Georges Vauquelin and Bengt von Mentzer

G Protein-coupled Receptors

Molecular Pharmacology

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G Protein-coupled Receptors

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Molecular Pharmacology From Academic Concept to Pharmaceutical Research

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Prof. Vauquelin's initial research dealt with the purification of β-adrenergic receptors (PhD, 1978) and the discrimination between agonist- and antagonist- β-adrenergic receptor interaction. Part of this work was performed in 1979-1980 at the Case Western Reserve University, Cleveland, during a post-doctoral stay in the lab of Prof. M. Maguire. Dr. Vauquelin's subsequent work focused on *in vitro* techniques. It included the identification and characterization of GPCRs and mechanisms of receptor regulation by physiological and patho-physiological conditions with a particular emphasis on receptors in the human CNS. Some of his research also dealt with the interaction of natural products from plants and animal venoms (conotoxins) with receptors and their ligands and with the investigation of membrane proteins with potential receptor-like activity. More recently, intact cell systems expressing either endogenous or transfected receptors (for neuropeptide Y, angiotensin II) have been used to compare ligand-receptor interactions by radioligand binding and functional measurements. Dr. Vauquelin is (co)author of over 180 publications in international journals.

Dr. von Mentzer's initial research dealt with mechanisms, classification and localization of β-adrenergic receptors involved in colon motility (PhD, 1985). Dr. von Mentzer's postdoctoral studies were focused on the understanding of the functional impact of drug-receptor interactions at the University of Leicester with Prof. Stefan Nahorski. These studies comprised the functional coupling of muscarinic receptors to phosphoinositol signalling pathways. During the ensuing 10 years, Dr. von Mentzer moved to the field of fatty acid metabolism, concentrating on the arachidonic acid pathway. These studies led to a joint collaboration with Prof. Arthur Spector at the University of Iowa and Prof. Jim Hamilton at Boston University. Dr. von Mentzer returned back to receptor pharmacology and developed a particular interest in neuropeptide research. His present occupation deals with drug development at AstraZeneca. He is an initiator of teamwork in several discovery/development projects related to gastrointestinal neuropeptide GPCRs and provides scientific support to project and group leaders at different stages in drug development.

Thinking must never submit itself,
neither to a dogma,
nor to a party,
nor to a passion,
nor to an interest,
nor to a preconceived idea,
nor to anything whatsoever,
except to the facts themselves,
because for it to submit to anything else would be the end of its existence.

Henri Poincaré (French mathematician and natural philosopher 1854–1912)

To Bengt Åblad and Enar Carlsson who introduced us into their world of knowledge, enthusiasm and wisdom, and greatly stimulated our eagerness to undertake this enterprise. Many thanks from both authors.

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Preface

This book provides, in the first place, useful scientific background to those involved in pre-clinical research and to those who need to function within multi-disciplinary teams in the pharmaceutical industry (from medical chemistry and molecular biology via pharmacology to drug marketing). Today's education of researchers in the pharmaceutical industry needs to be broad and insight into pharmacological issues needs to be warranted. In this respect, the focus on G protein-coupled receptors (GPCRs) is rationalised by the fact that they constitute major cellular targets for pharmaceutical drugs.

The aim is also to provide students and investigators with a basic interest in life sciences an insight into the fast evolving GPCR field. More generally speaking, to create knowledge in the domain of molecular pharmacology. In this respect, the book will also provide necessary background for the elaboration and reading of research papers on GPCRs and even information brochures about pharmaceutical drugs (which nowadays often describe their molecular mechanism of action).

To these ends:

1. much attention is devoted to the analysis and interpretation of experimental data (radioligand binding and functional assays)
2. the now widespread use of recombinant cell lines, receptor mutants and related artifices in drug research is critically evaluated
3. special attention is also devoted to trendy but often poorly understood concepts such as insurmountable antagonism, inverse agonism and allosteric interactions.

We are especially indebted to Ulla Schefström, Lena Ruehl and Ann-Marie Ashton for their excellent administrative and technical support.

Georges Vauquelin and Bengt von Mentzer
July 2007

Foreword

The greatest need for new textbooks occurs in areas that are rapidly changing. Such textbooks have to be written by scientists actually involved in the research which is advancing the field. It is most often very difficult to persuade experts in these fields to set time aside to spread their knowledge and to produce concise but instructive information. Georges Vauquelin and Bengt von Mentzer have a recognized expertise on G protein-coupled receptors. Their book on this matter is, therefore, unique. It is an updated work that covers the major aspects of the present knowledge concerning this very interesting class of receptors.

The science of GPCRs is one of the most attractive areas of modern biological research. As it is growing very rapidly, this book will be very useful for many workers in biological as well as clinical science dealing with these receptors. It should even help to stimulate new lines of research.

GPCRs are widely considered as interesting targets for drugs. In fact, some of them are already used as targets for very popular drugs such as beta-blockers, muscarinic agonists and antagonists, many psychotropic drugs and others. Very intense research on GPCRs is being conducted in academic as well as in pharmaceutical industry laboratories dealing with drug design and pharmaceutical innovation.

As a pharmacologist involved in experimental and clinical research, I consider this book to be essential reading. It should serve the needs of scientists of course, but also of graduate and postgraduate students in pharmacology, biochemistry and cell biology.

Pascal Bousquet
Professor of Pharmacology Medical School
Louis Pasteur University
Strasbourg
France

1 Chemical messengers and the cell membrane

Higher organisms are composed of a multitude of cell types; each possessing specialized physiological functions. Their harmonious co-existence is only possible if they can communicate with each other; i.e. if they can exchange information concerning their respective needs. Cell signalling can result either from direct interaction of a cell with its neighbour (*juxtacrine signalling*) or from the exchange of small molecules, i.e. '*chemical messengers*'.

These chemical messengers will only induce physiological responses in those cells which contain specific recognition proteins, i.e. '*receptors*'. As a general definition, receptors carry out two distinct functions:

- First they recognize the structure of one or several chemical messengers, which results in messenger–receptor binding.
- Next the messenger–receptor complex is able to generate a 'signal', which modulates specific metabolic pathways in the cell, resulting in a physiological response such as contraction or relaxation of muscle cells.

Based on their mode of transportation and their range of action, the chemical messengers can be divided into three major classes: hormones, neurotransmitters and local chemical mediators (Figure 1).

1.1 Endocrine signalling by hormones

Endocrine glands (or nerve endings for neurohormones) secrete hormones into the blood stream. They can be transported to almost any part of the body. For the hormones, receptors may thus be located in or on cells from distant tissues. Typical examples are: adrenaline which is secreted by the chromaffin cells of the adrenal medulla and which acts on a great number of tissues in the body and insulin which is secreted by the pancreatic β-cells and which acts on the liver and the adipose tissue. Since hormones are diluted in the bloodstream, they need to remain active at low

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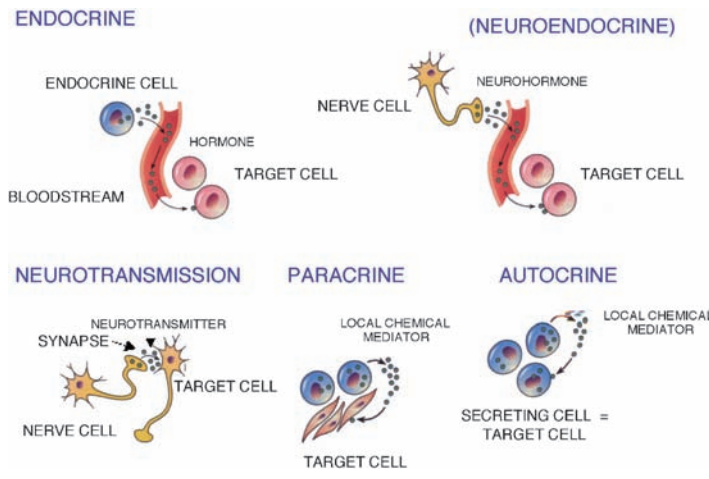


Figure 1 Signalling by hormones, neurotransmitters and local chemical mediators Reprinted from Seeley *et al.*, *Anatomy and Physiology*, 5th edn., © (2000), McGraw-Hill, with permission from the McGraw-Hill Companies.

concentrations (usually $< 10^{-8}$M). According to their site of production, common vertebrate hormones can be divided into: pituitary, hypothalamic, thyroid, parathyroid, digestive, pancreatic, placental, gonadal, adrenal cortical, adrenal medullary, liver, kidney, cardiac and pineal hormones (Table 1, Figure 2).

The brain exerts a profound effect on the endocrine system via the pituitary gland (hypophysis), which hangs by a short stalk from the hypothalamus (i.e. part of the brain, Figure 3). It comprises two major portions:

- The posterior pituitary gland is an extension of the nerve tissue of the hypothalamus. It secretes antidiuretic hormone and oxytocin into the bloodstream.
- The anterior pituitary gland is a glandular tissue. Its hormones are secreted in response to releasing neurohormones secreted by the hypothalamus (these are carried by the bloodstream in the direction of the anterior pituitary gland). Involved (neuro)hormones are listed in Table 2. The pituitary hormones then stimulate their target cells in the body to make other low molecular weight hormones. The end products of these cascades feedback-inhibit hormone production at hypothalamic and/or pituitary levels (Figure 4).

Most peptide hormones are synthesised as preprohormones and must be processed further to produce the finished hormone. The synthesis of insulin in the pancreatic β-cells constitutes a typical example (Figure 5). The first peptide synthesised is 'preproinsulin'. The first 23 amino acids of this protein (i.e. the pre-piece) are very hydrophobic and are required for the penetration of the protein into the endoplasmic reticulum. The pre-piece is rapidly cleaved off in the endoplasmic reticulum, to form

Table 1 Examples of hormones involved in major physiological control of body functions (Zubay, 1993).

