Ligand Regulation of β_1- and β_2- Adrenergic Receptors and their GFP-Tagged Forms

A thesis presented for the degree of

Doctor of Philosophy

by

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Abbreviations

α	α subunit of G proteins
A	adenosine
ADP	adenosine-5'-diphosphate
Ala (A)	alanine
Asn (N)	asparagine
Asp (D)	aspartate
AR	adrenergic receptor
Arg (R)	arginine
ATP	adenosine-5'-triphosphate
β	beta subunit of G proteins
β_1	beta-1
β_2	beta-2
β_3	beta-3
β_4	beta-4
B_{max}	maximum binding sites
βARK	β adrenergic receptor kinase
$\beta\gamma$	beta-gamma subunit of G proteins
BSA	bovine serum albumin
C	cytosine
CAM	constitutively active mutant
cAMP	adenosine 3':5'-cyclic monophosphate
cDNA	complementary DNA
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
cpm	counts per minute
Cys (C)	cystine
C terminus	carboxy terminus

DHA	dihydroalprenolol
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
dpm	disintegrations per minute
EC	extracellular
EC_{50}	concentration of agonist producing half-maximal response
EDTA	ethylenediamine tetra-acetic acid
fmol	femtomole
γ	gamma subunit
G	guanosine
G_i	inhibitory G protein
G_s	stimulatory G protein
$G_s\alpha_S$	long isoform of the inhibitory G protein α subunit
$G_s\alpha_S$	short isoform of the stimulatory G protein α subunit
GDP	guanosine 5'-diphosphate
GFP	green fluorescent protein
Gln (Q)	glutamine
Glu (E)	glutamate
Gly (G)	glycine
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
GTP	guanosine 5'-triphosphate
h	hour
^3H	tritium
HEPES	(N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])
His (H)	histidine
IBMX	isobutylmethylxanthine
IC	intracellular
Ile (I)	isoleucine

kDa	kiloDalton
K_d	dissociation constant
K_i	inhibitory constant
L	litre
LB	L-broth
Leu (L)	leucine
Lys (K)	lysine
μg	microgram
μl	microlitre
M	molar
MEM	minimal esential medium
mg	miligram
min	minute
ml	mililitre
N_A	Avagadro's number
NBCS	new born calf serum
nm	nanometre
N terminus	amino terminus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
pmol	picomole
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Ser (S)	serine
$t_{1/2}$	half-life (time)
T	thymidine

TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Thr (T)	thrionine
TM	transmembrane
Tris	tris(hydroxymethyl)aminomethane
Val (V)	valine
WT	wild-type

Summary

G protein coupled receptors (GPCRs), as the name suggests, require a coupling transducer called a G protein to enable conversion of extracellular stimuli into intracellular second messengers. Over 200 GPCRs with the same topological seven transmembrane structure have been identified along with their coupling G proteins. To understand more about GPCR signalling and particular components required to achieve efficient signalling, extensive studies have involved the β_2-AR signalling pathway. Receptor activation and subsequent receptor desensitisation and internalisation of the β_2-AR and GPCRs in general have proved a major area of interest.

Various types of ligand can bind to a GPCR such as agonist, inverse agonist and antagonist. Pharmacologists have struggled to clearly categorise these types of ligands. It was the aim of my first chapter to develop the basis for a GPCR ligand screen using the β_2-AR as a model system. Previously it has been demonstrated that a (constitutively active mutant) CAM-β_2-AR becomes up-regulated when treated with the inverse agonist, betaxolol for 24 h (MacEwan and Milligan, 1996a,b). In this study, a CAM-β_2-AR, C-terminally tagged with GFP was also found to be up-regulated by betaxolol. In fact a differential pattern of up-regulation was found to occur with a range of β-blockers. The pattern of up-regulation was found to correlate with the ability of the ligands to induce production of cAMP, thus acting as partial agonists at the CAM-β_2-AR-GFP construct. This developed the basis for a rapid screening assay for ligand regulation of this receptor.

Chapter 2 investigated the sequestration of the WT-β_1-AR, WT-β_2-AR and their GFP-tagged forms. It was shown that the β_1-AR constructs internalised more rapidly than the β_2-AR, but 10-20 % less maximal β_1-AR receptor was internalised compared to the β_2-AR. Tagging these receptors with GFP did not alter their

pharmacology but receptor sequestration was markedly impeded, indicating limitations to the use of GFP as a tagging molecule. Down-regulation of the WT-β_2-AR-GFP was also smaller than that of the WT-β_2-AR after 24 h treatment of agonist stimulation.

Receptor desensitisation and sequestration of the β_2-AR requires phosphorylation of the receptor by (protein kinase A) PKA and (GPCR kinases) GRKs. As the roles of these kinases and their sites of phosphorylation have been debated, a mutant form of the β_2-AR lacking all the potential C-terminal GRK phosphorylation sites (BARK$^-$-β_2-AR-GFP) was used to investigate this issue. The construct was found to internalise in response to isoprenlaine stimulation, this being at a faster rate than internalisation of WT-β_2-AR-GFP. Secondly, the PKA inhibitor Rp cAMP had no effect on internalisation of this mutant construct, WT-β_2-AR or WT-β_2-AR-GFP. These results suggest that PKA is not involved in sequestration of the β_2-AR and that other phosphorylation sites in the receptor may be responsible for this effect.

Chapter 1

Introduction

1.1 Cellular Signalling

Repeatedly, different cells in the tissues of the body are exposed to a variety of stimuli, including neurotransmitters, peptide molecules and ions, which ultimately produce many effects. To monitor these events and respond accordingly it is essential for the cell to have a direct mechanism for measuring its environment. The expression of cell surface receptors within the plasma membrane of the cell is one way of achieving the recognition of external stimuli and decoding them into a form recognisable to components inside of the cell. This is not merely a "one step" reaction but involves a series of separate steps linked together involving receptor, regulatory proteins and effectors to generate recognisable intracellular second messengers. The second messengers have the ability to bind and regulate certain enzymes within the cell leading ultimately to effects on cell growth, differentiation and division.

One of the most studied sub-types of receptor are the β-adrenergic receptors (β-ARs) which respond to circulating adrenaline and noradrenaline. As early as 1957 Rall and Sutherland observed that hormone bound β-AR allosterically regulated the effector molecule, adenylyl cyclase. This observation prompted intense study into the molecular mechanisms of this pathway which identified regulatory protein components that linked active β-AR to adenylyl cyclase. These components were found to have a requirement for GTP, and hence, were named GTP binding proteins or G proteins (Rodbell et al., 1971 a and b ; Gilman, 1987). These are heterotrimeric proteins with three subunits, α, β and γ. The β and γ subunits are tightly associated but the α subunit continually associates and dissociates from the protein complex depending on the requirements of the cell. In order to produce an intracellular second messenger signal, ligand bound β-AR associates with the α subunit of a G protein which in turn activate adenylyl cyclase to produce the second messenger cAMP. This second messenger is an allosteric effector which activates protein kinase A (PKA) to

produce a variety of cellular effects. The βγ complex may also be involved in certain signalling mechanisms but these are rather dated (Sternweis, 1994).

There are a range of cell surface receptors including growth factor receptors, with intrinsic tyrosine kinase activity, but by far the largest class are the seven trans-membrane spanning receptors that function via the heterotrimeric G proteins. The β-AR is a sub-family of this family of G protein coupled receptors (GPCRs). This sub-family consists of four sub-types, β_1, β_2, β_3 and β_4 -ARs. The aim of this chapter is to present as clear a picture as possible of what is known about the β-AR family, their coupling G proteins and the effector components of their signalling pathways. How these pathways are regulated shall be discussed, including the role of these receptors and their signalling in disease. An interesting feature of some GPCRs is their ability to induce formation of second messenger signals in the absence of agonist stimulation. These receptors are termed constitutively active mutant (CAM) GPCRs and their significance in the study of receptor activation and development of drug therapies will be discussed. The use of certain techniques used to probe these signalling pathways to develop insight into potential new therapies will also be presented.

1.2 The β-Adrenergic Receptor Sub-family

a) Introduction

The β-ARs effect their responses via the sympathetic nervous system and control a wide range of physiological responses. These include control of vascular tone, cardiac function, metabolism and behaviour. Stimulation of sympathetic nerve terminals induces a release of endogenous β-agonists, adrenaline and noradrenaline which activate β-ARs on postsynaptic sites.

Presently, there are four identified members of the β-AR family, these being sub-types β_1, β_2, β_3 and β_4. Before these sub-types were identified it was firstly noted that some tissues would respond differentially to the known β-agonists (isoprenaline, adrenaline and noradrenaline). Therefore, β-ARs were divided into two classes β_1 and β_2 -ARs, displaying a different order of potency to β-agonists, β_1 (isoprenaline > noradrenaline > adrenaline) versus β_2 (isoprenaline > adrenaline > noradrenaline). A third 'atypical' β-AR was subsequently identified now termed the β_3-AR (Emorine et al., 1989). This receptor exhibited a distinct pharmacological profile compared to the β_1 and β_2 -ARs, showing a lower affinity for classical β-agonists with the rank order of potency being, noradrenaline > isoprenaline > adrenaline. The β_1 and β_2 -AR antagonist CGP12177A acts as an agonist at the β_3-AR and is more potent at the human compared to the rat receptor (Liggett, 1992). No β_3 selective antagonist has been identified which can cause problems when trying to investigate this receptor, especially in tissues in which it is co-expressed with other β-ARs. There are some non-selective antagonists available, e.g. (-)-bupranolol, ICI118551 and CGP20712A. Recently, a fourth 'atypical' β_4-AR was discovered in rat atria by Kaumann and Lynham (1997). Kaumann had previously proposed the existence of this receptor in mammalian heart (Kaumann, 1989). It has been elegantly demonstrated that CGP12177A (a β_3-AR agonist and a β_1 and β_2 -AR antagonist) causes cardiostimulation and binds to cardiac putative β_4-ARs in both WT and β_3-AR knockout mice (Kaumann et al., 1998).

b) β-adrenergic receptor cloning

Three distinct mammalian β-AR cDNAs, β_1 (Frielle et al., 1987), β_2 (Dixon et al., 1986) and β_3 (Emorine et al., 1989) have been isolated. Both the β_1 and β_2 -AR genes are intronless and therefore, neither sub-type can generate diversity by differential mRNA splicing. In humans the β_3-AR gene consists of two exons and a

single intron (Granneman et al., 1992) and therefore, the gene can undergo differential splicing of pre-mRNA to generate multiple forms of the β_3-AR. In fact there are two distinct splice variants of the β_3-AR in humans varying only by the presence or absence of 6 C-terminal amino acids.

c)β-adrenergic receptor structure

All β-ARs are 7 transmembrane (TM) spanning receptors which couple through G proteins to elicit a response within the cell (Figure 1.1a). These 7 TM regions are linked by 3 extracellular and 3 intracellular loops. The protein is lodged in the plasma membrane of the cell so that the N-terminal region of the protein is extracellular and the C-terminal region projects into the cell.

High conservation between family members within the 7 TM α helical regions indicated that this area of the GPCR is important for ligand recognition. Each TM region has a specific orientation within the plasma membrane to form the ligand binding pocket (Figure 1.1b). Site directed mutagenesis studies have been used to identify specific residues involved in ligand recognition by GPCRs. Not surprisingly the most extensive research has concentrated on the β_2-AR. Strader et al., (1988, 1989) identified Asp^{113} in TM 3 as vital for both antagonist and agonist binding. The latter is also dictated by Ser^{204} and Ser^{205} of TM5. Also important for sterio-selectivity of catecholamines is Asn^{293} in TM6 of the β_2-AR (Wieland et al., 1996; Zurmond et al., 1999). From studies on the β_1-AR, TM4 is largely responsible for the 10 fold increase in affinity of noradrenaline at this receptor compared to the β_2-AR (Frielle et al., 1988; Dixon et al., 1989). Isogaya et al (1998, 1999) generated β_1/β_2-chimeric receptors to demonstrate that TM2 and TM7 of the β_2-AR are important in the binding of β_2-AR selective agonists, Tyr^{308} of TM7 being particularly important. As this is a Phe in the β_1-AR only Leu^{110}, Thr^{117} and Val^{120} in TM2 of this receptor

were found to dictate binding of ligands. Various β_3-AR mutants with residues altered in their TM domains have been studied (Gros et al., 1998). These included delVLA (deletion of Val, Leu and Ala in TM1), G53F (TM1), D117L (TM3) and N312A (TM6) β_3-AR mutants. Both TM1 mutations did not alter the binding of ligands or signalling of the receptor. The TM3 mutant exhibited suppressed ligand binding and adenylyl cyclase activation and the TM6 mutation led to alterations in the signal transduction pathway of the receptor.

Recently GPCRs have been described as two or even more independent folding units (Herbert et al., 1996; Tarasova et al., 1999). To demonstrate this split muscarinic receptors have been utilised, where the receptor is broken at the 3rd intracellular loop to generate an m3-trunk and an m3-tail (Jakubik and Wess, 1999). When they were co-expressed in COS-7 cells both m3 fragments specifically interacted and the presence of muscarinic ligands enhanced the association. This was a receptor specific interaction since fragments from other co-expressed receptor constructs did not associate with the m3 fragments. Three conserved proline residues located in TM5, 6 and 7 were identified as being essential for proper fragment association again indicating the importance of the TM domains in receptor structure conformations. A study on the lutenising hormone (LH) receptor demonstrated that co-expression of receptor units in HEK293 cells partially reconstituted ligand-induced signal generation (Osuga et al., 1997). An important role of TM1 was discovered in signalling of the LH receptor. The use of antagonist peptides to receptor TM regions has also proved useful to demonstrate the importance of TM association in the functioning of GPCRs. A peptide derived from TM6 of the β_2-AR, when co-expressed with β_2-ARs inhibited receptor activation and dimerisation (Herbert et al., 1996). This method has also been utilised by Tarasova et al., (1999) to identify regions important for functioning of the CCXR4 and CCR5 chemokine receptors.

Figure 1.1

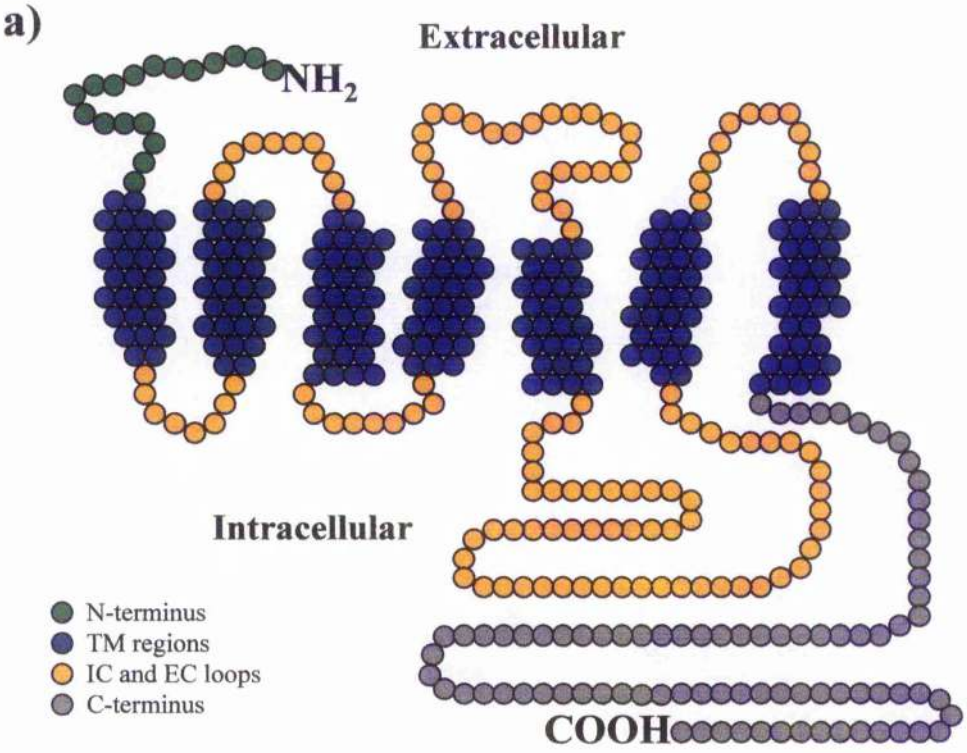
a) Topological representation of a typical G protein coupled receptor

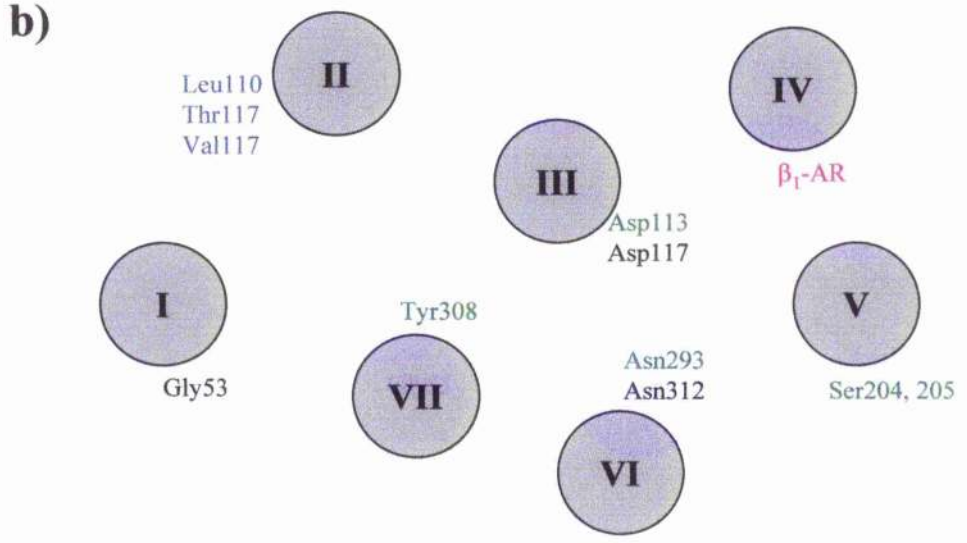
G protein coupled receptors (GPCRs) have 7 α helices forming the 7 transmembrane (TM) regions (blue), linked by 3 intracellular (IC) and 3 extracellular (EC) loops (orange). The N-terminal region (green) contains the sites for glycosylation and the C-terminal tail (grey) contains the site for palmitoylation.

b) Position of the 7 TM α helices in the cell plasma membrane and the specific residues important for ligand binding and signalling.

The view is from the intracellular side of the membrane. The residues found to be important for ligand binding and functioning of the receptor are indicated by colour, β_2-AR (green), β_3-AR (black), β_1- and β_2- AR (blue). TM5 is important in selective noradrenaline binding at the β_1-AR (pink).

Figure 1.1


a)
Extracellular
NH_2
Intracellular
N-terminus
TM regions
IC and EC loops
C-terminus
COOH


b)
II
Leu110
Thr117
Val117
IV
β_1-AR
III
Asp113
Asp117
I
Gly53
Tyr308
VII
V
Asn293
Asn312
Ser204, 205
VI

It is not only the TM domains that are important for regulation of receptor functions. The intracellular loops and the C-terminal tail are also important regions. These are the regions of the GPCR that are facing into the cell and are a prime target for intracellular signalling molecules. In the case of GPCRs these are firstly the G proteins. Two main areas of the β_2-AR have been identified as being important in coupling to G proteins (G_s or possibly G_i). These are the 3rd intracellular loop and the C-terminal tail also found to be targets for phosphorylation by cAMP dependent protein kinase (PKA) and G protein coupled receptor kinases (GRKs) (Benovic et al., 1985; Fredericks et al., 1996). These kinases assist in switching off of the signalling cascade by uncoupling the receptor from G protein. Intracellular loop 2 has also been identified as a regulatory region in this process (Jockers et al., 1996). Once desensitised the receptor can undergo down-regulation or resensitisation. For resensitisation to occur the receptor is internalised and dephosphorylated. Intracellular loops 1 and 2 and the C-terminal tail of the β_2-AR were found to regulate this process (Jockers et al., 1996). Also present in the C-terminal tail of the β_2-AR is a site for dynamic palmitoylation which regulates the ability of the receptor to be desensitised. A palmitoylation minus (C341G) mutant of the β_2-AR displayes increased basal phosphorylation and a decreased rate of agonist-promoted desensitisation (Moffett et al., 1993 and Section 1.6f).

d)β-adrenergic receptor function and signalling

The β-ARs are co-expressed in many tissues througout the human body but usually in a specific tissue one receptor type will predominate. In cardiac tissue the β_1-AR is predominantly expressed but the β_2-AR also plays important functional roles. The β_2-AR is predominantly expressed in respiratory airways and lung to control bronchial relaxation. The β_3-AR has important functions in adipose tissue and

lypolysis regulating many metabolic processes. The β_2-AR again has a role to play in this area of body metabolism (see Sections 1.7 a-e).

All three subtypes are coupled to adenylyl cyclase through the stimulatory G protein G_s to stimulate the generation of the second messenger cAMP, and this has been documented in Section 1.4. In heart the β_2-AR also regulates L-type Ca^{2+} channels possibly through G_i and stimulates phospholipase A_2 mediated by arachadonic acid (Skeberdis et al., 1997; Pavoine et al., 1999) (see Section 1.7b for details).

1.3 Guanine Nucleotide Binding Proteins (G proteins)

a) Introduction

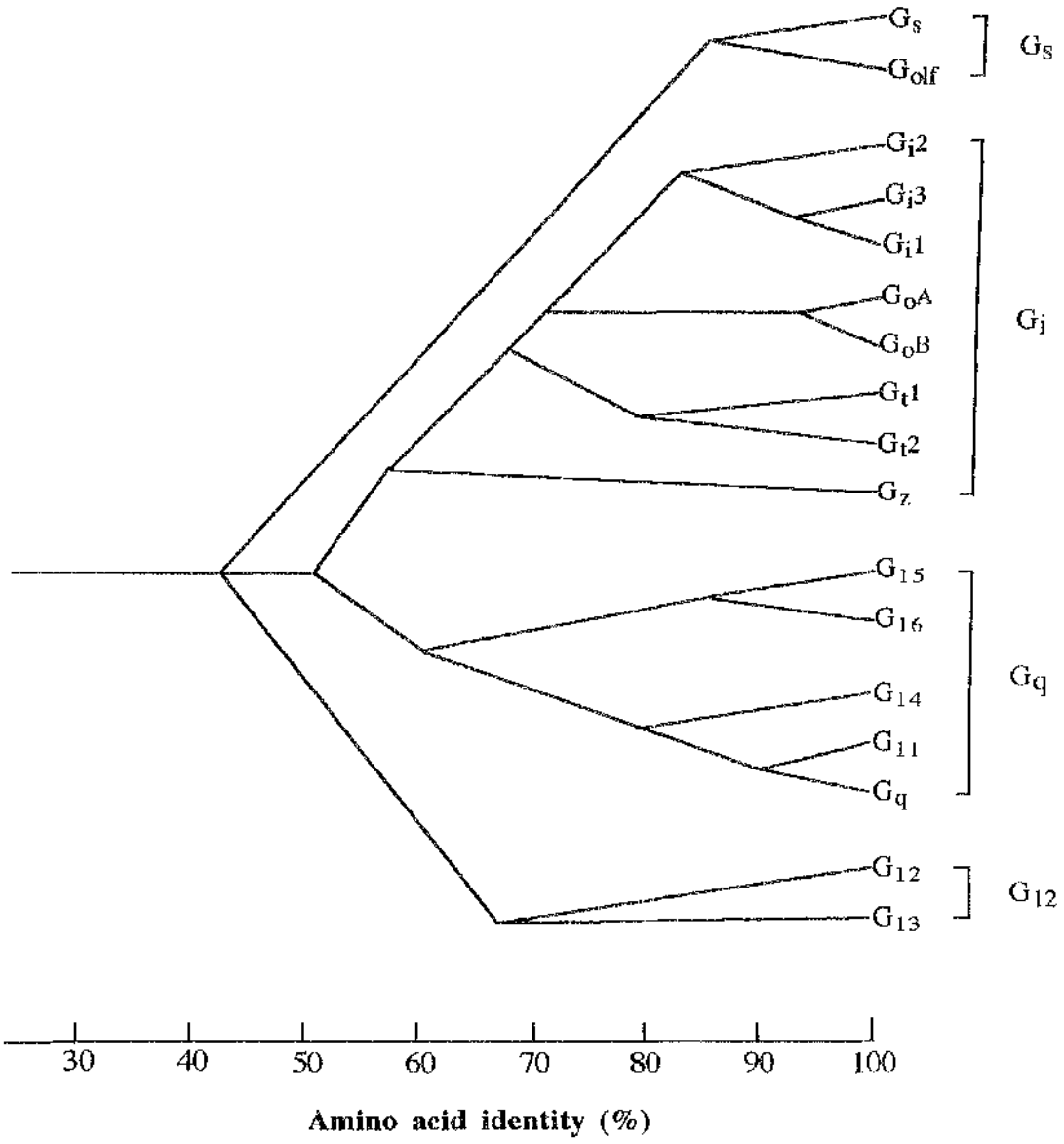
In 1971 Rodbell et al., discovered that hormone activated receptor linked signal transduction had a requirement for GTP. Study of these GTP dependent processes has revealed a protein component which binds and hydrolyses GTP (G proteins) to link ligand/hormone bound receptor to effectors which generate recognisable intracellular signals. These activate the appropriate cellular processes to achieve a specific cellular response. G proteins are therefore, signal transducers and those that couple to GPCRs consist of an α-subunit (39-52 kDa), which contains the guanine nucleotide binding site and intrinsic GTPase activity, and two tightly associated β (35–36 kDa) and γ (6-10 kDa) subunits forming a $\beta\gamma$ subunit complex (Gilman, 1987).

Figure 1.2

G protein α subunit amino acid identity

The relationship among mammalian Gα subunits is displayed. The α subunits are grouped by amino acid sequence identity and define four distinct classes of Gα subunits. The splice variants of $G_s\alpha$ are not shown (Simon et al., 1991).

Figure 1.2


G_s
G_{olf}
G_s
G_i2
G_i3
G_i1
G_oA
G_oB
G_t1
G_t2
G_z
G_i
G_{15}
G_{16}
G_{14}
G_{11}
G_q
G_q
G_{12}
G_{13}
G_{12}
30
40
50
60
70
80
90
100
Amino acid identity (%)

b) G protein subunits

More than 20 different G protein α subunits have been identified corresponding to 16 gene products divided into four functionally different classes termed G_s, G_i, G_q and G_{12} (Figure 1.2). All α subunits share sequence homology to a greater or lesser degree which reflects the similar function of these proteins. The G_s family includes $G_s\alpha$ and $G_{olf}\alpha$, and mediates the hormonal stimulation of adenylyl cyclase and closing of Ca^{2+} channels. The $G_i\alpha$ family include $G_i\alpha$ (α_{i1}, α_{i2}, α_{i3}), (Jones and Reed, 1987), $G_t\alpha$ (α_t and α_{t2}) (Lochrie et al., 1985; Tanabe et al., 1985; Yatsunami and Khorana, 1985; Medinski et al., 1985), $G_o\alpha$ (α_{oA}, α_{oB}) (Hsu et al., 1990; van Dongen et al., 1988), $G_{gust}\alpha$ and $G_z\alpha$. $G_i\alpha$ is generally involved in the inhibition of adenylyl cyclase and opening of K^+ channels, $G_t\alpha$, the rod outer segment G protein mediates the stimulation of cGMP phosphodiesterase and $G_o\alpha$, mediates Ca^{2+} channel closure and inhibition of phosphoinositide (PI) turnover. It is unclear which effectors are mediated by $G_{gust}\alpha$ but it is involved in recognition of taste, and $G_z\alpha$ has no clear function as yet but may inhibit type I and V adenylyl cyclases (Taussig and Gilman, 1995). The G_q family includes $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$, $G_{15}\alpha$ and $G_{16}\alpha$ (Strathmann and Simon, 1990; Simon et al., 1991;Wilkie et al., 1991) and are predominantly coupled to the stimulation of PI turnover. The last class contains $G_{12}\alpha$ and $G_{13}\alpha$ as members (Strathman and Simon, 1990).

In addition to the diversity among α chains, there are also multiple genes encoding at least 6 Gβ and 12 Gγ subunits. Five of the Gβ subunits share around 80% amino acid homology with the differences spread throughout their ~340 amino acid length (Simon et al., 1991; Watson et al., 1996). $G_{\beta 5}$ has only 53 % amino acid identity with other Gβ subunits. The 12 Gγ subunits share little sequence identity (Ray et al., 1996). These subunits tightly associate as a dimer complex which is thought of as a single functional monomer because the two subunits cannot be dissociated except with denaturants. If the known Gβ and Gγ subunits could combine to form Gβγ

dimers, there would be 72 potential combinations (Neer, 1997). However, Gβ and Gγ subunits show certain allowed associations in cells (Schmidt et al., 1992; Pronin et al., 1992; Muller et al., 1993; Yan et al., 1996).

c) Structural features of G proteins

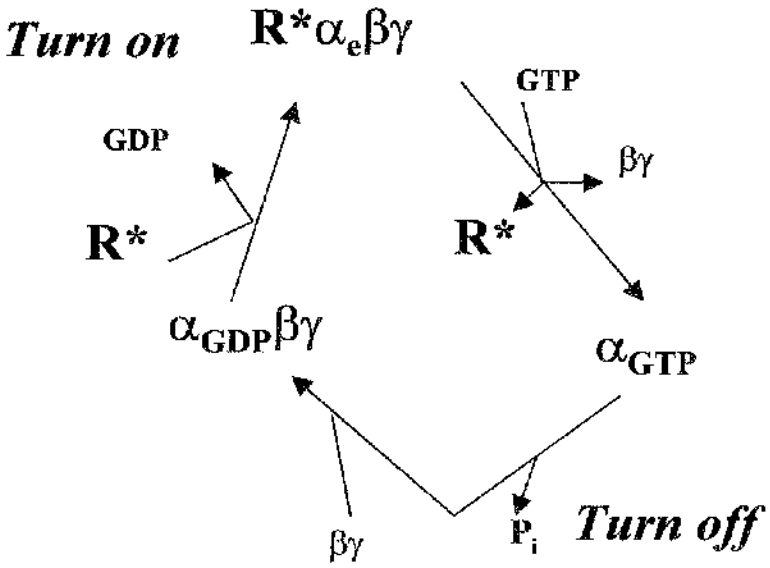
This super family of GTP hydrolases share a common structural core exemplified by a particular G protein $p21^{ras}$ (Ras). At the core of every G protein is the guanine nucleotide-binding site which binds and hydrolyses GTP to GDP. Both substrate and product stably bind the G protein. It is the α sub-unit of the G protein which contains this catalytic core able to hydrolyse GTP to GDP. It consists of 5 α helices surrounding a 6-stranded β-sheet and contains the consensus sequences involved in GTP binding. These bind the phosphate group and guanine ring of GTP. Also present in the core is a binding consensus site for Mg^{2+}, essential for catalysis (Sprang, 1997). The β subunit has an N-terminal helix followed by a repeating module of seven similar β-sheets, each with four antiparallel strands, that form the blades of a β-propeller structure. The γ subunit contains two helices and no inherent tertiary structure. The N-terminal helix of the γ subunit forms a coiled-coil with the N-terminal helix of the β subunit, whereas the remainder of this subunit interacts extensively with the β-propeller (Lambright et al., 1996; Sondek et al., 1996). The βγ complex acts as an inhibitor of GDP to GTP exchange at the α subunit and interaction occurs at two distinct interfaces. However, upon receptor activation by agonist ligand a conformational change occurs in the α subunit reducing its α helical content, as demonstrated for the $G_i\alpha$ subunit (Tanaka et al., 1998). Lambright et al., (1996) firstly demonstrated this conformational change in a refined 2.0 Å crystal structure of the $G_t\alpha$ subunit at two switch regions (I and II) but not in its βγ counterpart. Figure 1.3 demonstrates the switching on and off of G protein in the G protein cycle.

Figure 1.3

The G protein cycle

The GTPase cycle of trimeric G proteins. The 'turn-on' step begins when the activated receptor (R*) associates with the trimer ($\alpha GDP\beta\gamma$), causing dissociation of GDP. Then GTP binds to the complex of R* with the trimer in its 'empty' state ($\alpha_e\beta\gamma$), and the resulting GTP-induced conformational change causes αGTP to dissociate from R* and form $\beta\gamma$. After the 'turn-off' step (hydrolysis of bound GTP to GDP and inorganic phosphate P_i), αGDP reassociates with $\beta\gamma$ (from Iiri et al., 1998).

Figure 1.3



In order for G protein to couple to receptor it needs to be anchored to the membrane in some way. This is achieved by lipid modification with either myristate and/or palmitate (Casey, 1994, 1995; Milligan et al., 1995b; Ross, 1995). Only some G protein α subunits are co-translationally myristoylated (eg, $G_i\alpha$ and $G_o\alpha$). It has been debated whether this is merely an anchoring lipid or if it has a different regulatory role to play as has been proposed for the post-translational modification of palmitoylation. Palmitoylation occurs on all G protein α subunits (except α_t). As it is dynamic and appears to have a regulatory role in β_2-AR signalling it may also play a regulatory role at the level of the G protein.

d) Activation of $G_s\alpha$

As this study is concerned with β-AR signalling and its effects on adenylyl cyclase regulation, this section will concentrate on coupling of β-ARs to the stimulatory G protein $G_s\alpha$, which regulates the effector molecule adenylyl cyclase. However the $\beta\gamma$ subunit can induce certain effects acting synergistically with some forms of adenylyl cyclase. Most cells express two forms or splice variants of $G_s\alpha$, these being the long and the short forms ($G_s\alpha_L$ and $G_s\alpha_S$), with molecular weights of 45 and 42 kDa on SDS-PAGE gels, respectively. They differ by a 15 amino acid insert between the Ras-like domain and the α-helical domain and there is an exchange of Glu for Asp at position 72 of the polypeptide (Bray et al., 1986; Robishaw et al., 1986). Studies have been performed to determine if the long and short forms differentially interact with either receptors or adenylyl cyclase. Yagami, (1995) showed that a β-AR agonist/receptor complex catalysed the exchange from GDP to GTP on $G_s\alpha_L$ but not on $G_s\alpha_S$. It was also demonstrated that the glucagon receptor in rat liver shared $G_s\alpha_L$ with the β-AR but also coupled to $G_s\alpha_S$ (Yagami, 1995). As the guanine nucleotide site is between these two domains this change in the linker size may have an influence on the kinetics of these two splice variants. This indeed was found to be the case for

the β_2-AR signalling through these splice variant forms (Seifert et al., 1998). Fusion proteins between the β_2-AR and either $G_s\alpha_L$ or $G_s\alpha_S$ were used to study GPCR/G protein coupling in a one:one ratio. It was observed that the β_2-AR-$G_s\alpha_L$ form exhibited properties of a constitutively active GPCR.

e) G-protein stoichiometry

The IP prostanoid receptor is another GPCR which also stimulates production of second messenger cAMP. From studies on this receptor endogenously expressed in NG108-15 cells Kim et al., (1994) estimated the copies per cell of each member or the IP receptor/$G_s\alpha$/adenylyl cyclase signalling cascade to measure the stoichiometrical ratio of these proteins. Kim et al., (1994) found that for 100 000 copies of receptor per cell there were at least 10 times more $G_s\alpha$ molecules (1 250 000) but only 17 500 copies of adenylyl cyclase per cell. Therefore, adenylyl cyclase appears to be a limiting factor in the $G_s\alpha$/adenylyl cyclase cascade.

f) $G_i\alpha$, $G\beta\gamma$ and other effector pathways

The β_2-AR is not exclusively coupled to $G_s\alpha$ but can also couple to the inhibitory G protein to induce effects on other effector pathways. Daaka et al., (1997) presented a model for β_2-AR-mediated G protein switching to activate MAP kinase. Once β_2-AR stimulation induces activation of PKA, a negative feedback occurs where PKA phosphorylates the β_2-AR and uncouples it from $G_s\alpha$. The receptor is then able to couple to $G_i\alpha$, releasing $\beta\gamma$ subunits which then activate MAP kinase (Crespo et al., 1995; Daaka et al., 1997). A similar mechanism occurs in heart for PLA_2 activation where PKA activated by both β_1 and β_2 -ARs phosphorylates and uncouples the β_2-AR from $G_s\alpha$. Activation of PLA_2 can then occur (Pavoine et al., 1999). The β_2-AR

can also activate L-type Ca^{2+} channels but there is debate as to whether the channel is activated by PKA phosphorylation (Skeverdis et al., 1997) or is regulated through $G_i\alpha$ (Xiao et al., 1995). Other reports demonstrate the regulation of the β_2-AR by $G_i\alpha$ (Kuschel et al., 1999; Zou et al., 1999).

1.4 Adenylyl Cyclase

a) General overview

The second messenger cyclic 3', 5' adenosine monophosphate (cAMP) has played a central role in hormone signalling since the late 1950s when it was discovered by Sutherland et al., (1957). The adenylyl cyclases are the family of enzymes that convert intracellular ATP to cAMP. This is a multi-gene family (Table 1.1 modified from Houslay and Milligan, 1997) of which there are at least 9 isoforms (Taussig and Gilman, 1995), some of which have splice variant forms. The first adenylyl cyclase isoform (AC I) was purified from bovine brain using a forskolin affinity resin by Krupinski et al., (1989). Forskolin is a diterpene molecule that directly binds and activates all adenylyl cyclase isoforms except type IX (Yan et al., 1998). Six additional full length isoforms (ACs II - VI and VIII) were identified by application of low stringency hybridisation and PCR techniques (Feinstein et al., 1991; Bakalyar et al., 1990; Gao and Gilman, 1991; Katsushika et al., 1992; Premont et al., 1992; Yoshimura et al., 1992; Cali et al., 1994). Type VII adenylyl cyclase, identified by Krupinski et al., (1992), is a partial sequence of novel isoforms. All adenylyl cyclase isoforms have molecular weights of ~120 000 Da. Varied activation of adenylyl cyclases by both α and $\beta\gamma$ subunits of G proteins occurs upon agonist stimulation of GPCRs. Following generation of cAMP, activation of protein kinase A (PKA) occurs. This enzyme phosphorylates many cellular substrates to cause the onset of a

variety of molecular pathways to produce the desired physiological or biochemical responses within the cell.

b) Structure of adenylyl cyclases

All nine isoforms of adenylyl cyclase share a common structure of two transmembrane regions M_1 and M_2, and two cytoplasmic regions C_1 and C_2 (Krupinski et al 1989 Taussig and Gilman, 1995; Hurley, 1999). The transmembrane domains are each composed of 6 spanning α helices to form a common double motif. M_1 and M_2 are linked by the C_1 region and the C-terminal tail of the protein contains the C_2 region (Figure 1.4a from Houslay and Milligan, 1997). Unlike the GPCR family, it is the intracellular regions of adenylyl cyclases, C_1 and C_2, that show high homology between family members. In the GPCR family it is the transmembrane regions that are highly conserved between family members. The cytoplasmic regions provide the active site(s) and catalytic activity of the protein. Unlike C_1, the C_2 region of adenylyl cyclase exhibits catalytic activity but this is enhanced in the presence of C_1. It appears that the C_1 and C_2 regions form a heterodimer to form the active site(s) (Figure 1.4b). Two intensely hydrophobic pockets are formed at the end of each binding pocket when forskolin binds, acting as a glue to join together the two cytosolic subunits. The endogenous cellular substrate for this enzyme is ATP and this molecule has been shown to bind in this cleft by mutagenesis studies (Liu et al., 1997; Tang et al., 1995; Yan et al., 1997; Tucker et al., 1998; Sunahara et al., 1998). The ATP binding pocket is lined by hydrophobic residues of C_2, which pack around the purine ring, and charged residues of C_1 which interact with phosphate groups of ATP. Mg^{2+} is essential for this interaction and conversion of ATP to cAMP (Pieroni et al., 1995; Zimmermann et al., 1998).

Table 1.1 Differential regulation of the multiple forms of adenylyl cyclases

			← Regulators →				
Adenylyl Cyclase	Splice Variants	Chromosome Location	Ca^{2+}	G protein $\beta\gamma$	G protein αG_i	PKC	PKA
ACI		7p12-7p13	↑	↓	↓	↑	?
ACII		5p15.3	nc	↑(+ αG_s)	nc	↑	?
ACIII		2p22-2p24	↑(+ αG_s)	nc	?	↑	?
ACIV		14q11.2	nc	↑(+ αG_s)	nc	nc/↓	?
ACV	2	3q13.2-3q21	↓	nc	↓	nc/↑	↓
ACVI	2	12q12-12q13	↓	nc	↓	nc/↑	↓
ACVII		16q12-16q13	nc	(↑)	?	(↑)?	?
ACVIII		8q24.2	↑	(↓)	(↓)	?	?
ACIX		?	↓	nc	?	?	?

↑, signifies increase in activity; ↓, signifies decrease in activity; nc, signifies no change in activity; (+ αG_s), signifies synergism seen with αG_s.

Figure 1.4

a) A general topographical representation of adenylyl cyclases

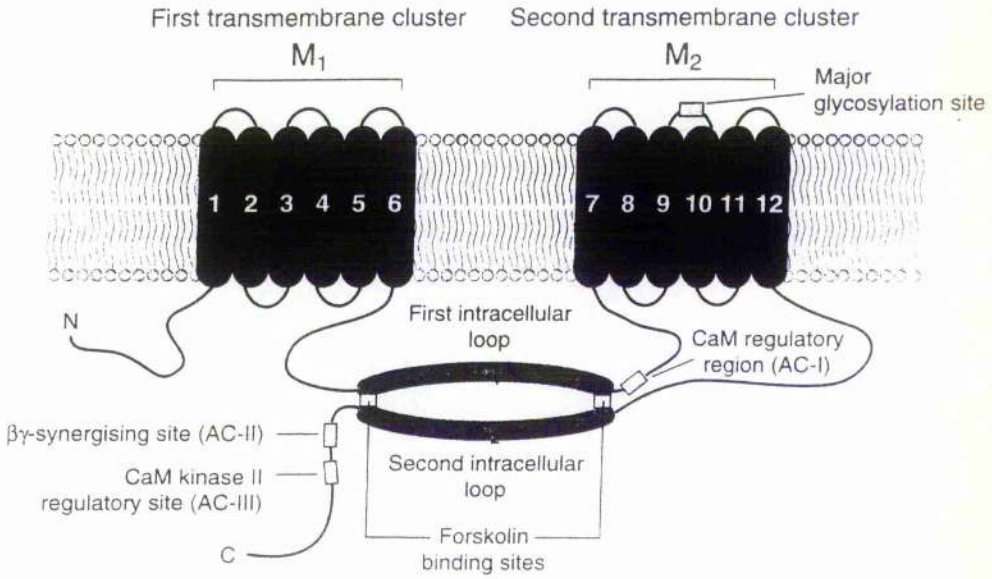
The putative structure of adenylyl cyclase has been deduced from sequence analysis, implying 12 transmembrane helices, and from functional studies that locate the catalytic and various regulatory sites to the intracellular cytosolic-located regions of the molecule (adapted from Houslay and Milligan, 1997).

b) The active site of adenylyl cyclases

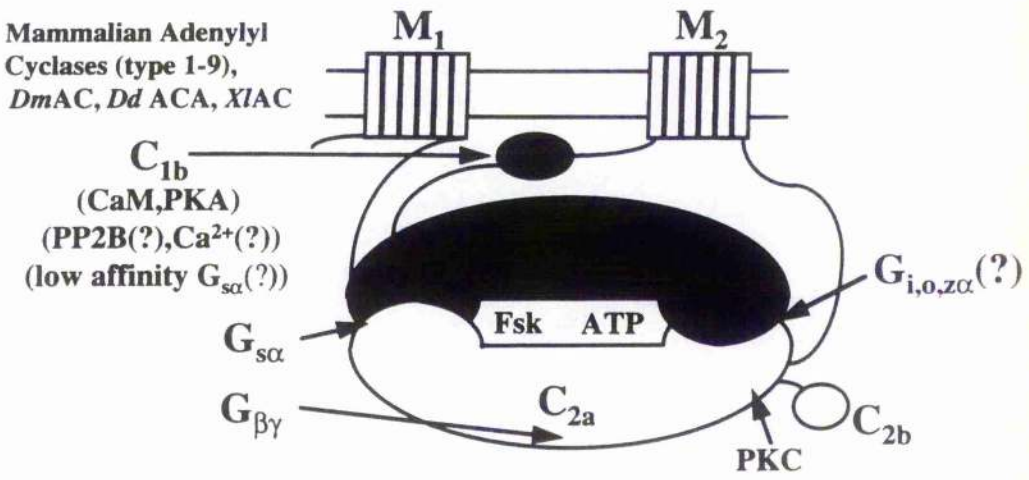
Regions C_{1a} and C_{2a} of adenylyl cyclases bind to form the active site for catalysis and binding of the regulators Fsk (forskolin) or ATP.

Figure 1.4

a)


First transmembrane cluster
Second transmembrane cluster
M1
M2
Major glycosylation site
1 2 3 4 5 6
7 8 9 10 11 12
N
First intracellular loop
CaM regulatory region (AC-I)
βγ-synergising site (AC-II)
CaM kinase II regulatory site (AC-III)
Second intracellular loop
Forskolin binding sites
C

b)


Mammalian Adenylyl Cyclases (type 1-9), DmAC, Dd ACA, XlAC
M1
M2
C1b
(CaM,PKA)
(PP2B(?),Ca2+(?))
(low affinity Gsα(?))
Gi,o,zα(?)
Fsk ATP
Gsα
Gβγ
C2a
C2b
PKC

c) Activation by G-protein subunits

Agonist activation of GPCRs induces activation of G proteins which in turn cause dissociation of βγ subunits from GTP bound α subunits. GTP-$G_s\alpha$ binds to and regulates all adenylyl cyclase isoforms at a crevice on the outside of C_2 and the N terminus of C_1. This binding can be similar to the "glue-like" mechanism of forskolin, but GTP-$G_s\alpha$ also appears to bind and regulate adenylyl cyclase by another mechanism involving a conformational change in the protein to allosterically regulate it (Yan et al., 1997; Tesmer et al., 1997). $G_i\alpha$ selectively inhibits adenylyl cyclase types V and VI and it may bind in a groove pseudo symmetrically related to the $G_s\alpha$ binding groove (Yan et al., 1997; Tesmer et al., 1997). The Gβγ subunit conditionally regulate several adenylyl cyclases including the type II isoform. Its binding site does not overlap with the $G_s\alpha$ binding site, consistent with the observation that Gβγ activates type II adenylyl cyclase when $G_s\alpha$ is bound (Chen et al., 1995).

d) Other regulatory mechanisms of adenylyl cyclases

The effects of Ca^{2+} on adenylyl cyclase isoforms are varied depending on which isoform is being targeted and what concentration of Ca^{2+} is being used. Types I, VIII and to some extent type III are markedly stimulated by nM concentrations of Ca^{2+}/calmodulin. The other isoforms (II, IV, V and VI) are insensitive to calmodulin. However, high concentrations of Ca^{2+} (100-1000 μM) result in inhibition of adenylyl cyclase isoforms due to competition for Mg^{2+} which is essential for catalysis (Taussig and Gilman, 1995).

Phosphorylation is a mechanism of regulation commonly exhibited by an extensive range of molecular signalling pathways. Adenylyl cyclases are indirectly regulated by

the phosphorylation of stimulated GPCRs by the second messenger activated and agonist-dependent kinases that phosphorylate these receptors during desensitisation (detailed in Section 1.6 b and c). The possibility of PKA phosphorylation of adenylyl cyclases as a negative feedback inhibition has been investigated but to little avail (Premont et al., 1992). PKC phosphorylation of adenylyl cyclases has been studied in more detail. Apparently PKC activates the enzymatic activity of type II and V adenylyl cyclases (Jacobowitz et al., 1993; Yoshimura et al, 1993; Lustig et al., 1993; Kawabe et al., 1994).

1.5 Receptor Activation Models

a) Constitutively active mutant receptors

It is well documented and accepted that agonist bound GPCR has a conformation or structure which is in an activated state, able to couple to its cognate G protein and induce activation of effector and second messenger production. For the last two decades it has been debated whether agonist binding induces a change in receptor conformation (Okuma et al., 1992) or if agonist binds to an already active receptor conformation (Leff, 1995). In favour of the theory proposed by Leff is the finding that certain GPCRs can couple to G proteins and induce second messenger production in the absence of agonist. These are termed constitutively active mutant (CAM) GPCRs. The agonist-independent activity of these GPCRs can be lowered by addition of inverse agonists indicating that these receptors are precoupled to G protein in the absence of agonist. They can also be further stimulated by addition of agonist. Therefore, GPCRs must exist together as inactive and active forms. Studies on these CAM GPCRs has led to better understanding of mechanisms by which GPCRs function. Secondly, various activated conformations for one GPCR may occur to

induce differential effector outputs (Kenakin, 1995; Leff et al., 1997). Several theories of receptor activation have been proposed and are summarised.

b) Two state receptor theory

This receptor theory has developed from consideration of ion channel states. The active state of the channel is one that allows the flow of ions and the inactive state is the closed channel. Therefore, it is predicted that all GPCRs exist as an equilibrium between two receptor conformations, i.e. an inactive form (R) and an active form (R*) (Leff, 1995). In a resting system the equilibrium between R and R* defines the basal activity of the effector cascade. Effective activation of G protein only occurs through R*. The position of the equilibrium between R and R* varies with individual receptors and is altered by the presence of receptor ligands (Figure 1.5 modified from Milligan et al., 1995). Agonists possess increased affinity for R* and, therefore, bind and stabilise this form to activate G protein and effector. Inverse agonists have the opposing property of binding and stabilising the R form to often reduce basal effector output. Antagonists bind GPCRs, but do not preferentially stabilise either form of the receptor. Figure 1.6a shows the two-state scheme of receptor activation by Chidiac et al., (1994). The influence of any auxiliary proteins is not directly considered in this model.

c) Ternary complex model

Unlike the two-state receptor theory, the ternary complex model takes into consideration the role of the coupling G protein in receptor activation. The receptor is also considered as a flexible entity since it is capable of producing more than one effect within the cell (DeLean et al., 1980; Costa et al., 1992). Figure 1.6b shows

that the receptor itself or agonist bound receptor can form complexes with G protein. The resulting agonist/receptor/G protein complex is the ternary complex. This model was revised to take into account the two-state receptor theory (Samama et al., 1993). In this model GPCRs still resonate between R and R* conformations and agonists do not directly drive the inactive receptor to assume the active conformation. In addition a transient R*-G complex occurs, whose formation is promoted by the binding of agonists to form a ternary complex of HR*-G where H represents agonist/hormone (Figure 1.6c). In this circumstance the GPCR is an allosterically regulated enzyme. The action of agonist increases the affinity of the receptor for the G protein and inverse agonists inhibit the formation of these complexes. Gardner, (1995) has described the flexibility of this model to demonstrate that agonists affect the affinity of the receptor for the G protein rather than the proportion of the receptors distributed between R and R*.

d) Three-state receptor model and the cubic ternary complex model

Study of GPCRs has indicated that they can exhibit altered pharmacology and can undergo differential coupling to multiple effector pathways. An agonist can act strongly through one arm of a signalling pathway but act as an inverse agonist in the other. Leff et al., (1997) have revised their two-state receptor model and propose a three-state receptor model in which two active conformations of the receptor exist, each being able to couple to a different G protein and effector pathway. They argue that in the two-state model of receptor activation, if the receptor is able to activate two different G proteins this would not allow for the altered pharmacology of receptors through different pathways. Therefore, in order for receptor promiscuity to result in altered agonist pharmacology it is necessary to propose more than one active receptor state. The three-state receptor model is shown in Figure 1.7a and a full explanation and derivation of the model is presented by Leff et al., (1997).

Figure 1.5

Ligands that bind to GPCRs

The diagram shows the position of the equilibrium between an inactive state R and an active state R* which varies with individual receptors and is altered by the presence of receptor ligands. Agonists function by stabilising R* while inverse agonists preferentially stabilise R. A continuum of ligands between full agonists and full inverse agonists is expected to exist, with antagonists being able to bind to receptor but having no preference for R or R*, or effect on equilibrium between these two forms.

Figure 1.5

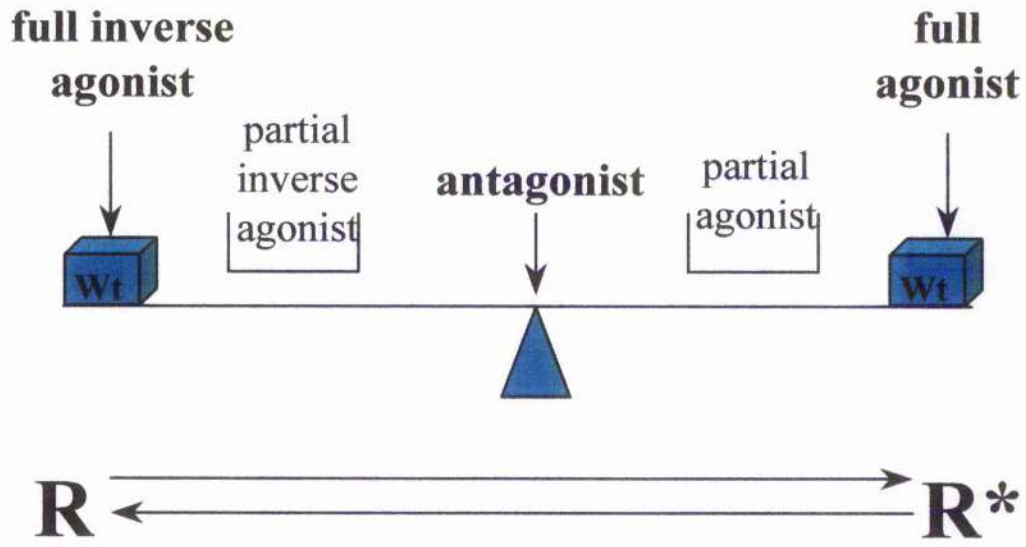

full inverse agonist
partial inverse agonist
antagonist
partial agonist
full agonist
Wt
Wt
R
R*

Figure 1.6

a) The two sate receptor theory

This depicts a system wherein a receptor spontaneously assumes either an inactive state (R) or and active state (R*), with the equilibrium between these being described by a unimolecular constant K_S ([R*]/[R]). A given ligand (H) binds to R and R*, to yield the association constants K_H (ie [HR]/[H][R]) and αK_H (ie [HR*]/[H][R*]), respectively; H is assumed to have no other effect on either R or R*. If $\alpha > 1$, then H binds with greater affinity to the active form of the receptor and increases the number of receptors in the active state (ie [R*] + [HR*]), thereby stimulating the activity of the receptor (ie H is an agonist). If $\alpha < 1$, the number of receptors in the inactive state is increased by H (ie H has a negative intrinsic activity). The influence of any auxiliary proteins is not directly considered.

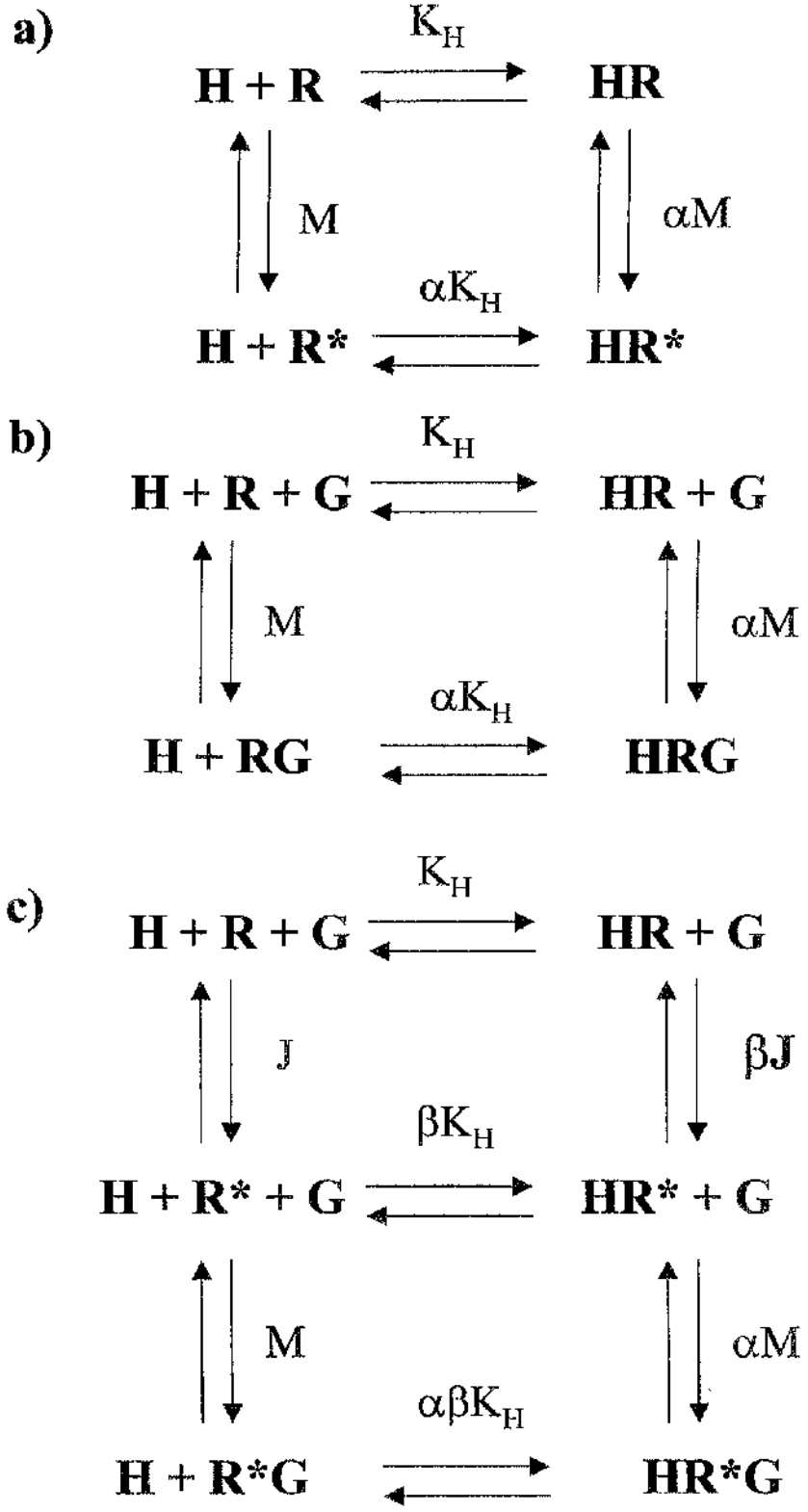
b) The ternary complex model

This model takes into account the role of the coupling G protein in receptor activation as the presence of guanine nucleotide appears to convert the receptor from a high to low affinity state. The receptor is considered as a flexible entity since it is capable of producing more than one effect within the cell. The scheme shows M as the affinity of R (receptor) for G (G protein), α as the efficacy of the ligand and K the receptor affinity of the ligand. K = [HR]/[H][R], M = [RG]/[R][G], α = [HRG][R]/[HR][RG].

c) The revised ternary complex model

This shows the extended or allosteric ternary complex model. This model introduces an explicit isomerisation step regulating the formation of the state of the receptor from R to R*, which is capable of binding to the G protein. J represents an equilibrium constant in receptor isomerisation. J = [R*]/[R], K = [HR]/[H][R], M = [R*G]/[R*][G], α = [HR*G][R*]/[HR*][R*G], β = [HR*][R]/[HR][R*].

Figure 1.6


a)
K_H
H + R
HR
M
αM
$αK_H$
H + R*
HR*
b)
K_H
H + R + G
HR + G
M
αM
$αK_H$
H + RG
HRG
c)
K_H
H + R + G
HR + G
J
βJ
$βK_H$
H + R* + G
HR* + G
M
αM
$αβK_H$
H + R*G
HR*G

In fact it has been proposed that a GPCR may have several active receptor states coupling to different G proteins (Kenakin, 1995). Kenakin proposed the theory of "agonist trafficking" in which each agonist may be able to select or activate its own specific active receptor state. These receptor states may possess differential ability to activate subsequent signalling pathways. Evidence for this idea comes from studies on the α_{1B}-AR, in which a single point mutation with the 20 different amino acids was carried out. All 20 mutant constructs were found to have different degrees of constitutive activity, the wild-type construct being quiescent (Kjelsberg et al., 1992). This indicated that the receptor could form many active states and that only one state is quiescent (ie the wild-type).

The three-state receptor model has been developed further to accommodate for coupling of different receptor conformations to different G proteins and effector pathways (Zuscik et al., 1998). This was derived from studies on the β_2-AR, which is routinely classed as a receptor which couples to $G_s\alpha$ and downstream cAMP production. Studies now indicate that the β_2-AR activates Na^+/H^+ exchange. A C116F mutant of the β_2-AR was engineered that exhibited the ability to constitutively activate Na^+/H^+ exchange while only maintaining competent coupling to cAMP. This indicated that this receptor could form multiple activation states that are G protein specific. A revised cubic ternary complex model was devised to explain the coupling of two distinct active receptor states coupling to two different G proteins (Figure 1.7b from Zuscik et al., 1998).

Figure 1.7

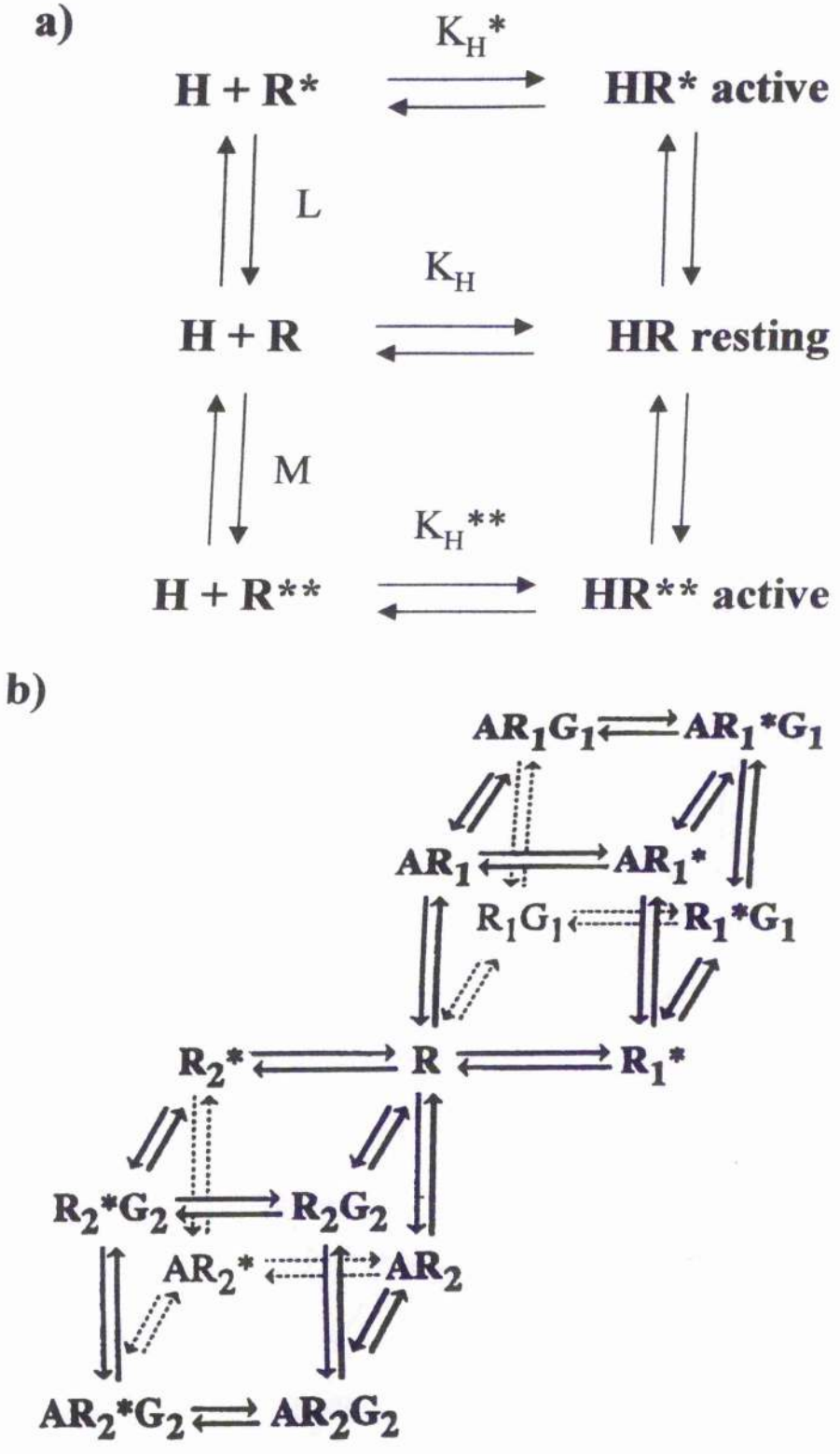
a) The three state receptor theory

It is hypothesised that the receptor exists in three states, an inactive or resting conformation, R, an active conformation, R*, which interacts with G protein, G1, and another active conformation, R**, which interacts with G protein, G2. In the absence of agonist/hormone H, the distribution of the receptors into the three states is governed by the equilibrium constants L = [R]/[R*] and M = [R]/[R**]. The activity of agonist is governed by the equilibrium dissociation constants K_H = [H][R]/[HR], K_H* = [H][R*]/[HR*] and K_H** = [H][R**]/[HR**], which determine its affinity for R, R* and R** respectively. The receptor is distributed amongst the three unoccupied and three occupied states, so that total receptor concentration is: R_{tot} = [R] + [R*] + [R**] + [HR] + [HR*] + [HR**].

b) The cubic ternary complex model

A single receptor can form two distinct activated conformations that selectively interact with different G proteins. A = agonist, R = receptor and G = G protein.

Figure 1.7


a)
K_H*
H + R*
HR* active
L
K_H
H + R
HR resting
M
K_H**
H + R**
HR** active
b)
AR_1G_1
AR_1*G_1
AR_1
AR_1*
R_1G_1
R_1*G_1
R_2*
R
R_1*
R_2*G_2
R_2G_2
AR_2*
AR_2
AR_2*G_2
AR_2G_2

e)The CAM-β_2-adrenergic receptor

The most studied CAM GPCR is a form of the human β_2-AR in which a short segment of the third intracellular loop was replaced with the corresponding region from the α_{1B}-AR (Samama et al., 1993; Samama et al., 1994). This work has stemmed from findings that the reciprocal mutation in the hamster α_{1B}-AR (part of the 3rd intracellular loop replaced by the corresponding region in the β_2-AR) produced a CAM form of this receptor (Cotecchia et al., 1990; Kjelsberg et al., 1992). As this receptor displays elevated basal adenylyl cyclase and GTPase activity, it has been investigated what properties determine the receptor's constitutive activity. From analysis on the structure of this CAM protein it was found that the same Cys which moves upon agonist binding to WT-β_2-AR is found closer to the ligand binding pocket than in the ligand-unoccupied WT receptor (Javitch et al., 1997).

Certain inverse agonists such as betaxolol and sotalol but not other β-blockers have been shown to reduce basal levels of CAM-β_2-AR induced cyclase activity. After long-term treatment of CAM-β_2-AR expressing cells with inverse agonists, an up-regulation of the receptor was detected and again this was not exhibited by other β-blockers (MacEwan and Milligan 1996a,b). As inverse agonists uniquely displayed these properties it was proposed that these ligands could potentially be put to therapeutic use in disease states.

1.6 Receptor Desensitisation Mechanisms

a) Introduction

Sustained exposure of cells expressing GPCRs to agonist ligands frequently results in a waning of response to the ligand. This effect is termed desensitisation and can be

viewed as a negative feed back loop mechanism, its role being to regulate the sensitivity of the receptor to the initial stimulus. For GPCRs desensitisation is a multi-step phenomenon (Lohse, 1993; Bohm et al., 1997). First, uncoupling of the receptor from the G protein occurs causing the receptor function to be desensitised. Sequestration of the receptor into an intracellular compartment then occurs followed by possible down-regulation, if the stimulation is chronically persistent. Down-regulation may involve a loss of receptor number due to degradation of the receptor protein and reduction of steady state mRNA. Desensitisation has been categorised as either homologous (agonist-specific) or heterologous (agonist-non specific).

b) Homologous Desensitisation

This phenomenon occurs only at receptors which have been stimulated by agonist (agonist specific) and consequently desensitises the subsequent response of the same receptor only. The proteins primarily found to be involved in homologous desensitisation are the G protein coupled receptor kinases (GRKs), of which there are currently six full length cDNAs encoding the members of the emerging GRK sub-family of Ser/Thr protein kinases (Table 1.2, Pitcher et al, 1998; Strader et al., 1995). The first GRK isolated was rhodopsin kinase (RK or GRK1). This GRK has limited tissue distribution being found in the retina of the eye. βARK1 (GRK2) and βARK2 (GRK3) are the β-adrenergic receptor kinases, originally given this name due to their initial characterisation as kinases able to phosphorylate and desensitise the β_2-AR (Fredericks et al., 1996) and the β_1-AR (Freedman et al., 1995). βARKs are not exclusive to the βAR system since they have also been reported to phosphorylate the muscarinic receptor (Kameyama, et al., 1993), the type 1A angiotensin receptor (Oppermann et al., 1996) and many other GPCRs. GRK4 has been reported to be found predominantly in the testis. Like GRKs 2 and 3, GRKs 5 and 6 are ubiquitously expressed. GRK5 has been reported to phosphorylate the β_2- and β_1-

Table 1.2 Molecular properties of G protein coupled receptor kinases (GRKs)

Family name	Common name	Membrane association	Tissue distribution	Chromosome Mapping	Features/ Regulation
GRK1	RK*	Farnesylation	Retina	13q34	Autophospho-rylation
GRK2	βARK1	$G_{\beta\gamma}$, acidic PL	Ubiquitous	11q13	PH domain, PKC, calmodulin
GRK3	βARK2	$G_{\beta\gamma}$ acidic PL	Ubiquitous	22q11	PH domain
GRK4	IT-11	Palmitoylation	Testis	4q16.3	Four splice variants
GRK5		PL binding	Ubiquitous	10q24-qter	Autophospho-rylation
GRK6		Palmitoylation	Ubiquitous	5q35	Calmodulin

*RK, rhodopsin kinase; PL, phospholipid; PH, plekstrin homology.

adrenergic receptors *in vitro* and *in vivo* (Fredericks et al. 1996; Rockman et al., 1996; Oppermann et al., 1996).

Structurally, GRKs contain a catalytic domain of 263 - 266 amino acids, flanked by a large N-terminal domain (~ 185 amino acids) with structural homology between GRK sub-types. The flanking C-terminal domain varies substantially between GRK sub-types. An interesting feature of GRKs 2 and 3 (βARKs) is their possession of a carboxy-terminal βγ binding domain (Inglese, et al., 1993). Other GRKs (RK and GRKs 4, 5 and 6) do not possess such a domain. Table 1.2 summarises the family of GRKs, their distribution and membrane association. The βγ binding domain recruits the kinase to the membrane through interactions with the βγ sub-units situated on the inner surface of the plasma membrane. Recent reports have shown this remarkable function of βγ sub-units to intimately link receptor activation to βARK-mediated desensitisation (Pitcher et al., 1992; Koch et al., 1994; Chen et al., 1995; Koch et al., 1993). Fushman et al., (1998) have examined this region and identified it as a pleckstrin homology (PH) domain, conferring binding specificity to βγ proteins, assisting recruitment of the protein to the plasma membrane. The solution structure and dynamics of the PH domain of GRK2 (βARK 1) show that it is capable of protein/protein interactions with βγ sub-units (Fushman et al., 1998). On binding of agonist, the receptor adopts the appropriate conformation releasing βγ subunits from the α subunit, both these factors in combination facilitate recruitment of the βARK and phosphorylation of the receptor. Since only the agonist-occupied or stimulated receptor has the appropriate conformation for the GRK to bind, this creates an agonist specific mechanism.

In the case of rhodopsin kinase, this protein has been shown to be unique in that it is the only GRK thus far reported to be isoprenylated (farnesylated) which facilitates anchorage of the kinase to the plasma membrane. Isoprenyl moieties are present on βγ subunits also, the γ subunit containing the geranyl-geranyl type of moiety. Studies

have reported that distinct β and γ subunit isoforms are present in G-proteins that may show a certain specificity for different GRKs (Schmidt et al., 1992; Pronin et al., 1992; Muller et al., 1993; Yan et al., 1996). This may be a possible means of directing different enzyme isoforms to different receptors although this has not been conclusively proved. GRK5 carries out its phosphorylation of the β-AR involving recruitment to the plasma-membrane through its phospholipid (PL) binding domain. The sites of phosphorylation in the β-AR by both GRK2 and GRK5 have been investigated extensively over the past decade and as yet there is still debate as to whether the sites identified *in vitro* are the sites of *in vivo* phosphorylation. It has even been postulated that additional or new sites of phosphorylation could be present in this receptor. Location of GRK2 and GRK5 phosphorylation sites within the extreme C-terminal tail (last 40 residues) of the β_2-AR have revealed that all are either Ser or Thr residues (Fredericks et al., 1996). However, from *in vivo* studies these residues do not appear to be important for desensitisation (Seibold et al., 1998). It has been suggested that four other residues in the C-terminal tail out with the last 40 amino acid residues investigated may be targets for *in vivo* phosphorylation. Hausdorff et al, (1991) have studied a mutant β_2-AR only containing mutations at these four amino acids (Ser 355, 356, 364 and Thr 360), and found that the *in vivo* sites of GRK phosphorylation could be among these.

