
-7M ISO
-6M ISO
WASH

Fig.5.2.1.b Cumulative concentration-responses to BRL37344 in human and rat β_3-adrenoceptor expressing CHO cells. Chambers contained the following cells:

A= rat β_3-adrenoceptor expressing CHO cells

B= rat β_3-adrenoceptor expressing CHO cells

C= rat β_3-adrenoceptor expressing CHO cells

E= human β_3-adrenoceptor expressing CHO cells

F= human β_3-adrenoceptor expressing CHO cells

G= human β_3-adrenoceptor expressing CHO cells

H= human β_3-adrenoceptor expressing CHO cells

Cells were first treated with 1 μM isoprenaline for 12 minutes. The cumulative concentration responses were then begun at 0.01 nM BRL37344 for 12 minutes, as shown on the legend for the x-axis, then increasing concentrations as shown, up to a maximal concentration of 1 μM BRL37344. Responses are expressed on the y-axis as the percentage change in acidification rate.

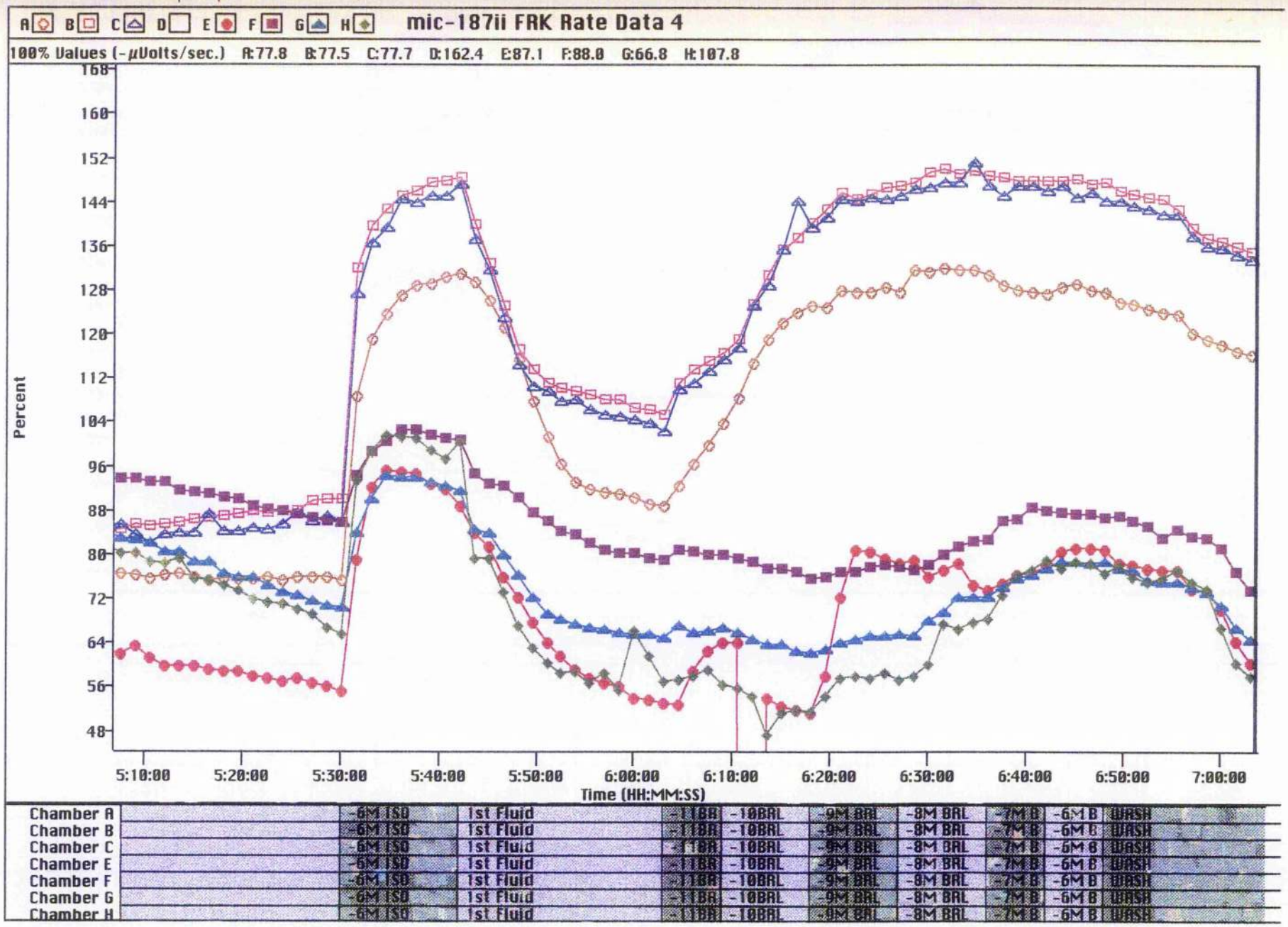

A B C D E F G H
mic-187ii FRK Rate Data 4
100% Values (-µVolts/sec.) A:77.8 B:77.5 C:77.7 D:162.4 E:87.1 F:88.0 G:66.8 H:107.8
Percent
168
160
152
144
136
128
120
112
104
96
88
80
72
64
56
48
5:10:00
5:20:00
5:30:00
5:40:00
5:50:00
6:00:00
6:10:00
6:20:00
6:30:00
6:40:00
6:50:00
7:00:00
Time (HH:MM:SS)
Chamber A
Chamber B
Chamber C
Chamber E
Chamber F
Chamber G
Chamber H
-6M ISO
1st Fluid
-10BRL
-9M BRL
-8M BRL
-7M B
-6M B
WASH

Fig.5.2.1.c Cumulative concentration-responses to CGP12177 in human and rat β_3-adrenoceptor expressing CHO cells. Chambers contained the following cells:

A= rat β_3-adrenoceptor expressing CHO cells

B= rat β_3-adrenoceptor expressing CHO cells

C= rat β_3-adrenoceptor expressing CHO cells

D = rat β_3 adrenoceptor expressing CHO cells

E= human β_3-adrenoceptor expressing CHO cells

F= human β_3-adrenoceptor expressing CHO cells

G= human β_3-adrenoceptor expressing CHO cells

H= human β_3-adrenoceptor expressing CHO cells

Cells were first treated with 0.1 nM CGP12177 for 12 minutes, as shown on the x-axis legend, then increasing concentrations as shown, up to 1 μM CGP12177. Responses are expressed on the y-axis as percentage change in acidification rate.

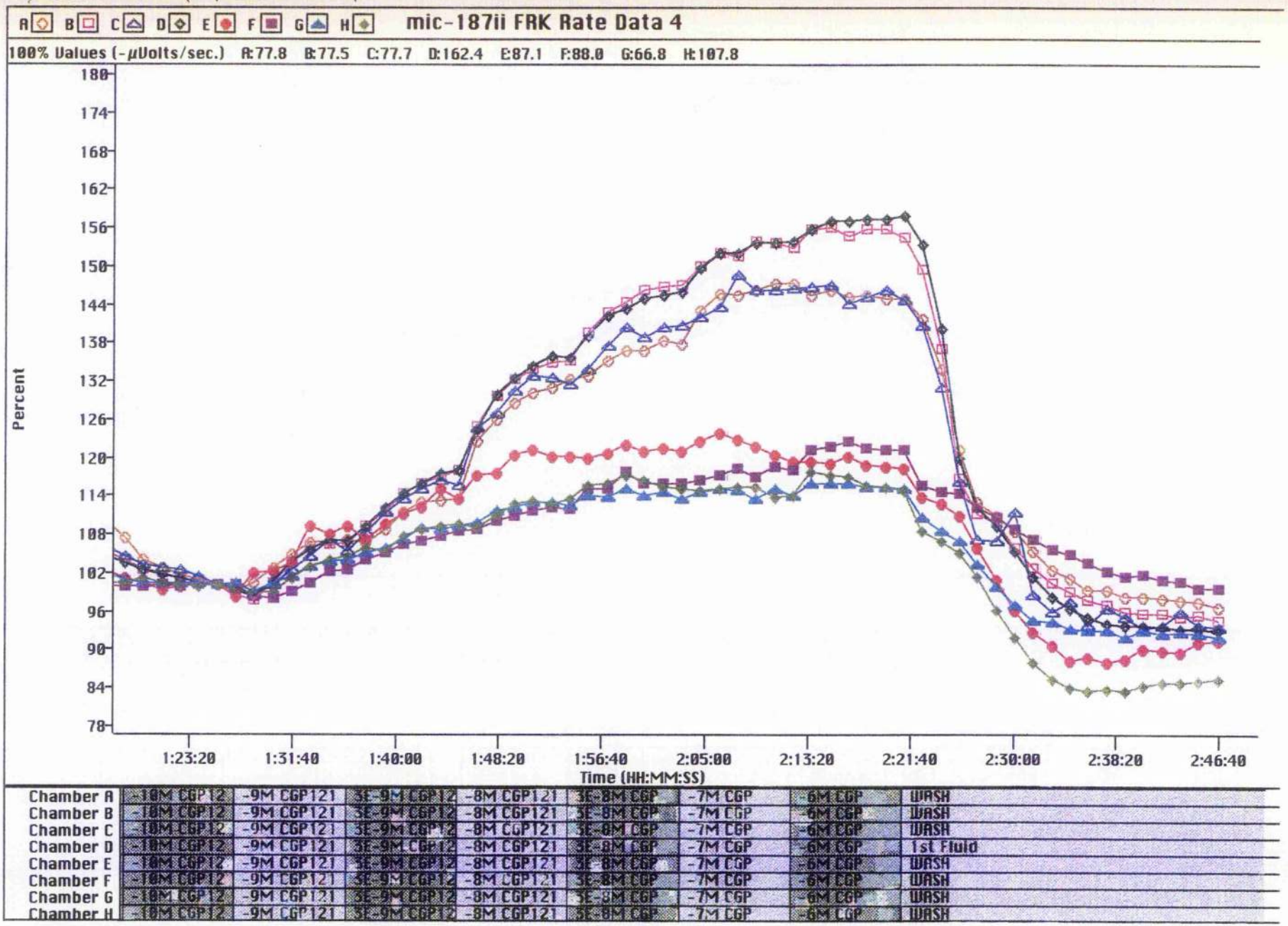

A B C D E F G H
mic-187ii FRK Rate Data 4
100% Values (-µVolts/sec.) A:77.8 B:77.5 C:77.7 D:162.4 E:87.1 F:88.0 G:66.8 H:107.8
Percent
180
174
168
162
156
150
144
138
132
126
120
114
108
102
96
90
84
78
1:23:20
1:31:40
1:40:00
1:48:20
1:56:40
2:05:00
2:13:20
2:21:40
2:30:00
2:38:20
2:46:40
Time (HH:MM:SS)
Chamber A -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP WASH
Chamber B -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP WASH
Chamber C -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP WASH
Chamber D -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP 1st Fluid
Chamber E -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP WASH
Chamber F -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP WASH
Chamber G -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP WASH
Chamber H -10M CGP12 -9M CGP121 3E-9M CGP12 -8M CGP121 3E-8M CGP -7M CGP -6M CGP WASH

Figure 5.2.2.a. Concentration-curve to BRL37344 in rat β_3-adrenoceptor expressing CHO cells. Results show plots from one set of cumulative concentrations.

Figure 5.2.2.b. Concentration-curve to isoprenaline in rat β_3-adrenoceptor expressing CHO cells. Results show plots from one set of cumulative concentrations.

Figures 5.2.2.c. Concentration-curve to CGP12177 in rat β_3-adrenoceptor expressing CHO cells. Results show plots from one set of cumulative concentrations.

Figure 5.2.2.a.

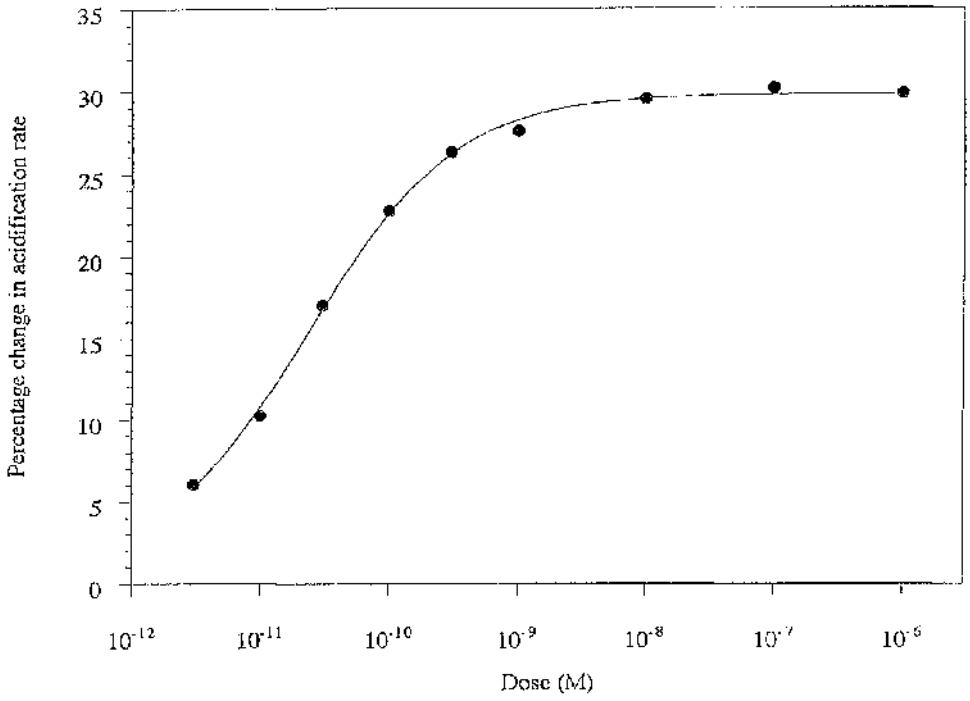


Figure 5.2.2.b.

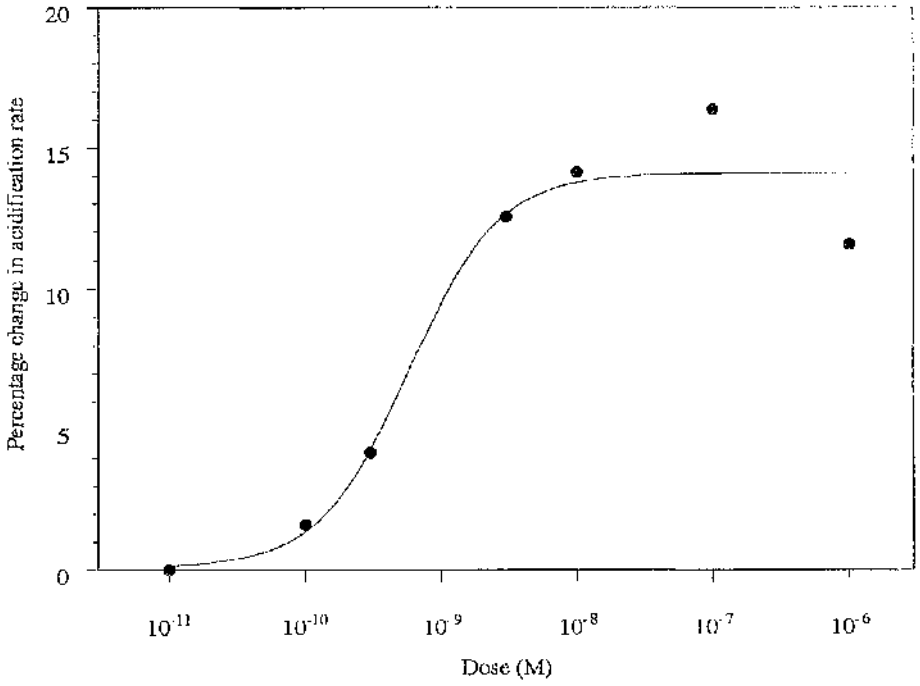


Figure 5.2.2.c.

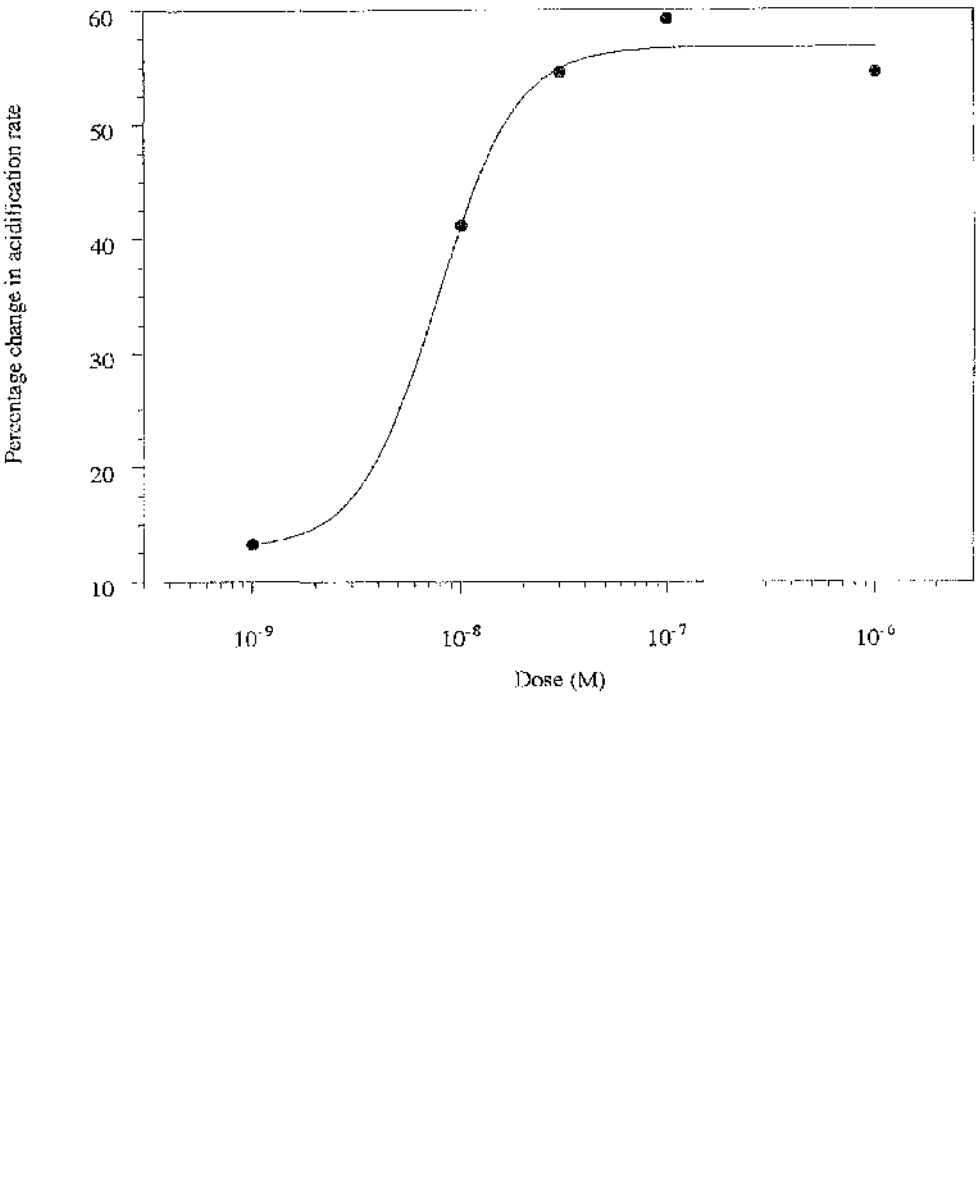

Percentage change in acidification rate
60
50
40
30
20
10
10^{-9}
10^{-8}
10^{-7}
10^{-6}
Dose (M)

Table 5.2.1. EC_{50} values for isoprenaline, BRL37344 and CGP12177 at the human and rat β_3-adrenoceptor transfected CHO cells. Means are calculated from 3-5 microphysiometer experiments, ± S.E.M.

	EC_{50} (nM)	
agonist	human β_3-adrenoceptor	rat β_3-adrenoceptor
isoprenaline	0.65 ± 0.07	1.24 ± 0.07
BRL37344	3.37 ± 1.30	0.02 ± 0.01
CGP12177	2.62 ± 0.74	6.40 ± 0.65

After cumulative concentration-responses were performed, an observation unique to the rat β_3-adrenoceptor CHO cells became apparent. After treatment of the rat β_3-adrenoceptor CHO cells with a maximally stimulating dose of BRL37344, then a washout period, it was noted that a sustained response occurred, in that the increased acidification rates did not return to basal levels, even after 1-2 hours of absence of the agonist. This observation was compared to the human β_3-adrenoceptor CHO cells which were found to return to basal levels of acidification rate immediately after BRL37344 was removed from medium. The sustained response was only seen in rat β_3-adrenoceptor responses to BRL37344, not upon removal of the other two agonists isoprenaline and CGP12177. Figure 5.2.3 shows the rate data from a microphysiometer trace, comparing the response to BRL37344 in the rat β_3-adrenoceptor and the human β_3-adrenoceptor.

To follow up the intriguing sustained response to BRL37344 found only in the rat β_3-adrenoceptor transfected CHO cells, experiments were performed to confirm that this was truly a β_3-adrenoceptor-specific occurrence, and that BRL37344 was not somehow activating the cells via another mechanism. It could possibly be a reflection of the higher affinity of BRL37344 for the rat versus the human β_3-adrenoceptor.

Rat and human β_3-adrenoceptor CHO cells were firstly treated with or without 10 μM propranolol, the β-adrenoceptor antagonist. All cells were then treated with a maximal dose of BRL37344 (0.1 μM and 1 nM in the human and rat β_3-adrenoceptor CHOs, respectively). Propranolol was still present in the medium of those chambers treated with BRL37344 after their pre-treatment with propranolol. Figure 5.2.4.a. shows the rate data for this experiment, resulting in the usual increase in acidification response to BRL37344 only in cells which had not received the antagonist. All cells treated with the antagonist showed

very little or no response to BRL37344 treatment. This proved that this sustained response to BRL37344 could be completely blocked by propranolol and was therefore a receptor specific response. Thus BRL37344 was only affecting the CHO cells' response via the β_3-adrenoceptor and no other mechanism. Using propranolol did not, however, answer the questions into why the sustained response only occurs in the rat β_3-adrenoceptor expressing CHO cells.

Propranolol (100 μM) was also added during the sustained response to BRL37344, in a separate experiment. After treatment with 1 μM BRL37344 for 6 minutes, followed by a washout, 100 μM propranolol was then added for 8 minutes (Figure 5.2.4.b). The effect of the addition of the β-adrenoceptor antagonist was to abolish the sustained response to BRL37344, and to return the acidification rate to basal levels. Upon removal of propranolol, the sustained response immediately returned. This, once again, indicated that the sustained response was a β_3-adrenoceptor specific response, and BRL37344 was not activating the cell by another process.