Hormone	Origin and structure	Major functions
	ADRENAL CORTICAL HORMONES	
GLUCOCORTICOIDS	Steroids; cortisol and corticosterone	Diverse effects on inflammation and protein synthesis
MINERALOCORTICOIDS	Steroids; aldosterone	Maintenance of salt balance
	ADRENAL MEDULLARY HORMONES	
ADRENALINE (EPINEPHRINE)	Derived from tyrosine	Glycogenolysis, lipid mobilization, smooth muscle contraction, cardiac function
NORADRENALINE (NOREPINEPHRINE)	Derived from tyrosine	lipid mobilization, arteriole contraction
	LIVER HORMONES	
ANGIOTENSIN II	Peptide (eight amino acids)	Responsible for essential hypertension (also indirectly via release of aldosterone from adrenal cells)
	KIDNEY HORMONES	
CALCITROL [1,25-(OH)2-vitamin D3]	Derived from 7-dehydrocholesterol	Maintenance of calcium and phosphorous hoemostasis
	PANCREATIC HORMONES	
INSULIN	Disulfide bonded dipeptide (21 and 30 amino acids)	Produced by β–cells of the pancreas, increases glucose uptake and utilization, increases lipogenesis, general anabolic effects
GLUCAGON	Peptide (29 amino acids)	Produced by α-cells of the pancreas, increases lipid mobilization and glycogenolysis to increase blood glucose levels
PANCREATIC POLYPEPTIDE	Peptide (36 amino acids)	Increases glycogenolysis, regulation of gastrointestinal activity
SOMATOSTATIN	Peptide (14 amino acids form)	Inhibition of glucagon and somatotropin release

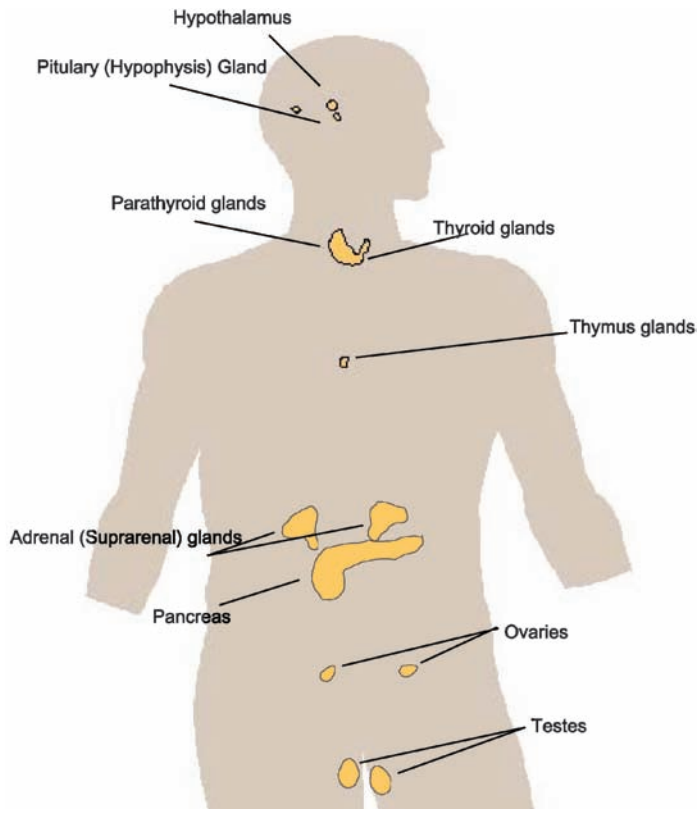


Figure 2 Localization of major endocrine glands in the human (both male and female gonads, testis and ovaries are shown).

'proinsulin', a 9000 dalton protein. Proinsulin is then transported in small vescicles to the golgi apparatus. Here, it is packaged into secretory granules along with enzymes that are responsible for its conversion to insulin. This involves folding, disulfide bond formation and cleaving of the extra piece in the middle of the proinsulin chain to produce the 5600 dalton, two-chain insulin molecule. This conversion begins in the golgi complex, continues within the secretory granules and is nearly complete at the time of secretion (Figure 5B).

Several hormones are not secreted in their active (i.e. receptor binding and stimulating) form. They need to be further processed on their way to, or even within, their target cells. As an example, the peptide hormone angiotensin II is one of the most potent vasoconstrictors known and large quantities of angiotensin II appear in the bloodstream as a response to a drop in the arterial pressure. Its synthesis constitutes an example of the complex interplay between different extracellular factors (Figure 6). The kidneys will start to release renin in the bloodstream when the arterial pressure falls. Renin itself is an enzyme that splits the end off angiotensinogen (a plasma proteins that is secreted by the liver), to release a decapeptide, angiotensin I. Within a few seconds, two

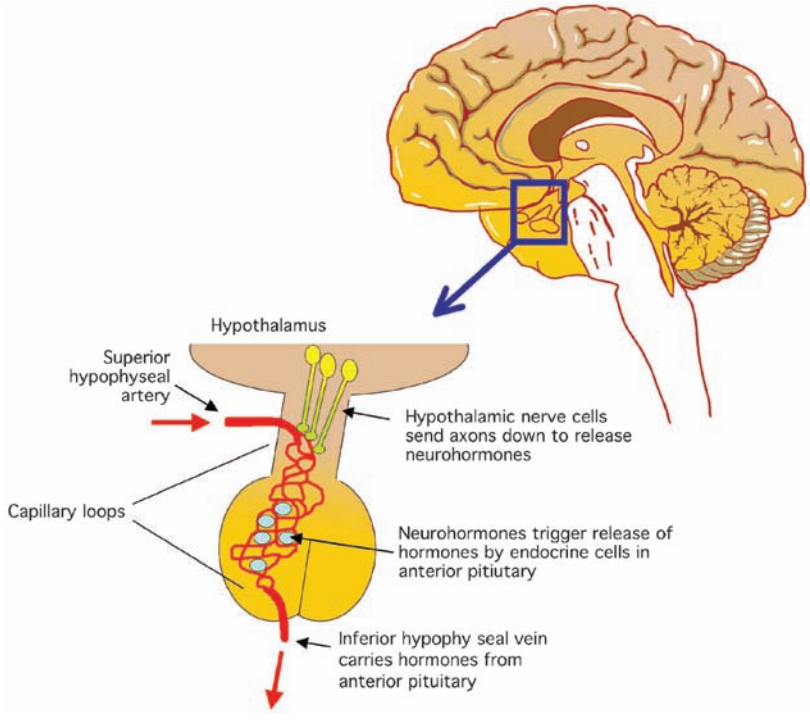


Figure 3 Connection of the endocrine system to the brain.

Table 2 Hormones by which the brain controls major body functions (Zubay, 1993).

Hormone	Origin and structure	Major functions
	HYPOTHALAMIC HORMONES	
CORTICOTROPIN-RELEASING FACTOR	Peptide (41 amino acids)	Acts on corticotrope to release ACTH and β-endorphin (lipotropin)
GONADOTROPIN-RELEASING FACTOR	Peptide (10 amino acids)	Acts on gonadotrope to release LH and FSH
PROLACTIN-RELEASING FACTOR	This may be TRH	Acts on lactotrope to release prolactin
PROLACTIN-RELEASE INHIBITING FACTOR	May be derived from GnRH precursor (56 amino acids)	Acts on lactotrope to inhibit prolactin release
GROWTH HORMONE-RELEASING FACTOR	Protein (40 and 44 amino acids)	Stimulates GH secretion

(*continued*)

Table 2 (*Continued*)

Hormone	Origin and structure	Major functions
SOMATOSTATIN	Peptide (14 and 28 amino acids)	Inhibits GH and TSH secretion
THYROTROPIN-RELEASING FACTOR	Peptide (three amino acids)	Stimulates TSH and prolactin secretion
	PITUITARY HORMONES	
OXYTOCIN	Peptide (nine amino acids)	Uterine contraction, causes milk ejection in lactating females, responds to suckling reflex and estradiol, lowers steroid synthesis in testes
VASOPRESSIN	Peptide (nine amino acids)	Blood pressure regulation, increases H_2O readsorption from distal tubules inkidney
MELANOCYTE-STIMULATING HORMONE	α peptide (13 amino acids) β peptide (18 amino acids) γ peptide (12 amino acids)	Pigmentation
CORTICOTROPIN (ACTH)	Polypeptide (39 amino acids)	Stimulates cells of adrenal gland to increase steroid synthesis and secretion
LIPOTROPIN	β peptide (93 amino acids) γ peptide (60 amino acids)	Increases fatty acid release from adipocytes
THYROTROPIN (thyroid-stimulating hormone)	α chain (96 amino acids) β chain (112 amino acids)	Acts on thyroid follicle cells to stimulate thyroid hormone synthesis
GROWTH HORMONE	Protein (191 amino acids)	General anabolic stimulant, increases release of insulin-like growth factor-I, cell growth and bone sulfation
PROLACTIN	Protein (197 amino acids)	Stimulates differentiation of secretory cells of mammary gland and stimulates milk synthesis
LUTEINIZING HORMONE	α chain (96 amino acids) β chain (121 amino acids)	Increases ovarian progesterone synthesis, testosterone synthesis
FOLLICLE-STIMULATING HORMONE	α chain (96 amino acids) β chain (120 amino acids)	Ovarian follicle development and ovulation, increases estrogen production and spermatogenesis

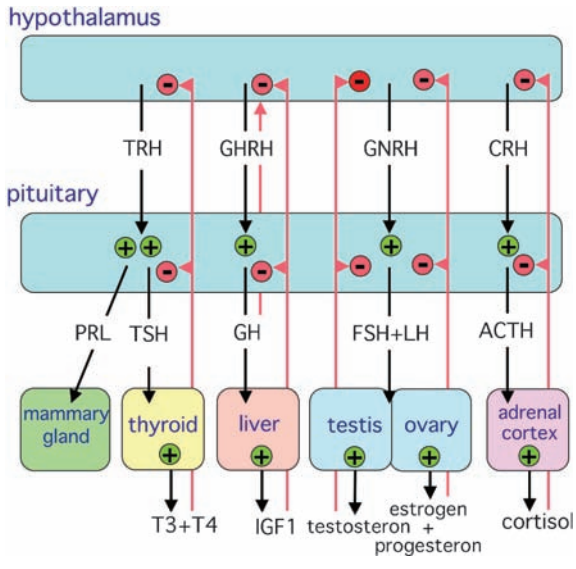


Figure 4 Control of hormone synthesis and secretion in the anterior pituitary. Example: follicle-stimulating hormone (FSH) is a protein acting on the gonads to stimulate the secretion of gonadal steroids.