GRK phosphorylation in isolation results in minimal desensitisation (Hausdorff et al., 1989) but proteins which assist in this desensitisation by binding to the phosphorylated receptor have been discovered. These are the functional co-factor arrestin proteins. Two arrestins, S arrestin (or arrestin) and cone arrestin are found in retina and are similar in substrate specificity (i.e. rhodopsin) (Craft et al., 1994). β-arrestin-1 and β-arrestin 2 were discovered following the revelation that the ability of βARK1 to desensitise the β_2-AR is reduced during purification. This indicated a loss of a co-factor required for efficient desensitisation and subsequently β-arrestin 1 and β-arrestin 2 were identified in a variety of tissues (Parruti et al., 1993). Binding of

these arretins to phosphorylated receptors in a 1:1 ratio disrupts the interaction of receptor with G protein and therefore, promotes homologous desensitisation (Lohse et al., 1993). In the β_2-AR system, β-arrestin increases βARK1 mediated desensitisation by ~10 fold (Pippig et al., 1993). It has also been reported that β-arrestins promote desensitisation of the β_1-AR (Freedman et al., 1995). In the basal state β-arrestin 1 is phosphorylated but undergoes a dephosphorylation when binding to agonist bound receptor (Lin et al., 1997). It is not clear whether this phosphorylation precedes or follows receptor binding. Once bound the arrestin protein acts as a clathrin adapter to induce internalisation of the protein (see Section 1.6g on receptor sequestration).

c) Heterologous Desensitisation

In common with homologous desensitisation, heterologous desensitisation also involves a phosphorylation mechanism but, by different kinases i.e. the agonist non-specific, or second messenger activated kinases, PKA and PKC (Chaung et al., 1996). Heterologous desensitisation, unlike homologous desensitisation, is a process whereby activation of one type of receptor causes desensitisation of other types of receptors also. For example, in the murine neuroblastoma x embryonic Chinese hamster brain NCB20 cells, transfected with the β_2-AR, stimulation with isoprenaline (a β-AR agonist) resulted in heterologous desensitisation of the endogenously expressed IP prostanoid receptor (Mullaney et al., 1995). In contrast exposure of cells to similar doses of iloprost (IP receptor agonist) did not result in any heterologous desensitisation of the β_2-AR. Therefore, not all GPCRs undergo heterologous desensitisation.

Distinct phosphorylation sites for second messenger activated kinases (PKA/PKC) have been investigated. Two consensus sites for PKA mediated phosphorylation

have been identified in the β_2-AR (Benovic et al., 1985; Hausdorf et al., 1989; Clark et al., 1989; Yuan et al., 1994). Firstly in the 3rd intracellular loop of the receptor (at serines 261 and 262) which is essential for G_s/receptor coupling and is the preferred site for PKA mediated phosphorylation. The second and less preferred site is in the N-terminal part of the C terminus (at serines 345 and 346), thought to play a part in receptor/G_s coupling. In fact PKC can phosphorylate these sites also. It appears that the number of consensus PKA phosphorylation sites present is correlated with the extent of heterologous desensitisation. The β_2-, β_1- and β_3-AR have two, one and no phosphorylation sites, respectively. β_3-ARs undergo little or no heterologous desensitisation whereas, the β_1-AR (Freedman et al., 1995) is intermediate and the β_2-AR undergoes extensive heterologous desensitisation. In this respect β_3-ARs can continue to respond to β-AR ligands if other co-expressed β-ARs are desensitised on stimulation by agonist.

e) Heterologous Regulation of Homologous Desensitisation

Previously it was considered that both homologous and heterologous desensitisation were completely separate events. This was shown in experiments on the PKA defective kin$^-$ S49 murine lymphoma cell line, which expresses the β_2-AR. Both wild type and kin$^-$ S49 cells express similar profiles of β-AR desensitisation of cAMP production and β_2-AR loss (Clark et al., 1988). The ability of the cAMP analogue 8-Br-cAMP to mimic the effect of adrenaline in decreasing β-AR agonist-induced adenylyl cyclase activity in membranes prepared from treated cells indicated that, along with the previous result, PKA is not involved in homologous desensitisation. However, Post et al., (1996) have demonstrated an important regulatory role for PKA in homologous desensitisation. By using a novel [^{3}H] forskolin binding assay which provides a direct means of measuring hormone-stimulated G_s/adenylyl cyclase interactions in both wild type and kin$^-$ S49 cells under desensitising conditions, it was

demonstrated that β_2-AR induced [^{3}H] forskolin binding decreased reflecting functional desensitisation of the β_2-AR pathway. The difference in the extent of desensitisation between the two cell types demonstrated a key role for PKA in homologous desensitisation.

The second messenger activated kinase, PKC, appears to regulate GRK expression in T cells but this appears to be a selective regulation among GRKs (De Blasi et al., 1995). Certain GRKs (GRK2) are phosphorylated by PKC which increases the activity of the GRK promoting increased desensitisation. Plekstrin homology domains may be the site of interaction between the GRK and PKC . Conversely, β-arrestin 1 expression is regulated by PKA through cellular levels of cAMP (Inglese et al., 1993; Chaung et al., 1996). Therefore, it can be concluded that second messenger activated kinases have various important regulatory roles to play in homologous desensitisation.

f) Involvement of Palmitoylation in Desensitisation

The importance of fatty acyl chains on signalling molecules has already been discussed when considering palmitoylation, a lipid modification of G proteins. This is a dynamic post-translational modification (Magee, 1990; Casey, 1994; Casey, 1995) and therefore, has the potential to be regulated. Most GPCRs are palmitoylated on Cys residues located in the proximal section of their C-terminal tail. This anchors the N-terminal portion of the cytoplasmic tail to the plasma membrane creating a fourth intracellular loop. Agonist exposure of the β_2-AR and the D1 dopaminergic receptor (Ng et al., 1994) alters this palmitoylated state and there is evidence that palmitoylation may effect the desensitisation of the receptor. One site of PKA phosphorylation is very close to the palmitoylated Cys, C341, on the β_2-AR and it has been proposed that depalmitoylation of the receptor may expose this site for

phosphorylation causing resultant desensitisation. In fact it has been demonstrated that the palmitoylated Cys 341 modulates phosphorylation of the β_2-AR by PKA (Moffett et al., 1996). This was indicated on finding that a C341G β_2-AR mutant which is not palmitoylated displays an elevated level of basal phosphorylation and a decreased rate of agonist-promoted desensitisation (Moffett et al., 1993). Therefore, it was proposed that concerted interactions between palmitoylation and phosphorylation could play an important role in regulation of β_2-AR function.

As documented earlier in this chapter (Section 1.3c) palmitoylation also plays a role in desensitisation at the level of the G protein Gα subunit (Milligan, 1993). All Gα subunits are palmitoylated and some have a requirement to be myristoylated. Palmitoylation of Gα subunits is also a dynamic process and therefore a site of regulation (Milligan et al., 1995b). After formation of the active α_s-GTP complex, depalmitoylation of the $G_s\alpha$ occurs releasing α_s from the membrane which may contribute to desensitisation of G protein signals.

A more recent report demonstrates that activation of a β_2-AR-$G_s\alpha$ chimera leads to rapid depalmitoylation and inhibition of repalmitoylation of both receptor and G protein (Loisel et al., 1999). By using a β_2-AR-$G_s\alpha$ chimera the receptor was permanently linked to $G_s\alpha$ and was therefore, in the active state (as if agonist bound) and could not be desensitised, internalised or down-regulated. Upon agonist activation both the receptor and $G_s\alpha$ portions of the chimera were depalmitoylated. However, forskolin activation of adenylyl cyclase did not induce depalmitoylation of the construct. Therefore, depalmitoylation is not regulated by second messenger production but by agonist activation. Repalmitoylation was therefore proposed not to occur at the active receptor/$G_s\alpha$ complex but at a later event in the pathway.

g) Receptor Sequestration

Once phosphorylated and uncoupled from G protein the β_2-AR receptor is found to internalise by a clathrin/dynamin mediated pathway assisted by β-arrestins which are clathrin adapters (Zhang et al., 1996, Lin et al., 1997). Originally, sequestration of GPCRs was thought to be a desensitisation mechanism since G_s was not found to be associated with receptors in the light membrane fractions and therefore, G protein/receptor coupling could not occur (Waldo et al., 1983). Evidence exists to disagree with this. Firstly, in most systems receptor sequestration is too slow compared to the rapid phosphorylation of receptor and uncoupling of G protein, and, hence, sequestered receptors will already have an impaired function (Roth et al., 1991). Secondly, in the majority of cell systems receptor sequestration is too limited to account for the extent of desensitisation observed (Lohse et al., 1990). In fact there has been an accumulation of evidence to indicate that sequestration is a mechanism for resensitisation of the receptor. This has been observed for the β_2-AR system where an agonist-induced internalisation of the receptor occurs (Yu et al., 1993; von Zastrow et al., 1994; Pippig et al., 1995). Removal of agonist or addition of antagonist causes redistribution of receptors to the cell surface indicating that the mechanism is agonist dependent. Following internalisation into endosomes/vesicles dephosphorylation of the β_2-AR by a vesicular membrane-associated form of the phosphatase PP-A2 occurs. This event only occurs under acidic pH and can be inhibited by NH_4Cl (Kreuger et al., 1997). The β_1-AR is also thought to internalise via a clathrin/dynamin dependent pathway but a recent study by Tang et al., (1999) have revealed a novel machanism for internalisation of this receptor. There is a 24 amino acid polyproline rich motif within the 3rd intracellular domain of the β_1-AR, and such motifs are known to mediate protein-protein interactions such as with Src homology (SH) 3 domains. An SH3 protein p4 was found to bind to the β_1-AR. These proteins also function sequentially in endocytic clthrin-coated vesicle formation and may act as an adapter protein by directing the β_1-AR to the endocytic machinery.

SH3/p4 has also been found to bind to dynamin *in vivo*. Therefore, the β_1-AR may be regulated by a different mechanism from the β_2-AR.

In parallel with the extent of heterologous desensitisation, the β_2-AR shows marked sequestration, whereas the β_1- and β_3-subtypes do not. These agonist regulated receptors may share or utilise the same endocytic machinery that mediates the constitutive endocytosis of a variety of receptors. How these receptors are targeted into the cell has been suggested by the finding of a conserved NPXXY motif present in many GPCRs which is homologous to constitutive endocytosis signals.

h) Down-regulation

Following chronic, long term exposure of cells to agonist, a ligand-dependent reduction of total receptor number may occur. This phenomenon is termed down-regulation and has been investigated quite rigorously over the past decade (Hadcock and Malbon, 1988a,b; Molenaar et al., 1990; Kompa et al., 1992; Gagnon et al., 1998; Jockers et al., 1999 and references therein). These studies have concentrated mainly on the β_2-AR system which have revealed that down-regulation involves at least two pathways. The first is the reduction in receptor mRNA steady-state level resulting from de stabilisation of the transcript (Hadcock and Malbon, 1988; Bouvier et al., 1989; Nantel et al., 1994; Danner et al., 1998).

The second pathway has been demonstrated through studies on the β-AR. This receptor was found to display a 40 to 50% down-regulation when hamster vas deferens DDT_1 MF-2 cells were treated for 1 h with isoprenaline, and this reduction in receptor level was maintained for 16 h as assessed by intact-cell [^{125}I] iodocyanopindolol binding (Hadcock and Malbona, 1988a,b). The rapid initial phase of agonist-specific down-regulation of the β-AR was predicted to either be caused by

a change in conformation of the receptor such that it could no longer bind and respond to agonist, or to be due to sequestration of the receptor away from the plasma membrane. Indeed, β-AR sequestration has been linked to down-regulation in a number of reports (Mahn et al., 1985; Gagnon et al., 1998; Kallal et al., 1998), claiming that internalised receptors are sent to lysosomes and degraded. However, there are contradictory views to this, stating that receptor sequestration is not linked to receptor down-regulation (Valiquette et al., 1990, Hausdorff et al., 1991, Campbell et al., 1991, Green et al., 1994, Barak et al., 1994; Jockers et al., 1999). From studies on L cells stably expressing a β_2-AR construct, a novel model of receptor down-regulation was presented by Jockers et al., (1999). As down-regulation of the β_2-AR still occurred in response to isoprenaline despite blockade of receptor endocytosis they postulated that a primary inactivation step may occur at the plasma membrane. The two contradictory models of β_2-AR down-regulation are presented in Figure 1.8.

Despite the ambiguities in the above process it is routinely agreed that the β_2-AR undergoes extensive (40 to 50 %) down-regulation following long-term agonist exposure. Studies on the β_1 and β_3 sub-types of β-AR are less well established. Long-term exposure of animals to β-AR agonists cause a loss of β_1- and β_2- ARs (Summers et al., 1997 and references therein). It has been reported that isoprenaline leads to preferential down-regulation of the β_2-AR (Summers et al., 1997). However, noradrenaline affects both β_1- and β_2- AR levels but not levels of β_3-AR when administered to hamsters (Carpene et al., 1993). It is generally accepted that the β_2-AR undergoes extensive down-regulation, whereas the β_3-AR undergoes very little down-regulation (Chambers et al., 1994), and the β_1-AR is intermediary between the two.

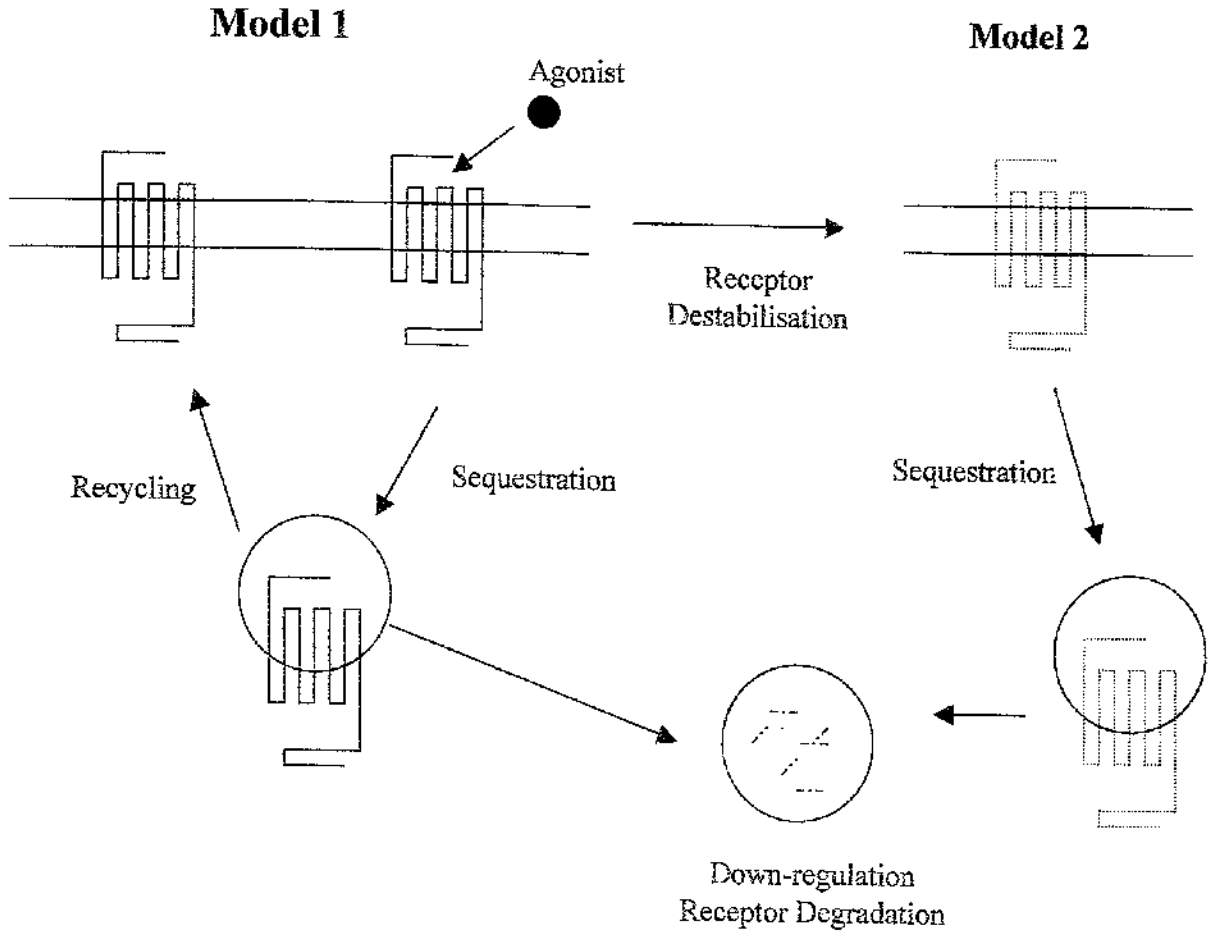
Figure 1.8

Models of GPCR down-regulation

Model 1: predicts that agonist binds to receptor and it is sequestered into early endosomes where it can be recycled. If the stimulation is chronically persistent the receptor is sent to lysosomes where it is degraded.

Model 2: predicts that the receptor is destabilised by agonist binding at the plasma membrane before it is sequestered. Following sequestration it is then degraded.

Figure 1.8


Model 1
Model 2
Agonist
Receptor
Destabilisation
Recycling
Sequestration
Sequestration
Down-regulation
Receptor Degradation

1.7 β-Adrenergic Receptors in Disease

a) Introduction

The sub-family of β-ARs are expressed in many tissues throughout the human body and are key targets within the autonomic nervous system, regulating a wide variety of physiological processes. The three types, β_1, β_2 and β_3 are differentially expressed and are each primarily important in the regulation of biological processes of specific organs. It is generally accepted that the β_1-AR is predominantly expressed in heart, the β_2-AR is predominantly expressed in lung and the β_3-AR is predominantly expressed in adipose tissue. All sub-types are linked to the production of the second messenger cAMP, a molecule which can activate and regulate many different signalling pathways in cells ultimately inducing an effect on a variety of physiological processes. In addition to adenylyl cyclase activation, the β_2-AR in cardiac cells can regulate other effectors, including voltage-sensitive calcium channels and sodium channels (Reiter, 1988; Skeberdis et al., 1997). In disease it is often these signalling pathways that can malfunction and endless efforts have been made to find the exact mechanisms involved to cause disease. This section will concentrate on some main areas of disease and the roles β-ARs play in these. These will include the involvement of the β_1 and β_2 -AR and to some extent the β_3-AR in heart failure, the contributory factors of asthma involving the β_2-AR, and the regulation of lipolysis by the β_3-AR, and associated problems to causing obesity and diabetes. The role the β_2-AR has to play in obesity and diabetes will also be discussed along with the regulation of blood pressure.

b) Cardiac Disease

Stimulation of β-ARs by sympathetic neuronal activation, by circulating catecholamines, or by adrenergic agonists increases heart rate (chronotropism), force of cardiac contraction (ionotropism), rate of cardiac relaxation (lusitropism) and automaticity (Post et al., 1999; Dzimiri, 1999). A common clinical condition is congestive heart failure (CHF) in which there is the loss of ability of the cardiac muscle to pump blood to tissues of the body. The severity of the condition is correlated with a rise in plasma catecholamines, but it is found that β-AR mediated responsiveness decreases in patients with CHF. Therapies are now being directed to the β-AR signalling pathway by firstly using disease induced animal models. It is unclear, however, how real these models are compared to the actual disease processes in human patients.

The mammalian heart primarily expresses the β_1-AR (75 - 85%) but these only mediate about 60% of ventricular contractility. A substantial number of β_2-AR can also be detected in cardiac tissue (Bristow et al., 1989). It has been reported that cardiac tissue also expresses both β_3- and β_4- ARs but their physiological relevance is unclear (Kaumann and Lynham, 1997; Kaumann et al., 1998). The β_3-AR apparently has a negative inotropic effect in cardiac tissue and this may be through activation of $G_i\alpha$ (Gauthier et al., 1996). At the end-stage of CHF the β_1-AR appears to be down-regulated with no decrease in β_2-AR levels, however it was found to be uncoupled from G protein. It is thought that this down-regulation is a way of protecting the heart from the rise in circulating catecholamine levels which occurs in CHF. It is at this stage that the β_2-AR response becomes predominant over that of the β_1-AR, in particular at low adrenaline concentrations. Therefore, the potential role for the β_2-AR to improve cardiac performance has been investigated. In healthy human heart both β_1 and β_2 -ARs are coupled to adenylyl cyclase regulating cAMP levels. However, during CHF β_1-ARs are down-regulated and the β_2-AR does not couple efficiently to

this effector, although, the positive inotropic effects of adrenaline and noradrenaline is increased >50% (Pavoine et al., 1999). It has now been identified that the β_2-AR can also regulate other signal transduction pathways in the heart. Pavoine et al., (1999) found that cAMP is the messenger of β_1-AR responses, but cell responses to β_2-AR stimulation are mediated by arachadonic acid release via phospholipase A_2 under cAMP control. Therefore, this β_2-AR signalling pathway through PLA_2 is regulated by the β_1-AR. The β_2-AR also controls the activation of L-type Ca^{2+} channels in cardiac myocytes through phosphorylation of the channel by PKA activated by second messenger cAMP (Skeverdis et al., 1997). However, this is contradictory to experiments by Xiao et al., (1995). They showed that activation of L-type Ca^{2+} channels through the β_2-AR was enhanced by pertussis toxin treatment indicating the role of a pertussis toxin sensitive G protein (possibly G_i) here, indicating a cAMP independent mechanism.

Polymorphisms of both the β_1-AR and β_2-AR have been identified. Green et al., (1993, 1994) have identified and pharmacologically characterised 4 polymorphic forms of the β_2-AR at positions 16, 27, 34 and 164. The Ile164 (T164I) variant was found in heart to be substantially uncoupled from G_s in both the non-agonist and agonist occupied forms. As the β_2-AR is also markedly uncoupled from G_s in CHF, individuals harbouring this receptor with heart failure may exhibit marked decompensation. Turki et al., (1996) have demonstrated that the Ile164 variant form when expressed in a transgenic model revealed a substantial impairment imposed by this polymorphism in cellular as well as intact heart function. Liggett et al., (1998) have extended this study to indicate that the Ile164 polymorphism of the β_2-AR can adversely affect the outcome of CHF. These findings prompted studies into identification of β_1-AR polymorphisms. Mason et al., (1999) have identified a G389R switch within a region of the β_1-AR important for receptor/G protein coupling, the resulting variant having enhanced receptor-G_s interaction with enhanced

activation of adenylyl cyclase. This may in some way have an influence on CHF and the extent of the disease.

Treatment of heart failure involves the use of β-blockers which function to inhibit the effects of high levels of circulating catecholamines which are continually stimulating β_1- and β_2- ARs to lead to CHF (reviewed by Doughty and Sharpe, 1997). The use of β-blockers in treatment have indicated an increase in ejection of blood from the left ventricle of the heart and an increase in left ventriclar volume. Studies which do not show this have been criticised for their short period of study. Metoprolol and carvedilol are two β-blockers which have been extensively studied but only carvedilol has been shown to be beneficial in survival rates (Doughty and Sharpe, 1997).

c) Asthma

Extensive studies on β_2-AR polymorphisms (at positions 16, 27, 36 and 164) and their involvement in asthma have been performed over the recent years due to high expression of this receptor in the lung. Endogenous catecholamines and administered β_2-agonists exert their primary effect on the β_2-AR of bronchial smooth muscle, resulting in relaxation and bronchial dilation. During asthma there is thought to be a malfunction of the β_2-AR signalling pathway since a substantial bronchial constriction occurs in this disease. Two of the β_2-AR polymorphisms have been studied in detail Gly16 (R16G) and Glu17 (Q27E). In 1995 Turki et al., identified a down-regulation of the β_2-AR at night in patients with nocturnal asthma. They found an over representation of a Gly16 allele of the β_2-AR in these patients. Therefore, it was concluded that the Gly16 polymorphism may play a role as an important genetic factor in asthma. However, when an asthmatic population as a whole was considered no role of this allele or the Glu27 or Ile164 alleles were identified as being the primary cause of asthma (Liggett, 1997).

When studying the response of these polymorphic forms of the β_2-AR to prolonged exposure to agonist it was found that the Gly16 variant underwent enhanced agonist-promoted down-regulation compared to WT-β_2-AR, whereas the Glu27 form was resistant to down-regulation (Green et al., 1994). Investigation into the roles of the Glu27 allele have indicated that this variant form of the β_2-AR provides a protective role against bronchial hyperactivity (the most common physiological abnormality found in asthmatic patients) (Hall et al., 1995). One way of treating this asthmatic response is to administer β_2-AR agonists since they enhance bronchial relaxation although there is debate as to the therapeutic benefits of these drugs.

d) Obesity and Diabetes

Obesity and non-insulin-dependent diabetes mellitus are two of the most common metabolic diseases and it appears that the β_3-AR may be linked to both. This receptor is the main receptor involved in the regulation of thermogenesis and lipolysis in brown and white adipose tissues in rodents (and possibly larger animals), so it is not surprising that it might have a role to play in these diseases (Strosberg et al., 1997a). In β_3-AR-deficient mice, a modest increase in body fat occurs which corresponds to reduced levels of β_3-AR mRNA in genetically modified *ob/ob* mice and *fa/fa* Zucker rats (Susulic et al., 1995; Charon et al., 1995). An Arg64 (W64R) polymorphism in the β_3-AR has been identified associated with obesity in Pima Indians and Japanese. In fact this mutation is observed in all populations of the world, but only a weak association, mostly in women, of the presence of the Arg64 variant with a number of symptoms related to the metabolic syndrome was detected. This polymorphism has also been associated with the early onset of non-insulin-dependent diabetes mellitus in a Finnish population, the Pima Indians, Mexican Americans and Japanese (Strosberg, 1997b). This single polymorphism in the β_3-AR is unlikely to be solely responsible for the onset of morbid obesity. Leptin is the product of the gene altered in *ob/ob*

mice and may play a role in regulating energy metabolism (Halaas et al., 1995). It is thought to activate the JAK-STAT signalling pathway in the hypothalamus to release noradrenaline, which would activate the β_3-AR. Agonists for the β_3-AR reduce mRNA levels of leptin and increase of leptin correlates with reduced β_3-AR activity. A negative feedback loop appears to operate here. However, very few defects in leptin or its receptor have been found in human populations. Large et al., (1997) have extended studies to investigate the role of the β_2-AR and its polymorphisms in obesity. The Glu27 variant in the homozygous form was found to be associated with increased body fat and enlarged fat cells, whereas the Gly16 variant improved adipocyte β_2-AR function. Mori et al., (1999) have also investigated the role of the Glu27 polymorphism in obesity in Japanese men. This variant of the β_2-AR was found to be significantly more frequent in the obese subgroup than the nonobese subgroup and was associated with weight gain in the subcutaneous fat area. Therefore, genetic variability in the human β_2-AR gene may be of importance in obesity.

e)Hypertension

Adrenergic neurotransmitters are important in the regulation of blood pressure and the β-AR functions to cause vessel relaxation and decreased resistance. β-ARs are known to contribute to the regulation of blood pressure through effects at several target sites, including the central nervous system, adrenergic nerve terminals, blood vessels, heart and kidney. Alterations in the sympathetic nervous system contributes to the development of systemic hypertension. The use of β-blockers appears to lower blood pressure in patients with hypertensive symptoms. The precise mechanisms by which this occurs has not been resolved.

1.8 Green Fluorescent Protein

Green fluorescent protein (GFP) is a 27 kDa polypeptide phosphoprotein from *Aequorea victoria* that emits green light with emission maximum of 509 nm upon fluorescent excitation at 488 nm. Its crystal sturucture has been reported by Oemö et al., (1996). Prior to its use as a bioluminescent reporter the study of cellular dynamics of GPCRs was limited to two main strategies.

a) Before GFP

The first strategy was the use of fluorescent labelling of GPCR ligands. There are obvious advantages of fluorescence over radioactivity measurements, however, there are also some disadvantages of this method. The pharmacological specificity and potency (if possible) of the fluorescently labelled ligand should be preserved but, unfortunately, the most appropriate ligands for this are antagonists. Atlas et al., (1976, 1978) have developed fluorescently labelled forms of the β-antagonist propranolol and a BIODIPY-CGP 12177 derivative has been developed by Heithier et al., (1994), which is also an antagonist at β_1- and β_2- ARs. Therefore, there are limited studies on agonist stimulations at GPCRs (in particular β-ARs) and their subsequent cellular trafficking. Quantitation and visualisation of specific ligand-receptor complexes in intact cells by fluorescence measurements have also been compormised by a too weak signal, high levels of tissue autofluorescence, and a lack of fluorescent staining specificity. Sometimes the cell can be swamped with flourescent ligand and thorough washing of the cells can be required if there is little change in fluorescence intensity upon binding of ligand to the GPCR. Some of these problems are being overcome and in time this method may be used for studying populations of receptors and their cycling within intact live tissue (McGrath et al., 1996).

Immunochemical detection of GPCRs is an alternative approach. This involves use of an antisera to certain peptide sequences within the GPCR or to a peptide tagging sequence engineered onto the N or C terminus of the protein. Many tags are available eg, HA, FLAG™, c-myc and VSV. Generally these tags do not alter the properties of the receptor. One disadvantage of this strategy is that fixed cells which have been permeabilised (to allow antibody access to potentially internalised GPCR) need to be used and, hence, this limits use of real time, dynamic, cell imaging (Milligan, 1999).

b) Studies using GFP as a bioluminescent reporter

The use of GFP as a bioluminescent reporter has overcome the dificulty in monitoring GPCR trafficking within living cells in real time. Several studies using GPCR-GFP fusion proteins has allowed time optical measurement of GPCR trafficking in response to agonist stimulation in live cells stably expressing the construct. These GPCRs include the β_2-AR (Barak et al., 1997; Kallal et al., 1998; McLean and Milligan, 1999) and a CAM-β_2-AR (McLean et al, 1999), the cholecystokinin type A receptor (Tarasova et al., 1997), the thyrotropin releasing hormone receptor (TRHR-1) (Milligan, 1998; Drmota et al., 1998, 1999), the α_{1A}-AR (Hirasawa et al., 1997), the α_{1B}-AR (Hirisawa et al., 1997; Awaji et al., 1998), the vasopressin V2 receptor (Schulein et al., 1998), the parathyroid hormone receptor (Conway et al., 1999), the CXCR-1 chemokine receptor (Barlic et al., 1999) and the Ca^{2+}-sensing receptor (Gama and Breitwieser, 1998).

Colocalisation experiments of some of these GFP-tagged receptors have been carried out with markers known to enter cells. For example, transferrin which enters cells *via* the constitutive endocytic pathway, has been labeled with various markers, which fluoresce red, (eg, rhodamine, Cys3, Texas red or Alexa^{495}) to look at its colocalisation with various receptors. This is to determine if the receptor being

studied enters the cell through the same pathway as the known pathway of transferrin receptor cell entry. Merging of the red (transferrin) and green (receptor) images produces a yellow image if the two proteins colocalise (Tarasova et al., 1997; Drmota et al., 1998; Kallal et al., 1998; Awaji et al., 1998). A second protein used for determining the fate of receptors is fluorescently labelled dextran which is preferentially sorted to late endosomes or lysosomes (Kallal et al., 1998).

Other colocalisation studies have been performed using GFP-tagged β-arrestin proteins. These are the proteins which assist GRKs in thier ability to phosphorylate and desensitise GPCRs in an agonist-dependent manner. This type of experiment was initiated by studies invoving a β-arrestin 2-GFP colocalising with the β_2-AR at the plasma membrane following agonist treatment of HEK293 cells transiently expressing the two constructs (Barak et al, 1997). The gonadotrophin releasing hormone (GnRH) receptor is resistant to short-term desensitisation. Following agonist stimulation at this receptor a β-arrestin 1-GFP construct was not redistributed to the plasma membrane (Vrecl et al., 1998). However, stimulation of the TRHR-1 receptor stably expressed in HEK293 cells did result in redistribution of a transiently expressed β-arrestin 1-GFP construct (Milligan et al., 1998; Vrecl et al., 1998; Groarke et al., 1999). Groarke et al., (1999) have also demonstrated that β-arrestin 1-GFP stably expressed in HEK293 cells is again redistributed when a transiently expressed TRHR-1 receptor is stimulated with TRH. Several other studies on GPCRs interacting with β-arrestins have been performed (Dery et al., 1999; Mhaouty-Kodja et al., 1999; Zhang et al., 1999).

c) Studies using GFP pairs

Until recently, it was not possible to label more than one protein at a time using GFP since there were no suitable variants of the protein that fluoresced at different

wavelengths. Therefore, the immunofluorescence studies in fixed cells, already outlined, would be the only option to study 2 proteins or more at any one time in a cell. However, there are now several GFP mutant forms available which emit different wavelengths of light giving different colours in addition to green light, eg. blue, cyan (Heim et al., 1994), yellow and red. Table 1.3 diplays the GFP variants used in double labelling experiments so far. W7/ECFP and 10C/EYFP have been used as double label pairs to label 2 distinct proteins in NRK cells. It was shown that a GFP variant 10C-labelled nuclear envelope marker, lamin B receptor (LBR-10C) could be detected in the same cell as a golgi complex marker, galactosyltransferase fused with the W7 variant of GFP. Cells were also imaged expressing LBR-10C and the chromatin marker, histone 2B fused to ECFP (H2B-ECFP) (Ellenberg et al, 1998; Ellenberg et al., 1999). GFP pairs have also been used for FRET (fluorescence resonance energy transfer) experiments in which fluorescent indicators for Ca^{2+} termed 'cameleons' were constructed. These consisted of tandem fusions of blue or cyan-emiting GFP, calmodulin, the calmodulin-binding peptide M13 and enhanced green or yellow-emiting GFP. Binding of Ca^{2+} caused calmodulin to wrap around M13 increasing FRET between the flanking GFPs (Miyawaki et al., 1997). Therefore, this development has opened up an exciting avenue for monitoring the interaction of proteins within living cell systems.

Table 1.3 Some GFP variants used in double labelling and their nomenclature[a].

Variant name	Colour	Ex/em	Amino acid changes	Commercial availability
p 4-3	blue	381/445	Y66H, Y145F	No
BFPsg50/ BFP	blue	387/450	F64L, Y66H, V163A	Q
EBFP	blue	380/440	F64L, S65T, Y66H, Y145F[c,d]	C
W2	cyan	432/480	Y66W, I123V, Y145H, H148R, M153T, V163N, N212K	No
W7	cyan	434/474	Y66W, N146I, M153T, V163A, N212K[b]	No
ECFP	cyan	434/474	K26R, F64L, S65T, Y66W, N146I, M153T, V163A, N164H, N212K[c,d]	C
S65T	green	489/511	S65T	C
GFPsg25/ rsGFP	green	473.509	F64L, S65C, I167T, K238N	Q
EGFP	green	488/507	F64L, S65T[c,d]	C
10C	yellow	514/527	S65G, V68L, S72A, T203Y	No
EYFP	yellow	514/527	S65G, V68L, S72A, T203Y[c,d]	C

[a]Abbreviations: Ex, excitation peak; Em, emission peak; GFP, green fluorescent protein. Suppliers: C, Clontech Inc., Palo Alto, CA, USA; Q, Quantum Biotechnologies, Montreal, Canada.

[b]Has the Q80R mutation that does not change the spectrum.

[c]Variant has one valine residue inserted at position 2, which is not counted.

[d]Variant has the H231L mutation that does not affect the spectral properties.

(Table adapted from Ellenberg et al., 1999)

Chapter 2

Methods

2.1 Materials

All reagents employed were of the highest grade possible and were obtained from the following suppliers.

a) General reagents, enzymes and kits

Alexis Corporation San Diego, CA, USA

[DL-Dithiothreitol] DTT

Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK

Ammonium persulphate (APS)

Calbiochem, CN Biosciences UK, Nottingham, UK

H89, G418, Rp cAMP.

Fisher Scientific, Loughborough, Leicestershire, UK

Sucrose, EDTA, glacial acetic acid, sodium chloride, calcium chloride, concentrated HCl, glucose, potassium di-hydrogen orthophosphate, magnesium sulphate, calcium chloride, HEPES, DMSO, sodium dodecyl sulphate, glycine, methanol, ethanol, sodium hydroxide, trichloro acetic acid.

ICN Pharmaceuticals Ltd., Basingstoke, Hants, UK

Betaxolol

Merck Ltd., Poole, Dorset, UK

Glycerol, potassium chloride, di-sodium hydrogen orthophosphate, bacto-agar, bacto-tryptone, bacto-yeast extract.

Promega UK Ltd., Southampton, UK

T4 ligase, *pfu*, CIAP, restriction enzymes, SV mini-prep kit, Wizard™ maxi-prep kit.

Quiagen, Crawley, West Sussex

Gel purification kit.

RBI, Natick, MA., USA

CGP12177

Roche Molecular Biochemicals/Boehringr Mannheim, Mannheim, Germany

DNA molecular weight marker X, Tris, restriction enzymes

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Agarose, bromophenol blue, forskolin, manganese chloride, ethidium bromide, DMEM (powder), poly-D-lysine, trypsin, Triton X-100, TEMED, acrylamide, bisacrylamide, Tween 20, isoprenaline, alprenolol, propranolol, labetolol, dihydroalprenolol, ICI118551, imidazole, cAMP, ATP, MOPS, rubidium chloride, Dowex, alumina, Ponceau S, bovine serum albumin, mineral oil, sodium tartrate, parpformaldehyde, phenol red, AEBSF [4-(2-Aminoethyl)benzenesulfonyl fluoride], apoprotinin, pepstatin A, leupeptin.

Whatman international Ltd., Maidstone, UK

GF/C Glass fibre filters.

b) Tissue culture plastic ware

Costar, Cambridge, MA., USA

75 cm^2 tissue culture flasks, 25 cm^2 tissue culture flasks, 6 cm and 10 cm tissue culture plates, 6, 12, 24 and 96 well plates, biofreeze vials.

Merck Ltd., Poole, Dorset, UK

Coverslips

c) Tissue culture reagents

GIBCO BRL, Life Technologies Ltd., Paisley, UK

New born calf serum, OPTIMEM, glutamine (200 mM), Lipofectamine transfection reagent.

Roche Molecular Biochemicals/Boehringer Mannheim, Mannheim, Germany

Dotap transfection reagent.

Sigma-Aldrich company Ltd., Poole, Dorset, UK

Dulbecco's modification of Eagle's medium (1 x), minimal essential medium (1 x).

d) Radiochemicals

Nycomed Amersham plc., Little Chalfont, Buckinghamshire, UK

1) [^{3}H] DHA (64 Ci/mmol)

(1-[*propyl*-2,3-^{3}H] Dihydroalprenolol)

2) (-)-[^{3}H]CGP-12177 (44 Ci/mmol)

[(-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H] Benzimidazol-2-one].

3) [2-^{3}H]Adenine (23 Ci/mmol, 1 mCi/ml)

e) Antisera

Clontech, Palo Alto, CA 94303-4230, USA

Anti-GFP - rabbit polyclonal IGg (1mg/ml), recognises GFP and various mutant forms.

New England Biolabs, Inc., Beverly, MA 01915-5599, USA

PhosphoPlus CREB (Ser133) Antibody kit.

Nycomed Amersham plc., Little Chalfont, Buckinghamshire, UK

Anti rabbit IgG (horse radish peroxidase)

Santa Cruz Biotechnology, Inc., Santa Cruz, California 95060, USA

Anti-β_1-AR (A-20) - rabbit polyclonal IgG (200 µg/ml), mapping to the C terminus of the human β_1-AR.

Anti-β_2-AR (H-20) - rabbit polyclonal IgG (200 µg/ml), mapping to the C terminus of the human β_2-AR. Cross reacts with β_3-ARs.

f) Oligonucleotides

Oswel DNA Service, Bolderwood, Southampton, UK

All oligonucleotides used to synthesise receptor-GFP constructs were diluted to a stock concentration of 100 pmol/mg and stored at -20 °C

2.2 Buffers and Reagents

a) Reagents for molecular biology

Gel loading buffer (6x)

For 10 ml:

Bromophenol Blue (2%)	1.25 ml
Sucrose	4 g

Dissolve in sterile H_2O and store at 4 °C

TAE buffer (50x)

Tris-acetate (40 mM)	242 g Tris
EDTA (1 mM)	100 ml of 0.01 M (pH 8)
glacial acetic acid	57.1 ml

Dilute to a final volume of 1 litre. This stock was diluted to a 1x solution when required.

Liquid broth (LB)

For 1 litre:

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Dissolve in deionised water and pH to 7.0. Sterilise by autoclaving at 126 °C.

b) Assay buffers

Phosphate buffered saline (10 x)

137 mM Na Cl	80 g
2.7 mM KCl	2 g
1.5 mM KH_2PO_4	2 g
8 mM Na_2HPO_4	11.4 g

Make up to 1 L in H_2O, pH 7.4. Dilute 1:10 to make a 1 x solution.

Binding wash buffer (TE)

75 mM Tris	45.4 g
1 mM EDTA	1.86 g

Make up to 5 L, pH 7.4 with concentrated HCl.

Binding assay buffer (TEM)

75 mM Tris	45.4 g
1 mM EDTA	1.86 g
12.5 mM $MgCl_2$	12.7 g

Make up to 5 L, pH 7.4 with concentrated HCl

Krebs-Ringer-HEPES buffer (KRH)

130 mM NaCl	7.0 g
5 mM KCl	0.37 g
1.2 mM $MgSO_4$	0.3 g
1.2 mM $CaCl_2$	0.26 g
20 mM HEPES	4.76 g
1.2 mM Na_2HPO_4	0.17 g
10 mM glucose	0.9 g
0.1 % (w/v) BSA	0.5 g

Make up to 500 ml in H_2O, pH 7.4.

Cyclase assay medium

(for 100 ml in H_2O)

DMEM	1.34 g
HEPES	0.48 g
glutamine (0.292 g/L)	1.00 ml
IBMX (100 mM)	1.00 ml

pH to 7.4 with 5 M KOH

Cyclase stop solution

(for 250 ml in H_2O)

TCA	12.5 g
cAMP	88 mg
ATP	137.5 mg

2.3 Molecular Biology

a) LB ampicillin agar plates

This has the same composition as LB but with bacto-agar (1.5% w/v) added. After autoclaving, it was left to cool before ampicillin was added to a final concentration of 50 μg/ml. The liquid LB agar was poured into 10 cm diameter petri dishes, and allowed to solidify before storing at 4 °C. LB ampicillin agar plates can be stored for up to 3 weeks without any loss of antibiotic activity.

b) Preparation of competent bacteria

The strain of *E. coli* used for transformation is DH5α, which can take up and express the vector pcDNA3 containing a particular cDNA. Multiple copies of this are produced by the bacteria which can be purified from cultures. For the DH5α to take up the DNA it has to be made competent. To make *E. coli* receptive or competent for foreign DNA entry the cells are treated with the following chemicals.

Solution 1 (for 100 ml)

1 M Potassium acetate	3.00 ml
1 M $RbCl_2$	1.00 ml
1 M $CaCl_2$	1.00 ml
1 M $MnCl_2$	5.00 ml
80% (w/v) glycerol	18.75 ml

The final volume was made up to 100 ml with deionised water and pH adjusted to 5.8 with 100 mM acetic acid. The solution was filter-sterilised and stored at 4°C.

Solution 2 (for 40 ml)

100 mM MOPS pH 6.5	4.00 ml
1 M $CaCl_2$	3.00 ml
1 M $RbCl_2$	0.40 ml
80% (w/v) glycerol	7.50 ml

The final volume was made up to 40 ml with deionised water and pH adjusted to 6.5 with conc HCl. The solution was filter-sterilised and stored at 4 °C.

DH5α cells were streaked out on an agar plate with no antibiotics and grown overnight at 37 °C. A single colony was grown in 5 ml of L broth overnight at 37 °C, and this was subed into 100 ml of L broth and grown until the optical density at 550 nm was 0.48. After chilling on ice for 5 min the cells were spun at 2-3 K for 10 min at 4 °C in 50 ml sterile falcon tubes. Each pellet was resuspended in 20 ml of solution 1 by pipetting, then chilled on ice for 5 min and spun as before. Each pellet was then resuspended in 2 ml of buffer 2 by pipetting and chilled on ice for a further 15 min. Cells were aliquoted and stored at -80 °C.

c) Transformation of competent cells with plasmid DNA

Between 10-100 ng of DNA was incubated with 50 µl of competent cells on ice for 15 min. The cells were then heated for 90 seconds at 42 °C and returned to ice for 2 min prior to addition of 800 µl of L broth. Cells were then allowed to recover by incubation at 37 °C for 1 h in a shaking incubator. After spinning at 13K for 5 min the cell pellet was resuspended in 200 µl of L broth and 50-200 µl was spread on an agar plate containing 50 µg/µl ampicillin. Plates were incubated overnight at 37 °C and transformed colonies selected and grown up overnight in 5 ml of L broth containing 50 µg/µl ampicillin.

d) Preparation of plasmid DNA

i) miniprep

Plasmid cDNA was prepared using the Promega™ Wizard Plus SV miniprep purification system. Briefly transformed bacterial cells from a 5 ml culture were spun and the pellet resuspended in resuspension solution [50 mM Tris-HCl, (pH7.5), 10 mM EDTA, 100 μg/ml RNase A], followed by cell lysis with lysis solution (0.2 M NaOH, 1 % SDS). The resulting lysate was then neutralised with neutralisation solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, pH4.2) to precipitate out unwanted chromosomal DNA. This was removed by centrifugation and the resulting supernatant was loaded onto a DNA purification column. Washing was performed with ethanol column wash (60 mM potassium acetate, 10 mM Tris-HCl, pH 7.5, 60 % ethanol) followed by elution with sterile water (100 μl at a concentration of 0.1-0.5 μg/μl)

ii) maxiprep

A similar method of purification was achieved but on a larger scale using the Promega™ maxiprep system. Plasmid cDNA was purified from a 500 ml culture to yield approximately 1 ml of DNA at a concentration between 0.5-2.0 μg/μl.

e) Quantitation of DNA

The concentration of DNA in a given sample was determined by measuring the absorbance at 260 nm (A_{260}) of a 1:200 dilution of the sample in sterile H_2O, assuming 1 absorbance unit was equivalent to 50 μg/ml of double stranded DNA. The purity of DNA was assessed by measuring the A_{280} in parallel and calculating the A_{260}:A_{280} ratio. A ratio of approximately 1.8 for DNA was considered to be sufficiently pure for use.

f) Digestion of plasmid DNA with restriction endonucleases

Plasmid DNA, generally 1 or 10 μg was digested in a volume of 10 or 50 μl respectively using buffer conditions recommended by the manufacturers instructions

and with 4-10 units of appropriate restriction enzymes at 37 °C for 4 hours to overnight. Specific digestions used in this study to make constructs are detailed in Section 2.4.

g) Separation of digested plasmid DNA by electrophoresis

Digested DNA was routinely analysed by agarose gel electrophoresis. Samples were prepared by addition of 6x loading buffer to a 1x final concentration. Samples between 0.5 and 5 kb were electrophoresed through 1 % (w/v) agarose gels containing 1 x TAE buffer and 2.5 mg/ml ethidium bromide. Electrophoresis was carried out towards the anode at 75-100 mA at room temperature in a horizontal electrophoresis tank containing 1 x TAE buffer. Ethidium bromide stained DNA fragments were visualised under UV light and photographed. Size was assessed by comparison with a 1 kb ladder. To isolate fragments for gel purification a sterile scalpel blade was used to excise the fragment from the gel.

h) Purification of DNA from agarose gels

To purify agarose gel fragments between 70 bp and 10 kb a Quiagen QIAquick gel purification kit was used. This involved melting of the gel fragment in QIAquick buffer (QG) followed by addition of isopropanol. The solution was loaded onto a purification column and washed with an ethanol based wash solution (PE). Elution of the DNA fragment was performed in sterile H_2O.

i) Phoshphatase treatment of DNA fragments

For one-site cloning procedures it was necessary to phosphatase treat the cut cloning vector so that it cannot recircularise. Removal of the 5' phosphate group was achieved by using calf intestinal alkaline phosphatase (CIAP from Promega). CIAP treatment was performed using 2.0 units of enzyme in a buffer (50 mM Tris-HCl, pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine) at 37 °C for 2 h. The reaction mixture was ran on a 1% agarose gel and the vector isolated by gel purification.

j) Ligation of DNA fragments

Ligation of vector DNA was routinely carried out overnight at 4 °C in a reaction volume of 20 µl containing 1x ligation buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DDT, 1 mM ATP), 3 units of T4 DNA ligase plus vector and insert DNA fragments. Reactions were performed using ratios of vector:insert of 1:1, 1:3 and 1:6. Ligated DNA was transformed as described in 2.3d.

k) Polymerase chain reaction

Amplifications were routinely performed in a reaction volume of 50 µl containing 20 ng of DNA template, dNTPs (0.2 mM each dATP, dCTP, dGTP and dTTP), 25 pmoles each of sense and antisense oligonucleotide primers, 1x thermophilic buffer (20 mM Tris-HCl, pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1 % Triton X-100, 10 µg/ml BSA). Samples were overlaid with light mineral oil to prevent evaporation, and the reaction was carried out on a Hybaid Omnigene thermal cycler. *Pfu* DNA polymerase (3 units) was added after the reaction mixture had been heated to 95 °C for 5 min. PCR conditions in preparation of receptor-GFP fusion constructs (Section 2.4) are outlined in Table 2.1.

2.4 Construction of GPCR-GFP fusion constructs

a) WT-β_2-AR-GFP

A *Hind*III-Signal-FLAG-β_2-AR-GFP-*Eco*RI construct was a kind gift from Glaxo Wellcome, Stevenage.

b) CAM-β_2-AR-GFP

Human wild-type β_2-AR in pcDNA3 was amplified by PCR using a *Hind*III-FLAG forward primer, 5'-AAAAAAAAGCTTGCCACCATGGACTACAAGGACGACGA-TGATAAGGGGCAACCCGGGAACGGC-3', and a *Bam*HI reverse primer, 5'-AA-

Table 2.1 PCR Conditions for construction of Receptor-GFP fusions

Template	Step 1 (1 cycle)	Step 2 (20 cycles)	Step 3 (1 cycle)
WT-β_1-AR	95 °C, 5 min	95 °C, 1 min 60 °C, 1 min 72 °C, 2 min	72 °C, 10 min
CAM-β_2-AR **BARK$^-$-β_2-AR**	95 °C, 5 min	95 °C, 1 min 55 °C, 1 min 72 °C, 2 min	72 °C, 10 min
GFP	95 °C, 5 min	95 °C, 1 min 60 °C, 1 min 72 °C, 4 min	72 °C, 10 min

All PCRs were performed using *pfu* DNA polymerase, in a final volume of 50µl. Reactions contained, 20 ng of DNA template, 25 pmoles of each primer, 0.2 mM dNTPs and 10 % DMSO.

AAAGGATCCTCCCGCCAGCAGTGAGTCATTTGTA-3'. This removed the stop codon and the initiating methionine (start codon) of the β_2-AR, with an initiator ATG being present in the N-terminally added FLAG epitope tag (ATG GAC TAC AAG GAC GAC GAT GAT AAG). The PCR product was digested with *Hind*III and *Bam*HI and the resulting fragment ligated into pcDNA3. The sequence encoding amino acids 172 to 291 was restricted from this construct using *Kpn*I/*Hpa*I and replaced by the equivalent region of the CAM-β_2-AR (Samama et al., 1993, 1994). A modified form of GFP (Zernicka-Goetz et al., 1997) was also amplified by PCR using a *Bam*HI forward primer, 5'-AAAAAGGATCCAGTAAAGGAGAAGAACTT-TTC-3', and an *Xba*I reverse primer, 5'-TGCTCTAGATTATTTGTATAGTTCATC-CATGCCATG-3'. This removed the initiating methionine of GFP, and the resulting PCR product was digested with *Bam*HI and *Xba*I and linked in frame to generate the CAM- β_2-AR construct.

c) WT-β_1-AR-GFP

Human WT-β_1-AR in pcDNA3 was amplified by PCR using a *Hind*III-FLAG forward primer, 5'-AAAAAAAAGCTTGCCACCATGGACTACAAGGACGACGA-TGATAAGGGCGCGGGGGTGCTC-3' and a *Bam*HI reverse primer 5'-AAAAAG-GATCCTCCCGCCCACCTTGGATTCCGAGGC-3'. This removed the stop codon and the initiating methionine (start codon) of the β_1-AR, with an initiator ATG being present in the N-terminally added FLAG epitope tag (ATG GAC TAC AAG GAC GAC GAT GAT AAG). The PCR product was digested with *Hind*III and *Bam*HI and the resulting fragment ligated into pcDNA3. The *Bam*HI/*Xba*I GFP fragment generated previously was linked in frame to generate the WT-β_1-AR-GFP.

d) BARK$^-$-β_2-AR-GFP

Human BARK$^-$-β_2-AR in pcDNA3 was amplified by PCR using a *Hind*III-FLAG forward primer, 5'-AAAAAAAAGCTTGCCACCATGGACTACAAGGACGACGA-TGATAAGGGGCAACCCGGGAACGGC-3', and a *Bam*HI reverse primer, 5'-AA-

AAAGGATCCTCCCGCCAGCAGTGCGTCATTTGC. This removed the stop codon and the initiating methionine (start codon) of the BARK$^-$-β_2-AR, with an initiator ATG being present in the N-terminally added FLAG epitope tag (ATG GAC TAC AAG GAC GAC GAT GAT AAG). The PCR product was digested with *Hind*III and *Bam*HI and the resulting fragment ligated into pcDNA3. The *Bam*HI/*Xba*I GFP fragment generated previously was linked in frame to generate the BARK$^-$-β_2-AR-GFP.

All constructs were sent to the Department of Genetics, University of Glasgow, UK for sequencing.

2.5 Routine Cell Culture

a) Cell growth

The parental cell line used for this study was Human Embryonic Kidney (HEK293) cells. It was grown in 75 cm^2 tissue culture flasks in Minimal Essential Medium (MEM) supplemented with 0.292 g/L L-glutamine and 10% Newborn Calf Serum (NBCS). Cells were incubated in cell culture incubators with a humidified atmosphere of 5 % CO_2/95 % air at 37 °C.

b) Cell subculture

Confluent cells were passaged using a sterile trypsin solution (0.1% w/v trypsin, 0.025% w/v EDTA, and 10 mM glucose). Growth media was removed from the cells and 2 ml of trypsin solution added. When the cells had detached from the surface of the flask, trypsinisation was stopped by the addition of 8 ml of growth medium. This cell suspension was mixed gently and split into fresh flasks or dishes as required.

c) Coating of plates with poly-D-lysine

50 mg of poly-D-lysine was diluted in 50 ml of sterile H_2O to make a 1 mg/ml solution which was stored at 4 °C. To coat tissue culture plates or coverslips the required amount was diluted 1:10. This resulting 0.1 mg/ml solution was left on plates or coverslips for 10 min then removed and the plates rinsed with sterile H_2O.

d) Transient transfections

Transient transfections of DNA into HEK293 cells were achieved using Lipofectamine™ reagent (Gibco Life Technologies) according to the manufacturers instructions.

i) Transfection of cells in 10 cm dishes

Briefly, cells were split into a required amount of 10 cm dishes and grown to 60-80% confluency. For each dish 10 µg of DNA was used. 0.1 mg/ml DNA stock solutions were made up in sterile water.

For a hypothetical transfection DNA mixes are as follows:

	Transfection	Mock
pcDNA3 (plasmid)	25 µl	100 µl
DNA	75 µl	-
Optimem	500 µl	500 µl
TOTAL	**600 µl**	**600 µl**

A Lipofectamine™/optimem mix was also made up in a ratio of 10:190. 600 µl of this was used for each transfection. The 600 µl DNA mix was incubated with the 600 µl of Lipofectamine™ mix for 30 min at room temperature. Once the cells were washed with optimem, 4.8 ml of optimem was added to each incubation and the resulting mixture added gently to the plate. After 5 h incubation in a cell culture incubator, 6 ml

of DMEM containing 20 % NBCS was added to the dish, and left overnight in the incubator. On the following morning, the DNA/Lipofectamine™ mixture was removed and replaced with about 10 ml of growth medium. The cells were incubated for a further 24 to 48 h before they were harvested or assayed.

ii) Transfection of cells on cover slips in 6 well plates

One µg of DNA was transfected into each well. A 0.1 mg/ml stock solution of DNA was prepared in sterile H_2O. For 1 cover slip the following mixes were prepared:

	DNA mix	Lipid mix
DNA (1 mg/ml)	15 µl	-
Lipofectamine™	-	5 µl
Optimem	35 µl	95 µl
TOTAL	**50 µl**	**100 µl**

These two solutions were combined and incubated at room temperature for 30 min. To stop the incubation 850 µl of optimem was added to the mixture and the resulting 1 ml added to the appropriate well after it had been washed with optimem. After 5 h, 2 ml of 10% NBCS/MEM was added to the well and left overnight. On the following morning the DNA/Lipofectamine mixture was removed and replaced with 3 ml of growth medium. The cells were left for a further 24 h before viewing on a fluorescent or confocal microscope.

e) Generation and maintenance of stable cell lines

Generation of stable cell lines involves selecting isolated colonies of cells which have incorporated the transfected DNA into their chromosomes. This is achieved using a selection antibiotic that kills all cells except those that have resistance conferred to them from the antibiotic resistance gene present in the plasmid carrying the cDNA to be expressed.

The transfection protocol for stable transfection is the same for that of transient transfection into 10 cm dishes except that 10 % NBCS/DMEM was used instead of optimem. After 24 h the medium was changed to normal cell culture medium (10% NBCS/MEM). After a further 24 h the stable transfections were split 1:4 into new 10 cm dishes in medium containing 1 mg/ml G418 sulphate. This medium was renewed every 3 days to maintain selection in order to obtain resistant clones. A plate of parental HEK293 cells of similar confluency to the transfected plates was used as a negative control to determine the rate of cell death. After 7 to 10 days, when all untransfected HEK293 cells in the control dish were dead, isolated clones of cells in the transfected dishes were picked. Approximately 50 clones for each transfection were picked by scraping with sterile blue tips and drawing up 0.5 ml of medium. The clones were transferred to a 24 well plate in 1 ml of G418 medium (1 mg/ml) per well. Medium again was renewed every 3 days.

Once the clones were confluent they were transferred to one well of a 6 well dish then to a 25 cm^2 flask and eventually into 2 x 75 cm^2 flasks. In the case of the GFP tagged constructs, clones from 24 well plates were split into one well of a 6 well plate and one well of a 6 well plate containing a poly-D-lysine coated cover slip. Each cover slip was examined in a fluorescent microscope and those clones which fluoresced expanded into a 25 cm^2 flask and eventually into a 75 cm^2 flask. For the non GFP-tagged constructs, one 75 cm^2 flask was harvested for assaying receptor levels and the other was expanded. Once the desired clones were obtained frozen cell stocks were made.

f) Preservation of cell lines

Stable cell lines were preserved in the earliest passage possible. Cells in 75 cm^2 flasks were grown to confluency before trypsinisation as in Section 2.5b. After addition of 8 ml of cell culture medium to the detached cells the resulting mixture was collected in a 10 ml falcon tube and centrifuged at 800 x g for 5 min. The cell pellet

was suspended in 1 ml of NBCS with 7.5 % DMSO (as a cryo-protectant). The cell suspension was transferred into a 1.5 ml cryovial. This was frozen overnight packed in cotton wool at -80 °C, and then transferred the following day to liquid nitrogen.

Cells to be resurrected were thawed at room temperature and resuspended in 10 ml of growth medium. After centrifugation at 800 x g for 5 min to remove traces of DMSO, the cell pellet was resuspended in 1 ml of growth medium and transferred to a new 75 cm^2 flask containing 10 ml of fresh growth medium.

g) Cell harvesting

Cells were harvested firstly by removing the growth medium and rinsing once in ice cold PBS buffer. In the case of cells having undergone long term drug treatments cells were washed 3 times. Using a disposable cell scraper, the cells were scraped off the base of the flask or 10 cm dish in 5 ml of PBS buffer. The cell suspension was collected into a 10 ml or 50 ml falcon tube along with a further 5 ml wash from the flask or plate. The tubes were centrifuged at 800 x g at 4 °C for 5 min. The cell pellets were stored at -80 °C until required for membrane preparation.

2.6 Protein Biochemistry

a) Production of crude plasma membranes

Frozen cell pellets were thawed and suspended in 5 volumes of ice-cold TE buffer, then homogenised with 50 strokes of a ground glass on teflon homogeniser to rupture the cells. Unbroken cells and nuclei were removed by centrifugation at low speed (1200 rpm) in a refrigerated microcentrifuge. The supernatant fraction was then centrifuged at 75 000 rpm for 30 min in a Beckman Optima TLX Ultracentrifuge (Palo Alto, CA) with a TLA100.2 rotor. The pellets were resuspended in TE and passed

through a syringe attached to a 25 gauge needle. Membranes were diluted to a final concentration of 1-5 mg/ml and stored at -80 °C until required.

b) Protein determination by BCA assay

To determine protein concentration of purified membrane preparations a method using bincinchoninic acid (BCA) and copper sulphate was used. Proteins reduce alkaline Cu(II) to Cu(I) in a concentration-dependent manner. Bicinchoninic acid is a highly specific chromogenic reagent for Cu(I), forming a purple complex with an absorbance maximum at 562 nm. The absorbance is directly proportional to the protein concentration. Known concentrations of bovine serum albumin (BSA) were used as standards (0.1-2.0 mg/ml).

REAGENT A

1 % (w/v) BCA	10 g
2 % (w/v) Na_2CO_3	20 g
0.16 % (w/v) sodium tartrate	1.6 g
0.4 % NaOH	4 g
0.95 % $NaHCO_3$	9.5 g

Make up to one litre with H_2O, pH11.25 with 50 % NaOH.

REAGENT B

4 % $CuSO_4$

Mix 49 parts A with 1 part B and add 200 µl to each well of a 96 well plate containing 10 µl of protein sample or standard. After incubation at 37 °C for 30 min, the absorbance was read.

c) Sample preparation for SDS-PAGE gel electrophoresis

i) Membrane protein samples were diluted to a final concentration of 2 mg/ml in TE buffer. This protein sample was then diluted in Laemmli buffer (5 M urea, 0.17 M

SDS, 0.4 M dithiothreitol, 50 mM Tris-HCl, pH 8.0 and 0.01 % bromophenol blue) to a final concentration of 1 mg/ml. After boiling the sample for 5-10 min 10 to 20 μg of protein was loaded into each well of a SDS-PAGE protein gel.

ii) Cell lysate samples were prepared as follows. Cells from a 6 cm dish were washed in PBS after the appropriate drug treatment. Cells were lysed in 500 μl of ice cold lysis buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 % (v/v) TX-100, 10 % (v/v) glycerol) containing protease inhibitors (final concentrations of 1 mM sodium vanadate, 1 mM AEBSF, 2 μg/ml apoprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin). For a further 20 min the extract was lysed in an Eppendorf tube while spinning on a rotating wheel at 4 °C. The sample was then centrifuged at 4 °C for 10 minutes at 13 K and 150 μl of the supernatant was added to 50 μl of 4x sample buffer (0.25 M Tris, 8 % (w/v) SDS, 50 % (v/v) glycerol, 0.4 M DTT, 0.02 % phenol red, pH 6.8). After boiling the samples for 10 min, 20 μl was loaded on the gel.

d) SDS-PAGE gel electrophoresis

Resolving polyacrylamide gel: 10 % (v/v) acrylamide, 0.27% (v/v) bisacrylamide, with 0.75 M Tris (pH8.8), 0.1 % (w/v) SDS, 0.01 % (w/v) ammonium persulphate and 0.001 % (v/v) TMED.

Stacking gel: 5 % (v/v) acrylamide, 0.13 % (v/v) bisacrylamide, with 0.25 M Tris (pH 6.8), 0.1 % (w/v) SDS, 0.01 % (w/v) ammonium persulphate and 0.001 % (v/v) TMED.

Running buffer: 25 mM Tris (pH 8.5), 0.192 M glycine and 0.1 % (w/v) SDS.

Samples were ran at 200 V using a Mini Protean II gel kit (Bio-Rad, Richmond, CA).

e) Western blotting

Following SDS-polyacrylamide gel electrophoresis, proteins were electrophoretically transferred onto nitrocellulose at ~100 V and 400 mA in transfer buffer (0.2 M glycine, 0.025 M Tris and 20 % (v/v) methanol). The membrane was then blocked

for 1 h in 3 % fat-free milk in PBS-T buffer (PBS containing 0.1 % (v/v) Tween 20). After a brief wash in PBS-T buffer, the membrane was incubated overnight at 4 °C with an appropriate primary antibody (anti-β_1-AR, anti-β_2-AR or anti-GFP) diluted at 1:2000 in PBS-T buffer containing 1 % fat-free milk. The primary antibody was then removed, and the blot was washed extensively in PBS-T buffer. Subsequent incubation with secondary antibody (donkey anti-rabbit IgG conjugated with horseradish peroxidase) diluted at 1:10 000 in PBS-T buffer containing 1 % fat-free milk, was performed for 2 h at room temperature. Following extensive washing in PBS-T buffer, the blot was visualised by enhance chemiluminescence. Exposure onto photosensitive film was performed. For immunodetection using the phosphoCREB antibody in Chapter 5, BSA was used instead of non-fat milk as specified by the PhosphoPlus CREB (Ser 133) Antibody Kit (New England Biolabs Inc.).

f) Sucrose-density gradient experiments

CAM-β_2-AR-GFP cells were collected by low speed centrifugation and washed in ice cold PBS. They were then resuspended in 2 ml of ice cold hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM EDTA) and homogenised for 5 min on ice in a Potter-Elvehjem (Teflon-glass) homogeniser. The resulting homogenate was adjusted to a final volume of about 7.5 ml with lysis buffer and centrifuged at 600 g for 5 min to remove nuclei and cell debris. A portion (7 ml) of this homogenate, containing approximately 20 mg of protein, was layered on to the top of a discontinuous sucrose-density gradient [5 ml each of 19 %, 23 %, 27 %, 31 %, 35% and 43 % (w/v) sucrose in hypotonic lysis buffer]. The gradient was centrifuged at 27 000 rev/min for 60 min in a Beckman SW 28 rotor at 4 °C. Seven 5 ml fractions were subsequently collected manually from the top of the gradient. The pellet was resuspended in 5 ml of lysis buffer and designated fraction 8. The fractions were stored at -80 °C until used.

Distribution of protein and marker-enzyme activities in this type of sucrose-density gradient has been described in detail previously (Svoboda et al., 1996; Svoboda et al., 1992). The first fraction (the first 5 ml from the top of the preformed sucrose gradient) represents the soluble cytosolic fraction, the second and third fractions are enriched in low-density membranes (light vesicles) and fractions 6 and 7 are preferentially enriched in plasma membranes.

g)Electrophoresis and immunoblot analysis

The proteins contained in the fractions were precipitated by incubation with 6 % (w/v) trichloroacetic acid on ice for 1 h. The precipitates were dissolved in a small volume of buffer B [1 M Tris (base), 0.5 M boric acid, 0,01 M EDTA] and then solubilised in an equal volume of Laemmli buffer. In order to resolve CAM-β_2-AR-GFP, a borate-based electrophoresis buffer system (Poduslo, 1981) was used, with modifications. Briefly, the resolving polyacrylamide gel was made with 10 % (w/v) acrylamide, 0.0625 % (w/v) bisacrylamide, 0.1 M Tris/0.1 M boric acid (pH 8.5), 0.0025 EDTA, 0.1 % (w/v) SDS, 0.005 % (w/v) TMED and 0.1 % (w/v) ammonium persulphate. The stacking gel was of the same composition, except that it contained 4 % (w/v) acrylamide. The borate electrophoresis running buffer was: 0.1 M Tris/0.1 M boric acid, 0.0025 EDTA, 0.1 % (w/v) SDS, pH 8.5. Electrophoresis was carried out at 150 V for 1 h using a Mini Protean II gel kit (Bio-Rad). After SDS-PAGE, proteins were electrophoretically transferred onto nitrocellulose at ~100 V and 400 mA in transfer buffer (0.2 M glycine, 0.025 M Tris and 20 % (v/v) methanol). The membrane was then blocked for 1 h in 3 % fat-free milk in PBS-T buffer (PBS containing 0.1 % (v/v) Tween 20). After a brief wash in PBS-T buffer, the membrane was incubated overnight at 4 °C with an appropriate primary antibody (anti-GFP) diluted at 1:2000 in PBS-T buffer containing 1 % fat-free milk. The primary antibody was then removed, and the blot was washed extensively in PBS-T buffer. Subsequent incubation with secondary antibody (donkey anti-rabbit IgG conjugated with horseradish peroxidase) diluted at 1:10 000 in PBS-T buffer

containing 1 % fat-free milk, was perfomred for 2 h at room temperature. Following extensive washing in PBS-T buffer, the blot was visualised by enhance chemiluminescence. Exposure onto photosensitive film was performed.

2.7 Assays

a) Membrane radioligand binding with [^{3}H] DHA

The expression of β-AR constructs in stable cell lines and transiently transfected cells were assessed by [^{3}H] DHA binding studies. These were performed in borosillicate glass tubes in triplicates, containing the following mix:

Membrane Protein (1mg/ml)	20 µl
Assay buffer (TEM)	60 µl
[^{3}H] DHA (~20 nM)	10 µl
Propranolol (β_2), betaxolol (β_1) (10^{-4} M) or H_2O	10 µl
Total Volume:	**100 µl**

Reactions were incubated for 45 min at 30 °C. Binding was stopped by vacuum filtration through GF/C filters. The filters were washed 3 times in ice-cold wash buffer to remove free radioligand from the membrane. Filters were inserted into vials containing 5 ml of liquid scintillant. After leaving the filters to soak the vials were counted in a Beckman LS6500 scintillation counter using the [^{3}H] counting channel. Specific binding was determined by subtracting the counts performed in the presence of propranolol or betaxolol (non-specific counts) from that with out (total counts). Receptor expression level (fmol/mg) was calculated by taking into consideration the specific activity of [^{3}H] DHA (64 Ci/mmol) and the amount of membrane protein used per tube.

The maximal binding (B_{max}) and the equilibrium dissociation constant (K_d) for DHA at the various β-AR constructs were assessed using increasing concentrations of [^{3}H] DHA (0.1-10 nM) with or without a single concentration of propranolol or betaxolol (10^{-5} M final) to measure non-specific binding. The binding affinity of the receptors for various β-drugs (isoprenaline, alprenolol and betaxolol) were assayed, using a single concentration of [^{3}H] DHA close to the K_d (~0.5-1.0 nM) and increasing concentrations of unlabelled drug (10^{-13} to 10^{-3} M).

b) Intact cell radioligand binding with [^{3}H] CGP 12177 and [^{3}H] DHA (in tubes)

To measure the receptor number within the plasma membrane of stably transfected intact cells the hydrophobic ligand [^{3}H] CGP 12177 was used. These were performed in borscillicate glass tubes in triplicates, containing the following mix:

50 000-200 000 intact cells in KRH	80 µl
[^{3}H] CGP 12177 (~100 nM)	10 µl
Propranolol (β_2), betaxolol (β_1) (10^{-4} M) or H_2O	10 µl
Total Volume:	**100** µl

Reactions were incubated for 2.5 h at 14 °C (adapted from Gagnon et al., 1998). Binding was stopped as for [^{3}H] DHA membrane binding and the specific binding determined as previously. Receptor number was calculated by taking into account the number of cells per assay tube, and the specific activity of [^{3}H] CGP 12177 (44 Ci/mmol). The fmoles of receptor per cell was converted to the number of receptors per cell by using Avagadro's number N_A.

The hydrophilic ligand [^{3}H] DHA was used to determine total cell membrane receptor. Incubations were set up as follows:

50 000-200 000 intact cells in KRH	80 µl
[^{3}H] DHA (~20 nM)	10 µl
Propranolol (β_2), betaxolol (β_1) (10^{-4} M) or H_2O	10 µl
Total Volume:	**100 µl**

Incubations were performed as for [^{3}H] DHA membrane binding and the calculations as above.

c) Intact cell radioligand binding with [^{3}H] CGP 12177 or [^{3}H] DHA (on 24 well plates)

Stably transfected cells were plated out into 24 well plates the day before the binding assay unless long term drug treatments were to be performed prior to binding. After the appropriate drug stimulations the cells were washed twice with ice cold PBS followed by addition of 200 µl of binding mix.

Binding mix (final concentrations):

10^{-5} M propranolol (β_2) or betaxolol (β_1) (or water-for total binding)

10 nM [^{3}H] CGP 12177 or 2 nM [^{3}H] DHA

in KRH buffer

[^{3}H] CGP 12177 bindings were performed on ice for 90 min and [^{3}H] DHA bindings in a 30 °C incubator for 45 min. Following incubation the binding medium was discarded and cells were washed once with ice cold PBS to wash excess [^{3}H] away. Addition of 0.5 ml of 0.5 mM EDTA/PBS helped detach the labelled cells from the plate. These were collected and put into vials containing 5 ml of scintillant along with a further 0.5 ml of PBS wash from each well. After capping and vortexing, the vials were counted in a Beckman LS6500 scintillation counter using the [^{3}H] counting channel. For each assay unlabelled cells were counted in a hemocytometer to calculate the receptor number for each clone.

All short term drug treatments were applied in KRH buffer in a 37 °C incubator/oven. Cell washes were performed in PBS . Over night drug treatments were performed in normal cell culture medium in a 37 °C incubator with 5% CO_2.

d) Intact cell adenylyl cyclase assay

This method has been taken from Wong, 1994.

i) Column preparation

Dowex: To prepare 100 columns, 200 g of Dowex (50WX 4-400) was washed once in 1 L of 1 M HCl, once in 1 L of 1 M NaOH and then several times in distilled H_2O until the residual wash was neutral pH 7. The washed Dowex was then made up to a volume of 200 ml and 2 ml of this solution was added to 100 glass wool stoppered columns pre-washed with distilled H_2O. Prior to use the columns were washed with 4 ml of HCl then with 14 ml of H_2O. Columns were stored at room temperature in H_2O.

Alumina: To prepare alumina cloumns 1 g of dry neutral alumina was added to glass wool stoppered columns and the columns washed with 12 ml of 1 M imidazole buffer (pH 7.3), followed by 15 ml of 0.1 M imidazole (pH 7.3) and then stored at room temperature in H_2O. On the day of use, each column was washed with 8 ml of 0.1 M imidazole (pH7.3). After use columns were washed with 6 ml of 0.1 M imidazole and then stored in H_2O.

ii) Assay conditions

Transiently or stably transfected cells were seeded into poly-D-lysine coated 24 well plates and were allowed to reattach overnight. Cells were then incubated in medium containing [^{3}H] adenine (1.0 µCi/well) for 16 to 24 h. On the day of assay the cell culture medium was removed and 2 ml of HEPES buffered DMEM added to wash away excess [^{3}H] adenine. Cells were then treated with 0.5 ml of agonist or 50 µM forskolin (positive control) in assay medium for 10 or 30 minutes at 37 °C. On

removal of agonist ice cold stop solution was added to stop the reaction and lyse the cells. After 30 minutes cells were scraped into the stop solution and the resulting mix transferred to Eppendorf tubes and spun for 5 min at 4 °C. The supernatant was applied to pre-washed Dowex columns over scintillation vials containing 4 ml of scintillant. 3 ml of H_2O was applied to the column to wash [^{3}H] ADP and ATP into these vials. The Dowex columns were then placed onto the Alumina columns, over a waste basin and 10 ml of H_2O applied. [^{3}H] cAMP was then eluted with 6 ml of 0.1 M imidazole, from the alumina columns over scintillation vials containing 8 ml of scintillant.

