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Biotechnology of β-Adrenergic Receptors

A. Donny Strosberg

Laboratoire d'Immuno-Pharmacologie Moléculaire, CNRS UPR 0415 and Université Paris VII, Institut Cochin de Génétique Moléculaire, 22, rue Méchain, 75014 Paris, France

Contents

Abstract

The emergence of Biotechnology has provided pharmacologists with a variety of methods for investigating the structure, the function, and the regulation of membrane-bound receptors with a precision that was not imagined even five years ago. These new tools have been developed and used to analyze the known catecholamine β_1- and β_2-adrenergic receptors and to discover and study a new subtype, the β_3 receptor. We review here the salient features of each of these three receptors, compare their structural and functional properties, and propose models to explain their differential regulation in time and space.

A whole family of proteins has now been found to share with the β-adrenergic receptors their most prominent features, including seven transmembrane domains and coupling with GTP-binding "G" proteins. We therefore propose that the biotechnology-based procedures developed for the β-adrenergic receptors will be well applicable to the other members of this "R_7G" family of receptors.

Index Entries: β-Adrenergic receptor subtypes; βAR-modulated adenylyl cyclase activation; cloning of βAR genes; expression of βAR genes in *E. coli*, in CHO, and in baculovirus-infected cells; regulation of βAR expression and function; the βAR ligand-binding region; sites of βAR–G_s protein interaction; glycosylation of βAR; phosphorylation; internalization; integrated model.

Introduction

The β-adrenergic catecholamine receptors constitute excellent models for the biotechnological analysis of receptor-modulated ligand-induced transmembrane signaling. They belong to the family of "R_7G proteins" (Strosberg, 1991), which transduce signals to such enzymes as adenylyl cyclase or phospholipase A or C, or modulate calcium or potassium ion channels, which are all coupled to GTP-binding regulatory proteins ("G proteins") and display the following general characteristics (Table 1): all the R_7G sequenced so far (Table 2) are constituted of a single polypeptide chain of 300–800 residues, often encoded by an intronless gene. This chain is composed of a glycosylated extracellular N-terminal portion, seven transmembrane (tm) segments connected by extracellular (e) and intracellular (i) loops. The C-terminal portion, which displays as much diversity and variation in length as the N-terminal region and the i_3 loop, also shares with i_3 a high incidence of Ser and Thr residues, which constitute potential targets for phosphorylations.

In this review, we will focus our attention on the structure–function relationship in the three β-adrenergic receptors and the mechanisms that regulate their expression. We will show how various biotechnology-derived procedures were applied to the analysis of these receptors and to associated GTP-binding G proteins and adenylyl cyclase. Because of the homology between the β-adrenergic receptors (βAR) and the other sequenced R_7G proteins, the application of these methods should lead to similar conclusions about their function. The first data emerging from the study of the five muscarinic acetylcholine receptor subtypes, and of the four serotonin and five dopamine receptor subtypes, already confirm that the expression and function of R_7G receptors are all exquisitely regulated at the levels of the genes, the mRNA, and the proteins by a variety of mechanisms (Strosberg, 1991). The existence of subtypes significantly contributes to differential activation in time and space by the natural agonists and effectors.

Biochemistry and Immunochemistry of the β-Adrenergic Receptors

α- and β-Adrenergic Receptors

The diversity of actions of catecholamines has been recognized since the beginning of the century. Vasoconstriction and vasodilatation in a

Table 1
Common Properties of the Members of the Family of R_7G Receptors

Structure	Function	Genetics
Single polypeptide chain	Coupled to a GTP-binding G protein	Frequently intronless genes
Seven tm domains	Agonist triggers various effectors through a G protein	Several subtypes encoded by highly homologous gene
Extracellular glycosylated N-terminus	Several subtypes bind the same natural agonist, but are selective for various synthetic ligands	
i_3 Loop or intracellular (or other e loops) C-terminus		
Three extra- and three intracellular loops	Subtypes couple to different G proteins and regulate different effectors	
Extensive diversity in sequence and length of N-terminus, i_3 loop, and C-terminus	Different receptors couple to the same G proteins and same effectors	

Table 2
Sequenced Members of the R_7G Family of G-Protein-Coupled Receptors

Nonpeptidic ligand	Peptide	Sensory
α-Adrenergic (1A, B, C; 2A, B, C)	Angiotensin II	Rhodopsin (blue, green, red)
Adenosine A1, A2	Bombesin	Olfactory (multiple)
β-adrenergic(β1, β2, β3)	Bradykinin	
Dopaminergic (D1–D5)	C5a anaphylatoxin	
Histamine H2	Endothelins (at least 3)	
Metabotropic glutaminergic	N-Formyl peptide	
Muscarinic cholinergic (m1–m5)	Neurotensin	
Platelet-activating factor	Tachykinins (Subst K, P, Neuro K)	
Serotoninergic (5HT-1A, 1C; 5HT-2)	Thrombin	
Thromboxane A2	Thyrotropin-releasing hormone	
Glutamic acid (metabotropic)	Vasopressin, oxytocin	
	FSH (Follicle-Stimulating Hormone)	
	LH-hCG (Lutobropin choriogonadotropin)	
	TSH (Thyroid Stimulating Hormone)	

variety of tissues and organs were proposed by hlquist (1948) to correlate with the presence of two major classe of receptors: α and β (Table 3).

Nearly 20 years later, in 1967, these classes were further subdivided (Lands et al., 1967) into α_1 and α_2, and β_1 and β_2 subtypes. The α_1AR were generally identified as postsynaptic and vasoconstrictive, whereas the α_2AR both retroactively inhibit noradrenaline release from sympathetic nerves and also are vasoconstrictive postsynaptically.

The β_1AR modulates the rate and strength of heartbeat, whereas β_2AR regulates relaxation of smooth muscles of vascular, uterine, and bronchial tissues. The lipolysis and thermogenesis in adipocytes and skeletal muscle were initially attributed to either β_1- or β_2AR, but were later correlated, at least in animals, with the presence of "atypical" βAR, which more recently have been designated as the β_3AR subtype (Emorine et al., 1989, 1991). For each subtype, additional physiological activities have been reported, including volume regulation of erythrocytes (in avian and amphibian erythrocytes), regulation of ion fluxes in heart, and so on.

Table 3
Correlation of α and β Receptors with Vasoconstriction and Vasodilation

Receptor subtype	Specificity of the Compound: Agonists order of efficiency	Specificity of the Compound: Antagonists	Localization and effect	Mechanism of action
α1 (A,B,C)	Adr ≥ Nor > Iso Selective agonists: methoxamine, phenylephrine	Prazosin	Generally postsynaptic (vasoconstriction)	Modification of Ca^{2+} flux
α2 (A,B,C)	Adr ≥ Nor > Iso Selective agonists: clonidine, tramazoline	Yohimbine Rauwolscine	Presynaptic (retroactive inhibition of the release noradrenaline from sympathetic nerves) Postsynaptic (vasoconstriction) Human and rabbit platelets Human and hamster adipocytes	Inhibition of adenylate cyclase
β1	Iso > Nor ≈ Adr	Practolol CGP 20712-A	Mammalian heart (contractility and speed) Avian erythrocytes	Stimulation of adenylate cyclase
β2	Iso > Adr >> Nor Selective agonist: procaterol	ICI 118551 butoxamine	Smooth, vascular, uterine, and bronchial muscles (relaxation) Amphibian erythrocytes	Stimulation of adenylate cyclase
β3 ("atypical")	Iso = Nor > Adr Selective agonist: BRL 37344		Fat tissue Striated muscles (lipolysis and thermogenesis)	Stimulation of adenylate cyclase

Physiological effects may result from opposing influences on different receptor systems present in the same cells but regulated independently. Adipocytes and cardiac myocytes express α- and β-adrenergic receptors. Noradrenaline, for instance, may at the same time stimulate adenylyl cyclase through binding to β_1AR and inhibit this enzyme through binding to α_2AR.

Subtypes were initially defined on the basis of tissue distribution, but the synthesis of a variety of specific ligands soon supported the detailed classification, based on orders of potencies of the natural agonists adrenaline and noradrenaline and of isoproterenol, studied in 1940 by Konzett, which still remains one of the most powerful synthetic agonists active on all α and βAR subtypes. Selective antagonists have been developed: prazosin is a potent α_1 antagonist, and yohimbine and raulwolscine are strong α_2 antagonists. Propranolol was one of the first, and is still one of the most used, β_1 and β_2 antagonist ("blocker"). A very extensive number of agonists and antagonists are now available for the detailed analysis of the βAR subtypes.

We summarize in Table 3 a few of the most used agonists and antagonists of α- and βAR, together with site of action and biochemical effect *(see below)*, and present in Table 4 a number of βAR ligands tested on all three human βAR.

At this time, three subtypes of α_1- and three of α_2-adrenergic receptors have been identified, mainly by cloning of their genes. The pharmacology of α_1- and α_2-adrenergic receptors has been extensively reviewed recently in two books edited, respectively, by Ruffolo (1987) and Limbird (1988), and will not be further discussed here. We will focus this review on the three β-adrenergic receptors.

Biochemistry of the βAR

Mechanism of Cyclase Activation

Binding of β-adrenergic agonists to the βAR leads to activation of the stimulatory G_s, triggering the replacement of GDP by GTP, and dissociation of G_s into its subunits α_s and βγ (Fig. 1).

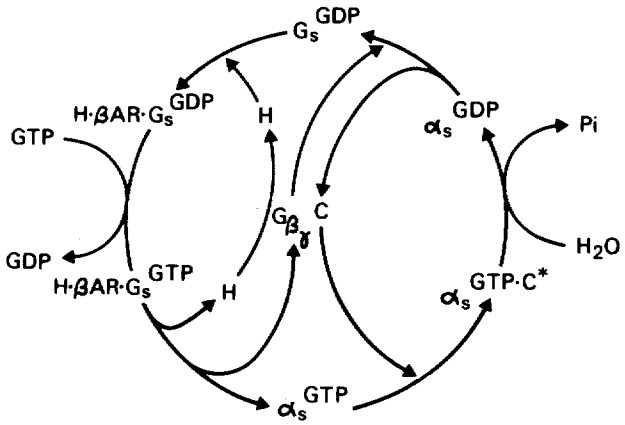


Fig. 1. Activation of adenylate cyclase by β-adrenergic receptors: The agonist (H)–bound β-adrenergic receptor (βAR), during its transient encounter with GDP-occupied G_s (G_s^{GDP}), induces the "opening" of the guanine nucleotide binding site. During this brief period, GDP dissociates from the open site and is replaced by incoming GTP; the agonist-bound receptor then dissociates from the GTP-loaded complex. The receptor-catalyzed nucleotide exchange results in the formation of an active, GTP-bound enzyme α_s, from which the βγ subunits dissociate. The α_s^{GTP} activates cyclase, which produces cAMP. Cleavage of GTP into GDP and Pi returns α_s to a form that recombines with βγ (modified after Gilman, 1986 and Levitski, 1988).

The βAR becomes phosphorylated by one or more kinases, and the activated GTP-bearing α_s goes on to activate adenylyl cyclase. The α_s subunit's GTPase activity converts the bound GTP into GDP and thus returns α_s to its ground state, allowing reassociation with the βγ subunits. At the same time, adenylyl cyclase returns to its basal level of activity (Fig. 1).

Ligands other than adrenaline, noradrenaline, or isoproterenol influence the three components of the β-adrenergic system: sodium may act on the receptor (Minuth and Jakobs, 1986), GTP and fluoride on G_s, and forskoline on adenylyl cyclase. At least two, and possibly as many as four, different kinases may phosphorylate the receptor: protein kinase A or C, β-adrenergic receptor kinase (Hausdorff et al., 1989), and possibly a yet-unidentified tyrosine kinase.

The effects of agonists may be mimicked by the end product of activated adenylyl cyclase:

Table 4
Relative Affinities of β-Adrenergic Ligands for the Three Human β-Adrenergic Receptors

RECEPTORS / LIGANDS	β1		β2		β3	
	Agonist	Antagonist	Agonist	Antagonist	Agonist	Antagonist
(-) ADRENALINE	●		●		○	
ALPRENOLOL		⊗		⊗	○	
BRL 28410	⊗		⊗		⊗	
BRL 37344	○		○		●	
BUTOXAMINE		○		●	(—)	
CGP 12177 (1)		⊗		⊗	○	
CGP 20712A		●		○		○
CLENBUTEROL	○		●		○	
DIHYDROALPRENOLOL (1)		⊗		⊗	(—)	
FENOTEROL	⊗		⊗		⊗	
HYDROXYBENZYLPINDOLOL		⊗		⊗	(NT)	
ICI 118551		○		●		○
IODOCYANOPINDOLOL (1)		●		●	○	
(-) ISOPROTERENOL	⊗		⊗		○	
(-) NORADRENALINE	●		○		●	
OXPRENOLOL		⊗		⊗	⊗	
PINDOLOL		⊗		⊗	⊗	
PRACTOLOL		●		○	(—)	
PRENALTEROL	⊗		⊗		⊗	
PROCATEROL	○		●		○	
(-) PROPRANOLOL		⊗		⊗	(—)	
SALBUTAMOL	○		●		○	
Iodocyanopindolol - diazirine (2)	●		●		○	
Iodoparazidobenzylcarazolol (2)	⊗		⊗		(NT)	

Results were obtained in competition binding experiments with [125] ICYP.

⊗ Equivalent affinity for all three βAR. ● Ligands which display an affinity which is at least five times higher for one of the subtypes than for any other .

○ Ligands displaying an affinity which is at least five time lower for one subtype than for one of the others (1) : Radiolabeled ligands used for direct binding studies, (2) : Ligands used for affinity labeling of the βARs (—) : Ligands which display no binding at concentration of ≤ 10 μM; (NT) : not tested (modified from Marullo, 1990).

cyclic AMP, which acts as a "second messenger" on a variety of proteins that it activates (Hadcock and Malbon, 1989). One of the sites of action of cAMP-dependent protein kinase A is the hormone-sensitive lipoprotein lipase of adipocytes, where a cAMP-activated protein kinase A causes phosphorylation of Ser^{563} (Holm et al., 1988).

The βAR Proteins

Before attempting to isolate βAR, methods were developed for characterizing the proteins by using radioactive ligands covalently bound to the receptor binding site through affinity labeling. The modified antagonists most extensively used for this were iodocyanopindolol diazirine (Burgermeister et al., 1982) and iodoparazidobenzyl carazolol (Lavin et al., 1982). Proof of specificity of the covalent modification was the total prevention by other unlabeled antagonists, such as propranolol. The labeling of receptors was done on either whole cells or membranes, and no purification step was required to visualize the receptors after subsequent solubilization and electrophoresis.

The affinity-labeling results provided the first evidence that the βAR were composed of single polypeptide chains of a mol wt ranging between 52 kDa (turkey erythrocyte) and 65 kDa (mammalian βAR). Proteolysis studies confirmed that a single binding site was labeled. Similar experiments on muscarinic acetylcholine, α-adrenergic, and dopaminergic receptors yielded essentially analogous results, suggesting, long before the cloning of the genes, that all these receptors coupled to GTP-binding proteins might possess similar structural properties.