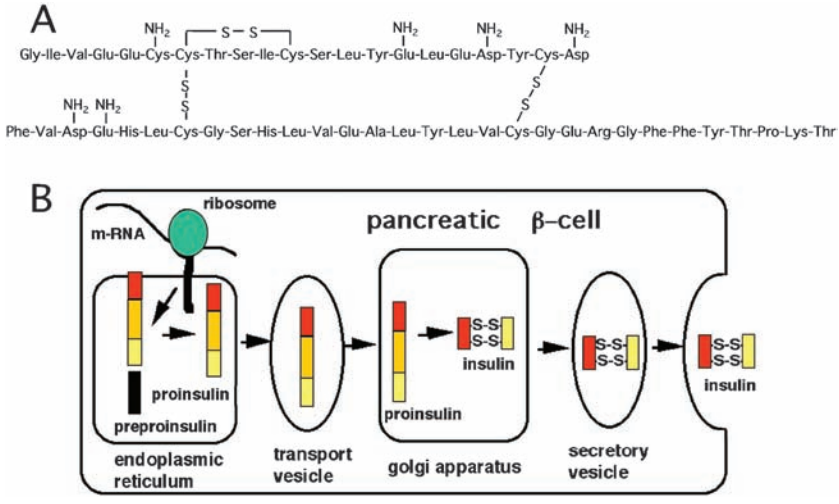


Figure 5 Structure (A) and synthesis (B) of insulin: preprohormones may be processed in cell to produce the hormone.

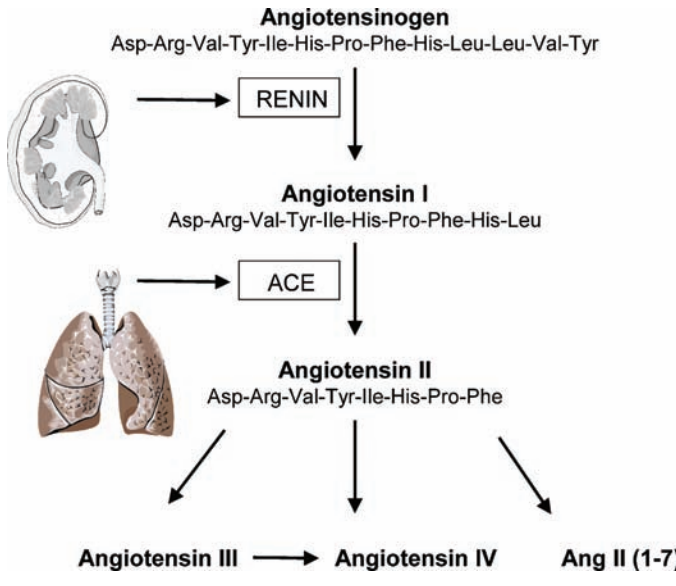


Figure 6 Classical synthesis of angiotensin II: prohormones may be processed on their way to the target cells.

additional amino acids are split from angiotensin I, to form the octapeptide angiotensin II. This conversion occurs almost entirely in the small vessels of the lungs and it is catalyzed by an enzyme called 'angiotensin converting enzyme' (ACE) that is present in the walls of these vessels. Angiotensin II persists in the blood for a minute or so (since it is rapidly inactivated by a number of different blood and tissue enzymes) and it will produce vasoconstriction by binding to specific receptors. Angiotensin II (Ang II) is further metabolized to angiotensin III, angiotensin IV and Ang II (1–7) (Figure 6).

1.2 The nervous system and synaptic signalling by neurotransmitters

The nervous system has two divisions:

- The *central nervous system (CNS)* consists of the brain and spinal cord.
- The *peripheral nervous system* consists of cranial and spinal nerves. It includes the autonomic nervous system.

The peripheral nervous system relays information to and from the central nervous system. The brain is the centre of activity that integrates this information and initiates responses.

The *autonomic nervous system* (part of the peripheral nervous system) has two divisions: sympathetic and parasympathetic. Often, they function in opposition to one

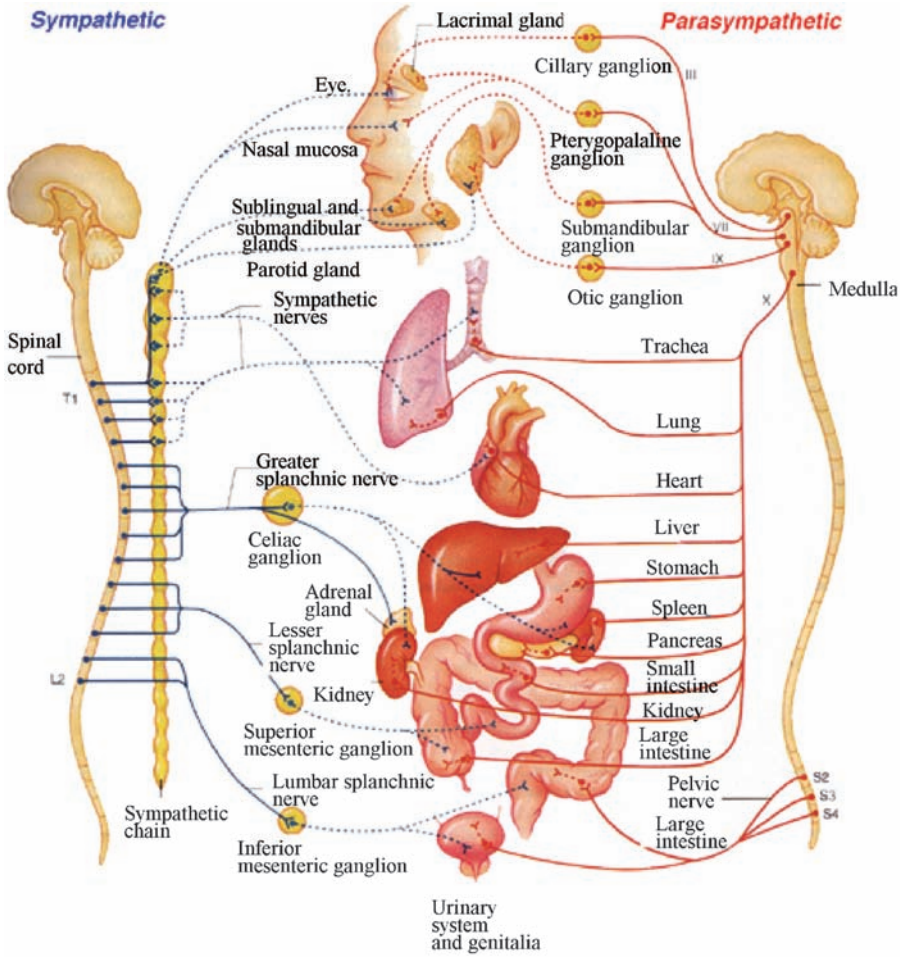
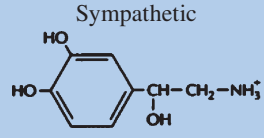
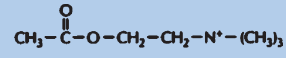


Figure 7 Divisions of the autonomic nervous system. Reprinted from Seeley *et al.*, *Anatomy and Physiology*, 5th edn., © (2000), McGraw-Hill, with permission from the McGraw-Hill Companies.

another; i.e. when an organ receives both sympathetic and parasympathetic impulses, the responses are opposites (Figure 7):

- The neurotransmitter noradrenaline (norepinephrine) is the main actor of the sympathetic system. This system is dominant in stress situations, which include anger and anxiety, as well as exercise. Such stress situations often involve the need for intense physical activity; i.e. the 'fight or flight response'. The involved physiological changes are listed in Table 3.
- The neurotransmitter acetylcholine is the main actor of the parasympathetic system (Table 3). This system dominates in relaxed (non-stress) situations to

Table 3 Autonomic control of body function.

Organ: Response	Sympathetic 	Parasympathetic 
Heart (cardiac muscle)	Increase rate	Decrease rate (to normal)
Bronchioles (smooth muscle)	Dilate	Constrict (to normal)
Iris (smooth muscle)	Pupil dilates	Pupil constricts (to normal)
Salivary glands	Decrease secretion	Increase secretion (to normal)
Stomach and intestines (smooth muscle)	Decrease peristalsis	Increase peristalsis for normal digestion
Stomach and intestines (glands)	Decrease secretion	Increase secretion for normal digestion
Internal anal sphincter	Contracts to prevent defecation	Relaxes for defecation
Urinary bladder (smooth muscle)	Relaxes to prevent urination	Contracts for normal urination
Internal urethral sphincter	Contracts to prevent urination	Relaxes to permit urination
Liver	Changes glycogen to glucose	None
Sweat glands	Increase secretion	None
Blood vessels in skin and viscera (smooth muscle)	Constrict	None
Blood vessels in skeletal muscle	Dilate	None
Adrenal glands	Increase secretion of adrenaline	None

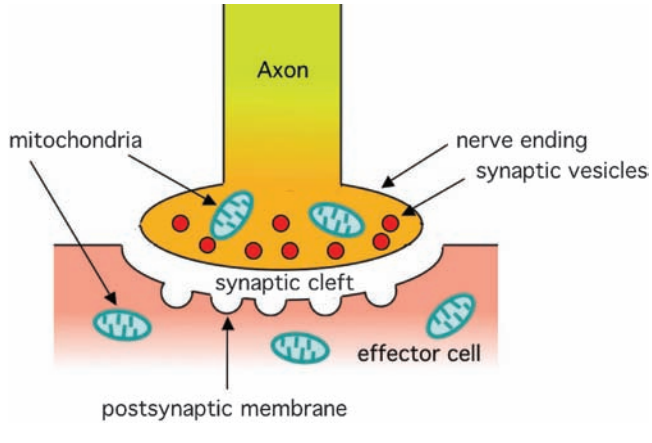


Figure 8 Anatomy of synapse/neuroeffector junction.

promote normal functioning of several organ systems. Digestion will be efficient and the heart will beat at a normal resting rate.