2.8 Confocal Laser Scanning Microscopy

Cells were observed using a laser scanning confocal microscope (Zeiss Axiovert 100: Zeiss Oberkochen, Germany) with Zeiss Plan-Apo 63 x 1.40 NA oil immersion objective, pinhole of 20, and electronic zoom 2 or 3. The GFP was excited using a 488-nm argon/krypton laser and detected with a 515- to 540-nm band pass filter. The images were manipulated with Universal Imaging MetaMorph software.

a) Fixed cell work

Cells grown on glass coverslips were washed with PBS and fixed for 20 min at room temperature using filter sterilised 4 % paraformaldehyde in PBS/5 % sucrose, pH 7.2. After one wash with PBS, the coverslips were mounted on microscope slides with 40 % glycerol in PBS.

b) Live cell work

When examining live cells, cells grown on a glass coverslip were maintained in Krebs-Ringer-HEPES buffer (KRH) and the temperature was maintained at 37 °C.

For time-course experiments the agonist isoprenaline was applied in KRH buffer at the appropriate concentration.

c) Spectrofluorimeter assay

Cells were seeded into black Costar view plates on the day before the experiment. On the day of the experiment, the media was removed from the cells and drug was added to the well in a final volume of 100 µl. The experiments were performed in phenol red-free F12 media containing 10 % fetal calf serum. A Spectrafluor Plus fluorimeter was used to read the plates, reading from the bottom at a gain of 100. A blank plate was initially read on the fluorimeter, and then the plates of cells were read at time 0 and after a 22 h incubation at 37 °C with drug. Results were calculated by subtracting the blank plate from the fluorescence values obtained to control for plate autofluorescence.

Chapter 3

Visualising Ligand Regulation of a Constitutively Active Mutant β_2-Adrenergic Receptor-Green Fluorescent Protein Fusion Protein

Chapter 3

3.1 Introduction

Binding of agonist to a G protein coupled receptor (GPCR) induces an allosteric transition within the receptor structure to produce a conformation of the receptor which is able to activate its cognate G protein and thus regulate the activity of down-stream effector enzymes. In the case of the β_2-adrenergic receptor (β_2-AR) for example, binding of agonist to the wild type receptor causes a structural modification in which movement of transmembrane helix 6 occurs, as measured by the position of Cys^{285} (Gether et al., 1997a). This phenomenon of agonist-induced formation of an active receptor conformation has led to acceptance of a modified ternary complex model where the active receptor (R*) and inactive receptor (R) are in equilibrium. In this model agonists preferentially bind to and stabilise R* shifting the equilibrium towards this form (Bond et al., 1995; Leff, 1995; Leff et al., 1997; Zuscik et al., 1998 and Chapter 1, Section 1.7).

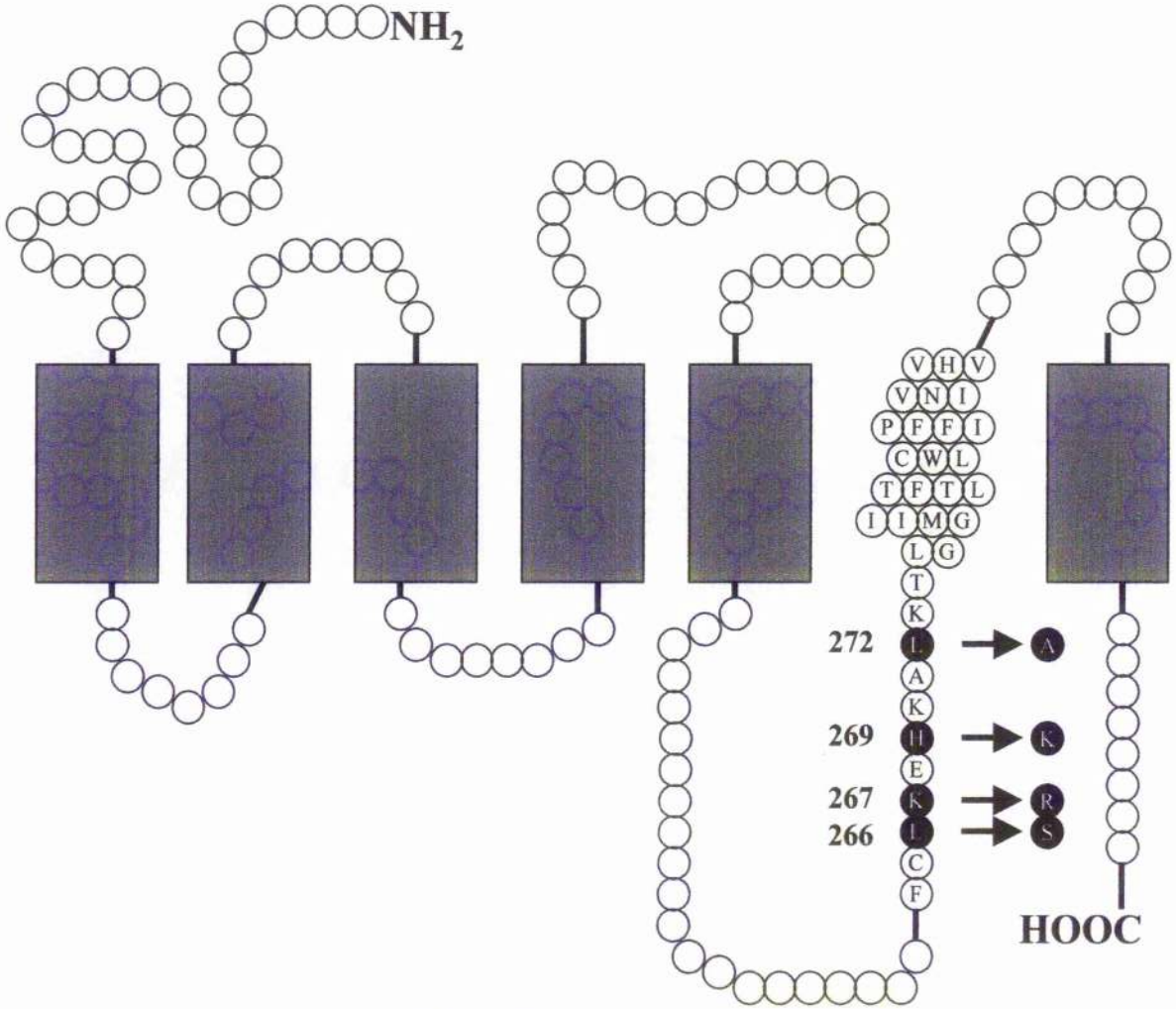
In endogenous receptor systems, in the absence of agonist there is always a small quantity of R* present which is able to couple to G protein generating a basal effector output. Receptor mutations have been studied which induce an agonist-independent shift in isomerisation equilibrium towards an R*-like conformation (Lefkowitz et al., 1993; Bond et al., 1995; Scheer and Cotecchia, 1997; Leurs et al. 1998). These receptors are termed constitutively active mutant (CAM) receptors and can generate agonist-independent signalling. Perhaps the most studied CAMGPCR is a form of the human β_2-AR in which a short segment of the third intracellular loop was replaced with the corresponding region from the α_{1B}-AR (Samama et al., 1993; Samama et al., 1994) (Figure 3.1). This work had stemmed from findings that the reciprocal mutation in the hamster α_{1B}-AR (part of the 3rd intracellular loop replaced by the

Figure 3.1

Structural model of the constitutively active mutant (CAM) β_2 adrenergic receptor.

Substitution of a short segment of the third intracellular loop of the β_2-AR with the corresponding region from the α_{1B}-AR renders the receptor constitutively active, resulting in stimulation of adenylyl cyclase in the absence of agonist-induced receptor activation. Solid circles represent the amino acids (in single letter code) of the WT-β_2-AR that were mutated. The amino acid residues mutated are shown to the right (Leu266Ser, Lys267Arg, His269Lys, and Leu272Ala).

Figure 3.1


NH2
V H V
V N I
P F F I
C W L
T F T L
I I M G
L G
T
K
272 L A
K
269 H K
E
267 K R
266 L S
C
F
HOOC

corresponding region in β_2-AR) generated a constitutively active mutant (Cotecchia et al., 1990; Kjelsberg et al., 1992).

The CAM-β_2-AR has been used to help clarify structural alterations upon binding and subsequent activation at the WT-β_2-AR. In the CAM form of this receptor the same Cys which moves upon agonist binding to WT-β_2-AR is found closer to the ligand binding pocket than in the ligand-unoccupied WT receptor (Javitch et al., 1997). Previous studies have found that overexpressed CAM-β_2-AR in mammalian cells displays increased agonist affinity, elevated basal adenylyl cyclase and GTPase activity and higher maximal agonist-stimulated adenylyl cyclase and GTPase activity than for WT-β_2-AR (Lefkowitz et al., 1993; Scheer and Cotecchia, 1997; Leurs et al, 1998). This has also been reported using the Sf9 insect cell expression system (Gether et al.,1997). Secondy, when conserved residue Asp-130 in TM3 of the β_2-AR was mutated to asparagine (D130N) a constitutively active receptor was formed. It was suggested from this that Asp-130 is an important part of the molecular switch that controls transition of the receptor between its active and inactive state. As TM6 is rearranged in the D130N mutant, the molecular switch, Asp-130, may govern the spatial disposition of TMs 3 and 6 (Rasmussen et al., 1999).

Of particular interest to my study are observations that sustained treatment of cells expressing the CAM-β_2-AR with certain "β-blockers", including betaxolol results in the subsequent detection of higher levels of [^{3}H] β_2-AR antagonist binding sites in membranes prepared from these cells (Pei et al., 1994; MacEwan and Milligan, 1996 a,b). Betaxolol has the characteristics of an inverse agonist (Pei et al., 1994; Samama et al., 1994; MacEwan and Milligan, 1996a,b)., i.e. a ligand that suppresses the basal signalling capacity of a GPCR. This effect appeared to be selective, because certain other β-blockers, including alprenolol, did not mimic these effects. Furthermore, the effects of the inverse agonist on CAM-β_2-AR levels were not related to their capacity

to suppress the GPCR-mediated activation of basal adenylyl cyclase activity (MacEwan and Milligan, 1996b).

In contrast to the effects on cellular levels of CAM-β_2-AR, it was noted that equivalent treatment with betaxolol had a much less dramatic effect on cellular levels of the wild type human-β_2-AR when it too was expressed in NG108-15 cells (MacEwan and Milligan 1996a). Because betaxolol treatment of CAM-β_2-AR-expressing cells had little effect on the levels of mRNA encoding this receptor (MacEwan and Milligan, 1996a), inverse agonists of the CAM-β_2-AR may function to stabilise an inherently unstable protein and thus decrease its rate of degradation. In the face of an apparently unchanged rate of synthesis, then, the inverse agonist causes an increase in steady-state levels of the GPCR. Kobilka and colleagues have gone further, indicating that any appropriate receptor ligand, whether agonist, neutral antagonist, or inverse agonist, may act to stabilise the structure of the purified CAM-β_2-AR and slow its denaturation (Gether et al., 1997 a,b).

In this chapter I set out to investigate whether ligand-induced up-regulation of the CAM-β_2-AR is restricted to compounds that display inverse agonism as reported by MacEwan and Milligan, or if Kobilka and colleagues' theory about ligand regulation at this receptor might be correct. In addition to using conventional methods such as [^{3}H] ligand binding and immunoblotting to measure receptor levels before and after drug treatments, it was decided that a direct visual means of detecting the receptor would be beneficial to the project. In the current study I used a PCR-based approach to construct forms of the WT- and CAM-β_2-AR that had a modified form of the 27kDa green fluorescent protein (GFP) derived from *Aequorea victoria* added in-frame to their C-terminal tail (detailed in methods Chapter 2, Section 2.4). Following transient or stable expression in cells these constructs could be directly visualised in a confocal microscope and changes in levels or distribution in response to various drug treatments provided an initial indication of ligand regulation at CAM-β_2-AR-GFP.

Initially, transient transfection of these constructs into HEK293 cells were performed to confirm that expression was obtained and that the constructs could be detected by fluorescence microscopy. Stable cell lines were then generated and single clones isolated for study i.e. a WT-β_2-AR clone, a WT-β_2-AR-GFP clone and a CAM-β_2-AR-GFP clone. All three clones were pharmacologically characterised for their ability to bind various β-ligands to examine whether GFP had any effect on ligand binding at each receptor. Expression levels of the three receptor constructs were also determined along with the K_d of [^{3}H] DHA for the receptor.

Up-regulation studies were firstly performed using the inverse agonist betaxolol. Measurements of up-regulation were achieved by a variety of methods, including confocal microscopy to quickly determine events occuring in the living cell after treatment, binding studies on membranes and intact cells with [^{3}H] DHA and [^{3}H] CGP12177, immunoblotting with antisera against the β_2-AR and GFP, and sucrose density gradient experiments. A selection of β-blockers were tested. These provided some interesting results suggesting the basis for a potentially rapid and efficient screening method in finding new GPCR ligands.

3.2 Construction and expression of wild-type and constitutively active mutant β_2-adrenergic receptor-green fluorescent protein fusion proteins

A PCR-based strategy was used to link a cDNA encoding a modified form of the GFP from *Aequorea victoria* with enhanced autofluorescence properties (Zernicka-Goetz et al., 1997) with cDNAs encoding both the FLAG-tagged WT-β_2-AR and a CAM form of this GPCR, produced by replacement of a small segment of the distal end of the third intracellular loop with the equivalent segment of the hamster α_{1B}-AR (Samama et al., 1993). These fusion proteins were anticipated to encode single open reading

frames in which the C terminus of the GPCR was linked directly to the N terminus of GFP (Figure 3.2a).

These fusion constructs were transiently transfected into HEK293 cells to confirm successful expression. Figure 3.2 illustrates confocal images obtained after successful expression of both WT- (b) and CAM- (c) β_2-AR-GFP fusion constructs. Each appeared to display a different cellular location, WT-β_2-AR-GFP being mainly plasma membrane located and CAM-β_2-AR-GFP being both at the plasma membrane but also with a large proportion of receptor expressed diffusely in intracellular membranes. From binding experiments using a single concentration of [^{3}H] DHA on membranes from four transient transfections (Figure 3.3), it was apparent that the CAM form of this receptor was routinely expressed at much lower levels than either the GFP- tagged or untagged forms of the WT receptor. However, its expression was slightly greater than the control experiment using empty vector pcDNA3. Previously CAM GPCRs have proven to be present at lower levels than their WT versions, possibly due to their higher instability and rapid turnover which may reflect their constitutive activity (Gether et al., 1997). Stable cell lines of each construct in HEK293 cells were then developed.

Once stable cell lines of WT-β_2-AR with and without GFP and of CAM-β_2-AR-GFP were established in HEK293 cells single clones of each were selected for study. A fluorescent microscope was utilised to directly screen for the receptor-GFP containing clones (Figures 3.4a,b). At least 70% of the putative WT-β_2-AR-GFP clones selected were positive but this was less than 40% for CAM-β_2-AR-GFP. There were marked differences in location and expression levels of receptor between the two constructs. In clones expressing the WT-β_2-AR-GFP construct, confocal microscopy performed on intact cells grown on glass coverslips demonstrated substantial amounts of the GFP-derived autofluorescence to be plasma membrane delineated (Figure 3.4a). Although clear plasma membrane-localised CAM-β_2-AR-GFP could be observed in

Figure 3.2

a) Schematic diagram of the cDNAs encoding the receptor-GFP fusion constructs generated for this study.

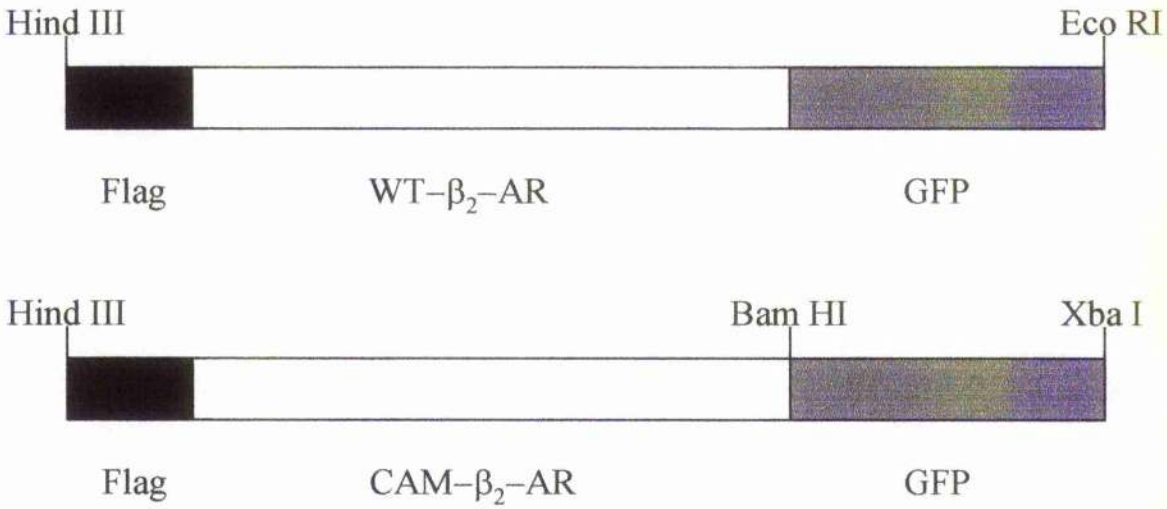
WT-β_2-AR-GFP was a kind gift from Glaxo Wellcome. CAM-β_2-AR-GFP was generated using a PCR based approach (see Chapter 2 Section 2.4).

b and c) Confocal analysis to determine the cellular location of WT- and CAM- β_2-AR-GFP following transient transfection in HEK 293 cells.

Following transient transfection of HEK293 cells on glass cover slips with WT- and CAM- β_2-AR-GFP cDNAs, cells were fixed, mounted on a microscope slide as described in Section 2.8a and then imaged by confocal microscopy, b) WT-β_2-AR-GFP and c) CAM-β_2-AR-GFP.

Figure 3.2

a)


Hind III
Eco RI
Flag
WT–β2–AR
GFP
Hind III
Bam HI
Xba I
Flag
CAM–β2–AR
GFP

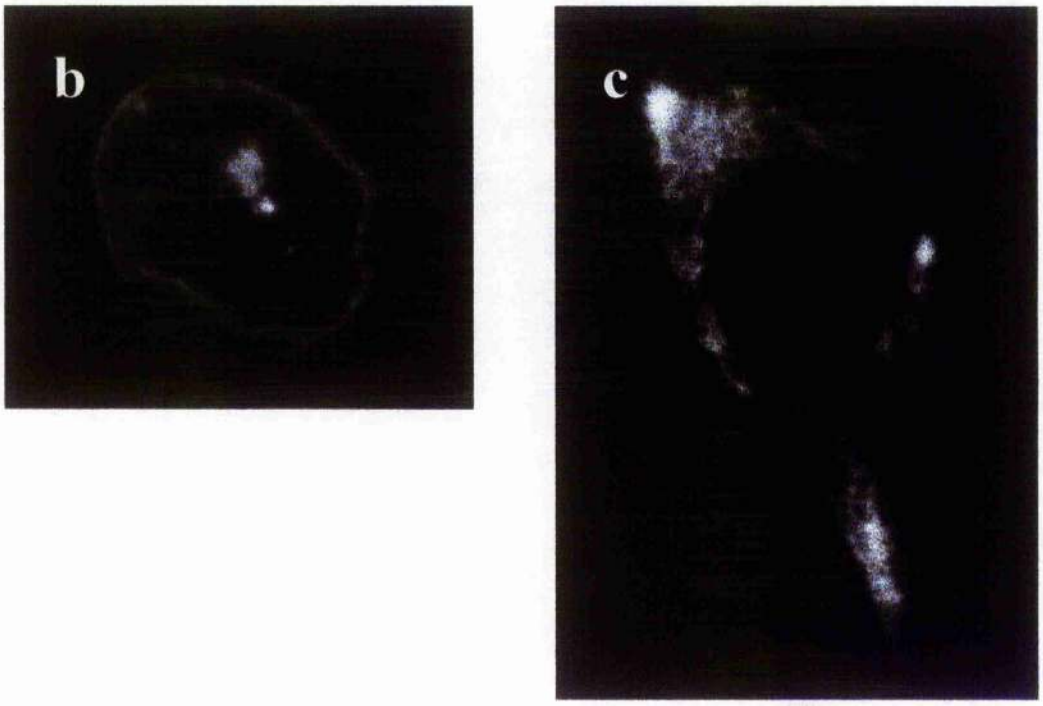

b
c

Figure 3.3

The WT-β_2-AR and WT-β_2-AR-GFP constructs are transiently expressed at higher levels than the CAM-β_2-AR-GFP construct.

Membranes (20 μg) prepared from HEK293 cells transiently expressing either the WT-β_2-AR, WT-β_2-AR-GFP or the CAM-β_2-AR-GFP constructs were analysed for their ability to bind a single, near saturating concentration of [^{3}H] DHA (2 nM). Both wild type receptor constructs were expressed at much higher levels than the CAM construct. Results are from one experiment performed in triplicate ± S.D. Similar results were obtained from two further experiments.

Figure 3.3

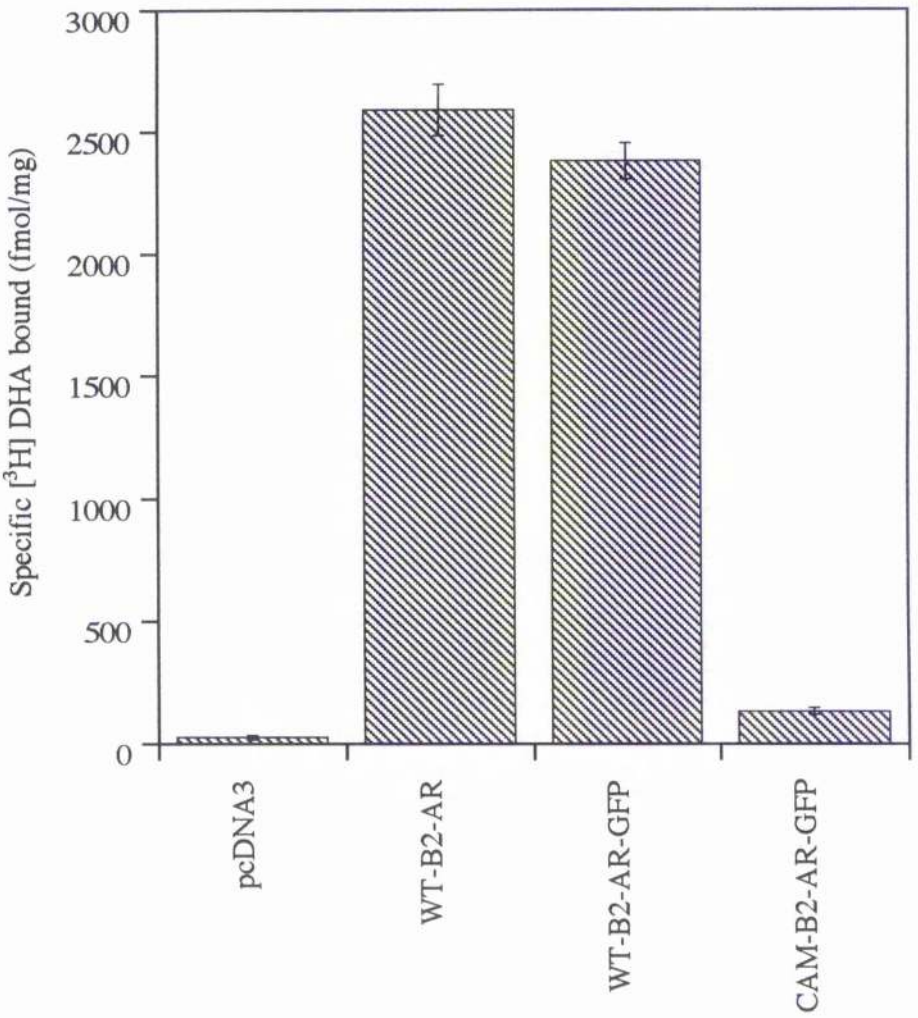

Specific [3H] DHA bound (fmol/mg)
3000
2500
2000
1500
1000
500
0
pcDNA3
WT-B2-AR
WT-B2-AR-GFP
CAM-B2-AR-GFP

Figure 3.4

a) WT-β_2-AR-GFP stable cell clones.

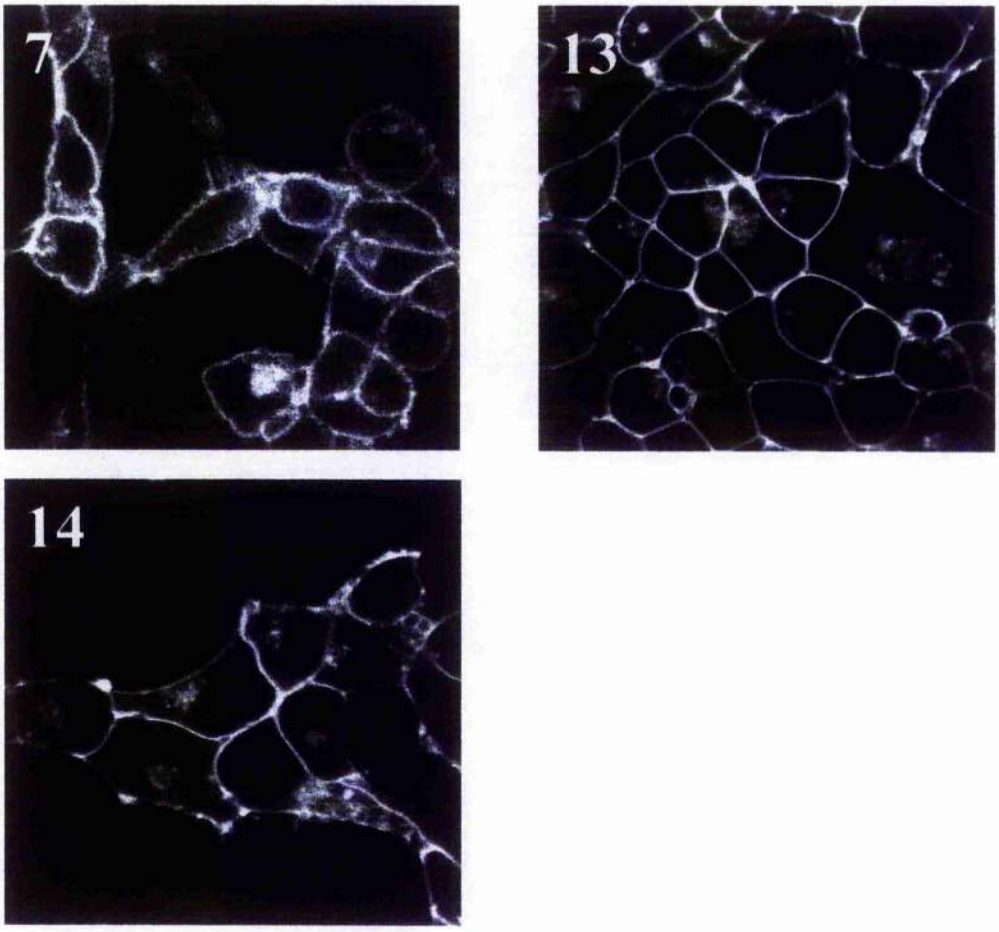
The WT-β_2-AR-GFP construct was expressed stably in HEK293 cells and individual clones were identified by live cell confocal microscopy. All positive clones were similar to the three clones (7,13,14) imaged with a largely plasma-membrane delineated autofluorescence.

b) CAM-β_2-AR-GFP stable cell clones.

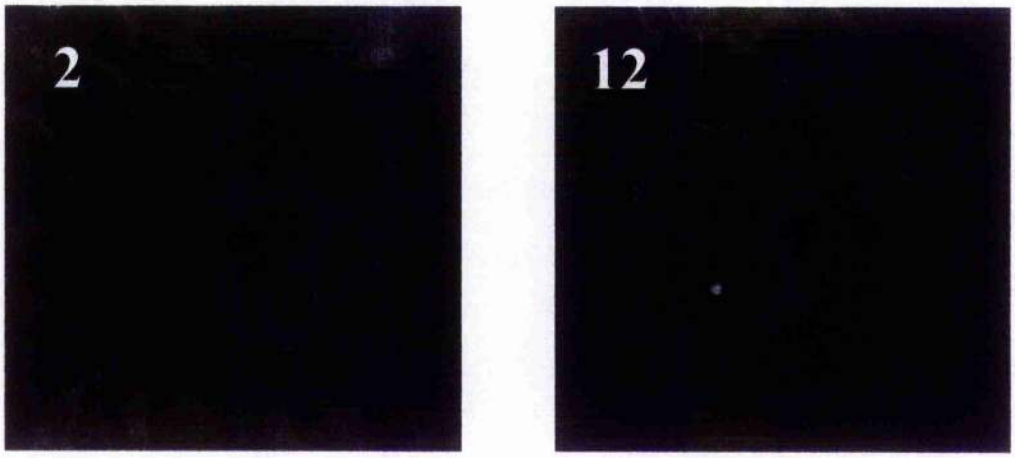
The CAM-β_2-AR-GFP construct was expressed stably in HEK293 cells and individual clones were identified by live cell confocal microscopy. All positive clones were similar to the two clones (2,12) imaged with some autofluorescence at the plasma membrane but a large proportion of receptor being distributed diffusely in intracellular membranes.

Figure 3.4

a) WT -β_2-AR-GFP clones



b) CAM-β_2-AR-GFP clones



stably expressing clones, there was a greater fraction of the GFP autofluorescence located intracellularly than for WT-β_2-AR-GFP (Figure 3.4b). These clones routinely had much less intense autofluorescence patterns than the WT-β_2-AR-GFP clones, a factor contributing to possibly why less CAM clones were detected. The non GFP-tagged WT-β_2-AR clones were initially screened by intact cell adenylyl cyclase assays. Figure 3.5 demonstrates ~1/3 of the WT-β_2-AR clones which were screened of which only 10-20 % were found to produce no significant response to isoprenaline. WT-β_2-AR clone #27 was selected for its ability to grow strongly and maintain a good level of receptor expression. WT-β_2-AR-GFP clone #13 was chosen for study based on clear plasma membrane delineated fluorescence and marked time-dependent receptor internalisation into discrete, punctate intracellular vesicles upon addition of the β-AR agonist isoprenaline (Figure 3.6). CAM-β_2-AR-GFP clone #2 demonstrated clear and easily detectible receptor compared to other clones isolated and for this reason was also chosen. Figure 3.7 gives an approximate indication of expression levels of each of these clones from using a single concentration of [^{3}H] DHA in binding studies. However, it was neccessary to carry out more detailed analysis to pharmacologically characterise these constructs.

3.3 Pharmacological characterisation

To determine more accuarately the receptor expression level of each clone saturation binding experiments with [^{3}H] DHA, an antagonist at the β_2-AR were carried out (Figures 3.8a, 3.9a and 3.10a). In agreement with the lower levels of autofluorescence in the CAM-β_2-AR-GFP-expressing clone compared to the WT-β_2-AR-GFP clone lower levels of [^{3}H] DHA-specific binding to membrane fractions isolated from the CAM-β_2-AR-GFP-expressing cells were measured (Table 3.1, B_{max} values). Higher expression of the GFP-tagged WT receptor was obtained than for the non-tagged receptor, a feature also exhibited by the β_1-AR (see Chapter 4).

Figure 3.5

Screening of WT-β_2-AR stable cell clones by intact cell adenylyl cyclase assays.

The WT-β_2-AR construct was expressed stably in HEK293 cells and individual positive clones were detected by intact cell adenylyl cyclase assays (Chapter 2, Section 2.7d). $5x10^{-5}$ M forskolin was used as a positive control for adenylyl cyclase activation and clones were selected on their ability to be stimulated by isoprenaline (10^{-5} M, 30 min). Data are means ± S.D. from a single experiment performed in triplicate.

Figure 3.5

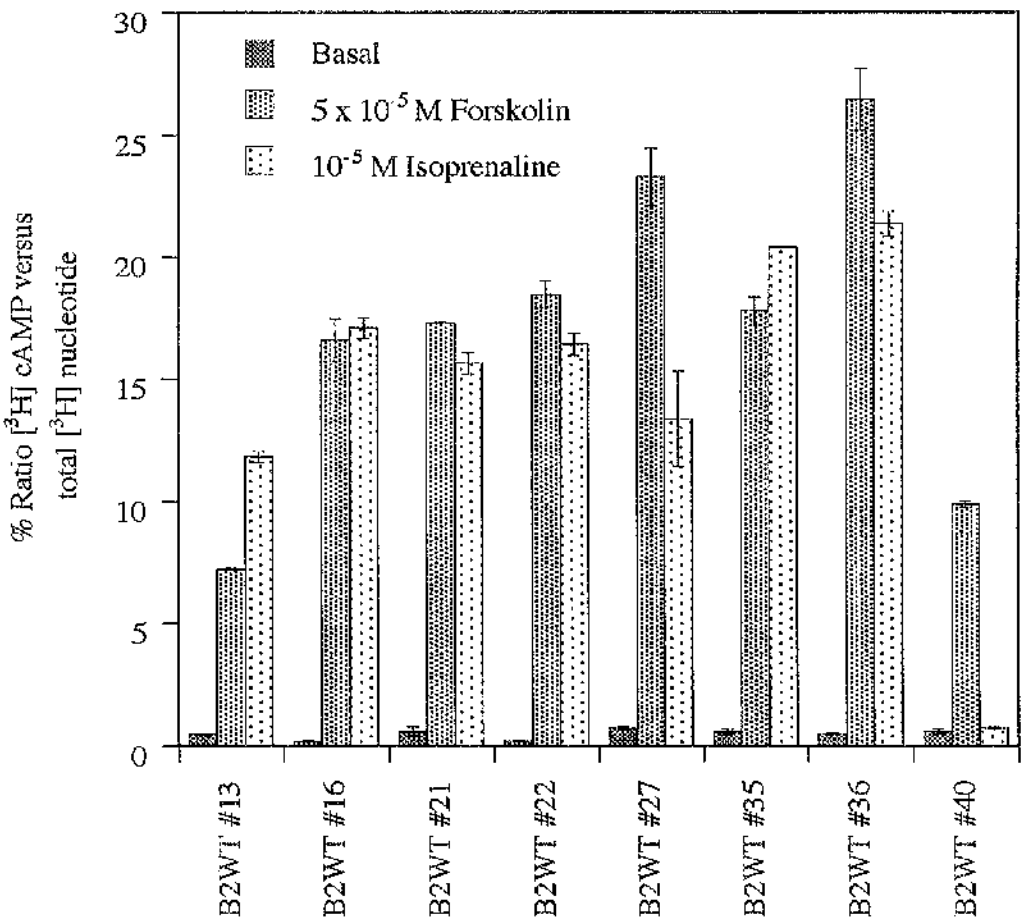

Basal
5 x 10-5 M Forskolin
10-5 M Isoprenaline
% Ratio [3H] cAMP versus total [3H] nucleotide
0
5
10
15
20
25
30
B2WT #13
B2WT #16
B2WT #21
B2WT #22
B2WT #27
B2WT #35
B2WT #36
B2WT #40

Figure 3.6

Internalisation of WT-β_2-AR-GFP by agonist stimulation.

A patch of WT-β_2-AR-GFP clone #13 cells were imaged in the confocal microscope in the absence of agonist (a) and following addition of 10^{-5} M isoprenaline for 10 (b), 15 (c), 20 (d), 30 (e) and 40 (f) minutes. This is representative of three separate experiments.

Figure 3.6

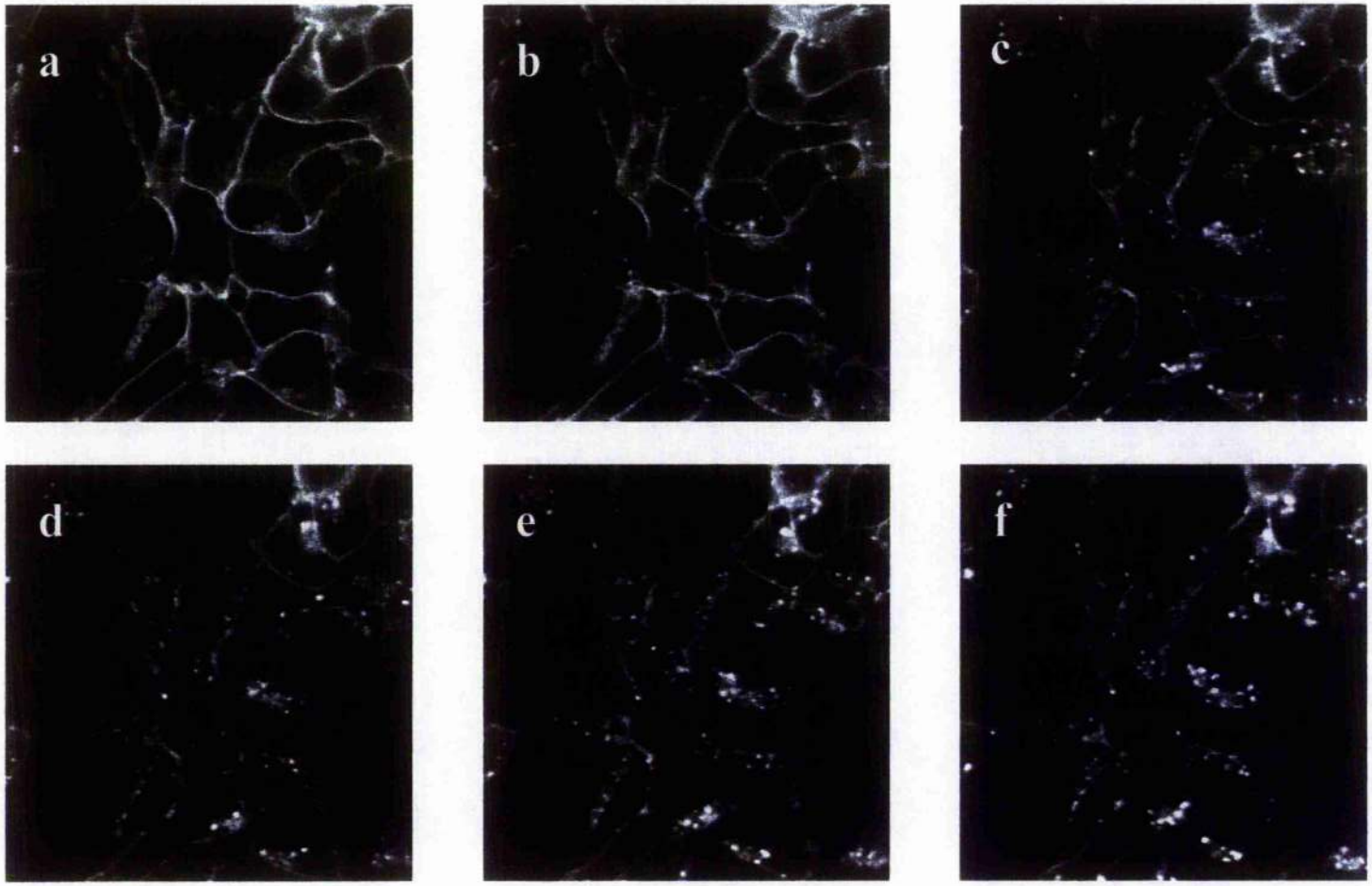


Figure 3.7

Estimation of receptor levels of β_2-AR clones from membrane binding studies with a single concentration of [^{3}H] DHA.

20μg of a membrane preparation from each cell line was used to estimate the approximate receptor level (fmol/mg). A close to saturating conentration of [^{3}H] DHA (2 nM) was used to determine total binding with 10^{-5} M propranolol as competing ligand to determine non-specific binding. Specific binding was used to estimate the receptor expression in each clone (fmol/mg). Data are presented as means ± S.E.M. n=3.

Figure 3.7

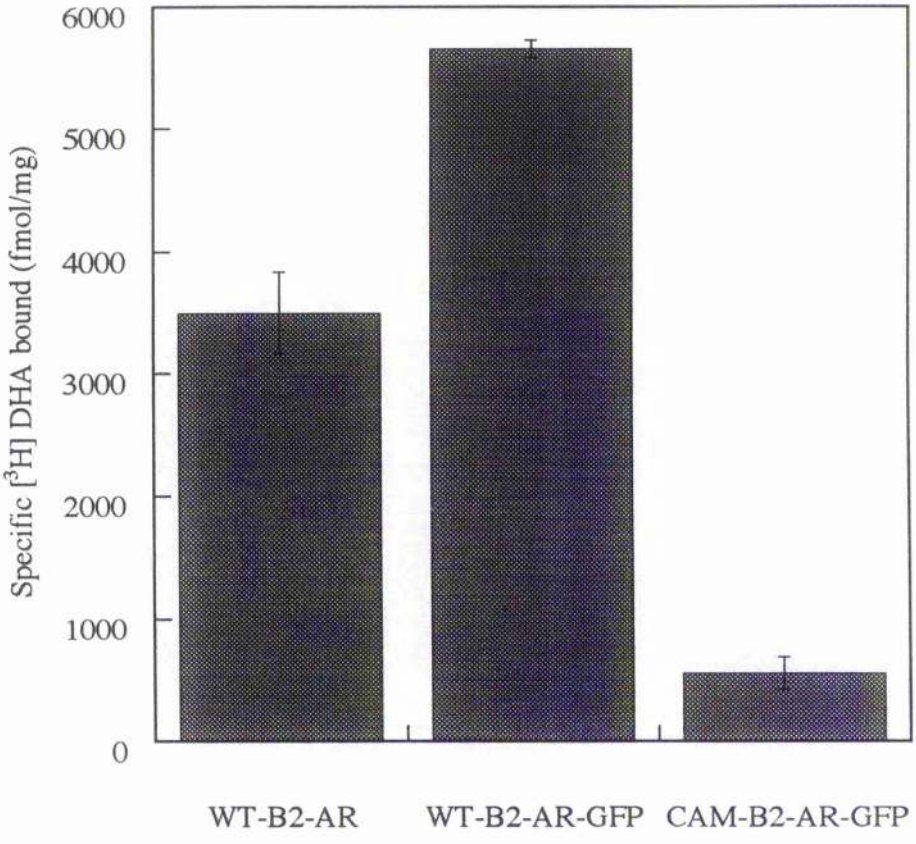

6000
5000
4000
3000
2000
1000
0
Specific [3H] DHA bound (fmol/mg)
WT-B2-AR
WT-B2-AR-GFP
CAM-B2-AR-GFP

Table 3.1

Ligand-binding characteristics of wild-type and CAM β_2-AR-GFP constructs.

Clone	K_d (nM)	B_{max} (pmol/mg)
WT-β_2-AR	0.62 ± 0.26	4.1 ± 0.9
WT-β_2-AR-GFP	0.75 ± 0.17	8.7 ± 0.7
CAM-β_2-AR-GFP	0.36 ± 0.13	1.0 ± 0.2

Data represent means ± S. D. from three independent experiments using [^{3}H] DHA as radioligand.

Table 3.2

Competition binding experiments at wild-type and CAM β_2-AR-GFP constructs.

Clone	K_i for isoprenaline (nM)	K_i for betaxolol (nM)	K_i for alprenolol (nM)
WT-β_2-AR	363 ± 82 (2)	344 ± 24 (2)	1.13 ± 0.2 (2)
WT-β_2-AR-GFP	782 ± 160 (3)	478 ± 16 (2)	2.50 ± 0.7 (2)
CAM-β_2-AR-GFP	24.2 ± 2.6 (3)	249 ± 14 (3)	0.72 ± 0.02 (3)

Data represent means ± S.E.M. from two or three independent experiments using [^{3}H] DHA as radioligand. The number of experiments are indicated in brackets.

The non-specific binding of [^{3}H] DHA at these receptor constructs, as determined by using a saturating concentration of propranolol (a β-AR blocker), was very low in this study (Figure 3.8a(ii), 3.9a(ii) and 3.10a(ii)). Saturation binding curves were converted into Scatchard plots for each receptor construct and results from these experiments indicated that the CAM-β_2-AR-GFP construct bound this ligand with an affinity similar to the wild-type β_2-AR-GFP (Table 3.1, K_d values and Figures 3.8b, 3.9b and 3.10b).

Competition for the specific binding of [^{3}H] DHA to membranes expressing WT-β_2-AR, WT-β_2-AR-GFP or CAM-β_2-AR-GFP with either betaxolol (an inverse agonist), alprenolol (an antagonist) or isoprenaline (an agonist) were performed to demonstrate that adding GFP to the C terminus of the receptor had little effect on basic receptor pharmacology (Table 3.2). When isoprenaline was used as the competing drug results indicated that this agonist had substantially higher affinity for CAM-β_2-AR-GFP (K_i = 24.2 ± 2.6 nM) than for WT-β_2-AR-GFP (K_i = 363 ± 82 nM) (Figure 3.11). As such, the previously noted high affinity of this agonist for the CAM-β_2-AR compared with WT-β_2-AR (Samama et al., 1993) was preserved following addition of GFP to the C-terminal tail of both of these GPCR variants. High affinity of the antagonist alprenolol and low affinity of the inverse agonist betaxolol for CAM-β_2-AR was also retained after addition of GFP as measured by the competition for the specific binding of [^{3}H] DHA by alprenolol or betaxolol at CAM-β_2-AR-GFP (Figure 3.12 and Table 3.2).

3.4 Ligand regulation of CAM-β_2-AR-GFP compared to the wild-type receptor constructs

From previous investigations long term treatment (≥24 h) of the CAM-β_2-AR causes an increase in expression levels of the receptor (Pei et al., 1994; MacEwan and

Figure 3.8

β_2-AR binding characteristics in WT-β_2-AR-expressing cells.

ai) A saturation binding study using increasing concentrations of [^{3}H] DHA was performed to measure specific binding (pmol/mg) in membranes from WT-β_2-AR cells.

aii) The same saturation binding study as in (ai) but showing total, specific and non-specific binding (d.p.m.).

b) Transformation of the specific binding data to generate a Scatchard plot. In the example displayed B_{max} was 3.31 pmol/mg and the K_d for [^{3}H] DHA was 0.36 nM.

Figure 3.8a(i)

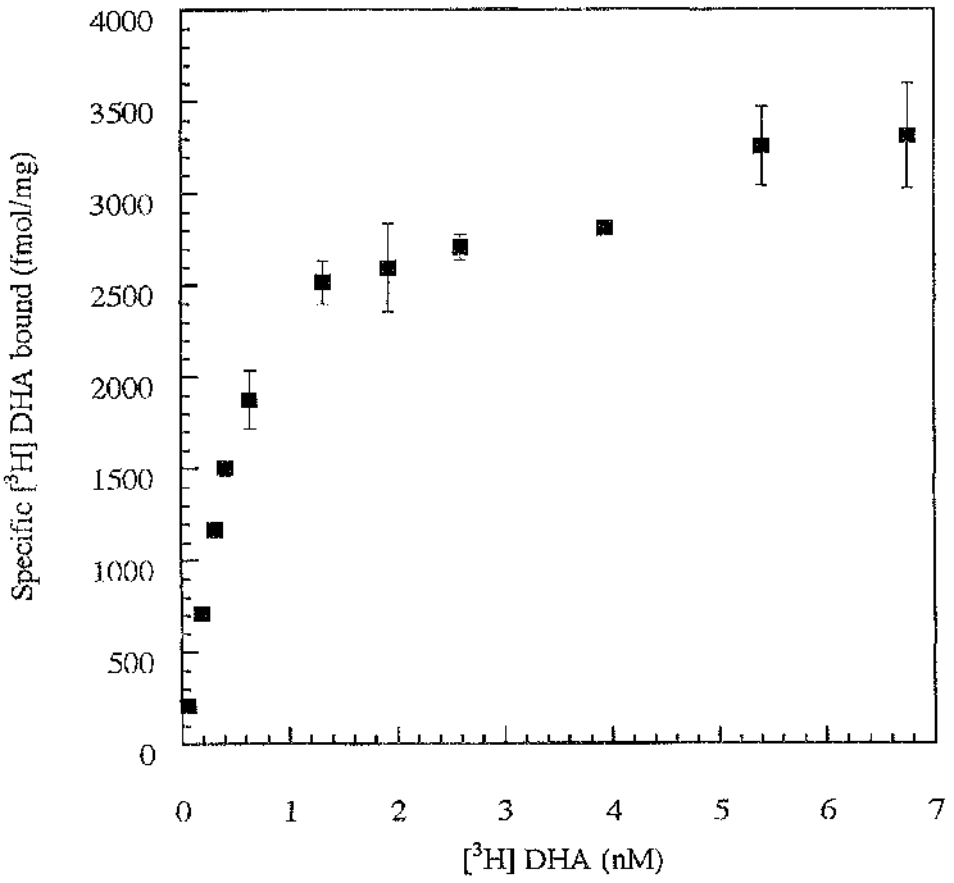

4000
3500
3000
2500
2000
1500
1000
500
0
Specific [3H] DHA bound (fmol/mg)
0
1
2
3
4
5
6
7
[3H] DHA (nM)

Figure 3.8a(ii)

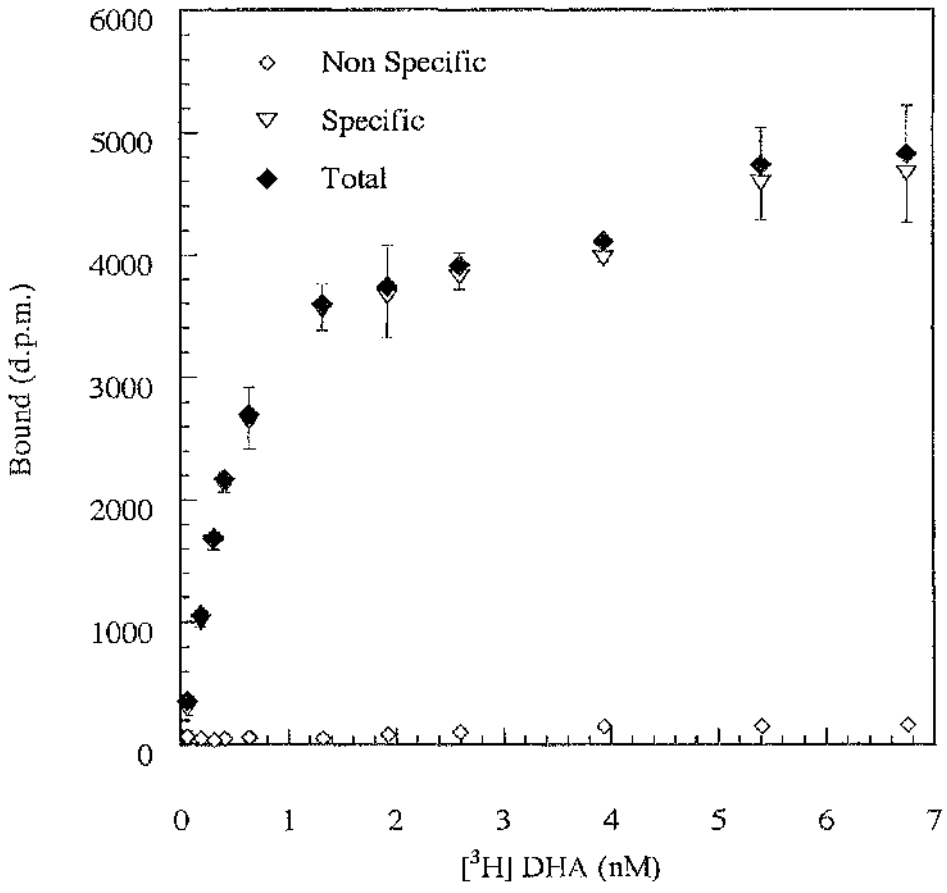


Figure 3.8b

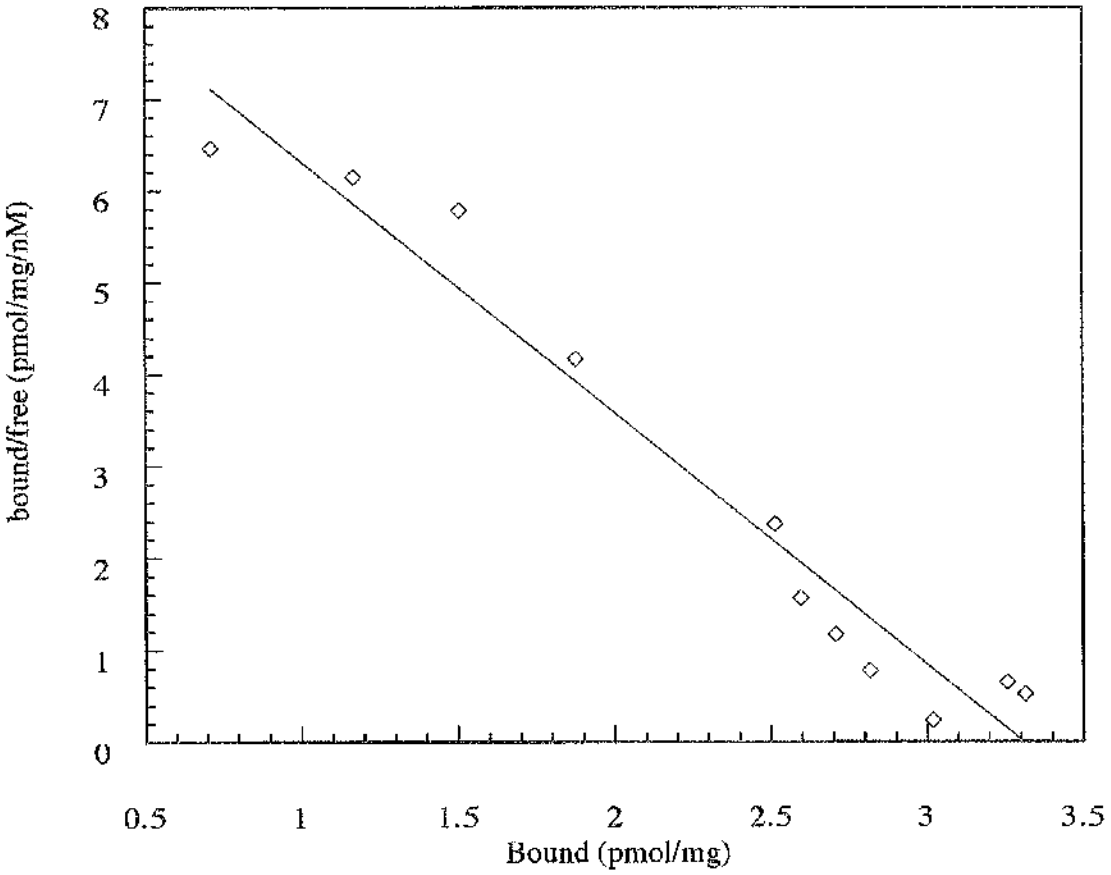


Figure 3.9

β_2-AR binding characteristics in WT-β_2-AR-GFP-expressing cells.

ai) A saturation binding study using increasing concentrations of [^{3}H] DHA was performed to measure specific binding (pmol/mg) in membranes from WT-β_2-AR-GFP cells.

aii) The same saturation binding study as in (ai) but showing total, specific and non-specific binding (d.p.m.).

b) Transformation of the specific binding data to generate a Scatchard plot. In the example displayed B_{max} was 9.24 pmol/mg and the K_d for [^{3}H] DHA was 0.87 nM.

Figure 3.9a(i)

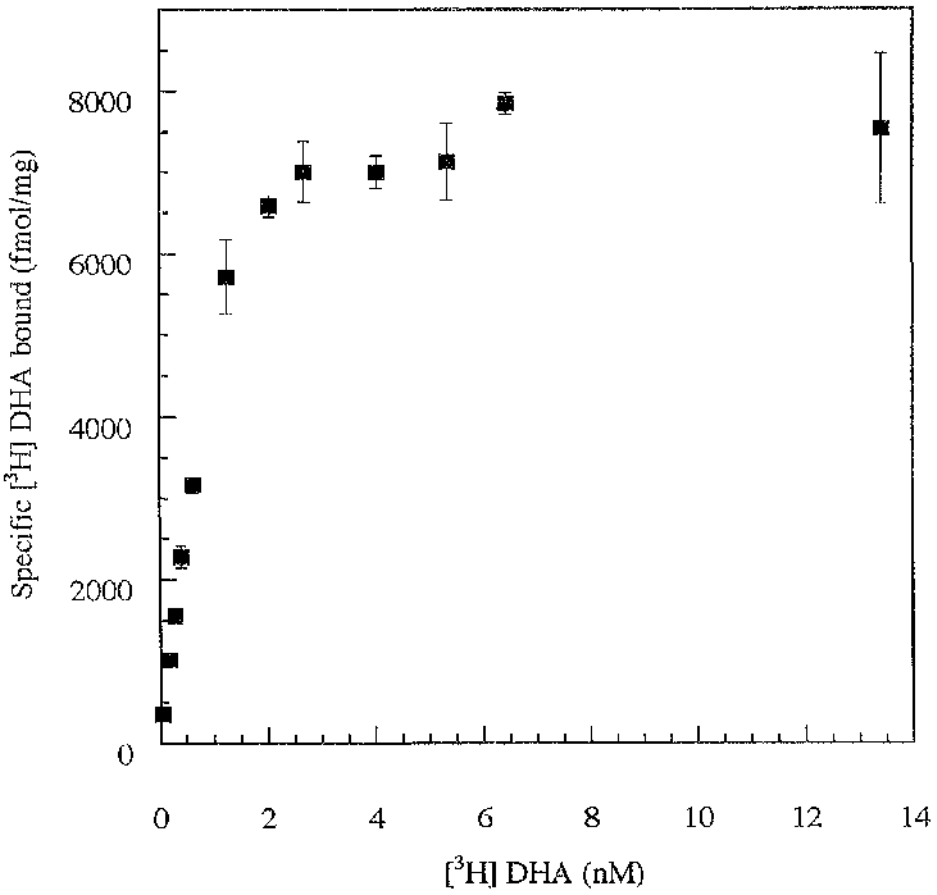

Specific [3H] DHA bound (fmol/mg)
8000
6000
4000
2000
0
0
2
4
6
8
10
12
14
[3H] DHA (nM)

Figure 3.9a(ii)

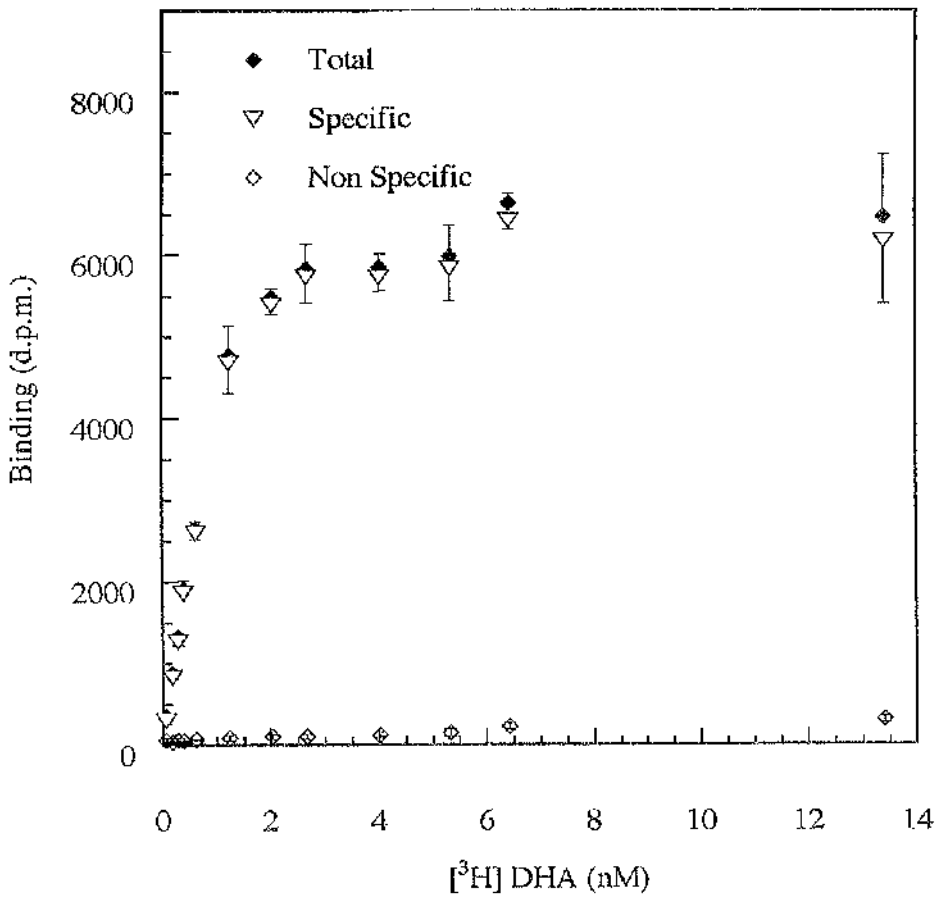


Figure 3.9b

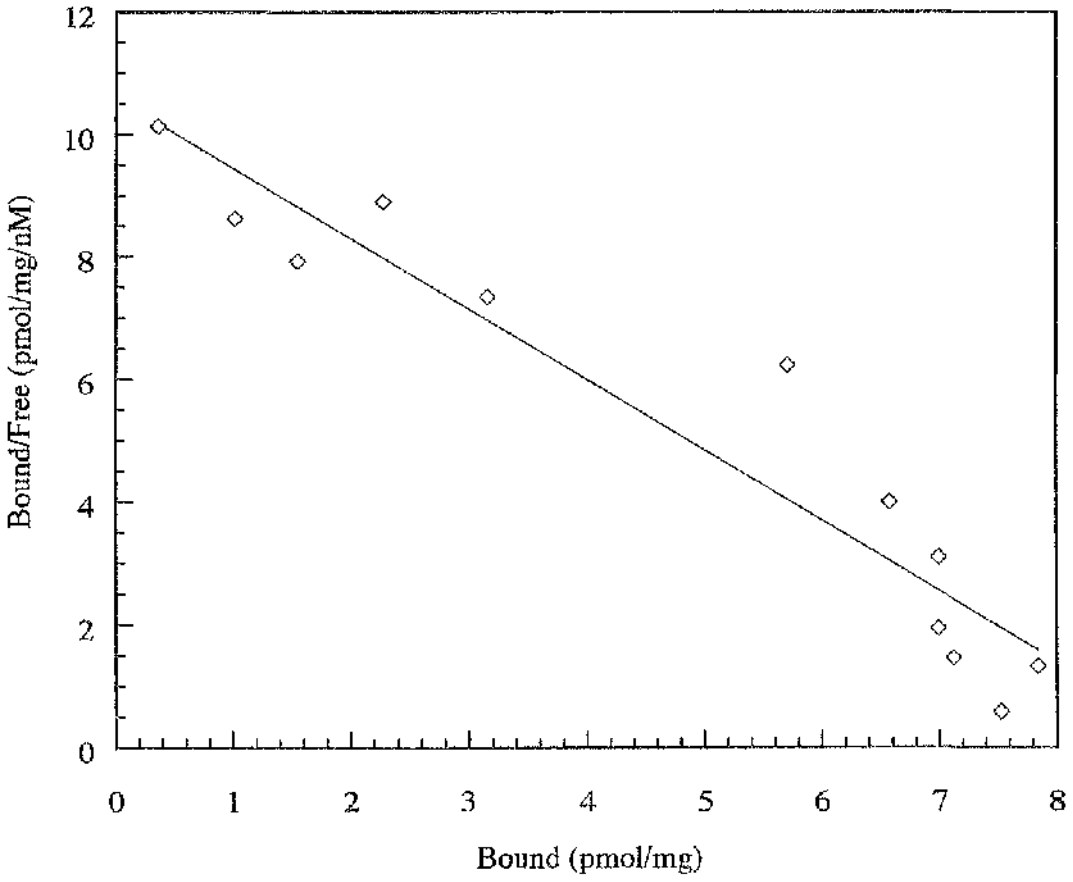


Figure 3.10

β_2-AR binding characteristics in CAM-β_2-AR-GFP-expressing cells.

ai) A saturation binding study using increasing concentrations of [^{3}H] DHA was performed to measure specific binding (pmol/mg) in membranes from CAM-β_2-AR-GFP cells.

aii) The same saturation binding study as in (ai) but showing total, specific and non-specific binding (d.p.m.).

b) Transformation of the specific binding data to generate a Scatchard plot. In the example displayed B_{max} was 0.74 pmol/mg and the K_d for [^{3}H] DHA was 0.23 nM.

Figure 3.10a(i)

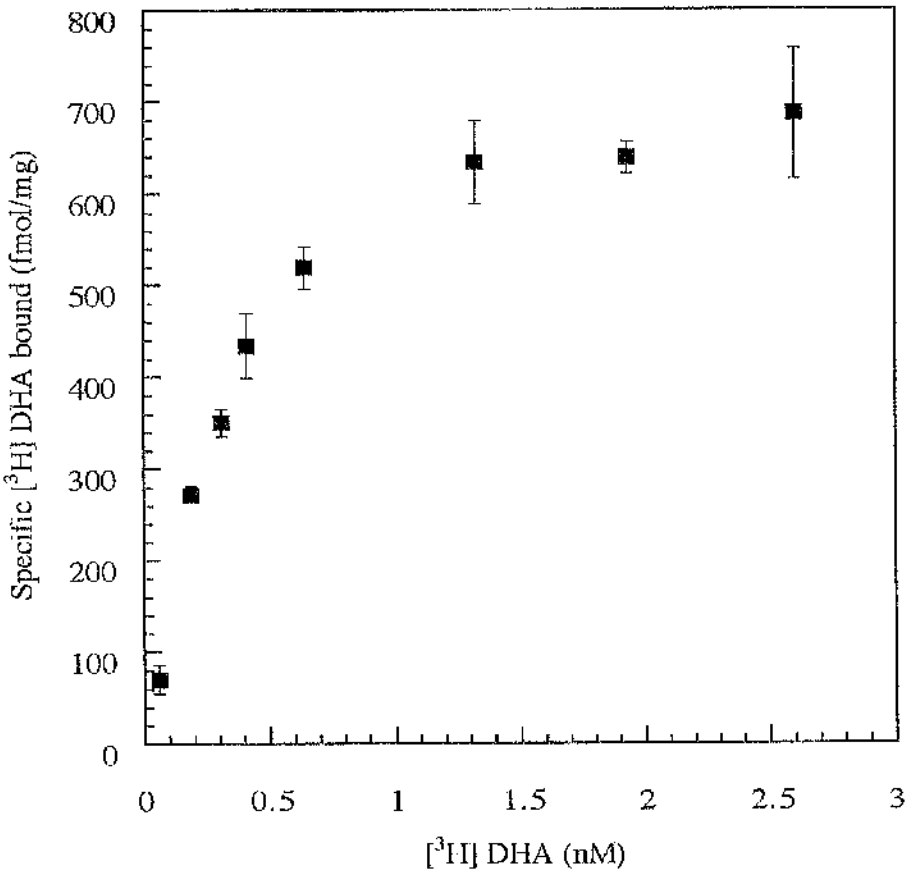


Figure 3.10a(ii)

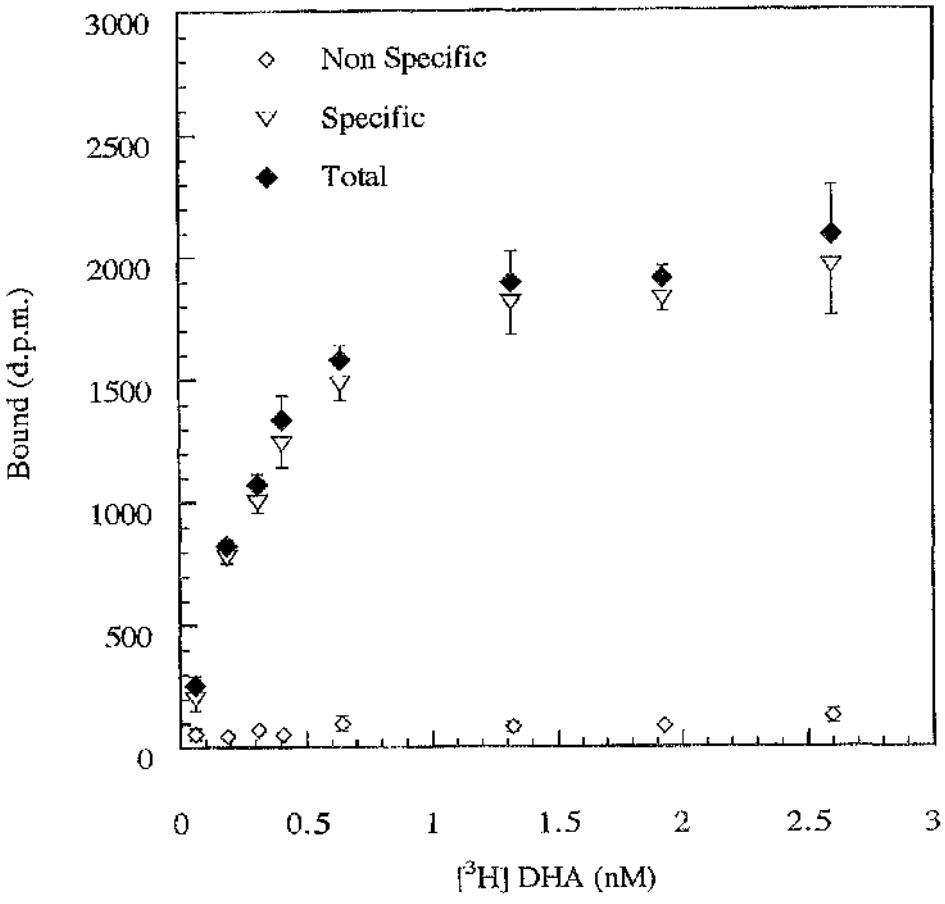


Figure 3.10b

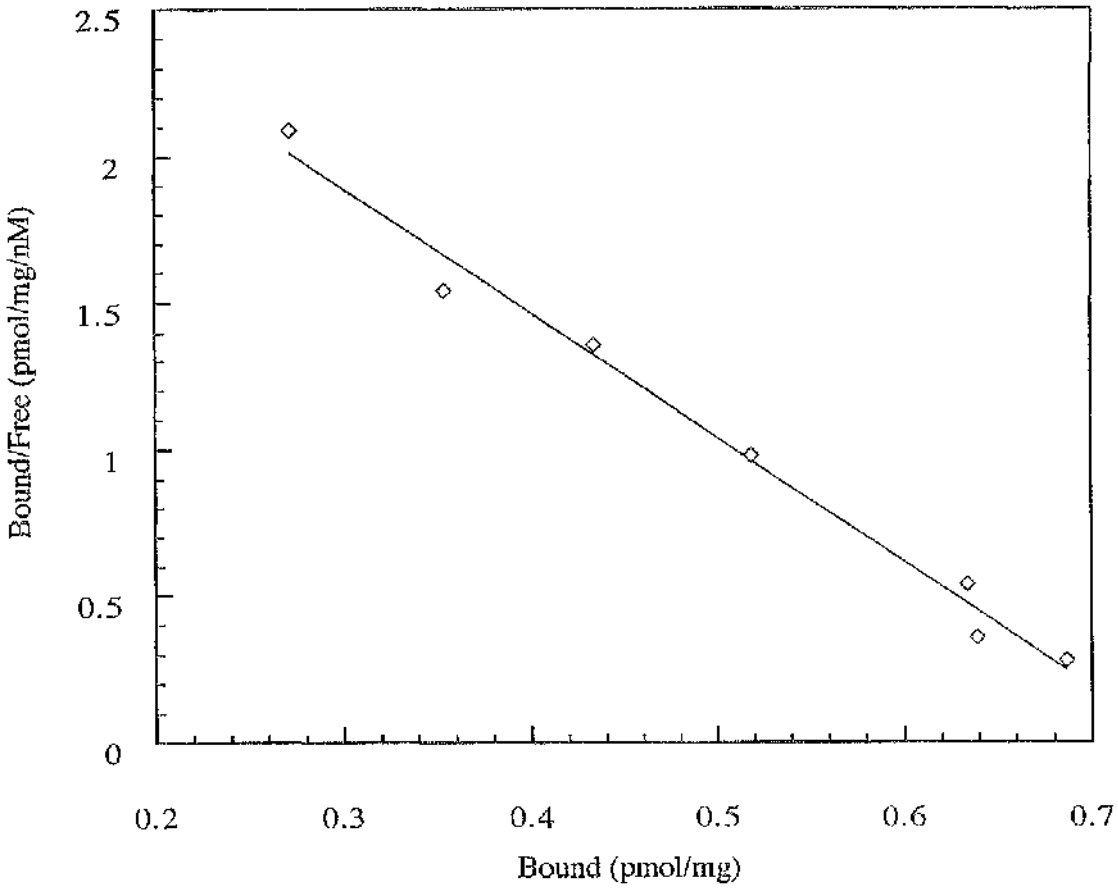


Figure 3.11

High affinity of isoprenaline for the CAM-β_2-AR is retained after addition of GFP.

Competition between [^{3}H] DHA (0.34 nM) and varying concentrations of isoprenaline for specific binding to membranes expressing either WT-β_2-AR-GFP (triangles) or CAM-β_2-AR-GFP (squares) was assesed. Results are from one experiment performed in triplicate ± S.D. Similar results were obtained from two further experiments.