Purification of the βAR

The purification of the βAR required several enrichment steps because of the scarcity of the receptors on any of the explored cell types. These steps were membrane isolation, solubilization, and purification, first by affinity chromatography, and then by high-performance liquid chromatography (HPLC). An exhaustive review discussing these successive steps has been published recently (Malbon, 1990). We will briefly summarize here the main features of the purification procedure.

Three sources were originally selected for purification of βAR: turkey erythrocytes, frog erythrocytes, and hamster lung. Membranes were prepared from each cell type or tissue, using for the erythrocytes, osmotic shock followed by salt gradient centrifugation, and for hamster lung tissue, conventional membrane preparation.

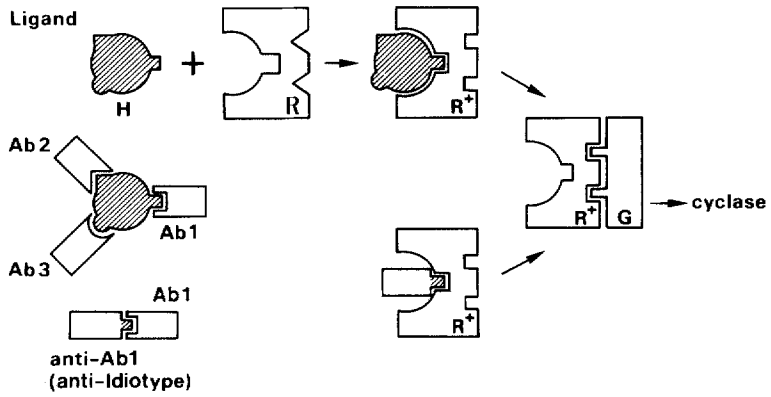
Membranes were solubilized with a variety of detergents. Among these, digitonin was soon recognized at the one that best preserved the ligand-binding activity, using tritiated dihydroalprenolol or radioiodinated cyanopindolol.

Turkey erythrocytes provided an abundant source of an adrenergic receptor coupled to a G_s protein and to adenylyl cyclase. Several milligrams of receptor could be affinity-purified using alprenolol–sepharose (Vauquelin et al., 1977, 1979) from a few liters of turkey blood. The precise pharmacological analysis of this receptor indicated that it was closer to β_1- than to β_2AR: like, although not pharmacologically identical to, the mammalian β_1AR.

The same procedure was later used by Caron et al. (1979) to purify the frog β_1AR and the hamster-lung β_2AR, and by Malbon (1990) to isolate the rat adipocyte β_1AR. Even though affinity chromatography resulted in considerable enrichment of the receptor preparation, the yield was low and contaminant proteins remained present. For these reasons, HPLC was used as a final step to obtain sufficient amounts of pure receptor, suitable for amino acid sequencing.

Immunochemistry of βAR

One of the goals of purifying βAR was to prepare antibodies. These would serve (1) to study topology to determine where the receptor is expressed in the cell and which parts are exposed to the solvent, and to see whether the binding site is recognized, activated, or blocked by antibodies, (2) to investigate whether the subtypes defined pharmacologically could be distinguished immunologically, (3) to follow expression in time and place (ontogeny) of the subtypes,



Scheme I. Schematic representation of the interactions between hormone (H), receptor (R and R+), G protein (G) and antibodies (Ab1, Ab2, and Ab3). The antigenic site is recognized by both the receptor and antibody Ab1.

(4) to study crossreactivity (phylogeny) between BARs from different species.

Antibodies Prepared Against Whole βAR

Several groups reported the preparation of antibodies against βAR. Fraser and Venter (1980) were the first to describe monoclonal antibodies directed against leukocyte βAR. Although these reagents appeared to display some of the expected properties, and were used appropriately, the cellular clones became rapidly extinct and the antibodies could not be studied further.

Polyclonal anti-βAR antibodies were prepared by Couraud et al. (1981), who showed for the first time that βAR could indeed be immunologically activated to stimulate adenylyl cyclase. To further dissect the immune response to βAR, monoclonal antibodies were prepared. Kaveri et al. (1987) thus described three antibodies derived from the spleen of mice immunized against human β_2AR coprecipitated from solubilized membranes with dihydroalprenolol coupled to bovine serum albumin. Using the immuno ("Western") blot procedure, antibodies recognized affinity-labeled receptor and served to analyze βAR expression at the surface of A431 cells by immuno- fluorescence and electron microscopy (Raposo et al., 1989). These antibodies, which were widely used by other groups, also reacted with human β_1AR and with βAR from other species, such as those expressed in rat C6 glioma cells (Kaveri et al., 1987) or chick heart. The three monoclonal antibodies (mABs) were unable to inhibit ligand binding or stimulate cyclase activation.

Chapot et al. (1989) prepared a MAb against the turkey erythrocyte βAR, purified by affinity chromatography on alprenolol–sepharose. This antibody, which also recognized this βAR in immunoblots even after affinity labeling, was used for detecting the receptor by immunofluorescence and reacted with both the 40- and 52-kDa forms of the turkey βAR. It had no effect on ligand binding or cyclase activation.

Antiidiotypic Antibodies Specific for βAR

Introduction. The difficulty of preparing sufficient amounts of purified receptor proteins to generate antireceptor antibodies prompted a number of groups to develop an alternative approach, by which one first prepares antiligand antibodies, which are then used to obtain anti-antiligand antibodies or anti-"idiotypic" antibodies, some of which, by virtue of being directed against the ligand binding site on the antibodies, will crossreact with the receptor for this ligand. (*see* Scheme I). Jerne developed the concept of the idiotypic network to explain the extraordinary capacity of the immune system to respond to any

antigenic challenge, and suggested that antibodies might actually mimic external antigens by constituting their "internal image." Although the structural basis of this molecular mimicry has not been established, a large number of well-characterized antiidiotypic antireceptor antibodies have now been described and are discussed in a good part of a recent volume of *Methods in Enzymology* (Langone, 1990; Strosberg, 1990a). The work done on the antiidiotypic anti-βAR antibodies will be summarized here.

Functional Properties of Anti-βAR Antiidiotypic Antibodies. Using both rabbit polyclonal antialprenolol and mouse monoclonal antialprenolol antibodies, antiidiotypic antibodies were generated and analyzed for their capacity to bind to βAR (reviewed in Strosberg, 1990b; Couraud and Strosberg, 1991). The antiidiotypes were shown to inhibit ligand binding, either to antialprenolol antibodies or to the βAR of turkey erythrocytes (Schreiber et al., 1980). The antiidiotypes directly bound to βAR on the surface of the cells. A monoclonal antiidiotype could be used to visualize downregulation of β_2AR in A431 cells after exposure to the agonist isoproterenol. The agonist-induced decrease in surface ligand binding sites, usually referred to as "sequestration" was accompanied by a strong reduction in binding of fluorescent antibody. This same antibody was used to reveal the β_2AR by immunoblot (Guillet et al., 1985) and to stimulate βAR-modulated adenylyl cyclase. For the rabbit polyclonal antiidiotypes, the results were, as expected, more complex. Schreiber et al. (1980) showed that these could, for some animals, intermittently stimulate cyclase. This apparent "cyclical" behavior correlated with the appearance, in the sera of the rabbits, of antibodies of the "third kind," i.e., antiantiidiotypic antibodies that displayed both antibody activity toward the antiidiotypic antibodies, and ligand-binding properties, as suit "internal image" antibodies (Couraud et al., 1983). The polyclonal antiidiotypic anti-βAR antibodies described by the group of Homcy et al. (1982) displayed only antagonistic activity towards the receptor.

Because of this variability in antibody specificity and the recent availability of purified and recombinant βAR, the antiidiotypic route became less attractive, and preparation of anti-βAR antibodies was done more routinely by immunizing with synthetic peptides or recombinant receptor. This procedure remains, however, a method of choice for other, less advanced systems where receptor has not yet been isolated and characterized at the molecular level.

Antibodies Prepared Against Synthetic βAR Peptides

The cloning and sequencing of the βAR (*see below*) led several groups to synthesize peptides, which were predicted, based on the presumed topology of the βAR, to correspond to solvent-exposed epitopes. The amino- (e_1) and carboxy-terminal (i_4) regions and the second extracellular (e_3) and the third intracellular (i_3) loops were privileged in these studies (*see* Fig. 2 for the locations of e_1, e_2, i_3, and i_4). Although the initial motivation was, for some, to use the antipeptide antibody to prove that the receptor protein was indeed synthesized, the immunological reagent provided the opportunity for further studies. The group of Dixon thus utilized an antibody raised against a C-terminal "wild-type" βAR peptide to characterize the internalization of the wild-type βAR and of a series of deletion mutants of hamster β_2AR (Strader et al., 1987b).

Wang et al. (1989) described the preparation of rabbit polyclonal antibodies against 12 synthetic peptides and used these in immunofluorescence experiments on cells first exposed to reducing and alkylating reagents. The same laboratory also reported that an antibody raised against the region in which rhodopsin interacts with the G_t protein, transducin, would also react with corresponding regions in mammalian βAR, even though the sequences are very dissimilar (Weiss et al., 1987). This very unexpected cross-reactivity contrasted with the findings of Magnusson et al. (1989, 1990), who prepared antibodies against the second extracellular loop (e_3) of the human β_1- and β_2AR, and demonstrated

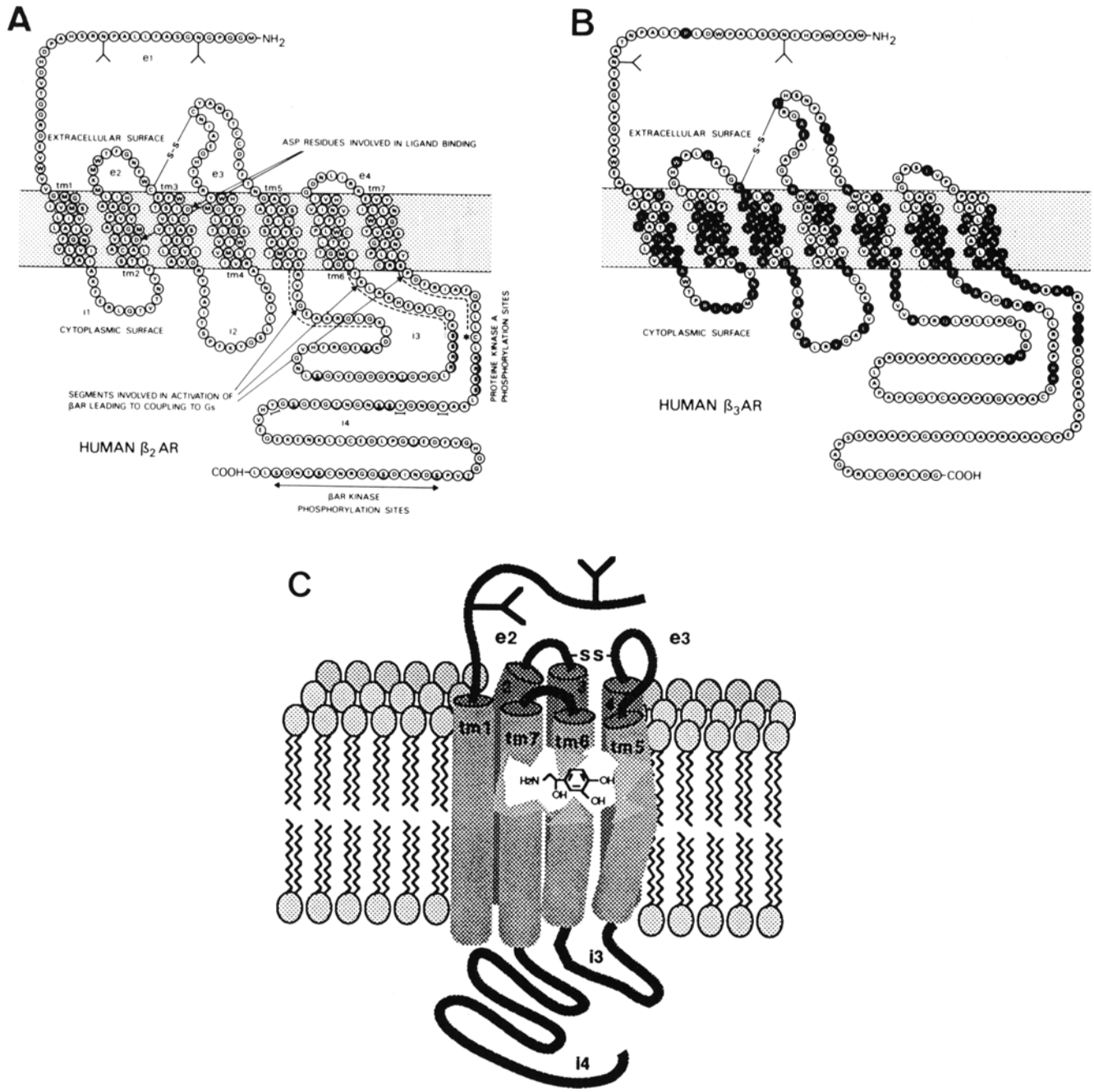


Fig. 2. Topological models for β_2- and β_3AR: primary structure of (**A**) the human β_2- and (**B**) β_3-adrenergic receptor and (**C**) proposed membrane topology of βAR. **A:** The sequences are represented in the one-letter code for amino acids. The single polypeptide chain is arranged according to the model for rhodopsin. The disulfide bond, essential for activity, linking Cys^{106} and Cys^{184} is represented by *-S-S-*. The two *N*-glycosylation sites in the amino-terminal portion of the protein are indicated by Y. The palmitoylated Cys^{341} residue in the *N*-terminus of the i_4 loop is indicated by an asterisk. Potential Ser and Thr phosphorylation sites are underlined. The three Tyr residues found in the i_4 of β_2-, but not of β_1- or β_3AR, are indicated by (—) signs. **B:** The residues that are conserved among the β_1-, β_2-, and β_3 subtypes are indicated in the β_3AR in black circles. **C:** Model for membrane organization of βAR. The ligand-binding region formed by seven transmembrane domains is buried in the lipidic bilayer (modified from Marullo, 1990).

exquisite specificity in recognition of the homologous peptides and corresponding receptors.

Autoantibodies Against βAR in Human Diseases

Introduction. Autoantibodies against membrane receptors have been well documented in a number of diseases (Harrison, 1985), including myasthenia gravis, in which antibodies interfere with the nicotinic acetylcholine receptor, and insulin-resistant diabetes, in which antibodies are formed against the insulin receptor. In the case of βAR, Venter's group reported the presence of autoantibodies in patients with asthma and allergic rhinitis (Venter et al., 1980). Sterin-Borda et al. (1984) described the presence of anti-βAR antibodies in sera of patients with cardiomyopathic complications of Chagas' disease, caused by the parasite *Trypanosoma cruzei*, and Kaveri et al. (1991) confirmed that, at least in one patient, such antibodies were specific for the β_1AR, which they detected by immunofluorescence and by immunoblot. These results prompted several groups to investigate the presence of such anti-βAR antibodies in the more common idiopathic cardiomyopathies (Limas et al., 1989; Magnusson et al., 1990). We will discuss these findings below.