Figure 5.2.3. Rate data from microphysiometer trace showing the sustained response to 1 μM BRL37344 in the rat β_3-adrenoceptor expressing CHO cells only. Chambers contained the following cells:

A= rat β_3-adrenoceptor expressing CHO cells

B= human β_3-adrenoceptor expressing CHO cells

C= rat β_3-adrenoceptor expressing CHO cells
D= rat β_3-adrenoceptor expressing CHO cells
E= rat β_3-adrenoceptor expressing CHO cells

F= human β_3-adrenoceptor expressing CHO cells
G= human β_3-adrenoceptor expressing CHO cells
H= human β_3-adrenoceptor expressing CHO cells

Chambers C to H were treated with 1 μM BRL37344 for 16 minutes, while chambers A and B were treated for the same time with 1 μM isoprenaline. The sustained response is clearly shown after the washout in chambers C, D and E, which contained rat β_3-adrenoceptor expressing CHO cells.

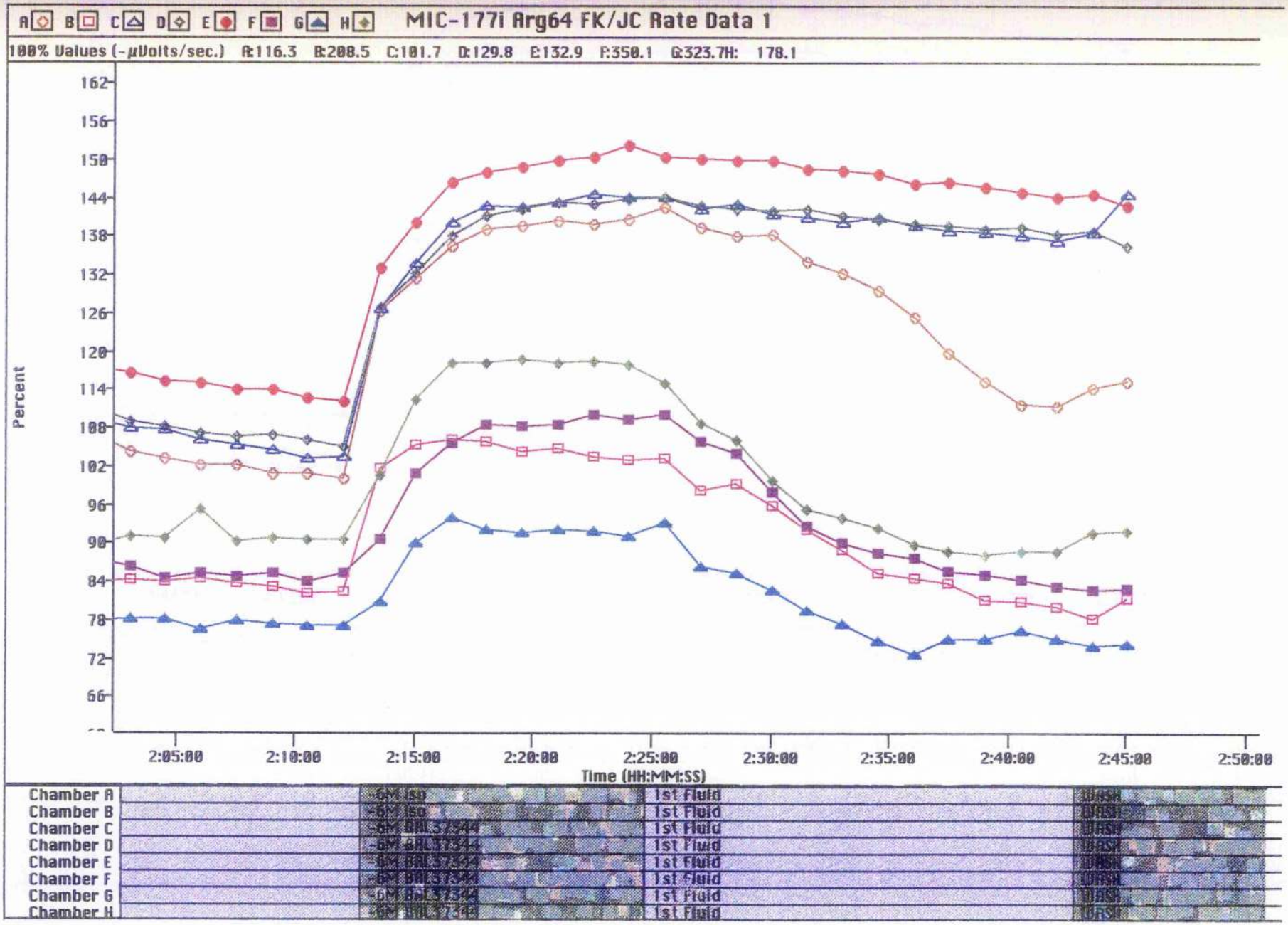

A B C D E F G H
MIC-177i Arg64 FK/JC Rate Data 1
100% Values (-µVolts/sec.) A:116.3 B:208.5 C:101.7 D:129.8 E:132.9 F:350.1 G:323.7H: 178.1
Percent
162
156
150
144
138
132
126
120
114
108
102
96
90
84
78
72
66
2:05:00
2:10:00
2:15:00
2:20:00
2:25:00
2:30:00
2:35:00
2:40:00
2:45:00
2:50:00
Time (HH:MM:SS)
Chamber A
Chamber B
Chamber C
Chamber D
Chamber E
Chamber F
Chamber G
Chamber H
1st Fluid

Figure 5.2.4.a and b. Rate data from microphysiometer showing blockade of the response to BRL37344. Chambers for 5.2.4.a. contained the following cells:

A= rat β_3-adrenoceptor expressing CHO cells

B= rat β_3-adrenoceptor expressing CHO cells

D= rat β_3-adrenoceptor expressing CHO cells

E= human β_3-adrenoceptor expressing CHO cells

F= human β_3-adrenoceptor expressing CHO cells

G= human β_3-adrenoceptor expressing CHO cells

H= human β_3-adrenoceptor expressing CHO cells

The legend below the x-axis shows chambers A, B, E and F with pre-treatments of cells with 10 μM propranolol, followed by the treatment with a maximally stimulating concentration of BRL37344 (1 nM for rat β_3-adrenoceptor expressing CHO cells and 100 nM for human β_3-adrenoceptor expressing CHO cells). Propranolol blocked the response to BRL37344 in human and rat β_3-adrenoceptor expressing CHO cells.

Chambers for Figure 5.2.4.b. contained the following cells:

A= rat β_3-adrenoceptor expressing CHO cells

B= rat β_3-adrenoceptor expressing CHO cells

Cells were treated for 6 minutes with 1 μM BRL37344, followed by washing out of this agonist completely, then approximately 20 minutes later, cells were further treated with 100 μM propranolol for 8 minutes. These treatments are shown on the Figure, underneath the x-axis values.

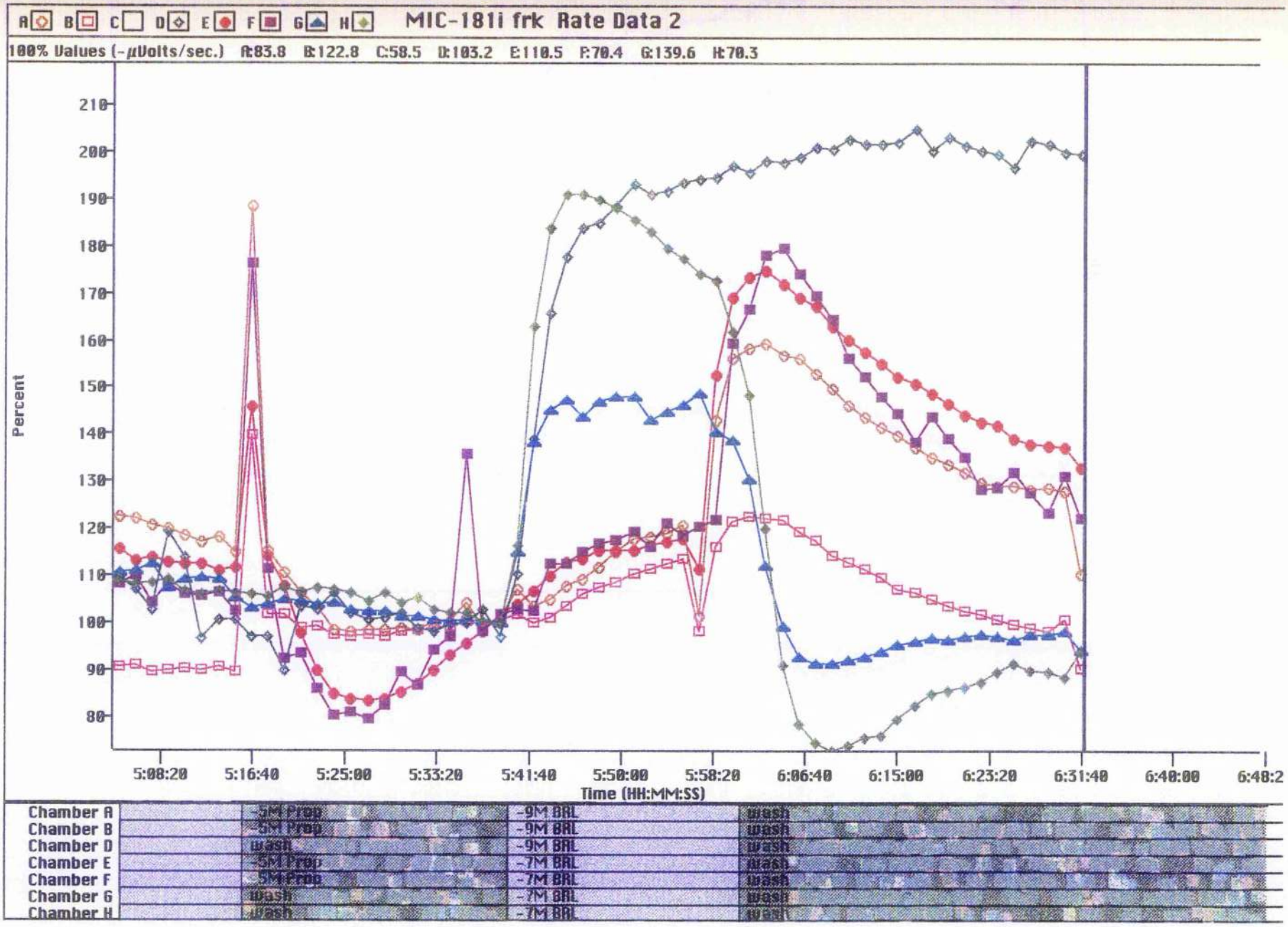
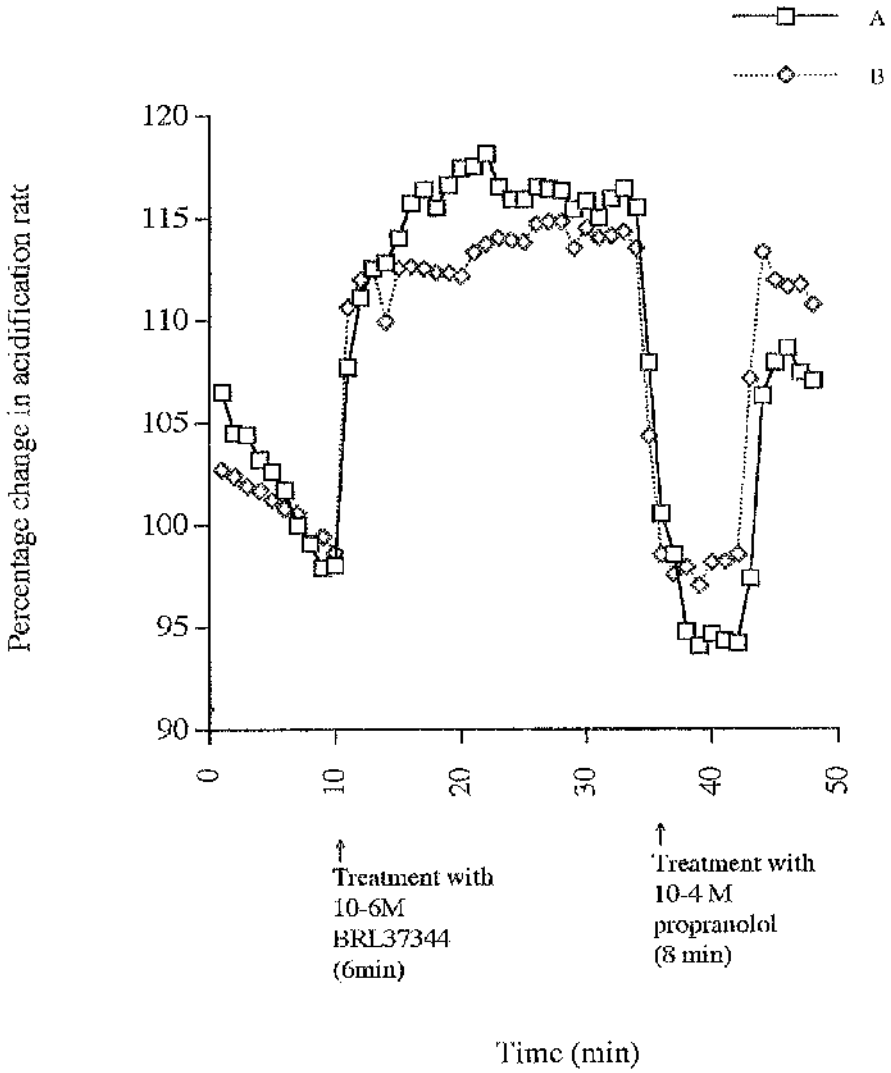

A B C D E F G H
MIC-181i frk Rate Data 2
100% Values (-µVolts/sec.) A:83.8 B:122.8 C:58.5 D:103.2 E:110.5 F:70.4 G:139.6 H:70.3
Percent
210
200
190
180
170
160
150
140
130
120
110
100
90
80
5:08:20
5:16:40
5:25:00
5:33:20
5:41:40
5:50:00
5:58:20
6:06:40
6:15:00
6:23:20
6:31:40
6:40:00
6:48:2
Time (HH:MM:SS)
Chamber A -5M Prop -9M BRL Wash
Chamber B -5M Prop -9M BRL Wash
Chamber D Wash -9M BRL Wash
Chamber E -5M Prop -7M BRL Wash
Chamber F -5M Prop -7M BRL Wash
Chamber G Wash -7M BRL Wash
Chamber H Wash -7M BRL Wash

Figure 5.2.4.b.


A
B
120
115
110
105
100
95
90
Percentage change in acidification rate
0
10
20
30
40
50
Treatment with 10-6M BRL37344 (6min)
Treatment with 10-4 M propranolol (8 min)
Time (min)

To assess the mechanisms of the cell's sustained response, an investigation focusing on the sodium/hydrogen exchanger (NHE) of cells was carried out on the microphysiometer. The profile of an acidification response to receptor activation on the microphysiometer is the summation of the contributions of individual cellular metabolic components that have been stimulated or inhibited by the presence of a ligand. The NHE is the major regulator of intracellular pH in mammalian cells. A ubiquitously expressed NHE subtype is named NHE1 and is stimulated by receptor tyrosine kinases and G-protein-coupled receptors (Hooley *et al*, 1996). It is probable that it is this NHE in the CHO cells that the hormones activate. A role for the G-protein α-subunit, $G\alpha_{13}$ in activation of NHE1 has been observed by Hooley *et al.* (1996). Most of the intracellular signalling pathways mediating receptor regulation of this exchanger, however, are poorly understood. NHE1 activity is stimulated by hormones, cytokines, and growth factors, increasing the rate of H^+ efflux, therefore resulting in an increase in pH_i . It can also be activated by hyperosmotic shock and cell adhesion. Receptor activation of NHE1 is associated with increased phosphorylation of the exchanger on serine residues, suggesting kinase-dependent regulatory mechanisms. The contribution of the NHE to the extracellular response can be determined using agents that directly interact with the exchange protein and inhibit its activity. The most commonly used inhibitors of the NHE are amiloride hydrochloride and its analogues.

Amiloride was added to both the rat and human β_3-adrenoceptor-transfected CHO cells after the treatment and removal of BRL37344, but also during the sustained response to BRL37344 seen in rat β_3-adrenoceptor CHO cells. As Figure 5.2.5. shows, on the immediate addition of a high concentration of amiloride (100 μM) to both rat and human β_3-adrenoceptor-transfected cells, the acidification rate drops back down towards basal levels. This suggests that

the Na^+/H^+ exchange system does play a part in the stimulatory response of the β_3-adrenoceptors recorded by the microphysiometer.

The effect of amiloride on basal levels was then assessed. This is shown in Figure 5.2.6. Amiloride was added after cells had been placed on the microphysiometer for 90 minutes, and its addition also produced a large drop in the acidification rate of the basal level. Upon amiloride removal, after an initial peak as protons are suddenly released from the cell, the rate returns to the original basal level. These results indicate that the Na^+/H^+ exchange is an integral part of the actual basal response recorded by the microphysiometer, and not just part of stimulation by agonists. The degree to which the NHE contributes to the stimulated response varies according to the particular receptor class. In the case of the β_3-adrenoceptors it does seem to contribute part of the response, and implies that this particular subtype of β-adrenoceptor is well-coupled to the NHE system in CHO cells.

Experiments were then performed where rat β_3-adrenoceptor expressing cells were placed on the microphysiometer and some chambers treated with amiloride in the medium from the start of the experiment, and others not treated with amiloride. It was my interest to observe what occurred upon stimulation with isoprenaline. This would assess the contribution of amiloride to the response on the microphysiometer. The resulting isoprenaline response is shown in Figure 5.2.7. The response was actually larger in those cells which have not received treatment at all with 100 μM amiloride, but whether there were slightly more cells in these chambers is difficult to prove. It does however seem that the three chambers left untreated by amiloride all had almost identical percentage changes in acidification (> 50%), and the three chambers treated showed less of a percentage change in acidification (>40% but not >45% change). It is difficult to assess accurately the amount of active cells in each chamber, but there does

seem to be quite a clear difference between the two sets of differently treated cells. This suggests that amiloride does play a role in the responses to agonists on the microphysiometer.

In parallel to these microphysiometer experiments, the sustained response was also studied using the cAMP accumulation 'flashplate' assay. Rat and human β_3-adrenoceptor CHOs were stimulated with 1 μM BRL37344 for 15 minutes, then thoroughly washed out (no phosphodiesterase inhibitor was included in the treatment). Cells were then left to recover from agonist treatment for up to two hours, with reactions being stopped with 10μl of 10% perchloric acid at different time points. Samples were then refrigerated overnight and neutralised the following day. As Figure 5.2.8. shows, cAMP levels continued to increase in the rat β_3-adrenoceptor CHO cells even after washout of BRL37344, but was also seen this time with the human β_3-adrenoceptor CHO cells too. Levels of up to 100 pmol/ml cAMP (per 30 μl sample) were observed after 2 hours in the rat β_3-adrenoceptor CHOs. The maximal time studied in the cAMP assay was 2 hours after washout of BRL37344, and at this time we cannot say whether the response is plateauing or still climbing. Two hours was also the approximate time point in the microphysiometer assay when responses of the rat β_3-adrenoceptor to BRL37344 finally returned to basal levels.

Two slightly differing types of CHO cells were used (due to different sources) for the original production of the stable rat and human β_3-adrenoceptor-transfected lines. The human β_3-adrenoceptor was originally transfected into CHO-K1 $dhfr^+$ cells, while the rat β_3-adrenoceptor had been transfected into CHO-K1 $dhfr^-$ cells. Therefore a pair of transient transfections were set up: the rat β_3-adrenoceptor transfected into CHO-K1 $dhfr^+$ cells and in parallel the human β_3-adrenoceptor into the same CHO cells. These transfected cells were then placed on the microphysiometer and treated with

isoprenaline and then BRL37344. Results, shown in Figure 5.2.9. prove conclusively that the difference in the two original types of CHO cells used can be ruled out for the production of the sustained effect to BRL37344 in the rat β_3-adrenoceptor.

Figure 5.2.5. Effect of addition of 100 μM amiloride on the sustained response to BRL37344. Treatments are shown at the base of the rate data. Chambers contained the following:

A= rat β_3-adrenoceptor expressing CHO cells

B= rat β_3-adrenoceptor expressing CHO cells

C= rat β_3-adrenoceptor expressing CHO cells

D= rat β_3-adrenoceptor expressing CHO cells

E= human β_3-adrenoceptor expressing CHO cells

F= human β_3-adrenoceptor expressing CHO cells

G= human β_3-adrenoceptor expressing CHO cells

H= human β_3-adrenoceptor expressing CHO cells

All chambers were treated with 1 μM BRL37344. Following this treatment 10 μM amiloride was added to chambers A, B, E and F after the BRL37344 had been washed out. This concentration of amiloride had no effect, but on 100 μM additions of amiloride to chambers A and B (containing rat β_3-adrenoceptor expressing CHO cells), the sustained response to BRL37344 was partially abolished. On removal of amiloride, the response returned to the level of the sustained response.