Figure 3.11

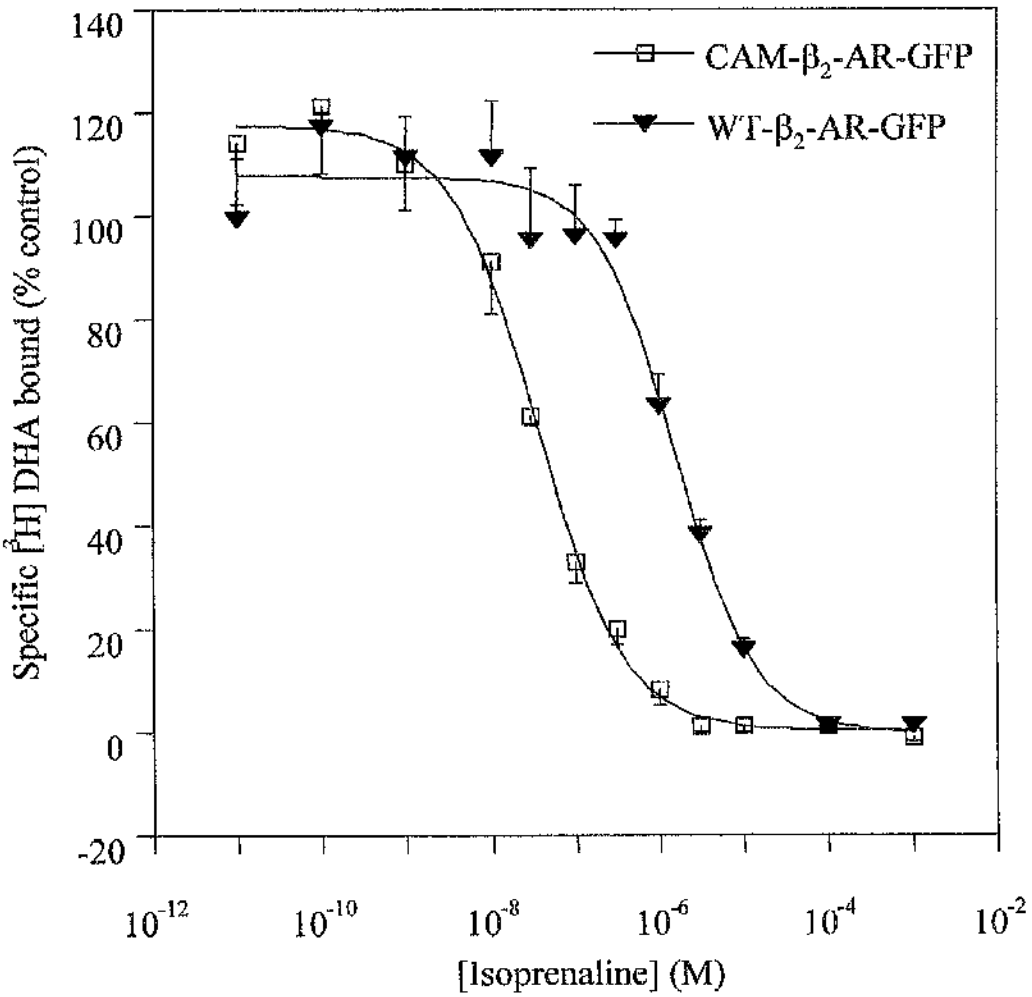
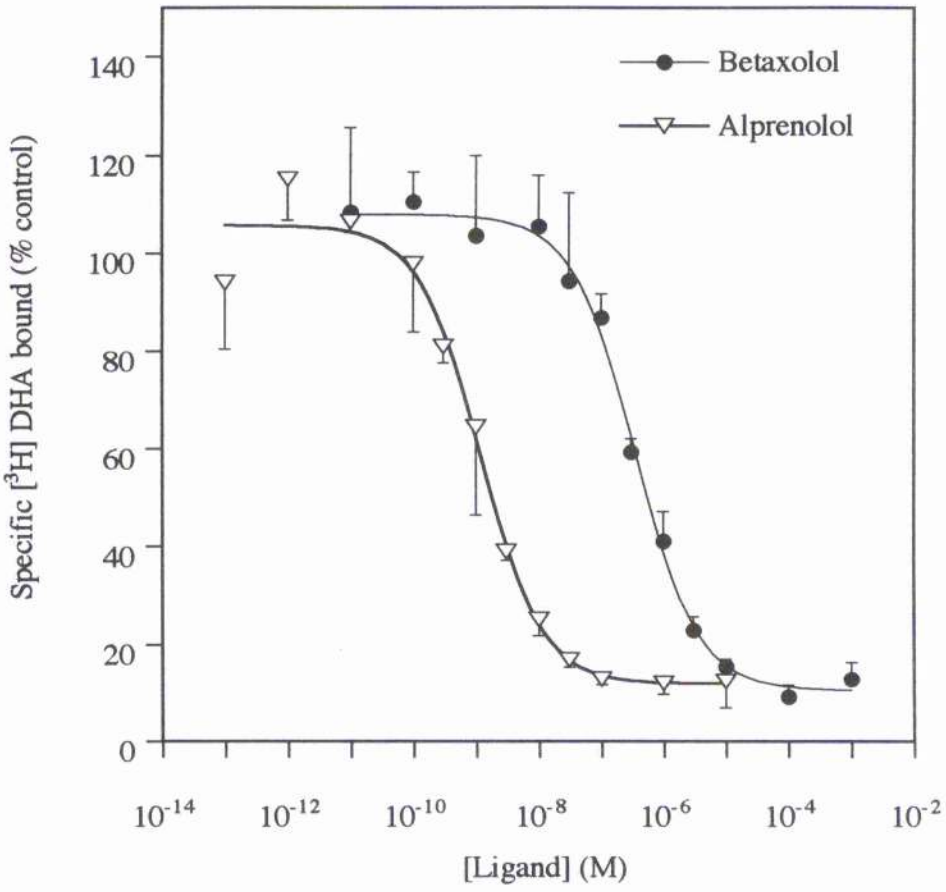

CAM-β2-AR-GFP
WT-β2-AR-GFP
Specific [3H] DHA bound (% control)
140
120
100
80
60
40
20
0
-20
10-12
10-10
10-8
10-6
10-4
10-2
[Isoprenaline] (M)

Figure 3.12

High affinity of alprenolol compared to betaxolol at CAM-β_2-AR.

Competition between [^{3}H] DHA (0.65nM) and varying concentrations of alprenolol (triangles) or betaxolol (circles) for specific binding to membranes expressing CAM-β_2-AR-GFP was assessed. Results are from one experiment performed in triplicate ± S.D. Similar results were obtained from two further experiments.

Figure 3.12


Betaxolol
Alprenolol
Specific [3H] DHA bound (% control)
140
120
100
80
60
40
20
0
10-14
10-12
10-10
10-8
10-6
10-4
10-2
[Ligand] (M)

Milligan, 1996a,b). CAM-β_2-AR-GFP cells were treated with or without 10^{-5} M betaxolol for 24 h and the effects monitored firstly by confocal microscopy (Figure 3.13a,b) where a marked increase in both plasma membrane-delineated and diffuse intracellular fluorescence was observed (Figure 3.13b). Washing of the cells followed by an intact cell ligand binding experiment with [^{3}H] DHA indicated a 2.75 ± 0.28 fold up-regulation of CAM-β_2-AR-GFP in response to betaxolol (Figure 3.13c). Similar results were obtained when the same experiment was performed on membrane preparations from the same cells (Figure 3.14). This figure also demonstrated that sustained treatment of cells expressing WT-β_2-AR or WT-β_2-AR-GFP with betaxolol failed to result in a significant up-regulation of the construct. That the increased GFP autofluorescence in response to treatment with betaxolol in cells expressing CAM-β_2-AR-GFP represented up-regulation of the GPCR-GFP fusion protein was further confirmed by immunoblotting studies on membranes of untreated and betaxolol-treated cells (Figure 3.15a, lanes 1 and 2, and Figure 3.15b, lanes 1 and 2). Antibodies against both the β_2-AR and against GFP indicated that betaxolol treatment substantially increased levels of a family of poorly resolved polypeptides that are likely to represent differentially glycosylated forms of the receptor (Figure 3.15), although the current studies cannot exclude that a degree of proteolytic degradation had occurred to produce this pattern. Little or no effect of betaxolol at the WT-β_2-AR (Figure 3.15b, lanes 3 and 4) or WT-β_2-AR-GFP (Figure 3.15a, lanes 3 and 4, and Figure 3.15b lanes 5 and 6) was detected by immunoblotting.

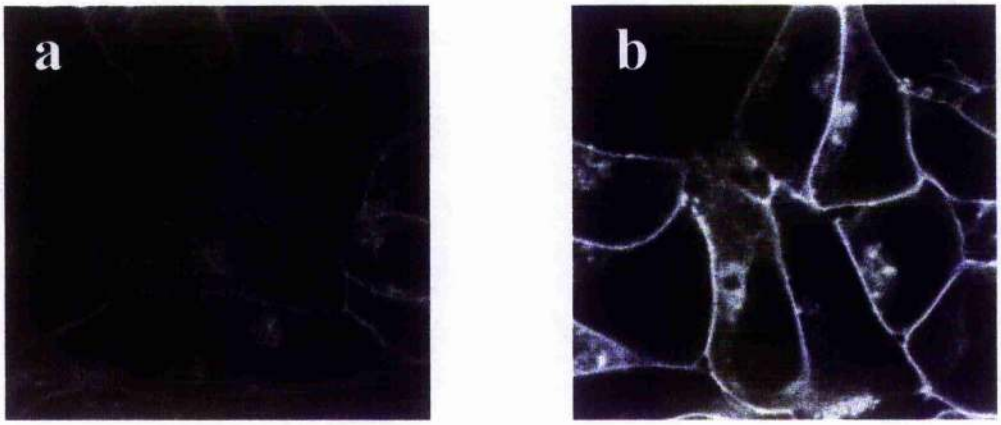
Up-regulation of GFP-autofluorescence was also observed by treatment of the CAM-β_2-AR-GFP-expressing cells with a range of other β-blockers including DHA, labetolol, and ICI118551 (24 h, each at a saturating dose of 10^{-5} M) (Figure 3.16). Pharmacological selectivity of this effect was apparent because it was not produced by treatment with the α_1-AR antagonist prazosin or the α_2-AR antagonist yohimbine (24 h, each at 10^{-5} M) (Figure 3.17). Although each of the β-blockers described above resulted in greater levels of autofluorescent signal, the pattern of distribution of

Figure 3.13

Up-regulation of CAM-β_2-AR-GFP by betaxolol.

Cells of a single clone were grown on glass coverslips in the absence (a) or presence (b) of 10^{-5} M betaxolol for 24 h. These cells were then visualised. In (c) the same clone was used where cells were untreated or treated with betaxolol (10^{-5} M for 24 h). These were then washed and used to measure the specific binding of [^{3}H] DHA in intact cells ([^{3}H] DHA is a lipophillic antagonist that crosses the plasma membrane and thus provides a measure of total cell levels of β_2-AR-binding sites). Data are presented as means ± S.E.M., $n = 3$.

Figure 3.13


a
b

c)

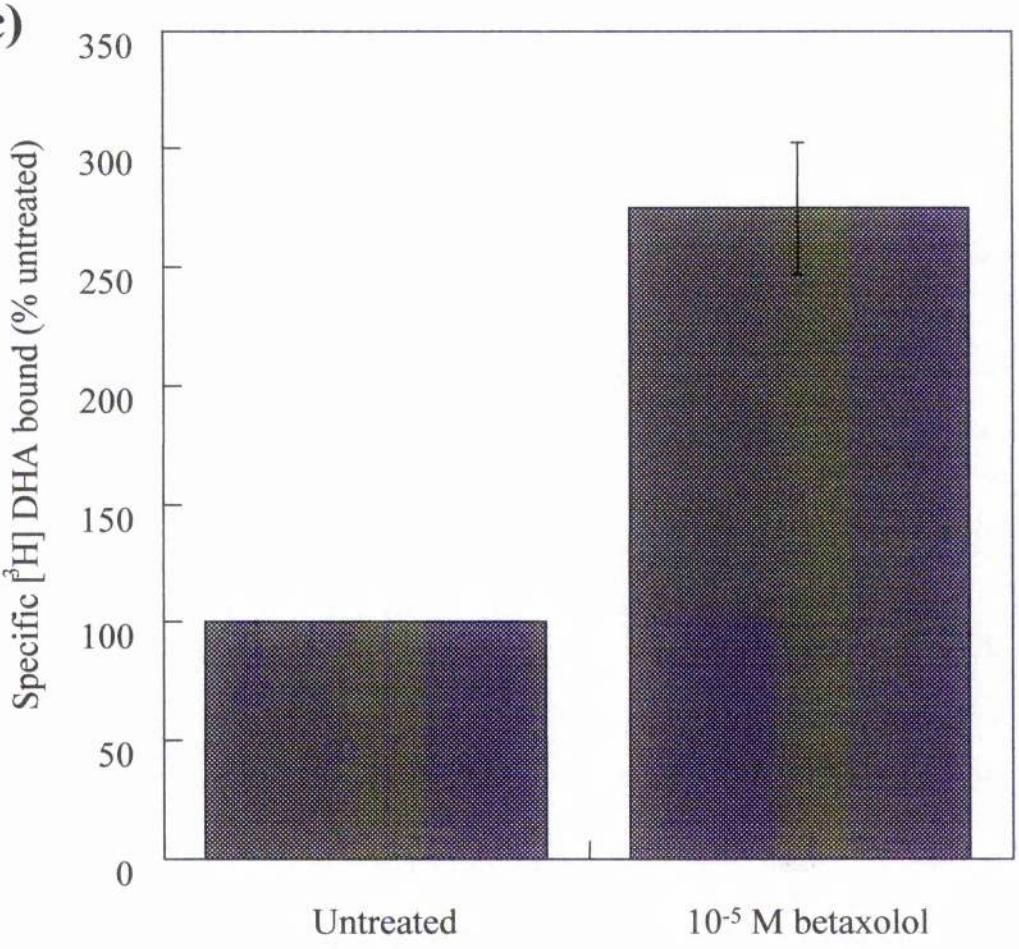

Specific [3H] DHA bound (% untreated)
350
300
250
200
150
100
50
0
Untreated
10-5 M betaxolol

Figure 3.14

Betaxolol treatment of β_2-AR clones: membrane binding studies.

Membrane fractions were prepared from cells expressing each β_2-AR construct, which had been maintained for 24 h in the absence or presence of betaxolol (10^{-5} M). 20 μg of each was used to measure the specific binding of [^{3}H] DHA. Data are represented as a % of the specific binding at untreated cells. Data are presented as means ± S.E.M. n=3

Figure 3.14

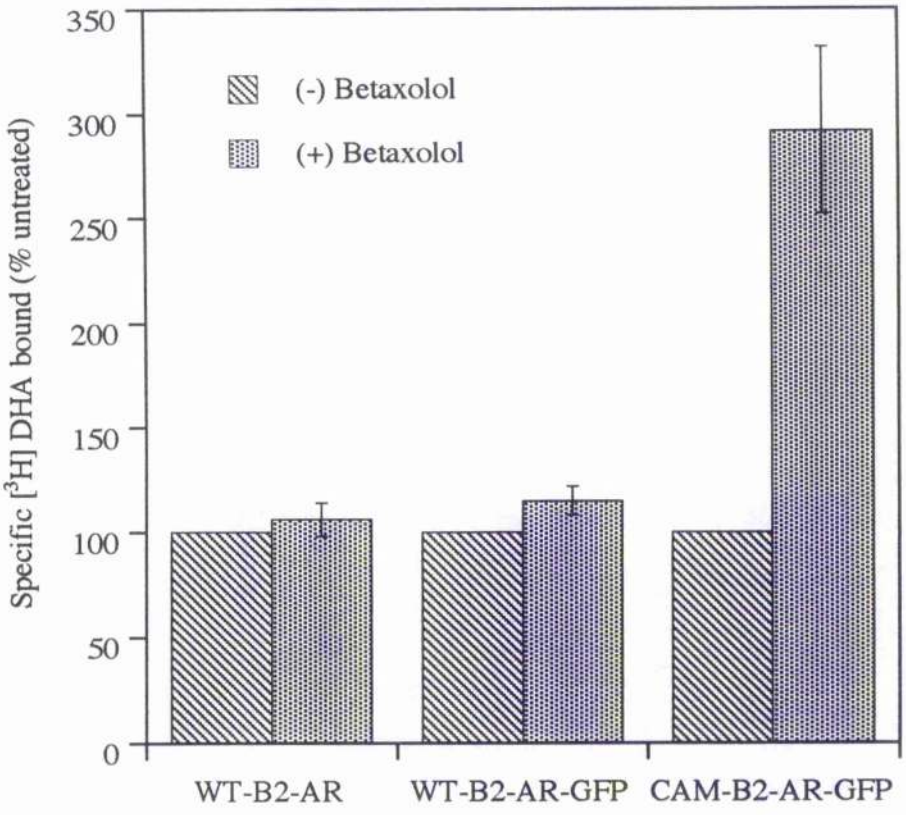

350
300
250
200
150
100
50
0
Specific [3H] DHA bound (% untreated)
(-) Betaxolol
(+) Betaxolol
WT-B2-AR
WT-B2-AR-GFP
CAM-B2-AR-GFP

Figure 3.15

Betaxolol treatment of β_2-AR clones: immunoblot studies.

Membrane fractions were prepared from cells expressing each β_2-AR construct, which had been maintained for 24 h in the absence or presence of betaxolol (10^{-5} M). Each gel was loaded with 30 μg of CAM-β_2-AR-GFP membranes, 10 μg of WT-β_2-AR membranes and 5 μg of WT-β_2-AR-GFP membranes in accordance with the differing expression level (B_{max} values) for each clone.

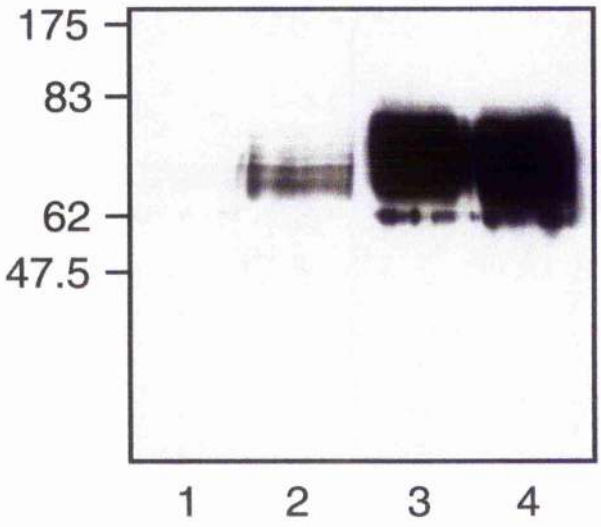
a) The anti-GFP immunoblot shows up-regulation of CAM-β_2-AR-GFP (lane 2) compared to untreated cells (lane 1). WT-β_2-AR-GFP expression (lane 3) is little modified by betaxolol over-night treatment (lane 4).

b) The anti-β_2-AR immunoblot shows up-regulation of CAM-β_2-AR (lane 2) compared to untreated cells (lane 1). Betaxolol has no effect on the expression of WT-β_2-AR (lanes 3 and 4) and little effect on expression of WT-β_2-AR-GFP (lanes 5 and 6).

A representative experiment of three is shown.

Figure 3.15

a)



b)

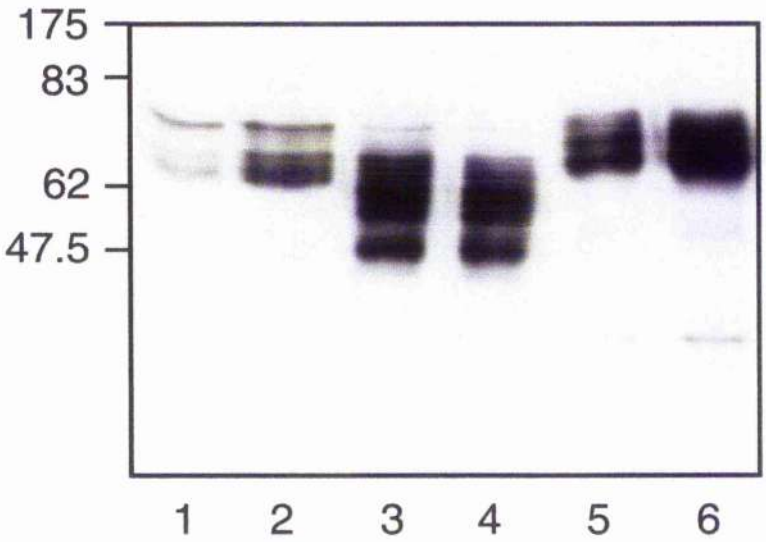


Figure 3.16

Up-regulation of CAM-β_2-AR-GFP by other β-AR blockers.

CAM-β_2-AR-GFP-expressing cells were exposed to no ligand (a), DHA (b), labetolol (c), or ICI118551 (d) (each at 10^{-5} M) for 24 h. The cells were then imaged by confocal microscopy. Results from a typical experiment are displayed.

Figure 3.16

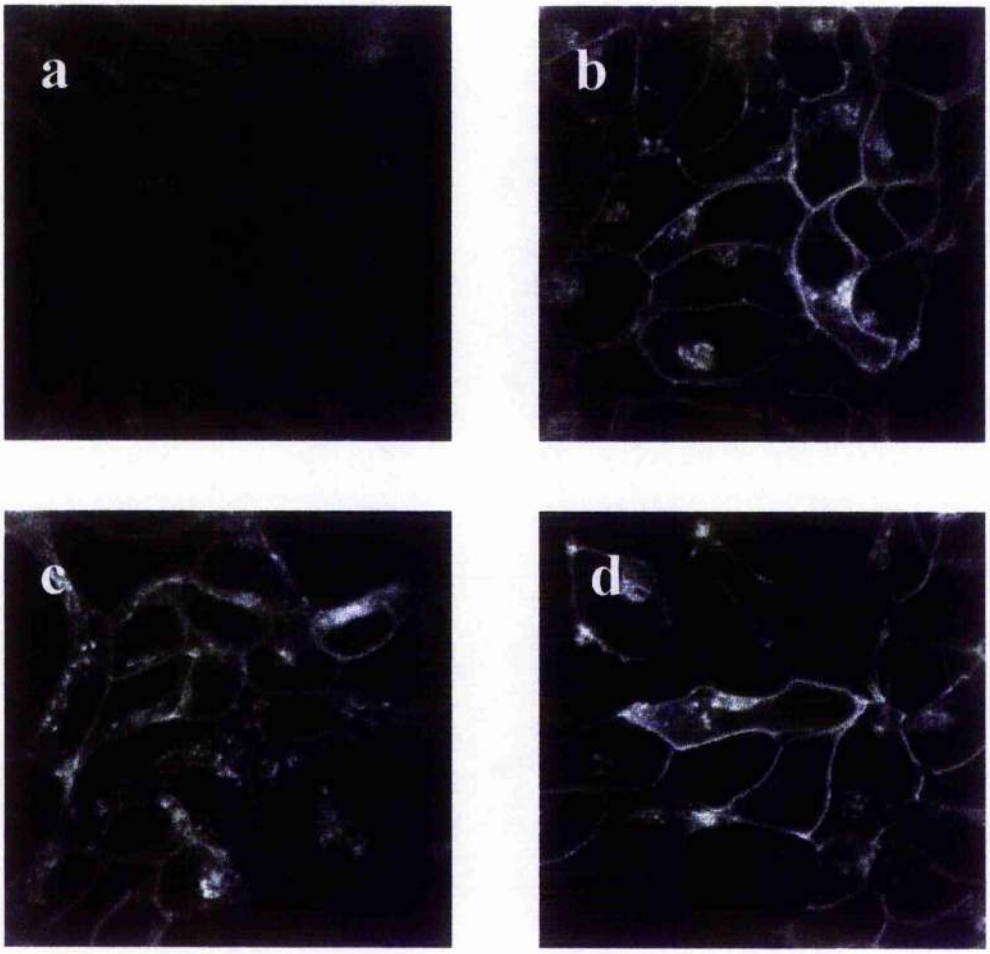
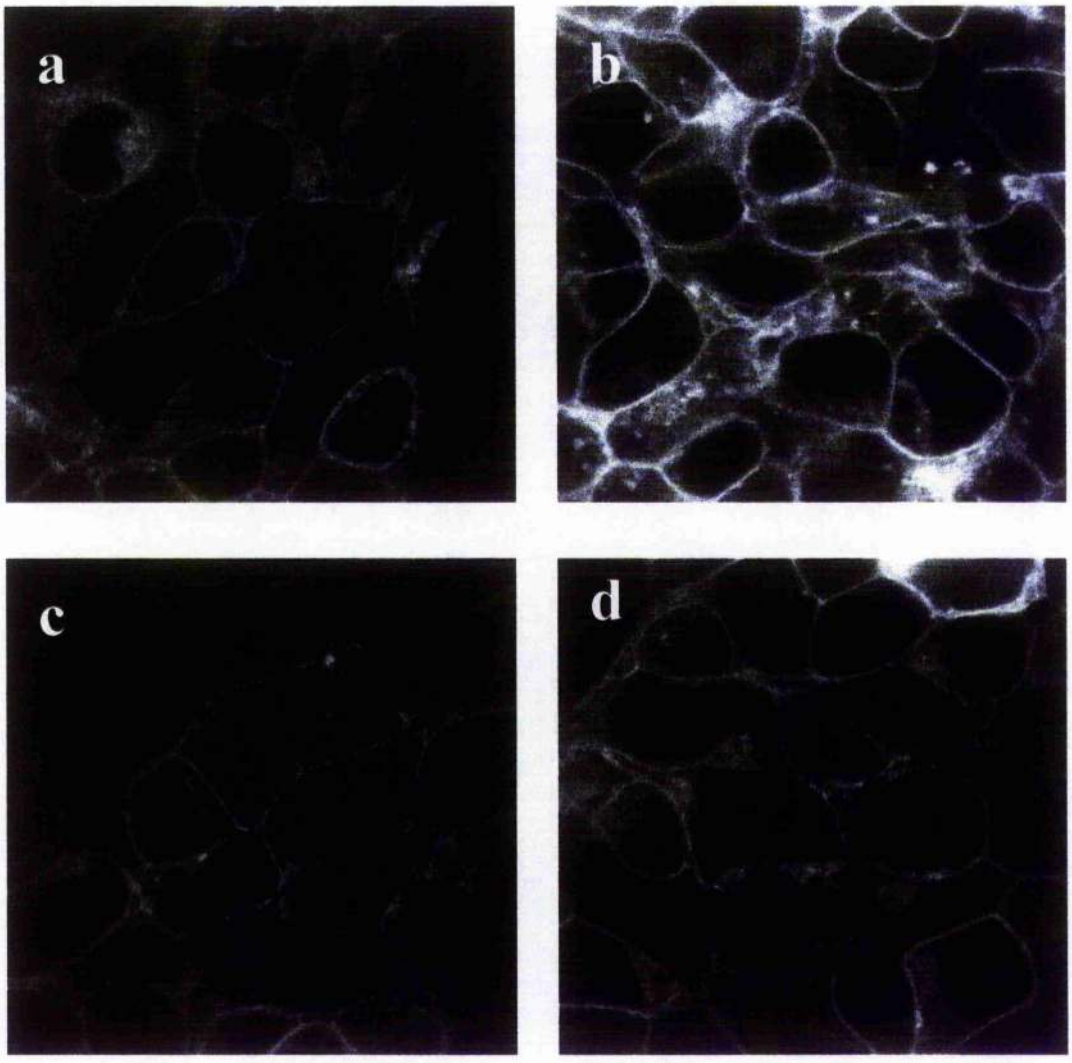


Figure 3.17

Pharmacological selectivity is maintained at CAM-β_2-AR-GFP.

CAM-β_2-AR-GFP-expressing cells were exposed to no ligand (a), betaxolol (b), prazosin (α_1-AR antagonist) (c), or yohimbine (α_2-AR antagonist) (d) for 24 h (each at 10^{-5} M). The cells were then imaged by confocal microscopy. Results from a typical experiment are displayed.

Figure 3.17



cellular CAM-β_2-AR-GFP was not identical. Both betaxolol and ICI118551 resulted in a large increase in homogenous plasma membrane-delineated fluorescence (Figure 3.16d,e). However, as with the untreated cells, a significant amount of predominantly diffuse, intracellular staining was observed, the level of which was greater than in the untreated cells (Figure3.16a,d,e). By contrast, after treatment with labetolol, although a substantial increase in plasma membrane CAM-β_2-AR-GFP was observed, there was also an increase in the intracellular signal. A significant fraction of this autofluorescent signal displayed a subplasma membrane, distinctly punctate localisation (Figure 3.16c) that appeared similar to the pattern produced by short-term treatment with the agonist isoprenaline (Figure 3.6). Long term treatment of this clone with the full agonist isoprenaline (10^{-5} M, 24 h) caused a large redistribution of receptor such that it became intracellular with a marked punctate pattern (Figure 3.18). To explore a possible basis for these differences, basal intact cell adenylyl cyclase activity and its regulation by a variety of ligands was assessed. Although basal cAMP level in these cells were low, both ICI118551 and betaxolol were able to reduce them further, indicating that these ligands function as inverse agonists at the CAM-β_2-AR-GFP. By contrast, alprenolol and DHA displayed partial agonism, and, in this system, a maximally effective concentration of labetolol was able to elevate cAMP levels to the same extent as isoprenaline (Figure 3.19).

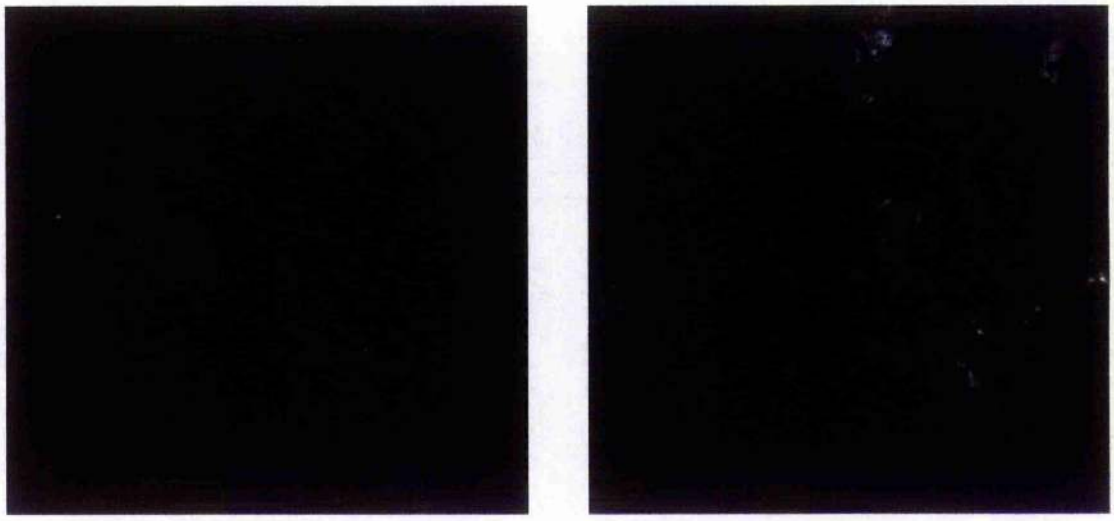
The capacity of betaxolol to alter the fluorescence intensity of CAM-β_2-AR-GFP-expressing cells could be detected and directly quantitated in a spectrofluorimeter after seeding of cells into wells of a 96-well microtiter plate (Figure 3.20, this experiment was carried out by Nicola Bevan, Glaxo Wellcome). This allowed concentration-response curves to betaxolol to be calculated conveniently. Up-regulation was observed with EC_{50} = 0.17 μM. This value was in good accordance with the measured K_i of betaxolol to bind to this GPCR-GFP construct as determined from competition binding experiments between [^{3}H] DHA and betaxolol (0.25 ± 0.01 μM) (Figure 3.12). This enhanced fluorescent signal was not simply due to the addition of

Figure 3.18

No up-regulation occurs upon treatment with agonist isoprenaline.

CAM-β_2-AR-GFP-expressing cells were exposed to no ligand, or isoprenaline (10^{-5} M) for 24 h. The cells were then imaged by confocal microscopy. Results from a typical experiment are displayed.

Figure 3.18



Basal **+ isoprenaline**

Figure 3.19

Ligand regulation of adenylyl cyclase activity in intact cells expressing CAM-β_2-AR-GFP.

Basal (1) adenylyl cyclase activity and its regulation by forskolin ($5x10^{-5}$ M; 2), isoprenaline (10^{-5} M; 3), betaxolol (4), and a range of β-blockers used in Figure 3.16 [labetolol (5), DHA (6), ICI118551 (7)] and alprenolol (8; all at 10^{-5} M) were assessed as described in Chapter 2 Section 2.7d. Data represent means ± S.D. of triplicate assays from a single representative experiment. Two additional assays produced similar results, although the extent of [^{3}H] nucleotide conversion varied over a 2-fold range between experiments.

Figure 3.19

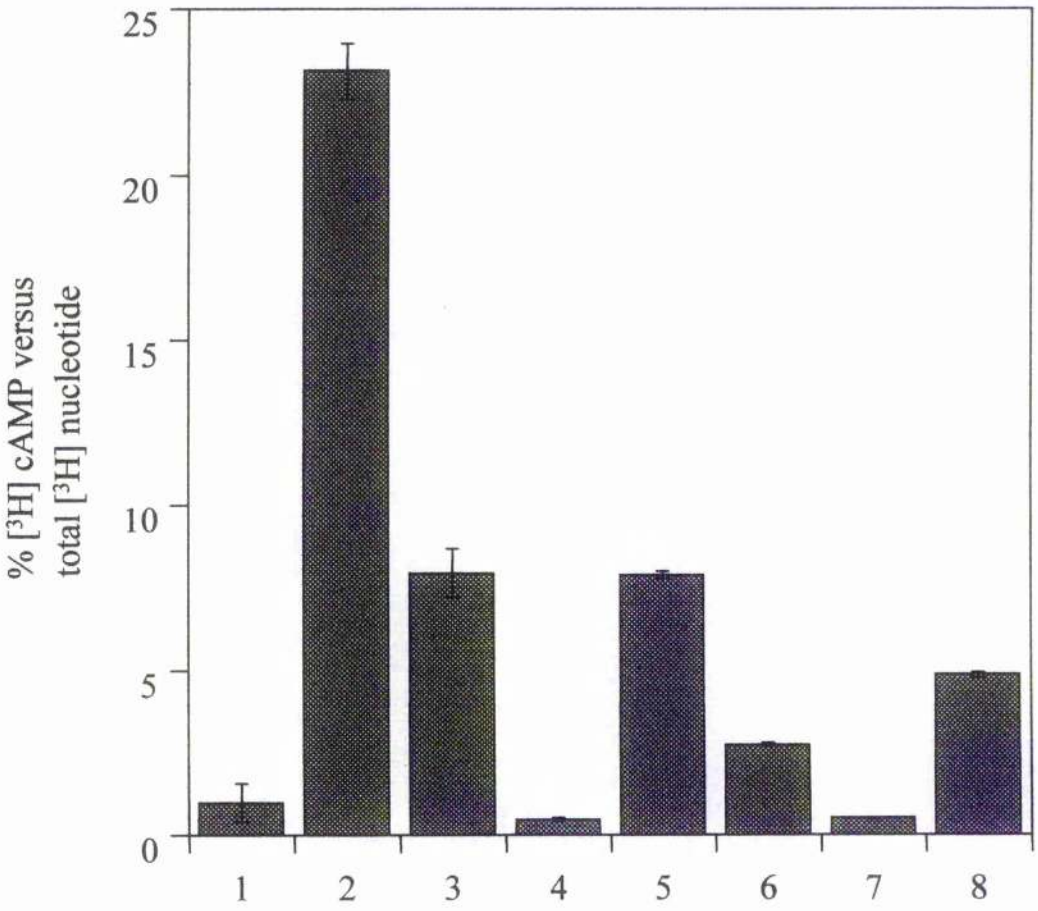
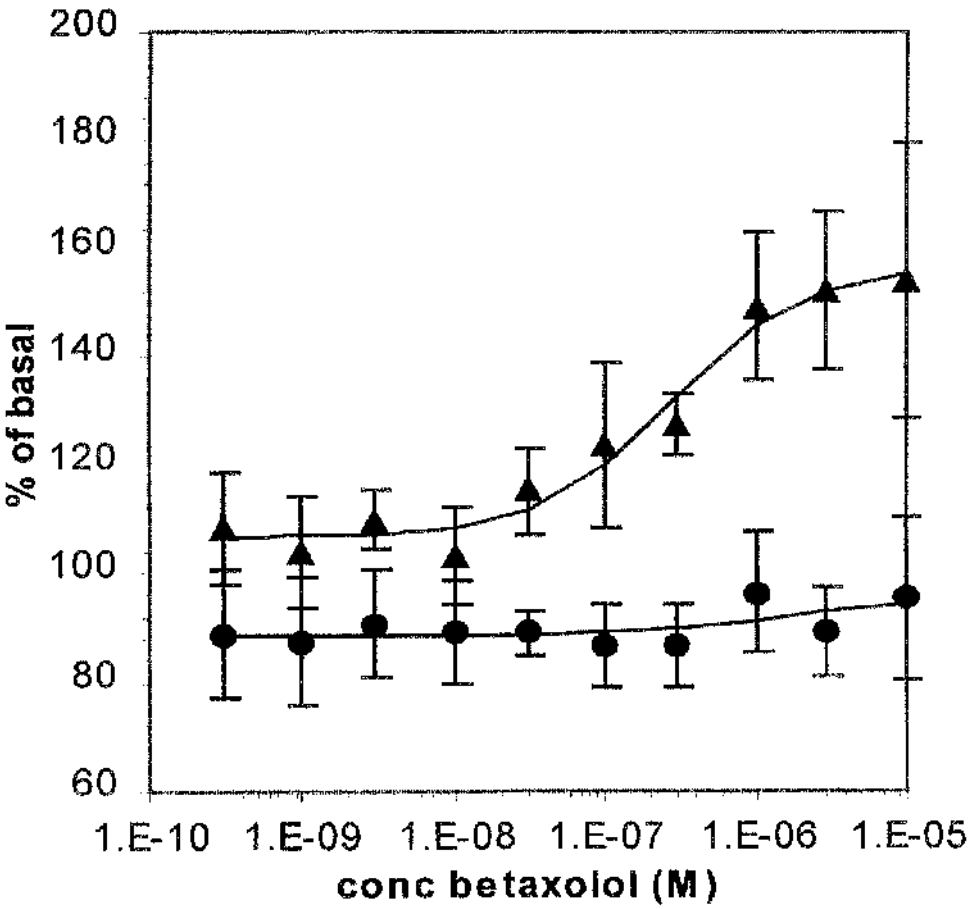


Figure 3.20

Concentration dependence of the up-regulation of CAM-β_2-AR-GFP by betaxolol.

Cells expressing CAM-β_2-AR-GFP were grown in wells of a 96-well microtiter plate. The cells were then exposed to varying concentrations of betaxolol, and fluorescence was measured on a Spectrofluor Plus fluorimeter either at 0 h (circles) or after 22 h (triangles). Values are the mean percentages ± S.E. of basal fluorescence from six experiments performed in duplicate.

Figure 3.20


200
180
160
140
120
100
80
60
% of basal
1.E-10
1.E-09
1.E-08
1.E-07
1.E-06
1.E-05
conc betaxolol (M)

the ligand, because no alteration in fluorescence intensity was recorded when betaxolol was added and fluorescence was measured immediately (Figure 3.20).

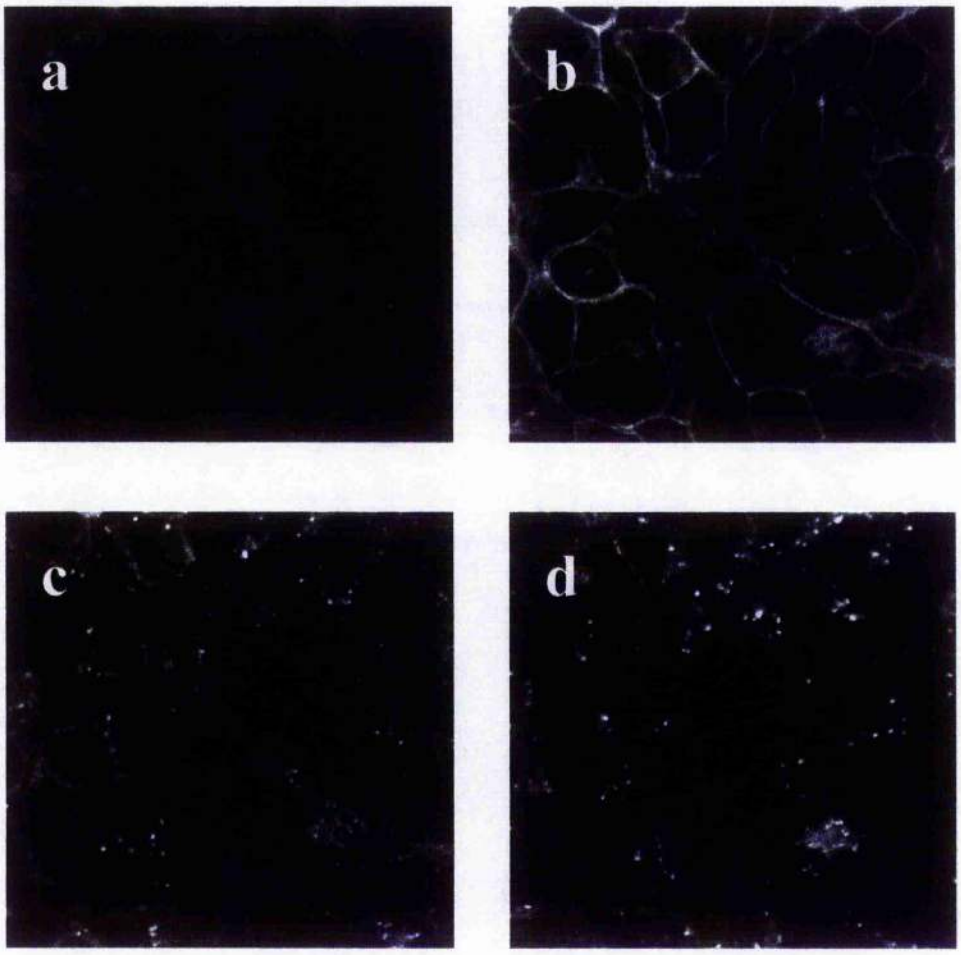
The betaxolol up-regulated CAM-β_2-AR-GFP was sensitive to agonist treatment. After the removal of betaxolol and its replacement by isoprenaline (10^{-5} M), rapid internalisation of the construct into intracellular, punctate vesicles was observed. This process could be visualised by confocal microscopy (Figure 3.21a-d) and was indistinguishable in phenotype from that recorded for WT-β_2-AR-GFP (Figure 3.6). [^{3}H] CGP12177 is a hydrophillic β-AR antagonist that is unable to cross the plasma membrane. Therefore, in intact cell-specific binding experiments, it identifies only the cell surface population of forms of β-ARs. Such intact cell binding studies were performed on naive cells expressing CAM-β_2-AR-GFP, those that had been treated with betaxolol (24h, 10^{-5} M), and such cells after replacement of betaxolol with isoprenaline (10^{-5} M) for 30 min. Cell surface up-regulated CAM-β_2-AR-GFP was essentially all internalised by short-term agonist treatment (Figure 3.21e). In conjunction with the confocal and binding experiments Jiri Novotny performed a series of sucrose density gradient experiments which showed up-regulation of CAM-β_2-AR-GFP by betaxolol (10^{-5} M, 18 h) at both the plasma membrane of these cells (Figure 3.22a (untreated), b (+ betaxolol), lane7) and in intracellular membranes (Figure 3.22a,b, lanes 3 and 4). Short term exposure of this clone to isoprenaline (10^{-5} M, 30 min) caused a redistribution of the CAM-β_2-AR-GFP construct from lane 7 to lanes 3,4 and 5 (Figure 3.22b,c). This can be interpreted as a movement of receptor from the plasma membrane to intracellular membranes (see Drmota et al., 1999), showing that CAM-β_2-AR-GFP can still respond to agonist after up-regulation.

Figure 3.21

Internalisation of up-regulated CAM-β_2-AR-GFP by isoprenaline.

Confocal studies: CAM-β_2-AR-GFP-expressing cells were untreated (a) or exposed to betaxolol (10^{-5} M, 24 h; b-d). After betaxolol treatment, the cells were washed, and isoprenaline (10^{-5} M) was added for 0 (b), 10 (c), or 30 (d) minutes. [^{3}H]CGP12177 binding studies: (e), cells, as above, were untreated, exposed to betaxolol (10^{-5} M, 24 h), or exposed to betaxolol followed by further exposure to isoprenaline (10^{-5} M, 30 min). Intact cells were then washed and used to measure the specific binding of [^{3}H]CGP12177. Data are presented as means ± S.E.M. n=3.

Figure 3.21


a
b
c
d

e)

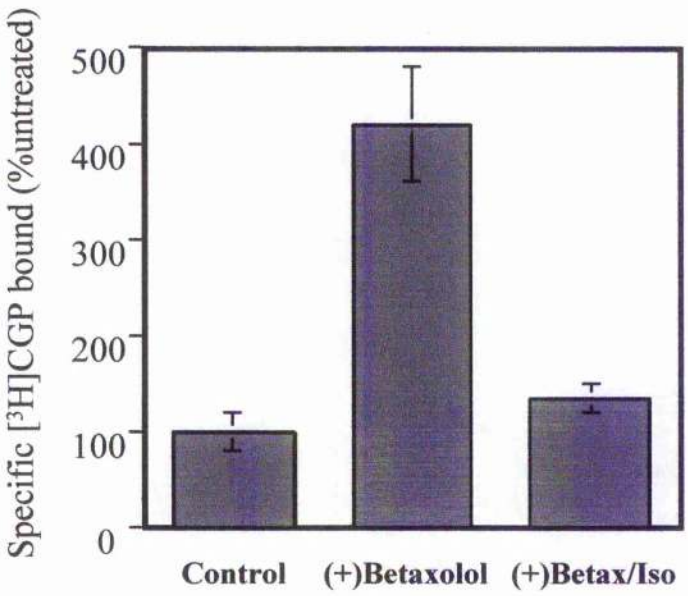
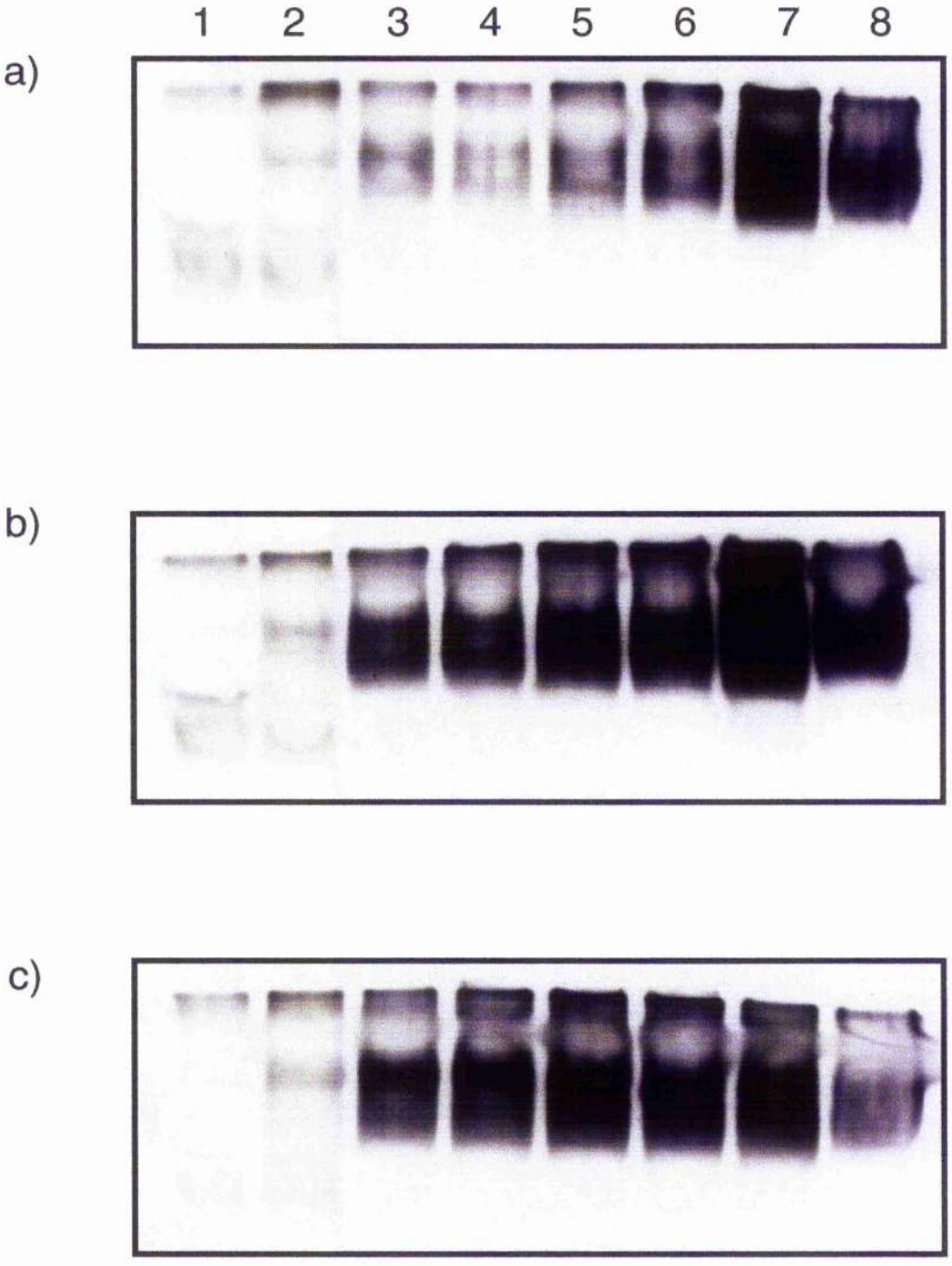

Specific [3H]CGP bound (%untreated)
500
400
300
200
100
0
Control
(+)Betaxolol
(+)Betax/Iso

Figure 3.22

Internalisation of up-regulated CAM-β_2-AR-GFP by isoprenaline as assessed by sucrose density gradient experiments.

CAM-β_2-AR-GFP-expressing cells were untreated (a) or exposed to betaxolol (10^{-5} M, 18 h; b). After betaxolol treatment, the cells were washed, and isoprenaline (10^{-5} M) was added for 30 minutes (c). The cells were fractioned on a sucrose density gradient and the fractions (1-8) collected. Equivalent amounts of fractions (1-8) for each cell sample (a, b and c) were then run on a borate based protein gel (Chapter 2, Section 2.6g). Once the protein was transferred to nitrocellulose the samples were probed with an anti-GFP antibody. A representative experiment is shown.

Figure 3.22



3.5 The β2-AR antagonist alprenolol up-regulates the CAM-β_2-AR-GFP construct in cells of this clone

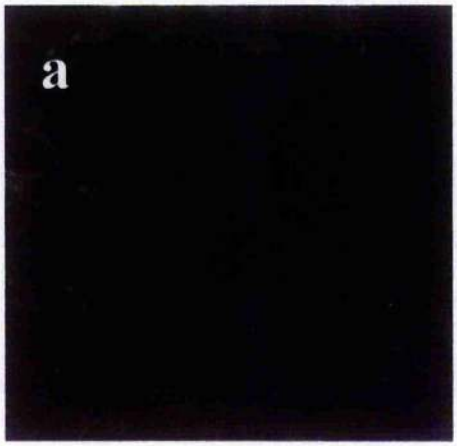
In previous studies using [^{3}H] ligand binding studies, sustained treatment of cells expressing the CAM-β_2-AR with alprenolol did not apparently produce an increase in cellular levels of the mutant protein (MacEwan and Milligan, 1996a,b). However, exposure of CAM-β_2-AR-GFP-expressing cells to a high concentration of alprenolol (24 h, 10^{-5} M) caused a clear increase in cellular fluorescence (Figure 3.23a,b). It was noted that alprenolol treatment also resulted in a distinctly punctate appearance of a fraction of the intracellularly located GPCR, as observed earlier following treatment with labetolol. In apparent contradiction to this, when cells were washed after alprenolol treatment (10^{-5} M, 24h) and the binding of a single concentration of [^{3}H] DHA assesed on intact cells, no up-regulation of the CAM-β_2-AR-GFP construct was detected (Figure 3.23c). To explore the basis for the apparent discrepancy of up-regulation of CAM-β_2-AR-GFP by alprenolol in the current studies but not CAM-β_2-AR in previous work, CAM-β_2-AR-GFP-expressing cells were exposed to a range of concentrations of alprenolol. The cellular autofluorescence pattern (Figure 3.24a) and, after extensive washing, the measured specific binding of [^{3}H] DHA to intact cells were monitored (Figure 3.24b). The [^{3}H] ligand binding studies demonstrated a clear increase in levels of the construct after treatment with concentrations of alprenolol between 10^{-10} M and 10^{-8} M. This reached a plateau on treatment with 10^{-7} M alprenolol and was greatly reduced by pretreatment with 10^{-5} M alprenolol (Figure 3.24b). Equivalent results were produced when using [^{3}H] CGP12177 as radioligand (Figure 3.24b). This suggests that residual alprenolol interferes with the binding assays but not the confocal analysis.

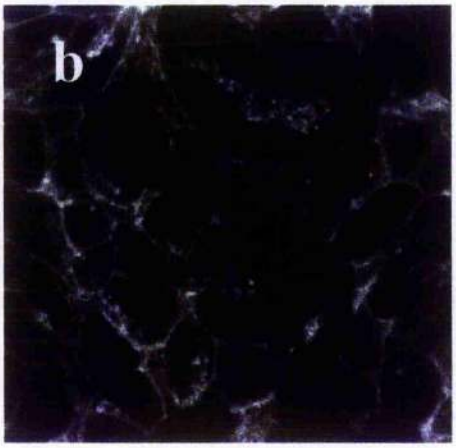
Figure 3.23

Up-regulation of CAM-β_2-AR-GFP by alprenolol is detected from confocal analysis but not by binding studies.

Confocal studies: CAM-β_2-AR-GFP-expressing cells were untreated (a) or exposed to alprenolol (10^{-5} M, 24 h; b) and then imaged on a confocal microscope. [^{3}H]DHA binding studies: Cells, as above, were untreated, or exposed to alprenolol or betaxolol (10^{-5} M, 24 h; c). Intact cells were then washed and used to measure the specific binding of [^{3}H]DHA. Data are presented as percentages of the untreated receptor level and are means ± S.E.M. n=3.

Figure 3.23





c) Intact cell-binding

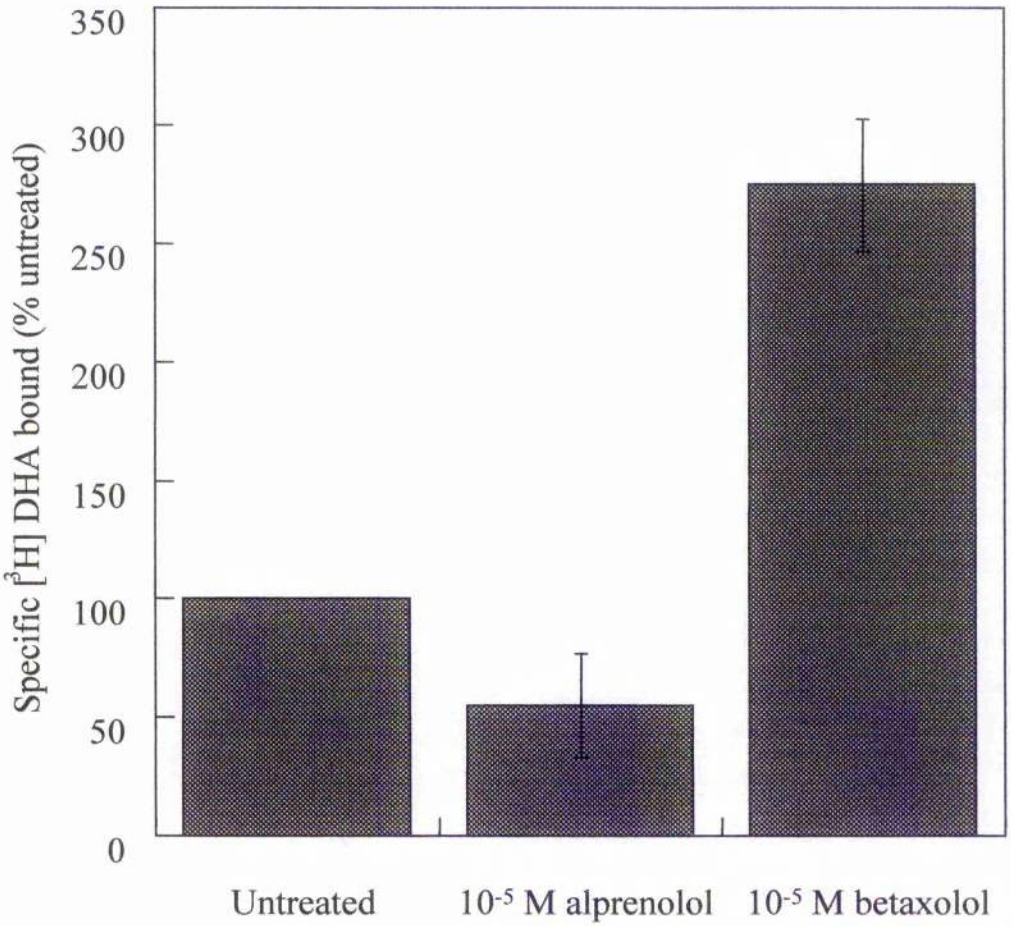


Figure 3.24

Concentration-dependence of the up-regulation of CAM-β_2-AR-GFP by alprenolol.

(a), confocal studies: CAM-β_2-AR-GFP-expressing cells were untreated or exposed to increasing concentrations of alprenolol; 10^{-10} M (i), 10^{-9} M (ii), 10^{-8} M (iii), 10^{-7} M (iv), and 10^{-5} M (v) for 24 h.

(b), binding studies: these cells were treated in dishes in the same way and subsequently washed. Intact cell-specific binding of a single concentration of either [^{3}H]DHA (squares) or [^{3}H]CGP12177 (triangles) was measured as described in Chapter 2 Section 2.7c to ascertain apparent levels of total cell receptor and cell surface receptor, respectively. Data represent means ± S.D. of triplicate assays from a single experiment that was representative of three performed.

Figure 3.24a - Confocal microscopy

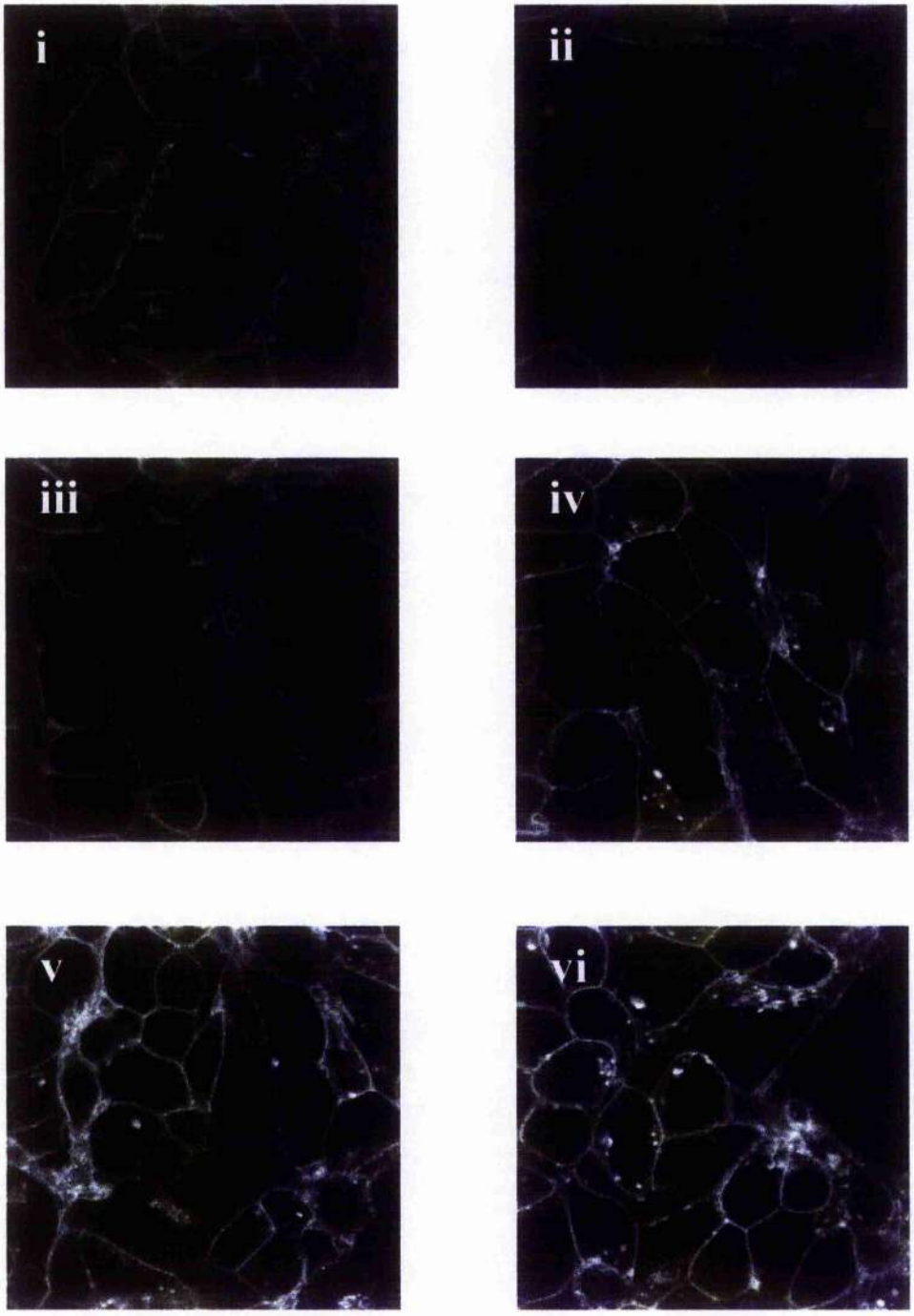


Figure 3.24b

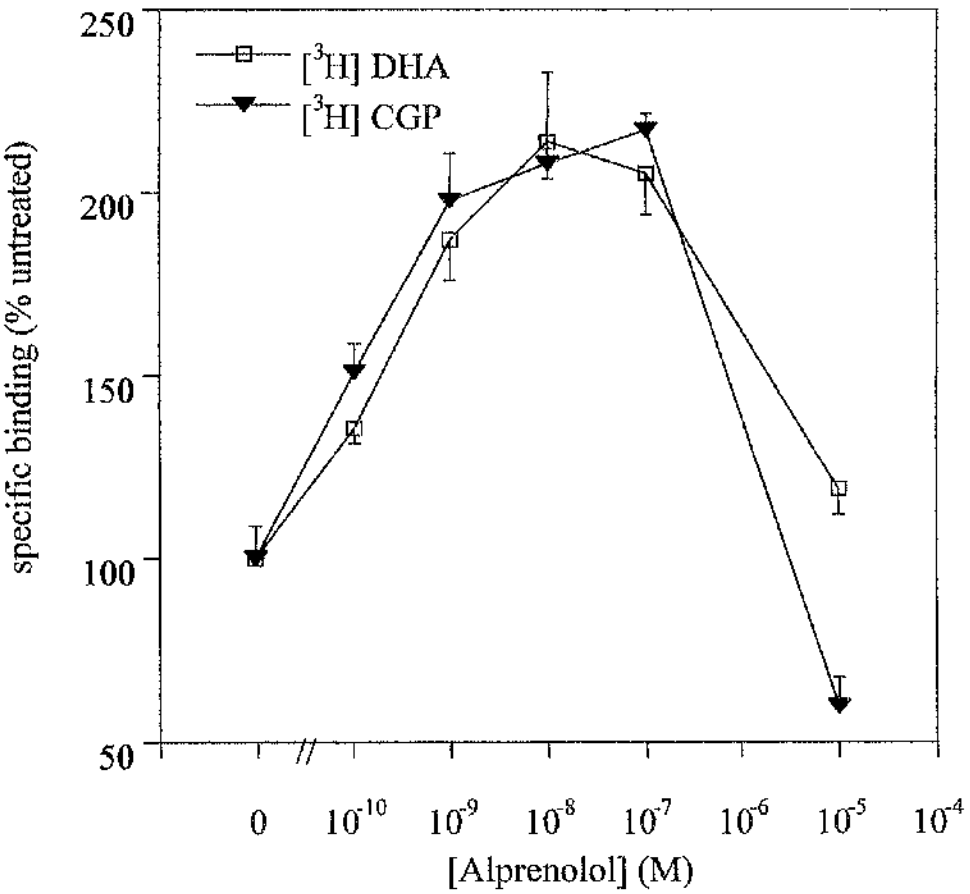
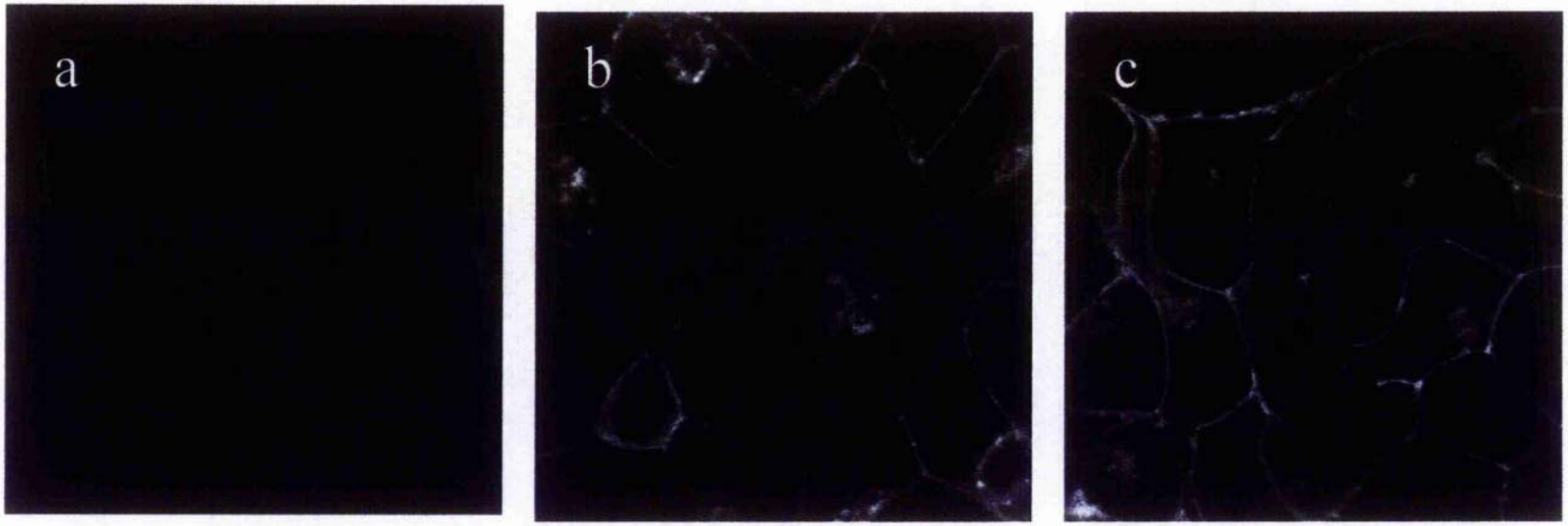

250
200
150
100
50
specific binding (% untreated)
[3H] DHA
[3H] CGP
0
10-10
10-9
10-8
10-7
10-6
10-5
10-4
[Alprenolol] (M)

Figure 3.25

Up-regulation of CAM-β_2-AR-GFP by the hydrophillic ligand CGP12177.

CAM-β_2-AR-GFP-expressing cells were untreated (a) or exposed to betaxolol (b) or CGP12177 (c) (10^{-5} M) for 24 h and then imaged. Results from a typical experiment are displayed.

Figure 3.25



3.6 The hydrophillic ligand CGP12177 up-regulates the CAM-β_2-AR-GFP construct

In this section I wanted to investigate whether it was essential for the agent causing up-regulation of the CAM-β_2-AR-GFP to cross the cell plasma membrane in order to induce its effect. It is well established that CGP12177 is a hydrophillic ligand that does not cross the plasma membrane. [^{3}H] CGP12177 is thus used to measure receptors on the cell surface as explained previously. CAM-β_2-AR-GFP cells were plated on glass coverslips and treated with or without CGP12177 or betaxolol (10^{-5} M, 24 h). Figure 3.25 shows that both drugs up-regulate this construct to similar extents providing evidence that a ligand which does not permeate the cell surface can also elicit up-regulation.

3.7 Discussion

An area of considerable interest in GPCR biology has been the observations that many GPCRs are not silent in the absence of agonist ligands but display constitutive activity (Lefkowitz et al., 1993; Scheer and Cotecchia, 1997; Leurs et al., 1998). A range of mutations of GPCRs have been reported to enhance the degree of constitutive activity. Such modified forms of a GPCR are thus believed to offer insights into conformational changes which may occur upon agonist binding to a wild type GPCR (Javitsch et al., 1997; Gether et al., 1997b). One of the most studied constitutively active mutant GPCRs is a form of the human β_2-AR in which a short segment of the distal region of the third intracellular loop was replaced by the equivalent section of the α_{1B}-AR (Samama et al., 1993, 1994; Gether et al., 1997a; Javitsch et al., 1997). As well as producing considerably greater agonist-independent stimulation of adenylyl cyclase activity than the wild type GPCR this CAM-β_2-AR has been shown to denature more readily than the WT-β_2-AR when purified and potentially to have a

markedly lower functional half-life (Gether et al., 1997a,b). In the present study I constructed and stably expressed a C-terminally GFP-tagged form of this CAM-β_2-AR to directly address such issues and to re-examine a series of reports which indicated that inverse agonists, but not neutral antagonists, cause up-regulation of the CAM-β_2-AR (MacEwan and Milligan, 1996a,b).

Although it might be anticipated that attachment of a 27kDa polypeptide to the end of a GPCR could significantly interfere with function, a series of reports have indicated the modified GPCRs to display essentially unaltered pharmacology and to interact with G proteins to initiate second messenger regulation (Barak et al., 1997; Kallal et al., 1998; Drmota et al., 1998,1999; Awaji et al., 1998; Tarasova et al., 1998). Such GFP-tagged constructs could also be used to explore the cellular distribution of the CAM-β_2-AR compared to the WT-β_2-AR.

MacEwan and Milligan have previously noted that prolonged treatment with either betaxolol or sotolol results in a substantial up-regulation of CAM-β_2-AR expressed stably in NG108-15 cells (MacEwan and Milligan, 1996a,b). Such conclusions were based on detection of increased levels of specific binding of [^{3}H]DHA following washing of the cells and membrane preparation. However, an equivalent up-regulation was not observed following pretreatment with alprenolol (MacEwan and Milligan, 1996). As betaxolol and sotolol both display characteristics of inverse agonists at the modified GPCR whilst alprenolol displays weak partial agonist function, an obvious conclusion was that the up-regulation reflected stabilisation of the CAM-GPCR in a manner dependent upon the inverse agonist characteristics of the ligands. However, in the current studies fluorescence analysis clearly indicated the CAM–β_2-AR-GFP construct to be up-regulated by alprenolol (Figure 3.23a,b and Figure 3.24) as well as by betaxolol (Figure 3.13) and a range of other "β-blockers" (Figure 3.16). The most likely explanation for this discrepancy is that the current fluorescence studies provide a direct monitor of the effect of the added ligands. By

contrast, the previous work required removal of the ligand, membrane preparation and subsequent [^{3}H]ligand binding studies. Betaxolol, as a β_1-AR-selective ligand has relatively low affinity for the CAM-β_2-AR whereas alprenolol has high affinity (Table 3.2). It could thus be anticipated that betaxolol would be effectively removed in washing regimens whereas this would be more difficult to achieve with a high affinity ligand. As such, it was possible that residual alprenolol would compete with [^{3}H]DHA in the subsequent binding experiments thus reducing the measured binding of a single concentration of [^{3}H]DHA. To approach this directly CAM-β_2-AR-GFP-expressing cells were treated for 24h with differing concentrations of alprenolol, subsequently washed and the specific binding of [^{3}H]DHA measured. Clear concentration-dependent up-regulation of [^{3}H]DHA binding was observed by prior treatment of the cells with concentrations of alprenolol up to 10^{-8} M. This plateaued at 10^{-7} M but at 10^{-5} M was essentially non-existent (Figure 3.24b). Such results would indeed be consistent with the competition model outlined above. Furthermore, equivalent results were obtained when the specific binding of a single concentration of the membrane impermeant antagonist [^{3}H]CGP12177 was measured following cellular pretreatment with varying concentrations of alprenolol (Figure 3.24b). Ligand-induced up-regulation of fluorescence associated with the CAM-β_2-AR-GFP retained pharmacological specificity. Levels of the GPCR construct were unaltered by sustained treatment of the cells with either the α_1-AR antagonist/inverse agonist prazosin or the α_2-AR antagonist/inverse agonist yohimbine. Morello et al., (2000), have suggested that V2 vasopressin receptor (V2R) mutants (shown to be associated with nephrogenic diabetes insipidus) do not fold properly and are located inside of the cell. Small cell-permeant V2R antagonists, but not impermeable antagonists were able to stabilise mutant V2Rs and allowed their maturation to the cell surface. This was not the case for the CAM-β_2-AR-GFP construct. The hydrophillic β-ligand, CGP12177, also produced an increase in receptor density suggesting that a stabilisation of an inherently unstable protein on the cell surface is occuring. This agrees with MacEwan and Milligan's findings that betaxolol treatment of NG108-15 cells stably expressing

the CAM-β_2-AR had no effect on the levels of mRNA encoding this receptor, indicating that betaxolol may be binding to the receptor and reducing the rate of receptor degradation. Contradictory to studies reporting that this receptor is also up-regulated by isoprenaline (10^{-4} M, 48h) (Gether et al., 1997), this was not found to be the case for the CAM-β_2-AR-GFP construct. However, this study used a 10 fold and sometimes a 100 fold smaller concentration of isoprenaline than used in the cited piece of work. Sf9 insect cells were also used whereas I used HEK293 cells, a mammalian cell expression system. These may be plausable explanations for the discrapancy.

Levels of the WT-β_2-AR-GFP construct were little affected by sustained treatment with β-blockers but as previously reported by others (Barak et al., 1997, Kallal et al., 1998) the agonist isoprenaline caused rapid internalisation of the construct into punctate vesicles and recycling of this construct to the plasma membrane could be achieved in rapid order by removal of the agonist and replacement with alprenolol (see Chapter 4, Section 4.5). It is well established that the CAM-β_2-AR does not function in an entirely agonist-independent manner (Samama et al., 1993, 1994; Stevens and Milligan, 1998), thus following betaxolol-mediated up-regulation of the CAM-β_2-AR-GFP, isoprenaline was also able to cause rapid internalisation of the construct into punctate vesicles in a manner similar to the WT-β_2-AR-GFP (Figures 3.21a-d). The sucrose density gradient experiment performed also provides a clear indication of this internalisation process (Figure 3.22).