Preparation of Selective Anti-β_1 and Anti-β_2 Antibodies. Magnusson et al. (1989) used computer programs to predict antigenic determinants in the amino acid sequences of the βAR. Two peptides corresponding to homologous sequences in the human β_1AR and β_2AR (e_3 loop, *see* Fig. 2) were used first as immunogens to prepare rabbit antibodies (Magnusson et al., 1989), and then as probes to detect receptor-specific antibodies in sera of patients with idiopathic dilated cardiomyopathy (Magnusson et al., 1990).

Anti-β_1AR Autoantibodies in Idiopathic Cardiomyopathy. Affinity-purified rabbit antibodies, raised against either β_1AR or β_2AR e_3 peptides, were shown by enzyme-linked immunoassay to be specific for the subtypes and to actually bind to cardiac tissue, which, in some immunized animals, appeared to be hypertrophied, as in dilated cardiomyopathy (Magnusson et al., 1989). When the β_1- and β_2AR e_3 peptides were used to screen human sera, it was shown that β_1AR-specific antibodies were present in 13 of 42 dilated cardiomyopathy patients, in none of 17 ischemic heart disease patients, and in four out of 34 healthy blood donors. The affinity-purified antibodies from patients inhibited noncompetitively the binding of iodocyanopindolol to the mostly β_1AR on rat C6 glioma cells. The antibodies recognized β_1AR by immunoblotting done with βAR produced in *Escherichia coli*, and bound *in situ* to human myocardiac tissue (Magnusson et al., 1990). These studies suggested that a subgroup of patients with idiopathic dilated cardiomyopathy developed in their sera autoantibodies directed against the e_3 loop of β_1AR. The functional effect of these antibodies was consistent with the selective β_1AR downregulation reported on failing human ventricular myocardium (Bristow et al., 1989).

Cloning and Expression of the βAR Genes

Cloning of the Genes

Mammalian β_2AR

Affinity-purified hamster-lung β_2AR provided enough starting material to sequence a few tryptic peptides, which led Dixon et al. (1986) to clone the cDNA encoding the β_2AR. The authors showed that the corresponding gene contained no introns, and that the predicted sequence of the protein contained the seven hydrophobic stretches homologous to those seen in rhodopsin and presumed to form the seven transmembrane domains actually observed in bacteriorhodopsin (Henderson and Unwin, 1975; Henderson et al., 1990). Expression of the cDNA in mammalian cells confirmed the ligand-binding properties of the β_2AR. The gene encoding the human receptor was cloned by Emorine et al. (1987) and Kobilka et al. (1987), and is presented in Fig. 2A in a one-dimensional drawing and in Fig. 2C in a spatial representation. It serves now as a model

for all other βAR and, in fact, for all R_7G proteins. When expressed in hamster or rabbit cells, it led to the synthesis of a protein that displayed β_2AR selectivity.

The Turkey Erythrocyte β_1AR Gene

The turkey erythrocyte receptor is a polypeptide of about 52 kDa; this mol wt is lower than the 65–68 kDa of the mammalian βARs. The difference is mainly from the sugar moiety: the turkey receptor contains only complex carbohydrates, whereas the human β_2 receptor contains both complex and oligomannose carbohydrates. This avian βAR is commonly purified as an active 40-kDa protein, apparently cleaved at both ends. The cDNA corresponding to the gene encoding the turkey erythrocyte receptor was cloned using oligonucleotide probes based on the partial sequence of a few tryptic peptides (Yarden et al., 1986). The deduced primary structure of the single polypeptide chain again displayed surprising homology with rhodopsin, particularly in the seven hydrophobic segments. It was, more expectedly, quite homologous to the mammalian β_2AR. The extracellular N-terminus and the intracellular carboxy-terminal regions, and the large cytoplasmic third loop, were much less conserved.

Cloning of the Human and Rat β_1AR Genes

The cloning of the human β_1AR did not actually come as a consequence of the cloning of the human β_2AR. Indeed, screening of a human genomic DNA library with the probe for the hamster-lung or human-placental β_2AR did not yield the expected β_1AR gene. Instead, it was the gene for the serotonin 5HT-1A receptor that was identified this way (Fargin et al., 1988). A sequence comparison revealed that the crosshybridization was most likely attributable to the near-identity of the transmembrane tm6 domain sequences. Only when the 5HT-1 A gene was used as a probe was the β_1AR gene actually isolated by crosshybridization (Frielle et al., 1987).

Two other groups achieved cloning of the human and rat β_1AR respectively, using crosshybridization. Emorine et al. (unpublished results) used the turkey erythrocyte β_1AR gene to clone the human β_1AR. Fraser et al. (1988) used the Drosophila β_2AR gene.

The comparison of the sequences indicates that the β_1AR from different species are more similar to each other than to the β_2- or β_3AR from the same species. This is most obvious in those regions that seem to harbor subtype-specific residues, such as the parts susceptible to phosphorylation by protein kinases or in contact with the G_s protein. Since this is true for each subtype one may conclude that differentiation into the three forms preceded speciation.

Cloning and Sequencing of the β_3AR Gene

The cloning and sequencing of the genes encoding the known β_1 and β_2 subtypes prompted us to reinvestigate the possibility that one or several other genes would encode additional βAR. Additional βAR subtypes had earlier been suggested to mediate the sympathetic control of various metabolic processes in the digestive tract (Bond and Clarke, 1987, 1988), adipose tissue (Wilson et al., 1984; De Vente et al., 1980; Bojanic et al., 1985), and skeletal muscle (Chaliss et al., 1988; Arch et al., 1989). The main common characteristic of these "atypical" βAR resided in the inability to be strongly antagonized by the β_1- and β_2AR antagonists, such as propranolol or alprenolol (reviewed in Zaagsma and Nahorski, 1990). In contrast, new agonists have been synthesized, which are potent stimulators of metabolic rate, adipose tissue lipolysis and thermogenesis, ileum relaxation, and soleus-muscle glycogen synthesis (Bond and Clarke, 1988; Wilson et al., 1984; Chaliss et al., 1988; Arch et al., 1989, 1984), although they have minimal effects at β_1 and β_2 sites.

By using as probes the genes coding for the turkey erythrocyte β_1AR and the human β_2AR, a human gene was indeed isolated, which encodes a third human βAR designated the "β_3AR" (Emorine et al., 1989) (Fig. 2B). Exposure of Chinese hamster ovary (CHO) cells transfected with this gene to adrenaline or noradrenaline pro-

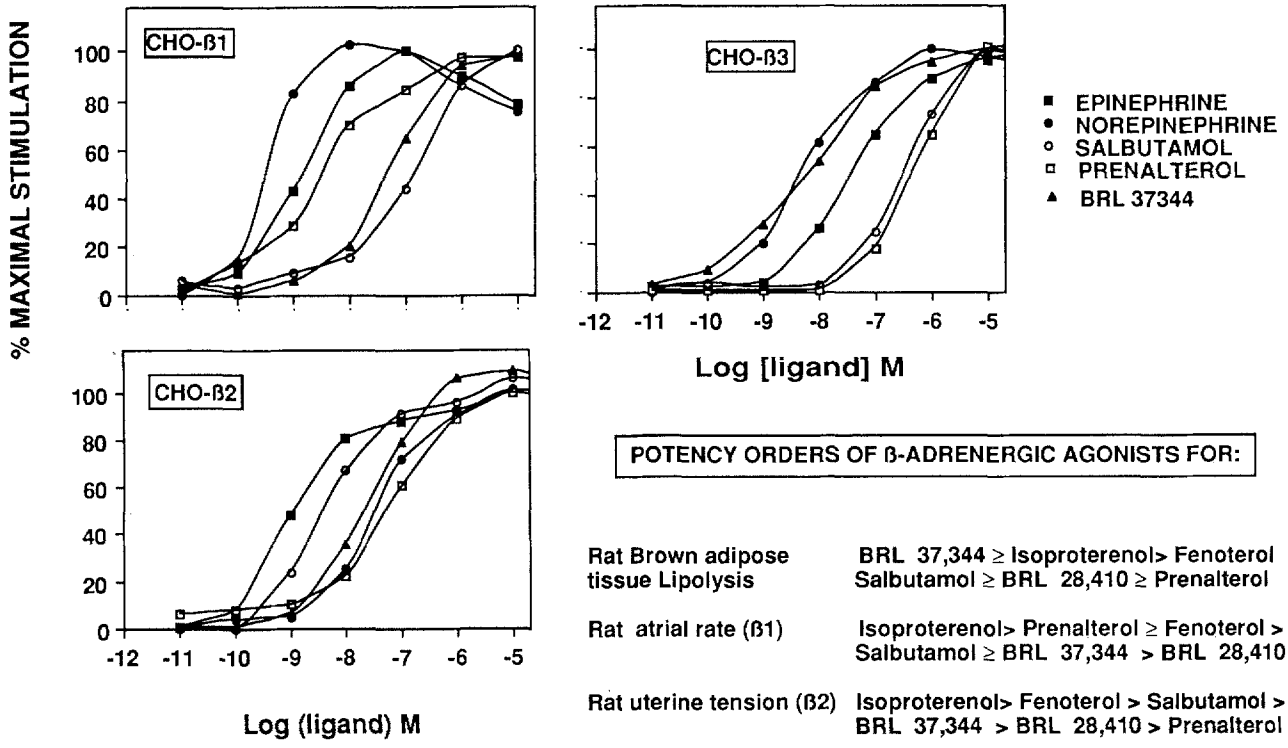


Fig. 3. Potency orders of the three human βAR for adenylyl cyclase stimulation and physiological responses. The three human βAR, each expressed separately in CHO cells, are here compared for their ability to modulate adenylyl cyclase through binding of five different agonists, and for their potency in stimulating rat brown adipose tissue (BAT) lipolysis, a typical β_3 response; rat atrial rate, a typical β_1 response; and rat uterine tension, a typical β_2 response (modified from Emorine et al., 1989).

moted the accumulation of cAMP (Fig. 3); only two of 11 classical βAR blockers weakly inhibited this effect, whereas three others (pindolol, oxprenolol, and CGP-12177) behaved as β_3AR agonists (Emorine et al., 1989; Fève et al., 1991). The potency order for stimulating cAMP accumulation in CHO β_3 paralleled that for modulating metabolic processes in tissues in which atypical adrenergic sites were thought to exist (Fig. 3).

Since most properties of the atypical βAR were described in rodents, we also cloned and sequenced the gene encoding the murine β_3AR (Nahmias et al., 1991). This β_3AR displays 85% homology to its human counterpart, especially in the transmembrane regions. Preliminary data on CHO cells transfected with the murine β_3AR suggest that its pharmacology is similar, but not identical, to that of the human β_3 (Nahmias et al., 1991) and to that of the "atypical" βAR expressed in murine 3T3-F442A cells (Fève et al., 1991). No molecular evidence for the existence of additional βAR genes has been found so far in either human or murine genomic DNA.

Cloning of the Other Components of the βAR-Cyclase System

βARK. Homologous desensitization of βAR has been shown to be associated with phosphorylation catalyzed by two, if not three, different kinases. One of these, βARK, phosphorylates only agonist-occupied receptor. The original name, "β-adrenergic receptor kinase" (βARK), resulted from the initial assumption that it phosphorylated

only this type of receptor. Later on, Benovic et al. (1987) actually showed that other R_7G proteins, including the α_2-adrenergic and the chick heart muscarinic receptors, could also be phosphorylated by this kinase, but the name was retained nevertheless.

This cAMP-independent kinase was purified and characterized by Benovic et al. (1987) and its gene was cloned two years later by the same group (1989). Several isoforms appear to exist. It is possible that each is associated with particular receptors, but this has not yet been established.

Cloning of β-Arrestin. Results obtained with purified βARK established that a cofactor was required to cause significant impairment of the capacity of phosphorylated βAR to bind, and thus to activate, G_s. The existence of such a cofactor had earlier been suggested by analogy with the rhodopsin–phosphodiesterase system in the retina. Phosphorylation of light-activated rhodopsin by rhodopsin kinase indeed required the binding of another protein "arrestin" to prevent activation from the relevant G_t protein, "transducin" (Wilden et al., 1986). Although this arrestin could, in large amounts, serve as a cofactor to βARK, the search for a βAR-specific arrestin was successfully performed by Lohse et al. (1990). The β-arrestin cDNA, isolated using the retinal arrestin cDNA as a probe, turned out to be a homologous protein of 48 kDa. It inhibited the signaling function of βARK-phosphorylated βAR by more than 75%, but had no effect on rhodopsin.

Cloning of G_s. The G_s protein interacting with βAR belongs to a large family of proteins, which includes G_t, or transducin, the protein that couples rhodopsin to phosphodiesterase; G_i, a group of proteins that regulate weak inhibition of adenylyl cyclase and modulate K^+ and Ca^{2+} channels; G_o, the "other" G proteins, G_p, G_q, and G_z, which modulate a number of effectors, including phospholipase C and A_2, and ion channels; and, finally, $G_{s,olf}$, which couples receptors for olfactory substances to olfactory-tissue-specific adenylyl cyclase (Reed, 1990). Although all G proteins are composed of three subunits, α, β, and γ, their polymorphism varies extensively: there is only one gene for α_s vs at least three for α_i. There are four translation products for α_s, but all appear to couple equally well to βAR.

Cloning of Adenylyl Cyclase. Adenylyl cyclase from bovine brain was isolated by affinity chromatography on an agarose matrix containing the cyclase activator, the diterpene forskolin (Pfeuffer et al., 1985). The eluate of this affinity gel could be further purified on immobilized calmodulin or wheat germ agglutinin, confirming that the enzyme was actually a glycoprotein, probably spanning the plasma membrane. The resulting 120-kDa protein catalyzed the synthesis of hormone-dependent cyclic AMP when reconstituted in phospholipid vesicles with homogeneous βAR and G_s (Feder et al., 1986).

After microsequencing several tryptic peptides of purified cyclase, corresponding synthetic oligonucleotides were used to isolate a cDNA that encoded the enzyme (Krupinski et al., 1989). Most of the deduced amino acid sequence of 1134 residues is distributed over two alternating sets of hydrophobic and hydrophilic domains. Each of the two large hydrophobic domains contains six transmembrane segments. The large hydrophilic domains are made of a sequence homologous to a single cytoplasmic domain of guanylyl cyclases, and may contain nucleotide-binding sites.