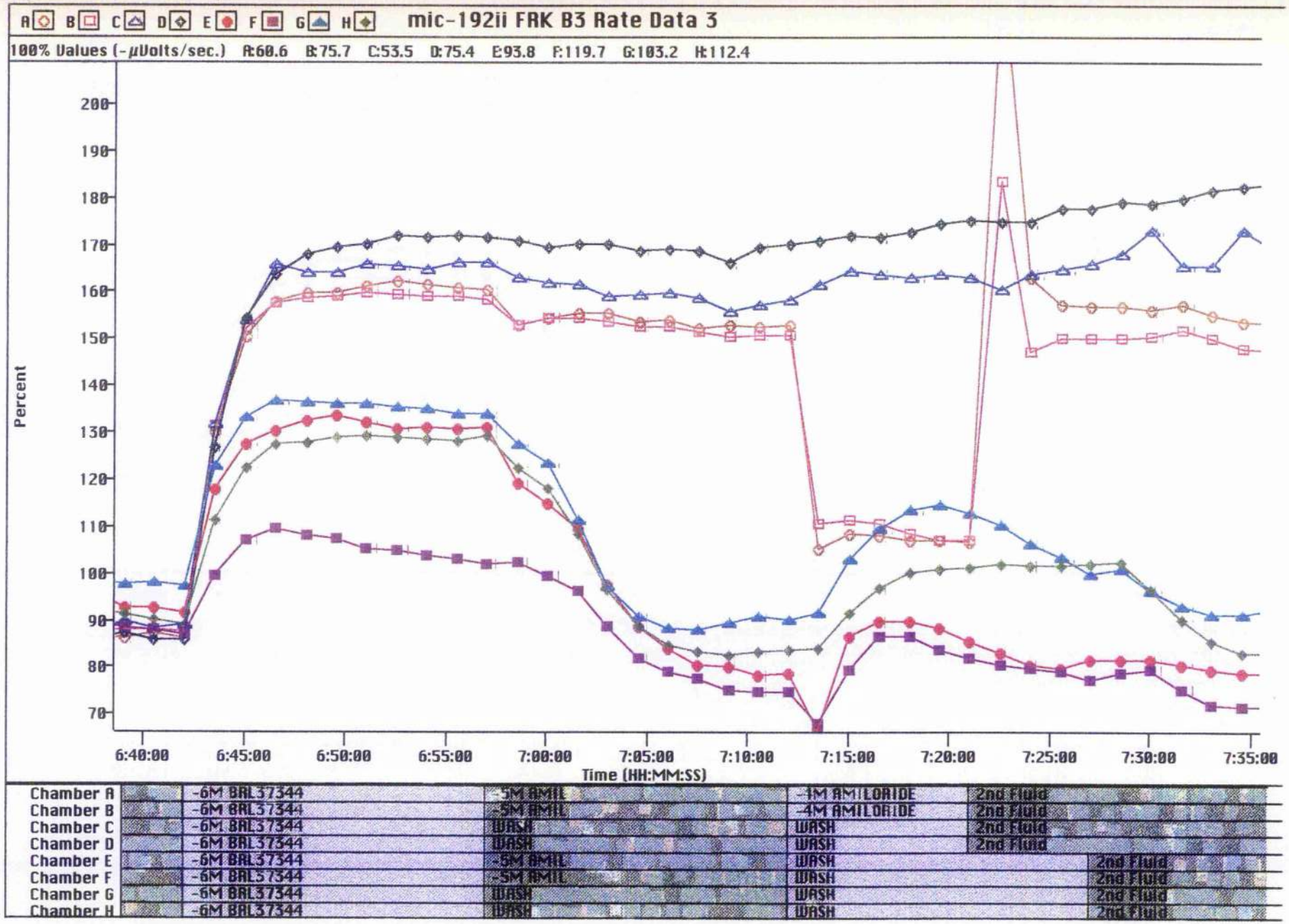

A B C D E F G H
mic-192ii FRK B3 Rate Data 3
100% Values (-µVolts/sec.) A:60.6 B:75.7 C:53.5 D:75.4 E:93.8 F:119.7 G:103.2 H:112.4
Percent
200
190
180
170
160
150
140
130
120
110
100
90
80
70
6:40:00
6:45:00
6:50:00
6:55:00
7:00:00
7:05:00
7:10:00
7:15:00
7:20:00
7:25:00
7:30:00
7:35:00
Time (HH:MM:SS)
Chamber A -6M BRL37344 -5M AMIL -1M AMILORIDE 2nd Fluid
Chamber B -6M BRL37344 -5M AMIL -4M AMILORIDE 2nd Fluid
Chamber C -6M BRL37344 WASH WASH 2nd Fluid
Chamber D -6M BRL37344 WASH WASH 2nd Fluid
Chamber E -6M BRL37344 -6M AMIL WASH 2nd Fluid
Chamber F -6M BRL37344 -5M AMIL WASH 2nd Fluid
Chamber G -6M BRL37344 WASH WASH 2nd Fluid
Chamber H -6M BRL37344 WASH WASH 2nd Fluid

Figure 5.2.6. Effect of amiloride on basal levels in rat and human β_3-adrenoceptor expressing CHO cells. Chambers contained the following:

B= rat β_3-adrenoceptor expressing CHO cells

C= rat β_3-adrenoceptor expressing CHO cells

G= human β_3-adrenoceptor expressing CHO cells

H= human β_3-adrenoceptor expressing CHO cells

On addition of 100 μM amiloride to chambers, the basal reponse was reduced.

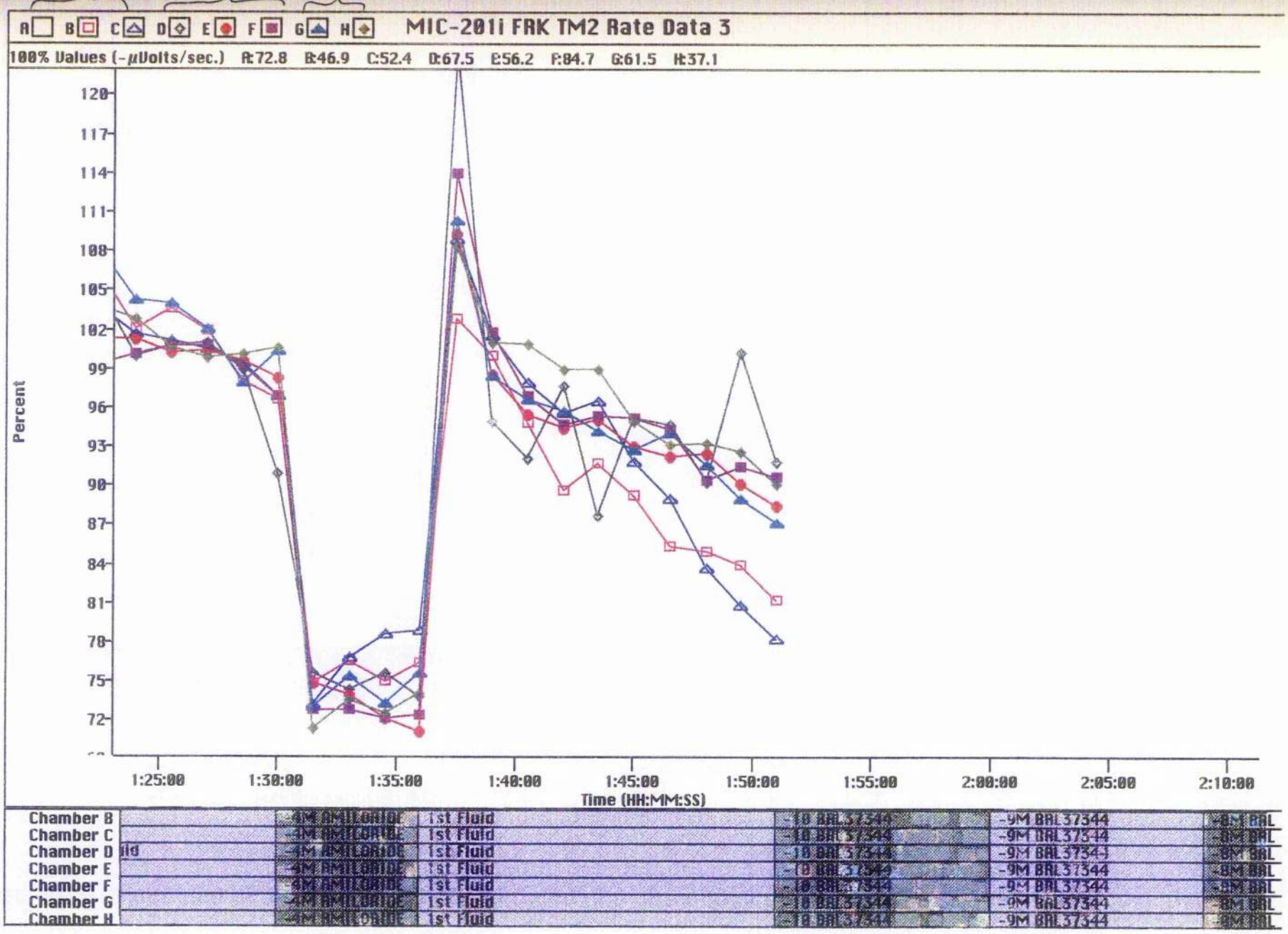

A B C D E F G H
MIC-201i FRK TM2 Rate Data 3
100% Values (-µVolts/sec.) A:72.8 B:46.9 C:52.4 D:67.5 E:56.2 F:84.7 G:61.5 H:37.1
Percent
120
117
114
111
108
105
102
99
96
93
90
87
84
81
78
75
72
1:25:00
1:30:00
1:35:00
1:40:00
1:45:00
1:50:00
1:55:00
2:00:00
2:05:00
2:10:00
Time (HH:MM:SS)
Chamber B
Chamber C
Chamber D
Chamber E
Chamber F
Chamber G
Chamber H
1st Fluid
-9M BAL37344

Figure 5.2.7. Effect of pre-treatment with amiloride (100 μM) on response to isoprenaline treatment in rat β_3-adrenoceptor expressing CHO cells. Chambers contained the following:

A= rat β_3-adrenoceptor exprcssing CHO cells, pre-treatment with 100 μM amiloride.

B= rat β_3-adrenoceptor expressing CHO cells, pre-treatment with 100 μM amiloride

D= rat β_3-adrenoceptor expressing CHO cells, pre-treatment with 100 μM amiloride

E= rat β_3-adrenoceptor expressing CHO cells

F= rat β_3-adrenoceptor expressing CHO cells

H= rat β_3-adrenoceptor expressing CHO cells

Cell pre-treated with 100 μM amiloride (chambers A, B and D) produced less of a percentage change in acidification rate to 1 μM isoprenaline than those chambers not pre-treated with amiloride.

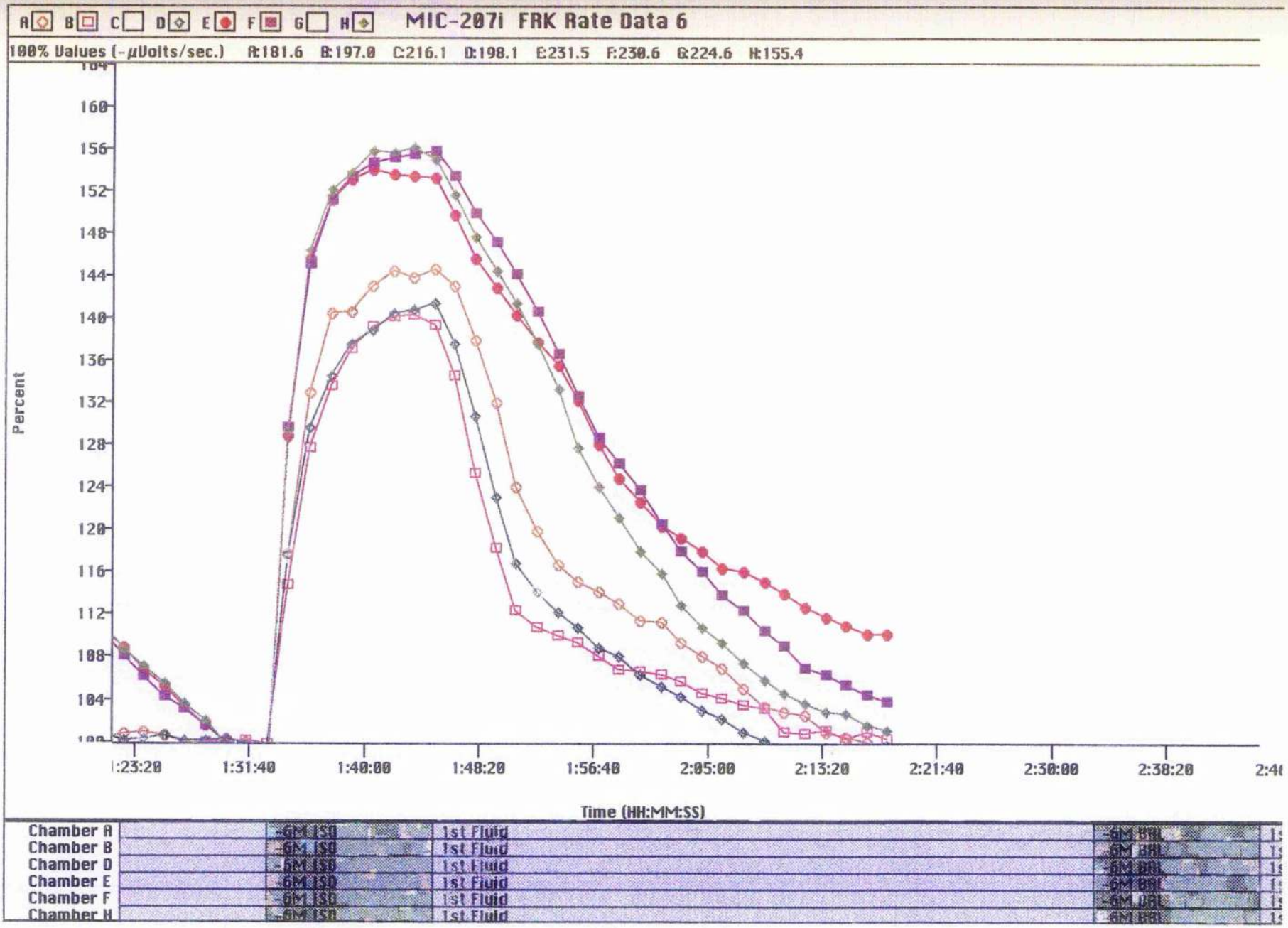

A B C D E F G H
MIC-207i FRK Rate Data 6
100% Values (-µVolts/sec.) A:181.6 B:197.0 C:216.1 D:198.1 E:231.5 F:230.6 G:224.6 H:155.4
Percent
160
156
152
148
144
140
136
132
128
124
120
116
112
108
104
1:23:20
1:31:40
1:40:00
1:48:20
1:56:40
2:05:00
2:13:20
2:21:40
2:30:00
2:38:20
Time (HH:MM:SS)
Chamber A
Chamber B
Chamber D
Chamber E
Chamber F
Chamber H
1st Fluid

Figure 5.2.8. Effect on cAMP accumulation in rat and human β_3-adrenoceptor expressing CHO cells, after pre-treatment with 1 μM BRL37344 for 15 minutes, then removal. Results for C15 (human β_3-adrenoceptor) CHO cells are shown as closed circles, while the rat β_3-adrenoceptor expressing CHO cell results are shown as open squares. The Figure shows the time from 0 minutes when treatment with BRL37344 was begun, then its removal after 16 minutes. Results are the mean + S.E.M. of triplicate samples.

Figure 5.2.8.

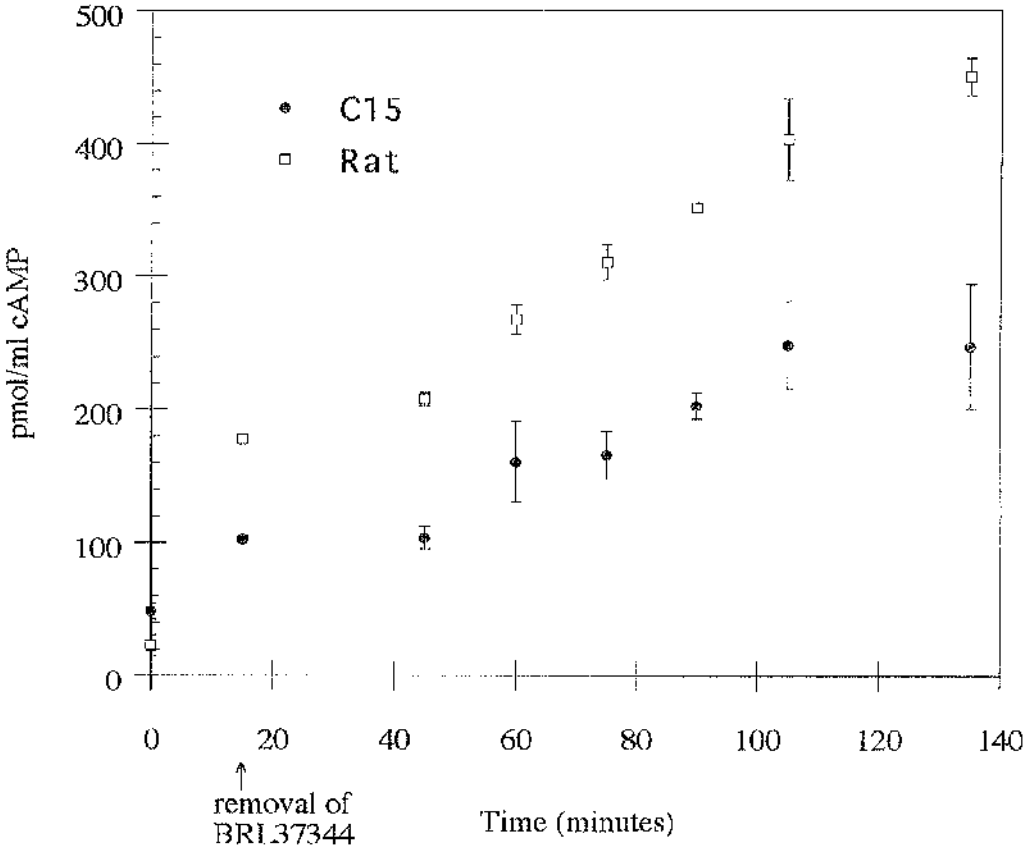

500
400
300
200
100
0
pmol/ml cAMP
C15
Rat
0
20
40
60
80
100
120
140
removal of
BRL37344
Time (minutes)

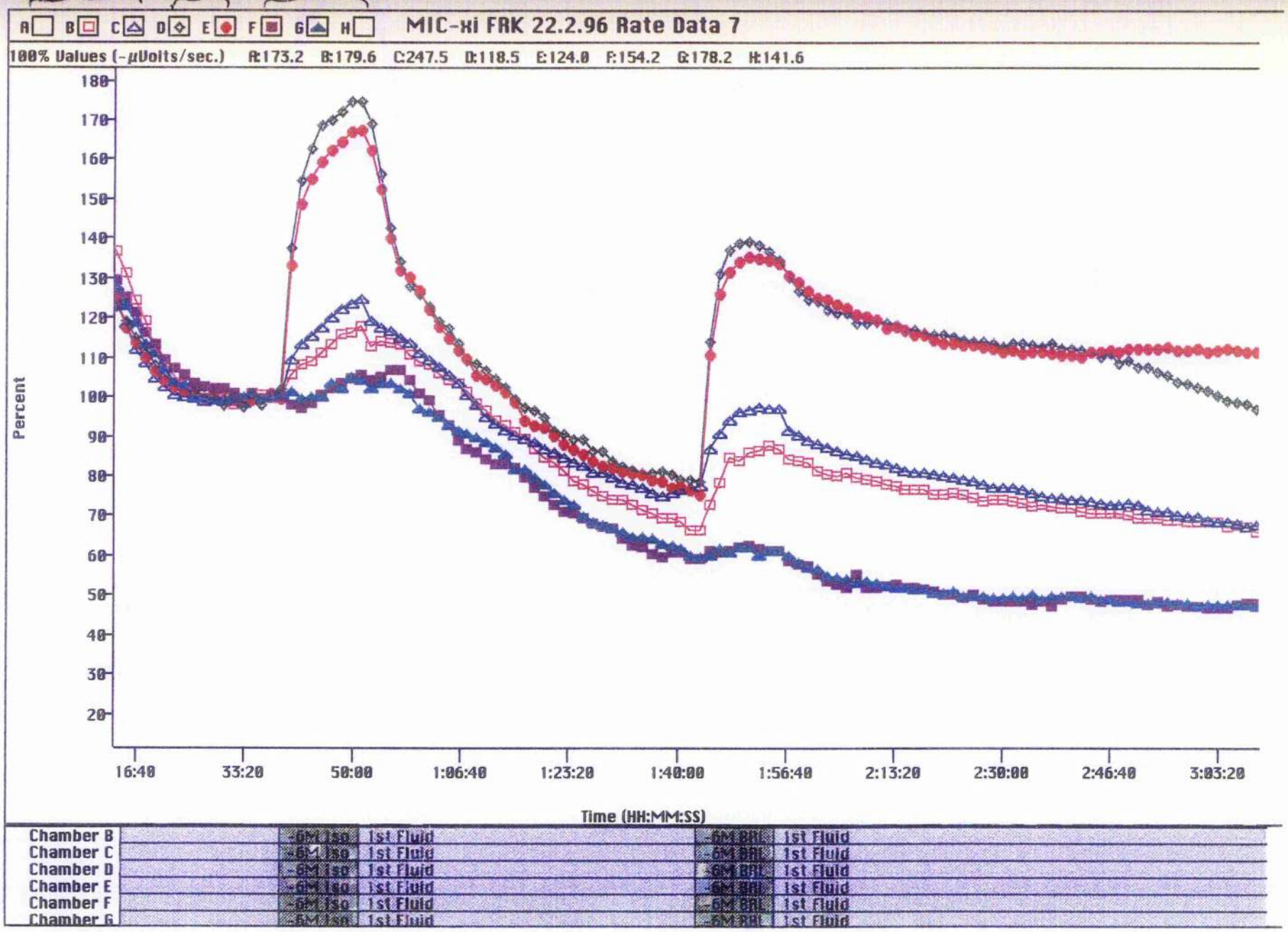
Figure 5.2.9. Effect of isoprenaline and BRL37344 on transient transfections of the rat and human β_3-adrenoceptor into CHO-K1 dhfr$^+$ cells, then compared to stable rat β_3-adrenoceptor expressing CHO cells. Treatments are shown below the x-axis. Chambers contained the following:

B= transient rat β_3-adrenoceptor expressing CHO-K1 dhfr$^+$ cells
C= transient rat β_3-adrenoceptor expressing CHO-K1 dhfr$^+$ cells

D= rat β_3-adrenoceptor expressing CHO cells (stable)
E= rat β_3-adrenoceptor expressing CHO cells (stable)

F= transient human β_3-adrenoceptor expressing CHO-K1 dhfr$^+$ cells
G= transient human β_3-adrenoceptor expressing CHO-K1 dhfr$^+$ cells

After treatment of chambers B to G with 1 μM isoprenaline, responses were seen in all cells. After treatment of chambers B to G with 1 μM BRL37344, transient rat β_3-adrenoceptor expressing CHO-K1 dhfr$^+$ cells produced the same sustained response to BRL37344 as stable rat β_3-adrenoceptor expressing CHO cells.


A B C D E F G H
MIC-xi FRK 22.2.96 Rate Data 7
100% Values (-µVolts/sec.) A:173.2 B:179.6 C:247.5 D:118.5 E:124.0 F:154.2 G:178.2 H:141.6
Percent
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
16:40
33:20
50:00
1:06:40
1:23:20
1:40:00
1:56:40
2:13:20
2:30:00
2:46:40
3:03:20
Time (HH:MM:SS)
Chamber B
Chamber C
Chamber D
Chamber E
Chamber F
Chamber G
1st Fluid

These unique results of the rat β_3-adrenoceptor expressing CHO cells' response to BRL37344 on the microphysiometer were used as a useful indicator in testing the mutants, i.e. a sustained response to BRL37344 would suggest "rat β_3-adrenoceptor-like" pharmacology over "human β_3-adrenoceptor-like" pharmacology.

Mutants were placed on the microphysiometer and treated with 1 μM isoprenaline and 1 μM BRL37344 for 12-15 minutes each. At this stage of the study all samples were produced from transient tranfections, not mass cultures which would be used later. Figure 5.2.10. shows the results of mutants TM6, TM7, TM1+7 and Trp64Arg. These mutants all showed typical human "β_3-adrenoceptor-like" pharmacology on the microphysiometer, in that responses to BRL37344 returned to normal basal values after BRL37344 was removed. Responses observed were approximately a 20% change in acidification rates.

TM1, TM2, TM4 and TM5 mutants were placed on the microphysiometer and stimulated with 1 μM isoprenaline and 1 μM BRL37344. These are shown in the rate data traces of Figure 5.2.11. Responses were approximately 20% changes in the acidification rate above basal levels, upon normalization of the data. TM1, TM4 and TM5 chimeras, like the first four mutants tested, showed the typical "human β_3-adrenoceptor-like" pharmacology, that is, return to basal levels immediately after washout of BRL37344 However, when the TM2 mutant was stimulated with 1 μM BRL37344, upon removal of the agonist it showed signs of a slower return to basal than any other mutant.