Treatment of CAM-β_2-AR-GFP-expressing cells with a range of "β-blockers" resulted in increased brightness of the cells as monitored in the confocal microscope. However, careful examination of the cells demonstrated differences in the distribution pattern of the up-regulated GPCR (Figures 3.16, 3.23a,b and 3.24a). Treatment with both betaxolol and ICI118551 resulted in an essentially uniform pattern of plasma membrane-delineated GPCR fluorescence. In contrast, alprenolol, to some degree, and more markedly labetolol, produced a pattern in which a fraction of the GPCR

signal was present with a punctate intracellular location, somewhat akin to the pattern observed after short term treatment with the agonist isoprenaline. It is known that compared to full agonists such as adrenaline or isoprenaline the relative intrinsic activity of partial agonists is more pronounced at the β_2-AR as expression levels are increased (MacEwan et al., 1995) and for the CAM-β_2-AR compared to the WT-β_2-AR at equal levels of expression (Samama et al., 1993). Relatively few ligands traditionally described as "antagonists" appear to be purely neutral in effect following binding within the crevice formed from the topological architecture of the seven transmembrane domains of GPCRs for catecholamines (Milligan et al., 1995; Milligan and Bond, 1997). Indeed, ligand stabilisation of particular conformations of a GPCR may be considered in a similar manner to the induced-fit models of enzyme-substrate interactions. Therefore, the bulk of "antagonists" will favour production of conformations less or more similar to agonist-induced conformations than the mean spectrum of populations present in the absence of ligand. They will, therefore, behave as either inverse agonists or partial agonists. If partial agonists, however, it might be expected that they would display poor intrinsic activity relative to classical agonists for that GPCR, or they would previously have been characterised as agonists rather than antagonists. Therefore, the capacity of the "β-blockers" employed in this study to regulate cAMP levels in intact cells expressing CAM-β_2-AR-GFP (Figure 3.19) was measured. Although the basal level of cAMP was relatively low in these cells both betaxolol and ICI118551 reduced this level further, a property consistent with their classification as inverse agonists. However, alprenolol displayed a clear ability to increase cAMP levels in intact CAM-β_2-AR-GFP-expressing cells, acting as a partial agonist when compared to isoprenaline. Perhaps surprisingly, labetolol was as effective as isoprenaline in stimulating intact cell adenylyl cyclase activity in these cells (Figure 3.19). This property of labetolol has been reported previously from experiments in whole cells (Samama et al., 1993; Chidac et al., 1994) dispite being inhibitory in membranes (Murray and Keenan, 1989). It thus appears that visual examination of the distribution of up-regulated CAM-β_2-AR following sustained

exposure to a selection of "β-blockers" can provide a useful indication of their functional pharmacological properties in whole cells .

Visual examination of the fluorescence of cells grown on individual coverslips with and without sustained treatment with the β-AR ligands was appropriate when examining a single concentration of ligand but not very suitable to attempt to generate quantitative concentration-response curves. However, the increase in cellular fluorescence of CAM-β_2-AR-GFP-expressing cells in response to treatment with betaxolol could also be monitored in a spectrofluorimeter. As such, cells grown in a 96 well microtitre plate could be used to generate EC_{50} values for the effect of betaxolol (Figure 3.20). The values obtained were in good accord with previous estimates for the up-regulation of non GFP-tagged CAM-β_2-AR and inhibition of basal adenylyl cyclase activity in membranes expressing the CAM-β_2-AR (MacEwan and Milligan 1996a) and with the K_i of betaxolol estimated from ligand binding experiments in these cells. Importantly, the increase in cellular fluorescence was not observed by simply adding betaxolol to the cells and immediately monitoring fluorescence intensity. As such it requires the time-dependent up-regulation of CAM-β_2-AR-GFP levels.

This study has demonstrated that the fluorescence methods utilised here are extremely advantageous over other methods, as ligand regulation can be easily detected or visualised without further manipulation of the cells. The quantitative feature of this fluorescence study in 96 well plates has now prompted the possibility of developing a rapid screening assay to identify new GPCRs and their regulatory ligands. To determine whether ligand regulation can be monitored by these techniques in other GPCR systems, studies are currently underway in our lab investigating ligand effects on the constitutively active α_{1B}-AR and a CAM-β_1-AR, both tagged with GFP. The advantages of GFP-tagging have been presented here but there are limitations in the sensitivity of the experimiment. However, this has led to extending the study on

CAM-β_2-AR to the use of other fluorescent tags such as *Renilla* luciferase and *Photinus* luciferase to develop a more sensitive GPCR and GPCR ligand screening assay.

Chapter 4

The Use of GFP Tagging to Compare Internalisation, Trafficking and Ligand Regulation of the WT-β_1-AR versus the WT-β_2-AR

Chapter 4

4.1 Introduction

The sub-family of β-adrenergic receptors (β-AR) are seven transmembrane spanning serpentine-like receptors regulated by transducer-like molecules called G proteins. These receptors therefore, belong to the super-family of signalling receptors termed G protein coupled receptors (GPCRs). In the case of the β_1- and β_2- AR sub-types of β-AR, upon agonist binding to receptor and subsequent receptor activation, these stimulate the effector molecule adenylyl cyclase (AC) through the stimulatory G protein α sub-unit ($G_{s\alpha}$) to increase intracellular levels of cAMP. β-ARs are activated by catecholamines and related molecules. Both β_1- and β_2- ARs mediate responses to noradrenaline released from sympathetic nerve terminals and to circulating adrenaline. Table 4.1 summarises some of the characteristics of these 2 sub-types of β-AR.

To elicit an effector output agonist ligand must bind to the receptor binding site. Tota and Strader (1990) have indicated that it is the seven transmembrane (TM) spanning regions of the β-AR which are arranged to form a binding pocket buried within the membrane bilayer. Further studies using deletion and mutagenesis approaches have identified key residues within β_1- and β_2- ARs important not only for binding agonist ligands but also neutral antagonists. For the β_2-AR, Asp^{113} located in TM3 is vital for both agonist and antagonist binding, whereas, key residues Ser^{204} and Ser^{207} in TM5 interact with two hydroxyl groups of the catechol ring of agonists (Strader et al., 1988, 1989). Wieland et al., (1996) and Zuurmond et al., (1999) have identified and studied respectively the responsibility of Asn^{293} in TM6 of the β_2-AR for stereoselectivity of catecholamines by virtue of its interaction with the β-OH in the aliphatic side chain. Work of the same nature has also been carried out on the β_1-AR reporting TM4 to be largely responsible for the 10 fold higher affinity of noradrenaline at this receptor compared to the β_2-AR (Frielle et al., 1988 and Dixon et

Table 4.1

Some important features of the human β_1-AR and the β_2-AR

	β_1-AR	β_2-AR
Location	cardiac tissue and kidney	smooth muscle - bronchus, intestine and uterus; skeletal muscle, heart, leukocytes, presynaptic nerves and brain
Amino acids	477	413
Molecular Weight	51233	46557
Glycosylation	Asn 15	Asn 6, Asn 15
Palmitoylation	Cys 392	Cys 341
Disulphide bonds	Cys 131-Cys 209	Cys 106-Cys 184
Agonists	isoprenaline, xamoterol, denopamine	isoprenaline, salmeterol, salbutamol, formeterol
Phosphorylation	1 PKA site in intracellular loop 3 10 potential βARK sites in C terminal tail	1 PKA site in C terminal tail 1 PKA site in intracellular loop 3 11 potential βARK sites in C terminal tail

al., 1989). A recent study by Isogaya et al., (1999) used a similar approach to look at agonist binding as Marullo et al., (1990) who reported that no TM region in the β-AR is responsible for selectivity of antagonists. From generating β_1/β_2-chimeric receptors TM2 and TM7 of the β_2-AR were found to be important for binding to β_2-AR selective agonists, Tyr^{308} of TM7 being particularly important. This residue is a Phe in the β_1-AR and agonist binding at this receptor is only determined by TM2; Leu^{110}, Thr^{117} and Val^{120} being the key residues involved (Isogaya et al., 1998, Isogaya et al., 1999).

Sustained exposure of β_1- and β_2- ARs to agonist ligands frequently results in a waning of response to the ligand, an effect termed desensitisation. This is a multi-step phenomenon designed to prevent hormonal overload, and has been well studied for the β_2-AR/G_s/AC system. First, uncoupling of the receptor from the G protein occurs causing receptor function to be attenuated. Sequestration of the receptor into an intracellular compartment then occurs followed by down-regulation, if the stimulation is chronically persistent.

Short-term desensitisation occurs within seconds to minutes and involves phosphorylation of the receptor by two classes of Ser/Thr kinases, cAMP dependent protein kinase A (PKA) and β-AR kinases (βARKs) also known as G protein coupled receptor kinases (GRKs), to uncouple the receptor from the stimulatory G protein, G_s (Lohse, 1993; Benovic et al., 1988 and Hausdorff et al., 1990). For the β_2-AR all of the 11 GRK phosphorylation sites have been proposed to reside in the distal portion of the C-terminal tail, whereas one PKA site located in the 3rd cytoplasmic loop and a second less characterised site in the proximal portion of the C terminal tail have been identified. There are 10 proposed GRK sites in the β_1-AR C-terminal tail and only one PKA site in the 3rd intracellular loop in an analogous position to the one in the β_2-AR (Frielle et al., 1987). Uncoupling of the receptor from G_s is assisted by binding of the phosphorylated receptor to inhibitory proteins called β-arrestins which are

recruited by GRK phosphorylated receptors. Zhou and Fishman, (1991) proposed that the β_1-AR could only be desensitised through a PKA-dependent mechanism however, GRK2 and GRK 5 have been shown to phosphorylate this receptor. Like β_2-AR desensitisation, β_1-AR desensitisation appears to be due to approximately equal contributions from GRKs and PKA (Freedman et al., 1995).

Once phosphorylated the receptor is found to internalise by a clathrin/dynamin-mediated pathway assisted by β-arrestins which are clathrin adapters (Zhang et al., 1996, Lin et al., 1997 and outlined in Chapter 1, Section 1.6b). This process was found not to be involved in desensitisation but in the resensitisation and recycling of the receptor (Yu et al., 1993). The receptor is resensitised so it can undergo another round of agonist activation. Following internalisation into endosomes/vesicles dephosphorylation of the β_2-AR by a vesicular membrane-associated form of the phosphatase PP-A2 occurs. This event only occurs under acidic pH and can be inhibited by NH_4Cl (Kreuger et al., 1997).

Elucidation of this sequence of events has resulted in an accepted model for the clathrin-dependent internalisation pathway exhibited by the β_2-AR (Figure 4.1). This is basically a receptor cycle to turn on and off the stimulus repeatedly. However, there is a mechanism to turn off the effect of a stimulus permanently. This is a process termed down-regulation, a pathway which is debated to either diverge from the receptor cycle (Figure 4.1, Kallal et al., 1998, Gagnon et al., 1998) or to be independent from receptor internalisation (Valiquette et al., 1990, Hausdorff et al., 1991, Campbell et al., 1991, Green et al., 1994, Barak et al., 1994). Down-regulation is demonstrable only after a few hours and involves receptor protein degradation and a reduction in steady state levels of receptor mRNA (Hadcock et al., 1988, 1989). Reports have shown that several GPCRs can be targeted to lysosomes

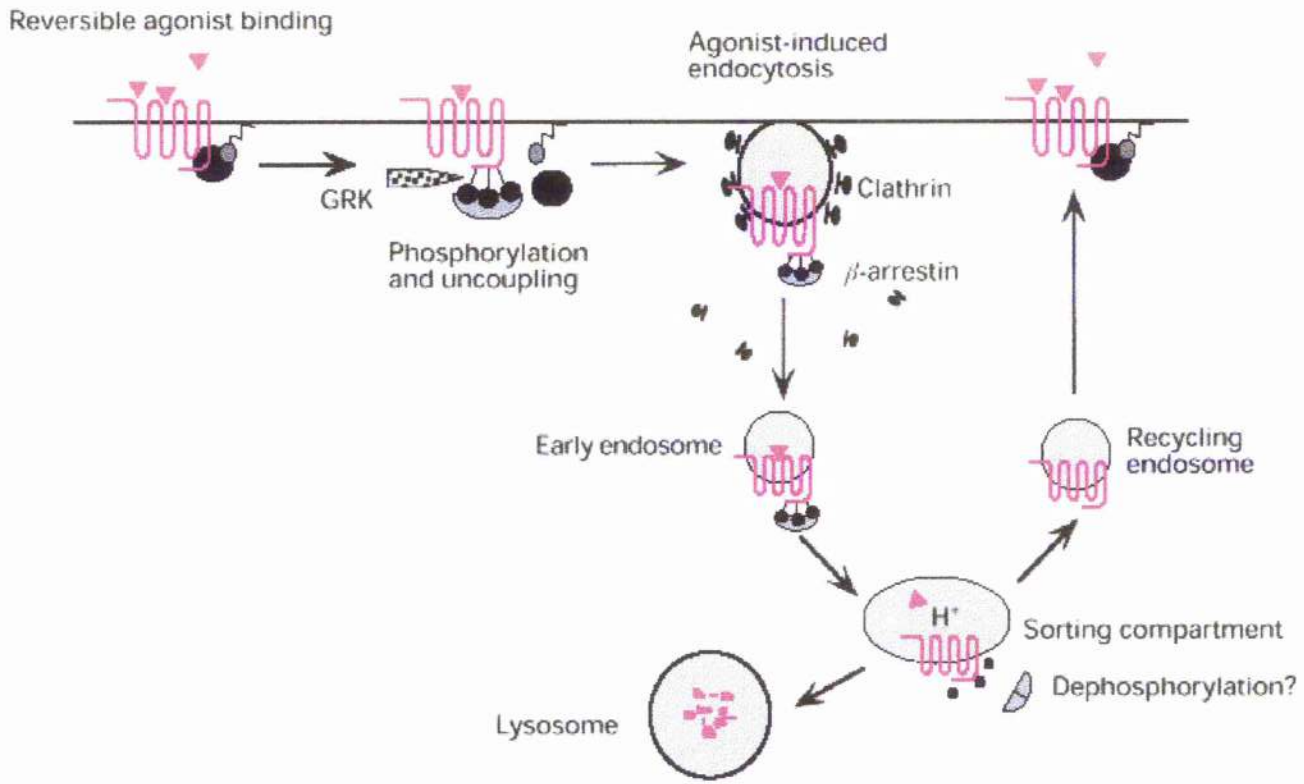
Figure 4.1

Accepted model of agonist stimulated GPCR sequestration and recycling.

Typical pathway for a neurotransmitter receptor, exemplified by the β_2-AR. Agonist binding which is reversible, is followed by receptor phosphorylation by GRKs, interaction with β-arrestins and uncoupling from G proteins, which mediate desensitisation. The ligand-receptor complex is internalised via clathrin into vesicles that soon shed their clathrin coat and become early endosomes. Ligand and receptor dissociate in an acidified perinuclear compartment. Endosomal phosphatases may dephosphorylate the receptor, allowing dissociation of β-arrestins. The ligand is degraded, whereas the receptor is recycled to the plasma membrane, where it can interact with ligands with high affinity. Resensitisation requires internalisation, processing and recycling of receptors.

From Bohm, Grady and Bunnett, Biochem. J., 1997, **322**, 1-18.

Figure 4.1


Reversible agonist binding
Agonist-induced endocytosis
GRK
Phosphorylation and uncoupling
Clathrin
β-arrestin
Early endosome
Recycling endosome
H+
Sorting compartment
Dephosphorylation?
Lysosome

in an agonist-dependent manner (Hein et al., 1994, Petrou et al., 1997, Tarasova et al., 1997) and this has also been reported for the β_2-AR tagged with GFP (Kallal et al., 1998). Gagnon et al., (1998) demonstrated that in HEK293 cells, receptor internalisation and down-regulation of the β_2-AR could be inhibited by the K44A dominant negative mutant of dynamin, a mutant known to block the pinching off of endocytic vesicles. When the same experiment was performed in HeLa cells (Gagnon et al., 1998) or L cells (Jockers et al., 1999), little or no effect on down-regulation was measured respectively. This discrepancy may be due to a difference in cell type but Jockers et al., (1999) have argued that the overexpression of K44A dynamin in HEK293 cells may also affect pathways other than endocytosis. They also argue that in L cells and A431 cells β_2-AR sites are lost after long term agonist exposure dispite blocking of lysosomal or proteasomal functions. They postulate that these cells may use an alternative mechanism for down-regulation possibly involving plasma membrane proteases.

In this chapter of work a comparison of ligand regulation of β_1-AR versus β_2-AR was attempted by using stable HEK293 cell systems expressing either β_1-AR or β_2-AR with or without GFP linked to their C-terminal tail. Previous reports have shown that addition of the 27kDa GFP polypeptide to a range of receptors does not alter the receptor pharmacology or interaction of the receptor construct with G-proteins to initiate a second messenger response (Barak et al., 1997; Kallal et al., 1998; Drmota et al., 1998,1999; Awaji et al., 1998; Tarasova et al., 1998). As previously shown in Chapter 3 ligand regulation of a CAM-β_2-AR-GFP construct stably expressed in HEK293 cells was monitored with the added aid of confocal microscopy.

Initially, transient transfections of the β_1-AR with or without linked GFP into HEK293 cells were performed to confirm that expression was obtained and that the GFP construct could be detected by fluorescence microscopy. Stable cell lines of HEK293 cells were then generated and single clones isolated for study i.e. a WT-β_1-

AR clone and a WT-β_1-AR-GFP clone. Both clones were pharmacologically characterised for their ability to bind the β-AR agonist isoprenaline and the β_1-AR selective antagonist betaxolol, to examine whether GFP had any effect on ligand binding at this receptor. Expression levels of the receptor constructs were also determined along with the K_d of [^{3}H] DHA for the receptor. Intact cell adenylyl cyclase assays were performed on both clones plus the two β_2-AR clones generated earlier in Chapter 3 to determine the EC_{50} for isoprenaline at each receptor construct.

Internalisation of the receptor-GFP constructs was determined visually and by [^{3}H] CGP12177 binding studies on intact cells. The rates of internalisation were determined and compared to wild-type receptor constructs. Confocal microscopy was also used to examine recycling of both WT-β_1-AR-GFP and WT-β_2-AR-GFP. Finally, analysis of receptor down-regulation was achieved by intact cell and membrane binding assays in conjunction with confocal microscopy to reveal differences in ligand regulation of these two sub-types of β-AR.

4.2 Construction and expression of wild-type β_1-adrenergic receptor and a green fluorescent protein-tagged form of this receptor in HEK293 cells.

A cDNA of the human β_1-AR was modified such that an 8 amino acid (DYKDDDDK) Flag™ epitope tag was added to the N terminus of the encoded protein (Flag-β_1-AR). This construct was further modified by a PCR-based strategy to link a cDNA encoding a modified form of the GFP from *Aequorea victoria* with enhanced autofluorescent properties (Zernicka-Goetz et al., 1997) to its C terminus (Figure 4.2a). This fusion protein was anticipated to encode a single open reading

Figure 4.2

a) Schematic diagram of the cDNA encoding the WT-β_1-AR-GFP fusion construct generated for this study.

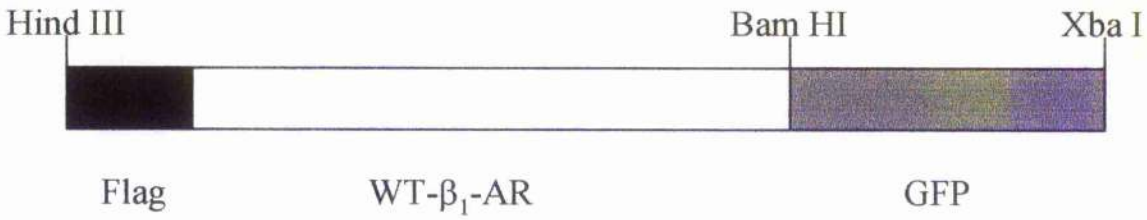
WT-β_1-AR-GFP was generated using a PCR based approach to link GFP to the N terminus of the WT-β_1-AR. (see Chapter 2 Section 2.4c).

b) Confocal analysis to determine the cellular location of WT-β_1-AR-GFP once transiently transfected into HEK293 cells.

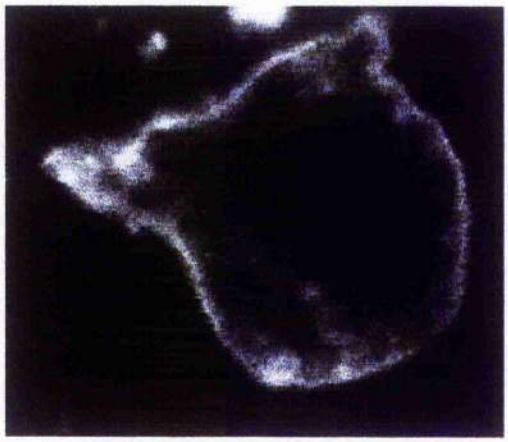
Following transient transfection of HEK293 cells on glass cover slips with the WT-β_1-AR-GFP cDNA, cells were fixed, mounted on a microscope slide as described in Section 2.8 and then imaged by confocal microscopy.

Figure 4.2

a)



b)



frame in which the C terminus of the GPCR was linked directly to the N terminus of GFP (Figure 4.2a).

Both GFP-tagged and non-GFP-tagged forms of this receptor were transiently transfected into HEK293 cells. When WT-β_1-AR-GFP transiently transfected HEK293 cells grown on a glass coverslip were examined in a confocal microscope, the cellular location of this construct was very similar to that of WT-β_2-AR-GFP (Figure 3.6, Section 3.2, Chapter 3), its fluorescence being distributed mainly at the plasma membrane of the cell (Figure 4.2b). From binding experiments using a single concentration of [^{3}H] DHA on membranes from HEK293 cells transiently transfected with pcDNA3, WT-β_1-AR, WT-β_1-AR-GFP, WT-β_2-AR or WT-β_2-AR-GFP (Figure 4.3), it was apparent that WT-β_1-AR-GFP could be expressed at a similar level to both the WT-β_2-AR and WT-β_2-AR-GFP constructs. The untagged form of the receptor was expressed at routinely lower levels than the GFP-tagged form. However, its expression was significantly greater than the control experiment using empty vector pcDNA3. Stable cell lines of each construct in HEK293 cells were then developed.

Once stable cell lines of WT-β_1-AR with and without linked GFP were established in HEK293 cells single clones of each were selected for study. A fluorescent microscope was utilised to directly screen for the WT-β_1-AR-GFP containing clones. Figure 4.4 shows confocal images from two positive clones grown on glass coverslips demonstrating that the WT-β_1-AR-GFP protein construct is targeted to the plasma membrane as substantial amounts of the GFP-derived autofluorescence is plasma membrane delineated. Disappointingly, only about 20% of the putative WT-β_1-AR-GFP clones selected were positive. However, clone #9, like WT-β_2-AR-GFP clone #13 (Figure 3.6), exhibited a marked time-dependent receptor internalisation into discrete, punctate intracellular vesicles upon addition of the β-AR agonist isoprenaline (Figure 4.5). WT-β_1-AR-GFP exhibited a smaller degree of

Figure 4.3

Expression levels of WT-β_1-AR and WT-β_1-AR-GFP from transient transfections in HEK293 cells as assessed by [^{3}H] DHA binding.

Membranes (20 μg) prepared from HEK293 cells transiently expressing either the WT-β_1-AR, WT-β_1-AR-GFP, WT-β_2-AR or WT-β_2-AR-GFP constructs were analysed for their ability to bind a single, near saturating, concentration of [^{3}H] DHA (2 nM). Results are from one experiment performed in triplicate ± S.D. Similar results were obtained form two further experiments.

Figure 4.3

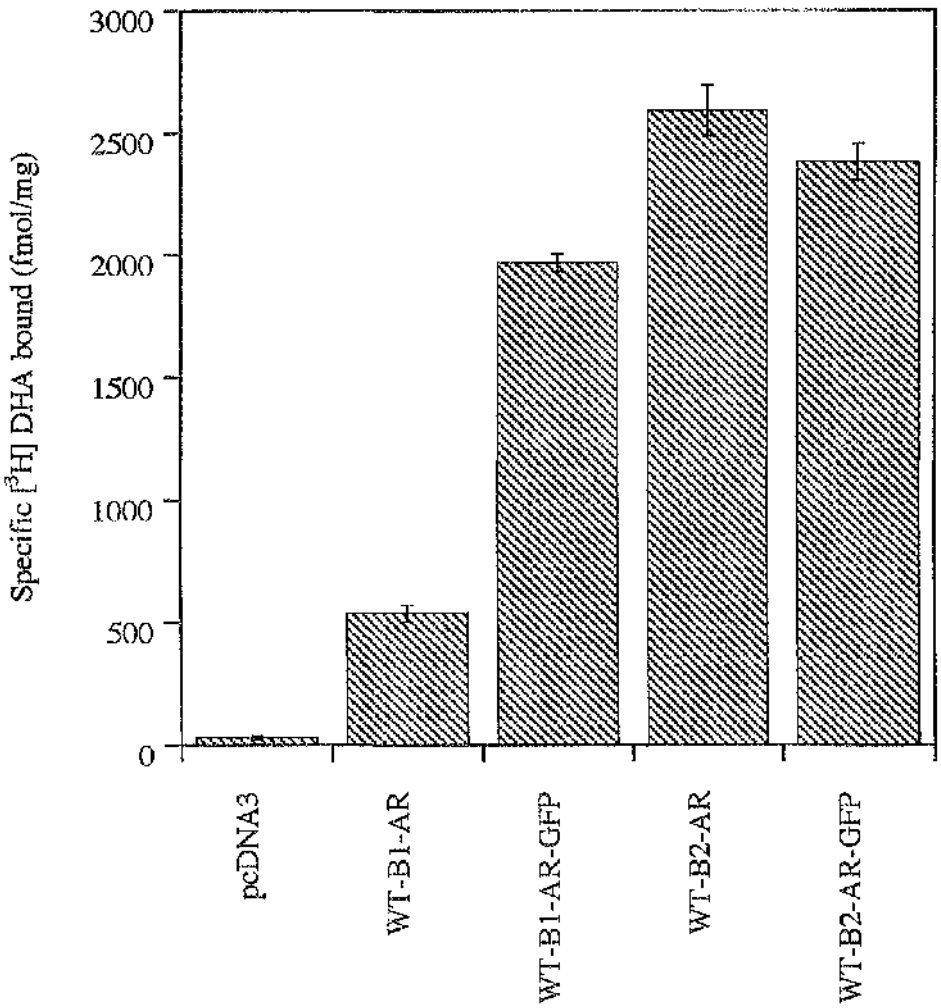
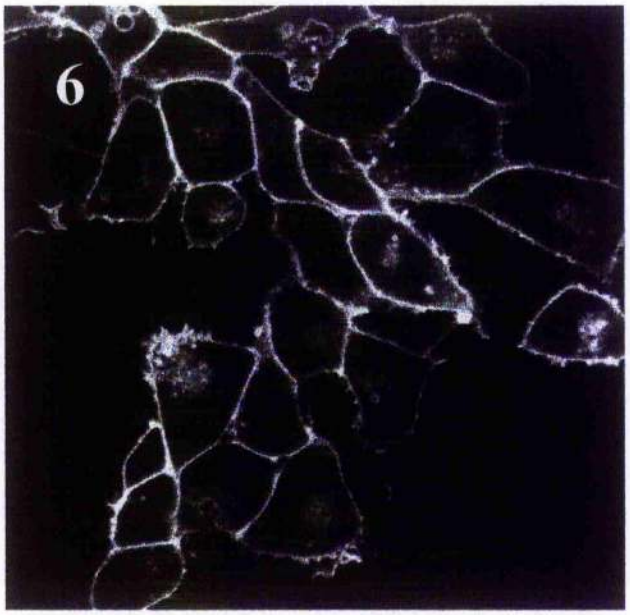

Specific [3H] DHA bound (fmol/mg)
3000
2500
2000
1500
1000
500
0
pcDNA3
WT-B1-AR
WT-B1-AR-GFP
WT-B2-AR
WT-B2-AR-GFP

Figure 4.4

WT-β_1-AR-GFP stable cell clones.

The WT-β_1-AR-GFP construct was expressed stably in HEK293 cells and individual clones were identified by live cell confocal microscopy. All positive clones were similar to the two clones (6 and 9) imaged with a largely plasma-membrane delineated auto fluorescence.

Figure 4.4



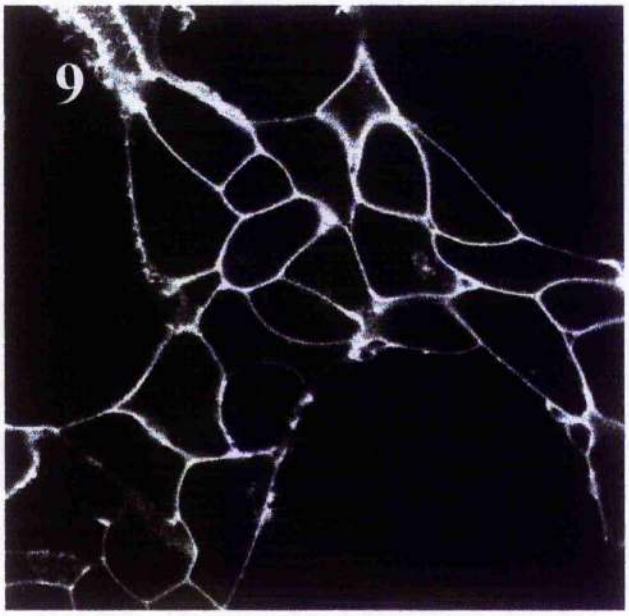
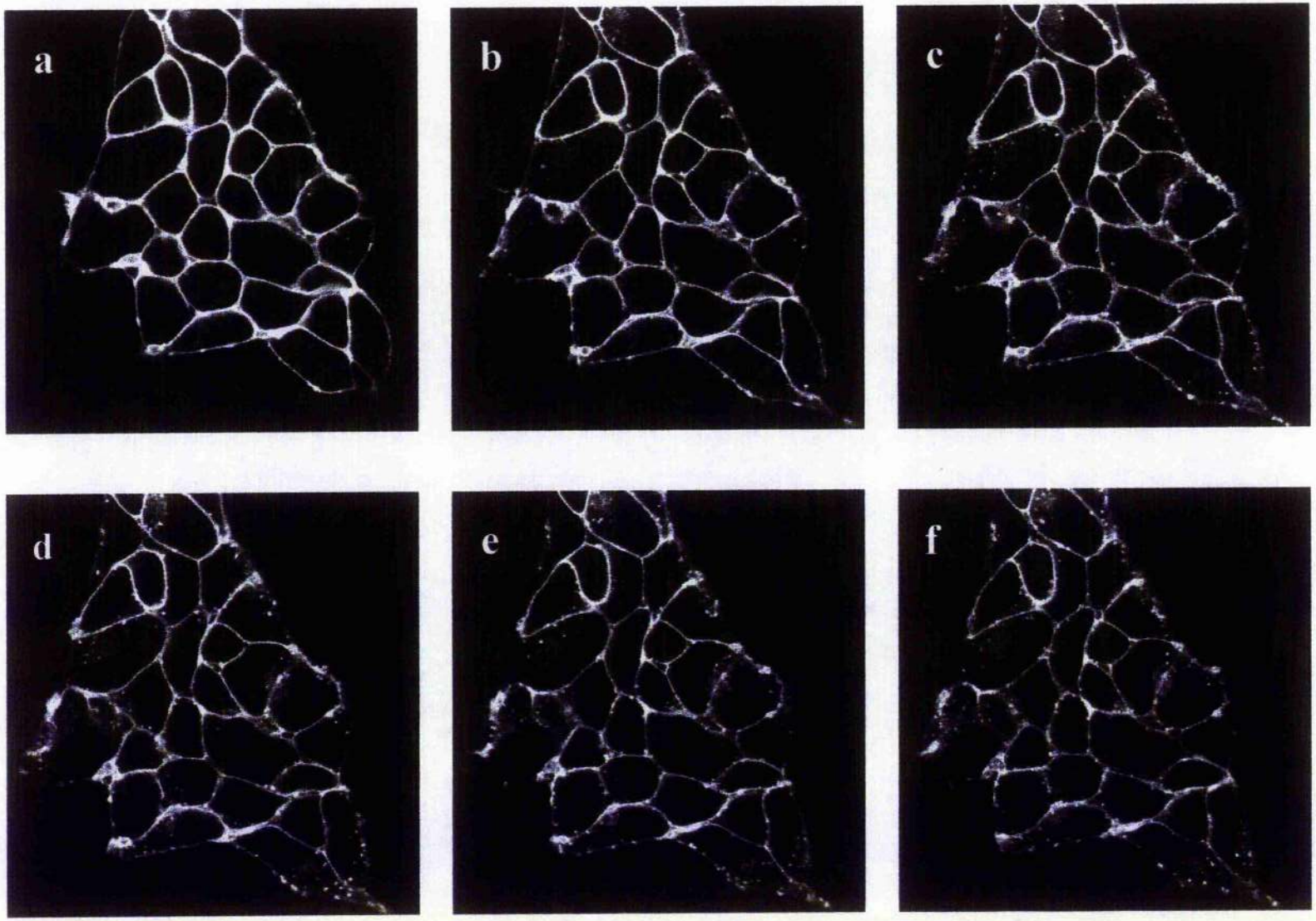


Figure 4.5

Internalisation of WT-β_1-AR-GFP by agonist stimulation.

A patch of WT-β_1-AR-GFP clone #9 cells were imaged in the confocal microscope in the absence of agonist (a) and following addition of 10^{-5} M isoprenaline for 10 (b), 20 (c), 30 (d), 40 (e) and 50 (f) minutes. This is representative of three separate experiments.

Figure 4.5



internalisation than WT-β_2-AR-GFP. Both constructs internalised fairly rapidly with a distinct punctate pattern appearing within 10 minutes of isoprenaline addition (Figure 3.6b and 4.5b). However, internalisation became maximal at about 30 minutes for WT-β_1-AR-GFP (Figure 4.5 c and d), but WT-β_2-AR-GFP internalised further with a 30 to 40 minute stimulation of isoprenaline. In order to make such conclusions from this data it was essential to investigate this more quantitatively (see Section 4.4).

The non-GFP-tagged WT-β_1-AR clones were initially screened by [^{3}H] DHA binding studies using a single concentration of radioligand. Figure 4.6a displays a selection of clones examined of which 30% were found to bind substantial amounts of a single concentration of [^{3}H] DHA and therefore, were assumed to be expressing WT-β_1-AR at reasonable levels (greater than 300 fmol/mg was determined as a reasonable level of β_1-AR). Three of the positive clones were used to make membranes for western blotting with an anti-β_1-AR antibody (Figure 4.6b). All three clones expressed a doublet of proteins with approximate molecular weights of 50 kDa and 60 kDa which may correlate with glycosylated and unglycosylated forms of the β_1-AR (Table 4.1 indicates that the unglycosylated form of the human β_1-AR is approximately 50 kDa). Clone #11 was selected due to strong immunodetection and a high level of [^{3}H] DHA binding compared to the other positive clones. Figure 4.7 gives an approximate indication of expression levels of clones #11 and #9 using a single concentration of [^{3}H] DHA in binding studies. As with the β_2-AR clones, it was necessary to perform more detailed analysis to pharmacologically characterise these constructs.

Figure 4.6

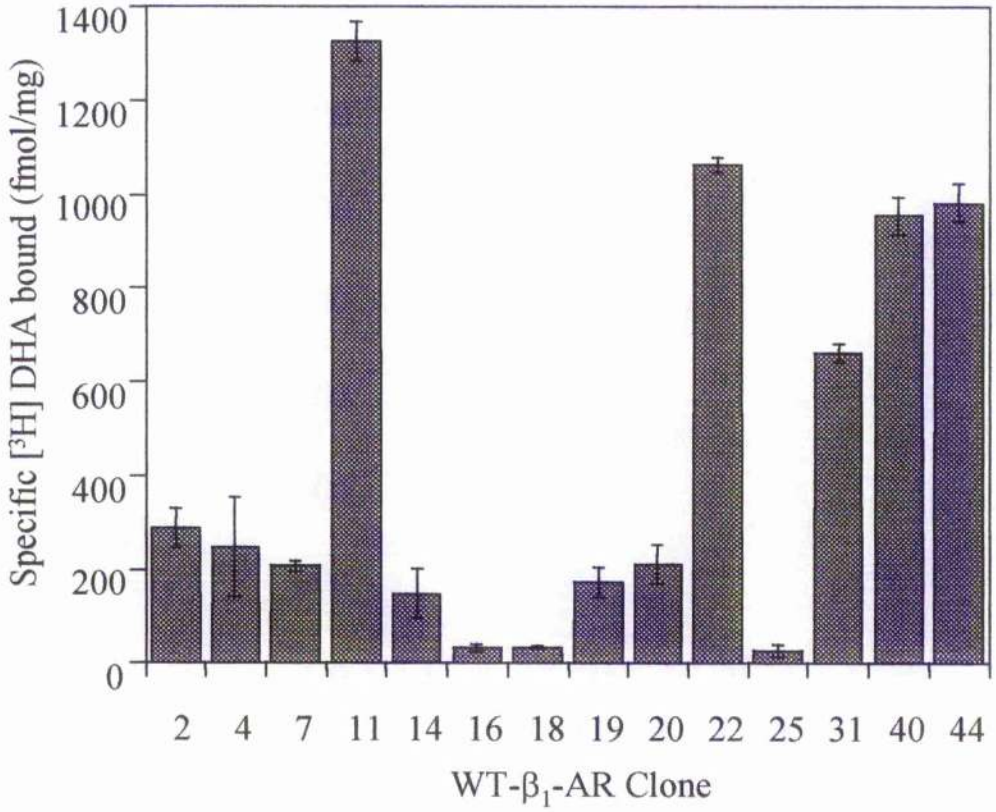
Screening of WT-β_1-AR stable cell clones

a) The WT-β_1-AR construct was expressed stably in HEK293 cells and individual positive clones were detected by membrane binding studies with a single concentration of [^{3}H] DHA (2 nM). A range of selected clones are shown. Data are means ± S.D. from a single experiment performed in triplicate.

b) 20 μg of membranes from clones 11 (1), 22 (3) and 40 (4) were run on an SDS protein gel along with membranes from WT-β_2-AR clone 27 (2). Nitrocellulose membranes were probed with anti-β_1-AR antibody to detect expressed β_1-AR.

Figure 4.6

a)


Specific [³H] DHA bound (fmol/mg)
1400
1200
1000
800
600
400
200
0
2 4 7 11 14 16 18 19 20 22 25 31 40 44
WT-β1-AR Clone

b)

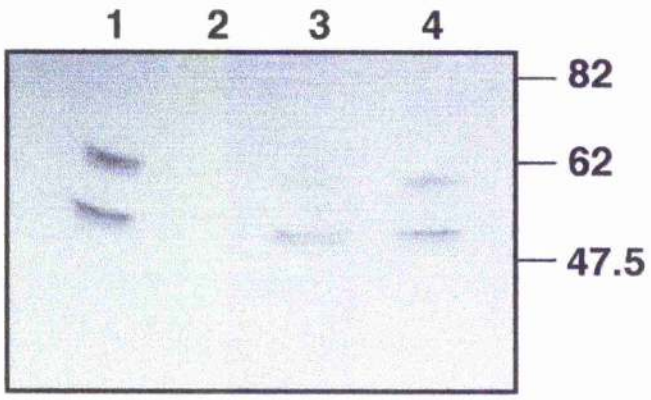

1 2 3 4
82
62
47.5

Figure 4.7

Estimation of receptor levels of β_1-AR clones from membrane binding studies using a single concentration of [^{3}H] DHA.

20 μg of membrane preparation from each cell line was used to estimate the approximate receptor level (fmol/mg). A close to saturating dose of [^{3}H] DHA (2 nM) was used to determine total binding with 10^{-5} M propranolol as competing ligand to determine non-specific binding. Specific binding was used to estimate the receptor expression in each clone (fmol/mg). Data are presented as means ± S.E.M. n=3.

Figure 4.7

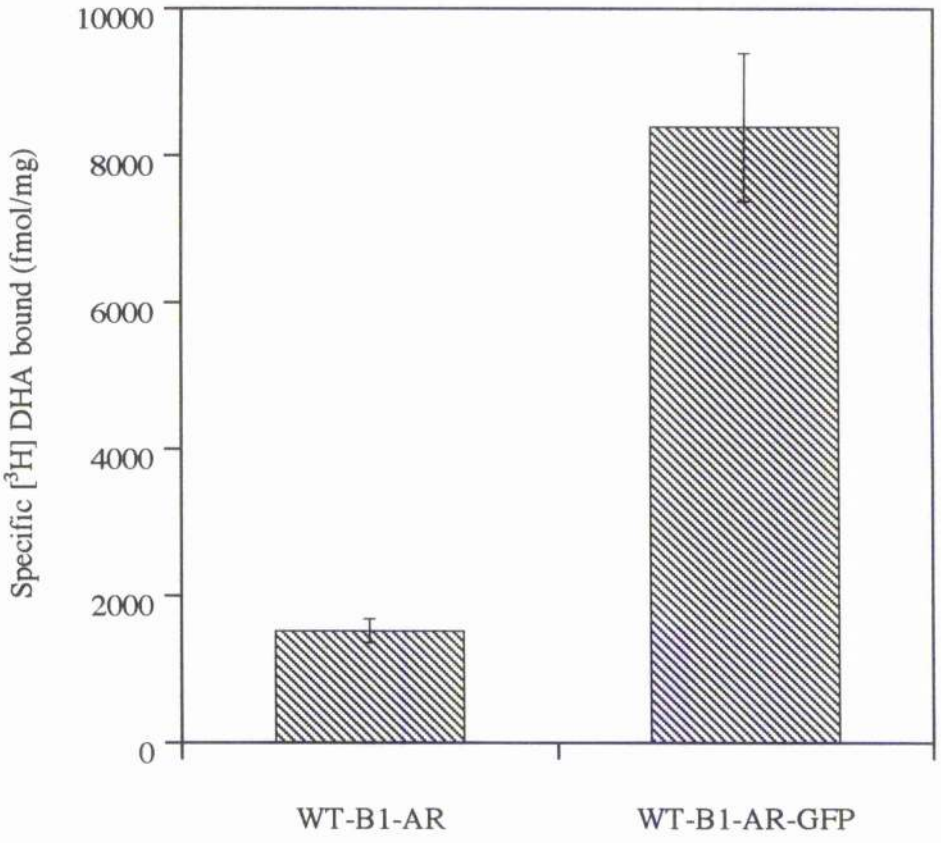

10000
8000
6000
4000
2000
0
Specific [3H] DHA bound (fmol/mg)
WT-B1-AR
WT-B1-AR-GFP

Table 4.2

Ligand-binding characteristics of GFP and non-GFP tagged forms of the β_1-AR and β_2-AR constructs.

Clone	K_d (nM)	B_{max} (pmol/mg)
WT-β_1-AR	1.08 ± 0.2	2.6 ± 0.4
WT-β_1-AR-GFP	1.81 ± 0.3	18.8 ± 2.5
WT-β_2-AR	0.62 ± 0.26	4.1 ± 0.9
WT-β_2-AR-GFP	0.75 ± 0.17	8.7 ± 0.7

Data represent means ± S.E.M. from three independent experiments using [^{3}H] DHA as radioligand.

Table 4.3

Competition binding experiments at GFP and non-GFP tagged forms of the β_1-AR construct.

Clone	K_i for isoprenaline (nM)	K_i for betaxolol (nM)
WT-β_1-AR	127 ± 10	16 ± 0.4
WT-β_1-AR-GFP	289 ± 64	25 ± 12

Data represent means ± range. from two independent experiments using [^{3}H] DHA as radioligand.

4.3 Pharmacological characterisation

Expression levels of WT-β_1-AR and WT-β_1-AR-GFP clones were determined more accurately by saturation binding experiments with [^{3}H] DHA (also an antagonist at the β_1-AR). WT-β_1-AR-GFP (Figure 4.9a(i)) was found to be expressed at much higher levels than WT-β_1-AR (Figure 4.8a(i)) (Table 4.2 B_{max} values). The ability of the GFP fusion construct to be expressed at higher levels than its non-GFP-tagged form was also apparent for the WT-β_2-AR. As for the WT-β_2-AR, the non-specific binding at both β_1-AR constructs was very low (Figures 4.8a(ii) and 4.9a(ii)). Saturation binding curves were converted into Scatchard plots for each receptor construct and results from these experiments indicated that both constructs bound the ligand [^{3}H] DHA with similar affinity (Table 4.2 and Figures 4.8b and 4.9b).

Competition for the specific binding of [^{3}H] DHA to membranes expressing WT-β_1-AR or WT-β_1-AR-GFP with either betaxolol (a β_1-AR antagonist) or isoprenaline (an agonist) were performed to demonstrate that adding GFP to the C terminus of the receptor had little effect on basic receptor pharmacology (Table 4.3). When betaxolol was used as a competing drug results indicated that both β_1-AR constructs bound this ligand with high affinity (Figure 4.10 a and b, and Table 4.3). Betaxolol is a β_1-AR selective antagonist and proved to bind to the β_1-AR constructs with 10-20 times higher affinity than the β_2-AR constructs (compare Tables 3.2 and 4.3). As such, the previously noted high affinity of the antagonist betaxolol for the β_1-AR was preserved following addition of GFP to the C-terminal tail. Isoprenaline was found to have approximately 3 fold higher affinity for the β_1-AR constructs compared to the β_2-AR constructs (compare Table 3.2 and 4.3), but little difference in the K_i for isoprenaline was detected between the two β_1-AR constructs (Figure 4.11 a, b).

Figure 4.8

β_1-AR binding characteristics in WT-β_1-AR-expressing cells.

a i) A saturation binding study using increasing concentrations of [^{3}H] DHA was performed to measure specific binding (pmol/mg) in membranes from WT-β_1-AR cells.

a ii) The same saturation binding study as in (a i) but showing total, specific and non-specific binding (d.p.m.).

b) Transformation of the specific binding data to generate a Scatchard plot. In the example displayed B_{max} was 3.21 pmol/mg and the K_d for [^{3}H] DHA was 1.35 nM.

Figure 4.8a(i)

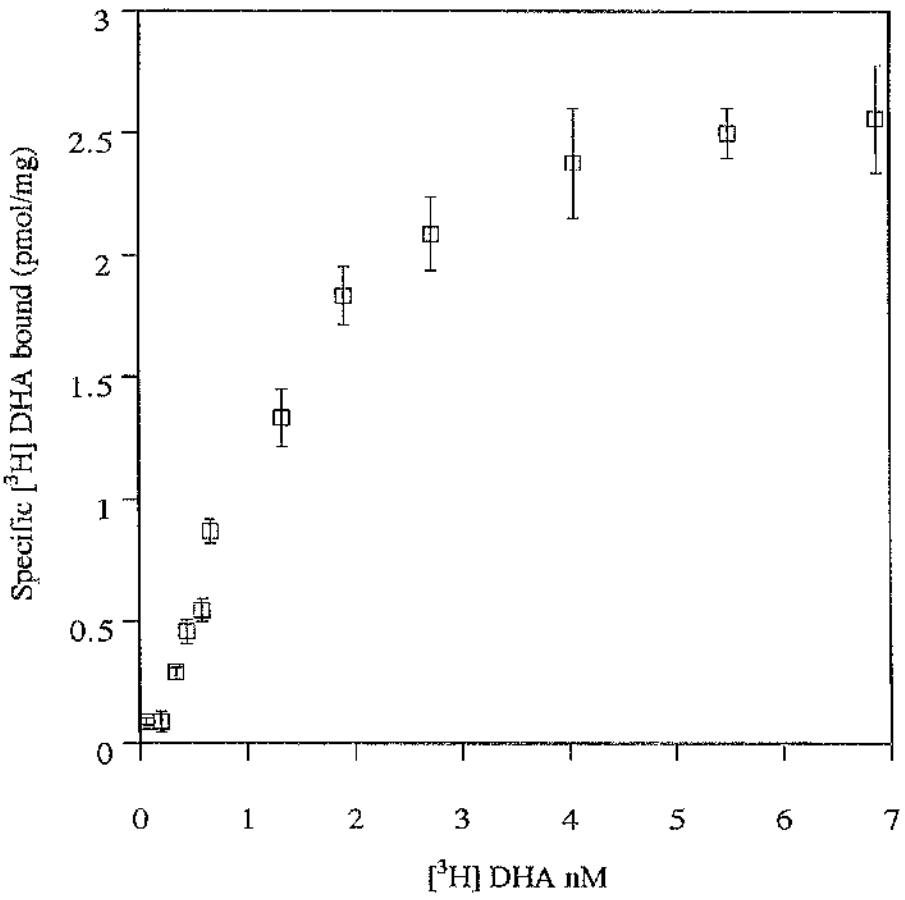


Figure 4.8a(ii)

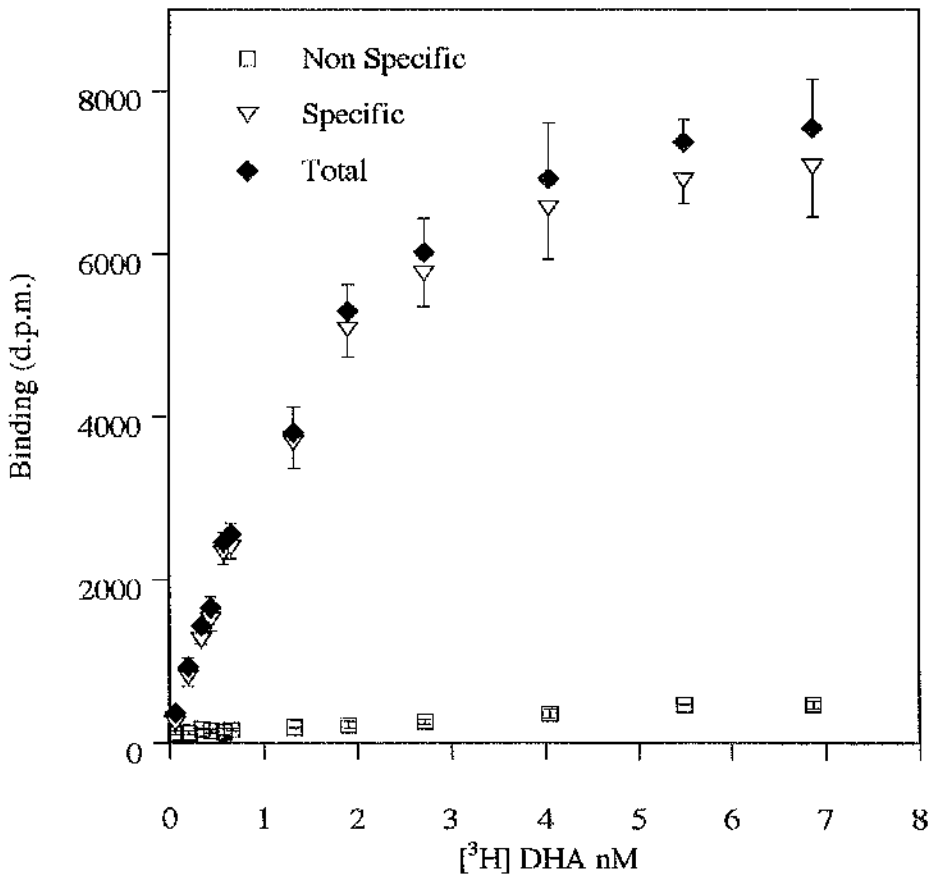


Figure 4.8b

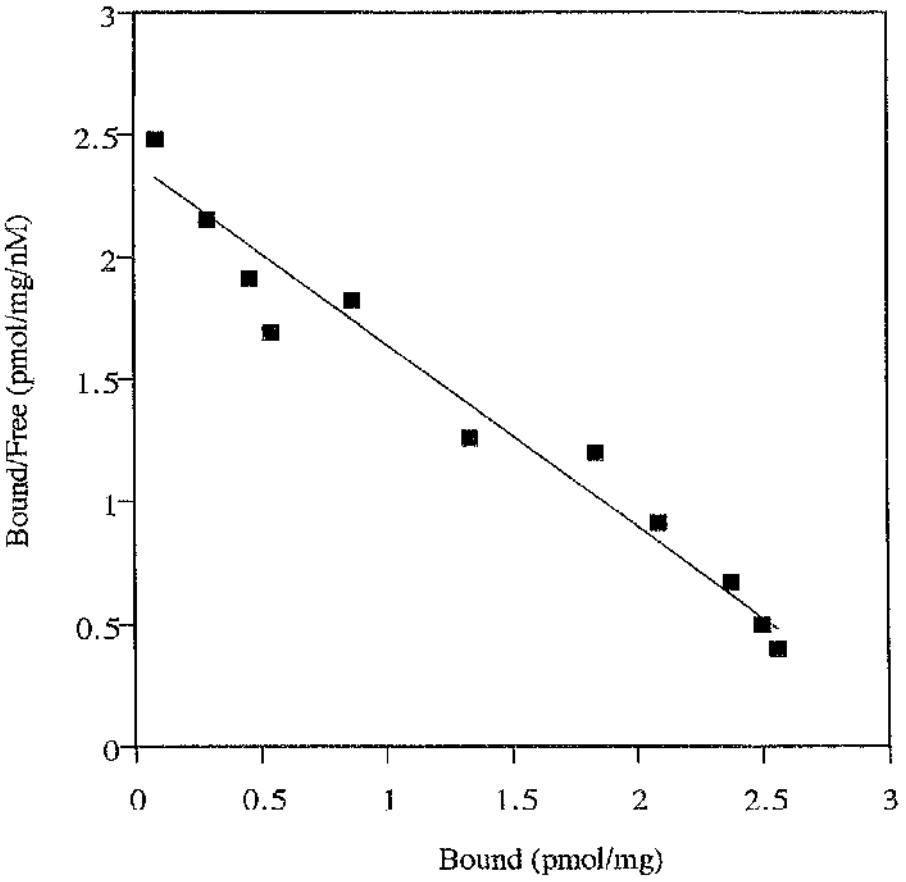


Figure 4.9

β_1-AR binding characteristics in WT-β_1-AR-GFP-expressing cells.

a i) A saturation binding study using increasing concentrations of [^{3}H] DHA was performed to measure specific binding (pmol/mg) in membranes from WT-β_1-AR-GFP cells.

a ii) The same saturation binding study as in (a i) but showing total, specific and non-specific binding (d.p.m.).

b) Transformation of the specific binding data to generate a Scatchard plot. In the example displayed B_{max} was 16.91 pmol/mg and the K_d for [^{3}H] DHA was 1.24 nM.

Figure 4.9a(i)

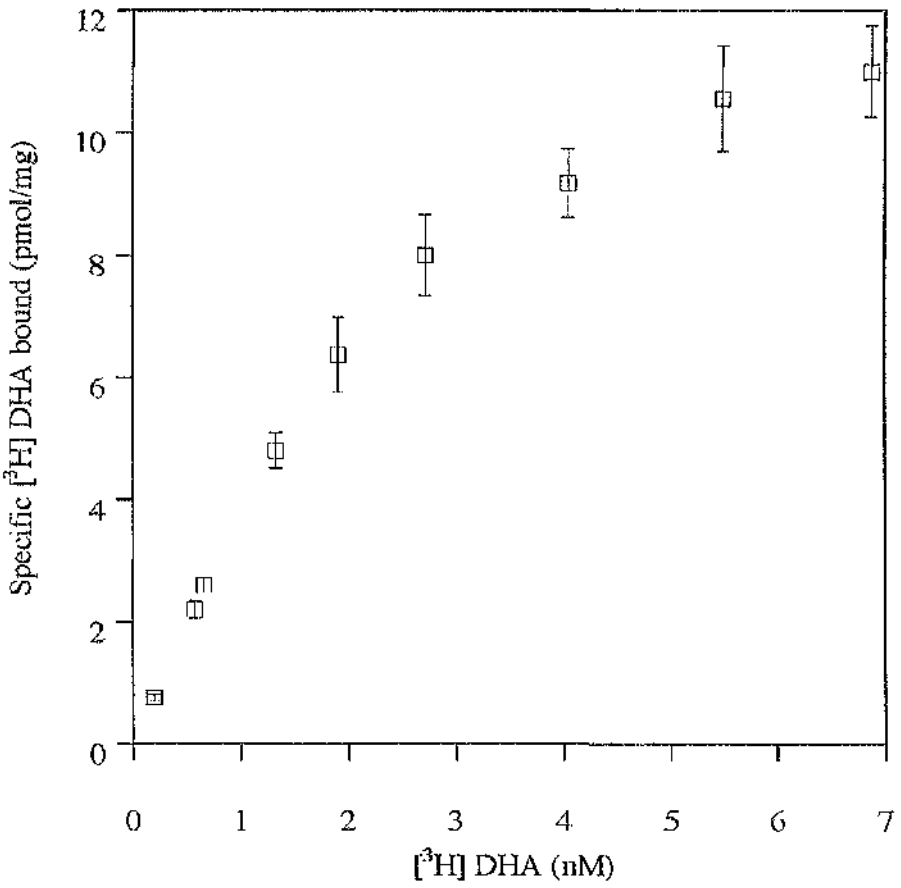


Figure 4.9a(ii)

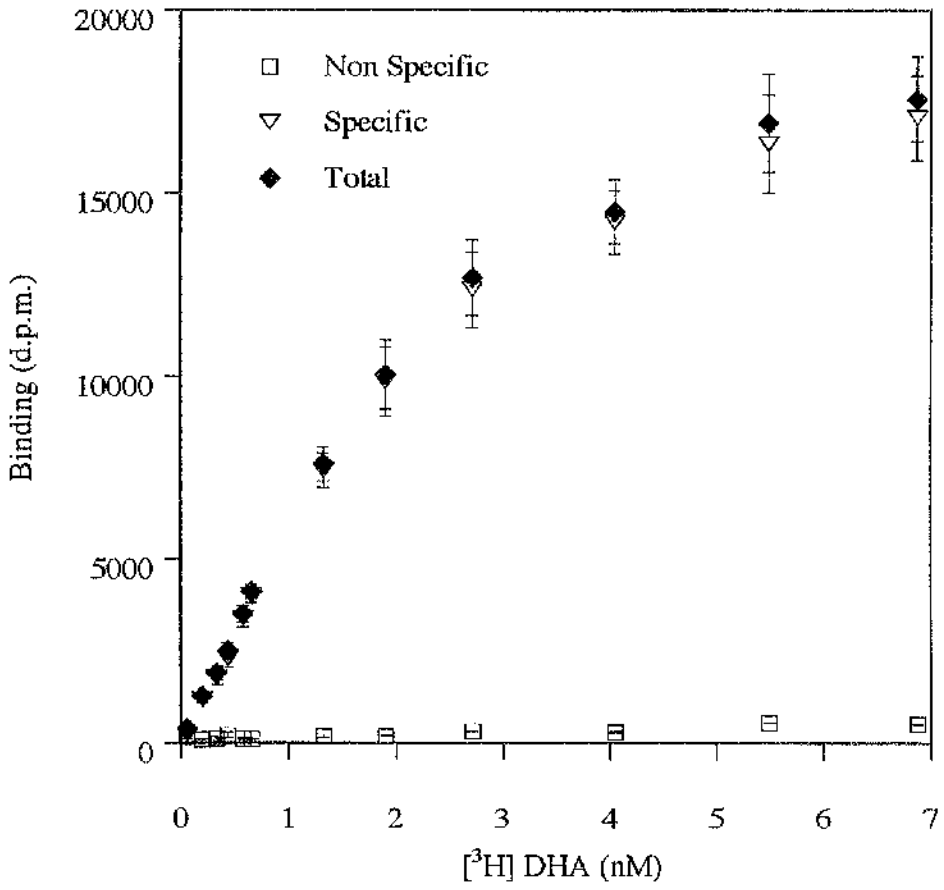


Figure 4.9b

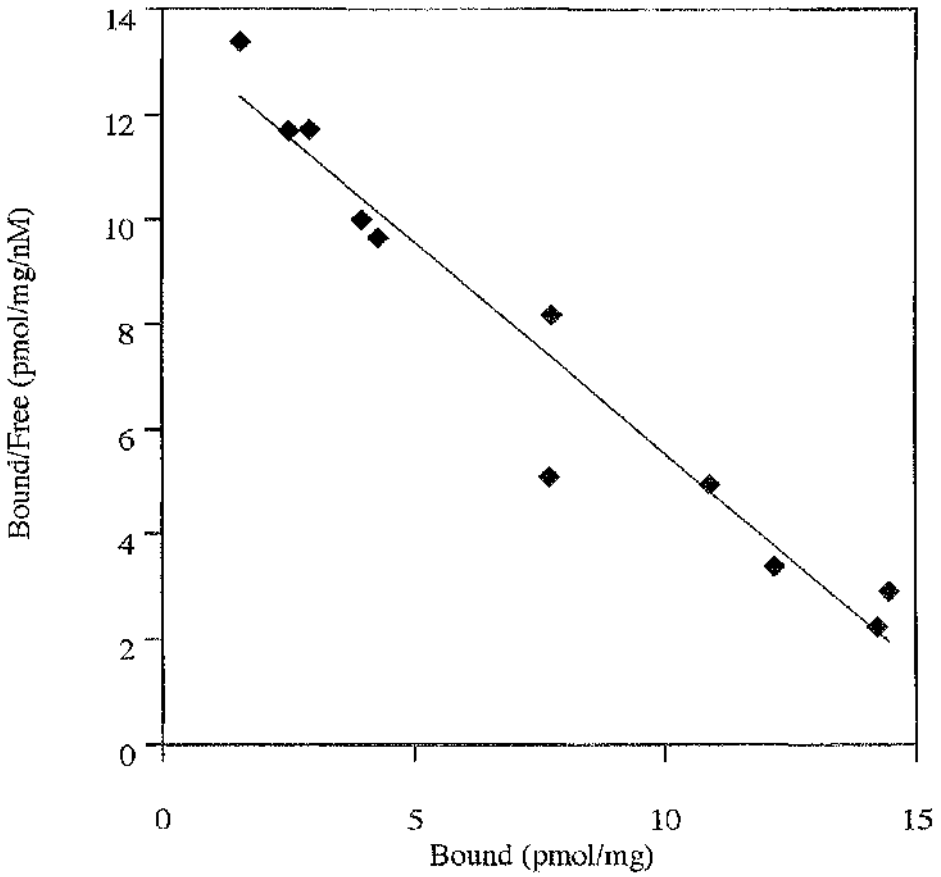


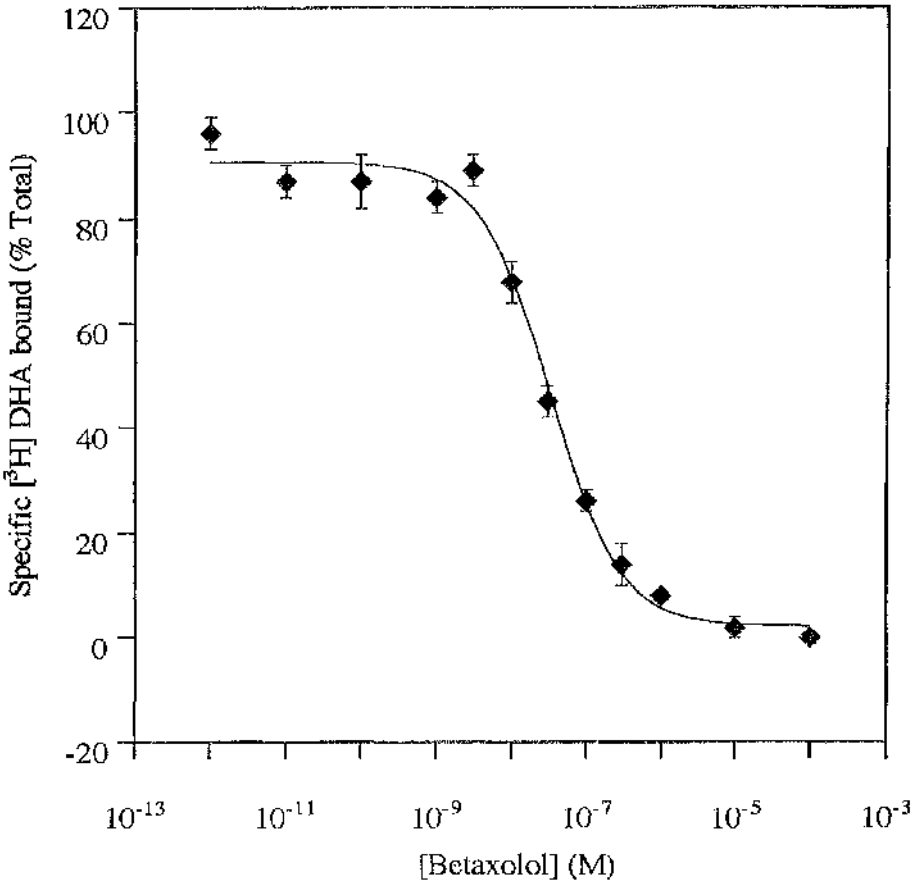
Figure 4.10

High affinity of the β_1-AR antagonist betaxolol for the WT-β_1-AR is retained after addition of GFP.

Competition between [^{3}H] DHA (1.2 nM (a) and 1.0 nM (b)) and varying concentrations of betaxolol for specific binding to membranes expressing either WT-β_1-AR, Hill coefficient = 0.9 (a); or WT-β_1-AR-GFP, Hill coefficient = 0.9 (b) was assessed. Similar results were obtained from one further experiment (Table 4.3).

Figure 4.10

a)



b)

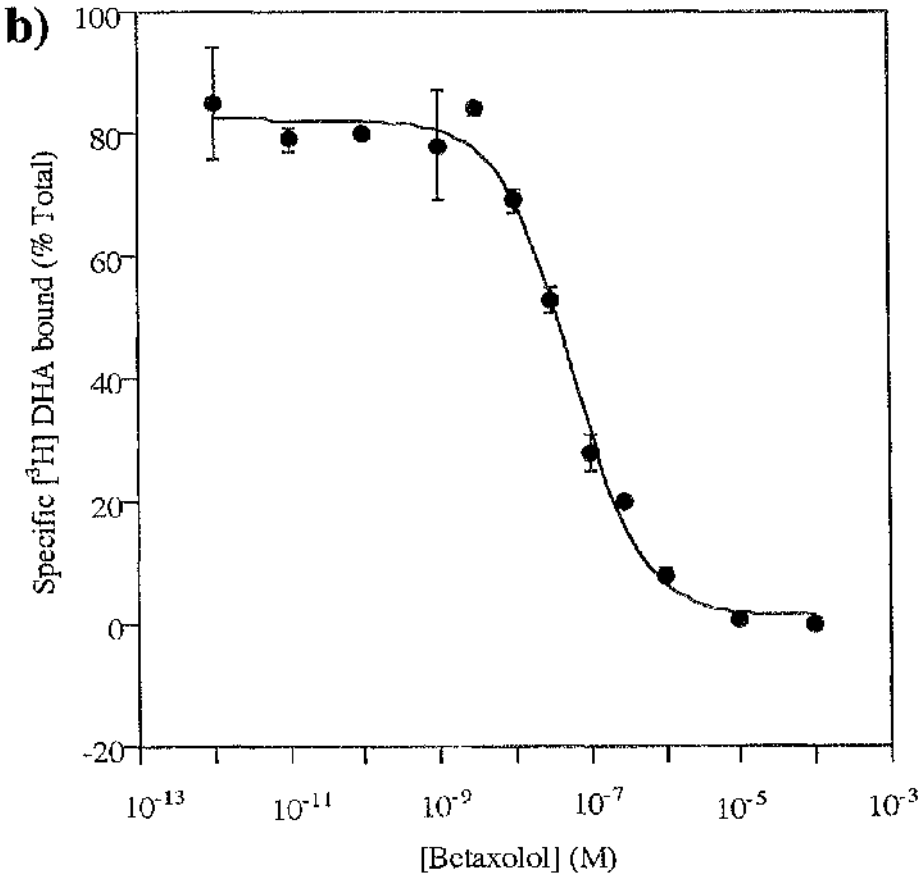
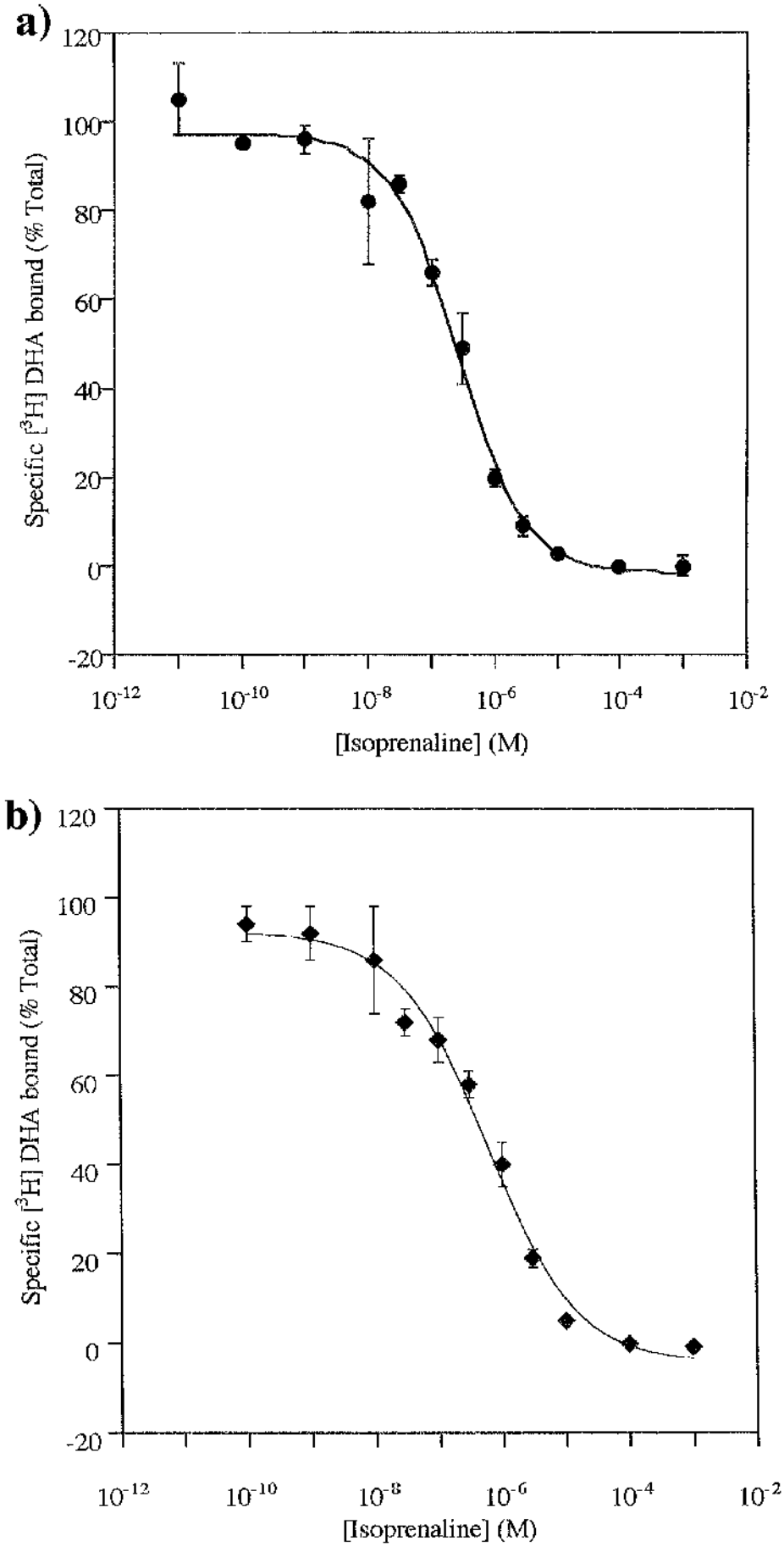


Figure 4.11

GFP has little effect on the affinity of isoprenaline for the WT-β_1-AR.

Competition between [^{3}H] DHA (1.0 nM (a) and 1.6 nM (b)) and varying concentrations of isoprenaline for specific binding to membranes expressing either WT-β_1-AR, Hill coefficient = 0.8 (a); or WT-β_1-AR-GFP, Hill coefficient = 0.6 (b) was assessed. Similar results were obtained from one further experiment (Table 4.3).

Figure 4.11


a)
120
100
80
60
40
20
0
-20
Specific [3H] DHA bound (% Total)
10-12
10-10
10-8
10-6
10-4
10-2
[Isoprenaline] (M)
b)
120
100
80
60
40
20
0
-20
Specific [3H] DHA bound (% Total)
10-12
10-10
10-8
10-6
10-4
10-2
[Isoprenaline] (M)

To compare the functionality of WT-β_2-AR-GFP and WT-β_2-AR the two clones were labelled with [^{3}H] adenine (1 μCi/ml, 24 h) and the capacity of isoprenaline to stimulate the generation of [^{3}H] cAMP measured. Both constructs allowed stimulation of [^{3}H] cAMP production with isoprenaline displaying a similar potency at WT-β_2-AR-GFP (EC_{50} = 1.43 ± 0.2 x 10^{-9} M) compared to WT-β_2-AR (EC_{50} = 3.82 ± 0.5 x 10^{-9} M). However in these selected clones, WT-β_2-AR-GFP gave a lower maximal isoprenaline stimulation than WT-β_2-AR (Figure 4.12a). A concentration-effect curve for isoprenaline was also generated for WT-β_1-AR and WT-β_1-AR-GFP expressing clones. Isoprenaline had a 10 times higher potency at the WT-β_1-AR-GFP construct (EC_{50} = 6.7 ± 0.2 x 10^{-9} M) compared to the WT-β_1-AR construct (EC_{50} = 7.1 ± 0.4 x 10^{-8} M) and a similar potency to the β_2-AR constructs examined above (Figure 4.12b).

4.4 Internalisation studies

In Chapter 3 and in Section 4.2 internalisation of WT-β_2-AR-GFP and WT-β_1-AR-GFP were shown respectively. It is impossible to determine accurately the time-course of internalisation from confocal images as this relies on judgement by eye. Therefore, intact cell binding studies were employed to quantitate more fully the time-course of internalisation of the two receptor-GFP constructs. This also allowed a comparison with the non-GFP-tagged receptors to be achieved. As internalisation of a cell surface receptor was to be measured, [^{3}H] DHA was not an appropriate radioligand for this assay as it is hydrophobic and can cross the plasma membrane. Therefore, any internalised receptor would still bind |^{3}H| DHA. |^{3}H| CGP121777 was an ideal choice for such studies as it is hydrophilic, so cannot cross plasma membranes, and is an antagonist at both the β_1-AR and the β_2-AR.