The topographical homology between adenylyl cyclase and plasma membrane dihydropyridine-sensitive L-type Ca^{2+} and Na^+ channels and transporters initially suggested that the enzyme also played a similar role: it could, for instance, both synthesize and transport cAMP from cells, as is observed in the slime mold Dictyostelium, which uses cAMP as an extracellular signal for chemotaxis, aggregation, and differentiation (Gerisch 1987). No evidence for such a function has been presented so far.

Expression of the βAR Genes

In Mammalian Cells

Natural Expression of βAR in Mammalian Cells. Two cell lines have been extensively used to char-

acterize natural expression of βAR. These are the S49 lymphoma and the A431 carcinoma cells. Considerable knowledge has been obtained from studying S49 mutants that displayed deficiency in coupling G proteins, deficiency in cyclase activation, or absence of particular kinases *(see below* [Regulation by cAMP]). The resulting findings lay at the basis of the model of Receptor-G protein-Cyclase interaction presented in previous sections.

Cell-biology-related information has been obtained mostly from the analysis of the expression of β_2AR in the human A431 cells. Antibodies raised against the receptor were used to follow agonist-induced internalization visualized by fluorescence and electron microscopy (Raposo et al., 1989). The study of pre-and postconfluent cells also underlined the important role of *N*-glycosylation in promoting expression of the βAR at the cell surface (Cervantes-Olivier et al., 1988).

Expression of Recombinant βAR. After the cloning of the cDNA and genes coding for the various βAR, several eukaryotic cells were used to express the corresponding proteins at the cell surface. Several groups used transient expression in COS cells as a convenient way to demonstrate the ability of recombinant βAR to bind ligands, to transduce signal to G protein, and finally to induce cyclase activation (Strader et al., 1987a; Dixon et al., 1988).

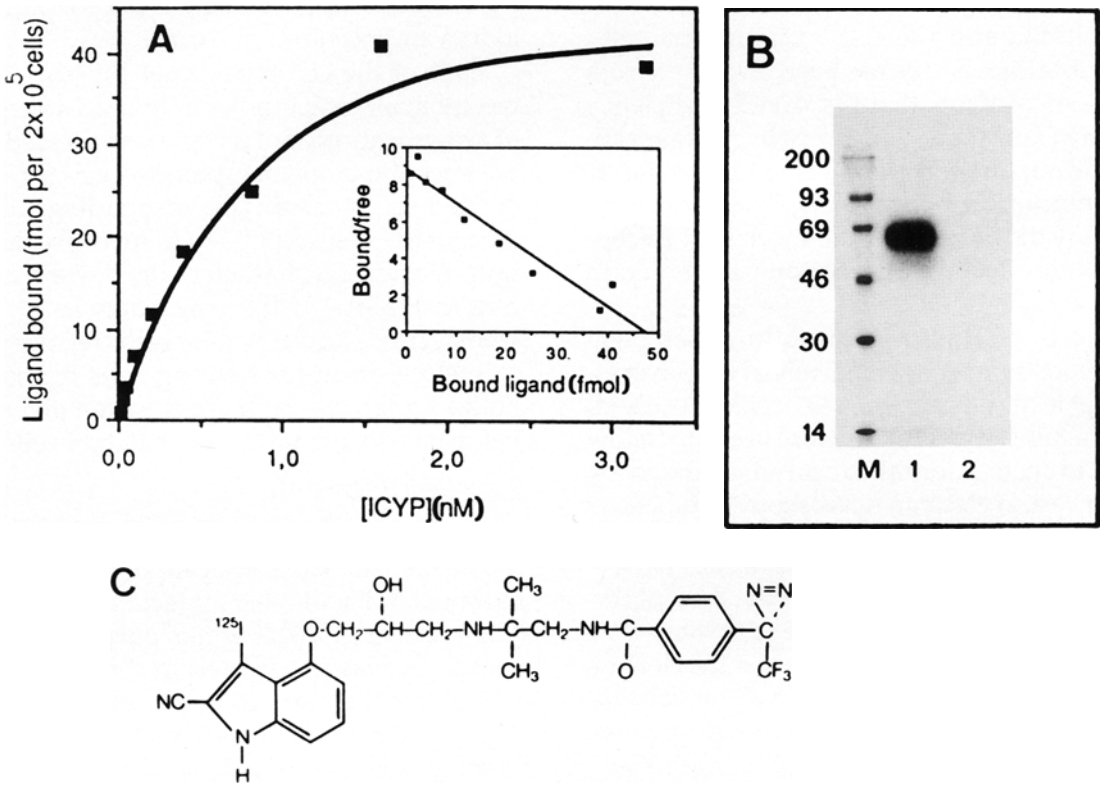
Transient expression requires renewed transfection of cloned cDNA or genes in cells for every experiment. When extensive characterization of the receptor in the cell is required, or when a stable, standardized system is used for systematic screening purposes, permanent cell lines were established. Several cell types were used for this purpose. These include CHO cells (Emorine et al., 1989; Tate et al., 1991), Chinese hamster kidney cells (Dixon et al., 1988), mouse L cells (Fraser et al., 1987). We compared, in Fig. 3, the properties of the three βAR subtypes, each expressed in different CHO cells. The data in Fig. 4 represent additional typical data obtained with β_3AR expressed in CHO cells: A ligand-binding saturation curve and corresponding Scatchard analysis, and an affinity-labeling characterization of the expressed receptor using the photoaffinity label iodocyano-pindolol diazirine.

Each of the cell types used for expression of recombinant receptors has its own advantage: Ease of growth, number of receptors expressed per cell, presence of appropriate G protein or effector, absence of other receptor subtypes responding to the same antagonist, or susceptibility to mycoplasma infection. A common characteristic, however, is relative instability of the constructs used for the transfection, and it is generally recommended to reclone receptor-bearing cells regularly and not to exceed 20–25 passages with a given clone, before starting from a fresh batch of cells.

Expression of βAR in Baculovirus-Infected Insect Cells

The hamster β_2AR was also expressed in high numbers in baculovirus-infected Sf9 insect cells (George et al., 1989). For this purpose, the receptor cDNA was transferred in the viral genome, which was then introduced in the Sf9 cells. This expression system is such that no stably transformed cells can be obtained, and each experiment requires a new infection step. Nevertheless, 1 L of Sf9 cells infected with the right recombinant virus may yield as much as 20–40 nmol of ligand-binding receptor, with specific activities in crude lysates of >30 pmol/mg of protein. Photoaffinity labeling revealed two major species of receptor with M_r 46 kDa (probably unglycosylated) and 48 kDa (possibly partially glycosylated), compared with 65 kDa in CHO cells.

Similar experiments done in other laboratories, including our own, yielded lower numbers of receptors per cell than those reported by George et al. (1989), but still confirmed the potential usefulness of the baculovirus-expressing system for mass production of βAR required for future crystallographic and other physicochemical analyses of the purified protein. We have thus expressed human β_1AR and β_2AR in Sf9 cells (N. Blin, personal communication). Parker et al. (1991) showed that turkey βAR expressed in Sf9 cells could actually stimulate endogenous arthropod adenylyl cyclase. βAR, and also muscarinic



(±)-3-[^{125}I]Iodocyanopindolol diazirine

Fig. 4. Isotherm and Scatchard analyses (inset) of ICYP binding to intact CHO-β_3 cells. **A:** Results represent the mean of two experiments done in duplicate (SEM = 0–9.4%). Under the conditions used, there were 46 ± 5 fmol of ICYP binding sites per 2×10^5 cells (K_d ± SEM = 490 ± 90 p*M*). **B:** Photoaffinity labeling of the β_3AR with [^{125}I] ICYPD alone (lane 1) or in the presence of 10^{-4}*M* ICI 118551 (lane 2). Size (in kilodaltons) of mol-mass markers (lane M) are indicated at the left (Emorine et al., 1989). **C:** Structure of iodocyanopindolol diazirine.

receptors, expressed in this system could be purified and coreconstituted with various purified G proteins in phospholipid vesicles.

Expression of βAR in Microorganisms

Transient and stable expression of recombinant βAR in mammalian cells was extensively used for characterizing ligand binding, G-protein coupling, and adenylyl cyclase activation. These properties do not seem to vary extensively with the cell type, but the establishment and maintenance of cell lines expressing βAR does require considerable effort and constant monitoring to prevent drift to nonexpression and to limit contamination, especially by mycoplasma. Mammalian cell culture, which is slow, also requires expensive equipment and skillful personnel. Finally, last but not least, mammalian cells possess their own receptors and effector proteins, which may interfere with the analysis of the products of the transfected genes.

Expression in E. coli. Because bacteriorhodopsin, which is also an R_7G protein, functions in the membranes of the *Halobacterium halobium*, it was

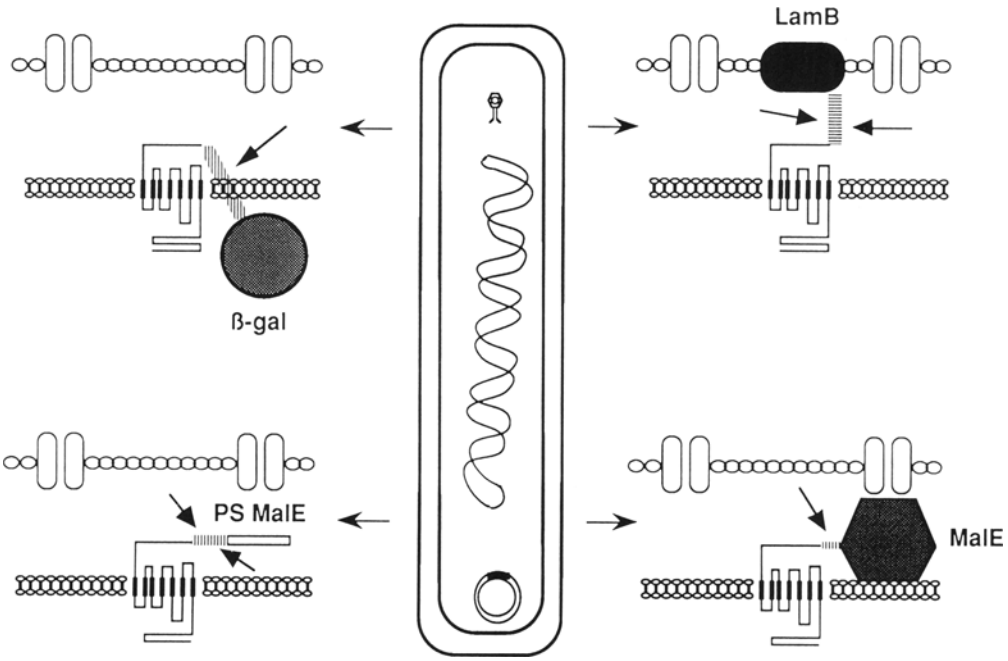


Fig. 5. Fusion partners used for expressing βAR in *E. coli*. We present here four of the fusion partners used for expressing βAR and other R_7G receptors in *E. coli*. βgal stands for β–galactosidase, and is shown in the cytoplasm; LamB is the external outer membrane receptor for phage λ; and MalE is the periplasmic maltose E protein involved in maltose transport. PSMalE is a small *N*-terminal fragment of MalE. The βAR is represented in its characteristic R_7G representation, as a protein mostly localized in the inner membrane. Arrows highlight possible proteolytic cleavage points at the junctions between the two fusion partners (modified from Marullo, 1990).

reasonable to expect that other R_7G might be functionally expressed in the membranes of bacteria, for instance, of *E. coli*. If produced, but not capable of binding ligands, these proteins would at the very least serve as immunogens to generate antibodies. We will discuss here the various vectors that were used to achieve this expression. They are summarized in Fig. 5.

The first experiments were done by fusing the gene coding for the human β_2AR with that for β-galactosidase. Ligand-binding receptor was indeed identified, mostly at the surface of the inner membrane (Marullo et al., 1988). Sizeable amounts of fusion protein were produced, but binding studies, affinity labeling, and specific antibodies revealed that only a small proportion generated active receptor, after the β-galactosidase partner was cleaved off. Each bacteria produced about 25 active receptor molecules, but because of the very low background, the binding properties could be well defined.

This result thus confirmed that unglycosylated receptor displays full ligand-binding properties, that correct disulfide bonding occurs in the bacteria, and that myristoylation and palmitoylation are not essential. Considerable progress was made using a number of vectors containing other fusion partners, including the gene coding for lamB, the receptor for the λ phage, and the gene for MalE, another protein involved in maltose transport in *E. coli* (Fig. 5). The number of active receptors was thus increased to 300 using these vectors, and several other βAR were shown to be functionally expressed in *E. coli* (Marullo et al., 1989; Chapot et al., 1990). In each case, the receptors could be specifically affinity-labeled using

iodocyanopindolol diazirine, and the receptors displayed the expected subtype-selective ligand-binding properties (Fig. 6).

The βAR was produced even when only the signal sequence of the MalE gene or no fusion partner at all was used and the receptors expressed under the control of the t7 polymerase promoter. This allowed us to label receptor proteins intrinsically by growing the bacteria in the presence of the antibiotic rifampicin while adding radiolabeled ^{35}S-cysteine. This procedure was extended to other types of R_7G proteins, including the serotonin 5HT-1A receptor (Bertin et al., in preparation). Fig. 7 shows various intrinsically labeled receptors.

The reason that active receptor proteins mostly displayed the molecular weight corresponding to their own polypeptide chain, without any contribution of the fusion partner, has not been thoroughly investigated. A likely cause may be the high susceptibility to proteolytic enzymes, which would be especially active on the area joining the two fusion partners. This region would be very exposed, since one of the partners is in the cytoplasm (β-galactosidase), in the periplasm (MalE), or in the outer membrane (LamB), whereas the hydrophobic βAR would mostly be in the inner membrane (Fig. 5).

The ligand-binding affinity for agonists is, in *E. coli*, always lower than that observed in mammalian cells. This is because mammalian cells contain a G_s protein that forms with the receptor and the agonist a ternary complex in which the ligand is bound with markedly higher affinity (one to two orders of magnitude) than to the receptor alone. *E. coli* does not contain this G_s. However, reconstitution experiments with recombinant G proteins also expressed in *E. coli*, at least for the α_s subunit, yielded R–G complexes that displayed the same ligand-binding affinity as reported for mammalian membranes (Freissmuth et al., 1991; Bertin et al., 1991).