For further confirmation of this 'rat β_3-adrenoceptor-like' behaviour, this TM2 mutant was then placed alongside the wild-type rat and human β_3-adrenoceptors on the microphysiometer. The results of this are shown in Figure 5.2.12.

When studied in direct comparison to the rat and human β_3-adrenoceptor CHOs, the TM2 mutant clearly showed the sustained response observed with the rat β_3-adrenoceptor. Its pharmacology suggested a much closer similarity to the rat β_3-adrenoceptor than that of the human β_3-adrenoceptor, of which the TM2 mutant is mainly comprised.

Figure 5.2.10. Responses to isoprenaline (1 μM), BRL37344 (1 μM) and noradrenaline (1 μM) in chimeras TM6, TM7, TM 1+7 and Trp64Arg. Treatments are shown below the x-axis and chambers contained the following:

A= TM6 chimera

B= TM6 chimera

C= TM7 chimera

E= TM1+7 chimera

F= TM1+7 chimera

G= Trp64Arg chimera

H= Trp64Arg chimera

All chambers containing chimeric β_3-adrenoceptor expressing CHO cells produced responses to isoprenaline, BRL37344 and noradrenaline similar to those seen in the human β_3-adrenoceptor expressing CHO cells, i.e. no sustained response to BRL37344.

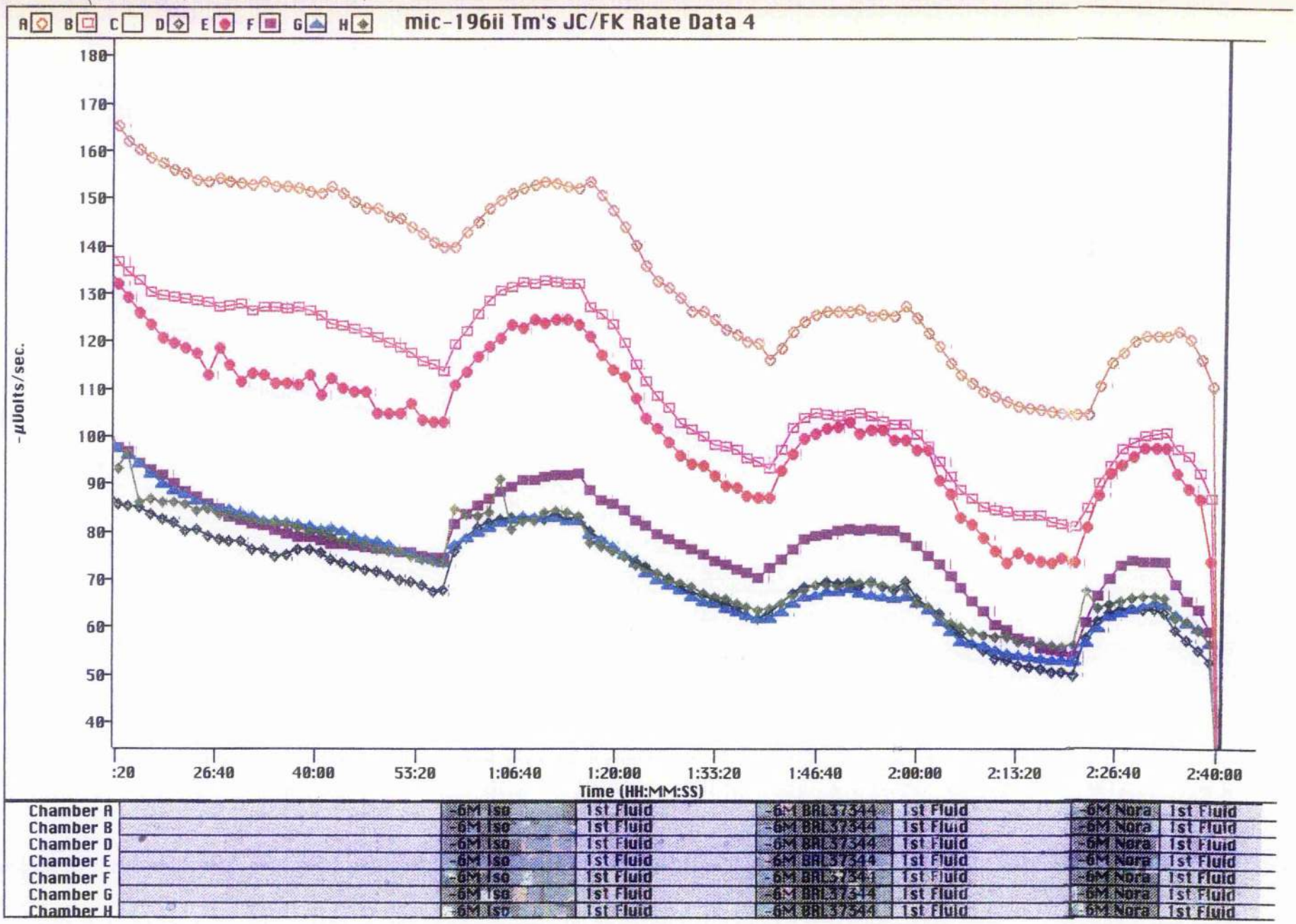

A B C D E F G H
mic-196ii Tm's JC/FK Rate Data 4
-µVolts/sec.
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
:20
26:40
40:00
53:20
1:06:40
1:20:00
1:33:20
1:46:40
2:00:00
2:13:20
2:26:40
2:40:00
Time (HH:MM:SS)
Chamber A -6M Iso 1st Fluid -6M BRL37344 1st Fluid -6M Nora 1st Fluid
Chamber B -6M Iso 1st Fluid -6M BRL37344 1st Fluid -6M Nora 1st Fluid
Chamber D -6M Iso 1st Fluid -6M BRL37344 1st Fluid -6M Nora 1st Fluid
Chamber E -6M Iso 1st Fluid -6M BRL37344 1st Fluid -6M Nora 1st Fluid
Chamber F -6M Iso 1st Fluid -6M BRL37344 1st Fluid -6M Nora 1st Fluid
Chamber G -6M Iso 1st Fluid -6M BRL37344 1st Fluid -6M Nora 1st Fluid
Chamber H -6M Iso 1st Fluid -6M BRL37344 1st Fluid -6M Nora 1st Fluid

Figure 5.2.11 Responses to isoprenaline (1 μM) and BRL37344 (1 μM) in chimeras TM1, TM2, TM4, and TM5. Treatments are shown below the x-axis and chambers contained the following cells:

A= TM1 chimera

B= TM1 chimera

C= TM2 chimera

D= TM2 chimera

F= TM4 chimera

G= TM5 chimera

H= TM5 chimera

The TM2 chimera showed the sustained response to BRL37344 similar to rat β_3-adrenoceptor expressing CHO cells, while all other chimeras tested here did not.

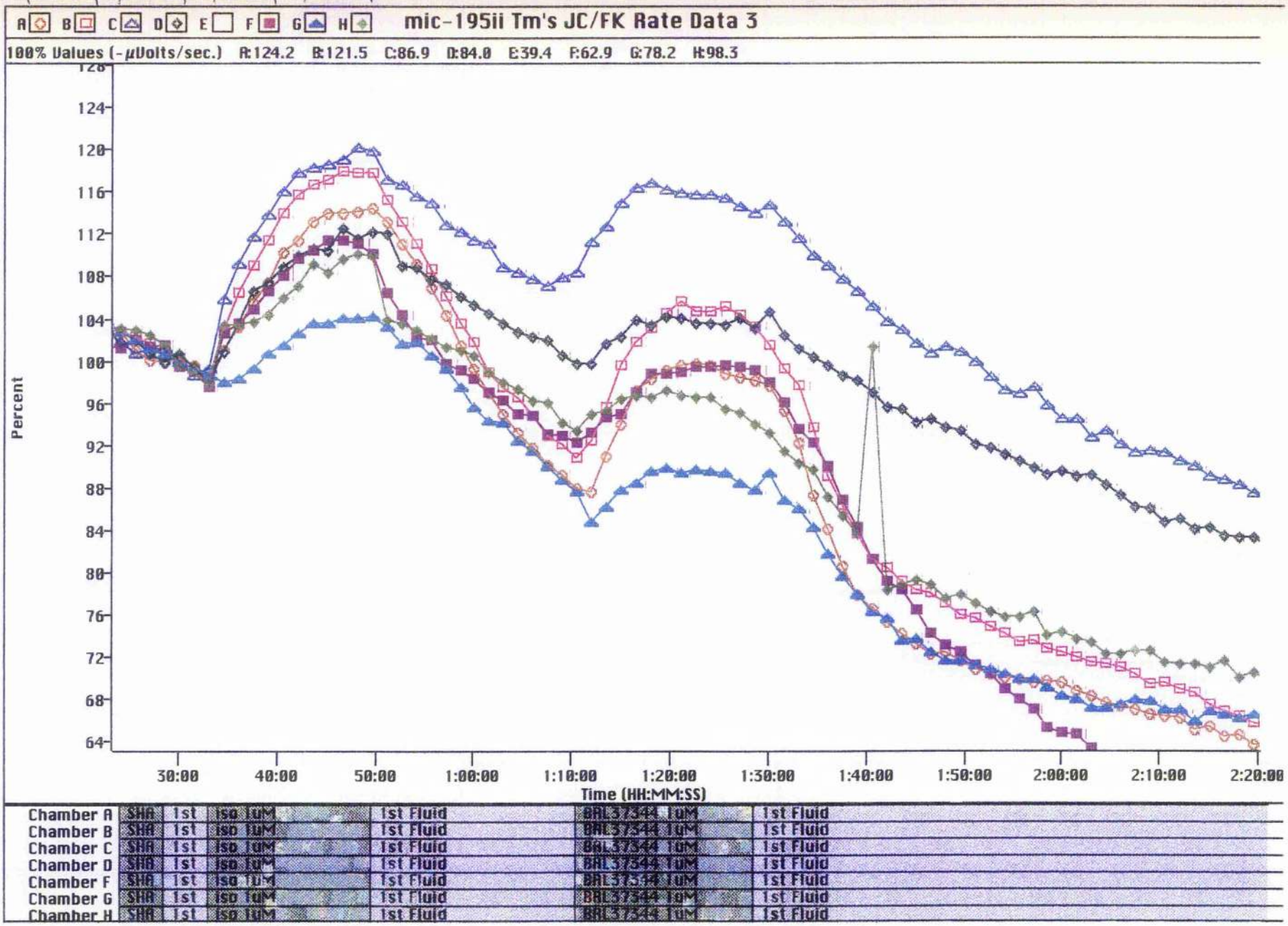

A B C D E F G H
mic-195ii Tm's JC/FK Rate Data 3
100% Values (-µVolts/sec.) A:124.2 B:121.5 C:86.9 D:84.0 E:39.4 F:62.9 G:78.2 H:98.3
Percent
124
120
116
112
108
104
100
96
92
88
84
80
76
72
68
64
30:00
40:00
50:00
1:00:00
1:10:00
1:20:00
1:30:00
1:40:00
1:50:00
2:00:00
2:10:00
2:20:00
Time (HH:MM:SS)
Chamber A SHR 1st Iso 1uM 1st Fluid BRL37344 1uM 1st Fluid
Chamber B SHR 1st Iso 1uM 1st Fluid BRL37344 1uM 1st Fluid
Chamber C SHR 1st Iso 1uM 1st Fluid BRL37344 1uM 1st Fluid
Chamber D SHR 1st Iso 1uM 1st Fluid BRL37344 1uM 1st Fluid
Chamber F SHR 1st Iso 1uM 1st Fluid BRL37344 1uM 1st Fluid
Chamber G SHR 1st Iso 1uM 1st Fluid BRL37344 1uM 1st Fluid
Chamber H SHR 1st Iso 1uM 1st Fluid BRL37344 1uM 1st Fluid

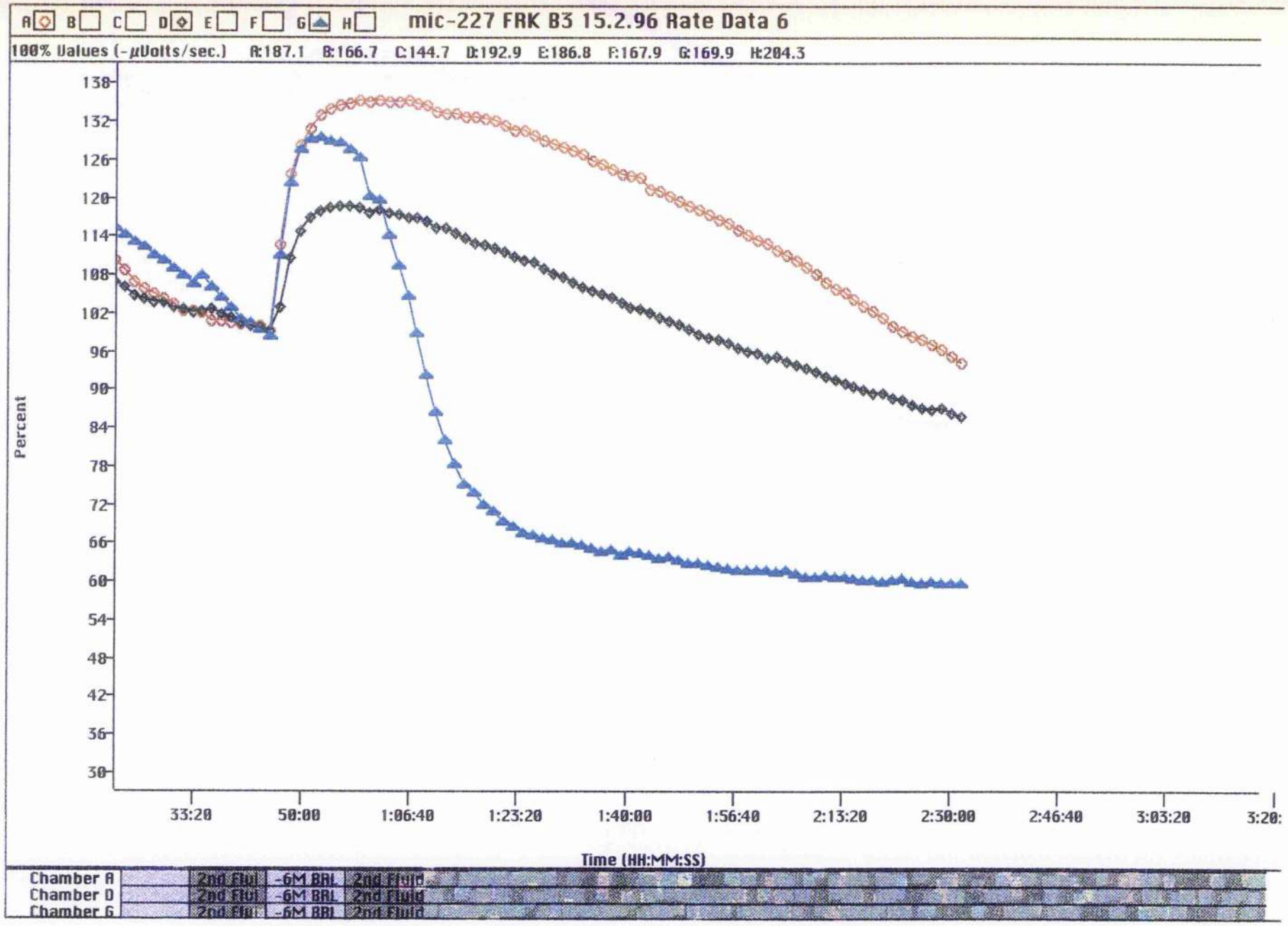
Figure 5.2.12. Responses to 1 μM BRL37344 in rat and human β_3-adrenoceptor expressing CHO cells, in comparison to the TM2 chimera. Treatment times are shown below the x-axis. Chambers contained the following cells:

A= rat β_3-adrenoceptor expressing CHO cells

D= TM2 chimera

G= human β_3-adrenoceptor expressing CHO cells

The rat β_3-adrenoceptor expressing CHO cells and TM2 chimeric β_3-adrenoceptor expressing CHO cells showed similar responses to to 1 μM BRL37344.


A B C D E F G H
mic-227 FRK B3 15.2.96 Rate Data 6
100% Values (-µVolts/sec.) A:187.1 B:166.7 C:144.7 D:192.9 E:186.8 F:167.9 G:169.9 H:204.3
Percent
138
132
126
120
114
108
102
96
90
84
78
72
66
60
54
48
42
36
30
33:20
50:00
1:06:40
1:23:20
1:40:00
1:56:40
2:13:20
2:30:00
2:46:40
3:03:20
3:20:
Time (HH:MM:SS)
Chamber A 2nd Fluid -6M BRL 2nd Fluid
Chamber D 2nd Fluid -6M BRL 2nd Fluid
Chamber G 2nd Fluid -6M BRL 2nd Fluid

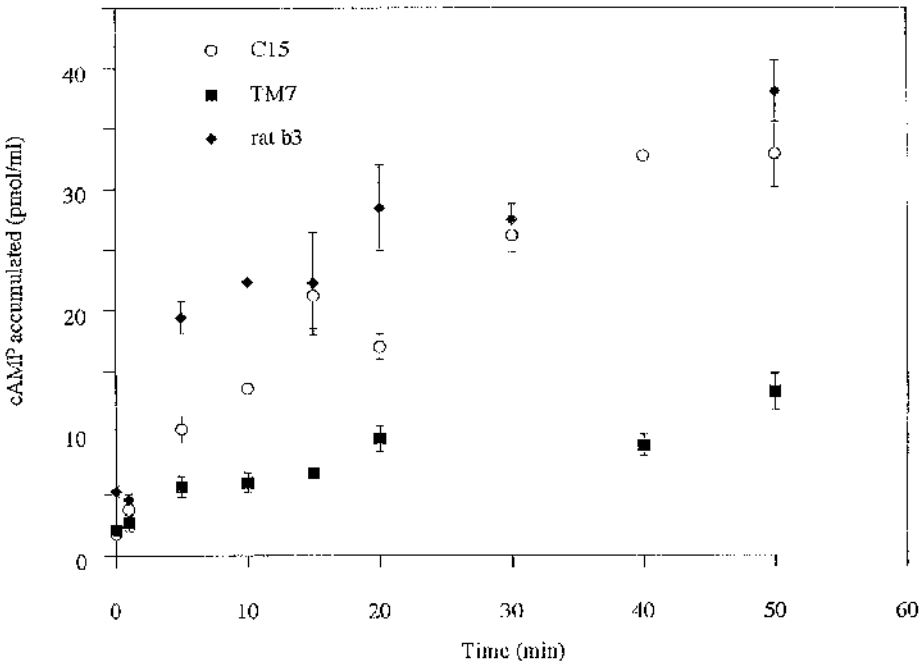
5.3. cAMP ACCUMULATION FLASHPLATE ASSAY RESULTS

Due to the relatively low changes in acidification rate of the CHO cells' responses to isoprenaline and BRL37344, it was proposed to perform full concentration-response curves in the mutant transiently transfected receptor expressing CHO cells using the highly sensitive cAMP flashplate assay, rather than the microphysiometer. The recorded response is also so far downstream on the microphysiometer that other metabolic responses in the cells may be masking results.

All cAMP assays were performed at room temperature, but the appropriate treatment time was investigated to ascertain the time used was on a linear scale. Figure 5.3.1. shows this time course performed by treatment of human β_3-adrenoceptor expressing CHO cells, rat β_3-adrenoceptor CHO expressing CHO cells and TM7 chimera CHO cells with a concentration of 1 μM isoprenaline for different times. The time course proved to be linear at least as far as 40 minutes, so all subsequent experiments involved treatments of cells with agonists for 30 minutes.

Figure 5.3.1. Time course of cAMP accumulation flashplate assay. Treatment of human β_3-adrenoceptor CHOs, rat β_3-adrenoceptor CHOs and TM7 chimera CHOs with 1 μM isoprenaline for different times proved to be linear up to 40 minutes.

Figure 5.3.1.


C15
TM7
rat b3
cAMP accumulated (pmol/ml)
0
10
20
30
40
0
10
20
30
40
50
60
Time (min)

Assays were performed for each individual mutant by treatment with different concentrations of isoprenaline, BRL37344 and CGP12177. As ascertained previously, a treatment time of 30 minutes was used and the assay carried out at room temperature. Concentration-response curves were plotted with cAMP levels shown in pmol/ml cAMP. Figures 5.3.2. to 5.3.11. show concentration-responses for each chimera to isoprenaline, BRL37344 and CGP12177, where the results are expressed as a percentage of the maximal response to isoprenaline. This was due to variation in values between different flashplate kits. Data are the means ± S.E.M of at least 3-5 experiments. These concentration-responses for the chimeric receptors can be compared with those for the human and rat β_3-adrenoceptor CHO cells in Figure.5.3.2. and 5.3.3.

Results from these concentration-response curves were then tabulated, in Table 5.3.1., showing EC_{50} values for each drug at the different chimeras and wild-types. A measure of the efficacy, defined as intrinsic activity (IA) is also plotted in Table 5.3.2. Values are the mean of three or more individual experiments, with S.E.M. shown.

Figure 5.3.2. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in human β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.3. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in rat β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.4 Concentration-responses to isoprenaline, BRL37344 and CGP12177 in TM1 chimera β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.5. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in TM2 chimera β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.6 Concentration-responses to isoprenaline, BRL37344 and CGP12177 in TM4 β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.7. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in TM5 chimera β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.2.

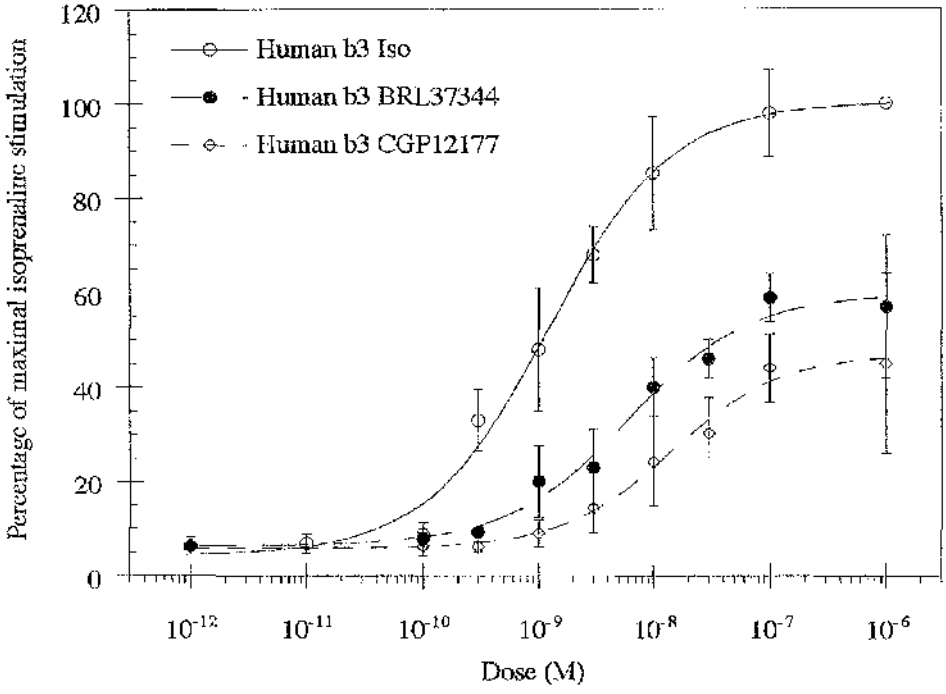


Figure 5.3.3.