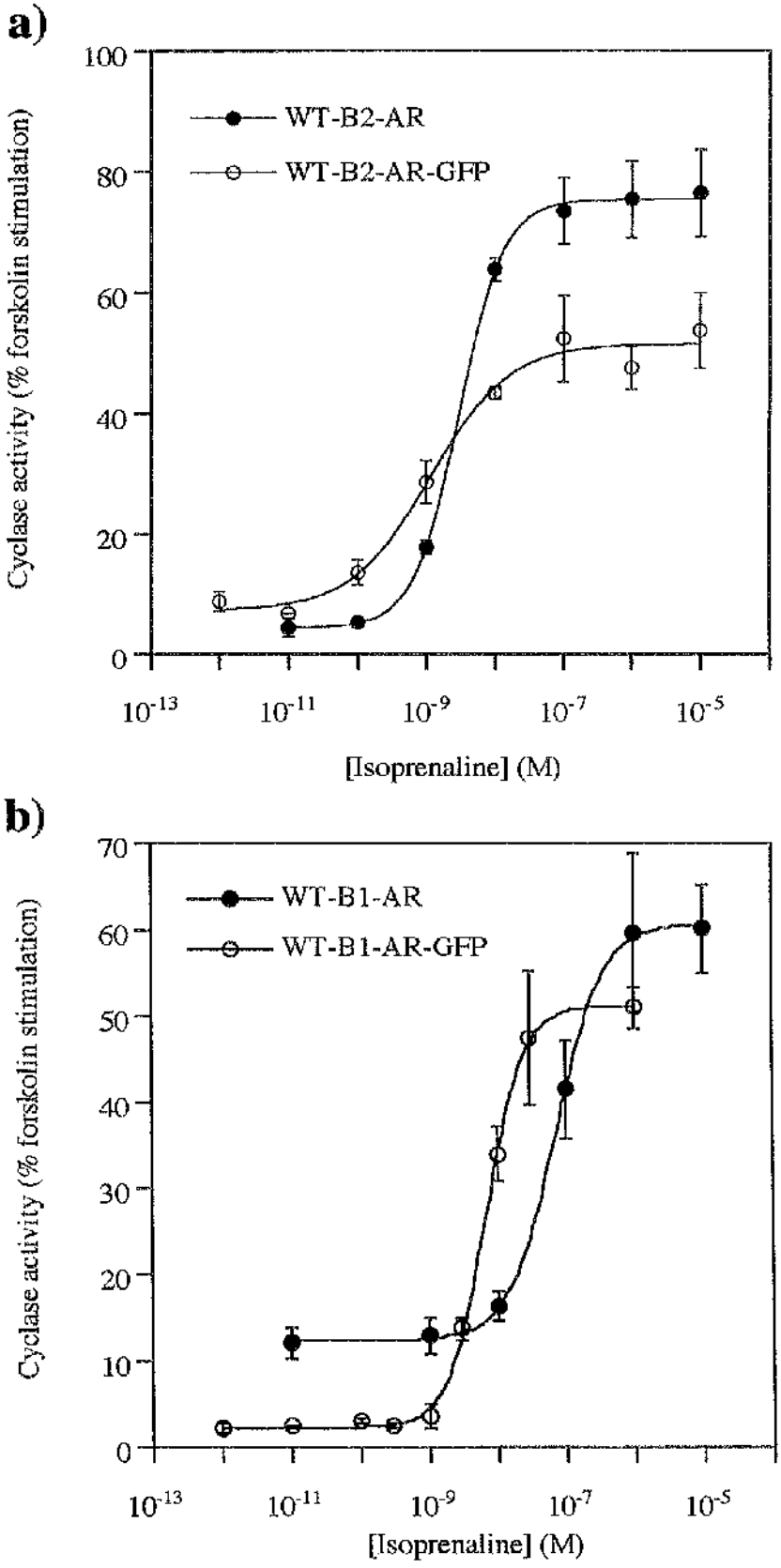
Figure 4.12

Isoprenaline stimulated regulation of adenylyl cyclase activity in intact cells expressing various β-AR constructs.

a) Basal adenylyl cyclase activity and its regulation by increasing concentrations of isoprenaline (10^{-12} M to 10^{-5} M) in WT-β_2-AR and WT-β_2-AR-GFP expressing cells was assessed as detailed in Chapter 2 Section 2.7d. Data represent means ± S.D. of triplicate assays from a single representative experiment. Two additional assays produced similar results. The average EC_{50} of isoprenaline ± S.E.M was calculated for both constructs. Isoprenaline stimulation gave an $EC_{50} = 3.8 \pm 0.9 \times 10^{-9}$ M at WT-β_2-AR, mean ± S.D., $n = 3$; and $EC_{50} = 1.4 \pm 0.3 \times 10^{-9}$ M at WT-β_2-AR-GFP, mean ± S.D., $n = 4$.

b) A similar dose-response to isoprenaline on WT-β_1-AR and WT-β_1-AR-GFP expressing cells. Results are from a single representative experiment performed in triplicate.

Figure 4.12


a)
Cyclase activity (% forskolin stimulation)
100
80
60
40
20
0
WT-B2-AR
WT-B2-AR-GFP
10^{-13}
10^{-11}
10^{-9}
10^{-7}
10^{-5}
[Isoprenaline] (M)
b)
Cyclase activity (% forskolin stimulation)
70
60
50
40
30
20
10
0
WT-B1-AR
WT-B1-AR-GFP
10^{-13}
10^{-11}
10^{-9}
10^{-7}
10^{-5}
[Isoprenaline] (M)

Confocal experiments involved treatment of cells grown in a monolayer on a glass coverslip. The intact cell binding experiments with [^{3}H] CGP12177 employed in Chapter 3 involved binding of the radioligand to cells in suspension (Gagnon et al., 1998; Orsini et al., 1998). As this is inconsistent with the confocal approach this binding assay was modified slightly to accommodate this discrepancy. Cells of each clone (WT-β_1-AR, WT-β_1-AR-GFP, WT-β_2-AR and WT-β_2-AR-GFP) were seeded into 24 well plates and were allowed to attach for 24 h. Time courses to monitor the rate of binding of [^{3}H] CGP12177 at 4°C were performed. A temperature of 4°C was used to prevent recycling of any internalised receptor. All four constructs bound [^{3}H] CGP12177 with similar rates (Figure 4.13a-d), maximal binding being achieved at approximately 60 minutes. It was decided that all [^{3}H] CGP12177 intact cell binding assays would be incubated for 90 minutes to insure that maximal binding was being achieved.

A second factor to consider was to ensure that all isoprenaline was efficiently washed away after treatment. Two experiments were performed on the β_2-AR-expressing cells to determine this (Figure 4.14 a and b). WT-β_2-AR and WT-β_2-AR-GFP cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for 30 minutes at 4°C to achieve association of ligand with cell surface receptor but to induce no receptor internalisation. Cells were then washed 0, 1, 2, 3, or 4 times and subsequently incubated at 4°C with [^{3}H] CGP12177 for 90 minutes (Figure 4.14a). It was apparent that binding of the agonist had occurred as unwashed cells exhibited a reduced binding of [^{3}H] CGP12177 indicating that residual isoprenaline was competing for binding and interfering with the assay (Figure 4.14a). One wash with buffer seemed to sufficiently wash away competing isoprenaline but it was decided that two washes would ensure all contaminating ligand was washed away. Providing all isoprenaline is washed away efficiently the hydrophobic ligand [^{3}H] DHA should be able to bind to any internalised receptor. Again WT-β_2-AR and WT-β_2-AR-GFP-expressing cells were treated for zero or 30 minutes with 10^{-5} M

Figure 4.13

Time-course of [^{3}H] CGP12177 binding to β_1- and β_2- adrenergic receptor expressing clones.

Cells of each clone were seeded into 24 well plates and the rate of binding of [^{3}H] CGP12177 (10 nM) to the WT-β_2-AR (a), WT-β_2-AR-GFP (b), WT-β_1-AR, (c) or WT-β_1-AR-GFP (d) was measured at 4°C.

Results are from a representative experiment performed in triplicate ± S.D.

Figure 4.13

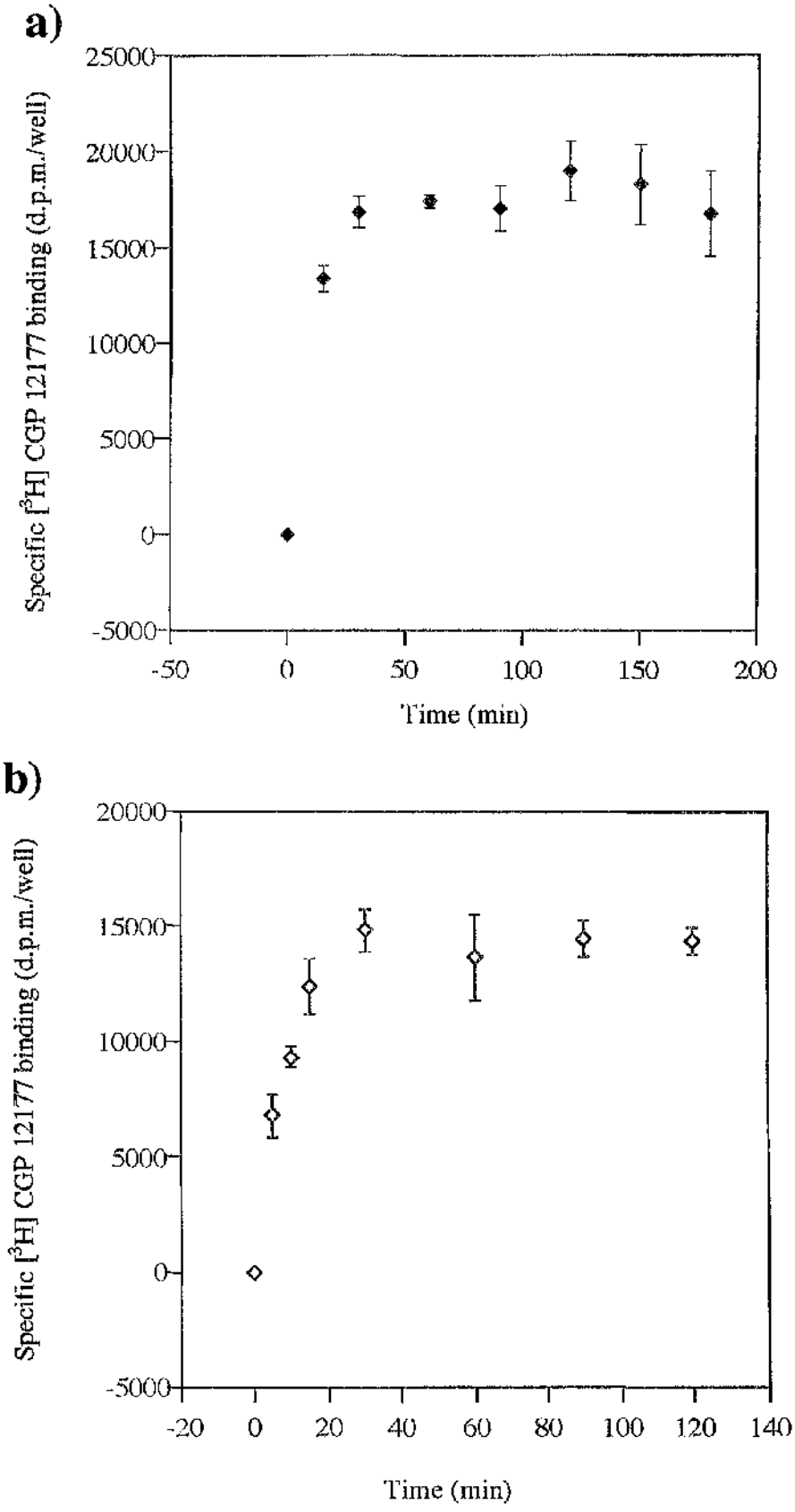
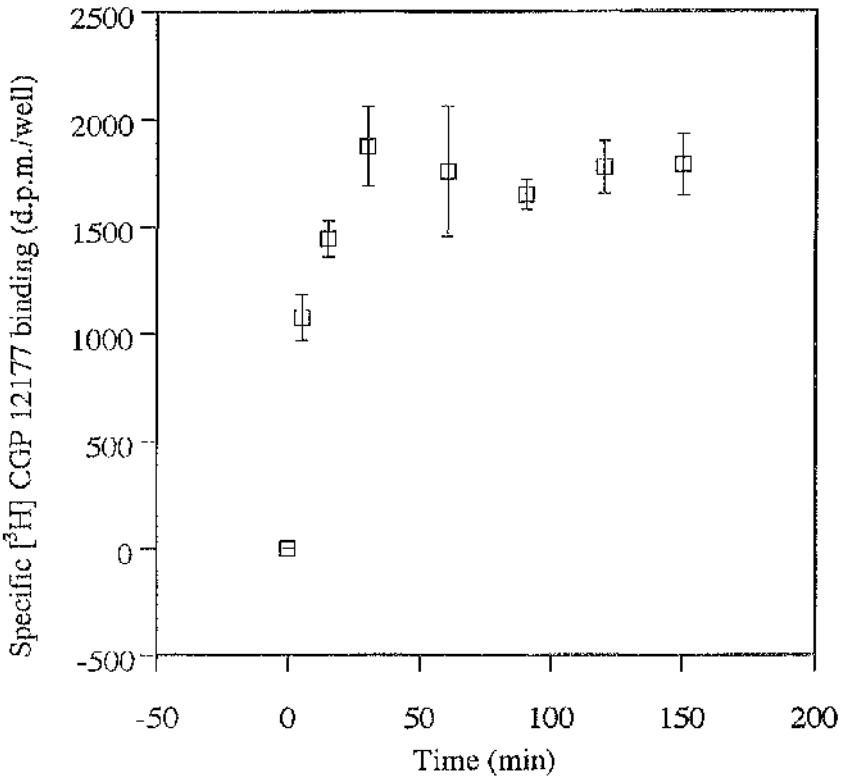

a)
Specific [3H] CGP 12177 binding (d.p.m./well)
25000
20000
15000
10000
5000
0
-5000
-50
0
50
100
150
200
Time (min)
b)
Specific [3H] CGP 12177 binding (d.p.m./well)
20000
15000
10000
5000
0
-5000
-20
0
20
40
60
80
100
120
140
Time (min)

Figure 4.13

c)



d)

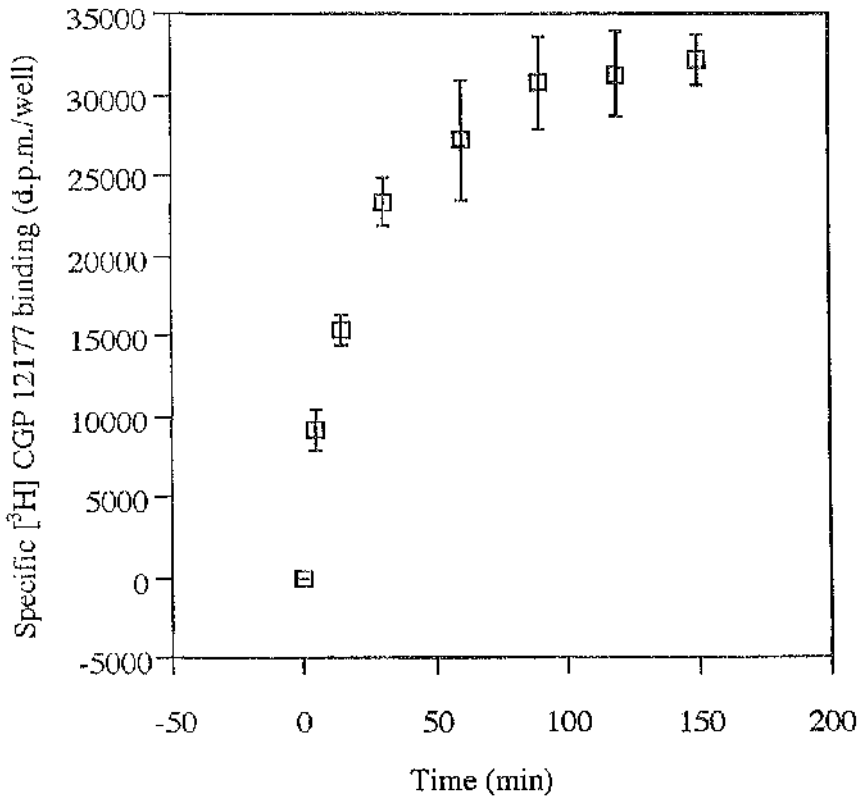


Figure 4.14

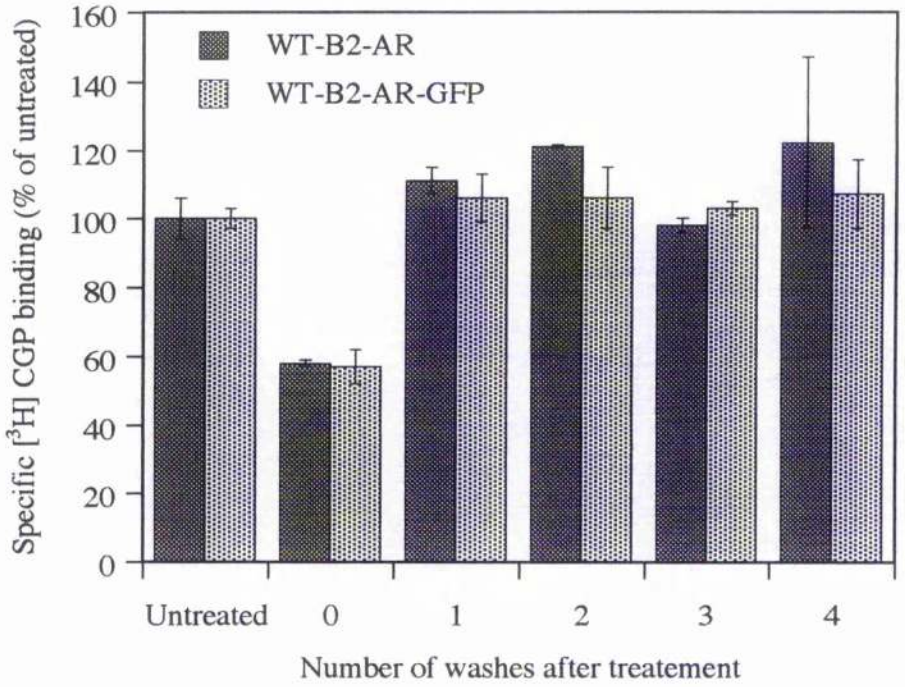
Optimisation of [^{3}H] CGP12177 binding assays.

a) To show that isoprenaline could efficiently be washed away, WT-β_2-AR, and WT-β_2-AR-GFP-expressing cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for 30 minutes at 4 °C. After washing the cells for 0, 1, 2, 3, or 4 times intact cell bindings were performed with 10 nM [^{3}H] CGP12177 at 4 °C for 90 minutes.

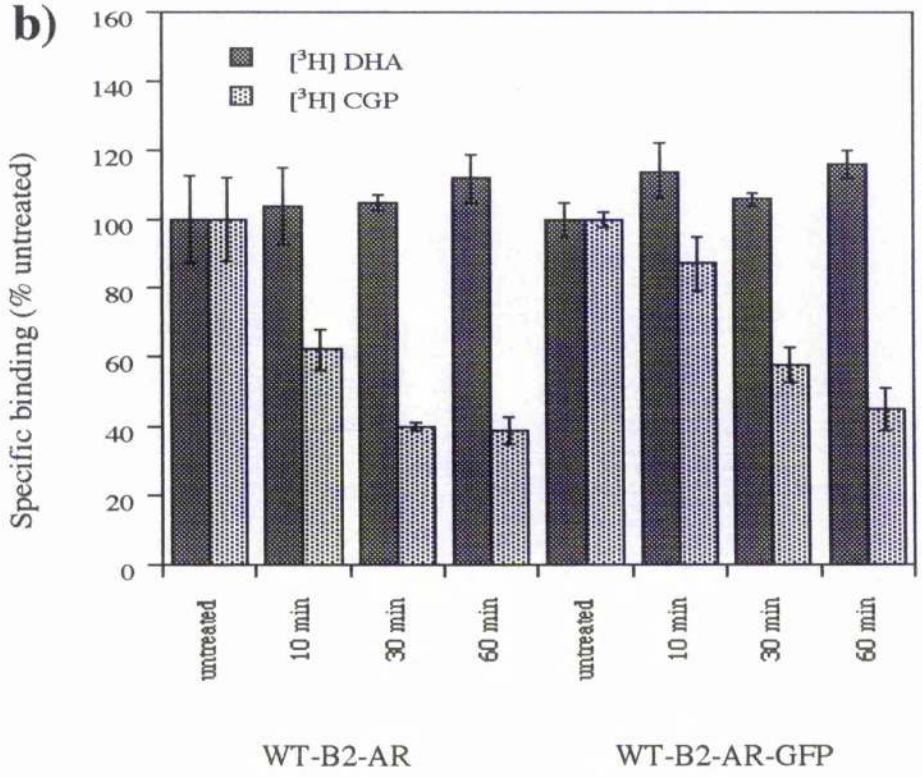
b) To show that internalised β_2-AR could still be measured, WT-β_2-AR, and WT-β_2-AR-GFP-expressing cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for 30 minutes at 37 °C. After two washes cells were incubated with [^{3}H] DHA for 45 minutes at 30 °C or [^{3}H] CGP12177 for 90 minutes at 4 °C. Results are from a representative experiment performed in triplicate ± S.D.

Figure 4.14

a)



b)



isoprenaline at 37°C to induce agonist-stimulated internalisation of the receptor constructs. Following two washes with ice cold buffer, intact cell bindings were performed for 90 minutes at 4°C with [^{3}H] CGP12177, and for 45 minutes at 30°C with [^{3}H] DHA (Figure 4.14b). A time-dependent internalisation of both receptor constructs occurred, as measured by loss of [^{3}H] CGP12177 binding, whereas total levels (cell surface and internalised) of the receptor were unchanged as monitored by the binding of [^{3}H] DHA (Figure 4.14b).

Time-dependent receptor internalisation in response to the agonist isoprenaline was now determined for the four clones. WT-β_2-AR-GFP produced little or no detectable internalisation 5 minute post-stimulation but internalised linearly up to 40 minutes, slightly plateauing off to 60 minutes (Figure 4.15). The WT-β_2-AR construct internalised more rapidly than WT-β_2-AR-GFP, its internalisation becoming maximal at 20 to 30 minutes of isoprenaline stimulation (Figure 4.14). Therefore, GFP appears to slow the ability of the β_2-AR receptor to internalise in response to agonist stimulation. This was also true for the β_1-AR constructs. WT-β_1-AR-GFP internalisation is maximal only at 40 minutes whereas the non-tagged construct only takes about 10 to 20 minutes to become maximally internalised (Figure 4.16). In agreement with the confocal data from Section 4.2 (Figures 3.6 and 4.6), the WT-β_1-AR internalised to a lesser extent (40 % internalised) than the WT-β_2-AR (70 % internalised) (Figure 4.17). These figures are also true for the GFP-tagged versions of these receptors except that only approximately 60 % of WT-β_2-AR-GFP was internalised at 60 minutes (Figure 4.18).

Figure 4.15

Isoprenaline-stimulated internalisation of WT-β_2-AR versus WT-β_2-AR-GFP.

WT-β_2-AR, and WT-β_2-AR-GFP-expressing cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for various time intervals. [^{3}H] CGP12177 binding was then performed to measure the rate of agonist stimulated internalisation of the two constructs with respect to each other. Results are represented as means ± S.E.M., n = 5 for WT-β_2-AR (diamonds) and n = 4 for WT-β_2-AR-GFP (squares).

Figure 4.15

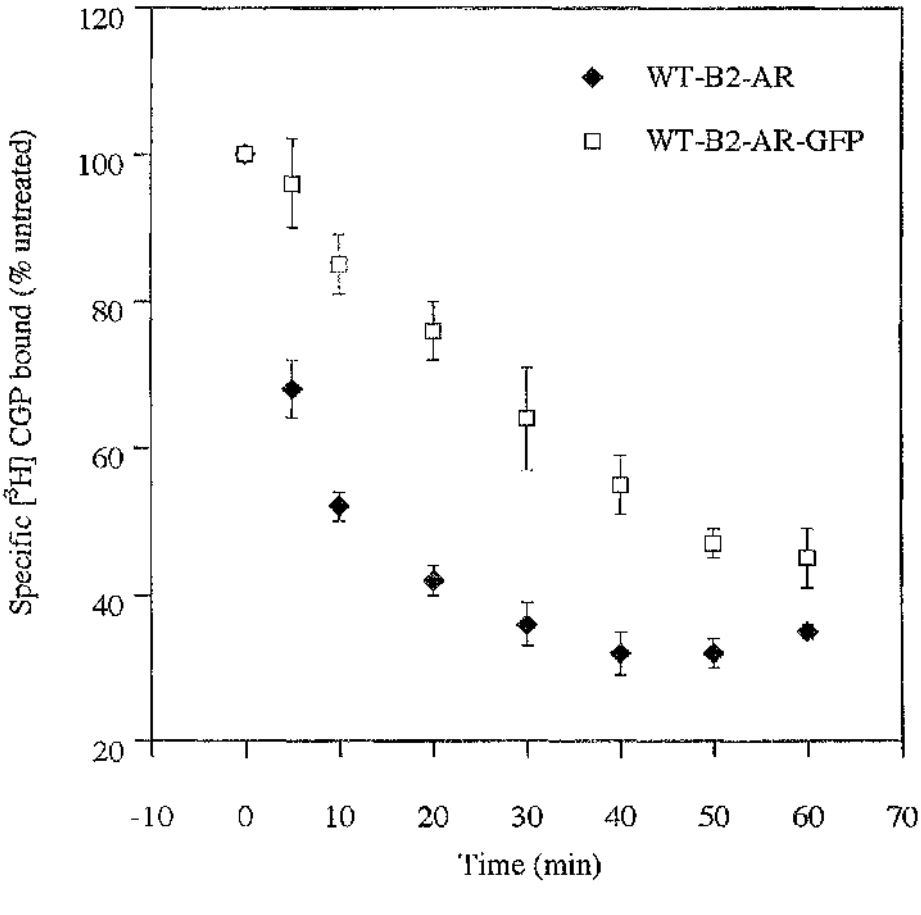

120
100
80
60
40
20
Specific [³H] CGP bound (% untreated)
WT-B2-AR
WT-B2-AR-GFP
-10
0
10
20
30
40
50
60
70
Time (min)

Figure 4.16

Isoprenaline-stimulated internalisation of WT-β_1-AR versus WT-β_1-AR-GFP.

WT-β_1-AR, and WT-β_1-AR-GFP-expressing cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for various time intervals. [^{3}H] CGP12177 binding was then performed to measure the rate of agonist stimulated internalisation of the two constructs with respect to each other. Results are represented as means ± S.E.M., n = 3 for WT-β_1-AR (diamonds) and n = 5 for WT-β_1-AR-GFP (squares).

Figure 4.16

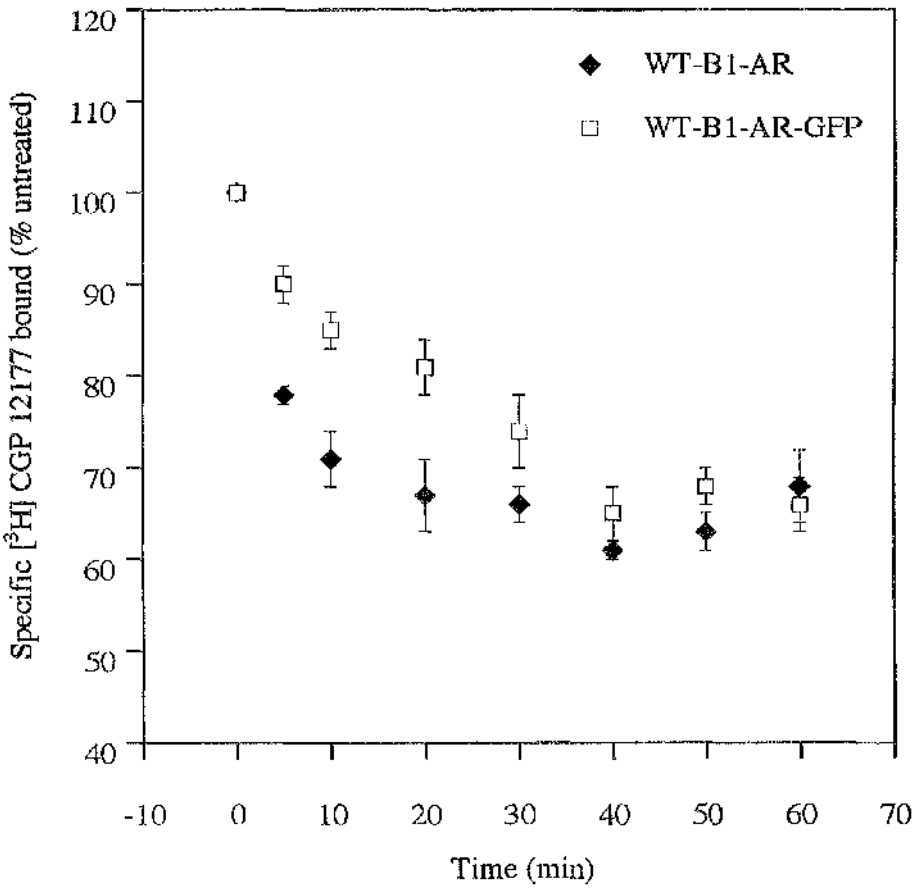

120
110
100
90
80
70
60
50
40
Specific [3H] CGP 12177 bound (% untreated)
WT-B1-AR
WT-B1-AR-GFP
-10
0
10
20
30
40
50
60
70
Time (min)

Figure 4.17

Isoprenaline-stimulated internalisation of WT-β_1-AR versus WT-β_2-AR.

WT-β_1-AR, and WT-β_2-AR-expressing cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for various time intervals. [^{3}H] CGP12177 binding was then performed to measure the rate of agonist stimulated internalisation of the two constructs with respect to each other. Results are represented as means ± S.E.M., $n = 3$ for WT-β_1-AR (diamonds) and $n = 5$ for WT-β_2-AR (squares).

Figure 4.17

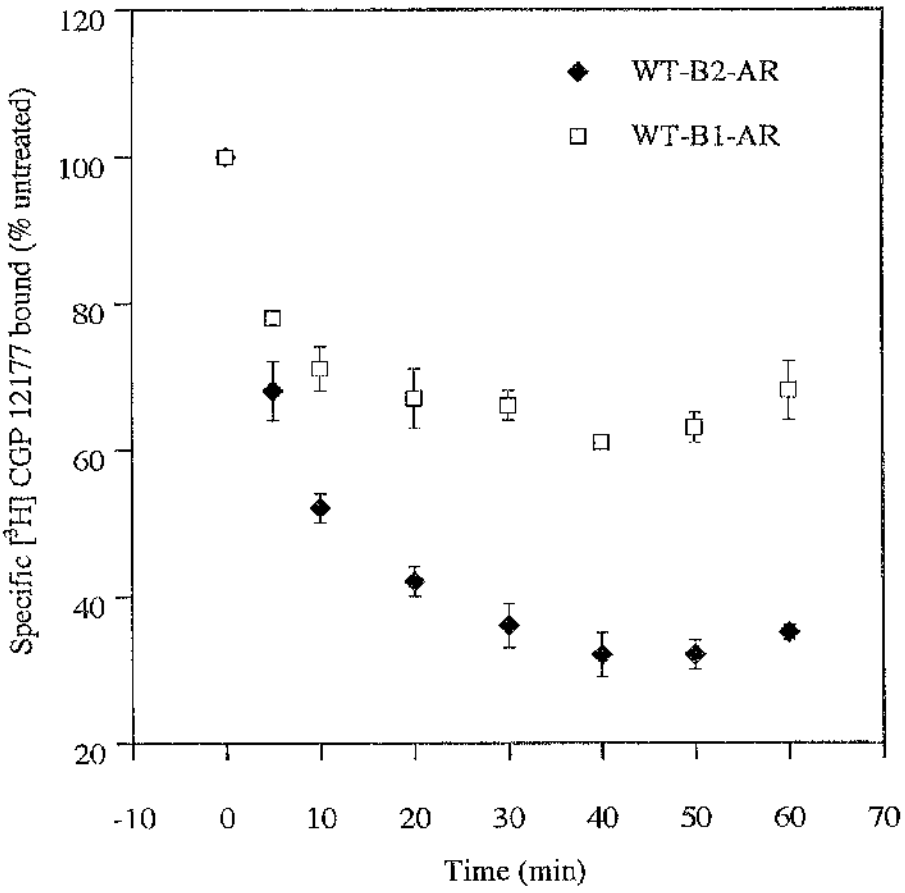
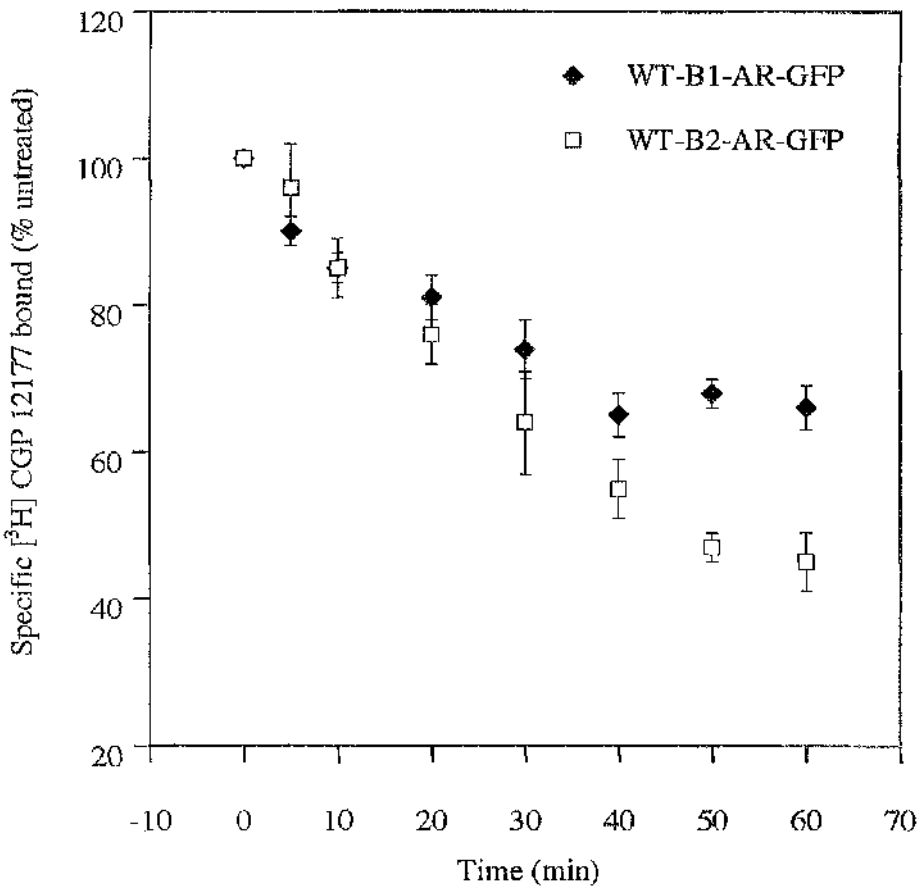

120
100
80
60
40
20
Specific [³H] CGP 12177 bound (% untreated)
WT-B2-AR
WT-B1-AR
-10
0
10
20
30
40
50
60
70
Time (min)

Figure 4.18

Isoprenaline-stimulated internalisation of WT-β_1-AR-GFP versus WT-β_2-AR-GFP.

WT-β_1-AR-GFP, and WT-β_2-AR-GFP-expressing cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for various time intervals. [^{3}H] CGP12177 binding was then performed to measure the rate of agonist stimulated internalisation of the two constructs with respect to each other. Results are represented as means ± S.E.M., $n = 5$ for WT-β_1-AR-GFP (diamonds) and $n = 4$ for WT-β_2-AR-GFP (squares).

Figure 4.18


120
100
80
60
40
20
Specific [3H] CGP 12177 bound (% untreated)
WT-B1-AR-GFP
WT-B2-AR-GFP
-10
0
10
20
30
40
50
60
70
Time (min)

4.5 Recycling of β-AR constructs

Internalisation of GPCRs occurs after the receptor becomes desensitised and can no longer respond to the stimulus. Internalisation is a prerequisite to resensitisation of the receptor which occurs in an intracellular compartment before returning to the cell surface to undergo another round of agonist stimulation. Tagging β-ARs with GFP was extremely useful when monitoring the recycling of these receptors. Firstly WT-β_2-AR-GFP-expressing cells were untreated or pretreated with 10^{-5} M isoprenaline for 30 minutes (Figure 4.19 a and b). The agonist was then removed by washing, and the β_2-AR selective antagonist alprenolol added at 10^{-5} M to prevent further internalisation of any recycled β_2-AR. After a 30 minute exposure to alprenolol a large proportion of the receptor had recycled back to the plasma membrane (Figure 4.19 c). This was more pronounced at 40 minutes (Figure 4,19d). Recycling of internalised WT-β_1-AR-GFP was also examined on cells pretreated with 10^{-5} M isoprenaline for 30 minutes (Figure 4.20 a and b). After a 10 minute exposure to betaxolol distinct recycling of receptor was noted (Figure 4.20c) which was even more pronounced at 30 minutes (Figure 4.20d).

4.6 Long term treatments with agonist isoprenaline

It has previously been documented that long term treatment (≥ 24 h) of cells expressing the β_2-AR with agonists such as isoprenaline can cause a substantial down-regulation of the receptor. The receptor is internalised and instead of being resensitised and recycled back to the plasma membrane the protein is trafficked to lysosomes where it is degraded. It seemed appropriate to determine that the GFP-tagged WT-β_2-AR, like the untagged form, could be down-regulated.

Figure 4.19

Recycling of WT-β_2-AR-GFP following isoprenaline-stimulated internalisation.

WT-β_2-AR-GFP-expressing cells were untreated (a) or pretreated with 10^{-5} M isoprenaline for 30 minutes (b-d). Following washing with 10^{-5} M alprenolol, receptor recycling was monitored at the indicated time points. 10^{-5} M alprenolol for 30 minutes (c) and 10^{-5} M alprenolol for 40 minutes (d).

Figure 4.19

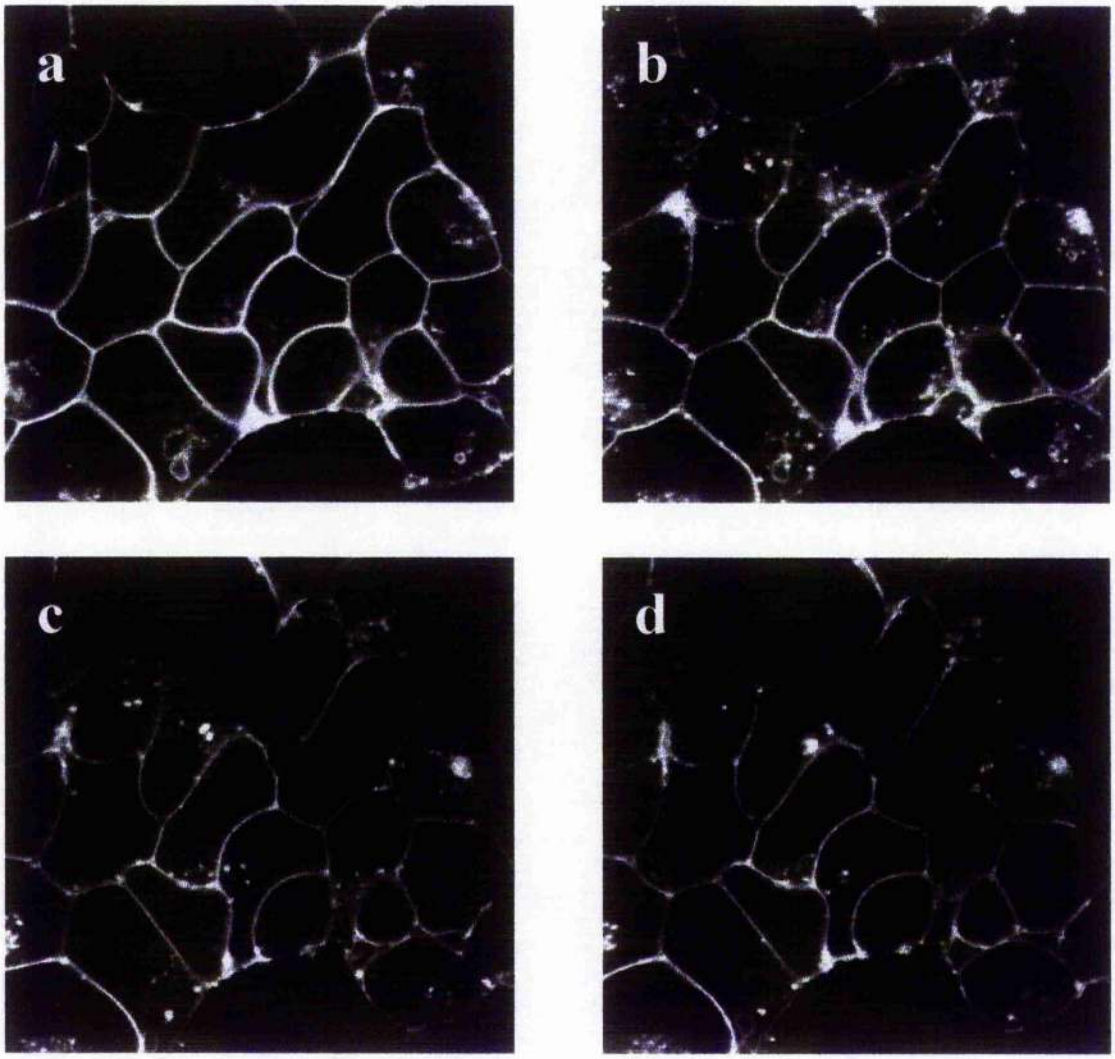
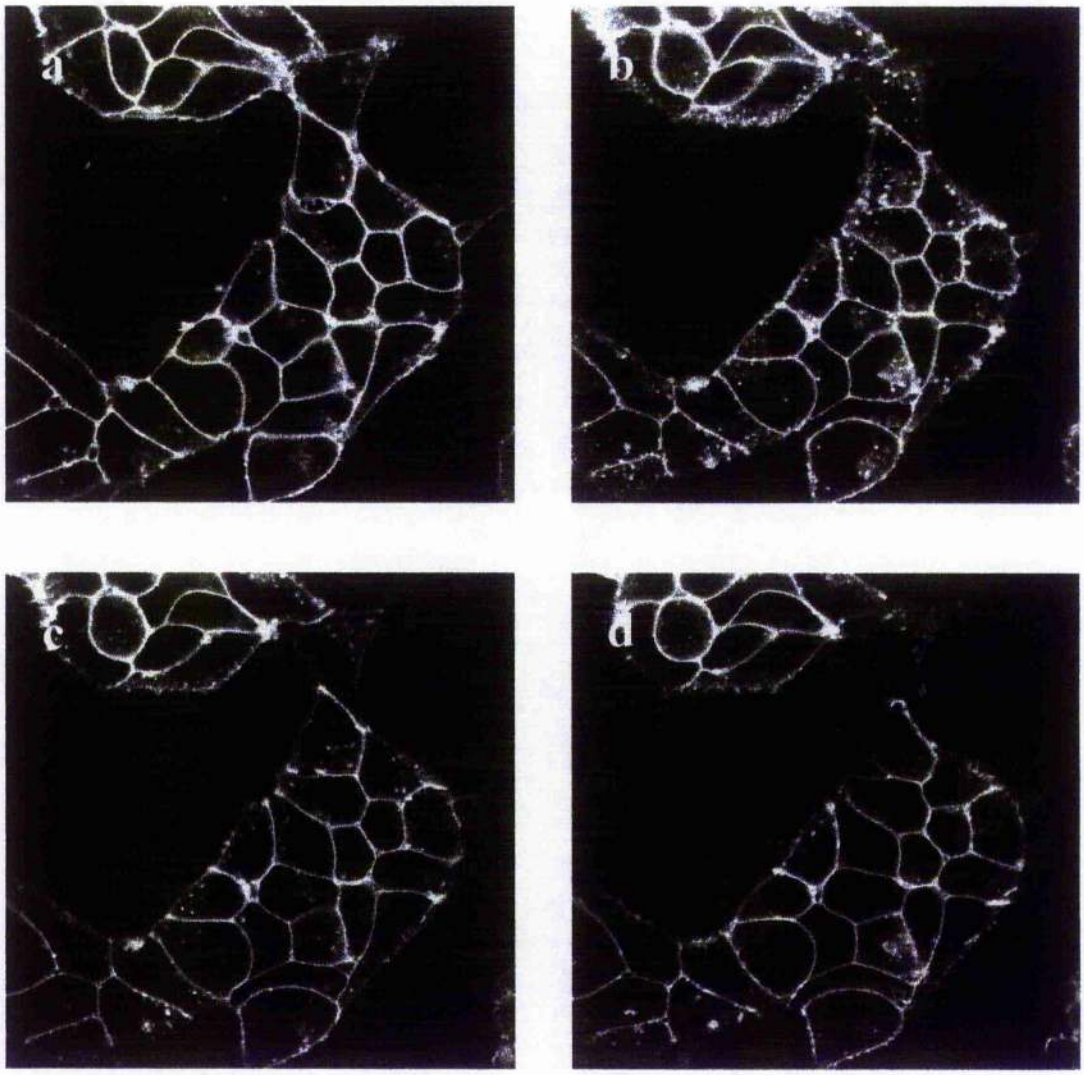


Figure 4.20

Recycling of WT-β_1-AR-GFP following isoprenaline-stimulated internalisation.

WT-β_1-AR-GFP-expressing cells were untreated (a) or pretreated with 10^{-5} M isoprenaline for 30 minutes (b-d). Following washing with 10^{-5} M betaxolol, receptor recycling was monitored at the indicated time points. 10^{-5} M betaxolol for 10 minutes (c) and 10^{-5} M betaxolol for 30 minutes (d).

Figure 4.20



WT-β_2-AR and WT-β_2-AR-GFP expressing cells were untreated or exposed to 10^{-5} M isoprenaline for 24 hours. After thoroughly washing the cells, intact cell binding was performed using a single concentration of [^{3}H] CGP12177 to measure cell surface receptor and [^{3}H] DHA to measure the extent of total receptor loss. As expected the WT-β_2-AR-expressing clone exhibited a large loss of receptor from the cell surface with a 40 ± 3 % (mean ± S.D) loss in total cell receptor (Figure 4.21a). Perhaps surprisingly, although the WT-β_2-AR-GFP-expressing clone displayed a massive loss of cell surface receptor 81 ± 9 % of total receptor was still detected from binding studies after agonist treatment (Figure 4.21a). [^{3}H] DHA binding on membranes from cells treated in the same way indicate that approximately 60 % of total WT-β_2-AR is lost but no loss of WT-β_2-AR-GFP occurs (Figure 4.21b). Due to this discrepancy confocal analysis of living WT-β_2-AR-GFP cells before and after treatment with isoprenaline was required. Following a 24 h treatment with 10^{-5} M isoprenaline the cells imaged demonstrated that WT-β_2-AR-GFP was certainly being lost from the cell surface but had associated into a massive aggregation within an intracellular compartment with very little or no protein degradation (Figure 4.21c).

Long term treatment with 10^{-5} M isoprenaline was also performed on the WT-β_1-AR-GFP clone (Figure 4.22). An 8 h treatment produced a marked punctate pattern of fluorescence within the cells with receptor still remaining at the cell surface (Figure 4.22b). This punctate pattern was also apparent at 24, 48, and 72 h of isoprenaline stimulation with a possible loss of receptor from the cell surface (Figure 4.22 d, e and f, respectively). Figures 4.22 a and c are images of these cells in the basal state. To attempt to clarify the results obtained here, ligand binding experiments on intact cells were performed on WT-β_1-AR-GFP and WT-β_1-AR using a single concentration of [^{3}H] radioligand ([^{3}H] DHA or [^{3}H] CGP12177) (Figure 4.23). Following treatment of WT-β_1-AR-GFP cells with 10^{-5} M isoprenaline for 24, 48 or 72 h, binding experiments with [^{3}H] DHA indicated that a slight up-regulation of the receptor construct had occurred at 24 h but essentially the levels of receptor remained near to

Figure 4.21

Down-regulation of β_2-AR-expressing clones

WT-β_2-AR and WT-β_2-AR-GFP-expressing cells were untreated or treated with 10^{-5} M isoprenaline for 24 h.

a) Cells were washed thoroughly and subsequently incubated with [^{3}H] DHA for 45 minutes at 30 °C or [^{3}H] CGP12177 for 90 minutes at 4 °C to determine the ratio of total receptor and cell surface receptor respectively. Results are represented as means ± S.E.M., $n = 3$ for WT-β_2-AR and $n = 3$ for WT-β_2-AR-GFP.

b) Cells were treated as stated and membranes prepared. [^{3}H] DHA binding experiments were performed on 20 μg of each membrane preparation and the expression level of each construct after treatment determined (fmol/mg). A representative experiment performed in triplicate is shown.

c) Untreated and treated WT-β_2-AR-GFP cells were imaged in a confocal microscope to show the location of the receptor-GFP construct before and after treatment.

Figure 4.21

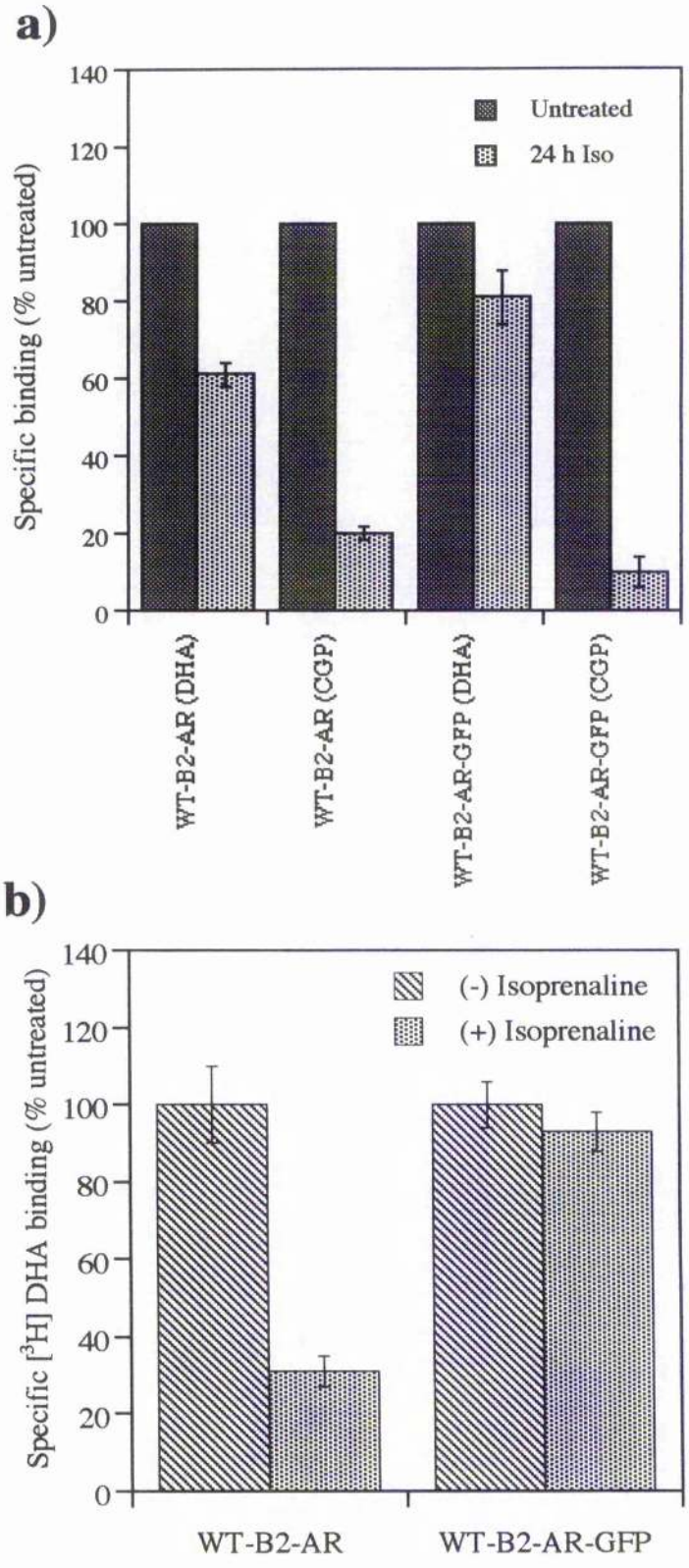
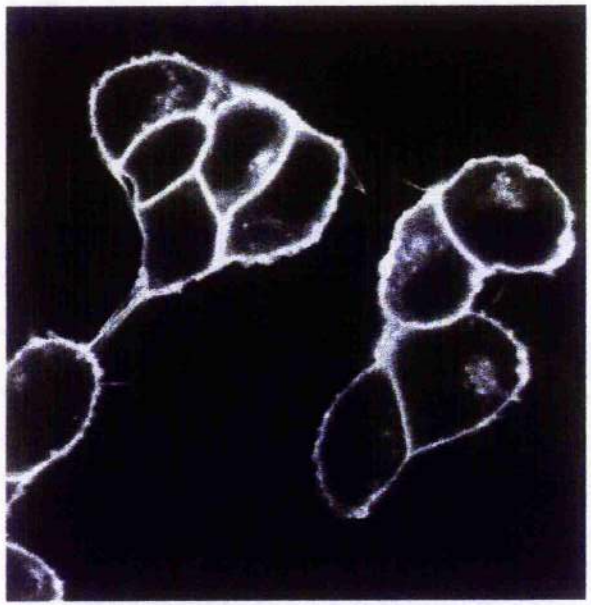
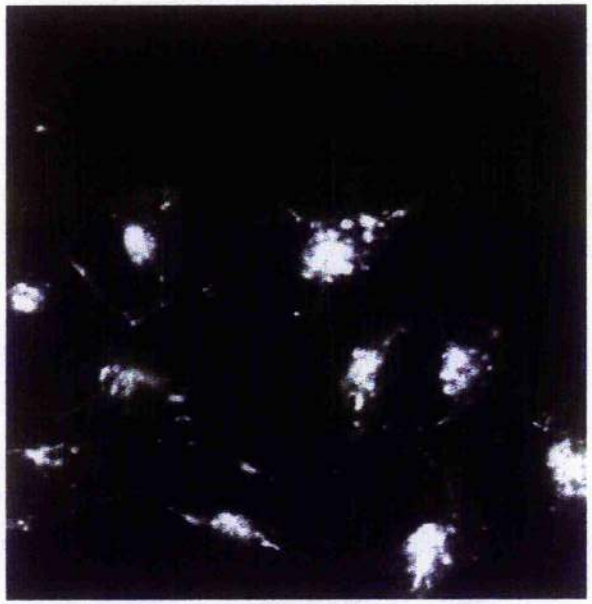

a)
Specific binding (% untreated)
140
120
100
80
60
40
20
0
Untreated
24 h Iso
WT-B2-AR (DHA)
WT-B2-AR (CGP)
WT-B2-AR-GFP (DHA)
WT-B2-AR-GFP (CGP)
b)
Specific [3H] DHA binding (% untreated)
140
120
100
80
60
40
20
0
(-) Isoprenaline
(+) Isoprenaline
WT-B2-AR
WT-B2-AR-GFP

Figure 4.21c



Untreated



10^{-5} M Isoprenaline, 24 h

Figure 4.22

Fluorescence studies of WT-β_1-AR-GFP-expressing cells after long term treatments with isoprenline.

WT-β_1-AR-GFP expressing cells were plated onto glass cover slips and untreated (a,c) or treated with 10^{-5} M isoprenaline for 8 h (b), 24 h (d), 48 h (e) and 72 h (f). The cells were then imaged in a confocal microscope.

Figure 4.22

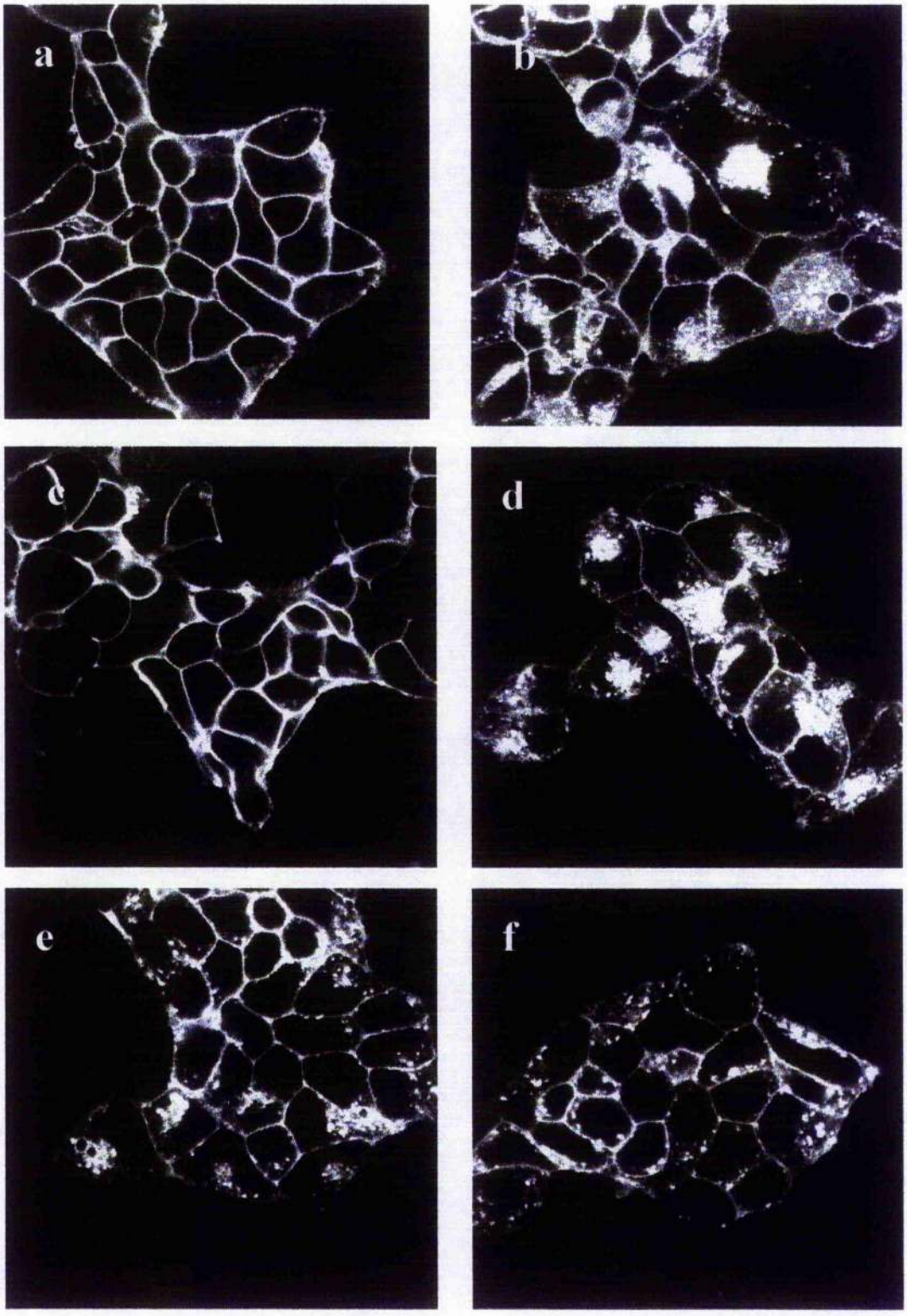
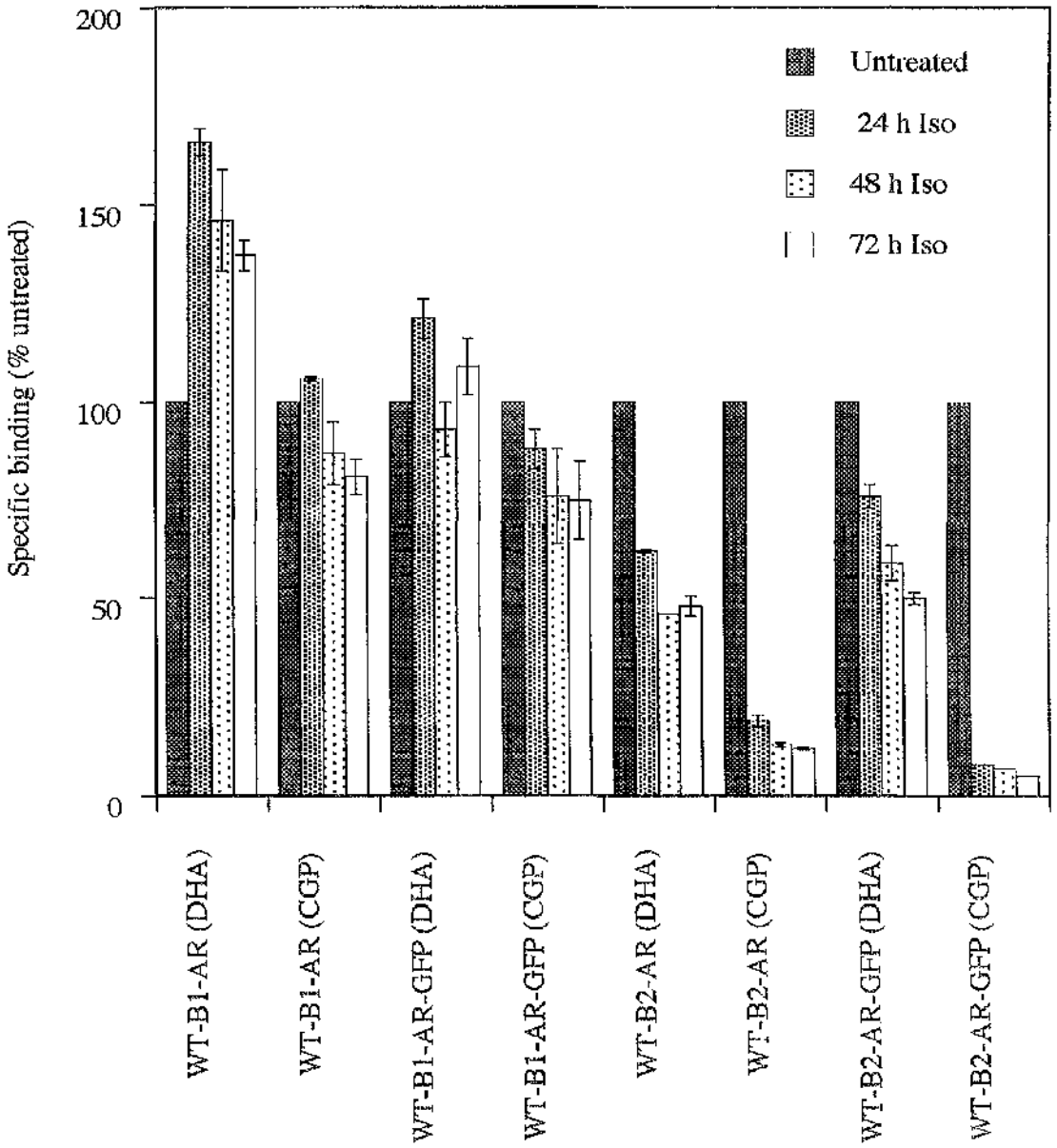


Figure 4.23

[^{3}H] ligand binding studies on WT-β_1-AR and WT-β_1-AR-GFP-expressing cells after long term treatments with isoprenaline.

WT-β_1-AR and WT-β_1-AR-GFP-expressing cells were untreated or treated in 24 well plates with 10^{-5} M isoprenaline for 24, 48 and 72 hours. After washing, the cells were incubated with [^{3}H] DHA for 45 minutes at 30 °C or [^{3}H] CGP12177 for 90 minutes at 4 °C to determine the ratio of total receptor and cell surface receptor respectively. Results are represented as means ± range, $n = 2$.

Figure 4.23


Specific binding (% untreated)
200
150
100
50
0
Untreated
24 h Iso
48 h Iso
72 h Iso
WT-B1-AR (DHA)
WT-B1-AR (CGP)
WT-B1-AR-GFP (DHA)
WT-B1-AR-GFP (CGP)
WT-B2-AR (DHA)
WT-B2-AR (CGP)
WT-B2-AR-GFP (DHA)
WT-B2-AR-GFP (CGP)

basal levels . From [^{3}H] CGP12177 binding an internalisation of WT-β_1-AR-GFP was measured correlating with the punctate pattern seen in Figure 4.22 d, e and f. WT-β_1-AR was up-regulated at 24 h but at 48 and 72 h again the receptor level stayed near the basal level of receptor. The [^{3}H] CGP12177 binding experiment predicts a slight internalisation of WT-β_1-AR (Figure 4.23). This is different to the WT-β_2-AR which undergoes a 40 % down-regulation. As seen previously, the WT-β_2-AR-GFP construct undergoes little down-regulation at 24 h but after 48 to 72 h isoprenaline stimulation at least 40 to 50 % of the receptor is destroyed in agreement with the data from the WT-β_2-AR. Essentially long term treatment of both β_1-AR constructs caused a slight redistribution of receptor from the plasma membrane but little change in total receptor level.

4.7 Discussion

Detailed studies have been performed on β_2-AR-expressing cell systems to characterise the β_2-AR/G_s/AC signaling pathway in response to agonist stimulation. Figure 4.1 and Section 4.1 present a generally accepted model of this process including subsequent receptor desensitisation, sequestration and resensitisation. Far fewer studies have examined the β_1-AR but as it shares 54 % amino acid homology with the β_2-AR, it might be expected to share regulatory mechanisms with the β_2-AR.

Previously, addition of the 27kDa GFP polypeptide has been utilised to present a clearer picture of not only β_2-AR regulation (Barak et al., 1997; Kallal et al., 1998) but also of the TRH receptor, the α_{1B}-AR and the cholecystokinin receptor (Drmota et al., 1998,1999; Awaji et al., 1998; Tarasova et al., 1998). All have indicated that the modified GPCRs display essentially unaltered pharmacology and are able to interact with their cognate G protein to initiate second messenger regulation. Barak et al., (1997) first used a GFP-tagged form of the β_2-AR to directly visualise the receptor and measure agonist and antagonist binding, agonist-stimulated adenylyl

cyclase activity, receptor phosphorylation and receptor internalisation compared to the WT-β_2-AR transiently expressed in HEK293 cells. Kallal et al., (1998) extended these studies using HeLa cells stably expressing either WT-β_2-AR or WT-β_2-AR-GFP. Both stressed the usefulness of GFP to monitor real time trafficking of the β_2-AR in response to agonist stimulation. As the use of GFP has been well established and is a popular technique to study the aforementioned GPCRs it was decided this approach should be applied to compare β_1-AR signaling with the already investigated β_2-AR system.

In Chapter 3, Section 3.3 it was shown that saturation binding studies with [^{3}H] DHA on the β_2-AR stably expressed in HEK293 cells confirmed previous data that the binding characteristics of antagonists at the GFP-tagged construct is not different from the untagged form of this receptor. This was also apparent from saturation binding experiments on GFP-tagged and untagged forms of the β_1-AR (Table 4.2). From competition binding experiments with betaxolol or isoprenaline, both β_1-AR constructs were shown to have similar affinities for each drug (Table 4.3 and Figures 4.10 and 4.11). This was previously found for the β_2-AR in Chapter 3 (Table 3.2 and Figures 3.11 and 3.12).

Both β_1- and β_2- AR constructs were functionally characterised using isoprenaline-stimulated intact cell adenylyl cyclase assays. From studies on the β_2-AR, over the past decade it is evident that the number of β_2-ARs expressed can have a dramatic effect on the kinetic parameters of adenylyl cyclase activation. Whaley et al., (1993) generated a series of L cell clones stably expressing the hamster and human β_2-AR over a 2000-fold range of receptor level (5 to 10000 fmol/mg of membrane protein). An increase in expression level of the β_2-AR was found to correlate with a decrease in EC_{50} for isoprenaline and with a slight increase in intrinsic activity (V_{max} or maximal output) for adenylyl cyclase activation. This work developed a mathematical prediction to analyse alterations in β-AR full agonist EC_{50} values with changes in

receptor number and has been extended by MacEwan and Milligan, (1995) to the study of partial agonist potency at the β_2-AR. Bouvier et al., (1988) have also observed similar results to Whaley et al., for the human β_2-AR expressed in chinese hamster fibroblast cells (CHW). However, the intrinsic activity was reduced markedly in cells expressing very high levels of receptor. Likewise, in a more recent study, when β_2-AR levels were increased in an inducible expression system of rat C_6 glioma cells stably expressing this receptor, the maximal cyclase output was decreased with a small shift in the dose-effect curve to the left (ie a reduction in EC_{50}) (Zhong et al., 1996). The mechanisms for this are not yet clear. In this present study isoprenaline exhibited a similar potency at the WT-β_2-AR to the WT-β_2-AR-GFP, in agreement with Kallal et al., (1998). However, the more highly expressed GFP construct gave a lower maximal response (20 to 30 % lower than that of the WT-β_2-AR). This is consistent with the above data from Bouvier et al., (1988) and Zohng et al., (1996).

In systems in which the effector species is quantitatively the limiting component of a signal transduction cascade, it is often observed that elevations in receptor number result in a leftward shift in the dose-effect curve (ie a reduction in EC_{50} value), consistant with the notion of a receptor population reserve. Isoprenaline was found to have a 10 times lower potency at the WT-β_1-AR than at the much more highly expressed WT-β_1-AR-GFP. The maximal cyclase activities for both constructs are similar but the 10 fold shift in EC_{50} would indicate a β_1-AR reserve. Zhong et al., (1996) show this for stably expressed β_1-AR in the inducible rat C_6 glioma cell exppression system. The EC_{50} for isoprenaline decreased with little effect on the maximal cylcase ouput when the receptor number was increased.

Alternatively, these results could be attributed to the addition of GFP to the C terminus of this receptor which may be interfering with activation of adenylyl cyclase. An additional factor to consider is that GFP has been engineered to be extremely stable in

mammalian cells and may stabilise the protein it is attached to, therefore, increasing expression levels of the receptor. In reality GFP may be a culprit here, causing stabilisation and increased expression of receptor, indirectly resulting in a variation of agonist potency.

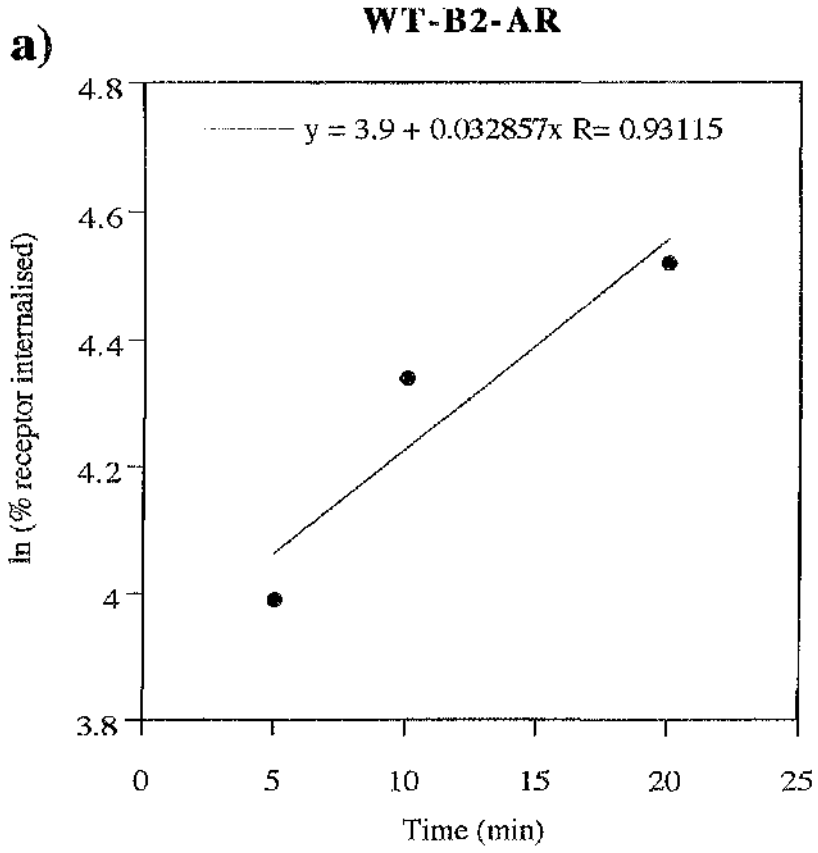
Internalisation studies provided more evidence that the use of GFP has to be treated with caution. From the earliest study on WT-β_2-AR-GFP, Barak et al., (1997) used flow cytometry to measure a 57 ± 5 % decrease in cell surface WT-β_2-AR after 30 minutes of isoprenaline exposure. WT-β_2-AR-GFP similarly exhibited a 62 ± 11 % loss from the cell surface. However, only one time point was investigated. Kallal et al., (1998) confirmed WT-β_2-AR-GFP internalisation by [^{3}H] CGP12177 binding in a HeLa cell line. A time-course of internalisation was performed in response to isoprenaline stimulation but not in parallel with WT-β_2-AR-expressing cells. The data in this present study clearly indicates that addition of GFP to WT-β_2-AR slows the internalisation of this receptor. Addition of GFP to WT-β_1-AR produced similar results (Figures 4.15 and 4.16). To estimate the $t_{1/2}$ of receptor internalisation for each receptor constuct it was neccessary to recalculate the raw data as the number of receptors gained within the cell at time x as a percentage of total receptor gained at 60 minutes. The natural log (ln) of each percentage value was then plotted against time and the $t_{1/2}$ at 50 % receptor internalisation was calculated from the equation of each line fit. Figure 4.24 and Table 4.4 demonstrates that WT-β_2-AR (a) internalises extremely rapidly with a calculated $t_{1/2}$ = 0.5 minutes and WT-β_2-AR-GFP (b) internalises much more slowly with a calculated $t_{1/2}$ = 25 minutes. WT-β_1-AR (c) internalises with a $t_{1/2}$ too rapid to be calculated with this data. A more detailed time course of internalisation is required with a large range of time points under 5 minutes. WT-β_1-AR-GFP (d) internalises more slowly than its untagged version but at a quicker rate than WT-β_2-AR-GFP with a calculated $t_{1/2}$ = 15 minutes. This, therefore, indicates that GFP is exerting an effect on receptor internalisation.