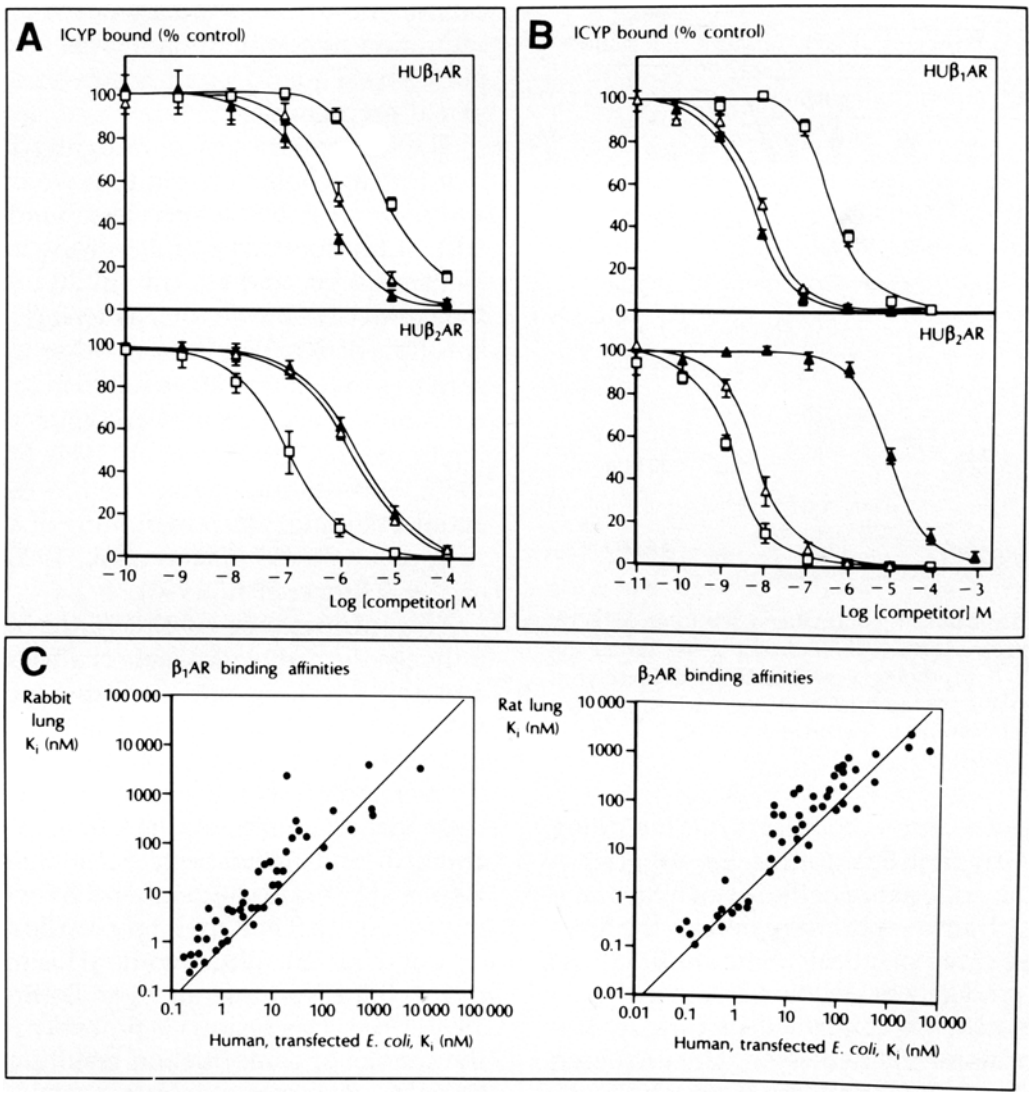
The *E. coli* expression system presents considerable advantages over mammalian culture when it comes to analyze chimeric receptors or mutants. Marullo et al. (1990) thus showed that one can rapidly evaluate in *E. coli* the binding properties of receptor proteins containing tm domains from both the β_1 and β_2 subtypes and obtain results similar to those seen in transient expression in COS-7 cells, which required considerably more time and effort. Breyer et al. (1990), using saturation hypermutation of restricted segments of the human β_2AR, analyzed 25 mutants expressed in *E. coli* and selected for retention of iodocyanopindolol binding activity. Screening was done by directly visualizing bacterial clones that bind radioiodinated ligand (Fig. 8). Binding of agonists was also performed, and mutants were found that retained antagonist, but not agonist, binding. Differential screening may thus provide a way to discover new ligand with either agonistic or antagonistic properties (Breyer et al., 1991).

Expression in Yeast. After it was shown that βAR could be functionally expressed in bacteria, a further step was taken by expressing the human β_2AR in the yeast *Saccharomyces cerevisiae* (King et al., 1990). Competitive ligand-binding experiments again demonstrated that the human βAR retained its characteristic affinities, specificity, and stereoselectivity, this time in yeast. Furthermore, coexpression of β_2AR and the α subunit from the rat G_s led to partial activation of the yeast pheromone response pathway by βAR-specific agonists, demonstrating that the coupled R and α_s complex could actually activate the downstream effector system (King et al., 1990). This system is normally activated through binding of the α or a mating factors to receptors that display the characteristic seven transmembrane domains of the R_7G proteins.

Structure–Function Analysis in βAR

The βAR Ligand-Binding Region

It was initially expected that the βAR ligand-binding site would reside in the extracellular portion of the protein, as is the case for other types of receptors. The homology of the primary structures of the βAR and of the other members of the



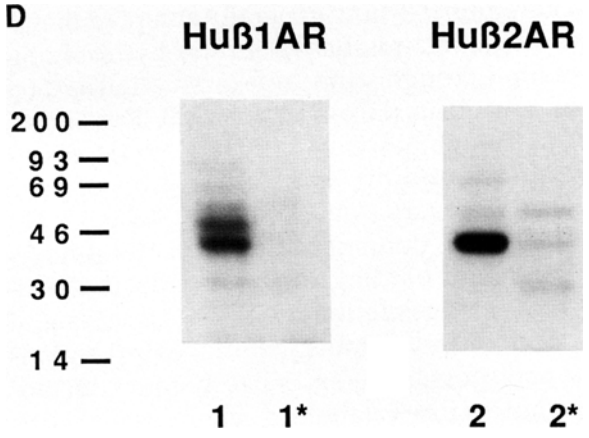


Fig. 6. Competition binding experiments performed on intact *E. coli* expressing β_1AR or β_2AR with β-adrenergic agonists (**A**) and antagonists (**B**). Data represent the mean of two independent experiments performed on the same bacterial culture; bars represent SD (n = 4–8). Nonspecific binding ranged from 1.4 to 6.9%. The order of affinity for both agonists and antagonists corresponds to that determined in mammalian tissue. **C**: Correlation of the affinities determined in bacteria, in rabbit (β_1), or in rat lung (β_2). **D**: Photoaffinity labeling of β_1AR and β_2AR on intact *E. coli*. Experiments were performed in the absence (lanes 1 and 2) or in the presence (lanes 1* and 2*) of propranolol in order to show the specific binding (Marullo et al., 1989; Strosberg and Leysen, 1991).

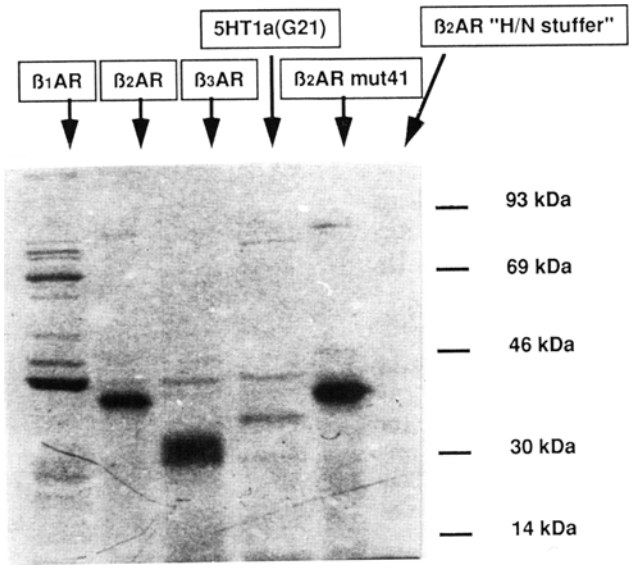


Fig. 7. Intrinsic labeling of bAR and serotonin 5HT-1A receptors expressed in *E. coli*. Receptor genes under the control of the t7 promoter were selectively expressed in the presence of the antibiotic rifampicin, which suppresses all other protein synthesis (Bertin et al., 1991).

R_7G family of receptors coupled to GTP-binding proteins with rhodopsin strongly suggested, however, the importance of the seven hydrophobic, possibly transmembrane regions in the function of these receptors. Proteolytic studies (Ross et al., 1988) and genetic deletion analyses (Dixon et al., 1986) actually confirmed that the hydrophobic core alone retained essentially unaltered βAR ligand-binding capability.

Therefore, it is now generally accepted that β-adrenergic ligands fit within the hydrophobic domains in roughly the same orientation as does retinal in opsin (Dixon et al., 1988; Ross et al., 1988). Though all seven domains are necessary for ligand binding, the contribution of each domain may vary (Marullo et al., 1989).

The actual demonstration that the β-adrenergic ligand-binding region is indeed constituted of residues lining the inside of several, if not all, of the seven transmembrane (tm) regions was made possible by several techniques, including photoaffinity labeling, chemical modification, site-directed mutagenesis, and limited saturation hypermutation. It was strongly supported by sequence comparisons and tridimensional predictions.

The first residues to be recognized as important for catecholamine binding were the Asp^{79} and Asp^{113} residues, which were found unexpectedly in tm domains 2 and 3 of the human and hamster β_2AR, and actually in all other neurotransmitter R_7G receptors, as well (Fig. 2). Substitution of Asp^{113} by most other amino acid residues, except for Glu, resulted in considerable reduction of both agonist and antagonist binding to the β_2AR (Dixon et al., 1988; Fraser et al., 1988). Substitution of Asp^{79} led to reduction of agonist binding, whereas binding of antagonists seemed unaltered (Dixon et al., 1988; Fraser et al., 1988; Breyer et al., 1990).

The importance of the ionic interaction between the carboxylate side chains of the Asp^{79} and Asp^{113} residues and the amino group of agonists was explored in great detail by the groups of Dixon, Fraser, and Lefkowitz, and our own (Breyer et al., 1990). For both residues it is now clear that no single residue can account for the ability to bind ligands *per se*, but, at the same time, Asp^{113} appears alone as essential for determining specificity. Thus, the Asp^{79} can be substituted by a number of residues without losing the capacity to bind β-adrenergic ligands (Breyer et al., 1990, 1991). This residue is probably involved in the change of conformation resulting in activation of G_s. This may also be true for Met^{82}, which may be substituted by Arg without significant loss of antagonist binding, but with reduced agonist binding (Breyer et al., 1991).

Replacement by mutagenesis of an Asp^{113} by a Glu in the hamster β_2AR converted the antagonists pindolol and oxprenolol into agonists (Strader et al., 1989). In the human β_3AR, position 113 is occupied by an Asp, but pindolol and oxprenolol are nevertheless recognized as agonists, rather than antagonists (Emorine et al., 1989).

More striking is the fact that one may actually substitute a Ser for Asp^{113} and observe weak

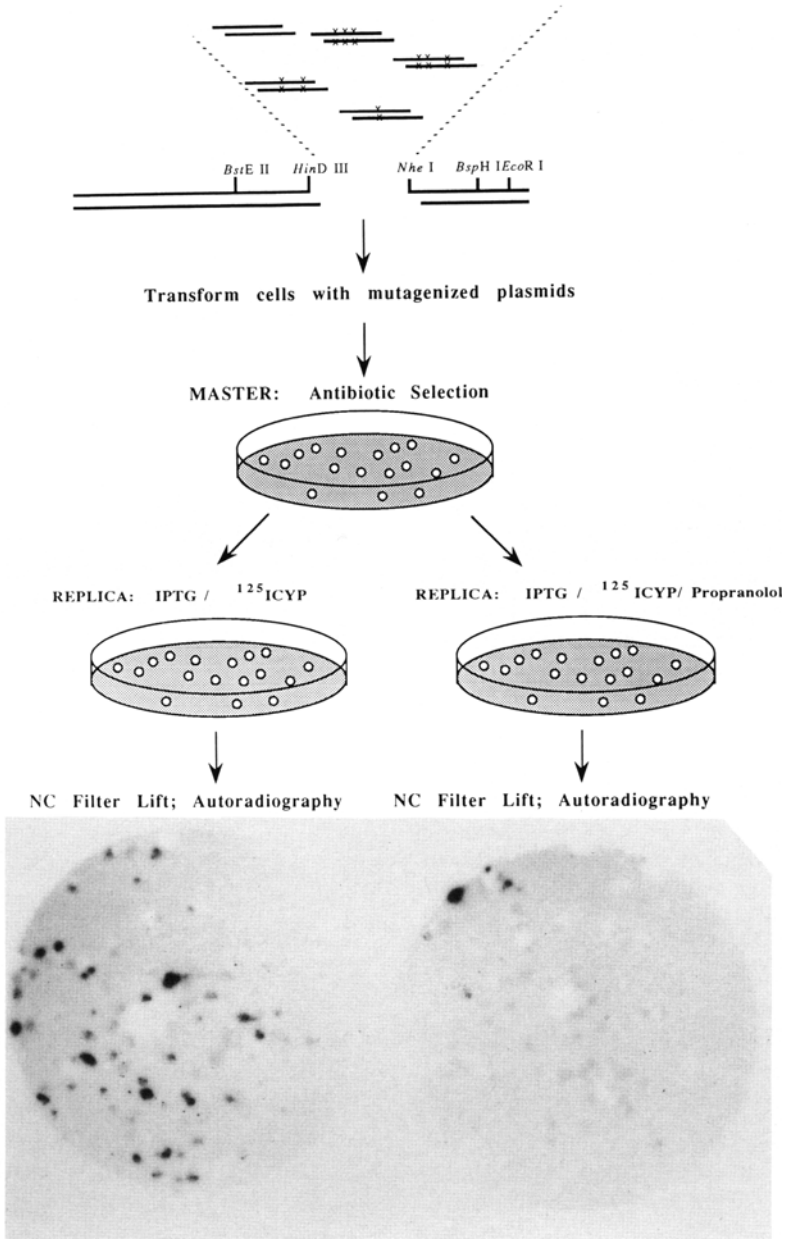


Fig. 8. Mutagenesis/screening strategy. A portion of β_2AR located in the second transmembrane region (position 76 to 83) was treated with mutagen and mutant receptors were screened for ^{125}ICYP ligand-binding activity. A population of oligonucleotide cassettes containing an average of 3.3 random changes throughout the cassette were synthesized. Only the first base of codon 83 was treated with mutagen, as it lies at the end of the cassette. Codon 83 had both a lower frequency and a restricted set of expected mutagenic substitutions: (Gly —> Arg or Gly —> Trp). Cassettes were ligated into a plasmid, thus reconstituting the β_2AR gene. *E. coli* were then transformed and screened for ^{125}ICYP binding activity under inducing conditions. Plates contain 20p*M* ^{125}ICYP alone, or 20p*M* ^{125}ICYP plus 10 μ*M d,l* propranolol. From Breyer et al., (1990).