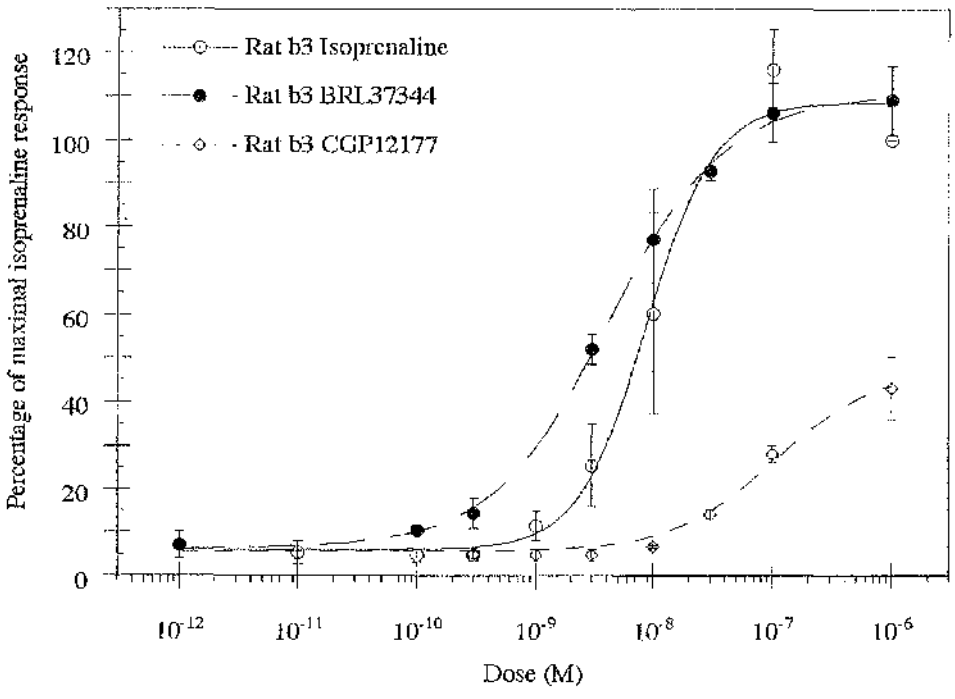


Figure 5.3.4.

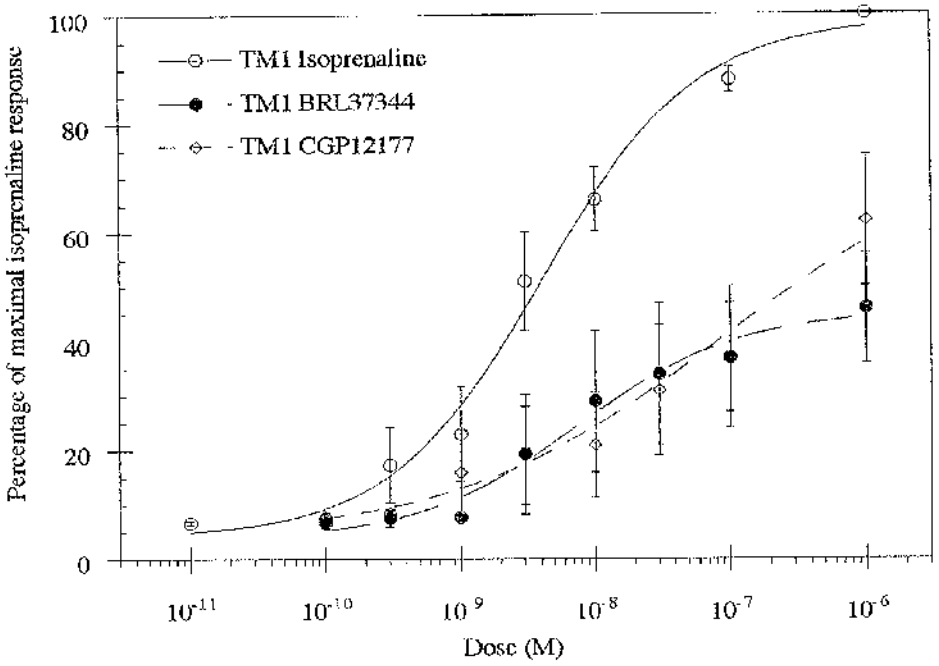


Figure 5.3.5.

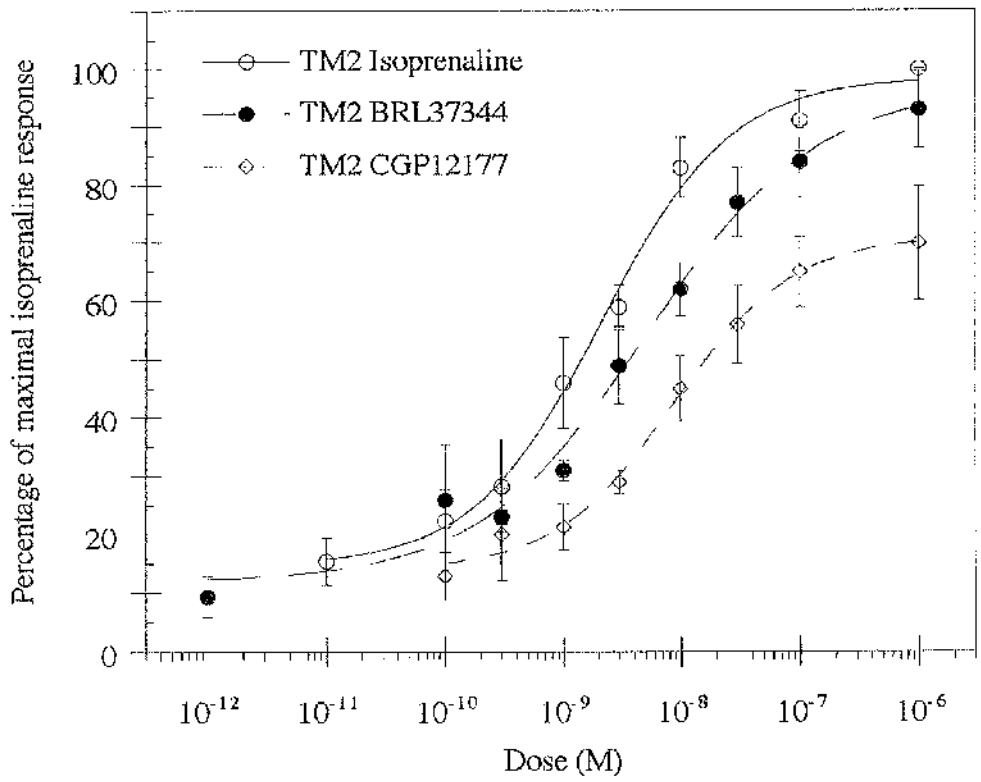


Figure 5.3.6.

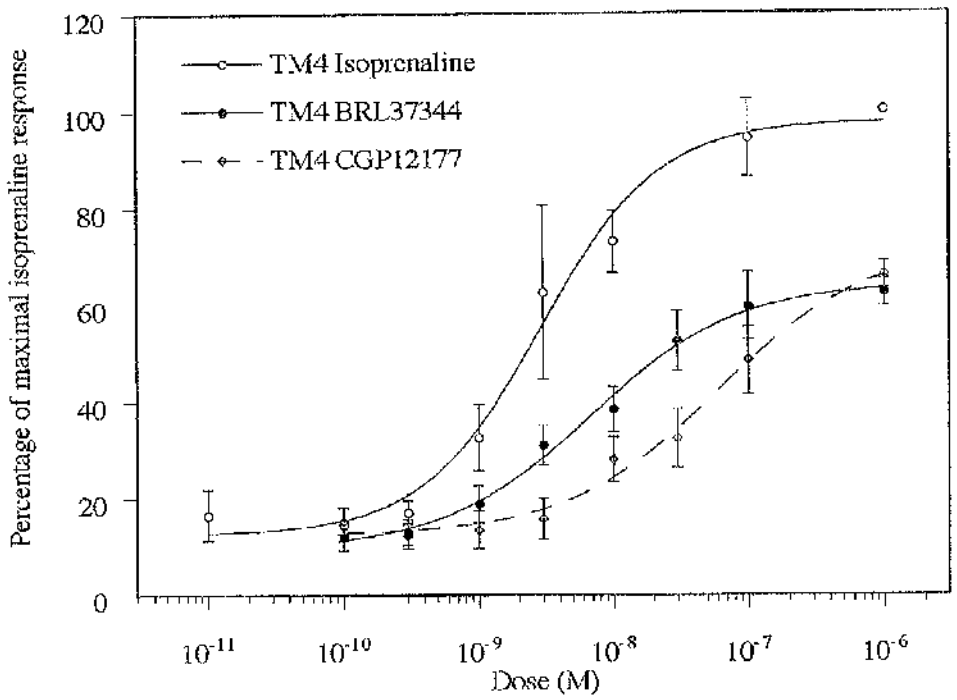


Figure 5.3.7.

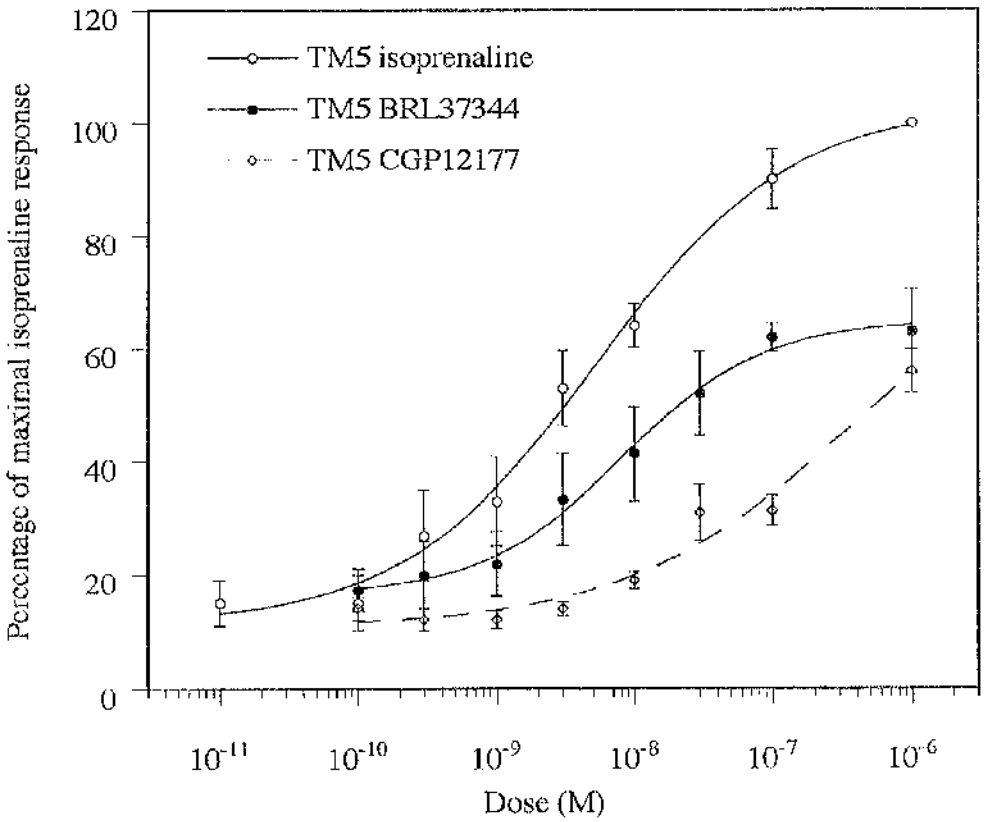


Figure 5.3.8. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in TM6 chimera β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.9. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in TM7 chimera β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.10. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in TM1+7 chimera β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.11. Concentration-responses to isoprenaline, BRL37344 and CGP12177 in Trp64Arg mutant β_3-adrenoceptor transfected CHO cells. Results are expressed as a percentage of the maximal isoprenaline response.

Figure 5.3.8.

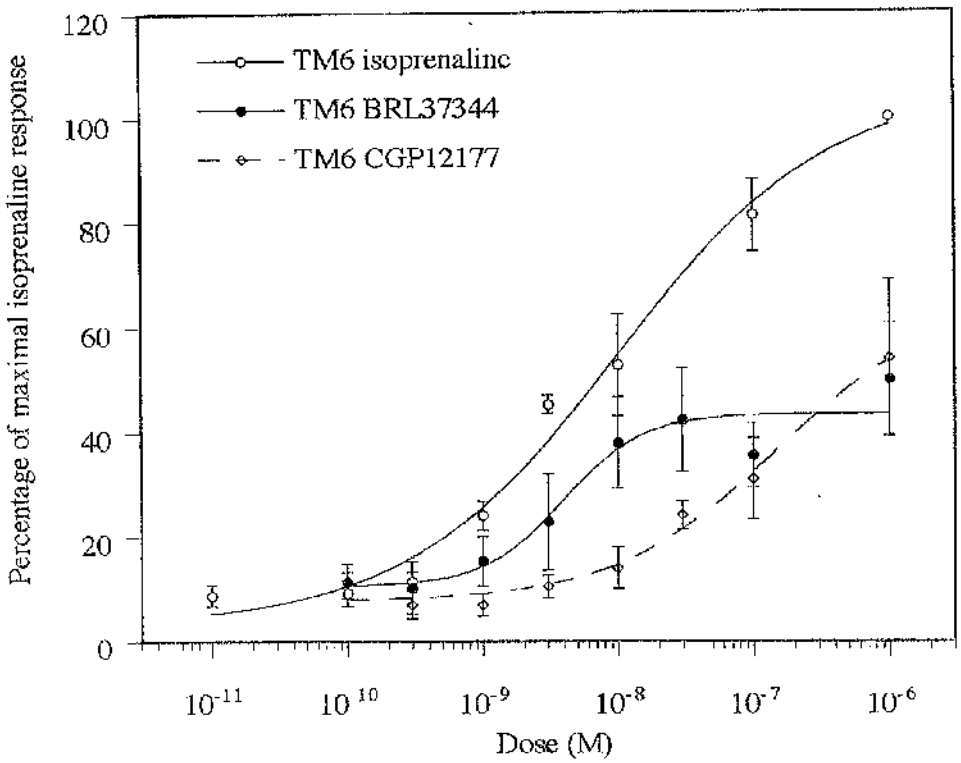


Figure 5.3.9.

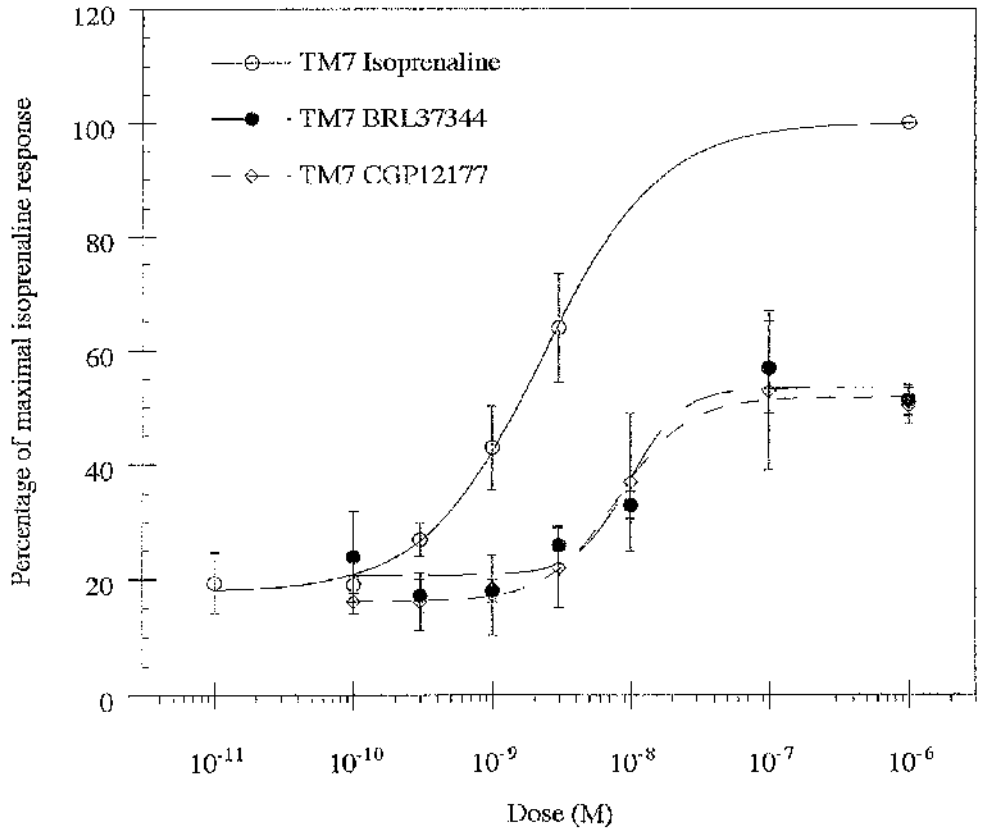


Figure 5.3.10.

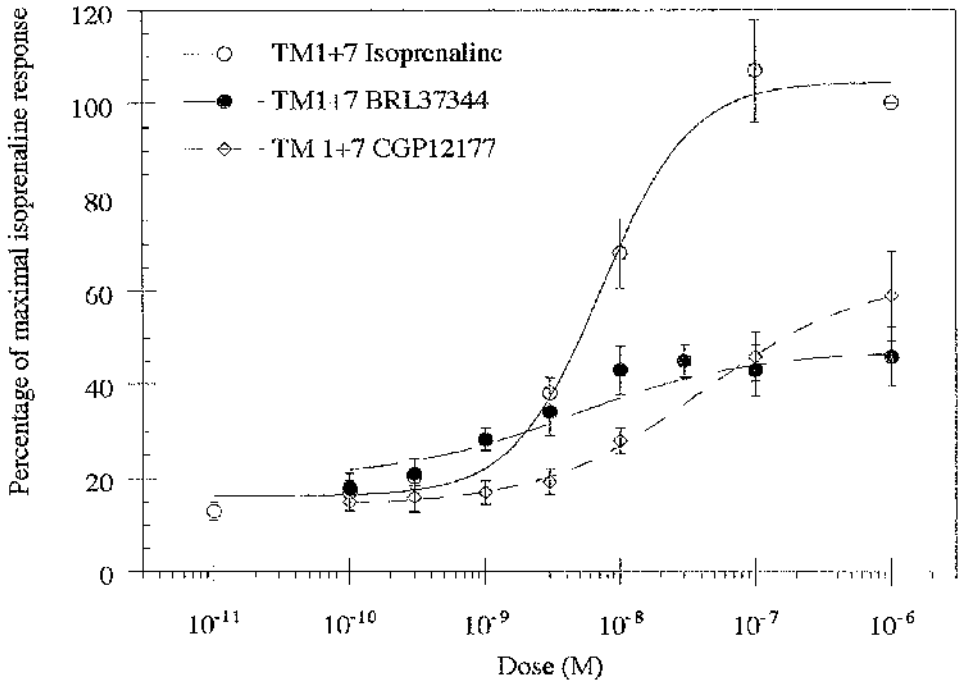


Figure 5.3.11.

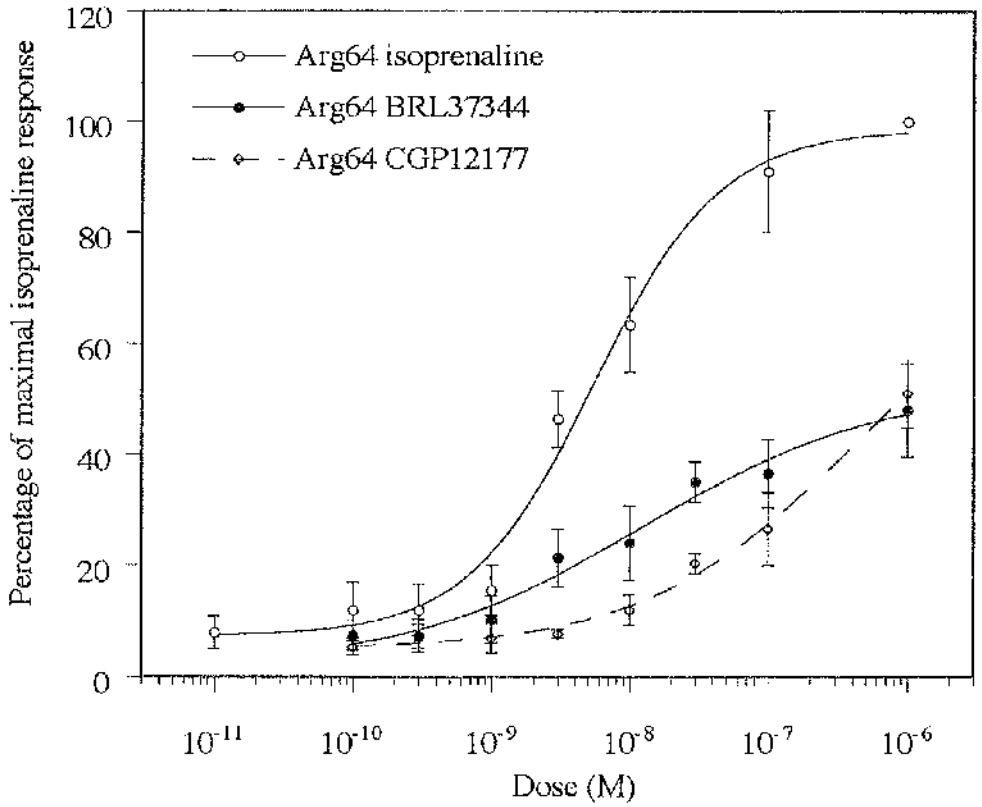


Table 5.3.1. EC_{50} values for isoprenaline, BRL37344 and CGP12177 at the rat, human and chimeric β_3-adrenoceptors. Means are estimated from 3-5 experiments.

	EC_{50} (nM)		
	isoprenaline	**BRL37344**	**CGP12177**
human β_3-adrenoceptor	1.4 ± 0.4*	9.1 ± 3.5	25.0 ± 8.0
rat β_3-adrenoceptor	7.0 ± 0.5	4.4 ± 2.2	82.0 ± 23.0**
TM1 chimera	3.7 ± 1.1	20.6 ± 12.0	213.0 ± 144.0
TM2 chimera	2.4 ± 10.7	6.4 ± 1.6	20.6 ± 4.1
TM4 chimera	2.4 ± 0.8	9.9 ± 3.1	68.0 ± 30.0
TM5 chimera	3.4 ± 1.2	9.9 ± 4.2	614.0 ± 265.0
TM6 chimera	8.3 ± 3.3	22.9 ± 16.0	116.0 ± 32.0
TM7 chimera	7.6 ± 5.3	7.9 ± 4.2	79.0 ± 67.0
TM1+7 chimera	7.33 ± 2.8	2.8 ± 1.2***	35.0 ± 12.0
Trp64Arg mutant	4.90 ± 1.1	31.0 ± 15.0	305.0 ± 153.0

* $p < 0.024$, where isoprenaline is significantly more potent than BRL37344.