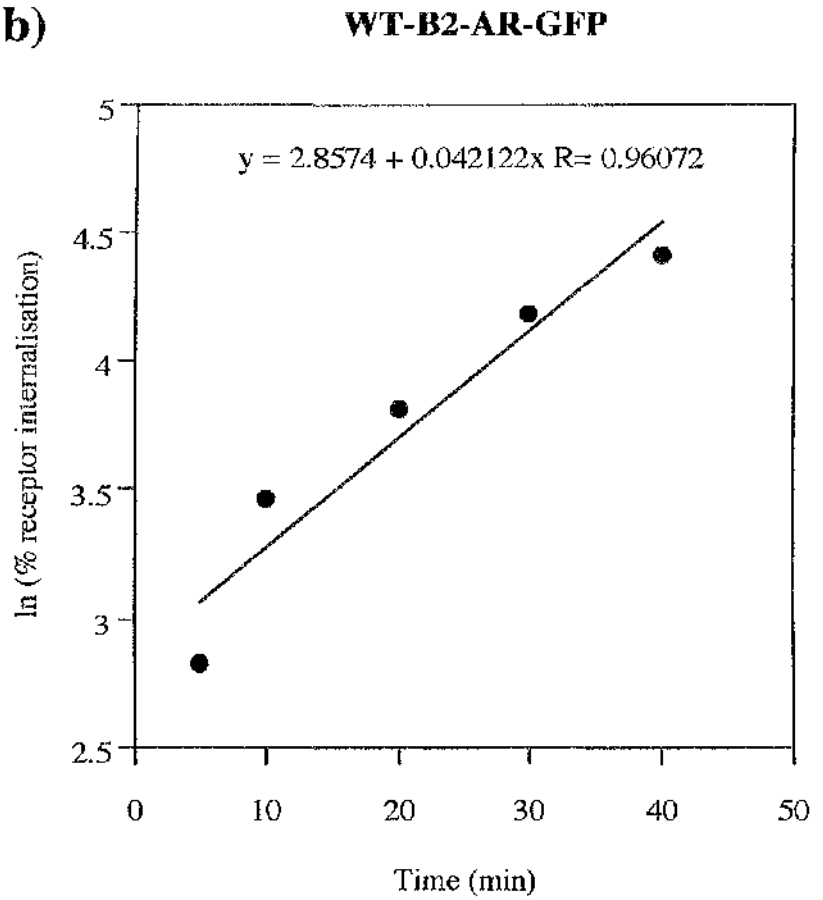
Figure 4.24

Estimation of intracellular receptor gain following isoprenaline stimulation of β-AR clones.

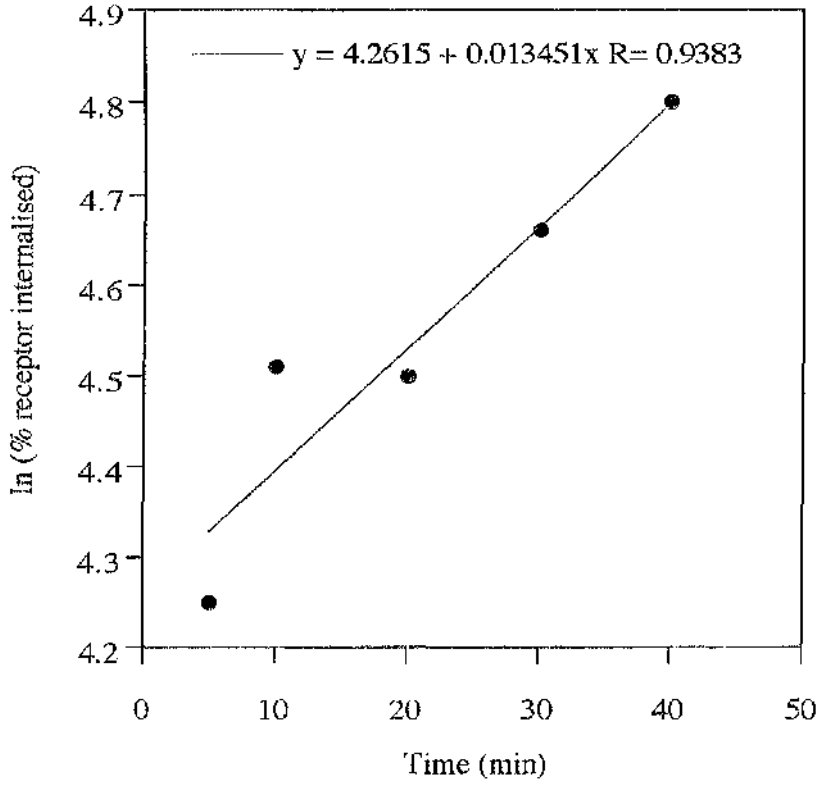
Raw data from the linear part of the curves in Figures 4.15, 4.16, 4.17 and 4.18 was used to calculate the number of receptors internalised as a percentage of total receptors internalised at 60 minutes isoprenaline stimulation. The natural logs of these values were then replotted against time. The equation of each line fit was used to estimate the $t_{1/2}$ of receptor internalisation for; a) WT-β_2-AR and WT-β_2-AR-GFP, b) WT-β_1-AR and WT-β_1-AR-GFP, $n = 3$ (Table 4.4).

Figure 4.24


a)
WT-B2-AR
y = 3.9 + 0.032857x R= 0.93115
ln (% receptor internalised)
4.8
4.6
4.4
4.2
4
3.8
0
5
10
15
20
25
Time (min)


b)
WT-B2-AR-GFP
y = 2.8574 + 0.042122x R= 0.96072
ln (% receptor internalisation)
5
4.5
4
3.5
3
2.5
0
10
20
30
40
50
Time (min)

c)


WT-B1-AR
y = 4.2615 + 0.013451x R= 0.9383
ln (% receptor internalised)
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
0
10
20
30
40
50
Time (min)

d)

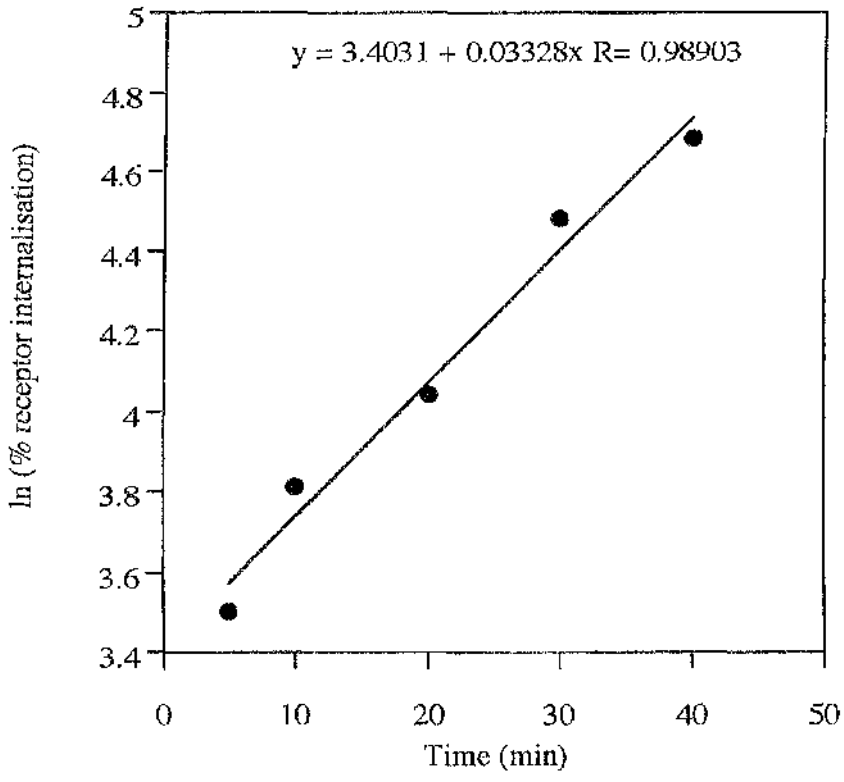

WT-B1-AR-GFP
y = 3.4031 + 0.03328x R= 0.98903
ln (% receptor internalisation)
5
4.8
4.6
4.4
4.2
4
3.8
3.6
3.4
0
10
20
30
40
50
Time (min)

Table 4.4

Rate of isoprenaline stimulated internalisation as $t_{1/2}$ values and % of receptor internalisation for the denoted constructs.

Clone	$t_{1/2}$ of internalisation (minutes)	Maximal receptor internalisation (%)
WT-β_1-AR	*N.D.	30
WT-β_1-AR-GFP	15	30
WT-β_2-AR	0.5	50
WT-β_2-AR-GFP	25	40

Values of $t_{1/2}$ were calculated for 50 % receptor internalisation from the equation of the line fits in Figure 4.24.

* N.D. = Not defined.

To attempt to understand how GFP may be achieving this, work by Jockers et al., (1996) may help form an explanation. The β_3-AR does not readily undergo rapid agonist-promoted desensitisation and internalisation (Ligget et al., 1993; Nantel et al., 1993; Chaudhry et al., 1994). It also has high sequence homology with the β_2-AR. Jockers et al., constructed a series of 15 β_3/β_2-AR chimeras to identify which regions of the β_2-AR are important in uncoupling and sequestration. It was revealed that the C-terminal tail, intracellular loop 3 and intracellular loop 2 of the β_2-AR added individually into the β_3-AR all partially restored the uncoupling phenotype and that their effects were additive to produce a desensitisation profile similar to that of the β_2-AR. The C-terminal tail, intracellular loop 2 and intracellular loop 1 all play additive roles in receptor sequestration, but not to restore it completely, whereas, intracellular loop 3 has a dominant negative effect. It may be that GFP interferes with the C-terminal tail and, therefore, slows internalisation. It is apparent however, that other regions in the β_2-AR must be involved for full sequestration of this receptor. It is evident that the β_1-AR internalises more rapidly and to a lesser degree than the β_2-AR (Figures 4.17 and 4.18). However, the latter observation is only apparent for the GFP-tagged constructs at later time points of 30 minutes or greater.

GFP's ability to slow down regulatory processes of receptors to which it is linked is again apparent when investigating long term treatment of receptor expressing HEK293 cells to the agonist isoprenaline. Gagnon et al., (1998) have previously reported that 40 to 50 % of the β_2-AR is lost from intact cells when stably expressed in HeLa cells (at 3-5 pmol/mg) and exposed to isoprenaline for 24 h. The present study shows that similar experiments on HEK293 cells stably expressing WT-β_2-AR produces results in accordance with Gagnon et al (Figure 4.21a). However, when WT-β_2-AR-GFP-expressing HEK293 cells are treated in exactly the same way, there is only a 10 to 30 % reduction in total receptor in whole cells. This is more pronounced in membrane binding assays (Figure 4.21b) where WT-β_2-AR-GFP was only reduced by about 10 %. Confocal analysis clearly indicates that the GFP-tagged receptor is held within an

intracellular compartment (probably the lysosomes) following a 24 h treatment with isoprenaline (Figure 4.21c). These results are contradictory to Kallal et al., (1998) who demonstrated a down-regulation profile for WT-β_2-AR-GFP in HeLa cells, similar to that reported by Gagnon et al., (1998) for the WT-β_2-AR also expressed in HeLa cells. The expression level of the GFP-tagged receptor in Kallal's study was substantially lower, only 200 fmol/mg. WT-β_2-AR-GFP is expressed at approximately 9 pmol/mg in the present study. This high expression level may be a determining factor in the extent of down-regulation of the receptor. When WT-β_2-AR-GFP-expressing cells are treated with agonist for 48 and 72 h, levels of β_2-AR antagonist binding sites do decrease to similar levels as WT-β_2-AR antagonist binding sites after 24 h of isoprenaline treatment (Figure 4.23). The data again demonstrate GFP's ability to compromise regulatory processes of the β_2-AR.

Previous reports on agonist regulation of β_1- and β_2- ARs have indicated significant differences in their uncoupling, sequestration and down-regulation patterns (Rousseau et al., 1996; Suzuki et al., 1992; Zhou et al., 1995). The β_3-AR undergoes very little desensitisation and down-regulation and it has been generally accepted that agonist stimulation of the β_1-AR leads to a desensitisation and down-regulation profile which is intermediary between the β_2- and β_3- ARs. From Figure 4.23 long term isoprenaline treatment produced a different profile of regulation for both GFP-tagged and non-GFP-tagged receptors. For WT-β_1-AR, the total receptor level appeared to increase initially and then decrease slightly but stayed above untreated levels of receptor. The GFP-tagged WT-β_1-AR, on the other hand, remained at similar levels of expression throughout treatment. In binding studies with [^{3}H] CGP12177 both constructs were lost from the cell surface at 48 and 72 h agonist treatment. The confocal images of WT-β_1-AR-GFP confirm this loss of receptor from the plasma membrane (Figure 4.22). Therefore, in this cell system it appears that the β_1-AR does not undergo agonist induced down-regulation.

The results of this chapter do demonstrate that GFP can be utilised as a helpful tool in monitoring the trafficking of GPCRs in response to agonist stimulation. It must be stressed that there are limitations, as with any method, in what can be interpreted from the results obtained. Highly expressing receptor systems are advantageous when trying to use a method with low sensitivity but the generated data should always be treated with caution. From using a range of methods in conjunction with one another a clearer picture of cellular receptor processing can be generated.

Chapter 5

Pharmacological Characterisation and Signalling of a C-terminally Mutated β_2-AR Tagged With GFP

Chapter 5

5.1 Introduction

For regulation of a cell's biological processes the phosphorylation and dephosphorylation of proteins or enzymes is a fundamental and extremely important mechanism. In mammalian cells these processes include, 1) metabolic pathways such as glycolysis, gluconeogenesis, glycogen synthesis/breakdown, fatty acid synthesis/breakdown etc., 2) cellular signaling pathways through GPCRs or tyrosine kinase receptors leading to activation of effectors such as adenylyl cyclase, PKA, PKC, MAPK etc., 3) phosphorylation of transcription factors, eg. c-jun, c-fos or CREB to induce effects on gene expression.

Chapter 4 presented a selection of the evidence surrounding the elucidation of an accepted model for receptor activation, desensitisation, internalisation and recycling or down-regulation of the β_2-AR sub-type of GPCR in response to agonist stimulation (Figure 4.1, Bohm et al., 1997). The processes of phosphorylation and dephosphorylation act as key molecular switches at various points in this cycle of events. In the process of receptor desensitisation, where a decreased responsiveness of receptor to agonist occurs, two distinct classes of kinases - second-messenger dependent kinases (PKA and PKC) and receptor specific protein kinases (GRKs) are involved. Phosphorylation of the β_2-AR by cAMP dependent protein kinase (PKA) has been pin-pointed at serines 261 and 262 in the third intracellular loop PKA consensus site (Clark et al., 1989; Yuan et al., 1994; Hausdorff et al., 1989) and at a second site at serines 345 and 346 in the C-terminal tail. 11 potential GRK phosphorylation sites at serine/threonine residues in the C-terminal tail of the β_2-AR have been identified by mutagenesis studies (Hausdorff et al., 1989; Bouvier et al., 1988). Currently 6 GRKs have been identified, all containing an N-terminal domain (residues 1-184), a catalytic domain (residues 185-456), a conserved C-terminal

autophosphorylation region (457-522) and a variable C-terminal region giving the GRK sub-type specificity (reviewed by Krupnick and Benovic, 1998; Bohm et al., 1997). In GRK2 (β-AR kinase-1 (βARK 1)) or GRK3 (βARK 2), the C-terminal variable region contains a pleckstrin homology (PH) domain, conferring binding specificity to $G_{\beta\gamma}$ proteins, assisting recruitment of the protein to the plasma membrane. Fushman et al., (1998) have presented the solution structure and dynamics of the PH domain of GRK2 (βARK 1) to show that it is capable of protein/protein interactions with $G_{\beta\gamma}$ sub-units. GRK5 has also been found to phosphorylate the β_2-AR, but does not contain a $G_{\beta\gamma}$ binding domain. Instead it is thought to associate with certain phospholipids in the plasma membrane to carry out its function (Kunapuli et al., 1993; Premont et al., 1994; Premont et al., 1995).

GRK phosphorylation is not sufficient to induce full desensitisation and uncoupling of receptor from activated G-protein. This is assisted by bifunctional, soluble proteins called arrestins. In the case of the β_2-AR, β-arrestins 1 or 2 are involved. The primary function of these proteins is to destroy the interaction of ligand bound receptor which has been phosphorylated by a GRK, with the heterotrimeric G protein α sub-unit to attenuate agonist activation (Lohse et al., 1990; Pippig et al., 1993). β-arrestins also regulate sequestration (internalisation) of several GPCRs including the β_2-AR by acting as clathrin adapters, the main component of clathrin coated pits of the endocytic receptor pathway (Figure 4.1). β-arrestin/arrestin chimeras defective in either receptor or clathrin binding do not support agonist-dependent internalisation of the β_2-AR (Goodman et al., 1996; Krupnick et al., 1997; Goodman et al., 1997). In the basal state β-arrestin 1 is a phospho-protein but is dephosphorylated on binding receptor and acting as a clathrin adapter (Lin et al., 1997). However, it is not clear whether β-arrestin 1 dephosphorylation precedes or follows receptor binding. Studies on GPCR internalisation regulated by β-arrestin 2 have revealed that β-arrestins dissociate from the β_2-AR following the redistribution of β-arrestins to coated pits (Zhang et al., 1999). This dissociation is proposed to facilitate dephosphorylation of

the β_2-AR by phosphatases in early endosomes so the receptor can be recycled back to the plasma membrane for another round of agonist stimulation (Krueger et al., 1997).

It appears that β-arrestin-mediated internalisation may be linked to downstream mitogenic signaling pathways. Lin et al., (1999) have demonstrated that β-arrestin 1 function is regulated by a negative feedback loop involving extracellular signal-regulated kinases (ERKs) through a protein termed MEK1. This was shown by using a dominant negative K97A mutant of MEK1 which increased β-arrestin 1-mediated sequestration. The same group have also demonstrated β-arrestin-dependent formation of β_2-AR-Src protein kinase complexes to generate a second wave of signal transduction in which the desensitised receptor functions as a critical structural component of a mitogenic signaling complex (Luttrell et al., 1999).

Of interest to this study are the sites of GRK phosphorylation and their role in β_2-AR internalisation. Fredericks et al. (1996), demonstrated *in vitro* phosphorylation of the β_2-AR by GRKs following reconstitution of recombinant β_2-AR into liposomes. Serines 396, 401 and 407, and threonine 384 in the C terminus of the β_2-AR were identified as the sites of GRK2 (βARK 1) phosphorylation whereas GRK5 also phosphorylated these residues in addition to threonine 398 and serine 411. It was hypothesised that these were also the sites of GRK phosphorylation in intact cells since the phosphorylation sites in the rhodopsin receptor identified *in vitro* were the same sites phosphorylated by rhodopsin kinase (RK or GRK1) *in vivo* (Palczewski, et al., 1995; Ohguro et al., 1995; Papac et al., 1993; McDowell et al., 1993). These findings also agreed with a previous study on the β_2-AR (Hausdorff et al., 1989). However, considerable debate has arisen surrounding the importance of these residues in receptor desensitisation and internalisation. Seibold et al., (1998) investigated desensitisation and internalisation of a range of β_2-AR constructs with mutations at the proposed GRK phosphorylation sites and PKA phosphorylation sites. Three mutant constructs of the β_2-AR were studied, PKA$^-$ with mutations at the 2 PKA consensus

sites (S261A, S262A, S345A, S346A), GRK2$^-$ constructed from the PKA$^-$ construct with additional mutations (T384A, S396A, S401A, S407A) and GRK5$^-$ constructed from GRK2$^-$ with additional mutations (T393A, S411A). All three mutants appeared to be rapidly phosphorylated and underwent a similar degree of agonist-induced desensitisation and internalisation compared to the wild-type β_2-AR. It was, therefore, proposed that the GRK site(s) that mediate the desensitisation and subsequent internalisation of the β_2-AR do not involve the sites identified by *in vitro* phosphorylation and that unidentified sites of phosphorylation are yet to be found. Indeed, Jockers et al., (1996) have implicated serines 137 and 143 in the intracellular loop 2 of the β_2-AR as potential phosphorylation sites of a different kinase such as cdc2 kinase. Serine 137 is contained within a potential cdc2 kinase phosphorylation consensus site S/TPXK/R. It was found that when the second intracellular loop of the β_2-AR was substituted into the β_3-AR, this markedly increased the ability of the receptor to undergo agonist-induced internalisation. Therefore, there may be a link with these potential phosphorylation sites and internalisation of the β_2-AR.

Available for study was a mutant β_2-AR in which all the 11 potential C-terminal GRK phosphorylation sites had been mutated to alanine or glycine (BARK$^-$-β_2-AR). This receptor construct was C-terminally tagged with GFP (BARK$^-$-β_2-AR-GFP) and stably expressed in HEK293 cells, the same cell expression system used by Seibold et al., (1998). The trafficking of this receptor in response to agonist stimulation was monitored to try and shed some light on the discrepancies reported above. Firstly, the construct was pharmacologically characterised for its ability to bind the β-AR agonist isoprenaline, the inverse agonist betaxolol and the antagonist alprenolol to determine any effect of the mutations on ligand binding. The expression level of BARK$^-$-β_2-AR-GFP was determined along with the K_d of [^{3}H] DHA for the receptor.

The internalisation profile of BARK$^-$-β_2-AR-GFP was compared to that of the WT-β_2-AR-GFP construct as measured by confocal microscopy and [^{3}H] CGP12177

binding studies on intact cells. Due to disagreement on the importance of PKA in β_2-AR desensitisation (Benovic et al., 1985 versus Seibold et al., 1998), the role of PKA in receptor internalisation was investigated by the use of two PKA activators, dibutyryl cAMP and 8-bromo cAMP and two PKA inhibitors H89 and Rp cAMP. The role of MEK1 in the regulation of β_2-AR endocytosis was also studied. Intact cell adenylyl cyclase assays were also performed to determine if this construct could generate a second messenger output and if so, to measure the EC_{50} for isoprenaline at this receptor construct.

5.2 Construction and expression of a green fluorescent protein-tagged form of the mutant BARK$^-$-β_2-AR in HEK293 cells.

A cDNA of the human β_2-AR containing glycine amino acid substitutions at all of its 11 potential GRK C-terminal ser/thr phosphorylation sites (BARK$^-$-β_2-AR in Figure 5.1), was modified such that an 8 amino acid (DYKDDDDK) Flag ™ epitope tag was added to the N terminus of the encoded protein (Flag-BARK$^-$-β_2-AR). This construct was further modified by a PCR-based strategy to link a cDNA encoding a modified form of the GFP from *Aequorea victoria* with enhanced autofluorescent properties (Zernicka-Goetz et al., 1997) to its C terminus (Figure 5.2a). This fusion protein (BARK$^-$-β_2-AR-GFP) was anticipated to encode a single open reading frame in which the C terminus of the GPCR was linked directly to the N terminus of GFP (Figure 5.2a).

Figure 5.1

Structural model of the WT-β_2-AR.

The seven transmembrane β_2-AR containing the 11 putative GRK (βARK) phosphorylation sites (black), the two identified PKA phosphorylation sites (grey) and the one identified palmitoylation site (light grey). The labeled GRK sites are those identified *in vitro* by Fredericks et al., (1996) to be phosphorylated by GRK2 and GRK5. All the potential GRK Ser/Thr phosphorylation sites in the C-terminal tail of the β_2-AR were mutated to glycines or alanines to produce a BARK$^-$-β_2-AR cDNA construct.

Figure 5.1

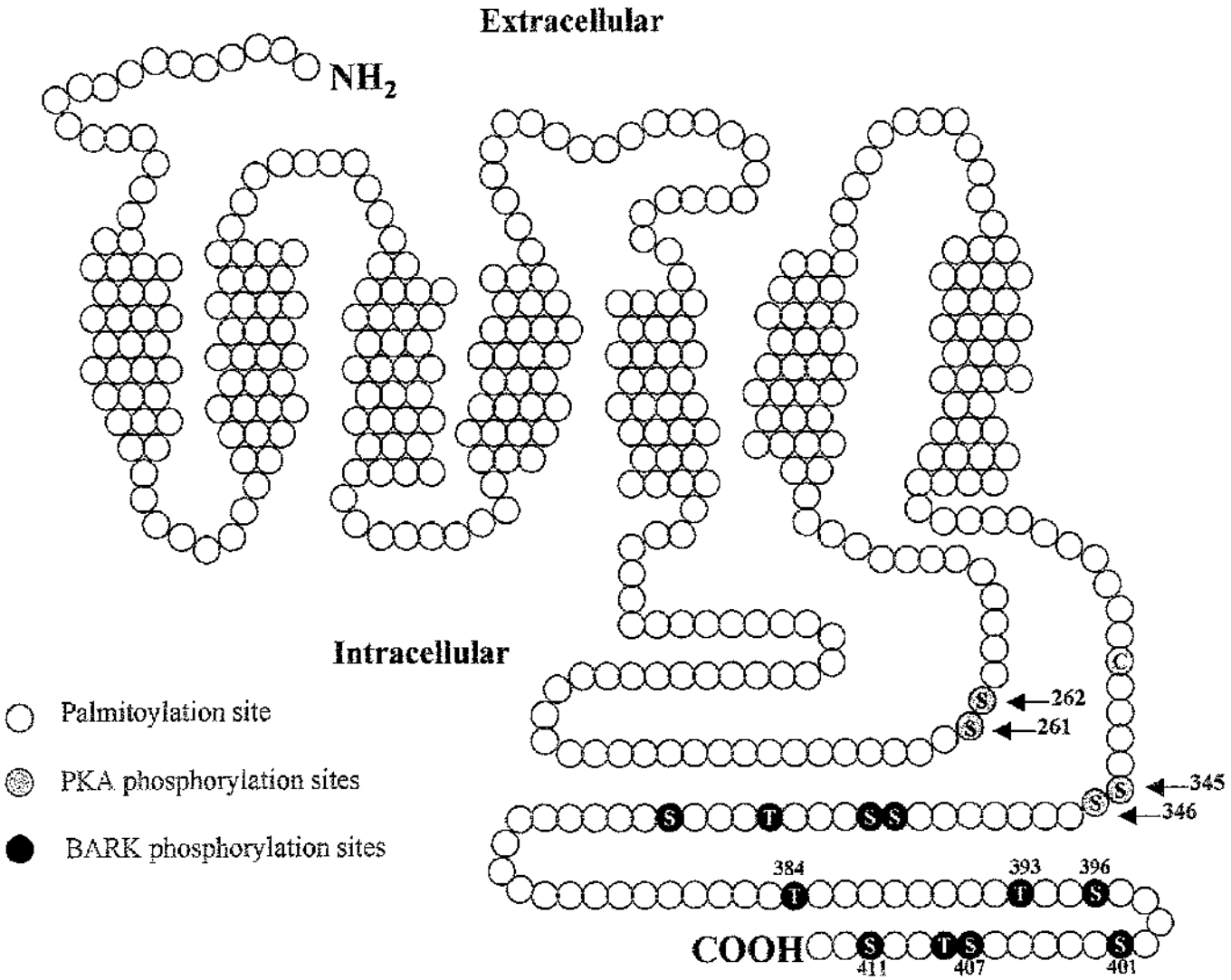

Extracellular
NH2
Intracellular
Palmitoylation site
PKA phosphorylation sites
BARK phosphorylation sites
C
S
262
S
261
S
S
345
346
S
T
S
S
384
T
393
T
396
S
COOH
S
411
T
S
407
S
401

Figure 5.2

a) Schematic diagram of the cDNA encoding the BARK^{-}-β_2-AR-GFP fusion construct generated for this study.

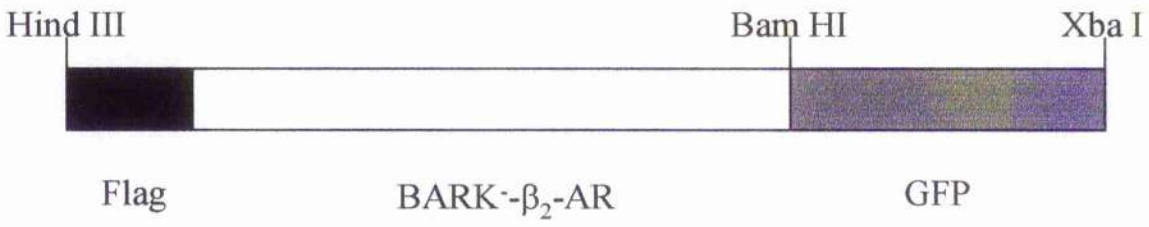
BARK^{-}-β_2-AR-GFP was generated using a PCR based approach to link GFP to the C terminus of a BARK^{-}-β_2-AR construct (Chapter 2, Section2.4d).

b) Confocal analysis of the cellular location of BARK^{-}-β_2-AR-GFP transiently transfected in HEK293 cells.

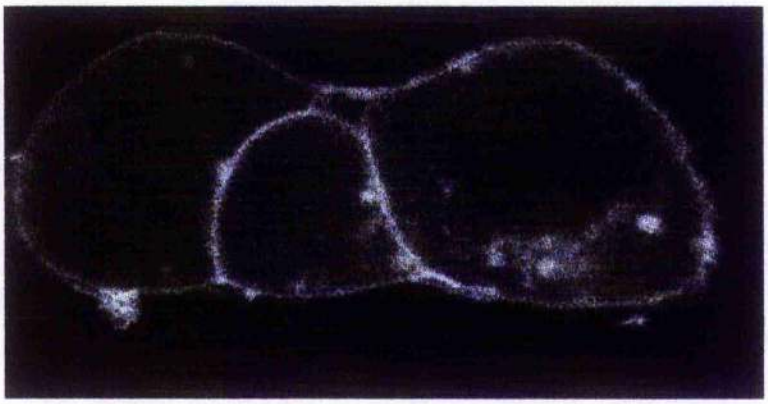
Following transient transfection of HEK293 cells on a glass coverslip with the BARK^{-}-β_2-AR-GFP cDNA, cells were fixed 48 h later, mounted on a microscope slide as described in Section 2.8 and then imaged by confocal microscopy.

Figure 5.2

a)



b)



One concern was that such a highly mutated form of the β_2-AR with GFP added onto its C terminus would have such structural alterations within the protein that it might not be targeted correctly to the plasma membrane. However, this was not the case. Transient transfection of the BARK$^-$-β_2-AR-GFP construct into HEK293 cells grown on a glass coverslip was followed by imaging in a confocal microscope. The cellular location of the construct was very similar to that of WT-β_2-AR-GFP (Figure 3.2b) and WT-β_1-AR-GFP (Figure 4.2b), its fluorescence being distributed mainly at the plasma membrane of the cell (Figure 5.2b). From binding experiments using a single concentration of [^{3}H] DHA on membranes from HEK293 cells transiently expressing empty vector pcDNA3, WT-β_2-AR, WT-β_2-AR-GFP or BARK$^-$-β_2-AR-GFP (Figure 5.3), it was apparent that the mutant receptor was routinely expressed at substantially lower levels than either of the non-mutated β_2-AR constructs. However, BARK$^-$-β_2-AR-GFP was expressed at levels to provide a significantly greater number of [^{3}H] DHA binding sites than the control experiment using empty vector pcDNA3. Stable cell lines of this mutant construct in HEK293 cells were then developed for comparison with the WT-β_2-AR-GFP stable cell clone already studied.

Once stable cell lines expressing BARK$^-$-β_2-AR-GFP were established in HEK293 cells single clones were selected for study. A fluorescent microscope was utilised to directly screen for positive clones. Only 2 out of 50 clones screened were positive indicating the difficulty in expressing this mutant construct. Clones #46 and #47 are shown in Figure 5.4a demonstrating that in these selected clones, the BARK$^-$-β_2-AR-GFP construct is targeted to the plasma membrane as a substantial amount of the GFP-derived autofluorescence is plasma membrane delineated. Clone #47 membranes were also subjected to western blot analysis using an anti-β_2-AR antibody directed to an amino acid sequence mapping to the C-terminal tail of the human β_2-AR. The blot in Figure 5.4b indicates that BARK$^-$-β_2-AR-GFP was detected by this antibody as a range of bands between 65 and 80 kDa even though it has a substantially altered C terminus. As with the β_2-AR constructs analysed in Chapter 3 and in Figure

Figure 5.3

Levels of BARK$^-$-β_2-AR-GFP compared to WT-β_2-AR and WT-β_2-AR-GFP following transient expression in HEK293 cells.

Membranes (20 μg) prepared from HEK293 cells transiently expressing either the WT-β_2-AR, WT-β_2-AR-GFP or BARK$^-$-β_2-AR-GFP constructs were analysed for their ability to bind a single concentration of [^{3}H] DHA (2 nM). Results are from one experiment performed in triplicate and are presented as means ± S.D. Similar results were obtained from two further experiments.

Figure 5.3

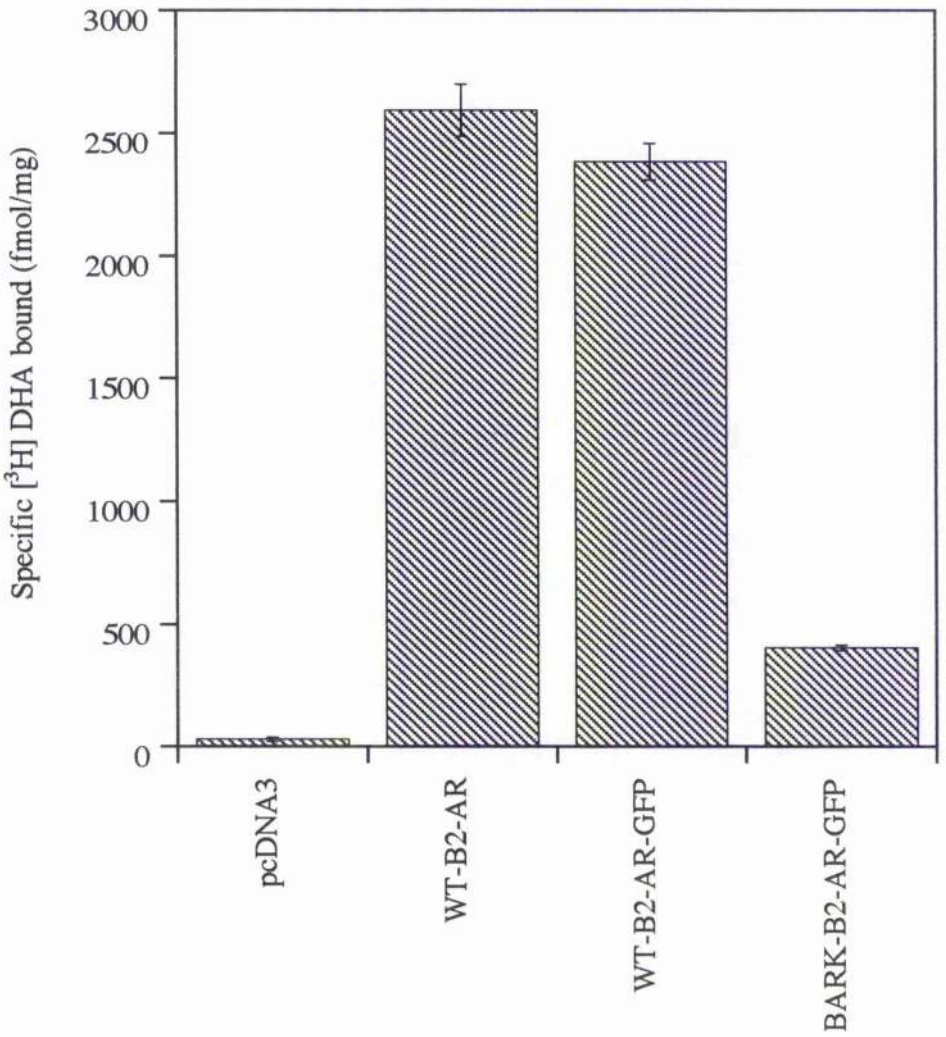

Specific [3H] DHA bound (fmol/mg)
3000
2500
2000
1500
1000
500
0
pcDNA3
WT-B2-AR
WT-B2-AR-GFP
BARK-B2-AR-GFP

Figure 5.4

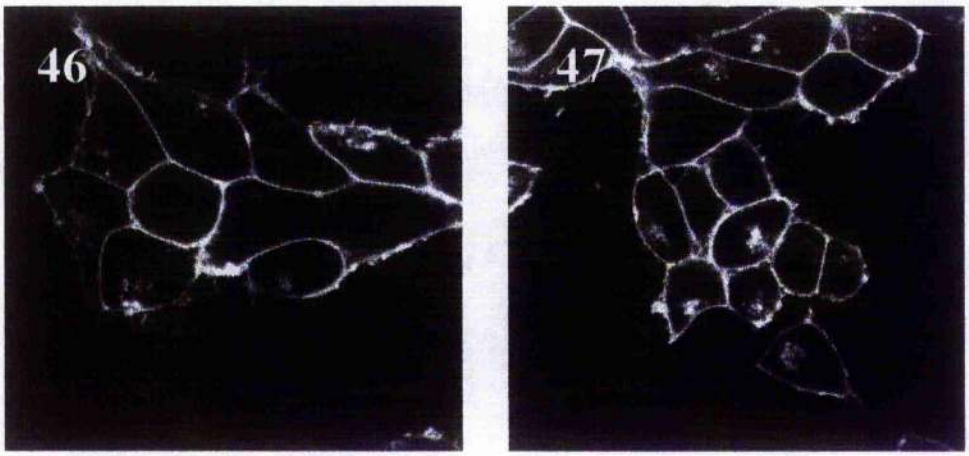
Cell clones stably expressing BARK^{-}-β_2-AR-GFP.

a) The BARK^{-}-β_2-AR-GFP construct was expressed stably in HEK293 cells and individual clones identified by live cell confocal microscopy. The two positive clones (#46 and #47) are imaged herein, with largely plasma membrane delineated autofluorescence.

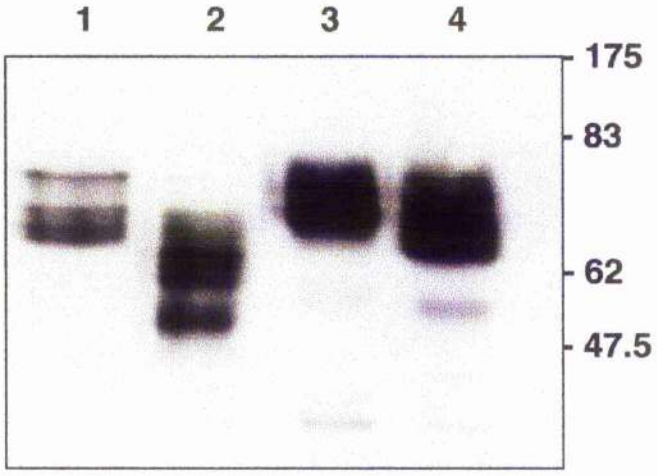
b) 15 μg of membrane preparations were loaded onto an SDS-PAGE protein gel. After transfer of the protein to a nitrocellulose membrane, the membrane was probed with an anti-β_2-AR antibody to show that all the β_2-AR constructs used in this study could be detected by this antibody, 1) CAM-β_2-AR-GFP, 2) WT-β_2-AR, 3) WT-β_2-AR-GFP and 4) BARK^{-}-β_2-AR-GFP.

Figure 5.4

a)



b)



5.4b, CAM-β_2-AR-GFP (1), WT-β_2-AR (2) and WT-β_2-AR-GFP (3), the range of bands for BARK$^-$-β_2-AR-GFP (4) probably correspond to differential glycosylation states of the protein, although protein degradation cannot be excluded. Figure 5.5 gives an approximate indication of expression levels of clone #47 compared to WT-β_2-AR-GFP clone #13 and WT-β_2-AR clone #27 using a single concentration of [^{3}H] DHA in membrane binding studies. It was now necessary to perform more detailed analysis to pharmacologically characterise this BARK$^-$-β_2-AR-GFP construct in comparison to WT-β_2-AR-GFP.

5.3 Pharmacological characterisation

Expression levels of BARK$^-$-β_2-AR-GFP clone #47 were determined more accurately by a saturation binding experiment with [^{3}H] DHA (Figure 5.6a(i)). BARK$^-$-β_2-AR-GFP was stably expressed at comparable levels to both WT-β_2-AR and WT-β_2-AR-GFP (Table 5.1, B_{max} values). As for all the previous constructs examined throughout this study, the non-specific binding of [^{3}H] DHA at BARK$^-$-β_2-AR-GFP using 10^{-5} M propranolol to compete for β_2-AR binding sites was very low (Figure 5.6a(ii)). The saturation binding curve was converted into a Scatchard plot and the K_d value calculated from the slope of the line indicated that this construct bound the ligand [^{3}H] DHA with similar affinity to both WT-β_2-AR constructs (Table 5.1 and Figure 5.6b).

Competition for the specific binding of [^{3}H] DHA to membranes expressing BARK$^-$-β_2-AR-GFP with either isoprenaline (an agonist), alprenolol (an antagonist) or betaxolol (an inverse agonist) were performed to determine whether the mutations introduced in the C terminus of the β_2-AR construct or addition of GFP had induced any alterations in basic receptor pharmacology (Table 5.2 and Figures 5.7, 5.8, and 5.9). High affinity for the antagonist alprenolol at this particular β_2-AR construct was

Figure 5.5

Estimation of receptor level in BARK$^-$-β_2-AR-GFP clone #47 from membrane binding studies using a single concentration of [^{3}H] DHA.

20 μg of membrane preparation from each of the denoted cell lines was used to estimate approximate receptor levels. A close to saturating concentration of [^{3}H] DHA (2 nM) was used to determine total binding with 10^{-5} M propranolol as competing ligand to determine non-specific binding. Specific binding was used to estimate the receptor expression in each clone (fmol/mg). Data are presented as means ± S.E.M., $n = 3$.

Figure 5.5

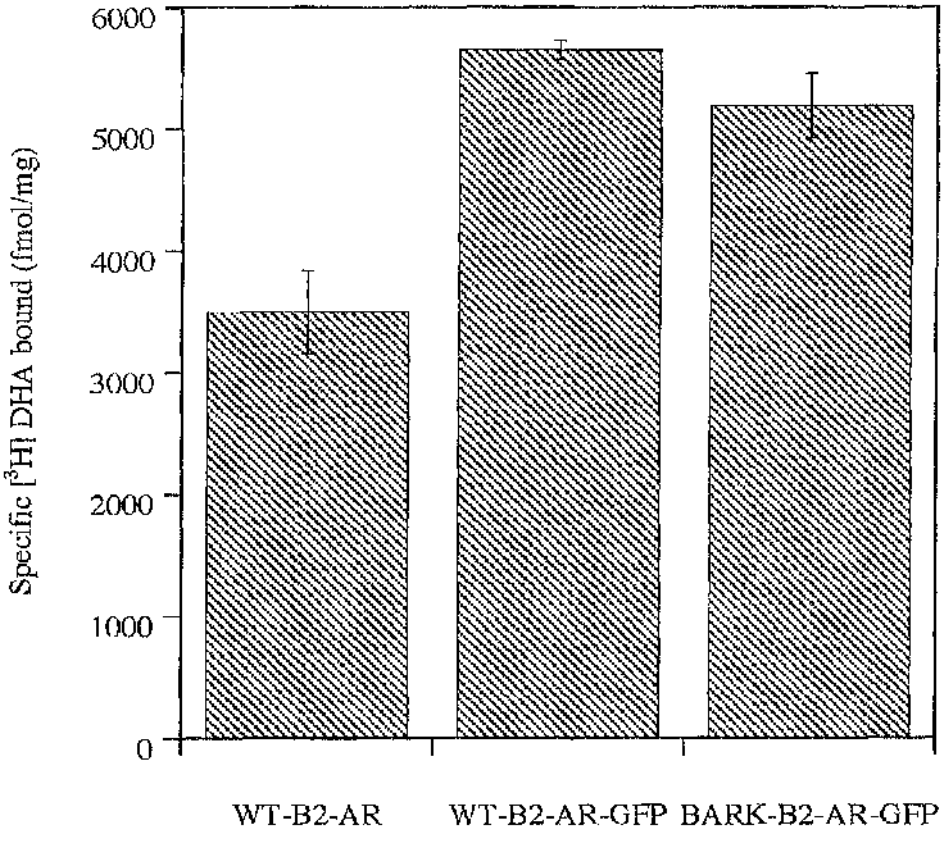

Specific [3H] DHA bound (fmol/mg)
6000
5000
4000
3000
2000
1000
0
WT-B2-AR
WT-B2-AR-GFP
BARK-B2-AR-GFP

Figure 5.6

Binding characteristics of BARK$^-$-β_2-AR-GFP-expressing cells.

a i) A saturation binding study using increasing concentrations of [^{3}H] DHA was performed to measure specific binding (pmol/mg) in membranes from BARK$^-$-β_2-AR-GFP cells.

a ii) The same saturation binding study as in (a i) but showing total, specific and non-specific binding (d.p.m.).

b) Transformation of the specific binding data to generate a Scatchard plot. In the example displayed the estimated B_{max} was 8.4 pmol/mg and the K_d for [^{3}H] DHA was 0.81 nM.

Figure 5.6a(i)

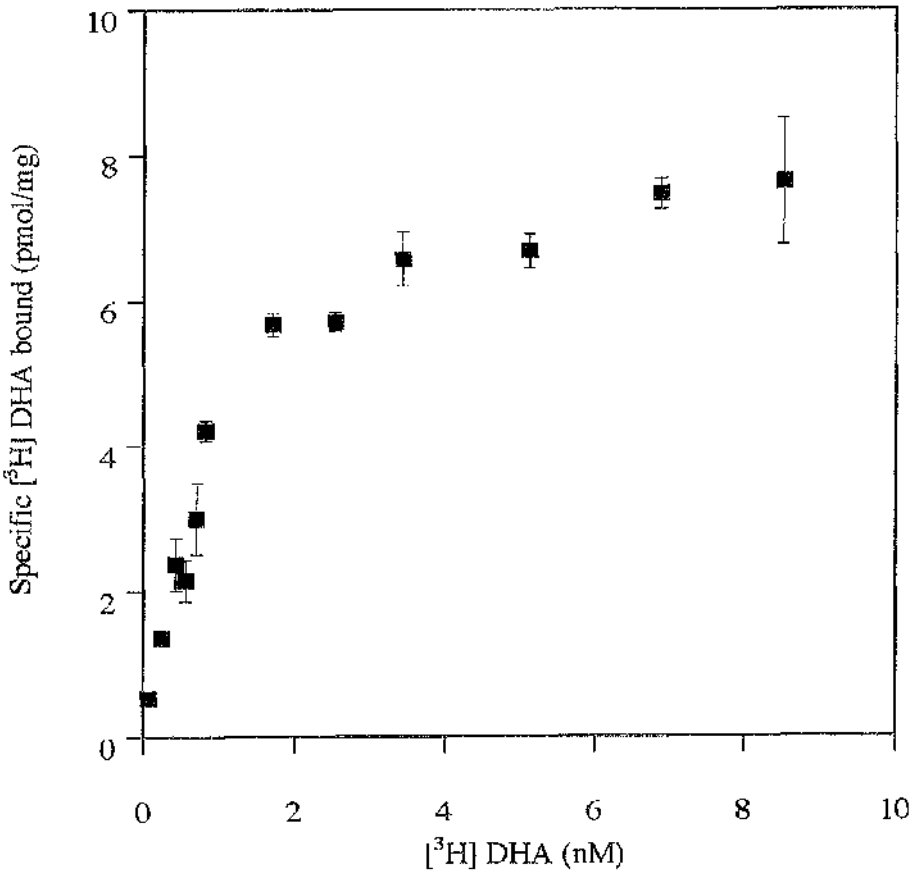


Figure 5.6a(ii)

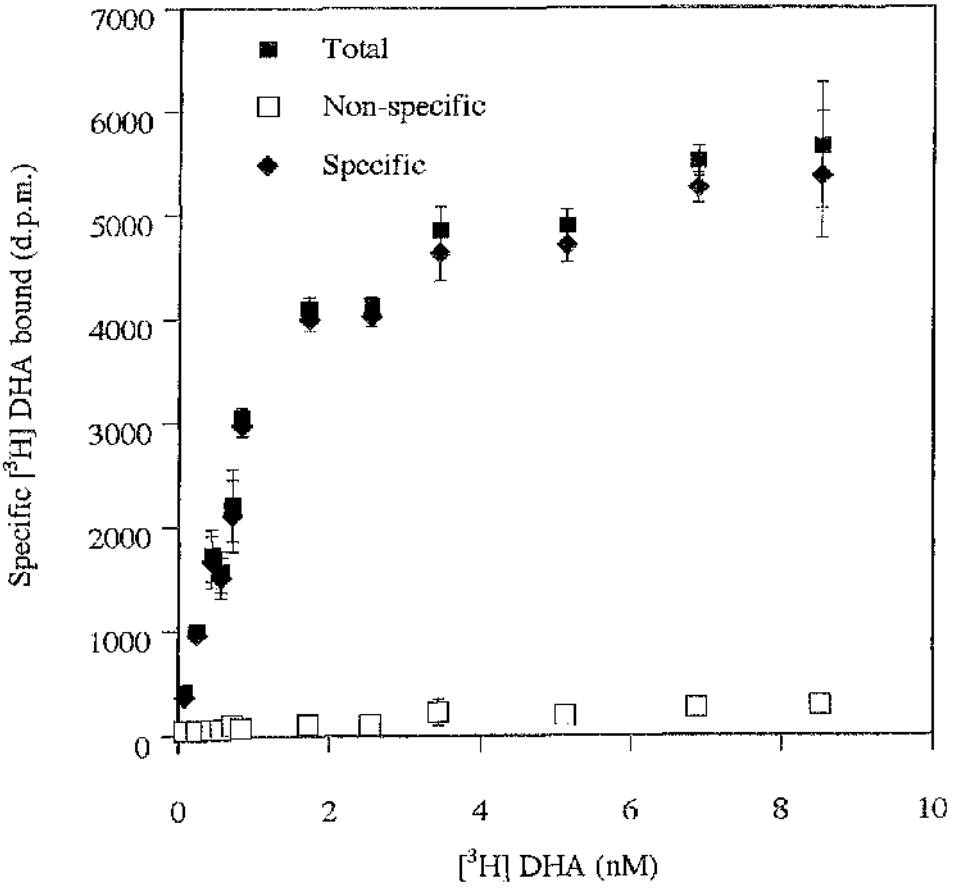


Figure 5.6b

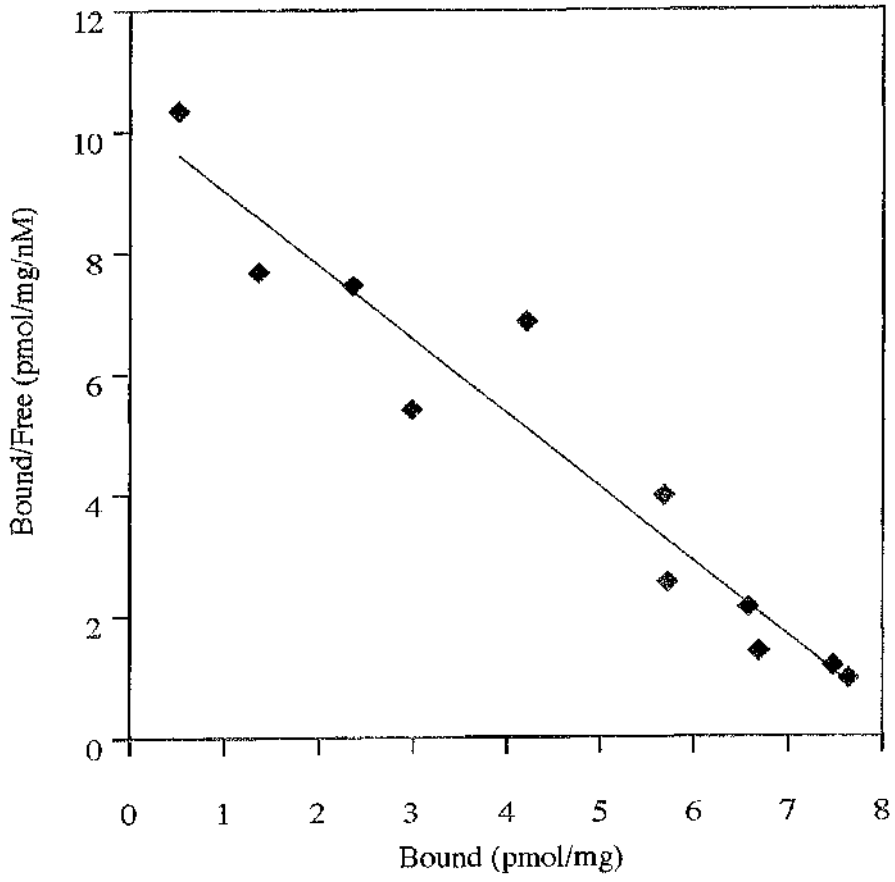


Table 5.1

Ligand binding charactheristics of GFP and non-GFP-tagged forms of WT-β_2-AR and of BARK$^-$-β_2-AR-GFP.

Clone	K_d (nM)	B_{max} (pmol/mg)
WT-β_2-AR	0.62 ± 0.26	4.1 ±0.9
WT-β_2-AR-GFP	0.75 ± 0.17	8.7 ± 0.7
BARK$^-$-β_2-AR-GFP	0.60 ± 0.25	6.7 ± 1.5

Data represent means ± .S.D. from three independent experiments using [^{3}H] DHA as radioligand.

Table 5.2

Competition binding experiments at GFP and non-GFP-tagged forms of WT-β_2-AR and at BARK$^-$-β_2-AR-GFP.

Clone	K_i for Isoprenline (nM)	K_i for Betaxolol (nM)	K_i for Alprenolol (nM)
WT-β_2-AR	363 ± 156 (2)	344 ± 34 (2)	1.1 ± 0.3 (2)
WT-β_2-AR-GFP	782 ± 277 (3)	478 ± 23 (2)	2.5 ± 1.0 (2)
BARK$^-$-β_2-AR-GFP	345 ± 22 (3)	345 ±64 (3)	1.1 ± 0.2 (3)

Data represent means ± S.D. from two or three independent experiments using [^{3}H] DHA as radioligand. The number of experiments are indicated in brackets.

maintained (Figure 5.7). The inverse agonist betaxolol displayed an affinity approximately 300 times lower than alprenolol at BARK$^-$-β_2-AR-GFP (Figure 5.8), which is consistent with the data obtained from competition binding studies on WT-β_2-AR-GFP (Table 5.2). A 2.3 fold higher affinity of isoprenaline at BARK$^-$-β_2-AR-GFP versus WT-β_2-AR-GFP was measured (Figure 5.9 and Table 5.2).

5.4 Internalisation studies

As BARK$^-$-β_2-AR-GFP is a modified version of the β_2-AR containing none of the known GRK/βARK phosphorylation sites it was postulated that this construct might undergo none or very little internalisation. It should be noted that the construct still contains the two identified PKA phosphorylation sites, one in the 3rd intracellular loop and one in the C terminus. Isoprenaline-induced internalisation studies were performed on BARK$^-$-β_2-AR-GFP-expressing cells in parallel with the same experiment on WT-β_2-AR-GFP-expressing cells. Confocal microscopy promptly indicated that the mutant BARK$^-$-β_2-AR-GFP construct did indeed internalise when a 10^{-5} M concentration of isoprenaline was added to the cells (Figure 5.10). In fact, the profile of internalisation looked substantially more rapid than that of the WT-β_2-AR-GFP construct (Figure 3.6). A distinct punctate pattern of fluorescence was seen inside the cell after 5 minutes of isoprenaline stimulation (Figure 5.10b). This became more pronounced at later times up to 30 minutes (figure 5.10 c to e). In Chapter 3, Figure 3.6, WT-β_2-AR-GFP only started to produce a detectable punctate pattern of internalisation at 10 minutes of isoprenaline stimulation. To accurately determine the differences in internalisation patterns between the two β_2-AR constructs it was necessary to perform [^{3}H] CGP12177 binding studies on intact cells of both clones.

Similar internalisation experiments to those detailed in Chapter 4, Section 4.4 were performed on BARK$^-$-β_2-AR-GFP-expressing cells. For a time-course of 0 to 60

Figure 5.7

Mutations within BARK$^-$-β_2-AR and addition of GFP to its C terminus does not alter the high affinity of the β_2-AR antagonist alprenolol.

Competition between [^{3}H] DHA (0.8 nM) and varying concentrations of alprenolol for specific binding to membranes expressing BARK$^-$-β_2-AR-GFP, Hill coefficient = 0.8. Similar results were obtained from two further experiments (Table 5.2).

Figure 5.7

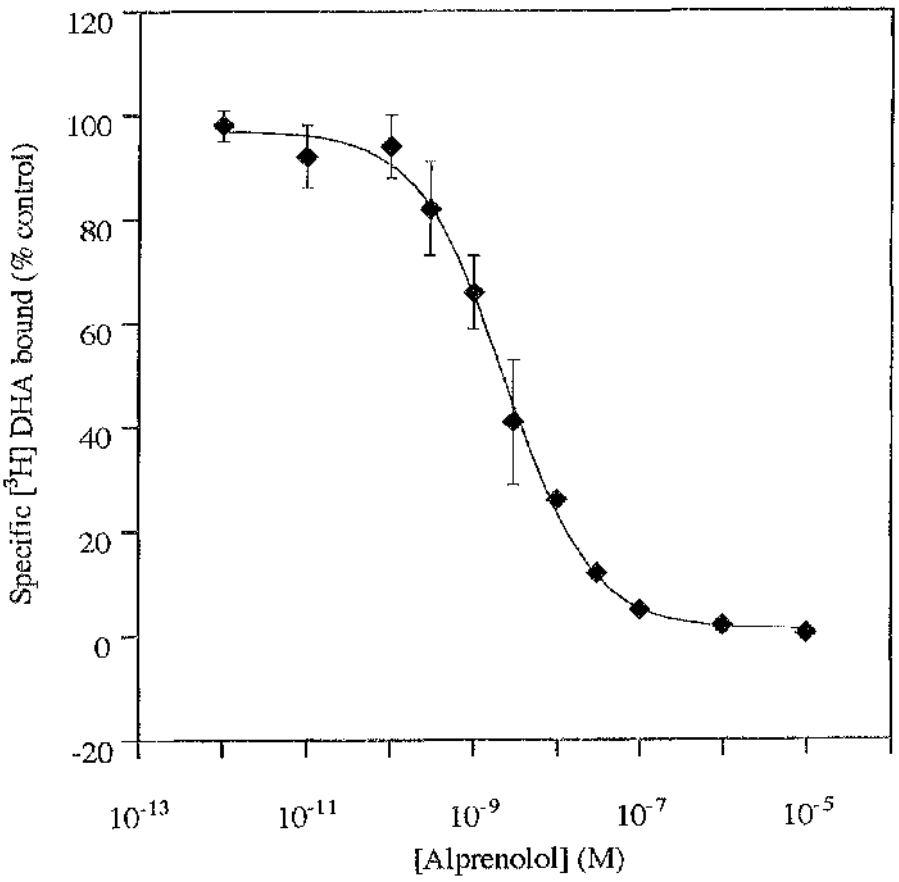

120
100
80
60
40
20
0
-20
Specific [3H] DHA bound (% control)
10-13
10-11
10-9
10-7
10-5
[Alprenolol] (M)

Figure 5.8

Betaxolol has a similar affinity at the BARK$^-$-β_2-AR-GFP construct as at the WT-β_2-AR-GFP construct.

Competition between [^{3}H] DHA (0.8 nM) and varying concentrations of betaxolol for specific binding to membranes expressing either BARK$^-$-β_2-AR-GFP, Hill coefficient = 0.9 or WT-β_2-AR-GFP, Hill coefficient = 0.8. Similar results were obtained from two further experiments (Table 5.2).

Figure 5.8

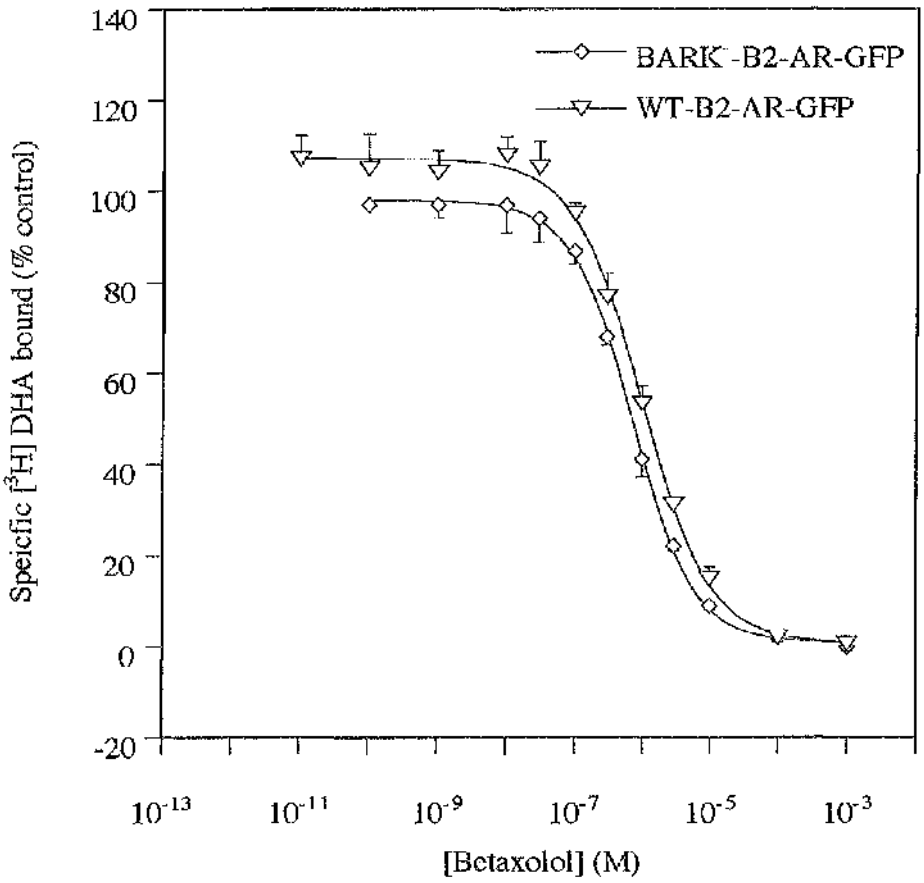

140
120
100
80
60
40
20
0
-20
Speicfic [3H] DHA bound (% control)
BARK -B2-AR-GFP
WT-B2-AR-GFP
10-13
10-11
10-9
10-7
10-5
10-3
[Betaxolol] (M)

Figure 5.9

Affinity for isoprenaline at the mutant BARK$^-$-β_2-AR-GFP construct.

Competition between [^{3}H] DHA (0.8 nM) and varying concentrations of isoprenaline for specific binding to membranes expressing BARK$^-$-β_2-AR-GFP, Hill coefficient = 0.7. Similar results were obtained from two further experiments (Table 5.2).

Figure 5.9

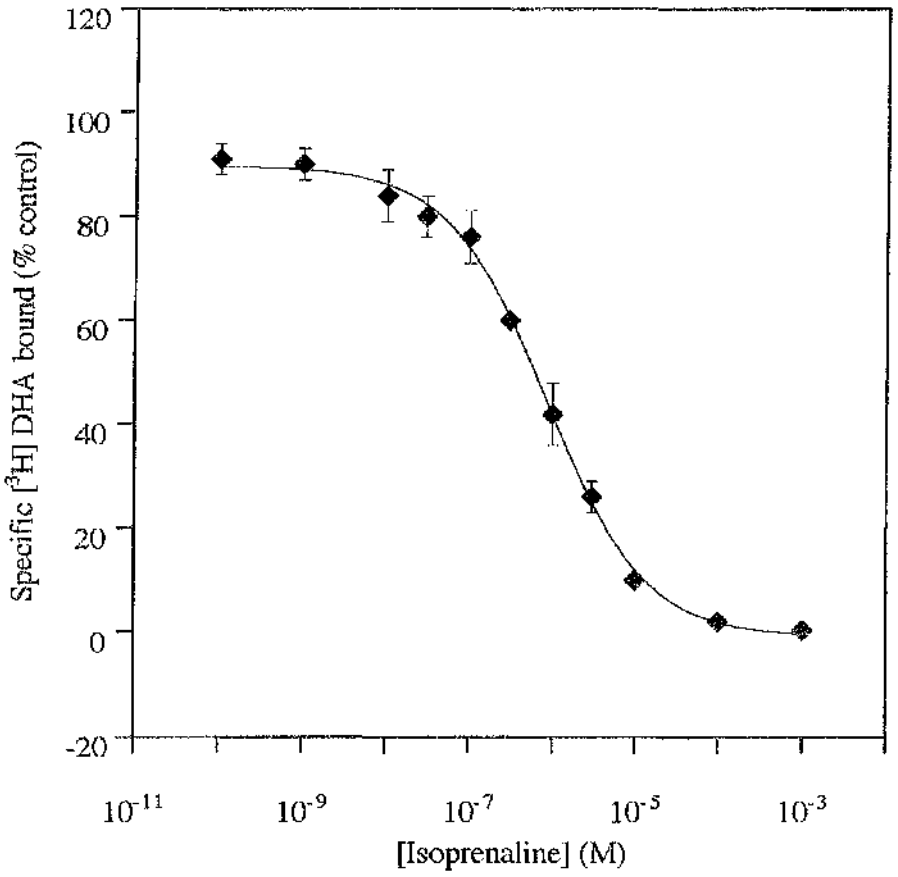
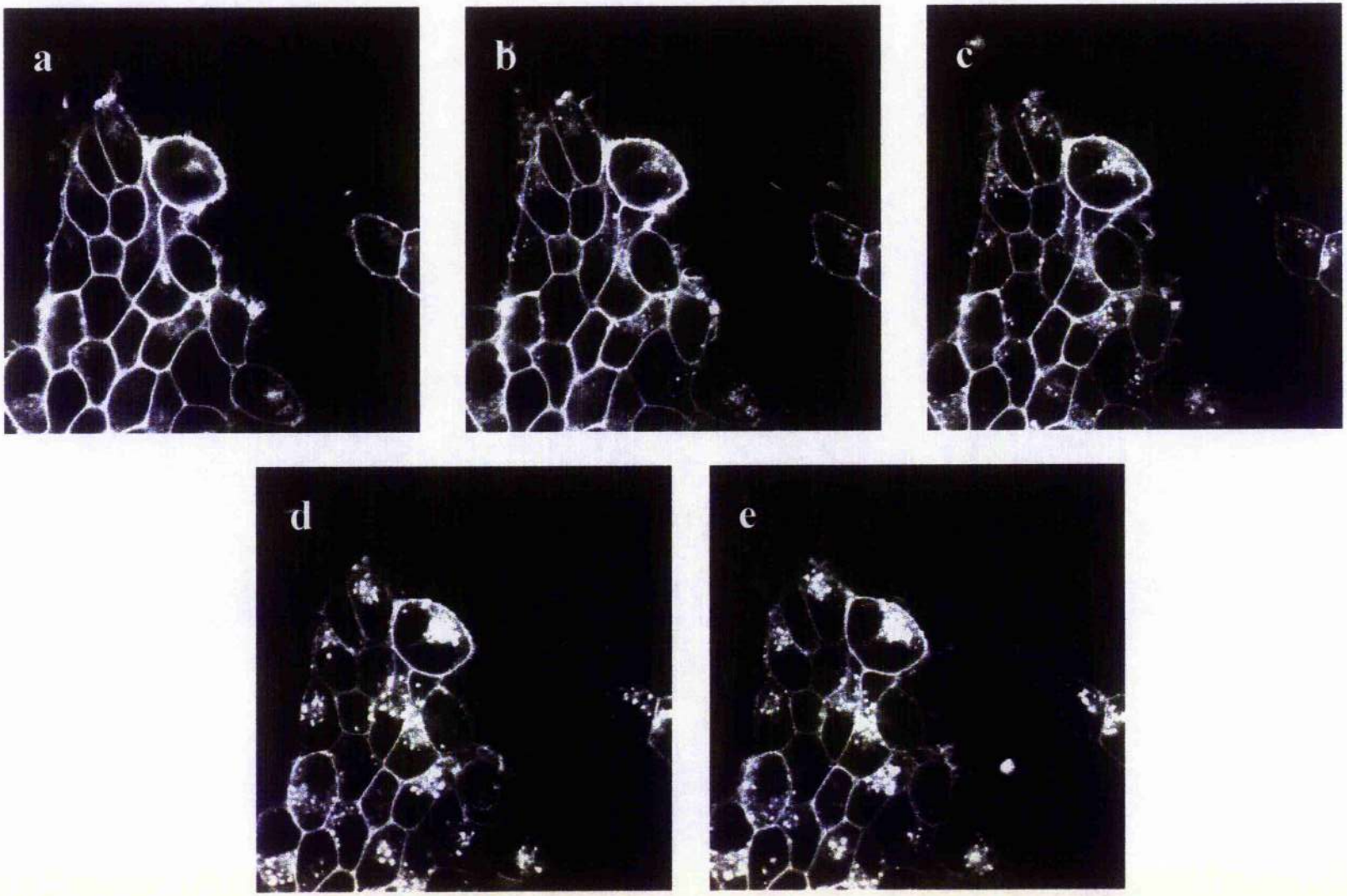

120
100
80
60
40
20
0
-20
Specific [3H] DHA bound (% control)
10-11
10-9
10-7
10-5
10-3
[Isoprenaline] (M)

Figure 5.10

Internalisation of BARK^{-}-β_2-AR-GFP by agonist stimulation.

A patch of BARK^{-}-β_2-AR-GFP clone #47 cells were imaged in the confocal microscope in the absence of agonist (a) and following addition of 10^{-5} M isoprenaline for 5 (b), 10 (c), 20 (d) and 30 (e) minutes. This is representative of two separate experiments.

Figure 5.10



minutes cells were treated with 10^{-5} M isoprenaline at 37°C, followed by a binding study with either [^{3}H] CGP12177 to measure plasma membrane receptors, or [^{3}H] DHA to measure total receptor (plasma membrane and internal receptor). Confirming the confocal data, BARK$^-$-β_2-AR-GFP underwent marked internalisation which was more rapid than WT-β_2-AR-GFP (Figure 5.11a). Conversion of the internalisation data to natural log plots indicated that isoprenaline-stimulated BARK$^-$-β_2-AR-GFP internalised with a $t_{1/2}$ = 10 minutes (Figure 5.11b). WT-β_2-AR-GFP had a substantially higher $t_{1/2}$ = 25 minutes (Table 4.4, Chapter 4). It is well known that the β_2-AR internalises through a clathrin-mediated pathway in HEK293 cells. To determine if BARK$^-$-β_2-AR-GFP also internalised through this pathway, 0.4 M sucrose was used to inhibit clathrin cage formation around internalisation vesicles and therefore, to impede receptor internalisation. As predicted, sucrose inhibited isoprenaline-stimulated internalisation of the WT-β_2-AR and WT-β_2-AR-GFP constructs (Figure 5.12). This was also apparent for BARK$^-$-β_2-AR-GFP indicating that this construct internalises through a clathrin-mediated pathway (Figure 5.12).

It has recently been reported that the function of β-arrestin 1 to facilitate clathrin-mediated endocytosis of the β_2-AR and to promote agonist-induced activation of extracellular signal-related kinases (ERK) is regulated by its phosphorylation/ dephosphorylation at Ser412 (Lin et al., 1999). ERK is regulated by a protein called MEK1 to negatively regulate receptor internalisation. This was demonstrated by using a dominant-negative K97A mutant of MEK1 which subsequently caused an increase in isoprenaline-stimulated β_2-AR internalisation in HEK293 cells. To further investigate this the present study used a MEK inhibitor, PD98059, to attempt to block the effect of MEK1 on ERK and therefore, induce further isoprenaline-induced internalisation of WT-β_2-AR, WT-β_2-AR-GFP and BARK$^-$-β_2-AR-GFP (Figure 5.13). However, no effect of the MEK1 inhibitor was seen in the denoted stable cell lines studied.

Figure 5.11

Isoprenaline-stimulated internalisation of BARK$^-$-β_2-AR-GFP versus WT-β_2-AR-GFP.

a) WT-β_2-AR-GFP, and BARK$^-$-β_2-AR-GFP-expressing cells were untreated or treated in 24 well plates at 37°C with 10^{-5} M isoprenaline for various time intervals. [^{3}H] CGP12177 binding was then performed for 90 minutes at 4°C to measure the rate of agonist-stimulated internalisation of the two constructs. Results are represented as means ± S.E.M., n =4 for WT-β_2-AR-GFP (squares) and n = 4 for BARK$^-$-β_2-AR-GFP (diamonds).

b) The internalisation data from the linear part of the curve for BARK$^-$-β_2-AR-GFP was converted to a natural log plot. The equation of the line fit was used to calculate the time at which 50 % of cell surface receptors were internalised. The calculated $t_{1/2}$= 10 minutes, n = 4.

Figure 5.11

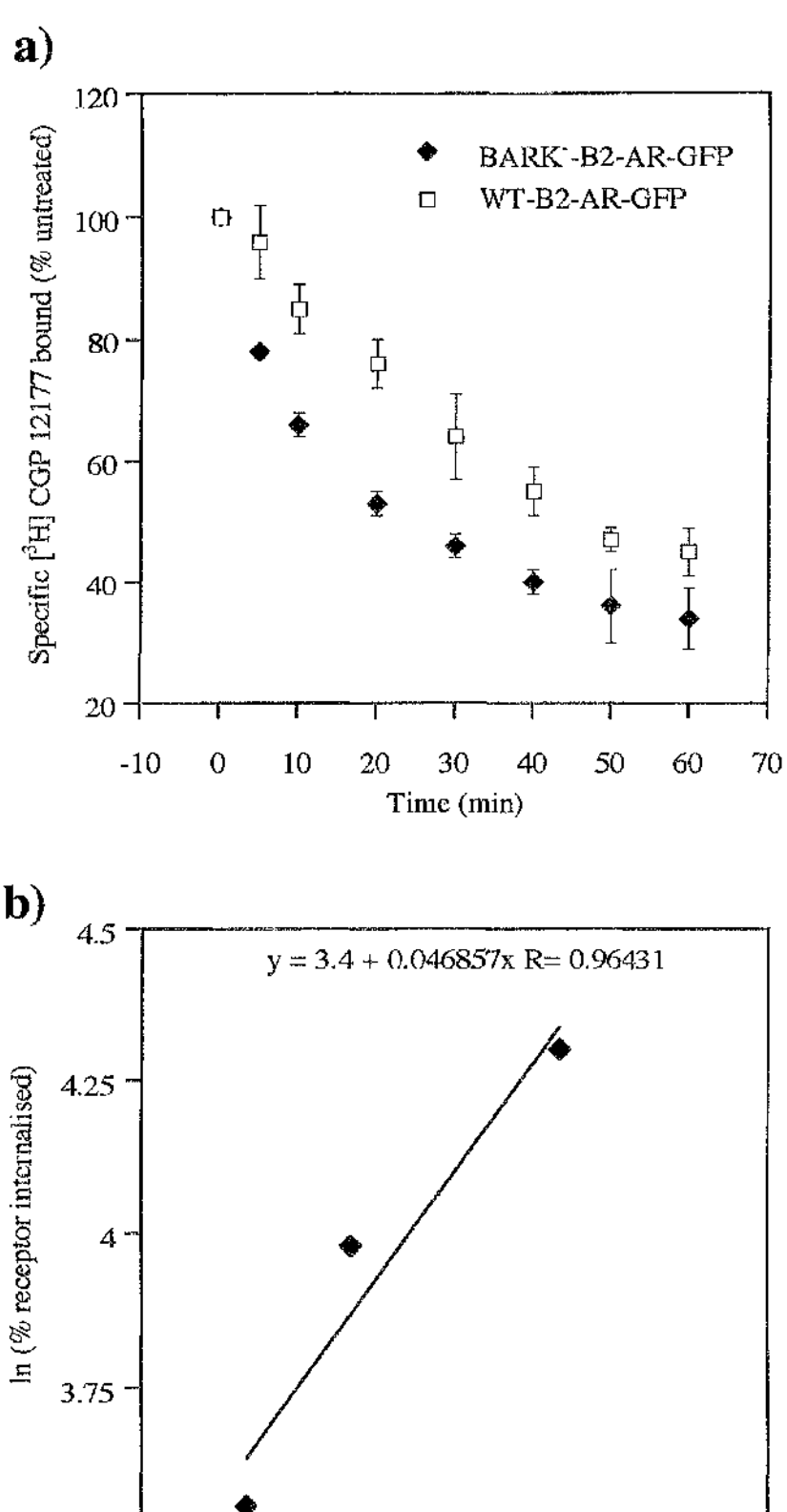

a)
120
100
80
60
40
20
Specific [3H] CGP 12177 bound (% untreated)
BARK-B2-AR-GFP
WT-B2-AR-GFP
-10
0
10
20
30
40
50
60
70
Time (min)
b)
4.5
4.25
4
3.75
3.5
ln (% receptor internalised)
y = 3.4 + 0.046857x R= 0.96431
0
5
10
15
20
25
30
Time (min)

Figure 5.12

0.4 M sucrose inhibits isoprenaline-induced internalisation of the denoted constructs.

The denoted cell clones were seeded into 24 well plates and were untreated or treated with 10^{-5} M isoprenaline, 0.4 M sucrose or 10^{-5} M isoprenaline + 0.4 M sucrose for 30 minutes at 37°C. After washing, the cells were incubated with 10 nM [^{3}H] CGP12177 for 90 minutes at 4°C to measure the effect of each treatment on the level of cell surface receptor. Results are from one experiment performed in triplicate ± S.D.