Neurons that transmit impulses to other cells do not actually touch them (Figure 8). To pass on information, neurotransmitters need to be secreted by axonal terminals of neurons. The small gap or space between the axon of one neuron and:

- The dendrites or cell body of the next neuron is called the synapse (or synaptic cleft).
- Muscle or gland cells is called the neuroeffector junction.

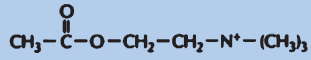
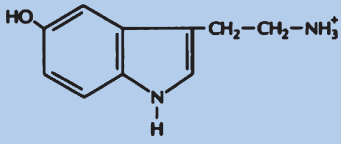
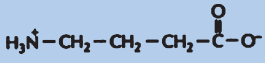
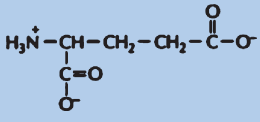
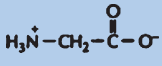
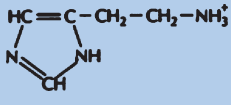
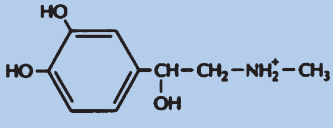
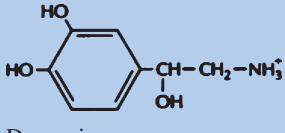
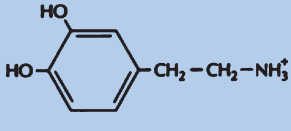
The receptors for neurotransmitters are present on the membrane from the innervated cells and may also be present on the nerve endings themselves (where, as 'autoreceptors', they are implicated in the autoregulation of neurotransmitter release).

The target cells for neurotransmitters are no more than 50 nm away from the nerve terminal (i.e. the width of a typical synaptic cleft). Diffusion of neurotransmitters over such small distances takes only a short period of time, so that this type of messenger is capable to induce cellular responses almost instantaneously (e.g. skeletal muscle cells may contract and relax again within milliseconds in response to the neurotransmitter acetylcholine). In contrast, the hormones may be required to travel over quite some distance before reaching their target cells, and this delays the onset of the responses. Another difference between neurotransmitters and hormones is that the concentration of the neurotransmitters may become fairly high in the synaptic cleft ($>10^{-4}$ M, due to the small volume of the synaptic cleft and the resulting limited dilution). Therefore, there is no need for neurotransmitters to be active at low concentrations.

Small molecule neurotransmitters

A first series of neurotransmitters consist of small molecules (Table 4). They consist of acetylcholine, amino acid derivatives (biogenic amines), amino acids themselves and even molecules (ATP, adenosine) that were initially thought to be strictly cytoplasmic constituents. As an example, dopamine is synthesized within the dopaminergic neuron (Figure 9). This synthesis starts with the amino acid tyrosine (obtained from the diet or from liver phenylalanine), which is converted to L-dopa by tyrosine hydroxylase (TH) in the presence of tetrahydrobiopterin as a cofactor. In other neurones and in chomaffin cells of the adrenal medulla, dopamine can further be transformed into noradrenaline/norepinephrine (neurotransmitter + hormone) and adrenaline/epinephrine (hormone). Dopamine, noradrenaline and adrenaline contain a catechol (*o*-dihydroxyphenyl) group and are therefore denoted as 'catecholamines' (Figure 9). Tyrosine hydroxylase is the rate-limiting enzyme in all catecholamine-secreting cells in the body. Those cells which are stained immunocytochemically for tyrosine hydroxylase are thus identified as those producing either dopamine or the other catecholamines noradrenaline and adrenaline.

Table 4 Small neurotransmitter molecules.

Neurotransmitters (small)	Derived from	Site of synthesis
Acetylcholine 	Choline	CNS, parasympathetic nerves
Serotonin = 5-Hydroxytryptamine (5-HT) 	Tryptophan	CNS, chromaffin cells of the gut, enteric cells
GABA 	Glutamate	CNS
Glutamate 		CNS
Aspartate		CNS
Glycine 		Spinal cord
Histamine 	Histidine	Hypothalamus
Adrenaline (epinephrine) 	Tyrosine	Adrenal medulla, some CNS cells
Noradrenaline (norepinephrine) 	Tyrosine	CNS, sympathetic nerves
Dopamine 	Tyrosine	CNS
Adenosine	ATP	CNS, peripheral nerves
ATP		Sympathetic, sensory and enteric nerves

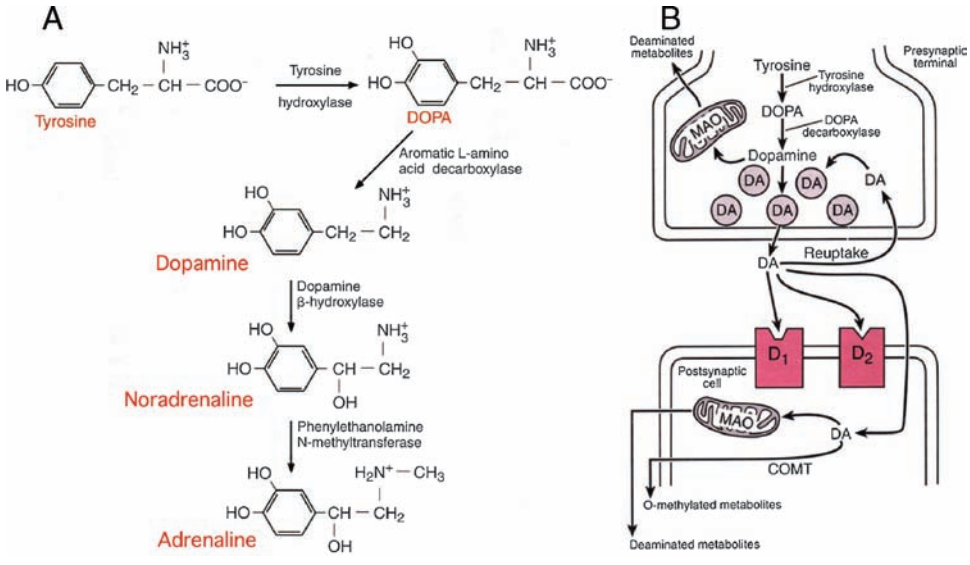


Figure 9 A: Catecholamine biosynthesis. B: Fate of dopamine (DA) in dopaminergic nerve. Reprinted from R.A. Rhoades and G.A. Tanner, *Medical Physiology*, 1st edn., © (1995), with permission from Lipincott Williams & Wilkins.

Neuropeptides

Many other neurotransmitters are derived from precursor proteins, the so-called peptide neurotransmitters. As many as 50 different peptides have been shown to exert their effects on neural cell function. Among them, β-endorphins are endogenous opiates (which also comprise the enkephalins and the dynorphins, Table 5). β-Endorphins have the same effects as opiate drugs such as morphine.

They can play a role in analgesia in response to stress and exercise. Other functions have been proposed for the β-endorphins, including regulation of body temperature, food intake and water balance. β-Endorphins and other endorphins arise from β-lipotropin, which itself is cleaved from an even larger precursor peptide, proopoiomelanocortin (POMC) (Figure 10). The primary protein product of the POMC gene is a 285 amino acid precursor that can undergo differential processing to yield at least eight signalling peptides (including adrenocorticotropic hormone 'ACTH' and α-melanocyte stimulating hormone 'α-MSH') dependent upon the location of synthesis and the stimulus leading to their production.

In general, neuropeptides are generated from precursor molecules produced in the rough endoplasmic reticulum (RER) and packaged in secretory vesicles or granules in the Golgi stacks. The granules are transported out from the cell body to the terminals (axonal transport) where they release their contents by exocytosis upon stimulation (Figure 11). In contrast, biogenic amines are produced in the cytosol of the cell body, axon and terminal and packaged by uptake in pre-formed granules or vesicles. As a

Table 5 Endogenous opiates and their structures.

Precursor	Endogenous opiate	Structure
Proopiomelanocortin (POMC)	β-Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Proenkephalin A	[Leu^5]Enkephalin	Tyr-Gly-Gly-Phe-Leu
	[Met^5]Enkephalin	Tyr-Gly-Gly-Phe-Met
	[Met^5]Enkephalin-Arg^6-Phe^7	Tyr-Gly-Gly-Phe-Met-Arg-Phe
Prodynorphin (proenkephalin B)	Dynorphin A (1-17)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gly
	Dynorphin A (1-13)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
	Dynorphin A (1-8)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile

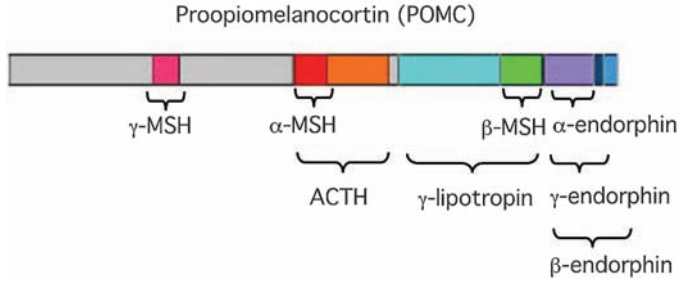


Figure 10 Peptide messengers derived from the POMC precursor protein.