** $p < 0.03$, where CGP12177 is significantly more potent in the human than the rat β_3-adrenoceptor.

*** $p < 0.100$ whereBRL37344 is significantly more potent at the TM1+7 chimera than at the human wild type β_3-adrenoceptor.

Table 5.3.2. Intrinsic activity (IA) values ± S.E.M. for isoprenaline, BRL37344 and CGP12177 at the rat, human and chimeric β_3-adrenoceptors. Estimates are made relative to the maximal level of isoprenaline stimulation in each cell line. Means result from at least three experiments.

	Intrinsic activity (IA)		
	isoprenaline	**BRL37344**	**CGP12177**
human β_3-adrenoceptor	1.00	0.49 ± 0.07	0.50 ± 0.10
rat β_3-adrenoceptor	1.00	1.08 ± 0.07*	0.39 ± 0.08
TM1 chimera	1.00	0.41 ± 0.08	0.57 ± 0.11
TM2 chimera	1.00	0.89 ± 0.06**	0.64 ± 0.13
TM4 chimera	1.00	0.59 ± 0.09	0.57 ± 0.18
TM5 chimera	1.00	0.58 ± 0.04	0.50 ± 0.04
TM6 chimera	1.00	0.48 ± 0.10	0.50 ± 0.16
TM7 chimera	1.00	0.40 ± 0.03	0.38 ± 0.07
TM1+7 chimera	1.00	0.38 ± 0.07	0.51 ± 0.11
Trp64Arg mutant	1.00	0.43 ± 0.08	0.47 ± 0.06

* $p < 0.004$ where values are significantly different from the human wild type β_3-adrenoceptor.

** $p < 0.006$ where values are significantly different from the human wild type β_3-adrenoceptor.

Once again, confirming results of the microphysiometer, the TM2 chimera was found to be the sole chimera showing 'rat β_3-adrenoceptor-like' pharmacology over human β_3-adrenoceptor pharmacology. The IA value for BRL37344 at the TM2 chimera was found to be significant when compared to the wild type human β_3-adrenoceptor ($p < 0.006$ using an unpaired Student's t-test). It was much closer to the IA value for the rat β_3-adrenoceptor of 1.08. This efficacy value for the rat β_3-adrenoceptor is significantly different when compared to the human β_3-adrenoceptor IA value for BRL37344 ($p < 0.004$ using an unpaired Student's t-test). BRL37344 was found to be almost a full agonist at the TM2 chimera, as it is at the rat β_3-adrenoceptor, while at all other mutants BRL37344 was only a partial agonist, similar to the human β_3-adrenoceptor. The compound CGP12177 was only a partial agonist at all chimeras and the rat and human β_3-adrenoceptor.

EC_{50} values for the chimeras were similar to that of the human β_3-adrenoceptor, with isoprenaline being the most potent, then BRL37344 and much less potent at the β_3-adrenoceptor, CGP12177. Isoprenaline was observed to be significantly more potent than BRL37344 at the human receptor ($p < 0.024$), and a tendency for this order of potency to be reversed was observed in the rat wild type receptor, although this did not reach a significant level. It was further observed that for the TM1+7 chimera the potency of BRL37344 was noticeably higher than isoprenaline (although this difference did not reach a statistically significant level), and furthermore, the potency of these two compounds was very similar in the TM7 chimera. It was also observed that the potency of BRL37344 was significantly lower than isoprenaline at the wild type human β_3-adrenoceptor ($p < 0.024$), the data indicates a clear trend to a rat β_3-adrenoceptor-like pharmacology in the TM7 and TM1+7 chimeras, which is especially clear in the TM1+7 mutant, but less so in the TM7 chimera alone. This conclusion is further supported by the fact that BRL37344 was

significantly more potent at the TM1+7 chimeric receptor than at the human wild type ($p < 0.1$), despite the fact that there was no significant difference in the receptor density between these two cell lines ($p < 0.46$).

5.4. ASSESSMENT OF RECEPTOR DENSITY

As all these mutants were produced from either transient transfections or mass cultures developed from transient transfections, it was important to establish the β_3-adrenoceptor receptor density (B_{max}) for chimeras, as the present position is only a comparison of transiently tranfected cell lines with stable cell lines. Apart from two mutants (TM1 and TM5) which were not developed into mass cultures and were always tested on the microphysiometer and flashplate assays as transients, all other receptor densities could be calculated from membranes of the mass cultures. [^{125}I]cyanopindolol was the radioligand of choice for specific binding to the β_3-adrenoceptors. This radioligand has Kd values for rat and human β_3-adrenoceptors of 1.6 ± 0.2 nM and 1.6 ± 0.7 nM, respectively. Membranes were generated for each chimera mass culture and then placed in the radioligand binding assay, as previously described in Chapter 2. Three increasing concentrations of the radioligand were used to identify B_{max} values, by plotting a saturation curve and using a Grafit program on an IBM PC computer to estimate B_{max} values. These receptor density values (B_{max}) are presented in Table 5.4.1. below. No specific binding was observed to non-transfected cells. It was observed that the B_{max} in all chimera-transfected cell lines was similar to, or significantly lower than that observed in human wild-type β_3-adrenoceptor expressing CHO cells.

It was of some concern that the intrinsic activity values recorded for the TM2 chimera CHO cells were higher than the other mutants and wild type human and rat β_3-adrenoceptor levels due to much higher levels of the β_3-adrenoceptor

being expressed in the chimeric cell lines. This table proves that an artefact due to differential expression is not the case in the TM2 chimera, showing all chimera receptor levels close to the stable wild-type rat and human β_3-adrenoceptor cell lines. I also observed that, despite the significantly lower B_{max} in the human wild type receptor expressing cells compared to the rat wild type receptor expressing cells ($p < 0.017$), shown in Table 5.4.1., CGP12177 was significantly more potent in the former than the latter ($p < 0.03$), indicating a pharmacological difference between these species homologues which is unlikely to be due to an artefact of differential receptor density. The significance level of the lower potency of CGP12177 in the chimera receptor expressing cell lines, as compared to the human wild type receptor expressing cells, has not been calculated as such differences would be impossible to distinguish from artefacts due to the lower receptor density in the chimeric receptor expressing cell lines.

Table 5.4.1. Receptor levels for the mutant β_3-adrenoceptors, rat and human β_3-adrenoceptor in the CHO cells. Significant differences from the human wild-type β_3-adrenoceptor are also shown.

Cells	**Receptor levels** fmol/mg membrane protein	**Significant difference from human wt β_3-adrenoceptor** (p value)
human β_3-adrenoceptor	217 ± 14	-
rat β_3-adrenoceptor	321 ± 35	0.017
TM2 chimera	103 ± 10	0.001
TM4 chimera	130 ± 23	0.012
TM6 chimera	61 ± 14	0.001
TM7 chimera	186 ± 36	Not significant
TM1+7 chimera	181 ± 49	Not significant
Trp64Arg mutant	90 ± 10	0.001

5.5 COMPUTATIONAL CHEMISTRY IMAGES CONSTRUCTED FOR THE RAT AND HUMAN β_3-ADRENOCEPTORS

To relate the physical arrangement of the transmembrane domains within the rat and human β_3-adrenoceptors to my findings, Drs. Brian Clarke and Frank Blaney in the Computational Chemistry Department at SmithKline Beecham kindly produced some images in relation to my work. The images were created using a protein modelling program called "QUANTA" from Molecular Simulations. The coordinates for the 7TM receptors were created using both a bacteriorhodopsin template and a rhodopsin template. Only rhodopsin is coupled to a G-protein. Coordinate building is carried out and a number of images produced. These Figures are shown below.

Figue 5.5.1. shows an image of the rat and human β_3-adrenoceptors (both side on and looking down from the top) with differences in the two structures highlighted in red. A much clearer image of the actual positioning of the altered amino acids are shown in Figure 5.5.2. This image is a top view of the β_3-adrenoceptor model from the extracellular side to the cytoplasmic side. It shows the two binding domains of the β_3-adrenoceptor. The ligand binding pocket could go all the way through the depth of the membrane and its diameter could vary depending on the relative orientations of the transmembrane domains. Ligands vary both in length and in width, and one might expect quite a large difference in the binding of a small, but very potent agonist such as noradrenaline and the more bulky ligands such as iodocyanopindolol, an agonist for the β_3-adrenoceptor but antagonist at both β_1- and β_2-adrenoceptors.

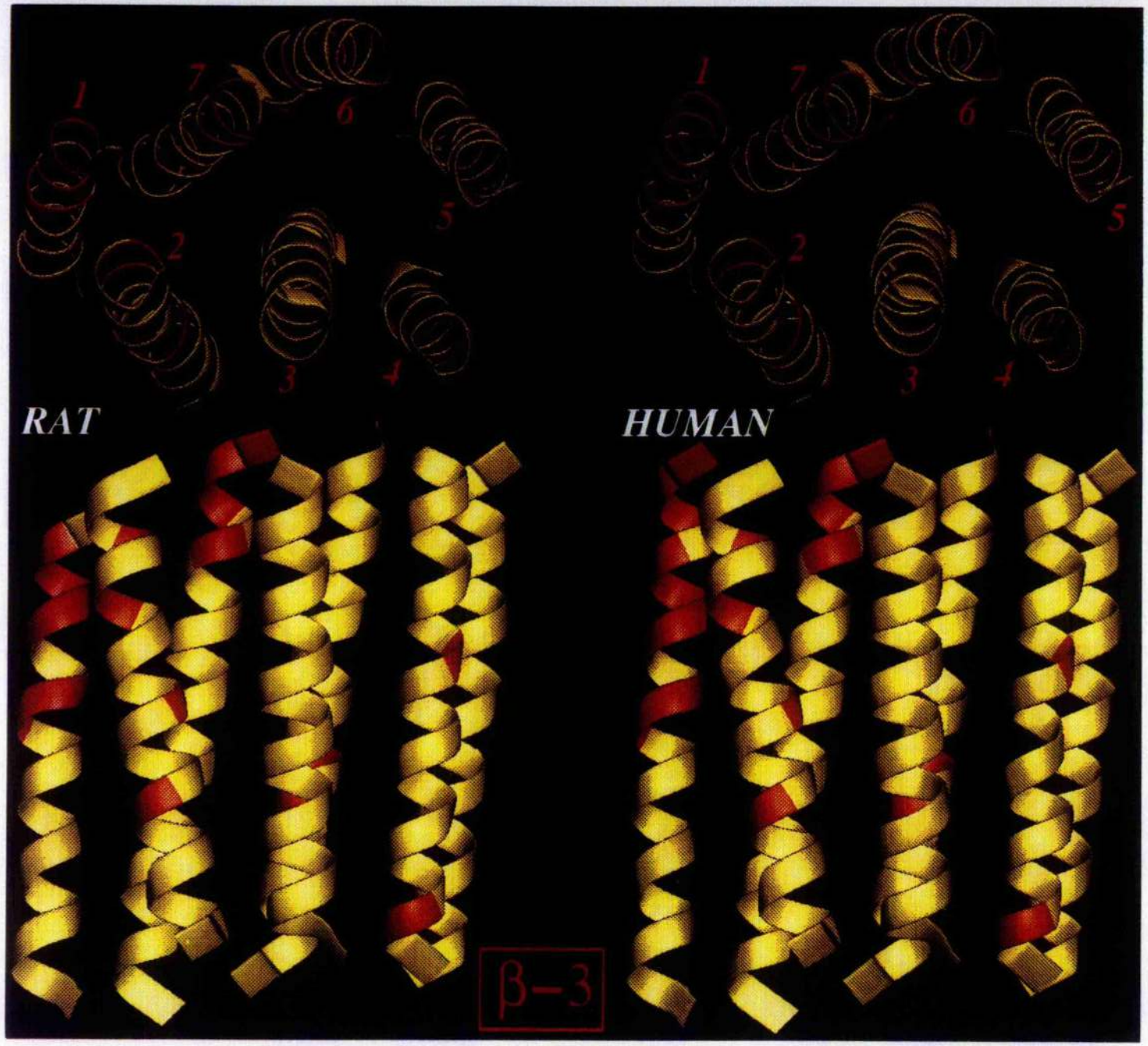
It is known that the first groove consisting of TM3, 4, 5, and 6 is devoted to ligand binding while the second groove (TM1, 2, 3, and 7) is mainly concerned with signal processing. For example noradrenaline binds mainly to serine residues on TM5 and an aspartate residue on TM3. The negative charge provided by aspartic acid (Asp^{113}) neutralizes the positive amino group of noradrenaline. The two serine residues (Ser^{204} and Ser^{207}) in the fifth domain donate hydrogen bonds to the catechol hydroxyl groups of noradrenaline. Figure 5.5.3. shows the side view of the human and rat β_3-adrenoceptor with amino acid changes between the two highlighted in red. As with Figure 5.5.2. it is clear how the amino acids interact between transmembrane regions, and therefore alter the conformation of the whole receptor. Figure 5.5.4. shows the structure of BRL37344, interestingly showing a fully-extended length of 14 Å, while the distance between the two grooves of the β_3-adrenoceptor is at least 20 Å. This suggests that in the case of BRL37344, if it binds in a similar fashion to isoprenaline in the first binding groove, it is unlikely to interact directly with the transmembrane 2 region to cause alterations from "human β_3-adrenoceptor-like" pharmacology to "rat β_3-adrenoceptor-like" pharmacology. These ideas will be elaborated on in the discussion section of this Chapter.

Figue 5.5.1. Image to show the rat and human β_3-adrenoceptor (both side on and observing from the top). Amino acid differences in the two structures are highlighted in red.

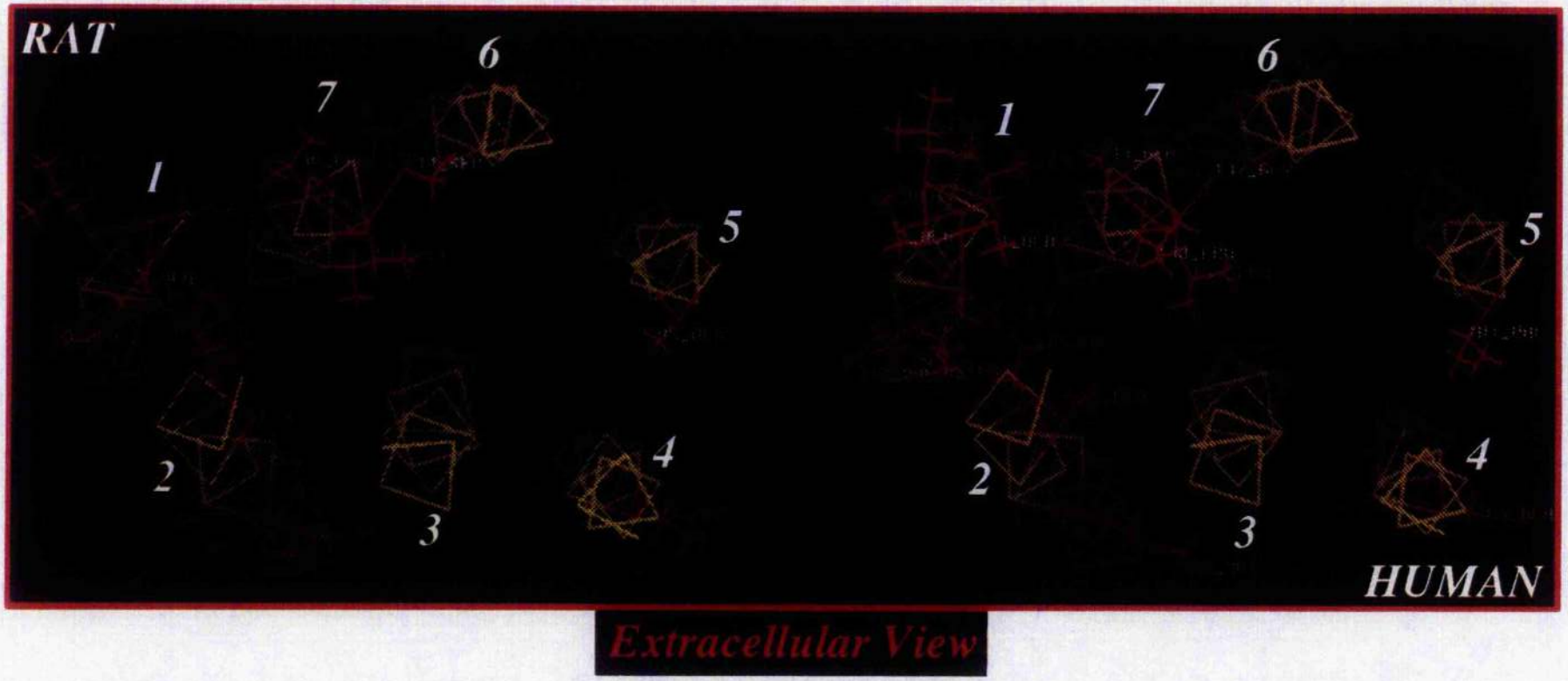
Figure 5.5.2. Image showing the extracellular view of the rat and human β_3-adrenoceptors. Individual amino acid changes are highlighted in red.

Figure 5.5.3. Image showing the side view of the rat and human β_3-adrenoceptor. Amino acid changes between the two are highlighted in red.

Figure 5.5.4. Structure of the β_3-adrenoceptor agonist BRL37344.

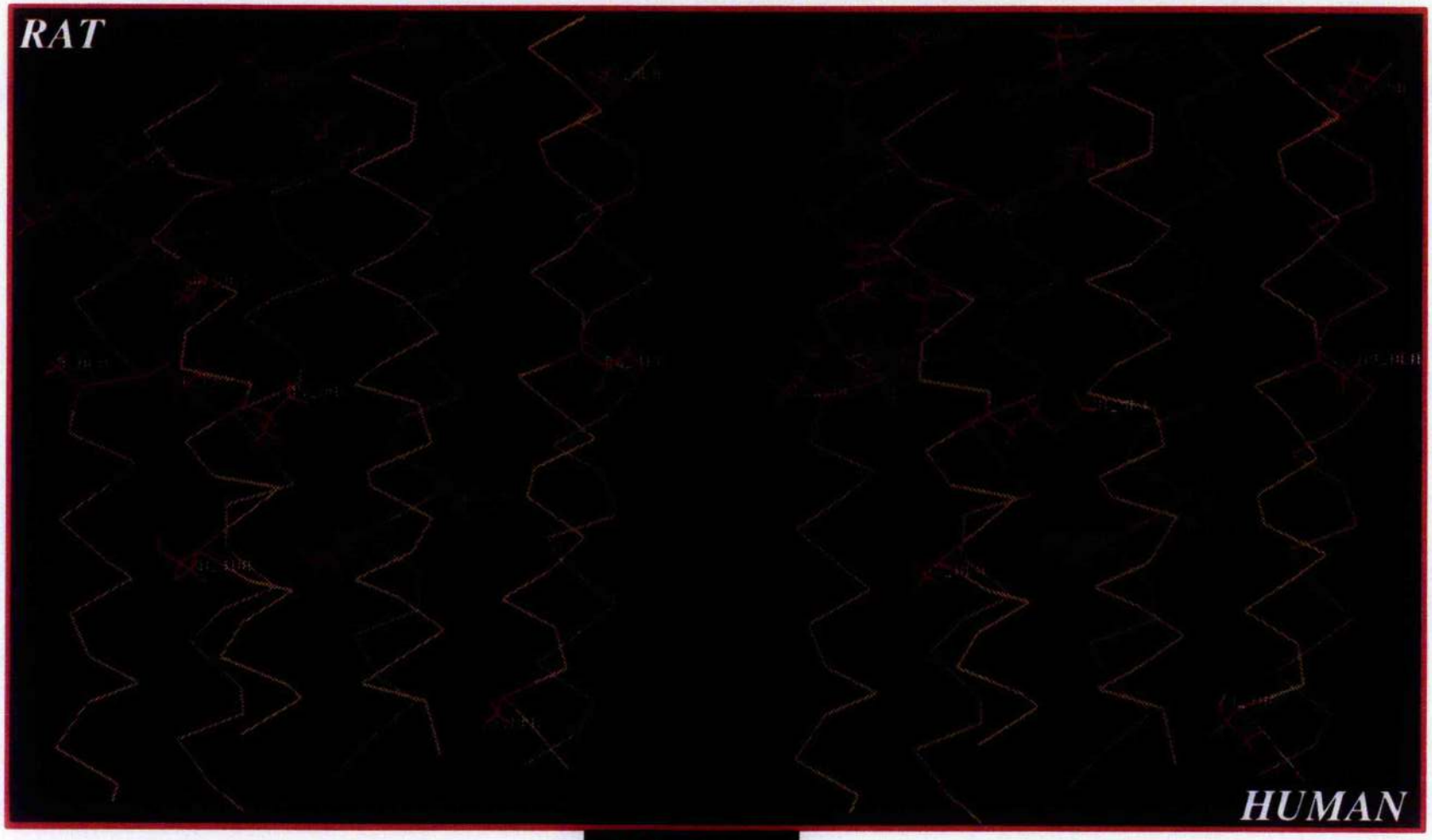

1
7
6
5
2
3
4
RAT
1
7
6
5
2
3
4
HUMAN
β–3

Red indicate residues that are <u>NOT</u> identical



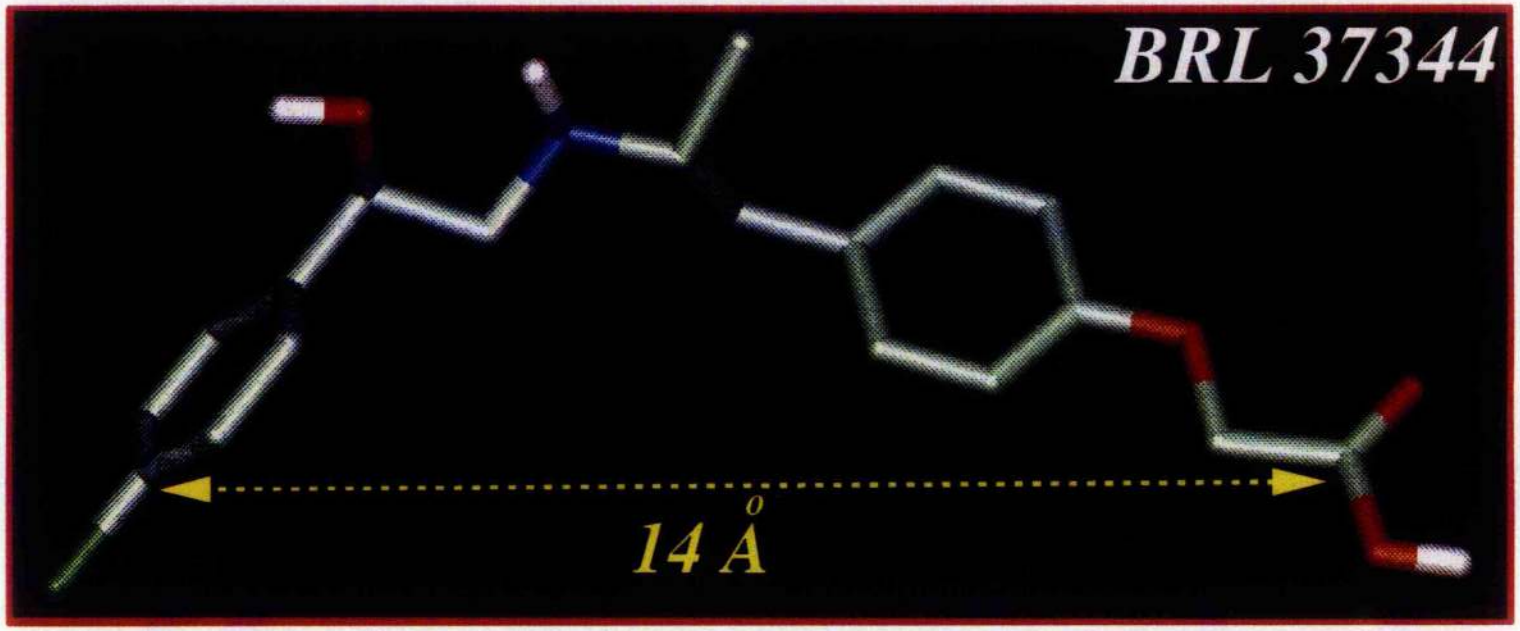
Comparison of Rat & Human Beta–3 Receptors (using a Rhodopsin Template Model)

Red indicate residues that are <u>NOT</u> identical



Side View

Comparison of Rat & Human Beta–3 Receptors (using a Rhodopsin Template Model)


BRL 37344
14 Å

5.6. DISCUSSION

Despite a marked similarity of sequence (some 79% amino acid identity overall, and 90% in the transmembrane regions), the two wild type cloned receptors in the present study, the rat and human β_3-adrenoceptors, have been found to have distinctively different pharmacology (Muzzin *et al*, 1991). This pharmacological difference is most clearly observed as a reversal in the rank order of intrinsic activity of compounds CGP12177 and BRL37344 between the two receptors (Liggett *et al*, 1992). The object of my current study, therefore, was to define more clearly the molecular basis for this difference in pharmacology.