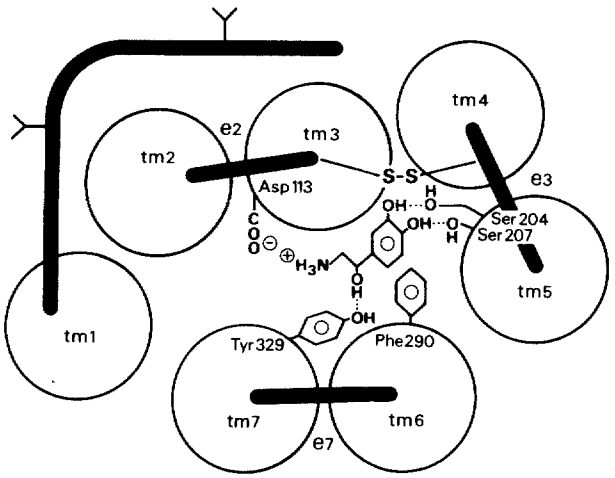


Fig. 9. A composite image of the β_2AR ligand-binding region. Proposed interactions in the ligand-binding region of the βAR viewed from the outside of the cell (modified after Dixon et al., 1988). All seven tm domains are essential for ligand binding. The ligand noradrenaline is shown surrounded by several of the amino acid side chains that were identified, by site–directed or photoaffinity labeling, to be involved in agonist binding. These are Asp^{113} in tm3, Ser^{204} and Ser^{205} in tm5, Phe^{290} in tm6, and Tyr^{329} in tm7. The essential disulfide bond (-S-S-) linking Cys^{106} (extracellular e_2 domain) and Cys^{184} (e_3 domain) is also represented. Asp^{79} (tm2), not represented here, is likely to be more important for signal transmission to G_s than for actual ligand binding, in which it is nevertheless involved. Whether all the interactions with the ligand occur simultaneously or sequentially is not known.

binding and cyclase activation with catechol containing esters and ketones, compounds that do not activate unmodified βAR (Strader et al., 1991). It is likely that in this case it is the hydroxyl side chain of Ser that forms a hydrogen bond with one of the hydroxyls on the catechol ring (Fig. 9).

Other residues are also involved in the binding of the ligands to βAR: Substitution of the Ser residues at positions 204, 207 (tm5), and 319 (tm7) by Ala, although without effect on antagonist binding, appeared to decrease the ability of the βAR to interact with agonists.

Interaction with the catechol ring of the ligand may also involve aromatic residues within the hydrophobic core of the βAR. The Phe^{290} in tm6 and Tyr^{329} in tm7 again appear to participate in agonist, but not antagonist, binding. Photoaffinity-labeling results suggest that His^{89} (tm2) and Trp^{330}, which belongs to tm7, may also be involved in ligand binding (Dohlman et al., 1988; Ross et al., 1988; Wong et al., 1988).

Structure–activity analysis of adrenergic ligands established that both the cationic amino moiety and the catechol ring are important for agonist activity. The negatively charged Asp^{113} would act as a counter ion for the amino group, and the aromatic Phe, Tyr, and Trp side chains may interact with the catechol ring, whereas the hydrophilic Ser residues might form hydrogen bonds with the hydroxyl groups on this same ring (Fig. 9). Very recently, energy transfer has been used to study the interaction between carazolol and the β_2AR (Tota et al., 1991). These fluorescence experiments provided physical evidence that the entire ligand-binding site is buried within the core of the protein.

This view of the ligand-binding site is not necessarily restricted to βAR, since several of the other sequenced members of the R_7G that interact with cationic ligands (e.g., dopamine, acetylcholine, serotonin, histamine) possess in their tm domains several identical residues at homologous positions to those implied in the βAR site. In contrast, the Asp at position 113 is absent from those R_7G receptors whose ligands are not amines, as is the case for the adenosine or tachykinin receptors, other neuropeptide receptors (e. g., bombesin), or the glycohormone LH, TSH, or FSH receptors. In this last group, the ligand-binding site appears to be located in the large 330-residue extracellular N-terminal region. In the recently described putative odorant receptors (Buck and Axel, 1991), Glu or Asp residues are found in both the tm2 and tm3 domains, confirming their important role in ligand binding and signal transmission.

The Sites of Interaction with G Proteins

G proteins are located at the cytoplasmic site of the plasma membrane and act intracellularly.

It was therefore expected that the regions of the βAR likely to interact with G proteins would be the internal loops, the lower portions of the transmembrane regions, and the intracellular C-terminal region. The fact that the turkey erythrocyte β_1AR could still modulate adenylyl cyclase activation via the G_s, even after removal of most of the carboxylic end, lowered the probability that this part played a major role in R–G_s interaction.

The groups of Dixon et al. and Kobilka et al. (1988) demonstrated, using a number of genetically engineered mutant receptors with truncations and deletions in the various parts of hamster or human β_2AR, that two relatively small segments appeared to be mainly responsible for interaction with G_s.

The deletion of the Arg^{222}-Val-Phe-Gln-Val-Ala-Lys-Arg^{229} sequence from the amino terminus of the i_3 loop of the hamster β_2AR resulted in the complete loss of agonist-mediated adenylyl cyclase activity, whereas the deletion of the 327–335 C-terminal portion of i_3 (Dixon et al., 1988) led to markedly reduced adenylyl cyclase stimulation. Similar conclusions were reached when Kobilka et al. (1988) delineated the domains involved in R–G coupling by analyzing chimeric receptors made of domains belonging to both α_2-adrenergic receptor, which inhibits cyclase upon agonist binding, and β_2AR, which stimulates cyclase. Although the changes were of a quite different nature and scope than those generated by Dixon et al.'s limited deletions, the results supported the idea that R–G coupling involves the i_3 loop. Additional studies have also documented the possibility that the much shorter i_2 loop and the part most proximal to the membrane of the carboxy terminus (Liggett et al., 1991) of the receptor may also be involved in the interaction with the G_s protein.

Lechleiter et al. (1990) analyzed the effect of substitutions of parts of m_3 between muscarinic acetylcholine receptor subtypes that either stimulate or inhibit adenylyl cyclase. Again, it appeared that the regions at the amino and carboxy termini of the i_3 loop are critical for interaction of the receptor with the G protein. Activation of a pertussis toxin insensitive G-protein pathway, leading to a rapid and transient release of intracellular Ca^{2+} characteristic of the m_3 subtype, could be specified by the transfer of as few as nine residues from the m_3 to the m_2 receptor. Transfer of no more than 21 residues from the m_2 to the m_3 subtypes were necessary to specify activation of a G protein sensitive to pertussis toxin, coupled to a slow and oscillatory Ca^{2+}-release pathway typical of the m_2 receptor (Lechleiter et al., 1990).

When the sequences essential for G activation were compared for receptors interacting with G_s protein, only relatively little homology was revealed, but Strader et al. (1987a) pointed out that both the *N*-terminus (221–228) and the C-terminus of i_3 in βAR may be predicted to form amphiphilic helices, and this is also the case in the muscarinic receptors. The i_3 loop is much smaller in the peptidergic receptors, such as those for the tachykinins, than in the neurotransmitter receptors. In the putative odorant receptors, the third cytoplasmic loop is only 17 amino acids long in 18 sequences examined. Of these, 11 exhibit a conserved sequence, which may suggest that these putative receptors all interact at this site with the same G protein (Buck and Axel, 1991). In the glutamate metabotropic receptor, which differs considerably from the other R_7G proteins, the i_3 loop is even smaller (13 residues). In contrast, the i_2 loop is much larger and does indeed contain typical amphiphilic-like sequences (Masu et al., 1991). In this receptor one might propose that it is the i_2 loop that is involved in interaction with a G protein.

The distribution of charged and hydrophobic residues in the helices in the relevant portions of i_2, i_3, and i_4 may actually determine G-protein specificity. Support for this hypothesis was found in the studies involving mastoparan. Mastoparan and similar small peptides can directly activate G proteins, possibly by mimicking portions of the receptor involved in R–G interaction, and this mimicry could find its origin in the fact that mastoparan, when incorporated in phospholipid micelles, adopts an amphiphilic helical conformation (Higashijima et al., 1988).

The Role of Glycosylation and Other Posttranslational Modifications

Glycosylation

The βAR contain significant amounts (up to 30% by apparent mol wt) of *N*-linked carbohydrate, which may be only complex, as in the turkey erythrocyte β_1AR (Cervantes-Olivier et al., 1985), or both complex and oligomannosidic (Cervantes-Olivier et al., 1988; Stiles et al., 1984). No evidence has been reported for *O*-glycosylation.

The potential consensus sites for *N*-glycosylation are found in both the amino-terminal and carboxy-terminal regions, as well as in several other parts of the protein; however, all indications suggest that in βAR only the *N*-terminal sites are actually glycosylated. In other R_7G receptors, the second or third extracellular loops were also found to contain *N*-glycosylation sites.

Glycosylation and Ligand Binding. Biosynthesis in the presence of inhibitors of partial (monensin) or complete (tunicamycin) *N*-glycosylation or enzymatic removal of the carbohydrate portion (Cervantes-Olivier et al., 1988) have no effect on ligand binding. Mutants of the hamster β_2AR in which the Asn^6 and Asn^{15} belonging to the two N-terminal consensus glycosylation sites are independently or simultaneously removed still show normal binding of both the agonist isoproterenol and the antagonist iodocyanopindolol (Dixon et al., 1988). Finally, each of the three human βAR displays normal and subtype-selective binding activity when expressed in *E. coli*, even though N-glycosylation is not expected to occur in bacteria, as confirmed by the molecular weights of the βAR, which correspond exactly to those expected for the polypeptide chains only. These findings thus established that glycosylation plays no role in binding of the ligands.

Other Roles for Glycosylation. The study of the expression of the human β_2AR expressed in A431 cells grown in the presence of monensin or tunicamycin revealed that transfer of the βAR through the cell and expression at the membrane may be markedly affected by changes in glycosylation (Cervantes-Olivier et al., 1988). It is also likely that carbohydrates may protect the receptor from proteolysis; this is particularly important, since these proteins have a very low turnover. Upon desensitization, βAR might undergo, when reaching endosomes, partial deglycosylation, which might increase susceptibility to degradation in lysosomes. Reglycosylation would signal return to the membrane before proteolysis has occurred. The sensitivity of the βAR polypeptide chain to enzymatic cleavage was well established when expressed in *E. coli*, in which no glycosylation occurs and where most receptor produced in the bacteria is inactive and probably proteolyzed before reaching the membrane (Marullo et al., 1988,1989; Chapot et al., 1990; Breyer et al., 1990).

Disulfide Bonds and Receptor Function

The βAR contain several cysteine residues, and it is likely that some might form disulfide bonds. The essential role of at least one of such bonds in ligand binding was established in 1979 by Vauquelin et al., who showed that reducing agents destroyed the activity of the β_1AR of turkey erythrocytes, but that the presence of agonists or antagonists prevented the loss of binding.

The group of Dixon et al. (1988) confirmed the role of Cys^{106} and Cys^{184} in maintaining ligand-binding activity, while showing that 10 of the other 12 Cys residues did not appear to be essential for βAR function.

Because Cys^{106} and Cys^{184} are completely conserved among all the R_7G receptors sequenced so far and loss of either or both of them results in similar effects, Dixon et al. concluded that these are the Cys residues that form the essential bond predicted to exist nearly a decade earlier by Vauquelin et al. (1980).

The role of this bond, which links the e_2 and e_3 domains (Fig. 2), is likely to reside in stabilizing a conformation that would shape the entrance of the ligand-binding pocket, thus providing, together with a number of negatively charged Glu and Asp residues, a favorable environment for the approaching positively charged catecholamine (Fig. 9).

Palmitoylation and Myristoylation

Palmitoylation. Another cysteine residue appears to play a role in stabilizing receptor interaction with the plasma membrane. This is Cys^{341} (Fig. 2) which, upon careful analysis by O'Dowd et al. (1989a), turned out to be palmitoylated in the hamster β_2AR. Since this residue is well conserved in the R_7G family of proteins, one may assume that this additional postsynthetic modification is also important for receptor function, even though its removal, by deletion, did not appear to affect ligand binding (Dixon et al., 1988). It may contribute to stabilizing R–G_s interaction by anchoring the C-terminal region in the membrane.

Myristoylation. One of the first observations made on the primary structure of the βAR was that these proteins could not be directly sequenced by Edman degradation. A "blocked" *N*-terminal residue was reported for several of the R_7G receptors, but the blocking group was not identified further. The sequences predicted after cloning of the corresponding genes consistently revealed the presence of an *N*-terminal Met-Gly dipeptide, which has been found to be myristoylated in a number of other proteins. Since this modification renders the α-amino group unavailable to the isothiocyanate reagent used in the Edman degradation, one may suggest that this is also what happens in the R_7G proteins. The role of this putative myristoylation has not, as yet, been elucidated.

Phosphorylation

Scrutiny of the sequences of the βAR reveals the presence, in varying numbers and positions, of Ser and Thr residues surrounded by positively charged Arg and Lys residues (Fig. 2 and Table 5). Such groupings of amino acids are the hallmark of sites of phosphorylation by protein kinases.

Two of these sequences characteristic for protein kinase A or C are found in the β_2AR at positions 257–264 and 341–348 (Table 5). One of these is found in a homologous region in the β_1AR; the β_3AR does not possess analogous residues in this part of the protein. In the C-terminal region, one finds in β_2 and β_1AR, respectively, 11 and 10 Thr and Ser residues; only three are seen in the β_3AR. These residues could be the target of another kinase, βARK, which is cAMP-independent.

Finally, the β_2AR, but not the β_1- or β_3AR, contains in its C-terminal region three tyrosine residues, one of which, Tyr^{366}, could well be the target of phosphorylation by a tyrosine kinase (Table 5). Evidence for the regulatory role of Tyr^{350} and Tyr^{354} has been provided by Valiquette et al. (1990).

It is not known whether the βAR actually undergoes phosphorylation in its "ground state," that is, in the absence of agonists; we will therefore discuss phosphorylation in the following section as a regulatory mechanism for posttranslational modification of the βAR.

Regulation of βAR Expression and Function

The simultaneous presence of three different βAR subtypes in a single cell type, such as adipocytes, clearly illustrates the complex regulation that each of these receptors must undergo to explain exquisite specific function in response to the same agonists.

Regulation probably occurs at three levels (Table 6): At the level of the genes, where several different regulatory sequences have been identified in the 5' and 3' flanking regions and where cAMP concentrations have been shown to regulate the transcription into mRNA; at the level of the proteins, where posttranslational modifications, such as various types of phosphorylation, occur in response to agonist binding; and finally at the level of the cell, where the onset of desensitization and downregulation starts with microaggregation, sequestration, and internalization of the receptor–agonist complexes.

In this section, "desensitization" refers to decrease in receptor function and "downregulation" to decrease in receptor expression. These phenomena are described as "homologous" when induced by the relevant agonist and "heterologous" when caused by compounds that do not act through the receptor.