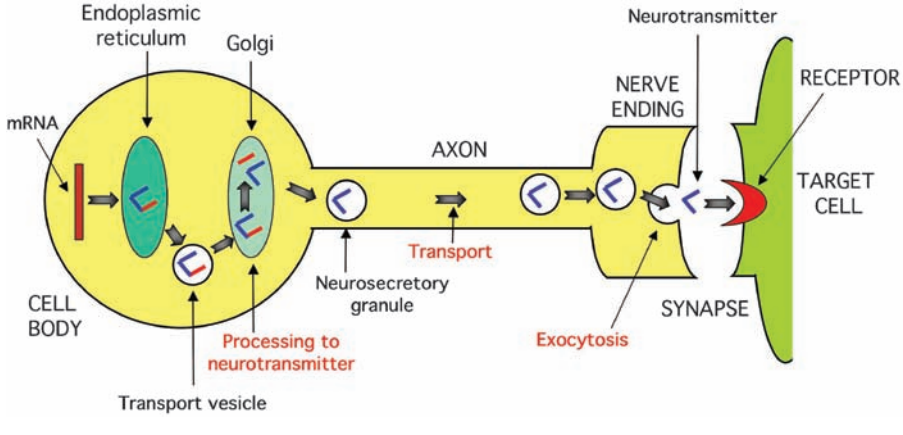


Figure 11 Biosynthesis of neuropeptides in neurons.

result, amines and peptides may co-exist in granules in varying proportions although their proportions in molecular terms may vary depending on the circumstances.

1.3 Paracrine signalling by local chemical messengers

Local chemical messengers are only active in the vicinity of the cells from which they are secreted. Extracellular enzymes rapidly destroy many of them or they are even very unstable of their own. Hence, they are quickly transformed into inactive metabolites so that they can only diffuse over a short distance. Some cells respond to signalling molecules that they themselves produce (autocrine signalling). In this respect, it is noteworthy that abnormal autocrine signalling frequently contributes to the uncontrolled growth of cancer cells. In this situation, a cancer cell produces a growth factor to which it also responds, thereby continuously driving its own proliferation.

Well-known local chemical mediators are histamine and prostaglandins. Histamine is secreted by mast cells (present in connective tissues throughout the body) when stimulated by injury, local infection or certain immunological reactions. It will cause local blood vessels to dilate and become leaky. This will facilitate the access of serum proteins (antibodies!) and phagocytic white blood cells to the site of injury.

Prostaglandins make part of the eicosanoids, a family of fatty acid derivatives, which also include prostacyclin and the thromboxanes (Figure 12). Many biological responses have been ascribed to these molecules, including smooth muscle contraction, platelet aggregation and inflammation. All eicosanoids are synthesized from arachidonic acid, which is formed from phospholipids.

The first step in the pathway leading to synthesis of either prostaglandins or thromboxanes is the conversion of arachidonic acid to prostaglandin H_2. Interestingly,

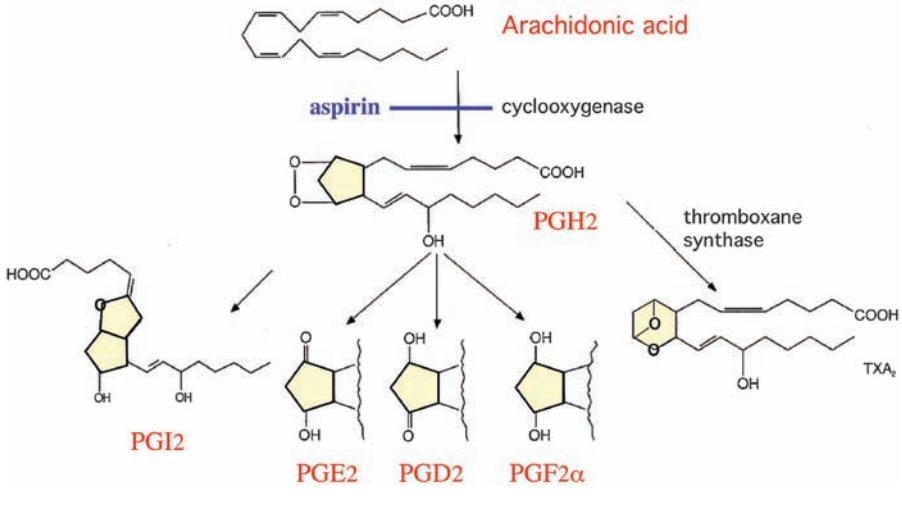


Figure 12 Structure and synthesis of principal prostaglandins. Reprinted from L.B. Wingard, T.M. Brody, J. Larner and A. Schwartz (1991), *Human Pharmacology: Molecular to Clinical*, p.234. Copyright (1991), with permission from Elsevier.

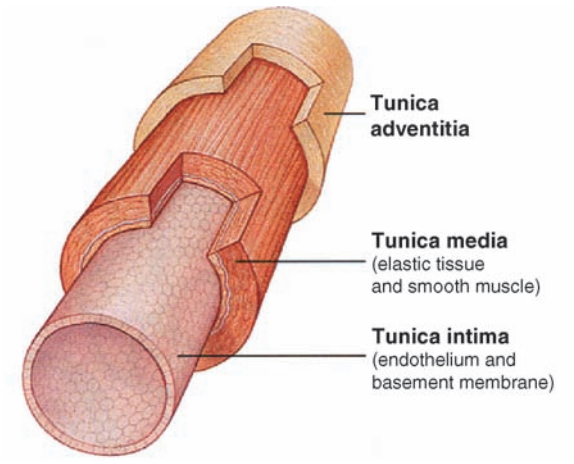


Figure 13 *Structure of an elastic artery.* Reprinted from Seeley *et al., Anatomy and Physiology* 5th edn., © (2000), McGraw-Hill, with permission from the McGraw-Hill Companies.

the enzyme that catalyzes this reaction (cyclooxygenase) is the target of anti-inflammatory drugs like aspirin. By inhibiting synthesis of the prostaglandins, aspirin reduces inflammation and pain. By inhibiting synthesis of thromboxane, aspirin also reduces platelet aggregation and blood clotting.

The vascular endothelial cells are now recognized to play an important paracrine role in controlling the contractile status of large blood vessels (which are composed of an internal layer of endothelial cells – facing the blood stream – and surrounding smooth muscle tissue) (Figure 13). In response to several external stimuli, they are able to produce a relaxing factor (EDRF, i.e. endothelium-derived relaxing factor), which diffuses to the surrounding smooth muscle. EDRF has been identified as the highly unstable nitric oxide radical ($NO^{\bullet}$). This area of research started with the discovery that, under specific conditions, the relaxation of pieces (strips) of aorta is strictly dependent on the presence of endothelium (which is usually not the case in isolated blood vessels unless special care is taken during preparation). Interestingly, organic nitrates have been used in clinical medicine for more than 100 years to produce vasodilatation and this action can now be explained by their ability to mimic the smooth muscle relaxing action of $NO^{\bullet}$.

When their level is increased in the endothelial cells, Ca^{2+} ions bind to nitric oxide synthase, an enzyme that acts on arginine to produce $NO^{\bullet}$. $NO^{\bullet}$ diffuses out of the endothelial cell and into the smooth muscle cell where it combines with the guanylyl cyclase enzyme. This enzyme converts GTP to cyclic GMP, which causes the smooth muscle cell to relax (Figure 14).

1.4 Hydrophobicity: effect on release and transport of messengers

The cell membrane constitutes a hydrophobic barrier between the cell and its environment. It is constituted of a double layer of *lipids* (mainly phospholipids but also

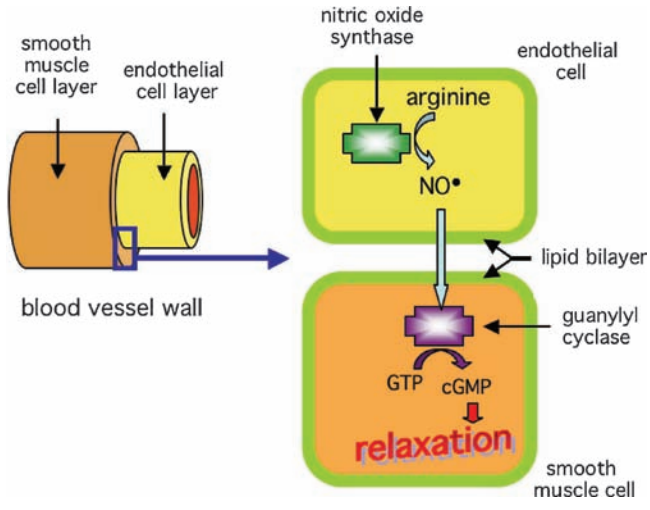


Figure 14 Synthesis and action of NO•.

glycolipids and cholesterol) wherein proteins are able to 'float'. The membrane lipids (Figure 15) are amphitatic molecules; i.e. they possess a hydrophobic and a hydrophilic part. For the phospholipids, the hydrophobic part is constituted of two long (up to 20 C atoms) hydrocarbon chains. The glycerol moiety, the phosphate group and the attached residue (ethanolamine, inositol, serine and choline) constitute the hydrophilic

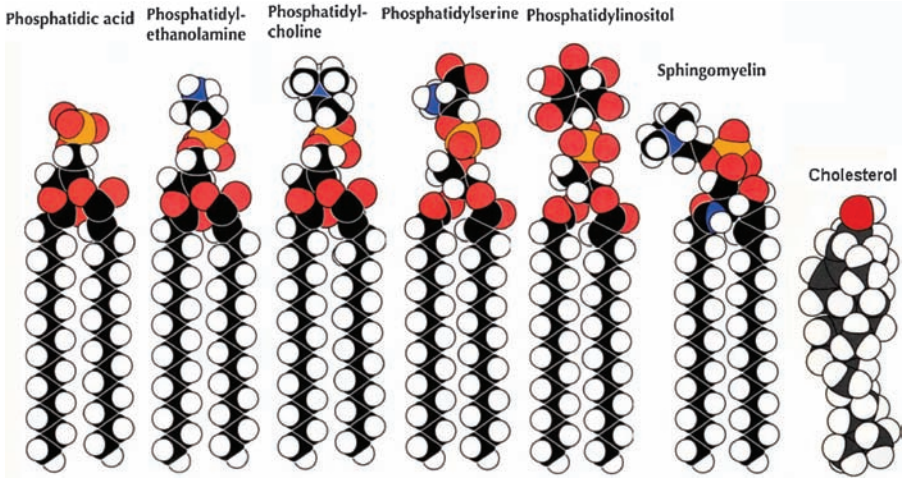


Figure 15 *Major lipids in membranes of eukaryotic cells*. Reprinted from Geoffrey M.Cooper, *The Cell: A Molecular Approach*, 4th edn., © (2007), with permission from ASM Press, Washington D.C.