In my study, I deliberately chose to monitor effects of the various mutations by a functional measure of receptor activity, such as cAMP accumulation, in order to assess the sum effects of both binding and intrinsic efficacy differences between mutants constructed. With this in mind, chimeric receptors were constructed rather than mutants with individually altered residues, as I would have expected this former approach to result in more global changes in receptor structure than single residue changes might have expected to give. This was important as the molecular basis for differences in intrinsic efficacy are likely to be due to more diffuse structural differences between receptors than would normally be the case for binding affinity alone. In addition, I considered it important to adopt a functional assay as part of the strategy as the possibility of accessory binding sites (i.e. not the recognized catecholamine binding pocket) for non-catecholamine agonists such as BRL37344 (as suggested by Liggett *et al*, 1992) may possibly complicate competition binding data with radiolabelled antagonists such as [^{125}I]-CYP. Ideally, direct binding studies with radiolabelled [^{3}H]BRL37344 (Muzzin *et al*, 1991) would be less complex to interpret,but, due to the low affinity and specific activity of this radiolabel, and

the relatively low receptor density of the cell lines used in my study, its use was prevented.

It is well documented that receptor density levels are directly related to potency and efficacy of agonists (Kenakin *et al*, 1987), a finding which has previously been confirmed in many cloned receptor systems (Whaley *et al*, 1993, George *et al*, 1988, MacEwan *et al*, 1995), including the β_3-adrenoceptor expressed in CHO cells (Wilson *et al*, 1996). This was also shown in Chapter 4. For this reason I have taken care in this study to minimize, as far as possible, the complication of differential receptor expression. B_{max} values from all cell lines used in this study were comparable, and where a crucial difference in pharmacology is suggested by the data, we have only drawn conclusions from the data where the receptor density is at a level which might be expected to yield artefactual differences in the data opposite to that actually observed.

One of the clearest differences between rat and human pharmacology observed in the present study is that BRL37344 has a significantly higher intrinsic activity in the rat than in the human wild type receptor, and has a significantly lower potency than isoprenaline in the human wild type receptor. In the TM2 chimera the potency of isoprenaline and BRL37344 are not significantly different from one another, however, BRL37344 has a significantly higher intrinsic activity at the TM2 chimera than in the human wild type receptor. This is a difference between the TM2 chimera and human wild type receptor which is unlikely to arise as a result of differential expression of the receptor, since the higher level of expression of the human wild type receptor would be expected to lead to the same or a higher intrinsic activity of a compound which showed no selectivity between the two receptors. I can therefore conclude that the TM2 region is a key molecular determinant of the rat versus human selective efficacy of

BRL37344. The intrinsic activity of CGP12177 at the human wild type receptor was not significantly different from the rat wild type receptor or any of the transmembrane chimeras. The significantly lower potency of CGP12177 in the rat cell line compared to the human is the reverse of the pharmacology one would expect to observe if there were no receptor density-independent differences between these two cell lines. These data are therefore consistent with CGP12177 exhibiting a pharmacological difference between rat and human β_3-adrenoceptor, which is independent of receptor density. In view of this finding, one might predict that rat-like CGP12177 pharmacology in the TM chimera cell lines would also be indicated by a lower potency of CGP12177 compared to the human wild type. Unfortunately, since the receptor densities in the TM chimera cell lines are all similar or lower than that of the human wild type it is not possible to determine whether the lower potency of CGP12177 observed in some of the TM chimera lines is due to an artefact of receptor density differences or a real tendency to rat-like pharmacology. For this reason the level of significance has not been calculated for these Figures.

The molecular basis of pharmacology at catecholamine receptors has been extensively studied by deletion and site directed mutagenesis, largely with the β_2-adrenoceptor (Strader *et al*, 1989). These studies have allowed the proposal of a ligand binding pocket for catecholamines where the catechol moiety of the endogenous agonist is bound via two serine residues in TM5 (Strader *et al*, 1989), and the amine moiety is bound via an aspartate residue in TM3 (Strader *et al*, 1988). In conventional models of GPCR structure based on that of bacteriorhodopsin (Hibert *et al*, 1991) this would suggest that the catecholamine binding pocket is formed in a region within the receptor bordered by TM's 3, 4, 5, and 6, and possibly TM7, with TM's 1, 2, and possibly 7 more distantly located. Further studies have helped to confirm this picture to some extent by showing that residues Ser in TM4 and Phe in TM6 are also of importance in the

binding of ligand (Strader *et al*, 1987, Dixon *et al*, 1988). However, a residue in TM2, Asp^{79}, which would be expected to be quite distant to the catecholamine binding pocket, has also been shown by a number of workers (Chung *et al*, 1988, Breyer *et al*, 1990, Strader *et al*, 1988) to be of importance in agonist binding. Interestingly, changes to this residue do not influence antagonist binding, only agonist binding. This finding led Strader *et al* (1988) to suggest that, although agonist and antagonist binding sites may well be largely overlapping, they are not necessarily identical. Strosberg (1993) has interpreted these same data by proposing that Asp^{79} on TM2 is only indirectly involved in binding of agonist, and that switching of the phenyl moiety of Tyr^{316} in TM7 from binding to Asp^{113} in TM3 to binding to Asp^{79} in TM2 is a key initial step in G-protein coupling. Strosberg (1993) therefore proposed in this model that the TM domains more distant from the catecholamine binding site may normally serve in a signal processing role rather than directly in binding catecholamine (Strosberg , 1993).

This model has also proved useful for generating a working hypothesis to explain the β_3 versus β_1- and β_2-adrenoceptor subtype selectivity of BRL37344; it is proposed that the bulky residue side chain of Phe^{49} in β_2-adrenoceptor may prevent access of BRL37344 to the signal processing region of the β_2-adrenoceptor, but no steric impediment would be expected to exist in the case of the β_3-adrenoceptor, where the equivalent residue is a smaller residue, glycine (Strosberg , 1993). The data in the current study may also be explained in terms of this hypothesis. For example, a number of the human to rat changes in the chimeras which occur towards the cytoplasmic side of the receptor may conceivably increase the degree of access to BRL37344 to the signal processing pocket of the receptor (e.g. Asp^{94} to Gly in TM2 and Pro^{326} to Gly in TM7). It must be recognized, however, that some of these changes would be expected to lead to greater steric hindrance (e.g. Gly^{325} to

Ser in TM7). While the complete binding pocket of the β_3-adrenoceptor, including both grooves is approximately 20-25 Å in size, the ligand BRL37344 is approximately 14 Å. This would imply that if it 'docks' with the β_3-adrenoceptor using similar residues and mechanisms to isoprenaline, and, unlike its positioning within the β_2-adrenoceptor could exist in its most extended conformation, BRL37344 is still unlikely to be able to interact directly with the TM2 region.

Recently, the basis for this hypothesis as a general explanation for the selectivity of BRL37344 at the β_3-adrenoceptor has been called into question from a study (Guan *et al*, 1995) where the β_3 vs β_1/β_2 subtype selective affinity of BRL37344 was investigated by using a series of β_2/β_3 chimeras. These studies suggested that TM5 is a key molecular determinant responsible for the higher affinity binding of BRL37344 at β_3-adrenoceptors when compared to β_2-adrenoceptors. My overall conclusion must therefore be that the major structural determinants of the rat vs human species selective efficacy of BRL37344, but not necessarily that of subtype selectivity, are located within TM region 2. This is a transmembrane region which, as mentioned earlier, is either not normally, or at least indirectly, associated with catecholamine binding.

My investigation into the sustained response to BRL37344 at the rat β_3-adrenoceptor only, was inconclusive, in that the actual mechanism of action was not uncovered. It was possible to conclude, however, that somehow, via the β_3-adrenoceptor specifically (the sustained response was blocked completely by propranolol) BRL37344 was able to 'switch on' the rat β_3-adrenoceptor for long periods of time. Results from the cAMP accumulation 'flashplate' were also inconclusive, and further studies would be necessary. For example, from my preliminary investigations, I showed that the cAMP levels continued increasing after BRL37344 removal, but this occurred in both

the rat and human β_3-adrenoceptor expressing CHO cells, the latter originally not showing the sustained response to BRL37344. It would be useful to continue these studies, perhaps checking how effective the washing out of BRL37344 was at the time. This could easily be performed by running experiments with control cells in parallel, where no washing out of BRL37344 takes place.

In the Trp64Arg chimera, although cellular levels of this chimera were lower than the wildtype human and rat β_3-adrenoceptors, there was no deviation in potency or intrinsic activity values away from those values for the wild type human β_3-adrenoceptor. This suggested that there was no alteration in the response to β_3-agonists by this mutant of the human β_3-adrenoceptor as compared to the wild type human β_3-adrenoceptor. Therefore, groups of the population with this mutation of the β_3-adrenoceptor, whether homologous or heterologous, would be expected to still respond to the same treatment as those without this mutation.

Overall from this Chapter, I have successfully identified a region of importance in the rat and human β_3-adrenoceptor, the TM2 region, which results in alterations in pharmacology from human-like pharmacology to rat-like pharmacology. However, we can conclude, from my studies and other groups, that this region, due to its known position, is not likely to interact directly with agonists, but it is more probable that it causes more overall changes within the β_3-adrenoceptor.

CHAPTER 6.

CONCLUSIONS

Within this Chapter I will discuss the conclusions which can be drawn from my investigations. I will, in certain areas, suggest possibilities for further approaches and studies, and assess what can be drawn from results from three very different Chapters.

While the β_1- and β_2-adrenoceptors have been studied in greater detail, less is known about the third β-adrenoceptor, especially the factors involved in its regulation. Other studies have investigated the down-regulation of receptors and their associated G-protein, finding that down-regulation of both polypeptides follow a similar time course, and that co-down-regulation occurred. So, for example, while my first Chapter investigated some aspects of desensitization in all three β-adrenoceptors, during the course of investigations it was interesting to observe that while the β_3-adrenoceptor polypeptide did not itself down-regulate, the associated G_s, down-regulated extensively. This provided further evidence, little of which was in existence already, for the growing argument that the receptor and G-protein do not necessarily have to co-down-regulate. The latter process of co-down-regulation is certainly the case for the β_1- and β_2-adrenoceptors. The reason for the down-regulation of the β_1- and β_2-adrenoceptors compared to the β_3-adrenoceptor, lies, presumably, in the presence of a number of phosphorylation sites in the C-terminal tail of the β_1- and β_2-adrenoceptors. As mentioned in Chapter 1, phosphorylation of multiple serine and threonine residues in the third cytoplasmic loop and C-terminal tail of the β_1- and β_2-adrenoceptor by regulatory kinases impairs the ability of the receptor to couple to G_s, therefore rendering the adrenoceptor desensitized. This is one of the rapid forms of desensitization. Evidence to contradict co-down-regulation of receptor and G-protein has recently emerged, however, by mutation of threonines in the C-terminal tail of the M_3 muscarinic receptor (in which these mutations prevent the M_3 receptor down-

regulation), resulting in down-regulation of $G_q\alpha/G_{11}\alpha$ similar to the wild-type M_3 receptor (Van de Westerlo *et al*, 1995) to occur. This strongly suggests that G-protein down-regulation, although often seen to follow a similar time-course of down-regulation to the receptor (see Chapter 3 investigations on β_1- and β_2-adrenoceptor expressing CHO cells), could perhaps not be directly coupled to receptor down-regulation.

Further studies in Chapter 3 also investigated loss of G_s from the plasma membrane, whether it was removed to the cytosol, or rapidly packaged into vesicles and degraded. After conflicting reports from other studies (Ransnas *et al*, 1989, Kvapil *et al*, 1994), my work led to my assumptions that, as I could not show that the G_s was present in the cytosol, it was most likely to be rapidly transferred to vesicles and degraded (Perkins *et al*, 1991, Von Zastrow *et al*, 1992, Kvapil *et al*, 1994). In such vesicular compartments, it is thought that receptors are dephosphorylated and reactivated, and perhaps therefore, would indicate a similar method for G-proteins, perhaps being co-sequestered with the receptor. All evidence does, therefore, seem to point to the G-protein having to be newly synthesised. There is a continuous turnover of proteins in cells, and therefore it may not be too surprising that Gs should be degraded and resynthesised in a similar way. My work remains still preliminary and inconclusive, while other groups have also produced conflicting reports for both receptors and G-proteins (Wu *et al*, 1995, Barak *et al*, 1995). A number of studies, using such *de novo* synthesis blockers as cycloheximide, will be necessary and the progress of more studies with detection of light vesicle fractions and their contents, will enable the further investigations of G-protein localization upon agonist-induced down-regulation.

While there is clear evidence for alterations in receptor mRNA levels (Hadcock *et al*, 1988, 1989, Nishikawa *et al*, 1993, Bouvier *et al*, 1989, Feve *et al*, 1991, Thomas *et al*, 1992) this is not the case for G-proteins. While Hadcock *et al* (1988, 1989) demonstrated that homologous and heterologous down-regulation occurred at the level of receptor mRNA, Nishikawa *et al* (1993), found that agonist effected alterations in β_1- and β_2-adrenoceptor mRNA levels. From my investigations in Chapter 3, I was able to rule out the possibility of regulation of G_s at the level of G_s mRNA in the β_1-adrenoceptor expressing CHO cells. This observation was in agreement with other groups, such as McKenzie and Milligan, (1990) and Mullaney *et al*, (1995). It would imply therefore, that transcriptional or translational control of $G_s\alpha$ is not a major mechanism in the overall desensitization process.

Work in Chapter 3 on the rate of down-regulation of G_s in the three β-adrenoceptor expressing CHO cell lines started investigations into areas of work studied more thoroughly in Chapter 4, that is, the effect of receptor density on elements of regulation. In Chapter 3 I looked at receptor expression on G-protein down-regulation. The fact that the β_1-adrenoceptor expressing CHO cells had been transfected with greater levels of β_1-adrenoceptors than the other cell lines expressing β_2- and β_3-adrenoceptors, resulted in a much more rapid down-regulation of G_s in β_1-adrenoceptor expressing CHO cells than the other cell lines. It may also have resulted in an alteration of isoprenaline potency at the β_1-adrenoceptor, as it is expected that isoprenaline would have a higher potency at the β_2-adrenoceptor. This could explain why, in my investigations, the agonist isoprenaline showed a higher potency for the β_1-adrenoceptor over the β_2-adrenoceptor. This could therefore, have been caused by the differences of receptor density of the respective

adrenoceptors in CHO cells (Whaley *et al*, 1994, Kim *et al*, 1994, MacEwan *et al*, 1995), as expression of receptor reserve would be anticipated to move concentration response curves to the left. The effect of receptor expression on rate of G-protein down-regulation was also shown when G_s down-regulation was examined in two β_3-adrenoceptor expressing CHO cell lines. While the D43 β_3-adrenoceptor expressing CHO cell line (3000 ± 400 fmol/mg membrane protein) showed down-regulation of G_s following various concentrations of isoprenaline, the C15 β_3-adrenoceptor expressing CHO cell line (390 ± 83 fmol/mg membrane protein) did not.

Investigations in Chapter 4 moved on to assessments of effect of receptor density on agonist potency and efficacy. As mentioned earlier, it has been little researched before (Whaley *et al*, 1994, Kim *et al*, 1994, MacEwan *et al*, 1995) but it is extremely vital that investigators note differences in receptor density in tissues and cells, when comparing results from their own data with that of other groups. In my study of three NCB20 neuroblastoma cell lines expressing different levels of the β_2-adrenoceptor, after treatments with the agonists isoprenaline, salbutamol, ephedrine and adrenaline, a common trend in results was noticed. This trend of effect by agonists was most clearly obtained in results from concentration-effect curves for isoprenaline and salbutamol, while results with ephedrine and adrenaline were more preliminary and incomplete. These showed that as receptor density increased in NCB20 cells, so the EC_{50} value decreased, thus indicating that potency was increasing. Work with salbutamol showed a similar, if not quite as clear pattern. When intrinsic activity values were also obtained for the agonists, and compared to that of the full agonist isoprenaline, it proved that while salbutamol was only a so-called "partial" agonist (intrinsic activity values less than 1) at the cell line with the lowest

β_2-adrenoceptor density (L9 NCB20 cells), it acted as a full agonist (close to or equal to an intrinsic activity value of 1) at the cell lines containing higher receptor densities (D1 and D4 NCB20 cells). This was also the case for ephedrine, while the physiological agonist adrenaline was a full agonist at all three cell lines. This is to be expected for adrenaline, as it is often used as the referral full agonist (as was isoprenaline in my studies), to which other intrinsic activity values of other agonists, are compared. These results proved that the definition of a "full" agonist is very much dependent on the tissue studied and levels of receptors present. Such terms as a "partial" agonist should therefore be carefully considered when applied to a compound, as my results showed a compound acting as a partial agonist at one tissue may act as a full agonist at another.

While it is possible to predict pharmacological relationships via mathematical theories, it is important to prove these theories practically too. My work follows previously successful studies such as those by MacEwan *et al*, (1995) and Whaley *et al*, (1994). Whaley *et al*, (1994) predicted, and demonstrated experimentally, that potency increased for adrenaline with increasing receptor number. These previous investigations, along with my present studies, provide novel insights into receptor theory and predictions of behaviour of full and partial agonists, and as previously detailed, are important for pharmacological approaches to experiments and analysis of data and results.

Results in Chapter 5 centred around the discovery that from the production of eight chimeric constructs, in which mutations of the human β_3-adrenoceptor were produced, the TM2 region chimera (where all regions are as the human β_3-adrenoceptor, except for the TM2 region which is the sequence of the rat β_3-adrenoceptor) was found to alter the pharmacology

of the human β_3-adrenoceptor to the rat β_3-adrenoceptor. Results for this TM2 chimera, using the cAMP accumulation flashplate assay, showed an increase in IA values from those of the human β_3-adrenoceptor for BRL37344, to IA values comparable to those of the rat β_3-adrenoceptor.

Another qualitative effect linking this chimera to the pharmacology of the rat rather than human β_3-adrenoceptor was noted on observations from microphysiometer studies. While the human β_3-adrenoceptor expressing CHO cells showed no sustained response to BRL37344 on its removal from medium, the rat β_3-adrenoceptor and, from all the chimeras tested, the TM2 chimera alone, showed a sustained response. This was a further indication that the TM2 chimera assumed pharmacological similarities to the rat β_3-adrenoceptor. The sustained response, not reported by any other investigators at the rat β_3-adrenoceptor, would seem to indicate a β_3-adrenoceptor specific effect (blockade of sustained response effected by propranolol). It can only be assumed that different residues in the rat β_3-adrenoceptor, and present in the TM2 region, effect the sustained response upon BRL37344 binding.

While other groups have mainly linked TM5 and TM3 as important regions for agonist binding in the β_2- and β_3-adrenoceptors (Strader *et al* 1988, 1989a), further investigators have also indicated the importance of one of the residues in the TM2 region, Asp^{79} (Chung *et al*,1988, Breyer *et al*, 1990, Strader *et al*, 1988) in agonist binding. Changes to these residues do not affect antagonist binding, only agonist binding. From this work, and also with the assistance of computer modelling of the receptor structure and BRL37344, it must be assumed that TM2 region does not directly interact with agonist (as not situated in the 'binding groove'), but causes more global changes to the receptor conformation.

CHAPTER 7.

REFERENCES

Adie, E.J., Mullaney, I., McKenzie, F.R., Milligan, G., (1992)*Biochem. J.* **285**, 529-536.

Adie, E.J., Milligan, G., (1994a)*Biochem.J.* **300**,709-715.

Adie, E.J. (1994b) *Biochem.J.* **303**, 803-808.

Ahlquist, R.P. (1948) *Am.J.Physiol.* **153**, 586-600.

Arbeeny, C.M., Meyers, D.S., Hillyer, D.E., Bergquist, K.E., (1994) *Int.J. Obes.* **18**, Suppl 2.4.abstract.

Arch, J.R.S. (1984) Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature* **309**.

Arch, J.R.S. (1993) *Medicinal Research Reviews.* **13**, 663-729.

Arner, P., Kriegholm, E., Engfeldt, P., (1991) *J.Pharmacol.Exp.Ther.* **259**, 317-322.

Balch, W.E. (1989) *J. Biol. Chem.* **264**, 16965-16968.

Barak, L.S., Tiberi, M., Freedman, N.J., Kwatra, M.M., Lefkowitz, R.J., Caron, M.G., (1994) *J. Biol. Chem.* **269**, 2790-2795.

Barak, L.S., Menard, L., Ferguson, S.S.G., Colapietro, A-M., Caron, M.G., (1995) *Biochemistry* **34**, 15407-15414.