Table 5
Potential Sites of Phosphorylation of Human βAR Subtypes

	Potential PKA or C site, third cytoplasmic loop		Potential βARK sites Carboxyl tail	
			Total	S + T
β2AR	^{257}GLRRSSKF	^{341}CLRRSSLK	73	11
β1AR	^{307}GKRRPSRL	^{393}CARRAARR	85	10
β3AR	^{275}CGRRPARL	^{363}CGRRLPPE	40	3
	Potential Tyr kinase site			
β2AR	^{348}KAYGNGYSSNGNTGEQSGYHVEQEKEN			
β1AR	^{399}RRRHATHGDRPRASGCLARPGPPPSPG			
β3AR	^{367}PPEPCAAARPALFPSGVPAARSSPAQP			

Table 6
Regulatory Mechanisms Involving βAR

Regulation at the level of the genes	Regulation at the level of the receptor proteins	Regulation at the level of the cell
Binding of effectors to signal sequences in the noncoding 5'and 3' flanking regions, e.g., GRE in the β_2AR, CRE Levels of mRNA	Phosphorylation of Ser or Thr residues by protein kinase A or by β-adrenergic receptor kinase, or of Tyr residues by an undentified tyrosine kinase Interaction with different G proteins	Sequestration, internalization, degradation, or reexpression at the cell surface

Regulation at the Gene Level

Regulatory Sequences in the βAR Genes

Each of the intronless genes coding for the three βAR subtypes is located on a different chromosome (the β_1-, β_2-, and β_3AR are located on human chromosome 10, 5, and 8, respectively), clearly suggesting that their regulatory environment is quite different as well. The comparison of the nucleotide sequences of the 5' flanking regions of the β_1-, β_2-, and β_3AR (Fig. 10) reveals that the genes contain a reverse sequence for the CAAT-box binding protein that, for β_2- and β_3AR, lies a few tens of nucleotides upstream from an A/T-rich region reminiscent of a TATA box. Additional CAAT boxes are found in the β_1- and β_3AR genes. Several sites for initiation of mRNA synthesis have actually been localized in the β_2AR gene (Emorine et al.,1987; Kobilka et al., 1987), and it is likely that transcription probably begins at homologous positions in the other βAR genes.

An ATG translation initiation codon followed by a short open reading frame, potentially encoding 16–19 amino acid residues, is found between the mRNA start sites and the structural gene of the β_2- and β_3AR, respectively. Removal of this ATG codon from the β_2AR gene increases receptor expression tenfold. Potential recognition sites for transcription factor NF1 and for proteins binding to the CACCC sequence are also common to both genes. Glucocorticoid- and cAMP-responsive elements (GRE and CRE), as well as sites for transcription factor Sp1, are found in the β_1- and β_2AR promoters (Emorine et al., 1991).

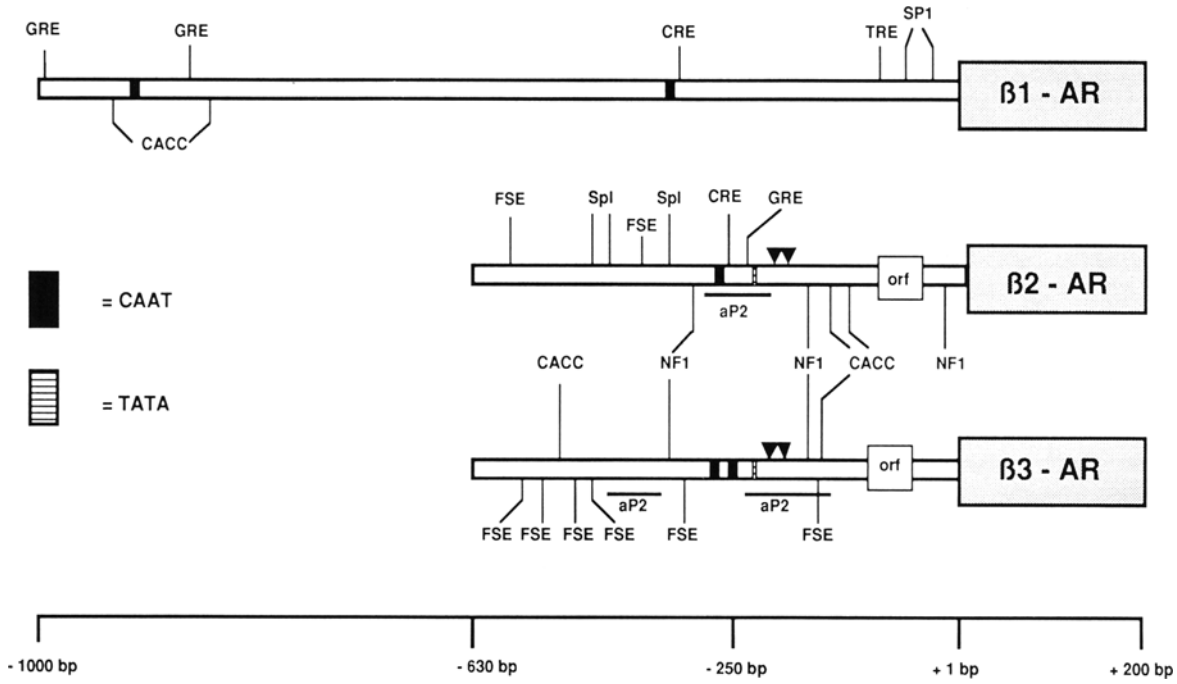


Fig. 10. Schematic structures of the human βAR promoters. We compare here the schematic and partial structures for the β_1-, β_2-, and β_3AR promoters. The coding regions are indicated by shaded boxes. GRE = glucocorticoid-responsive element; CRE = cyclic AMP responsive element; TRE = TPA-responsive element; FSE = fat-specific element; NF1, Sp1, and CACCC are recognition sites, respectively, for transcription factors NF1 and Sp1, and proteins binding the CACCC sequence; and aP2 = sequences partly homologous to the promoter of the gene encoding adipocyte P2 lipid-binding protein. The more commonly found CAAT and TATA boxes are also indicated (Emorine et al., 1991, unpublished observations).

Two regions of the β_3AR gene display close to 55% sequence homology with part of the promoter of the gene for the adipocyte P2 lipid-binding protein. Similar sequence homologies also exist within the β_2AR promoter region, but are restricted to shorter fragments. Murine 3T3-F442A adipocytes express several nuclear factors that bind specifically to the aP2 promoter fragment. Some of these factors recognize a 14-nucleotide-long "fat-specific element" (FSE), which also occurs within promoters of several genes that participate in adipose differentiation (Phillips et al., 1986; Bandhari et al., 1988). Approximations of such motifs (65–75% homology are found in the human βAR promoters: six in the β_3 and two in the β_2AR gene. Recognition sites for other adipocyte factors have been shown to exist in the aP2 promoter (Distel et al., 1987) and may also occur in the β_3- and β_2AR.

Multifactorial Control of Subtype Expression

The structural similarities and differences between the promoters of the β_1-, β_2-, and β_3 genes are probably reflected by specific actions of regulatory factors on the level of expression of each βAR gene. During tissue development, several factors and regulators of transcription interact to modulate the level of expression of various genes. Some factors may be cell- and differentiation-dependent and thus allow selective regulation of adrenergic sensitivity in accordance with cellular function.

Basal expression of βAR genes involves ubiquitous factors, such as those for CAAT and TATA elements, and may be further regulated by several modulators of transcription. For many other genes, in which regulatory elements (NF1, GRE,

CRE, and the like) are often present as close inverted repeats, efficient modulation of mRNA synthesis requires dimerization of transcription factors. An interesting observation is that in the β_1-, β_2-, and β_3 genes these same types of sequences appear as monomers, not as inverted repeats. These features of the β_1-, β_2-, and β_3AR promoters, and the occurrence of an additional ATG codon upstream from that for the receptors, may thus result in low levels of β_1-, β_2-, and β_3AR basal expression. Because many transcription factors can interact synergistically to generate their effects, it is possible that cooperation of two heterologous factors, instead of dimerization of a unique factor, is required for efficient modulation of βAR gene expression. Such modulation would require the cooperation of specific factors whose presence depends on tissue origin and environmental stimuli. Some regulatory elements might specifically modulate the expression of a single βAR subtype, whereas other factors would be active on all three. Even in this latter case, the possibility of positive or negative interactions among various modulators of βAR gene expression could lead to quantitative differences in the expression of each βAR gene. Such mechanisms could allow various specialized cells to modulate their adrenergic sensitivity independently in response to changes in hormonal and environmental conditions. The differences between the coding and flanking regions of the three human βAR subtypes are likely to be reflected in the ability of glucocorticoids and other gene-modulating agents to regulate the expression of the genes differentially.

The comparison of the mouse, hamster, and human β_2AR reveals a significantly higher homology between the 5' and 3' flanking regions than between the coding regions of these receptors, confirming the importance of the regulatory elements present in the noncoding regions. Although the human and murine β_2AR contain numerous GRE sites, both in the flanking and the coding regions, only one was found in the human β_1AR noncoding region and none in the β_3AR gene.

Regulation by Dexamethasone

Heterologous hormonal regulation of the β_2AR by steroids has been abundantly investigated, and it had been suggested that control of β_2AR gene transcription was the underlying mechanism. The availability of the cloned gene allowed this hypothesis to be verified.

In SV40 Transformed, Transfected Lymphoid Cells. Emorine et al. (1987) transfected the human β_2AR gene, under the control of its own promoter, into SV40 transformed rabbit lymphoid cells, which normally do not express this type of receptor. The authors showed that dexamethasone considerably increased β_2AR-regulated adenylyl cyclase activity, in line with the presence in the sequence of the gene of the GRE, likely sites of interaction with the glucocorticoid receptor. Replacement of the coding region of the βAR by the reporter enzyme chloramphenicol acetyl transferase did not allow, however, the determination of which of the GRE sequences in the β_2AR gene was actually responsible for regulation by dexamethasone (Emorine, unpublished results).

The transcriptional activation of the β_2AR gene by glucocorticoids was confirmed by Collins et al. (1988), who used the hamster β_2AR gene as a probe to demonstrate the increase of mRNA after treatment of hamster smooth-muscle cells with triamcinolone.

In Adipocytelike Differentiated 3T3-F442A Cells. The murine adipocyte-like differentiated 3T3-F442A cells express the three βAR subtypes. Fève et al. (1990, 1991) have shown that in the fibroblastlike preadipocyte form, these cells express low amounts of β_1AR mRNA. Upon differentiation, these β_1AR mRNA levels increased about fivefold and high amounts of β_3AR mRNA were also produced. Low levels of β_2AR mRNA appeared as well. When dexamethasone was supplied to preadipocytes and maintained throughout the differentiation process, the β_1- and β_3AR messages were totally depressed, but the β_2AR expression was stimulated about 40 times above that of

preadipocytes. In contrast, when dexamethasone was supplied to fully mature adipocytes, it stimulated β_2AR mRNA levels by a factor of only 2–3, although it still depressed β_1- and β_3AR mRNA expression. In smooth-muscle tissues, glucocorticoids also stimulated the expression of β_2AR mRNA two- to threefold (Fève et al., 1991).

Dexamethasone thus had a much stronger potency to stimulate β_2AR expression in preadipocytes than in differentiated adipocytes or smooth-muscle tissues. As suggested by the presence in the β_2AR promoter region of a GRE in the vicinity of aP2 promoter-like sequences, this could reflect synergistic cooperation of glucocorticoid receptors with preadipocyte-specific factors and/or other factors active on the β_2AR promoter. Positive interactions beween glucocorticoid receptors and transcription factors NF1 and Sp1 or those binding to CACCC and CAAT boxes have already been observed.

Cooperation of several factors binding to the aP2 promoterlike sequences of the β_3AR gene could be sufficient to strongly promote β_3AR expression during adipose differentiation. No sequence matching those proposed for negative regulatory GRE was detected in the β_3AR gene, but the nucleotide sequences involved in this type of regulation have not been extensively studied. The inhibitory effects of dexamethasone on β_3AR expression could also result indirectly from its action on other genes, whose products may control the transcription of the β_3AR gene.

Regulation by cAMP

It is generally recognized that βAR turnover is very low. The remarkable stability of these receptors contrasts with the exquisite sensitivity to various regulatory factors. We have already underlined the crucial role of agonists in this regulation, which will be further developed when we discuss postsynthetic modification of the proteins and, most prominently, phosphorylation (*see below*).

Although specific short-term desensitization of the β_3AR, through action of agonists, is likely to act at the level of the proteins, prolonged action of agonists also results in the decrease of βAR mRNA levels, and this effect is probably a result of the increase of intracellular cAMP. Other agents that enhance cAMP levels, through alternative R_7G systems (leading to "heterologous" desensitization) or by directly acting on G protein (cholera toxin) or cyclase (forskolin), also stimulate sharp decreases in mRNA levels (Hadcock and Malbon, 1988). Hadcock et al. (1989) investigated this agonist-promoted downregulation of βAR mRNA by studying S49 mouse lymphoma variants either having mutations in G_s, such as "unc" (in which $G_{s\alpha}$ is uncoupled from the βAR), "cyc$^-$" (no $G_{s\alpha}$), "H21a" ($G_{s\alpha}$ coupled to receptor, but not to cyclase), or deficient in the cAMP-dependent protein kinase A, the "kin" mutant. The authors concluded that the kinase activity was required for downregulation of mRNA, although elevated cAMP is not, and that functional R–G_s coupling is essential, since the unc or cyc$^-$ variants did not downregulate mRNA in response to agonist.

Regulation by a cAMP-Independent Pathway

The most surprising conclusion from these studies, based on DNA excess solution hybridization, was that the H21a cells were capable of agonist-promoted downregulation of βAR mRNA levels even in the absence of agonist-induced cyclase, suggesting the existence of another G_s-responsive effector in these cells.

Maguire and Erdos (1980) had shown earlier that regulation of magnesium transport requires functional R–G_s coupling, but not G_s–cyclase coupling. Calcium-channel activity, also regulated via βAR–G_s coupling, had been reported not to require cyclase activation, but to be regulated by protein kinase A (Yatani et al., 1988; Yatani and Brown, 1989; Mattera et al., 1989). Hoffmann (personal communication) recently suggested that in smooth muscle, but not in cardiac tissue, G_s could be directly coupled to a slow L-calcium channel.

Regulation at the Level of the βAR Proteins

Several structural features of the βAR outlined above (Cloning and Expression of the βAR Genes) suggest that regulatory mechanisms could act at the level of the βAR proteins, which are targets for glycosylation, myristoylation, palmitoylation, and, above all, phosphorylation. In addition, various parts of the βAR were shown to interact specifically with GTP-binding proteins. We will discuss here the role of these various sequences.

Regulation of Ligand Binding by G_s Protein

In the ternary complex of agonist, receptor, and G_s, the agonists are bound with markedly higher affinity than they are to the receptor alone. This increased affinity is not observed in the absence of G_s, as is the case in the S49 cyc⁻ mutant or when the βAR is expressed in *E. coli*, but may be restored by reconstitution with recombinant α_s and added βγ (Freissmuth et al., 1991). The residues responsible for R–G interaction have been identified both in the receptor (*see above* [The Sites of Interaction with G Proteins]) and in the G protein, and corresponding G-protein-derived peptides have been shown to inhibit the interaction (Hamm et al., 1988).