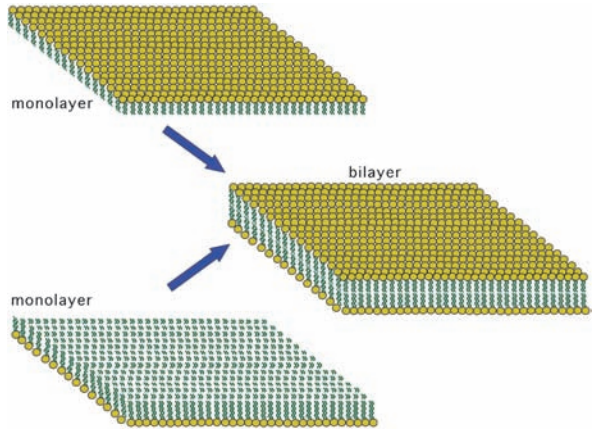


Figure 16 Assembly and structure of a phospholipid bilayer.

part. In an aqueous environment lipids tend to aggregate with their hydrophobic regions towards the inside and the polar head groups forming the interface with the water phase (Singer and Nicolson, 1972). Since the lipids have a cylindrical shape, their juxtaposition will result in the formation of a 'flat' lipid bilayer (Figure 16). As a result of its structure, the inner part of the cell membrane will form a hydrophobic barrier (i.e. composed of cholesterol and of the hydrocarbon chains of phospho- and glycolipids) for any compound that would like to enter the cell (Figure 17).

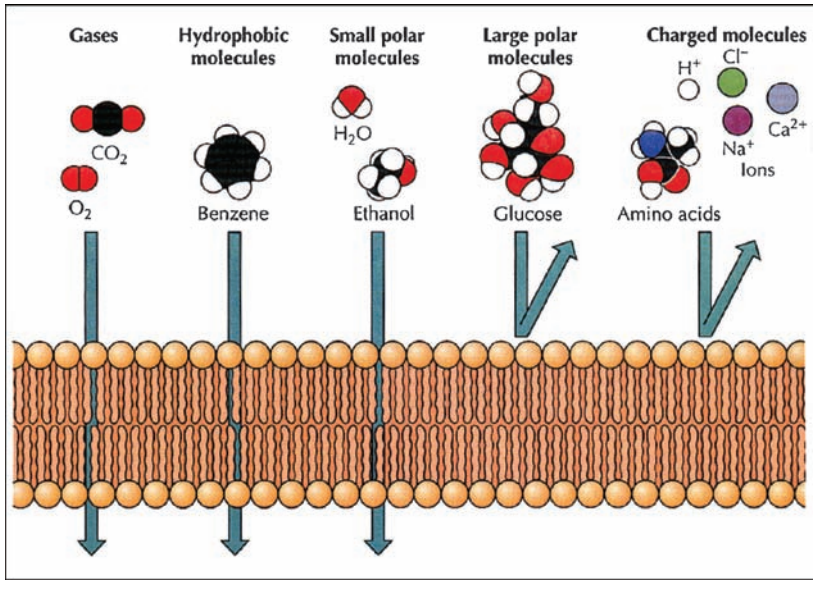


Figure 17 Permeability of the membrane lipid bilayer. Reprinted from Geoffrey M. Cooper, *The Cell: A Molecular Approach*, 4th edn., © (2007), with permission from ASM Press, Washington D.C.

Table 6 Examples of second messengers which stimulate release of local messengers for effect transmisson.

Structure	Hormone	Neurotransmitter	Local messenger
Small and hydrophobic	Steroids, thyroid hormones, vitamin D, retinoic acid		NO•, eicosanoids
Small polar/charged	e.g. adrenalin	e.g. dopamin	e.g. histamine
Large polar/charged	e.g. insulin	e.g. enkephalins	e.g. growth factors

Chemical messengers have varying degrees of hydrophobicity (Table 6). This will profoundly affect their release from the cells in which they are produced, as well as their transport to the receptor.

A limited number of hormones are lipophilic compounds with low-molecular weight: the steroid and thyroid hormones and also retinoic acid and the active metabolite of vitamin D_3. Steroid hormones are derived from cholesterol (Figure 18). They are synthetized mainly by endocrine glands such as the gonads (testis and ovary), the adrenals and (during gestation) by the fetoplacental unit.

They all possess the backbone from cholesterol and, at first sight, their structures seem to be pretty much alike. However, they show up to be quite different molecules, when we look at their three-dimensional representation. Accordingly, each steroid hormone can be recognized by a specific receptor. With respect to their biological function, one can distinguish between:

- Female sex steroids: the estrogens (e.g. estradiol) and progestins (e.g. progesteron) are secreted by the ovaries (depending on the stage of the ovarian cycle).
- Male sex steroids: androgens (e.g. testosteron) are produced in the testis and adrenals.
- Corticosteroids (e.g. the mineralocorticoid aldosterone and glucocorticoid cortisol) are produced in the adrenals.

These hormones are hydrophobic enough to freely diffuse across the membrane of the endocrine cells (Figure 19A). Since they cannot be stored in these cells, their secretion can only be modulated by factors which control their synthesis. For example, the transformation of cholesterol into pregnenolon (the first step in the synthesis of all steroid hormones, and occurring in the mitochondria) is under tight external control. Because of their limited solubility in water, hydrophobic hormones are attached to transport proteins (e.g. thyroid binding globulin for thyroid hormones, cortisol-binding globulin for cortisol, etc.) during their journey in the bloodstream. They can diffuse across the plasma membrane of every cell, but only target cells (i.e. those cells that possess the required receptor) will respond. The lifetime of these hormones is also exceptionally long; steroids persist in the blood for hours and thyroid hormones even for days. This

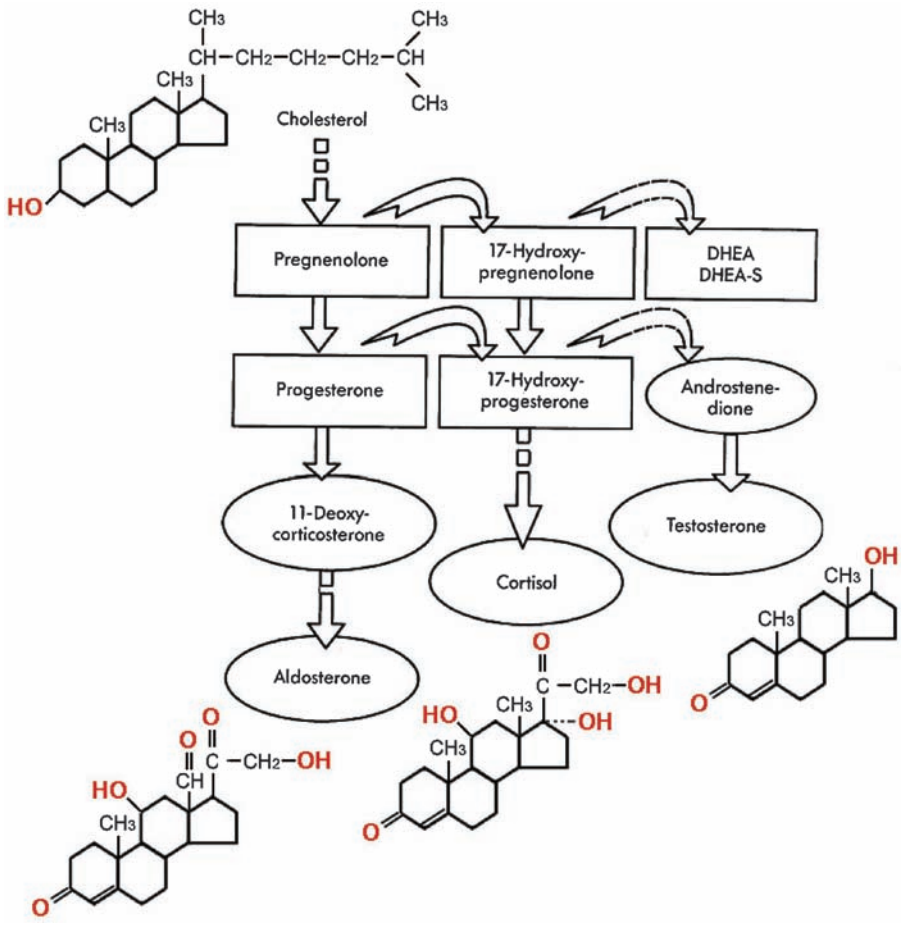


Figure 18 *Synthesis of major steroid hormones by the adrenal cortex*. Reprinted from R. Montgomery, T.W. Conway and A.A. Spector, *Biochemistry. A Case-Oriented Approach*, 5th edn., p. 811. Copyright (1990), with permission from Elsevier.

causes no problem to the organism, since the elicited effects are also long lasting. Receptors for steroids and other hydrophobic hormones are present inside target cells (Figure 19A). A typical target cell for steroid hormones contains about 10 000 receptors. In the absence of hormone, some receptors reside in the cytosol and will move to the nuclear compartment in the presence of the appropriate steroid hormone while others already reside in the nuclear compartment. Interestingly, although eicosanoids (thomboxanes, prostaglandins, leukotrienes) are fatty acid-derived lipids, they interact with receptors that make up part of the plasma membrane (and whose recognition sites are facing the extracellular side of the membrane).