Figure 5.12

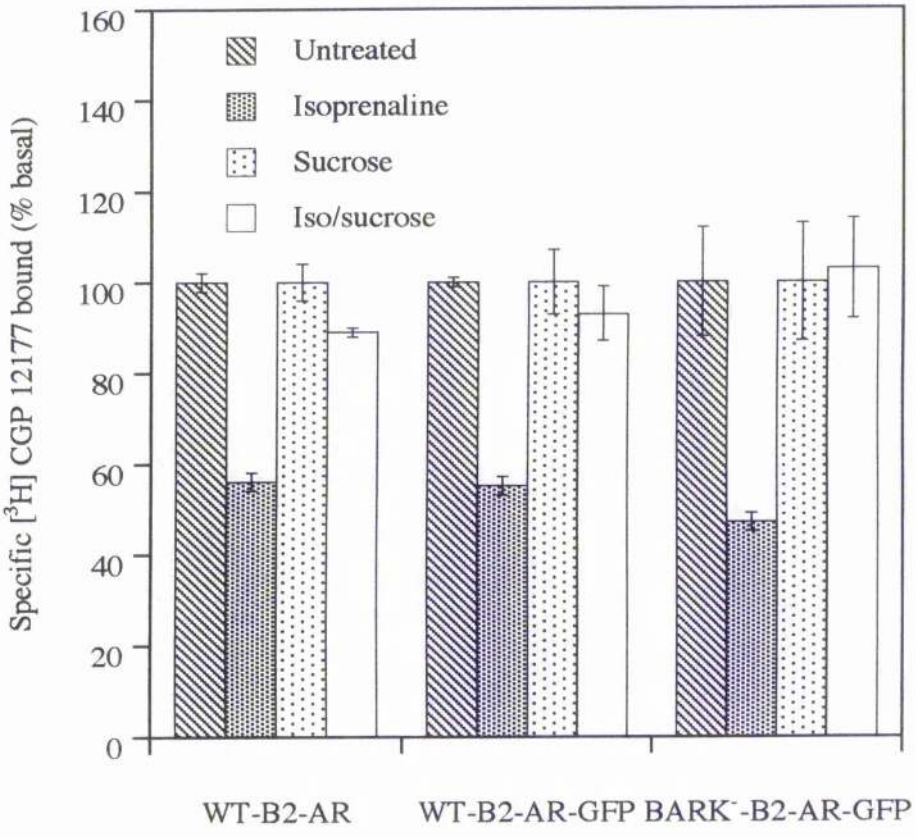
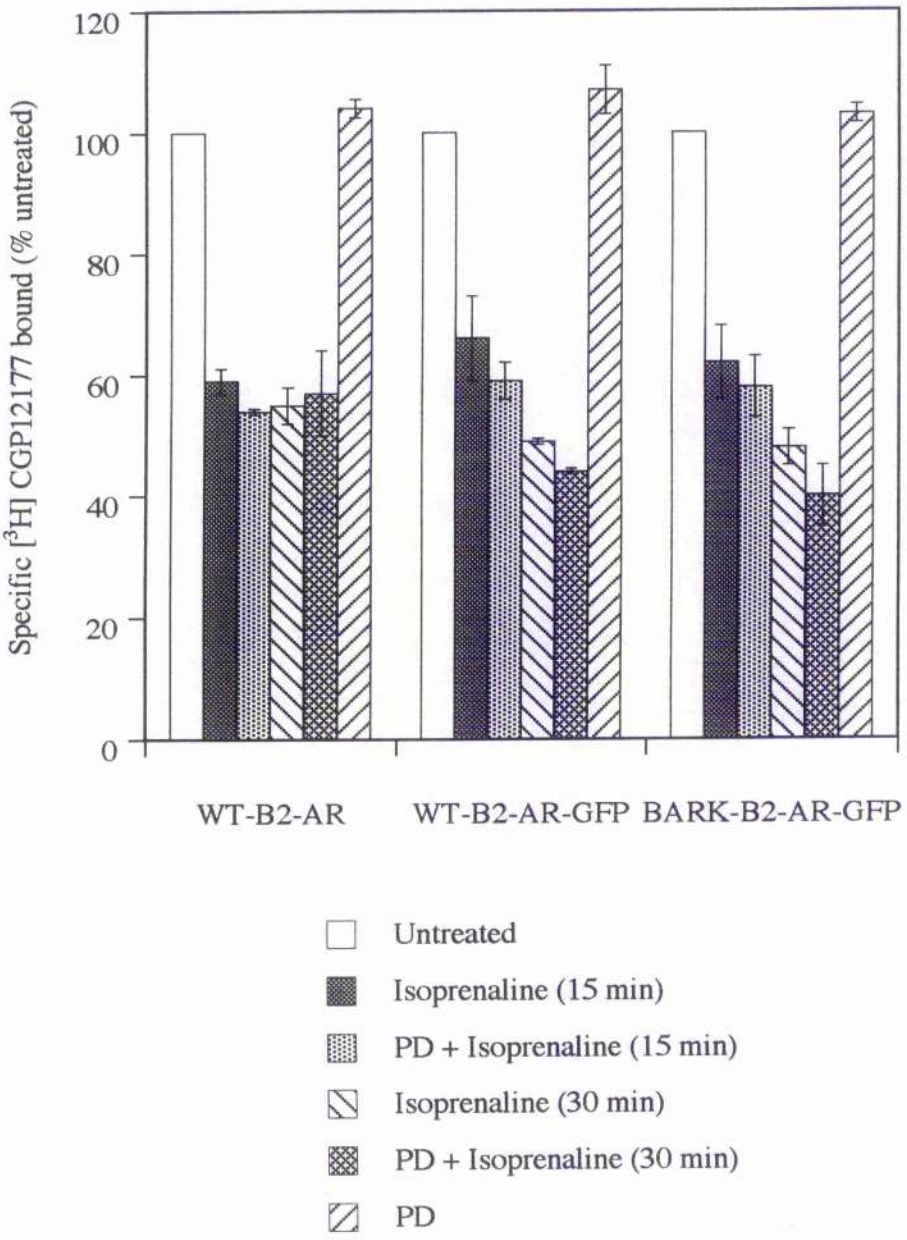

Specific [3H] CGP 12177 bound (% basal)
160
140
120
100
80
60
40
20
0
Untreated
Isoprenaline
Sucrose
Iso/sucrose
WT-B2-AR
WT-B2-AR-GFP
BARK-B2-AR-GFP

Figure 5.13

MEK inhibitor PD98059 has no effect on isoprenaline-induced internalisation of the β_2-AR.

The denoted cell clones were seeded into 24 well plates and were pre-incubated with or without PD98059 (50 μM) for 10 minutes. The cells were then incubated with 10^{-5} M isoprenaline for 15 or 30 minutes at 37°C. After washing, the cells were incubated with 10 nM [^{3}H] CGP12177 for 90 minutes at 4°C to measure the level of cell surface receptor following the various treatments. Results are means ± range., $n = 2$.

Figure 5.13


120
100
80
60
40
20
0
Specific [3H] CGP12177 bound (% untreated)
WT-B2-AR
WT-B2-AR-GFP
BARK-B2-AR-GFP
Untreated
Isoprenaline (15 min)
PD + Isoprenaline (15 min)
Isoprenaline (30 min)
PD + Isoprenaline (30 min)
PD

5.5 How significant a role does PKA play in internalisation of the β_2-AR ?

The BARK$^-$-β_2-AR-GFP construct does not contain the 11 potential GRK phosphorylation sites but does contain the two identified PKA phosphorylation sites. It may be reasonable to predict that these sites of phosphorylation could be responsible for the agonist-induced internalisation exhibited by BARK$^-$-β_2-AR-GFP, and that a loss of regulation may have occurred by removing the GRK phosphorylation sites to cause an increase in the rate of agonist-induced internalisation. Dibutyryl cAMP and 8-bromo-cAMP are cAMP analogues which can directly bind to and activate PKA. These reagents were used on WT-β_2-AR, WT-β_2-AR-GFP and BARK$^-$-β_2-AR-GFP expressing cells to attempt to induce agonist-independent receptor internalisation through phosphorylation by directly activated PKA (Figure 5.14). After a 60 minute incubation with either of these PKA activators, no receptor internalisation was detected by [^{3}H] CGP12177 intact cell binding in either clone. Incubation with 10^{-5} M isoprenaline for 60 minutes induced 60 to 70 % receptor internalisation (Figure 5.14). However, this may indicate that only PKA activated through agonist bound receptor may be able to phosphorylate and internalise receptors. Therefore the problem was approached from a different angle using PKA inhibitors.

The PKA inhibitor H89 has been used routinely (Chijiwa et al., 1990). BARK$^-$-β_2-AR-GFP-expressing cells were untreated or pretreated with H89 (15 minutes) to allow the inhibitor to enter into the cells and bind to PKA. A time-course of isoprenaline-stimulated internalisation for 0 to 60 minutes was then performed. As H89 is dissolved in DMSO a control experiment using DMSO in the incubation buffer was performed showing that this agent had no effect on the ability of isoprenaline to induce internalisation of the receptor construct (Figure 5.15). H89 appeared to substantially impede agonist-induced receptor internalisation (Figure 5.15). Contrary to this result, when a second inhibitor called Rp cAMP, was used, no inhibition of agonist-induced

Figure 5.14

Direct activation of PKA does not induce internalisation of β_2-AR constructs.

The denoted cell clones were seeded into 24 well plates and were untreated or treated with 10^{-5} M isoprenaline, 1 mM di-butyryl cAMP or 1 mM 8-bromo cAMP for 60 minutes at 37°C. After washing, the cells were incubated with 10 nM [^{3}H] CGP12177 for 90 minutes at 4°C to measure the effect of each treatment on the level of cell surface receptor. Results are from one experiment performed in triplicate ± S.D.

Figure 5.14

a)

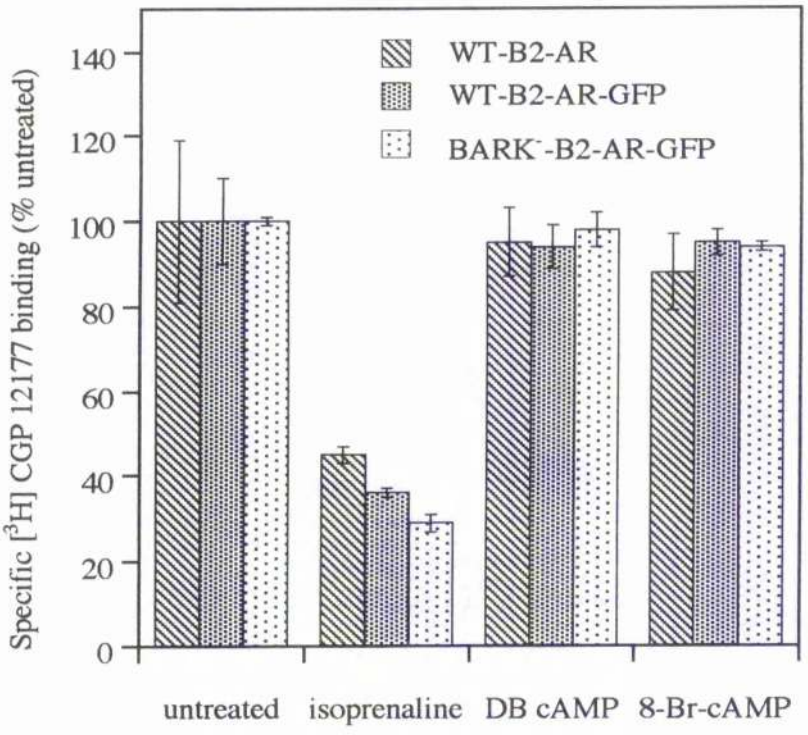
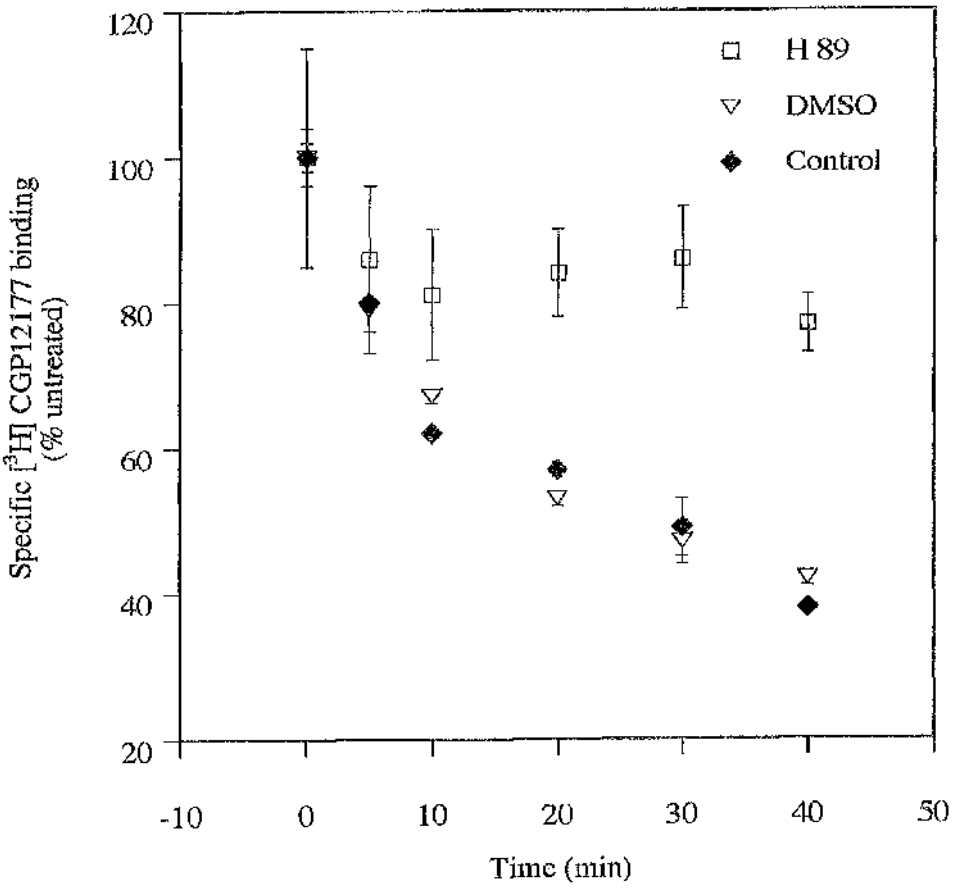

WT-B2-AR
WT-B2-AR-GFP
BARK⁻-B2-AR-GFP
Specific [³H] CGP 12177 binding (% untreated)
140
120
100
80
60
40
20
0
untreated
isoprenaline
DB cAMP
8-Br-cAMP

Figure 5.15

PKA inhibitor H89 appears to markedly reduce isoprenaline-induced internalisation of BARK$^-$-β_2-AR-GFP.

BARK$^-$-β_2-AR-GFP cells were seeded into 24 well plates and were pre-incubated with or without the PKA inhibitor H89 (10 μM) for 15 minutes. The cells were then incubated with 10^{-5} M isoprenaline at 37°C for a time course of 0 to 60 minutes. After washing, agonist-induced internalisation was measured by an intact cell binding study with 10 nM [^{3}H] CGP12177 at 4°C for 90 minutes. Results are from one experiment performed in triplicate ± S.D.

Figure 5.15


120
100
80
60
40
20
-10
0
10
20
30
40
50
Time (min)
Specific [3H] CGP12177 binding
(% untreated)
H 89
DMSO
Control

receptor internalisation was measured for either WT-β_2-AR, WT-β_2-AR-GFP or BARK$^-$-β_2-AR-GFP. Rp cAMP alone had no effect on the cells (Figure 5.16a). As PKA is an agonist-independent protein kinase, (GRK activation requires agonist bound to receptor) its effects may be more pronounced at low concentrations of agonist. The previous experiments used a high, saturating isoprenaline concentration (10^{-5} M). A second experiment was performed where WT-β_2-AR-expressing cells were untreated or treated with 10^{-5}, 10^{-7} or 10^{-9} M isoprenaline for 30 minutes. Approximately 50 % of receptor was internalised with 10^{-5} and 10^{-7} M isoprenaline but this could not be detected by treatment with 10^{-9} M isoprenaline (Figure 5.16b). When cells were pre-incubated with Rp cAMP for 15 minutes and then stimulated in the same way, again Rp cAMP had no effect on isoprenaline-induced internalisation at the higher concentration of 10^{-5} M or even at a 100 fold lower concentration of isoprenaline.

The ambiguity in the results using the two different PKA inhibitors was recently explained by Penn et al., (1999). It was shown in this study that H89 acts as an antagonist at both the β_1-AR and β_2-AR. Competition binding of [^{125}I] iodopindolol established K_i values of ~180 nM and 350 nM for H89 antagonism of the β_1-AR and β_2-AR, respectively. Penn et al., (1999) demonstrated that a 10^{-5} M concentration of H89 can markedly reduce the number of [^{125}I] iodopindolol binding sites in BEAS-2B cells stably expressing the β_2-AR. As H89 was routinely used at a concentration of 3 x 10^{-5} M (100 fold higher than the K_i at the β_2-AR), in the present study, it is not surprising that it appeared to impede agonist-induced internalisation when in fact, it was competing for binding of isoprenaline. To show H89s ability to act as an antagonist at the β_2-AR a binding study with [^{3}H] DHA on WT-β_2-AR and BARK$^-$-β_2-AR-GFP expressing cell membranes was performed which indicated that H89 bound to the β_2-AR in a concentration-dependent manner. Although 10^{-5} M H89 was shown to reduce [^{125}I] iodopindolol binding sites, it only blocked 70 to 80% of β_2-AR sites when using [^{3}H] DHA as the competing ligand (Figure 5.17). 3 x 10^{-5} M

Figure 5.16

PKA inhibitor Rp cAMP has no effect on isoprenaline-induced internalisation of the denoted β_2-AR constructs.

The denoted cell clones were pre-incubated for 15 minutes with or without the PKA inhibitor Rp cAMP (100 μM). The cells were then treated with agonist as indicated.

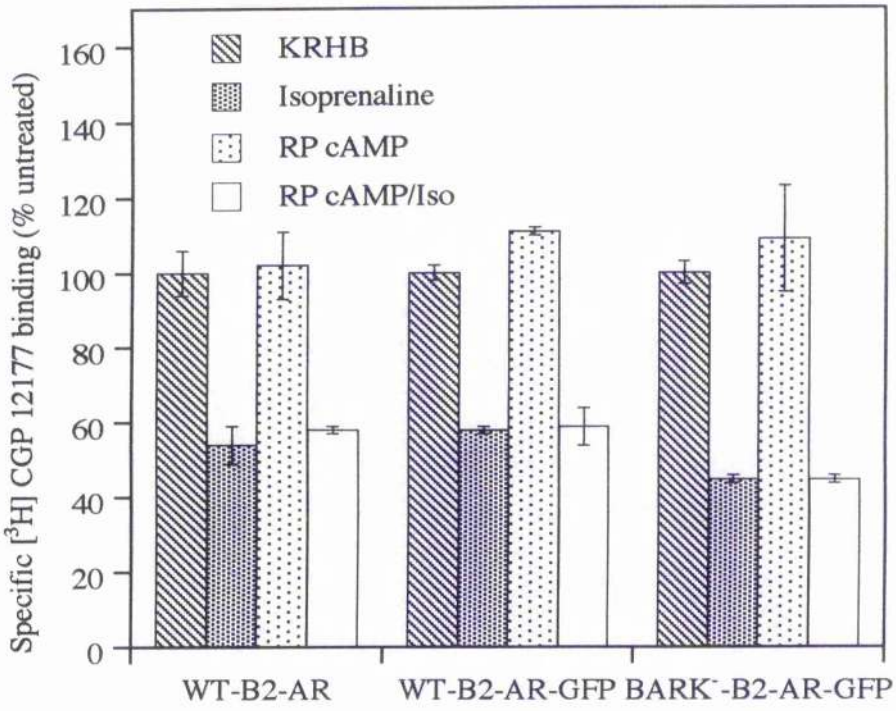
a) The denoted cell clones were treated or untreated with 10^{-5} M isopreanline for 30 minutes at 37°C.

b) WT-β_2-AR cells were untreated or treated with 10^{-5}, 10^{-7} or 10^{-9} M isoprenaline for 30 minutes at 37°C.

After washing, the cells were incubated with 10 nM [^{3}H] CGP12177 for 90 minutes at 4°C to measure the level of cell surface receptor following each treatment.

Figure 5.16

a)



b)

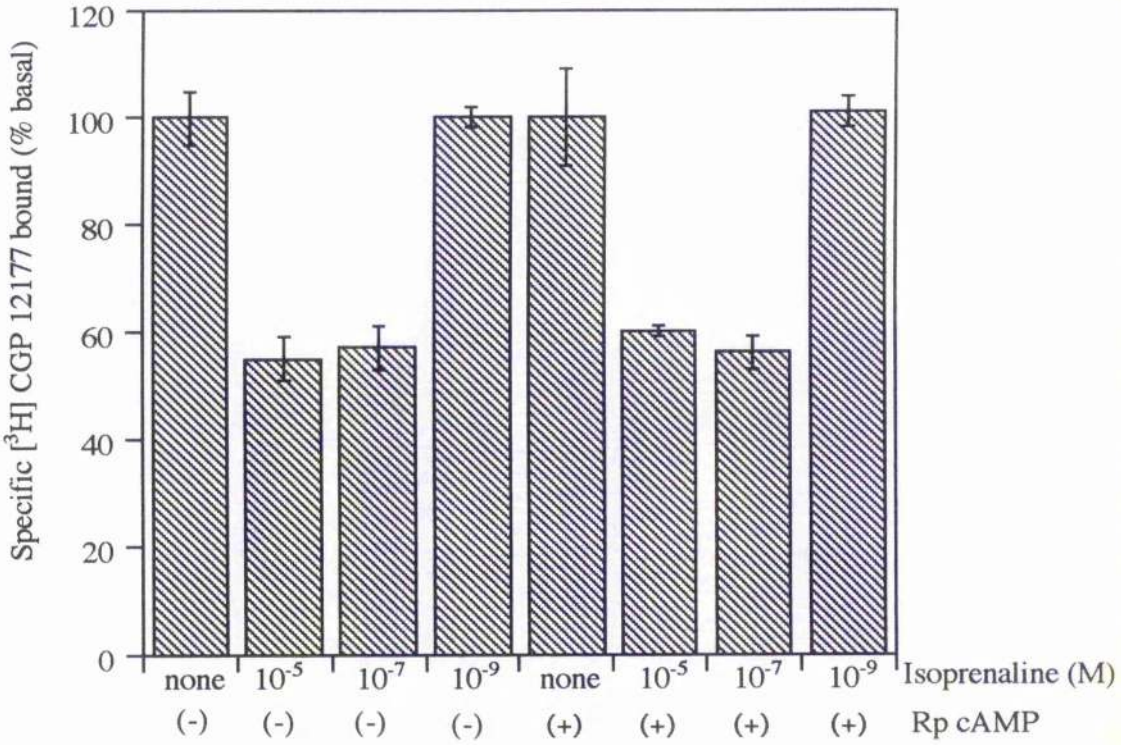
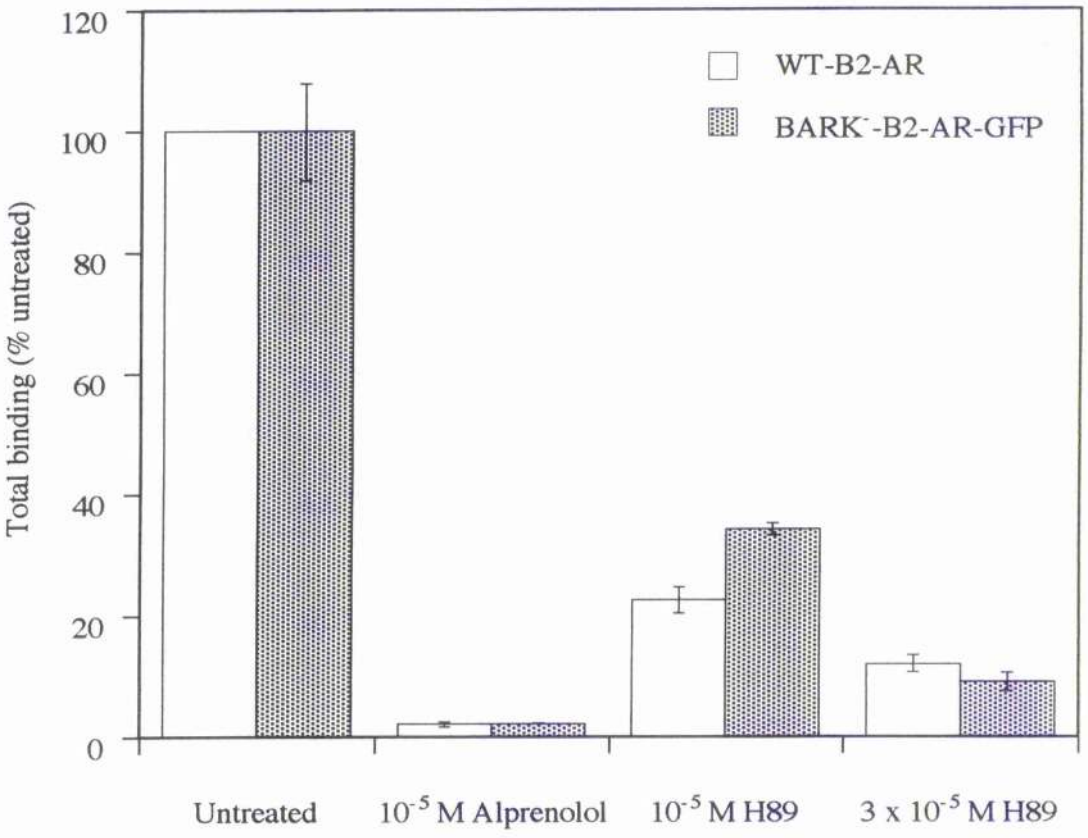


Figure 5.17

The PKA inhibitor, H89, binds to WT-β_2-AR and BARK$^-$-β_2-AR-GFP in a concentration-dependent manner.

20 μg of WT-β_2-AR or BARK$^-$-β_2-AR-GFP membranes were used in a [^{3}H] DHA binding study to demonstrate competition binding of 10^{-5} or 3 x 10^{-5} M H89 compared to the β_2-AR selective antagonist alprenolol (10^{-5} M). Results are from experiments performed in triplicate and are represented as means ± range., n = 1 for BARK$^-$-β_2-AR-GFP, n = 2 for WT-β_2-AR.

Figure 5.17


120
100
80
60
40
20
0
Total binding (% untreated)
WT-B2-AR
BARK-B2-AR-GFP
Untreated
10-5 M Alprenolol
10-5 M H89
3 x 10-5 M H89

H89 was still not as effective an antagonist as 10^{-5} M alprenolol but did block up to 90% of [^{3}H] DHA binding sites (Figure 5.17). A second experiment with CAM-β_2-AR-GFP-expressing cells (developed in Chapter 3) was performed (Figure 5.18). These cells were untreated (a) or treated with 10^{-5} M H89 (b), 3 x 10^{-5} M H89 (c), or 10^{-4} M Rp cAMP (d) for 24 h. The cells were then imaged in a confocal microscope. An up-regulation of CAM-β_2-AR-GFP was detected with H89 (Figure 5.18 b and c) similar to the up-regulation observed when using 10^{-5} M betaxolol (c). However, Rp cAMP induced no up-regulation of CAM-β_2-AR-GFP (d) indicating that it is the antagonistic effect of H89 and not its inhibition of PKA which caused receptor up-regulation. From an intact cell binding study on CAM-β_2-AR-GFP-expressing cells (Figure 5.18f) treated in the same way as previously, 10^{-5} M H89 induced up-regulation of the receptor to the same extent as 10^{-5} M betaxolol. 3 x 10^{-5} M H89 also up-regulated the receptor but to a lesser extent. This may be due to insufficient washing away of a higher concentration of H89 (Figure 5.18f).

As both PKA activators and PKA inhibitors had negative effects in the above experiments, no positive control was present to determine if these reagents were functioning properly in these cells. Some PKA inhibitors can be cell specific. It was attempted to monitor down-stream of PKA and examine phosphorylation of one of its target proteins, CREB using the Phospho Plus CREB (Ser133) antibody kit. A phospho-CREB antibody directed to Ser133 of CREB was used to detect changes in the levels of phospho-CREB following various drug treatments (Figure 5.19). However, little could be taken from the data (Figure 5.19a). The basal level of CREB phosphorylation was very high in untreated WT-β_2-AR-expressing cell lysates (Figures 5.19 a and b lane 1) compared to the provided negative control of SK-N-MC cells (Figure 5.19b, lane 3). Even the levels of phosphorylated CREB in cell extracts from SK-N-MC cells treated with the adenylyl cyclase activator forskolin (Figure 5.19b, lane 4) appeared lower than the levels of phosphorylated CREB from untreated WT-β_2-AR-expressing HEK293 cell extracts (Figure 5.19b, lane 1).

Figure 5.18

The PKA inhibitor H89 up-regulates the CAM-β_2-AR-GFP construct.

CAM-β_2-AR-GFP-expressing cells were plated onto glass coverslips and were untreated (a) or treated with 10^{-5} M H89 (b), 3 x 10^{-5} M H89 (c), 10^{-4} M Rp cAMP (d) or 10^{-5} M betaxolol (e) for 24 h at 37°C. the cells were then imaged on a confocal microscope.

f) Cells of the same clone were seeded into a 24 well plate and treated as above. After washing the cells, [^{3}H] DHA binding was performed at 30°C for 45 minutes to measure the level of CAM-β_2-AR-GFP expression following treatment. Results are from a single experiment performed in duplicate and are represented as means ± range.

Figure 5.18

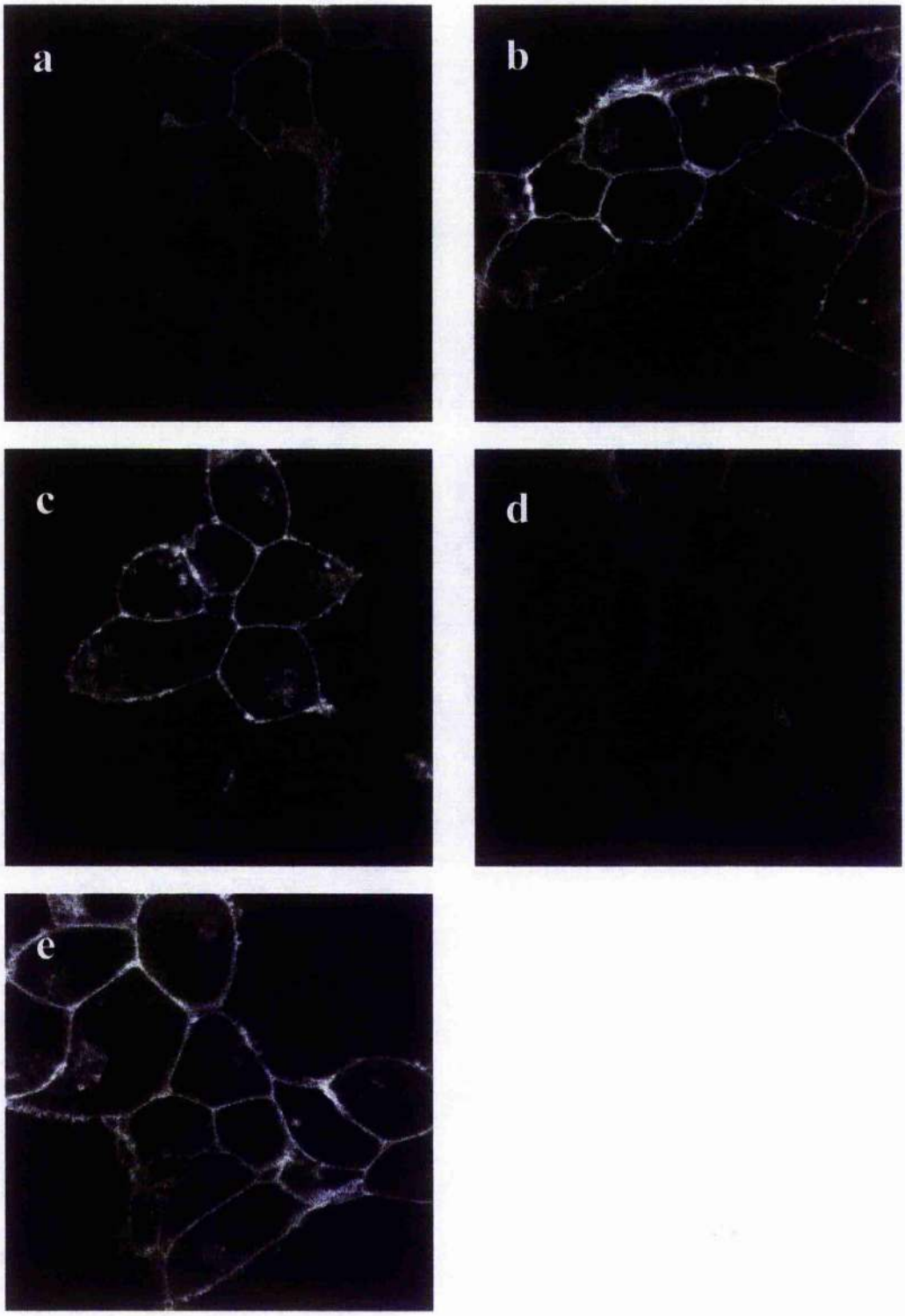


Figure 5.18 f)

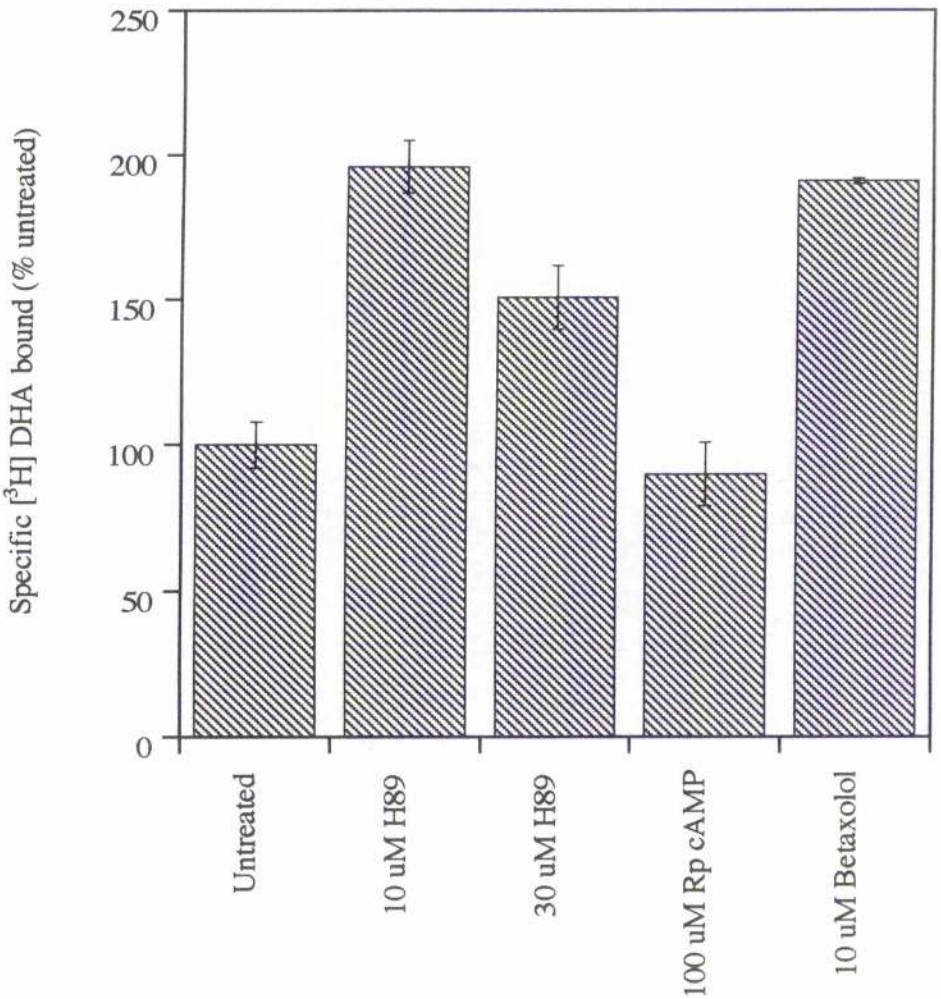
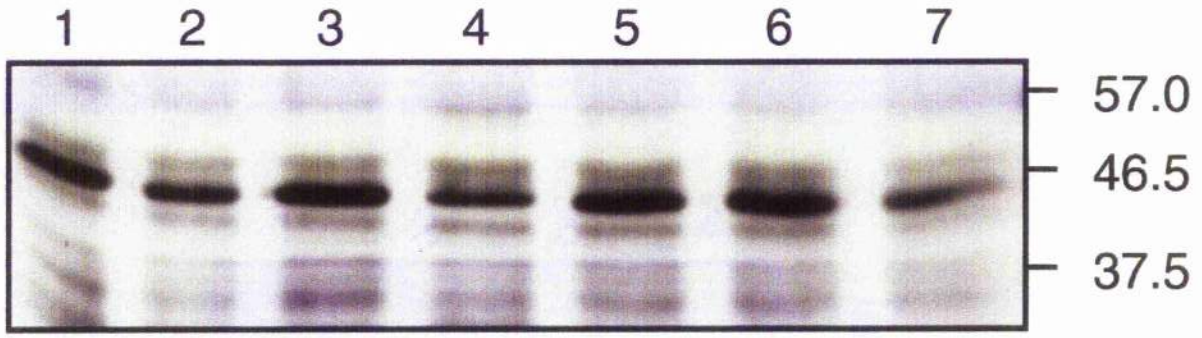

Specific [3H] DHA bound (% untreated)
250
200
150
100
50
0
Untreated
10 uM H89
30 uM H89
100 uM Rp cAMP
10 uM Betaxolol

Figure 5.19

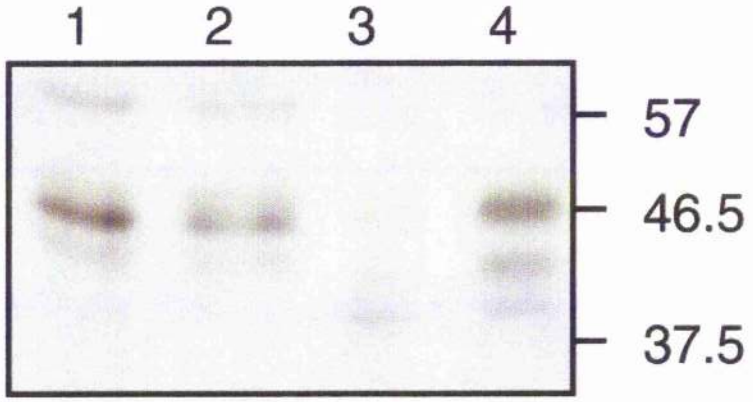
Phosphorylation of CREB at serine 133 in WT-β_2-AR-expressing cells. Confluent WT-β_2-AR-expressing cells in 6 cm dishes were pretreated with or without 100 μM Rp cAMP for 15 minutes, then with the denoted stimulant applied for 30 minutes. 15 μl of cell lysates prepared from these cells were loaded onto a 10% SDS-PAGE gel. Following transfer of protein to nitrocellulose membrane, the membrane was probed with a phospho-CREB antibody to indirectly determine the level of PKA activity by phosphorylation of serine 133; a) untreated (1), 1 mM dibutyryl cAMP (2), 100 μM Rp cAMP + dibutyryl cAMP (3), 10^{-5} M isoprenaline (4), 10^{-8} M isoprenaline (5), Rp cAMP + 10^{-5} M isoprenaline (6), Rp cAMP (7); b) untreated (1), dibutyryl cAMP (2), 10 μl untreated SK-N-MC cell lysate (3), forskolin treated SK-N-MC cell lysate (4).

Figure 5.19

a)



b)



5.6 Activation of adenylyl cyclase by agonist-stimulated BARK$^-$-β_2-AR-GFP

Internalisation of BARK$^-$-β_2-AR-GFP indicated that this receptor may be able to produce a second messenger output. As no effect of PKA was detected in the previous set of experiments it was anticipated that BARK$^-$-β_2-AR-GFP would not produce cAMP as a second messenger through adenylyl cyclase activation. However, when intact BARK$^-$-β_2-AR-GFP-expressing cells were labelled with [^{3}H] adenine and isoprenaline-stimulated production of [^{3}H] cAMP measured, isoprenaline induced a concentration-dependent increase in levels of cAMP with an $EC_{50} = 6.9 \pm 1.3 \times 10^{-8}$ M (mean ± S.D., $n = 2$) (Figure 5.20). This is 10 fold higher than the EC_{50} for isoprenaline at WT-β_2-AR-GFP (Chapter 4, Section 4.3).

5.7 Sustained treatment of BARK$^-$-β_2-AR-GFP-expressing cells with isoprenaline.

To assess if BARK$^-$-β_2-AR-GFP underwent agonist-mediated down-regulation. BARK$^-$-β_2-AR-GFP cells were untreated or treated with 10^{-5} M isoprenaline for 24 h washed, and membranes prepared. From membrane binding studies using a single concentration of [^{3}H] DHA, BARK$^-$-β_2-AR-GFP appeared to undergo substantially less down-regulation than WT-β_2-AR but may have down-regulated to a greater extent than WT-β_2-AR-GFP (Figure 5.21a). BARK$^-$-β_2-AR-GFP cells treated as above were imaged in a confocal microscope (Figure 5.21b). An aggregation of BARK$^-$-β_2-AR-GFP was detected after isoprenaline treatment. This aggregation did not appear to be so pronounced as the aggregation of WT-β_2-AR-GFP after 24 h isoprenaline treatment (Figure 4.20).

Figure 5.20

Isoprenaline-stimulated regulation of adenylyl cyclase activity in intact cells expressing the BARK$^-$-β_2-AR-GFP construct.

Basal adenylyl cyclase activity and its regulation by increasing concentrations of isoprenaline in BARK$^-$-β_2-AR-GFP-expressing cells was assessed as detailed in Chapter 2 Section 2.7d. Data represent means ± S.D. of triplicate assays from a single representative experiment. One additional assay produced similar results. The average EC_{50} of isoprenaline was calculated for BARK$^-$-β_2-AR-GFP as $EC_{50} = 6.9 \pm 0.9 \times 10^{-8}$ M, mean ± range, $n = 2$.

Figure 5.20

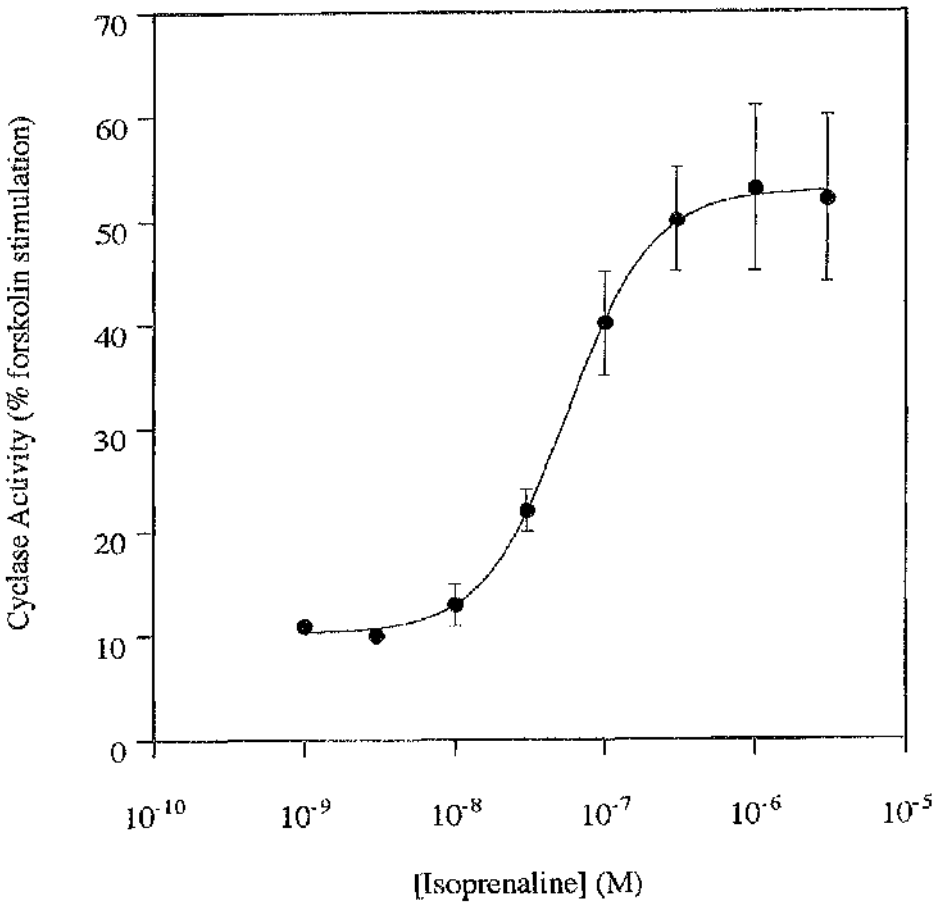

70
60
50
40
30
20
10
0
Cyclase Acivity (% forskolin stimulation)
10-10
10-9
10-8
10-7
10-6
10-5
[Isoprenaline] (M)

Figure 5.21

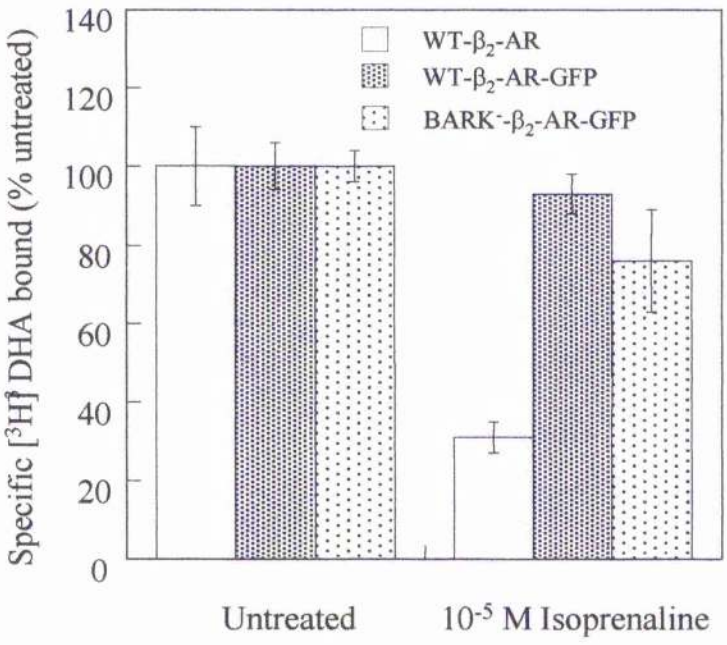
Overnight treatment of BARK^{-}-β_2-AR-GFP with isoprenaline.

a) BARK^{-}-β_2-AR-GFP-expressing cells were treated with or without 10^{-5} M isoprenaline for 24 h and membranes prepared. 20 μg of each membrane preparation was used in binding studies with a single concentration of [^{3}H] DHA (2 nM) to assess the levels of BARK^{-}-β_2-AR-GFP in each sample.

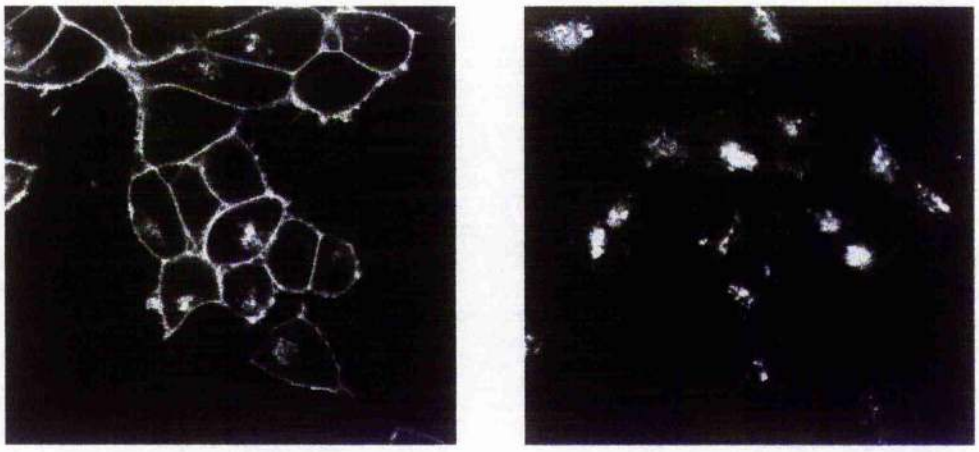
b) Cells of the same clone were plated onto glass coverslips and treated as above. The cells were then viewed on a confocal microscope and imaged to assess the distribution of BARK^{-}-β_2-AR-GFP.

Figure 5.21

a)



b)



Basal

+ 10^{-5} M Isoprenaline

5.8 Discussion

For over a decade the processes of GPCR agonist activation, desensitisation, internalisation and recycling or down-regulation have attracted the attention of many research groups. The most studied receptor has been the β_2-AR and as yet the full details of the above receptor cycle (Figure 4.1) have not been fully elucidated. It is generally accepted that upon agonist activation of the β_2-AR, phosphorylation of its C-terminal tail occurs by GRK 2,3 or 5 and second-messenger activated kinase PKA to uncouple the receptor from G protein and initiate its desensitisation.

There has been debate as to which residues of the β_2-AR are actually phosphorylated by GRKs *in vivo*. The sites found *in vitro* by Fredericks et al., (1996) to be phosphorylated in the β_2-AR by GRK2 and GRK5 (Ser 396, 401, 407, 411 and Thr 384, 398) were found not to be the sites phosphorylated *in vivo* by Seibold et al. (1998). A mutant construct with both PKA consensus sites and all the GRK2 and GRK5 serine/threonine phosphorylation sites mutated to alanine, was still found to be phosphorylated, undergo desensitisation and agonist-induced internalisation. The authors postulated that other sites within the β_2-AR C-terminal tail could be *in vivo* sites of GRK phosphorylation (Ser 355, 356, 364 and Thr 360) as Fredericks et al., only analysed residues 374-413 of the β_2-AR which contained only 7 potential GRK phosphorylation sites. All 11 of the serine/threonine residues found in the β_2-AR C-terminal tail , from amino acid 355 to 413, have been implicated as possible sites of GRK phosphorylation. Decreased desensitisation and phosphorylation has been reported for a mutant β_2-AR containing substitutions at all 11 C-terminal serine/threonine sites stably expressed in Chinese hamster fibroblast (CHW) cells (Hausdorff et al., 1989; Bouvier et al., 1988). Hausdoff et al., (1991) also studied a β_2-AR construct with only serines 355, 356, and 364 and threonine 360 substituted for either glycine or alanine and indicated that the *in vivo* sites of phosphorylation could be among these.

To investigate these discrepancies a mutant β_2-AR with the 11 potential GRK C-terminal phosphorylation sites mutated to alanine or glycine was utilised. To aid investigations the mutant receptor was C-terminally tagged with the 27 kDa GFP polypeptide so that confocal analysis of the protein could be achieved to visually monitor the receptor. The GFP-tagged mutant β_2-AR (BARK$^-$-β_2-AR-GFP) was stably transfected into HEK 293 cells and pharmacologically characterised to determine if mutations in the C-terminal tail of the β_2-AR or addition of GFP caused any effect on basic receptor pharmacology. Although expressed at relatively low levels in transient transfections, clones stably expressing this form of the β_2-AR at levels comparable to the WT-β_2-AR-GFP construct were isolated. The K_d for [^{3}H] DHA at the mutant receptor was similar to WT-β_2-AR-GFP (Table 5.1). Affinity for the agonist isoprenaline, inverse agonist betaxolol or antagonist alprenolol did not dramatically change (Table 5.2). Therefore, addition of GFP or the mutations introduced in the C-terminal tail of the receptor did not alter the basic receptor pharmacology of the receptor.

Analysis by confocal microscopy of BARK$^-$-β_2-AR-GFP in response to agonist stimulation indicated that this receptor construct rapidly internalised following addition of 10^{-5} M isoprenaline (Figure 5.10). This confirmed findings by Hausdorff et al., (1989) that a similar construct also internalised in CHW cells. However, the internalisation profile of BARK$^-$-β_2-AR-GFP ($t_{1/2}$ = 10 minutes) was faster than that of WT-β_2-AR-GFP ($t_{1/2}$ = 25 minutes) as assessed in [^{3}H] CGP12177 intact cell binding studies (Figure 5.11a and Table 4.4). A distinct punctate pattern of BARK$^-$-β_2-AR-GFP receptor internalisation was detected at 5 minutes whereas internalised WT-β_2-AR-GFP was only detectable at 10 minutes in the confocal microscope (Figure 5.10b and 4.6b respectively). A factor to consider here is that the BARK$^-$-β_2-AR-GFP construct may not be able to recycle as efficiently as the WT coustruct. Inhibitors of the recycling machinery were not used in the internalisation studies above and therefore, a faster rate of internalisation detected for the BARK$^-$-β_2-AR-GFP

construct could be an effect of inefficient recycling of this construct. It appears, however, that BARK^{-}-β_2-AR-GFP also internalises by a clathrin-dependent mechanism since 0.4 M sucrose inhibited agonist-stimulated internalisation of this construct (Figure 5.12).

As the BARK^{-}-β_2-AR-GFP construct still internalised in response to isoprenaline stimulation a potential role for PKA was investigated. The importance of PKA in the process of β_2-AR desensitisation and internalisation has been hotly debated, some groups agreeing with its role in receptor desensitisation (Post et al., 1996; Lohse et al., 1990; Benovic et al., 1985; Hausdorff et al., 1989; Moffet et al., 1996) and others finding little effect of PKA phosphorylation on β_2-AR desensitisation and internalisation (Seibold et al., 1998; Green et al.,1981). The use of the direct PKA activators, dibutyryl cAMP and 8-bromo cAMP, to attempt to induce internalisation of WT-β_2-AR, WT-β_2-AR-GFP or BARK^{-}-β_2-AR-GFP in the absence of agonist demonstrated that either PKA phosphorylation of the receptor had no effect on receptor internalisation, or that only PKA activated through agonist bound receptor may be able to internalise receptors (Figure 5.14).

When the previously described PKA inhibitor H89 was used to treat BARK^{-}-β_2-AR-GFP-expressing cells internalisation of the construct in response to agonist isoprenaline was inhibited by approximately 40% (Figure 5.15). However, this was found not to be due to inhibition of PKA, but by the antagonistic properties of H89 at the β_2-AR (Penn et al., 1999). H89 has a measured K_i = 350 nM for the β_2-AR stably expressed in BEAS-2B cells. This is comparable to the binding affinities of betaxolol and isoprenaline at the WT-β_2-AR (Table 5.2). As BARK^{-}-β_2-AR-GFP-expressing cells were pre-incubated with H89 at a concentration 100 times greater than its K_i, it is not surprising that it was able to compete for binding of isoprenaline and thus impede agonist-induced receptor internalisation. H89 was demonstrated directly to be a ligand at the WT-β_2-AR construct when included in a [^{3}H] DHA binding

study. 10^{-5} and 3 x 10^{-5} M H89 reduced binding of [^{3}H] DHA at the WT-β_2-AR by 80 and 90% respectively and at the BARK$^-$-β_2-AR-GFP by 70 and 90% respectively (Figure 5.17). Moreover H89, unlike Rp cAMP, induced up-regulation of CAM-β_2-AR-GFP following a 24 h treatment, an effect anticipated for β-blockers from the data of Chapter 3.

A second inhibitor, Rp cAMP, had no effect on agonist-induced receptor internalisation of the β_2-AR (Figure 5.16a) which could indicate that PKA has no role in internalisation of the β_2-AR or that this inhibitor is cell specific and does not function in HEK293 cells. Hausdorff et al., (1989) found that mutating the two PKA consensus sites in the β_2-AR to glycine residues produced a receptor which still underwent agonist-promoted internalisation, but exhibited decreased phosphorylation at low concentrations of isoprenaline (10 nM) and reduced desensitisation at low and high (2 μM) concentrations of isoprenaline. This would indicate that internalisation of GPCRs can still occur when phosphorylation and desensitisation of the receptor are impeded. The data presented in this study using Rp cAMP as an inhibitor of PKA would indicate this to be the case as receptor internalisation still occured in the presence of this inhibitor, even at low concentrations of isoprenaline (Figure 5.16 a and b). Seibold et al., (1998) also measured agonist-induced internalisation by adrenaline of a PKA$^-$ mutant of the β_2-AR, however no effect on desensitisation of the receptor was measured. Rapid phosphorylation of this construct was also detected after 1 minute of adrenaline treatment, however GRKs could have phosphorylated the receptor since a high concentration of agonist was used. Interestingly, a β_2-AR mutant lacking the *in vitro* phosphorylation sites (Fredericks et al., 1996) and both PKA consensus sites was still phosphorylated upon agonist stimulation. This would indicate new sites of *in vivo* phosphorylation present at other regions of the β_2-AR. In fact, additional sites of phosphorylation in intracellular loop 2 of the β_2-AR have been postulated from a study by Jockers et al., (1996). This study investigated the differences between the desensitisation and sequestration patterns of the β_2-AR

compared to the β_3-AR. The β_3-AR undergoes very little desensitisation and sequestration in response to agonist stimulation whereas the β_2-AR is well known to undergo rapid desensitisation and internalisation. This group wanted to find the components of the β_2-AR responsible for these processes. They constructed a series of 15 β_3/β_2-AR chimeras to identify which regions of the β_2-AR are important for uncoupling and sequestration. It was revealed that the C-terminal tail, intracellular loop 3 and intracellular loop 2 of the β_2-AR added individually into the β_3-AR all partially restored the uncoupling phenotype and that their effects were additive to produce a desensitisation profile similar to that of the β_2-AR. The C-terminal tail, intracellular loop 2 and intracellular loop 1 all played additive roles in receptor sequestration, but did not restore it completely, whereas, intracellular loop 3 had a dominant negative effect. They identified two potential sites of phosphorylation at serines 137 and 143 in intracellular loop 2 of the β_2-AR. Serine 137 is contained in the potential phosphorylation consensus site S/TPKK/R, which has been shown to be the preferred site for cdc2 kinase. It may be interesting to investigate mutation of these two sites to glycine or alanine within the BARK$^-$-β_2-AR-GFP construct to find if this abolishes agonist-induced desensitisation and sequestration.

As the BARK$^-$-β_2-AR-GFP construct underwent agonist-stimulated internalisation, experiments were performed to determine if the construct could generate a second messenger output. cAMP was generated in a concentration-dependent manner upon addition of isoprenaline (Figure 5.20). The EC_{50} was 10 times higher than at the WT-β_2-AR-GFP construct indicating that isoprenaline has a lower potency at the BARK$^-$-β_2-AR-GFP construct. This result was rather surprising as previous experiments in this study indicated no role for PKA in receptor internalisation of the β_2-AR. A possible explanation for this is that Rp cAMP does not function in these cells, a factor which was not determined from the phospho-CREB experiments performed (Figure 5.19). Conversely, PKA phosphorylation may not be a functional aspect of β_2-AR internalisation and may only be involved in the uncoupling and desensitisaiton of the

receptor since mutation of PKA consensus sites within the β_2-AR has produced a receptor construct that still internalises but exhibits reduced desensitisation (Hausdorff et al., 1989). However this was not found by Seibold et al., (1998). When down-regulation of the BARK$^-$-β_2-AR-GFP construct was analysed, a 24 h treatment with isoprenaline (10^{-5} M) appeared to cause a slight decrease in total receptor levels compared to WT-β_2-AR-GFP (Figure 5.21).

Despite the reported role of ERK regulation of β-arrestin 1 through MEK1 (Lin et al., 1999), and feedback inhibition of GRK2 activity by ERKs (Pitcher et al., 1999) an inhibitor of MEK1 (PD98059), had no effect on agonist-induced internalisation of WT-β_2-AR-GFP (Figure 5.13). The inhibitor was expected to promote further internalisation of the β_2-AR. This result therefore, is not consistent with the reported findings.

It is apparent from this chapter of work that there is still controversy surrounding β_2-AR phosphorylation mechanisms which induce receptor uncoupling, desensitisation and sequestration in response to agonist stimulation. The results herein have not reached definite conclusions about these processes but they have presented suggested avenues for further investigation. As the BARK$^-$-β_2-AR-GFP construct was shown to internalise through the clathrin-mediated pathway (Figure 5.14) it should be further investigated if β-arrestins can colocalise with this receptor using confocal microscopy (Groarke et al., 1999; Vrecl et al., 1998; Zhang et al., 1999; Milligan, 1999). The sites of phosphorylation on the β_2-AR need to be investigated further to fully determine which residues and which protein kinases are regulating desensitisation and sequestration. There may be distinct residues for distinct processes since mutation of certain residues in the C terminus of the β_2-AR has appeared to have an effect on phosphorylation and desensitisation of the receptor but no effect on sequestration. The potential role of serines 137 and 143 in intracellular loop 2 of the β_2-AR could

hint at new mechanisms of regulation of this receptor and should be further investigated.

Chapter 6

Final Discussion

Over the past few decades an understanding of cellular signalling processes has been formulated but attempts are still being made to characterise these events more fully. A variety of stimuli including hormones, neurotransmitters, ions, odourants and photons of light are able to activate receptors expressed on the surface of a cell. Detection of these signals by cell surface receptors initiates a series of events within the cell to produce ultimate effects on cell growth, differentiation and division.

One of the largest families of cell surface expressed receptors are the GPCRs. Over two hundred of these receptors have been identified and all have the same basic hydrophobicity profile of 7 α helices to form 7 transmembrane spanning regions linked by 3 extracellular and 3 intracellular loops. The extracellular N terminus contains sites for glycosylation. The 7 transmembrane regions are important for recognition and binding of ligands. Coupling to intracellular signalling processes is in part achieved by the intracellular loops, mainly intracellular loop 3, and also by the C-terminal tail.

In order to produce effector activation ligand bound receptor requires a G protein which is a heterotrimer of α, β and γ subunits. In the resting state receptor is coupled to GDP-bound G protein and agonist binding and activation induces a conformational change in the G protein to exchange GDP for GTP and promote dissociation of the βγ dimer from the active GTP-bound α subunit/receptor complex. Once the α subunit interacts and activates effector, GTP is hydrolysed to GDP promoting reformation of the heterotrimeric G protein complex (Gilman, 1984; Gilman, 1987). Several α, β and γ variants have been identified conferring different functional roles of G proteins. Both the GTP-bound α subunit and the βγ complex can influence the activity of cellular effector systems including adenylyl cyclase, phospholipase isoforms, various ion channels and MAP kinase activity, differentially or in combination.

This study has concentrated on two sub-types of the β-AR sub-family of GPCRs, these being the β_1- and β_2- ARs which couple through the stimulatory G protein $G_s\alpha$

to activate adenylyl cyclase and formation of the second messenger cAMP. Some receptors display the ability to activate effector independent of agonist stimulation. These are termed constitutively active receptors and can frequently be produced from mutations in their amino acid sequence. A selection of these receptors include a CAM form of the β_2-AR (Samama et al., 1993, 1994), the α_{1B}-AR (Cotecchia et al., 1990; Kjelsberg et al., 1992), the thyrotropin receptor (Parma et al, 1993), the leuteinizing hormone receptor (Shenker et al., 1993), the dopamine D_1 and D_5 receptors (Tiberi and Caron, 1994) and $5HT_{1D\alpha}$, $5HT_{1D\beta}$ and $5HT_{2C}$ receptors (Barker et al., 1994; Thomas et al., 1994).

In Chapter 3 a CAM form of the β_2-AR (CAM-β_2-AR) was examined in a HEK293 cell system to investigate its potential use for drug screening. MacEwan and Milligan (1996a,b) had previously demonstrated that this CAM-β_2-AR expressed stably in NG108-15 cells became up-regulated when exposed to saturating doses of the β_2-AR inverse agonists sotalol and betaxolol for 24 h. This characteristic was not exhibited by agonist or antagonist treatment and this was therefore, proposed as a potential drug screen for inverse agonists at this receptor. However, other studies have indicated that any β-ligand whether agonist, antagonist or inverse agonist can stabilise the CAM-β_2-AR and thus up-regulate it (Gether et al., 1997a,b). It was the aim of this study to develop the basis of a rapid drug screening assay with some degree of accuracy and efficiency. As it is now possible and extremely popular to tag GPCRs with GFP this approach was implemented in the study and proved advantageous for a drug screen over other methods. Stable expression of a CAM-β_2-AR-GFP construct in HEK293 cells and overnight treatment with several β-blockers indicated that not only inverse agonists but antagonists also induced up-regulation of the receptor. The different ratios of internal to plasma membrane up-regulated receptor demonstrated the differential regulation by these selected β-blockers. These patterns of up-regulation appeared to correlate with the ability of the drug to stimulate adenylyl cyclase activity through the receptor. Inverse agonists such as betaxolol and ICI118551, which did

not stimulate adenylyl cyclase, resulted in an increase in homogenous plasma membrane-delineated fluorescence with diffuse, intracellular staining as in untreated CAM-β_2-AR-GFP-expressing cells. In contrast, although labetolol produced a substantial increase in plasma membrane CAM-β_2-AR-GFP there was also an increase in intracellular signal with a marked punctate pattern, similar to the pattern produced in WT-β_2-AR-GFP-expressing cells exposed to agonist isoprenaline for a short period of time. Labetolol was able to elevate cAMP levels to the same extent as isoprenaline. However, isoprenaline did not up-regulate the CAM-β_2-AR-GFP construct.

In contrast to MacEwan and Milligan (1996a,b), alprenolol caused up-regulation of CAM-β_2-AR-GFP. Inefficient washing away of this high affinity ligand meant that residual alprenolol was able to compete for radioligand binding to the receptor, reducing the level of detectable receptor in MacEwan and Milligan's study. Monitoring fluorescence of a GFP-tagged GPCR is one way of overcoming the problem of using high concentrations of ligands. Therefore, effects of ligands can be directly screened without such complications. It may be argued that western blotting of the protein is just as an efficient way to look at ligand regulation. However, this method gives no indication of the cellular location of the protein. Cell fractions need to be ran on sucrose density gradients and then probed with antibody to determine protein location within the cell. This method is extremely time consuming so the prospect of being able to rapidly and directly visualise the level and location of receptor within the cell is understandably advantageous.

As initial fluorescence studies on the confocal microscope were not quantitative this prompted development of an assay to measure the extent of up-regulation using a spectrofluorimeter in 96 well format, which would produce numerical data for analysis of levels of up-regulation. From preliminary data, the EC_{50} for up-regulation of the CAM-β_2-AR-GFP construct by betaxolol was calculated to be in close agreement with the K_i of this drug at this receptor. However, the output of

fluorescence in this assay is quite limited and now constructs of the CAM-β_2-AR have been made with more suitable tags such as *Renilla* and *Photinus* luciferase to produce a better signal output (Ramsay and Milligan, unpublished results).

GFP tagging also proved useful in Chapter 4 when assessing the effect of short-term agonist stimulation at both the β_1- and β_2- ARs. Successful GFP-tagging has been exhibited for a number of receptors; eg, the β_2-AR (Barak et al., 1997; Kallal et al., 1998; McLean and Milligan, 1999) and a CAM-β_2-AR (McLean et al, 1999), the cholecystokinin type A receptor (Tarasova et al., 1997), the thyrotropin releasing hormone receptor (TRHR-1) (Milligan, 1998; Drmota et al., 1998, 1999), the α_{1A}-AR (Hirasawa et al., 1997), the α_{1B}-AR (Hirisawa et al., 1997; Awaji et al., 1998), the vasopressin V2 receptor (Schulein et al., 1998), the parathyroid hormone receptor (Conway et al., 1999), the CXCR-1 chemokine receptor (Barlic et al., 1999) and the Ca^{2+}-sensing receptor (Gama and Breitwieser, 1998).

Optical time imaging of agonist-stimulated receptor internalisation was achieved directly on the confocal microscope for the β_1- and β_2- ARs, however, limitations to the method were apparent. A quantitative measurement of receptor internalisation was achieved by [^{3}H] ligand binding studies. By using the hydrophillic ligand [^{3}H] CGP12177 a loss of cell surface receptor following agonist stimulation was measured. On comparing time-courses of internalisation between the GFP and non-GFP-tagged constructs for both β_1- and β_2- AR constructs, GFP substantially impeded the ability of the receptor to internalise. Overall the GFP-tagged receptors exhibited a similar pharmacological profile to their non-tagged versions and WT-β_1-AR-GFP like WT-β_1-AR internalised to a lesser extent than WT-β_2-AR-GFP and WT-β_2-AR respectively. The differences of β_1- versus β_2- AR internalisation may be attributed to a different process of internalisation (Tang et al., 1999). However, the kinetic properties of this internalisation process was the feature altered by GFP a finding not previously reported from studies using GFP-tagged receptors. Secondly,

down-regulation of the β_2-AR in response to a 24 h treatment with agonist isoprenaline was impared by the presence of GFP at the C terminus of the construct. This chapter of work, therefore pointed out the limitations of using GFP as a tool when analysing functional and kinetic parameters of GPCRs, in this case the β_1- and β_2- ARs.

In my final chapter the issue of β_2-AR sequestration in response to agonist stimulation was addressed. As yet the exact events of the desensitisation and sequestration of the β_2-AR following agonist stimulation are still to be resolved. Debate centres around several issues. What residues of the receptor are responsible for desensitisation and sequestration, and are the same or unique residues involved in each of these processes? Jockers et al., (1996) demonstrated that intracellular loop 3 of the β_2-AR is partly responsible for desensitisation of the receptor but has a dominant negative effect on sequestration and therefore, the 2 events are not related. This would indicate that one particular region of the β_2-AR may not be involved in both processes of desensitisation and sequestration. A GFP-tagged form of the β_2-AR with all the 11 potential GRK phosphorylation sites mutated from ser/thr to gly/ala underwent a marked time-dependent internalisation in response to isoprenaline stimulation. The rate of internalisation was faster for the BARK$^-$-β_2-AR-GFP construct than for the unmodified form of the receptor. If phosphorylation is critical for receptor internalisation in this case then either PKA is responsible or the receptor contains unidentified phosphorylation sites. The PKA inhibitor Rp cAMP had no effect on isoprenaline-induced BARK$^-$-β_2-AR-GFP internalisation, which may indicate, that other sites of phosphorylation are present in the receptor construct. Jockers et al., (1996) identified two potential phosphorylation sites at ser 137 and 143 in intracellular loop 2 of the β_2-AR. The role of PKA in β_2-AR desensitisation and sequestration has also been hotly debated. Seibold et al., (1998) have found no role of this kinase in desensitisation or sequestration which is contradictory to Hausdorff et al., (1989) who demonstrated that a β_2-AR lacking a PKA consensus site exhibited reduced

desensitisation but still internalised in response to agonist stimulation. Other groups (Post et al., 1996; Lohse et al., 1990; Benovic et al., 1995; Moffet et al., 1996) also agree with Hausdorff. Although the BARK$^-$-β_2-AR-GFP construct does indeed internalise, the rapidity of internalisation may be an indication of impeded recycling of the receptor. If this receptor construct cannot recycle efficiently, internalised receptor may aggregate in intracellular membrane compartments and, therefore, it may look as if more of the BARK$^-$-β_2-AR-GFP construct has internalised at earlier time points compared to the WT-β_2-AR-GFP construct which can efficiently recycle. As recycling of BARK$^-$-β_2-AR-GFP was not investigated by removal of agonist or addition of antagonist in this study, the recycling capacity of this mutant receptor in contrast to the WT construct should be investigated. Secondly, the sites of *in vivo* phosphorylation need to be further assessed and related to their role in desensitisation and sequestration. Another issue to consider is whether this construct still internalises through the clathrin/dynamin-mediated pathway. However, experiments with sucrose did indicate that this construct internalised through a clathrin-mediated pathway in response to agonist stimulation.

This study has highlighted the advantages of using GFP-tagging but like any method it should not be used in isolation. It is clearly apparent that GFP can affect the functioning of the receptor and in this study has provided no direct answers to the questions surrounding β_2- versus β_1- AR sequestration processes. However, in the area of drug screening, GFP, its variants and other fluorescent tags (luciferases) look to be a new and efficient way of analysing drug effects at different GPCRs. To conclude, GFP is not a be all and end all but is a helpful tool for investigating protein mobilisation and signalling.

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