We still have no good explanation for how ligand binding leads to G_s activation and how this correlates with increased affinity for agonists. This possibly bidirectional interaction may reflect mutual conformational effects. A number of other R_7G proteins are coupled to G_s and may also undergo GTP-induced changes in agonist affinity. Scrutiny of the regions in β_2AR known to interact with G_s did not reveal telling homologies in sequence, but the i_3 loops were generally shorter in these R_7G proteins than in receptors coupled to other G proteins.

Regulation of Receptor Function by Phosphorylation

Exposure of cells to agonists leads to loss of sensitivity, in part because of receptor sequestration, a rapid and transient event, and receptor downregulation, a slower event that requires longer exposure to agonists. Lefkowitz et al. (1990) have recently summarized current developments in understanding mechanisms of rapid desensitization involving receptor phosphorylation, which actually precedes sequestration and internalization (Fig. 11).

Binding of agonist to the receptor activates G_s and thus adenylyl cyclase, leading to increase of cAMP concentration. This would in turn activate PKA (Protein Kinase A) or PKC (Protein Kinase C), which would phosphorylate βAR at Arg-Arg-Ser-Ser sites adjacent to the regions of the receptor involved in coupling the G_s.

At high concentrations of agonist, βARK may phosphorylate the Ser and Thr residues of the C-terminus of agonist-occupied β_2AR and possibly of β_1AR, thus contributing to disruption of coupling between the receptor and G_s; this may require the presence of β-arrestin, which may act as a cofactor. The exact specificities of βARK and β-arrestin remain to be determined.

Thus, at least two, and possibly as many as four, types of protein kinases appear to participate in receptor regulation via phosphorylation. βARK would mediate homologous desensitization that is caused by specific agonists and act only on agonist-occupied receptor, and PKA or PKC would mediate heterologous desensitization caused by various classes of cyclase activators and active on different receptor systems. The role of the tyrosine kinase is not yet defined, but it could act on a tyrosine residue close to several charged residues (*see* Table 5) in the C-terminus of β_2AR.

Although a small amount of desensitization may still occur even after receptor phosphorylation is completely prevented, either by mutagenesis (Hausdorff et al., 1989) or by specific kinase inhibition (Lohse et al., 1989), it is likely that phosphorylation plays a major role in the onset of loss of responsiveness to agonists.

Phosphorylation precedes sequestration,which may be followed by either reexpression of the receptor at the surface or downregulation by

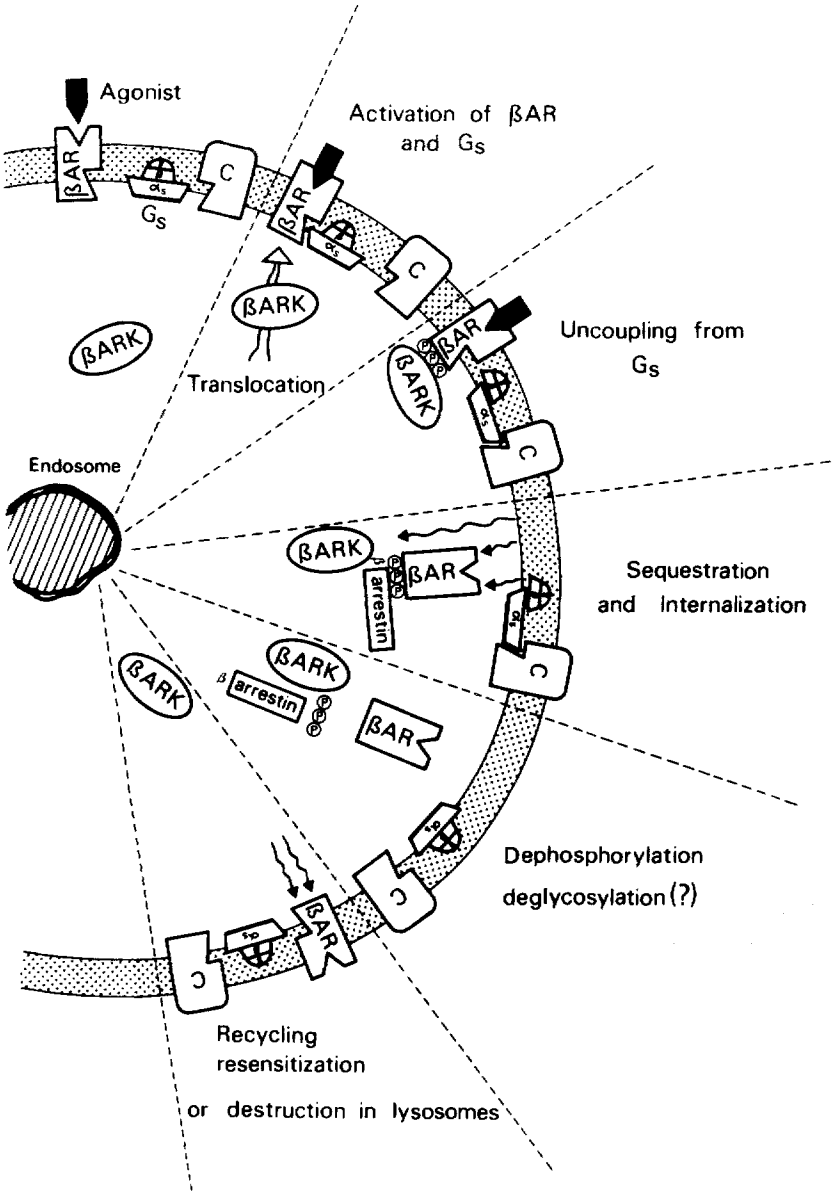


Fig. 11. Model for homologous desensitization of βAR. In this scheme of successive events, binding of agonist to the βAR is followed by activation of G_s, which leads to dissociation into α_s, β, and γ subunits. The activated α_s will stimulate cycles (C), and agonist-bound βAR becomes phosphorylated by βARK as well as by PKA or PKC (not shown). Phosphorylated βAR is sequestered and internalized while β-arrestin prevents its further interaction with α_s. Internalized βAR proceeds to endosomes and either to lysosomes or back to the membrane, after dephosphorylation and, possibly, reglycosylation. Meanwhile, α_s, having hydrolyzed bound GTP into GDP, reassociates with βγ and returns to ground state, as does cyclase (modified from Lefkowitz et al., 1990).

internalization and transfer into endosomes and lysosomes, as we will discuss below.

Regulation of Receptors at the Level of the Cell

Mutant forms of β_2AR that miss phosphorylation sites for either PKA or βARK still desensitize to an extent comparable to that of wild-type β_2AR, after exposure to agonist for several hours. This downregulation of receptor is not completely independent of coupling sites to G_s, since mutants defective in α_s or in coupling sites show impaired downregulation (reviewed in O'Dowd et al., 1989b).

The mechanism of downregulation is clearly linked to the internalization of receptor, first in pits, then in vesicles, then in endosomes, and, finally, in lysosomes, where receptor degradation may occur. It is not yet clear how sequestration and internalization are related, but there is little doubt that diminution in number of binding sites is a quantitative measurement of the overall phenomenon.

The internalization process of β_2AR in human A431 epidermoid carcinoma cells treated with the agonist isoproterenol was studied by Raposo et al. (1989), using a specific anti-β_2AR monoclonal antibody. It was thus shown (Fig. 12) that receptor microaggregation was followed by concentration of receptor in pits and vesicles that were devoid of clathrin, in stark contrast with what was previously observed for receptors for growth factors (e.g., epidermal growth factor), insulin, or low-density lipoprotein (LDL). Internalization via uncoated "smooth" pits and vesicles had earlier been reported for another R_7G protein, namely, the m_2 muscarinic receptor in CCL137 cells (Raposo et al., 1987). Another R_7G-like protein, the receptor for the mating factor in yeast, had been described to be internalized, albeit at a slower rate, even in mutants devoid of clathrin (Payne et al., 1988). This suggests that these three proteins, and possibly all other members of the family, use a common pathway that does not require clathrin to maintain the receptors in the membrane during formation of pits and vesicles and transfer to endosomes. This major difference from other receptor families, which are internalized via clathrin-coated structures, could possibly be caused by the considerable number of hydrophobic regions in R_7G proteins compared with the single transmembrane domain of the other receptors.

Tyrosine residues located in the cytoplasmic domains of several membrane receptors, such as the LDL and mannose-6-phosphate receptors, have been described as playing an important role in the agonist-induced internalization. Replacement of Tyr^{350} and Tyr^{354} of the human β_2AR by Ala residues through site-directed mutagenesis did not affect agonist-induced sequestration, but strongly decreased isoproterenol-induced downregulation of the β_2AR mutant expressed in transformed Chinese fibroblasts (Valiquette et al., 1990), confirming the role of these Tyr residues in receptor internalization.

Integrated Model

An integrated model for the βAR–ligand interaction may now be summarized as follows: Extracellular positively charged β-adrenergic agonists, such as the natural adrenaline or noradrenaline, or the synthetic isoproterenol, are attracted through mainly negatively charged Asp and Glu residues toward the entrance of a pocket constituted by the seven tm domains. The agonists would then progressively penetrate deeper into this pocket by interacting successively with a number of polar, hydrophilic, and hydrophobic amino acid residues. Antagonists may inhibit this binding and progression. The binding of the agonist triggers a change of conformation that alters the interaction between receptor and G_s protein, leading to displacement of GDP by GTP from G_s and decreased affinity of the receptor for the agonist. Although βAR becomes phosphorylated and internalized, dissociation of G_s into α_s and $\beta\gamma$ results in activation of adenylyl cyclase, cleavage of GTP into GDP and Pi, inorganic phosphate, and reassociation of α_s with $\beta\gamma$. All data available today suggest that similar, if

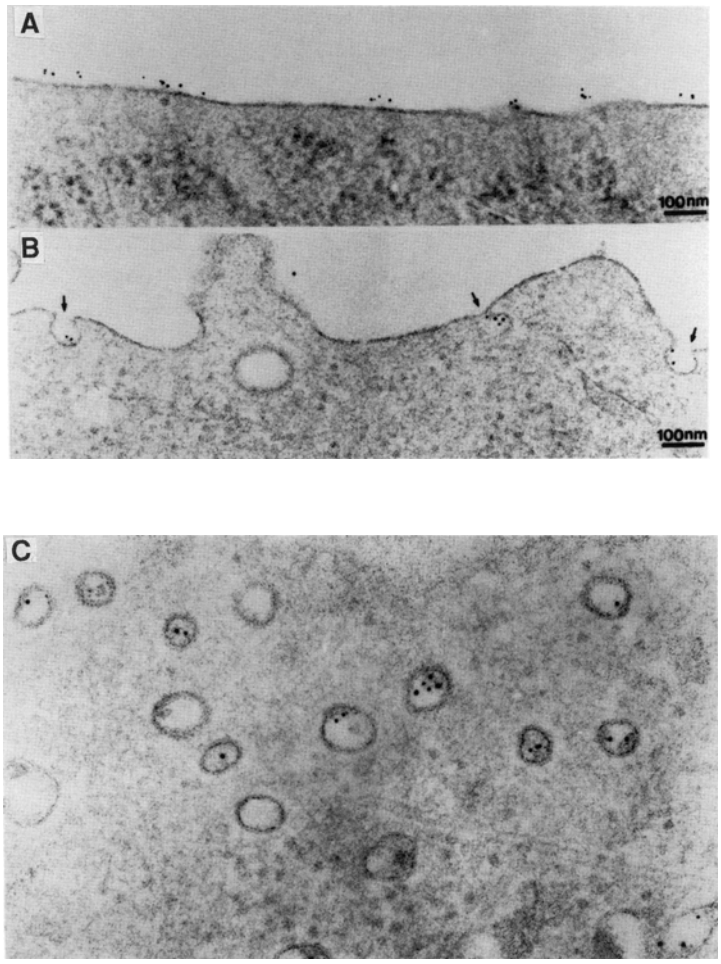


Fig. 12. Internalization of the β_2AR. A431 cells were incubated at 4°C with the anti-β_2AR antibody–anti-IgG–gold complex. **A:** When cells were fixed without further incubation at 37°C, gold particles were associated with the plasma membranes and were present as individual particles or clusters. **B:** After a 10-min incubation at 37°C, gold particles were seen in noncoated membrane invaginations (arrows). **C:** A431 cells were incubated with the anti-β_2AR antibody–anti-IgG–gold complex at 4°C and transferred to 37°C, and isoproterenol was added to the incubation medium for 30 min. The section, tangentially cut to the cell surface, shows a large number of non-clathrin-coated invaginations and vesicles containing βAR–BRK2–anti-IgG–gold complex. Bars, 100 nm (from Raposo et al., 1989).

not identical, mechanisms operate for the other R_7G receptors interacting with small neurotransmitters. No data have been reported yet for the small peptide receptors, but no compelling evidence opposes similar pathways for these ligands.

The R_7G receptors binding the large glycohormones, such as TSH, LH, or FSH, clearly work differently, since it has been known for a while, and now formally proven by using deletion mutants, that the binding site is entirely contained within the large extracellular *N*-terminal region.

Activation of the relevant G protein, however, requires interaction with an intact R_7G-like core, again suggesting that a similar mechanism will underly this signal transmission.

Conclusions and Perspectives

The studies reported in this review demonstrate that biotechnology has contributed to a significant extent to our progress in understanding how catecholamines act on β-adrenergic receptors to trigger adenylyl cyclase and induce a variety of physiological effects. Protein microsequencing of affinity-chromatography-purified receptors has led to synthesis of oligonucleotides, which in turn allowed the cloning of the receptor cDNA and genes. Expression of these genes, transfected in cells grown in vitro, and analyses of the resulting receptor, sometimes after site-directed mutagenesis, using radiolabeled ligands and fluorochrome- or enzyme-labeled monoclonal antibodies, suggested mechanisms of action for βAR that have now become models for the study of several of the numerous other R_7G receptors.

Finally, the availability of recombinant receptors and corresponding mRNA, DNA probes, and monoclonal antibodies have also provided tools for exploring new directions in detecting and treating human diseases.

Future areas of research on the βAR and other R_7G proteins will extend from protein chemistry and crystallographic analysis of receptor–ligand complexes to studies of the regulation of the subtype expression in normal, experimental, and pathologic situations. Recombinant receptors and corresponding effectors will replace the tissues, cells, or membranes currently used not only in binding assays, but also in the evaluation of the agonist or antagonistic properties of natural or synthetic peptides. Used as antigens, these recombinant proteins will also serve to detect specific antibodies in the sera of patients with autoimmune disorders. The availability of the genes will now provide the tools to explore the genetic polymorphism in the human population and possibly relate inherited susceptibility to disease to dysfunction of receptor or effector variants. Development of new drugs resulting from our increased understanding of receptor–effector interaction may follow new avenues, possibly aimed at regulating gene expression.

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