Many local chemical mediators, most hormones and all neurotransmitters are hydrophilic. Their structure is diverse, ranging from small molecules to relatively large

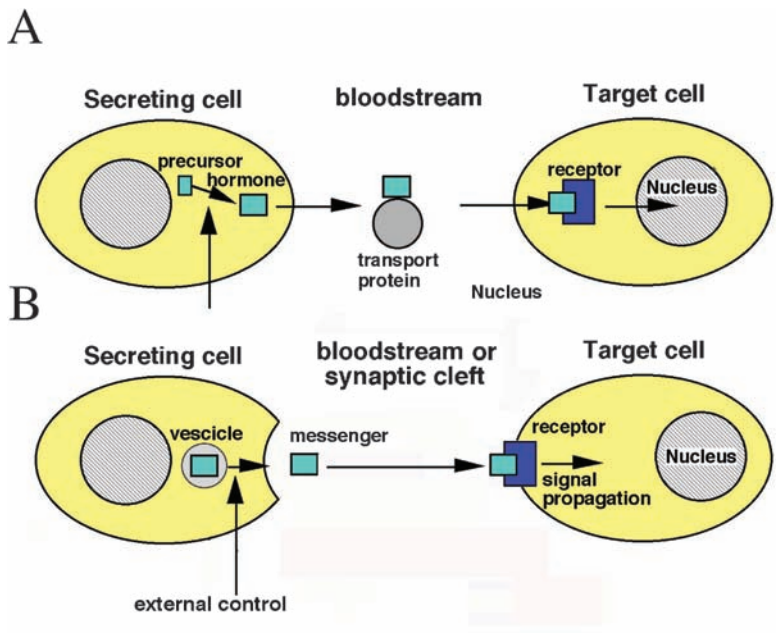


Figure 19 A: Secretion, transport and recognition of hydrophobic hormones. B: Secretion, transport and recognition of hydrophilic and peptide messengers.

polypeptides (Table 6). Because of their hydrophilicity, these molecules cannot cross any cell membrane. This has profound consequences for their mode of release, their transportation and the subcellular localization of their receptors (Figure 19B). Since these substances cannot diffuse across the membrane from the nerve ending, paracrine or endocrine cell, these hydrophilic molecules can only be released by the process of '*exocytosis*'; i.e. by the following steps:

- The chemical messengers are first stored inside small vescicles (possessing a lipid bilayer envelope) in the secretory cell. This enables the secretory cells to build up a reserve of messenger molecules.
- Fusion of the vesicles with the plasma membrane of the secretory cell results in the liberation of the chemical messengers. This exocytotic secretion of messenger molecules is rapid (within milliseconds) and under the control of factors which regulate the fusion of the vescicles with the cell membrane. At the nerve endings (Figure 20), for example, an invading action potential will depolarize the plasma membrane. Voltage-gated calcium channels open, allowing influx of calcium down its concentration gradient. The increased intracellular calcium promotes fusion of transmitter-containing synaptic vesicles with plasma membrane, resulting in exocytosis of vesicular contents. Calcium channels rapidly inactivate and the intracellular calcium is returned to normal by sequestration into mitochondria and active extrusion from the cell.

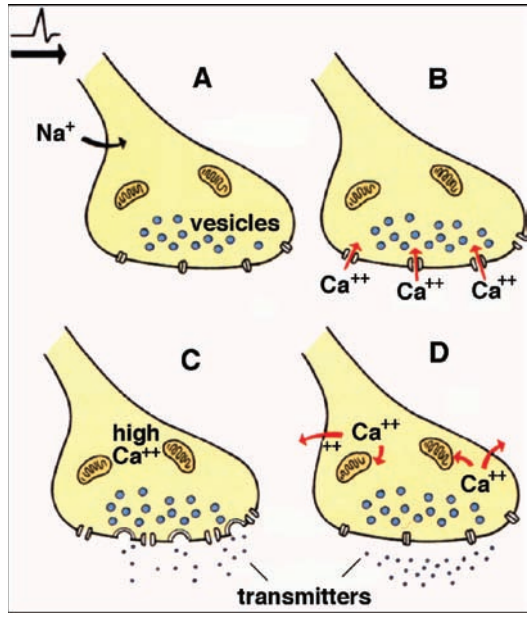


Figure 20 Molecular events involved in neurotransmitter release. A–D represents sequence of events. Reprinted from L.B. Wingard, T.M. Brody, J. larner and A. Schwartz (1991) *Human Pharmacology: Molecular to Clinical*, p. 234. Copyright (1991), with permission from Elsevier.

Most of the hydrophilic hormones are removed and/or broken down to inactive metabolites within minutes after entering into the blood, and local chemical mediators and neurotransmitters are removed from the extracellular space even faster: within seconds or milliseconds. Since the hydrophilic messengers cannot cross the cell membrane, the recognition sites of their receptors need face the extracellular side of the plasma membrane. The binding of a messenger molecule constitutes 'information' which these receptors will transfer across the cell membrane, either on their own or in association with other membrane proteins.

Large and/or charged hormones often need to leave the blood vessels to reach their target cells. This means that they cross the capillaries that supply blood to the various tissues. In the periphery, this is possible because there are gaps between the endothelial cells through which the hydrophilic messengers can diffuse to reach other tissues. In the brain, however, the endothelial cells form a continuous wall (Figure 21). Free transfer of hydrophilic messengers between the bloodstream and the brain is therefore rendered impossible (because they need to cross the hydrophobic cell membranes of the endothelial cells). The endothelial cells in the brain thus constitute a barrier, called the '*blood–brain barrier*', to circulating messengers. The reason for such a hydrophobic barrier resides in the fact that the extracellular concentrations of hormones, amino acids or ions undergo frequent small fluctuations. To prevent uncontrolled nervous activity, the brain must be kept rigorously isolated from such transient changes in the composition of the blood.

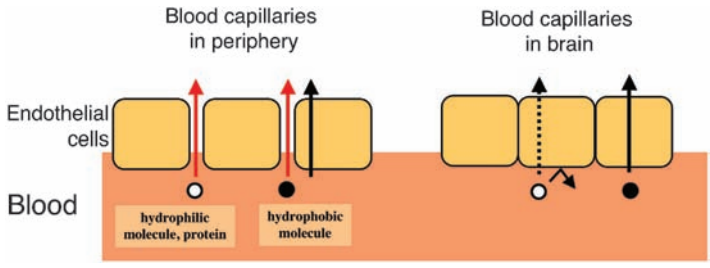


Figure 21 Origin and implications of the blood–brain barrier.

1.5 Membrane proteins and membrane receptors

Membrane proteins have diverse functions. Some of them consolidate the structure of the membrane, whereas others have more active functions, such as recognition (hormone, neurotransmitter and antibody receptors), pumping (ATPases), regulation of the inflow of ions and metabolites (calcium-gate, glucose channel) and enzymatic functions (phospholipases, adenylate cyclase). With regard to their degree of incorporation into the plasma membrane, proteins can be divided into two categories (Figure 22):

- The *intrinsic* (or integral) proteins are inserted in, and sometimes even extend through the membrane. They possess hydrophobic domains, which allow them to

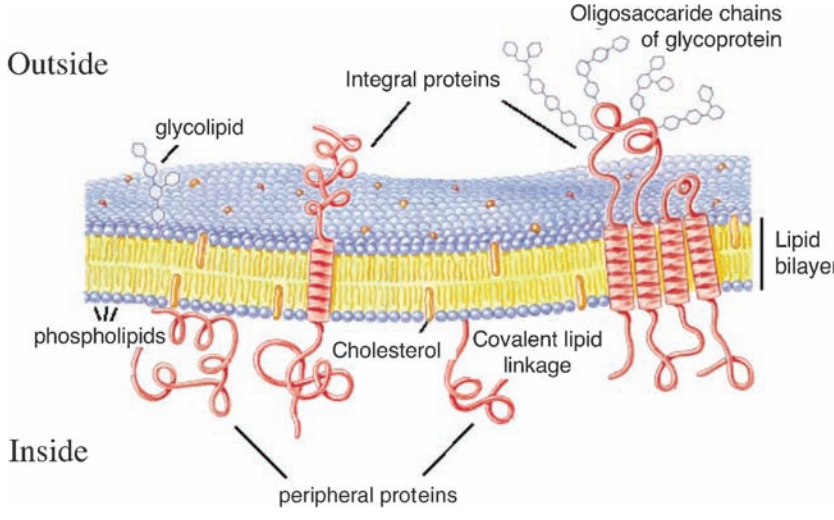


Figure 22 Structure of the plasma membrane: Integral proteins are embedded in the lipid bilayer by hydrophobic interactions between the amino acid residues protruding from their alpha helices and the hydrophobic tailes of the lipids. (Nelson and Cox, 2000). *Principles of Biochemistry* by Albert L. Lehninger, *et al.* © 2000, 1993, 1982 by Worth Publishers. Used with permission.

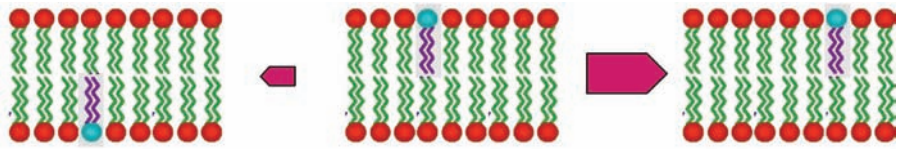


Figure 23 Phospholipid movements in the membrane. Easy: rapid lateral diffusion within the plane of membrane, difficult: "flip-flop" from one face of the bilayer to the other.