Benovic, J.L., Pike, L.J., Cerione, R.A., Staniszewski, C., Yoshimasa, T., Codina, J., Caron, M.G., Lefkowitz, R.J., (1985) *J. Biol. Chem.* **260**, 7094-7101.

Benovic, J.L., Bouvier, M., Caron, M.G., Lefkowitz, R.J., (1988) *Ann.Rev.Cell Biol.* **4**, 405-428.

Bensaid, M., Kaghad, M., Rodriguez, Le Fur, G., Caput, D., (1993) **318**, 223-226.

Berkowitz, D.E., Nardone, N.,Smiley, R.M., Price, D.T., Kreutter, D.K., Fremeau, R.T., Schwinn, D.A., (1995) *Eur.J.Pharmacol.* **289**, 223-228.

Bouvier, M., Leeblundberg, L.M.F., Benovic, J.L., Caron, M.G., Lefkowitz, R.J., (1987) *J Biol. Chem.* **262**, 3106-3113.

Bouvier, M., Hausdorff, W.P., De Blasi, A., O'Dowd, B.F., Kobilka, B.K., Caron, M.G., Lefkowitz, R.J., (1988) *Nature* **333**.

Bouvier, M., Collins, S., O'Dowd, B.F., Campbell, P.T., Deblasi, R.J., (1989) *J. Biol. Chem.* **264**, 16786-16792.

Bouvier, M., Guilbault, N., Bonin, H., (1991). *FEBS* **279**, 243-248.

Breyer, R.M., Strosberg, A.D., Guillet, J.G., (1990) *EMBO* **9**, 2679-2684.

Brodde, O-E., (1991) *Pharmacol. Rev.* **43**, 203-242.

Brodde, O-E., (1993) *Pharmacol.&.Ther.* **60**, 405-430.

Carlsson, E., Ablad, B., Brandstrom, A., Carlsson, B., (1972) *Life Sci.* **11**, 953-958.

Chambers, J., Park, J., Cronk, D., Chapman, C., Kennedy, F.R., Wilson, S., Milligan, G., (1994) *Biochem.J.* **303**, 973-979.

Chang, F.H., Bourne, H., (1989) *J.Biol.Chem.* **264**, 5352-5357.

Chen, X.H., Harden, T.K., Nicholas, R.A., (1994) *J.Biol.Chem.* **269**, 24810-24819.

Cheung, A.H., Sigal, I.S., Dixon, R.A.F., Strader, C.D., (1989) *Mol. Pharmacol.* **35**, 132-138.

Chung, F-Z., Wang, C-D., Potter, P.C., Venter, J.C., Fraser, C.M., (1988) *J.Biol.Chem.***263**, 4052-4055

Clapham, D.E.(1996) *Nature* **379**.

Clark, R.B., Goka, T.J., Green, D.A., Barber, R., Butcher, R.W., (1982). *Mol.Pharmacol.* **22**, 609-613.

Clement, K., Vaisse, C., Manning, B.S., Basdevant, A., Guy-Grand, B., Reuz, J., Silver, K.D., Shuldiner, A.R., Froguel, P., Strosberg, A.D., (1995).*New England Journal of Medicine* **333**, 352-354.

Collins, S., Bouvier, M., Bolanowski, M.A., Caron, M.G., Lefkowitz, R.J., (1989) *Proc. Natl.Acad.Sci.(USA).* **86**, 4853-4857.

Collins, S., Altschmied, J., Herbsman, O., Caron, M.G., Mellon, P.L., Lefkowitz, R.J., (1990) *J.Biol.Chem.* **265**, 19330-19335.

Collins, S., Caron, M.G., Lefkowitz, R.J., (1991) *Annual Rev. of Physiol.* **53**, 497-508.

Collins, S., Daniel, K.W., Rohlfs, E.M., Ramkumar, V., Taylor, I.L., Gettys, T.W., (1994) *Mol.Endocrinol* **8**, 518-27.

Connacher, A.A., Bennet, W.M., Jung, R.T., (1992) *Am.J.Clin Nutr.* **55** Suppl:258S-261S.

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

Dupree, P, Parton, R.G., Raposo, G., Kurzchalia, T.V., Simons, K., (1993) *EMBO J*. **12**, 1597-1605.

Emorine, L.J. (1989) *Science*.**245**, 1118-1121.

Ferguson, S.G., Menard, L., Barak, L.S., Koch, W.J., Colapietro, A-M., Caron, M.G., (1995) *J.Biol.Chem.*, **270**, 24872-24879.

Feve, B. (1991) *J. Biol. Chem.* **266**, 20329-20336.

Freedman, N.J., Liggett, S.B., Drachman, D.E., Pei, G., Caron, M.G., Lefkowitz, R.J., (1995) *J.Biol.Chem.***270**, 17953-17961.

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

Frielle, T., Caron, M.G., Lefkowitz, R.J., (1989) *Clinical Chem* **35**, 721-725.

George, S.T., Berrios, M., Hadcock, J.R., Wang, H.Y., Malbon, C.C., (1988) *Biochemical and Biophysical Research Communications*. **150**, 665-672.

Goldsmith, P., Backlund, P.S., Rossiter, K., Carter, A., Milligan, G., Unson, C.G., Spiegel, A.M., (1988) *Biochemistry* **27**, 7085-7090.

Granneman, J.G., Lahners, K.N., Chaudhry, A., (1991) *Mol. Pharmacol.* **40**, 895-899.

Granneman, J.G. , Lahners,K.N., Rao, D.D., (1992a) *Mol. Pharmacol.* **42**, 964-970.

Granneman, J.G., Lahners, K.N., (1992b) *Endocrinology* **130**, 109-114.

Granneman,J.G., (1992c) *J.Pharmacol and Exp.Ther.* **261**, 638-642.

Green, S.A., Holt, B.D., Liggett, S.B., (1992) *Mol.Pharmacol.* **41**, 889-893.

Guan, X-M., Amend, A., Strader,C., (1995) *Mol.Pharmacol.* **48**, 492-498.

Habener, J.F., (1990) *Mol.Endocrinol.* **3**, 1087-1094.

Hadcock, J.R., Malbon, C.C., (1988) *Proc.Natl.Acad.Sci.(USA)* **85**, 5021-5025.

Hadcock, J.R., Ros, M., Malbon, C.C., (1989) *J. Biol. Chem.* **264**, 13956-13961.

Harden, T.K., Cotton, C.V., Waldo, G.L., Lutton, J.K., Perkins, J.P., (1980), *Science* **210**, 441-443.

Hausdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G., Lefkowitz, R.J., (1989) *J.Biol.Chem.*, **264**, 12657-12665.

Hausdorff, W.P., Caron, M.G., Lefkowitz, R.J., (1990)*FASEB.***4**, 2880-2891.

Hertel, C., Muller, P., Portenier, M., Staehelin, M., (1983) *Biochem.J.* **216**, 669-674.

Hertel, C., Coulter, S.J., Perkins, J.P., (1986) *J.Biol.Chem.* **261**, 5974-5980.

Hertel, C., Nunally, M.H., Wong, S.K.F., Murphy, E.A., Ross, E.M., Perkins, J.P., (1990) *J.Biol.Chem.* **265**, 17988-17994.

Hibert, M.F., Trumpp-Kallmeyer, S., Bruinvels, A., Hoflack, J., (1991). *Mol.Pharmacol.***40**, 8-15.

Himms-Hagen, J., Cui, J., Danforth.E.Jr., Taatjes, D.J., Lang, S.S., Waters, B.L., Claus, T.H., (1994) *Am.J. Physiol* **226**, R1371-R1382.

Hooley, R., Yu, C-Y., Symons, M., Barber, D.L., (1996) *J. Biol. Chem.* **271**, 6152-6158.

Hoyer, D., Boddeke, H.W.G.M., (1993) *Trends in Pharmacol.Sci.* **14**, 270-275.

Inglese, J., Freedman, N.J., Koch, W.J., Lefkowitz, R.J., (1993) *J.Biol.Chem.* **268**, 23735-23738.

Johnson, R.A., Salomon, Y., (1991) *Methods in Enzymology* **195**, 3-21.

Jones, T.L.Z., Simonds, W.F., Merendino, J.J., Brann, M.R., Spiegel, A.M., (1990) *Proc. Natl. Acad. Sci. (USA)* **87**, 568-572.

Kaumann, A-J., (1991) *Cardiovascular drugs and therapy* **5**, 549-560.

Kelly, E., Keen, M., Nobbs, P., MacDermott, J., (1990) *Br.J.Pharmacol.* **100**, 223-230.

Kenakin, T.P., (1987) *Raven Press*, New York.

Kenakin, T.P., (1989) *Trends in Pharmacol.Sci.***10**, 18-22.

Kim, G-D., Adie, E, Milligan, G., (1994a) *Eur.J. Biochem* **219**, 135-143.

Kim, G-D, Milligan, G., (1994b) *FEBS* **355**, 166-170.

Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., Wittig, B., (1992) *Nature*, **358**, 424-426.

Kobilka, B.K., MacGregor, C., Daniel, K., Kobilka, T.S., Caron, M.G., (1987) *J.Biol.Chem.* **262**, 15796-15802.

Kobilka, (1988) *Science* **240**, 1310-1316.

Kopp, U., DiBona, G., (1983) *Am.J.Physiol.***244**, F418-F424.

Koski, G., Klee, M., (1981) *Proc.Natl.Acad.Sci. (USA)* **78**, 4185-4189.

Kozasa, T., Itoh, H, Tsukamoto, T., Kaziro, Y., (1988) *Proc. Natl. Acad. Sci. (USA)* **85**, 2081-2085.

Kunkel, L.M., Lalande, M., Monaco, A.P., Flint, A., Middlesworth, W., Latt, S.A., (1985) *Gene* **33**, 251-258.

Kvapil, P., Novotny, J., Svoboda, P., Ransnas, L.A., (1994) *Eur.J.Biochem* **226**, 193-199.

Laemmli, U.K., (1970) *Nature* **227**, 680-685.

Lambright, D.G., Sondek, J., Bohm, A., Skiba, N.P., Hamm,H.E., Sigler, P.B., (1996) *Nature* **379**.

Lefkowitz, R.J., Wessels, M.R., Stadel, J.M., (1980) *Curr.Top.Cell.Reg.***17**, 205-230.

Levis, M.J., Bourne, H.R., (1992) *J. Cell Biol.* **119**, 1297-1307.

Liggett,.S.B. (1992) *Mol.Pharmacol.* **42**, 634-637.

Liggett,S., Freedman, N.J., Schwinn, D.A., Lefkowitz, R.J., (1993) *Proc.Natl.Acad.Sci .(USA)* **90**, 3665-3669.

Lohse, M.J., Lefkowitz, R.J., Caron, M.G., Benovic, J.L., (1989) *Proc. Natl. Acad. Sci. (USA)* **86**, 3011-3015.

Lohse, M.J., Benovic, J.L., Caron, M.G., Lefkowitz, R.J., (1990) *J.Biol.Chem.***265**, 3202-3209.

Lohse, M.J. (1993) *BBA.* **1179**, 171-188.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., (1951) *J.Biol.Chem.***193**, 265-275.

MacEwan, D.J., Kim, G.D., Milligan, G., (1995) *Mol. Pharm* **48**, 316-325.

McKenzie, F.R., Milligan, G., (1990) *J. Biochem.* **265**, 17084-17094.

Milligan, G.,Unson, C.G., (1989) *Biochem. J.* **260**, 837-841.

Milligan, G., Green, A., (1991) *Trends in Pharmacol. Sci.* **12**, 207-209.

Milligan, G. (1993)*Trends in Pharmacol. Sci.*, **14**, 239-244.

Milligan, G., Svoboda, P, Brown, C., (1994) *Biochem.Pharmacol.* **48**, 1059-1071.

Milligan, G., Parenti, M., Magee, A.I., (1995) *Trends in Pharmacol. Sci.* **20**, 181-186.

Minneman, K.P., Weiland, G.A., Molinoff, P.B., (1980) *Mol.Pharm.*, **17**, 1-7.

Mitchell, F., Buckley, N.J., Milligan, G., (1993) *Biochem.J.* **293**, 495-499.

Mitchell, T.H., Ellis, R.D., Smith, S.A., Robb, G., Cawthorne, M.A., (1989) *Int.J.Obes* . **13**, 757-766.

Moinat, M., Deng, C., Muzzin, P., Assimacopoulos-Jeannet, F., Seydoux, J., Dulloo, A.G., Giacobino, J-P. (1995) *FEBS Letters* **373**, 131-134.

Mullaney, I., Shah, B.H., Wise, A., Milligan, G., (1995) *J.Neurochem.* **65**, 545-553.

Mumby, S.M., Kahn, R.A., Manning, D.R., Gilman, A.G., (1986) *Proc Natl Acad Sci (USA)* **83**, 265-269.

Mumby, S.M., Heuckeroth, R.O., Gordon, J.I., Gilman, A.G., (1990) *Proc Natl Acad Sci (USA)* **87**, 728-732.

Muzzin,P., Revelli, J-P., Kuhne, F., Gocayne, J.D., McCombie, W.R., Venter, J.C., Giacobino, J-P., Fraser, C.M., (1991) *J. Biol. Chem.* **266**, 24053-24058.

Nahmias, C. (1991) *EMBO J.* **10**, 3721-3727.

Nantel,F.,Bonin,H.,Emorine,L.J.,Zilberfarb,V.,Strosberg,A.D.,Bouvier, M.,Marullo,S., (1993) *Mol.Pharmacol.* **43**, 548-555.

Nantel, F., Marullo, S., Krief, S., Strosberg, A.D., Bouvier, M., (1994) *J. Biol. Chem.* **269**, 13148-13155.

Nelson, C.A., Seamon, K.B., (1986) *Federation Proceedings* **45**, 1525.

Neubig, R.R, (1994) Membrane organization in G-protein mechanisms, *FASEB* **8**.

Nishikawa, M., Mak, J.C.W., Shirasaki, H., Barnes, P., (1993) *Eur.J.Pharmacol* **247**, 131-138.

Northup, J.K., Sternweis, P.C., Smigel, M.D., Schleifer, L.S., Ross, E.M., Gilman, A.G., (1980) *Proc. Natl. Acad. Sci. (USA)* **77**, 6516-6520.

O'Dowd, B.F., Hnatowich, M., Regan, J.W., Leader, W.M., Caron, M.C., (1988) *J. Biol.Chem.* **263**, 15985-15992.

Ohe, M., Munakata, M., Hizawa, N., Itoh, A., Doi, I., Yamaguchi, E., Homma, Y., Kawakami, Y., (1995) *Thorax* **50**, 353-359.

Onorato, J.J., Palczewski, K., Regan, J.W., Caron, M.G., Lefkowitz, R.J., Benovic, J.L., (1991) *Biochem.* **30**, 5118-5125.

Perkins, J.P., Tocws, M.C., Harden, T.K., (1984) *Adv.Cyclic.Nucleotide Res.***17**, 37.

Pert, C.B., Snyder, S.H., (1973) Proc.Natl.Acad.Sci. (USA) **70**, 2243-2247.

Pfeuffer, T., Metzger, H., (1982) *FEBS Letters* **146**, 369-375.

Pietri-Rouxel, F., Lenzen, G., Kapoor, A., Drumare, M-F., Archimbault, P., Strosberg, A.D., Manning, B.St.J., (1995) *Eur.J.Biochem.* **230**, 350-358.

Pippig, S., Andexinger, S., Lohse, M.J., (1995) *Mol.Pharmacol.***47**, 666-676.

Rall, T.W., Sutherland, E.W., Berthet, J., (1957) *J.Biol.Chem.* **224**, 463-475.

Ransnaes, L.A. (1989) *Proc. Natl. Acad. Sci. (USA)* **86**, 7900-7903.

Raposo, G., Dunia, I., Delvaier-Klutchko, C., Kaveri, S., Strosberg, A.D., Benedetti, E.L., (1989) *Eur.Cell.Biol.***50**, 340-352.

Rasenick, M.M., Wang, N., (1988) *J.Neurochem.* **51**, 300-311.

Rasenick, M.M., Wang, N., Yan, K., (1990) *Adv.Second Messenger Phosphoprotein Res.***24**, 381-386.

Rooney, T.A., Hager, R., Thomas, A.P., (1991) *J.Biol.Chem.* **266**, 15068-15074.

Roth, N., Campbell, P.T., Caron, M.G., Lefkowitz, R.J., Lohse, M.J., (1991) *Proc. Nat. Acad. Sci. (USA)* **14**, 6201-6204.

Rubenstein, R.C., Linder, M.E., Ross, E.M., (1991) *Biochemistry* **30**, 10769-10777.

Saffitz, J.E., Liggett, S.B., (1992) *Circulation Research* **70**, 1320-1325.

Salomon, Y., (1979) *Adv.Cyclic Nucleotide Res.* **10**, 35-55.

Samama, P., Cotecchia, S., Costa, T., Lefkowitz, R.J., (1993) *J.Biol.Chem.* **268**, 4625-4636.

Samama, P., Pei, G., Costa, T., Cotecchia, S., Lefkowitz, R.J., (1994) *Mol.Pharm.* **45**, 390-394.

Sargiacomo, M., Sudol, M., Tang, Z-L., Lisanti, M.P., (1993) *J.Cell.Biol.* **122**, 789-807.

Seamon, K.B., Padgett, W., Daly, J.W., (1981) *Proc. Natl. Acad. Sci. (USA)* **78**, 3363-3367.

Severs, N.J., (1988) *J.Cell.Sci* **90**, 341.

Shear, M., Insel, P.A., Melmon, K.L., Coffino, P., (1976) *J. Biol. Chem.* **251**, 7572-7576.

Sibley, D.R. (1986) *Proc. Natl. Acad. Sci. (USA)* **83**, 9408-9412.

Stephenson, R.P., *Br.J. Pharmacol.* (1956) **11**, 379-393.

Strader, C.D., Dixon, R.A.F., Cheung, A.H., Candelore, M.R., Blake, A.D., Sigal, I.R., (1987) *J.Biol.Chem.* **262**, 16439-16443.

Strader, C.D., Sigal, I.S., Candelore, M.R., Rands, E., Hill, W.S., Dixon, R.A.F. (1988)*J.Biol.Chem.* **263**, 10267-10271.

Strasser, R.H., Sibley, D.R., Lefkowitz, R.J., (1986) *Biochemistry* **25**, 1371-1377.

Strosberg, A.D., (1993) *Protein Science* 2, 1198-1209.

Susulic, S., Frederich, R.C., Lawitts, J.A., et al (1995). In: Profram and abstracts of the 77th annual meeting of the Endocrine Society, June 14-17.

Susulic, V.S., Lawitts, J.A., Flier, J.S., Lowell, B.B., (1994) *Int.J.Obes.* 18 Suppl 2.5.abstract.

Suzuki, T., Nguyen, C.T., Nantel, F., Bonin, H., Valiquette, M., Frielle, T., Bouvier, M., (1992) *Mol.Pharmacol* **41**, 542-548.

Svoboda, P., Milligan, G., (1994) *Eur.J.Biochem* **224**, 455-462.

Svoboda, P., Kim, G.D., Grassie, MA., Eidne, KA., Milligan, G., (1996) *Mol.Pharm.* **49**, 646-655.

Thomas, R.F., Holt, B.D., Schwinn, D.A., Liggett, S.B., (1992) *Proc.Natl.Acad.Sci.(USA).* **89**, 4490-4494.

Thompson, P., Findlay, J.B.C., (1984) *BiochemJ.* **220**, 773-780.

Touhara, K., Inglese, J., Pitcher, J.A., Shaw, G., Lefkowitz, R.J., (1994) *J. Biol. Chem.* **269**, 10217-10220.

Turki, J., Pak, J., Green, S.A., Martin, R.J., Liggett, S.B., (1995) *J.Clin. Investigation* **95**, 1635-1641.

Valiquette, M., Bonin, H., Hnatowich, M., Caron, M.G., Lefkowitz, R.J., Bouvier, M., (1990) *Proc. Natl. Acad. Sci.(USA)* **87**, 5089-5093.

Van de Westerlo, E., Yang, J., Williams, J.A., (1995) *Biochem.J.* **310**, 559-563.

Vaziri, C., Downes, C.P., (1992) *Biochem.J.* **284**, 917-922.

Von Zastrow, M., Kobilka, B.K., (1992) *J.Biol.Chem.* **267**, 3530-3538.

Waldo, G.L., Northup, J.K., Perkins, J.P., Harden, T.K., (1983) *J.Biol. Chem.* **258**, 3900-3908.

Walston, J., Silver, K., Bogardus, C., Knowler, W.C., Celi, F.S., Austin, S., Manning, B., Strosberg, A.D., Stern, M.P., Raben, N., Sorkin, J.D., Roth, J., Shuldiner, A.R., (1995) *New England Journal of Medicine* **333**, 343-347.

Wang, H-Y., Lipfert, L., Malbon, C., Bahouth, S., (1989) *J.Biol. Chem*, **264**, 14424-14431.

Wang, N., Rasenick, M.M., (1991) *Biochemistry* **30**, 10957-10965.

Wedegaertner, P.B., Bourne, H.R. (1994) *Cell* **77**, 1063-1070.

Whaley, S.B., Yuan, N., Birnbaumer, L., Clark, R.B., Barber, R., (1994) *Mol. Pharmacol.* **45**, 481-489.

Widen, E., Lehto, M., Kanninen, T., Walston, J., Shudiner, A.R., Groop, L.C., (1995) *The New England Journal of Medicine*. 348-351.

Wilson, S., Chambers, J.K., Park, J.E., Ladurner, A., Cronk, D.W., Chapman, C.G., Kallender, H., Browne, M.J., Murphy, G.J., Young, P.W. In Press (1996).

Wu, D., Kuang, Y., Wu, Y., Jiang, H., (1995) *J.Biol.Chem.* **270**, 16008-16010.

Yagami, T., (1995) *Mol.Pharmacol* .**48**, 849-854.

Yamaki, K., Tsuda, M., Kikuchi, T., Chen, K.H., Huang, K.P., Shinohara, T., (1990) *J. Biol. Chem.* **265**, 20757-20762.

Yamamoto, K.K., Gonzalez, G.A., Biggs, W.H., Montminy, M.R., (1988)*Nature* **334**, 494-498.

Yu, S.S., Lefkowitz, R.J., Hausdorff, W.P., (1993). *J. Biol. Chem.* **268**, 337-341.

Zaagsma, J., Naborski, S.R., (1990) *Trends in Pharmacol. Sci* **11**,3-7.

Zhang, Y.Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., (1994) *Nature* **372**, 425-432.

Addendum:

Dohlman, H.G., Bouvier, M., Benovic, J.L., Caron, M.G., Lefkowitz, R.J., (1987), *J.Biol.Chem*, **262**, 14282-14288.

Eason, M.G., Liggett, S.B., (1992), *J.Biol.Chem.*, **267**, 25473-25479.

Widen, E., Lehto, M., Kanninen, T., Walston, J., Shudiner, A.R., Groop, L.C., (1995) *The New England Journal of Medicine*, **333**, 348-351.