'sink' into the hydrophobic core of the lipid bilayer. Proteins (such as cell surface receptors) which extend through the membrane have one or more hydrophobic regions of about 25 amino acids long that pass through the membrane (i.e. as an alpha helix, with the hydrophobic amino acid residues in contact with the hydrophobic tails of the lipid molecules) and hydrophilic regions that are exposed to the water at the intra- and extracellular faces of the membrane.

- The *extrinsic* (or external) proteins are 'loosely' associated to membrane lipids and/or intrinsic proteins via ionic bonds, sometimes involving divalent cationic bridges.

In 1972, Singer and Nicholson proposed their 'fluid mosaic' model wherein both lipids and intrinsic proteins are allowed to diffuse freely within the plane of the membrane. However, both are not allowed to 'flip-flop' from one face of the membrane to the other (Figure 23). Whereas some proteins may form stable complexes, other can undergo dynamic interactions with each other.

During the past two decades, a considerable amount of information has been gathered about the structure of membrane-located receptors as well as about the molecular mechanisms by which they are able to transfer information across the cell membrane. These receptors are all intrinsic proteins which extend through the membrane. Their binding site for the chemical messengers is exposed at (or at least accessible from) the extracellular side of the plasma membrane. However, they are unable to translocate these messenger molecules across the membrane. Instead, they transfer 'information' (i.e. the presence of a bound messenger) across the membrane by undergoing a conformational change. This conformational change will alter certain pathways in the cell metabolism, and this will give rise to the 'cellular response'. These cell-surface receptors can be divided into three major classes according to the mechanism that they use for the transfer of information (Figure 24):

- (A) Direct control of membrane ion channel activity (receptor makes part of an ion channel).
- (B) Interaction with effector components (enzymes, ion channels) via mainly G proteins.
- (C) Direct control of enzymatic activity (i.e. the receptor possesses intrinsic tyrosine kinase activity or regulates the activity of an associated kinase).

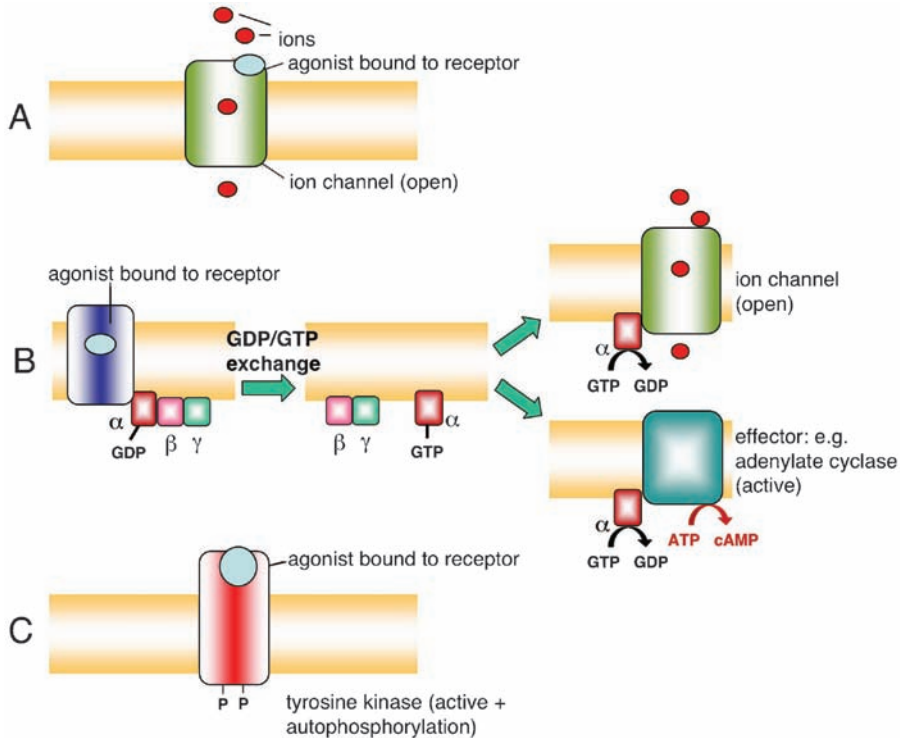


Figure 24 Major mechanisms for receptor-mediated transfer of information across the cell membrane.

1.6 Ligand–receptor interactions

Neurotransmission and hormonal information is extremely important for the well being of higher organisms. It is therefore not surprising that certain diseases may result from (or be associated with) anomalies in hormone- or neurotransmitter concentrations, or from the inability of their target cells to respond adequately. Administration of the messengers themselves (e.g. insulin) and of synthetic analogues is therefore often carried out to counteract these pathophysiological conditions, and in some instances also to alter normal physiological conditions (e.g. contraception). An important branch of the activities of the pharmaceutical industry is therefore implicated in the development of drugs which mimic or block the action of natural messengers:

- *The agonists.* These compounds bind to the receptors and elicit the physiological responses. They include the endogenous messengers and synthetic molecules.
- *The antagonists* or '*blockers*'. These compounds are all synthetic or derived from other organisms (e.g. present in plants and animal toxins). They bind to the receptor but this interaction does not elicit the physiological response. The

most common antagonists compete in a reversible fashion with the endogenous messengers for binding to their receptors, thereby preventing the target cells responding to the presence of these messengers (Figure 25). The binding sites for such competitive antagonists overlap at least in part with the binding site of the endogenous messenger.

The '*binding site*' of each receptor possesses a unique spatial arrangement of amino acid residues with which certain parts of the '*ligand*' (i.e. messenger or drug) can interact. The strength of such interactions differs from one drug to another, so that the affinity of a receptor is different for every drug. The order of affinities (often called '*order of potencies*') of a series of drugs for a specific receptor (i.e. its 'pharmacological profile') therefore serves as a useful 'fingerprint' for that receptor. Such fingerprints allow:

- The positive identification of a receptor.
- The discrimination of one receptor from another.
- The discovery of new receptors.

Messengers are often capable of recognizing a whole series of different receptors. These receptors are often specific for that messenger (e.g. the receptors for acetylcholine bind no other messenger), and they are usually referred to as '*receptor subtypes*'. Occasionally, such a receptor family may be shared by a limited number of messengers (e.g. the adrenergic receptors can be stimulated by both adrenaline and noradrenaline, but by no other messenger).

Agonists and antagonists are of medical interest if they show pronounced affinity and selectivity towards one or more specific receptors. The discovery of such drugs usually requires the synthesis of a considerable number of structurally related molecules and the screening of their toxicity and biological activity. The derived structure–toxicity and structure–activity relationships can then be used for the design of even more efficient compounds. In the past, most of the structure–activity relationships were carried out

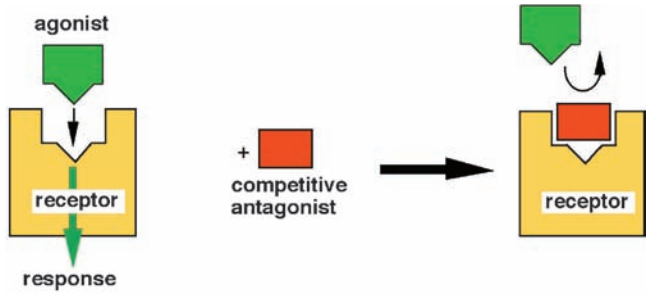


Figure 25 Agonists and competitive antagonists.

by measuring the drug-induced physiological responses *in vivo* or in intact tissues or organs. The last decades have, however, been characterized by the development of biochemical techniques such as radioligand binding and cell-based functional assays to investigate drug–receptor interactions. This allows the fast screening of the affinity of newly synthesized drugs for the receptor, or receptors, of interest. Sections B and C only deal with competitive antagonists. Alternative types of 'antagonism' (i.e. inverse agonists, allosteric and insurmountable antagonists) will be discussed later.

2 Radioligand binding studies

2.1 Technical aspects of radioligand binding

For a long time, hormone and neurotransmitter receptors remained abstract concepts whose existence was proposed only to explain pharmacological effects on target tissues. Since the mid-seventies, it has become possible to investigate of the receptor molecules themselves by the means of radioligand binding. This technique also allows the direct evaluation of the binding properties of any compound for a given membrane-bound receptor. Very often, radioligand binding experiments are performed with more-or-less purified cell membranes (Figure 26).

Radioligand binding is initiated by the incubation of cells, cell homogenates or purified plasma membrane preparations with an adequate radioactively labelled drug; the '*radioligand*'. Adequate radioligands can be selected out of the wide variety of commercially available agonists and antagonists. Obviously, these radioligands should display high affininty and selectivity towards the receptor of interest. If no such radioligands are available, ligands can be custom-labelled by the investigator (for radioiodination) or by specialized institutions:

- Tritium [^{3}H] and iodine [^{125}I] are the most frequently used isotopes. Because of the long half-life of tritium (12.3 years), the tritiated ligand does not have to be resynthesized or repurchased frequently. They can be stored for a rather long time but, nevertheless, care should be taken to check for radiation-induced ligand degradation. In addition, because of the relatively low specific radioactivity of tritium (29 Ci/mmol), tritiated ligands are only suitable when the biological material contains sufficient amounts of the desired receptor.
- If not, radioiodinated ligands are more suitable because of the relatively high specific radioactivity of ^{125}I (2125 Ci/mmol). However, the short half-life (60 days), the exposure of the investigator to gamma rays and the fact that the pharmacological and physicochemical properties of the iodinated ligand may deviate considerably from those of the original ligand constitute major drawbacks of this isotope.

G Protein-Coupled Receptors: Molecular Pharmacology From Academic Concept to Pharmaceutical Research
Georges Vauquelin and Bengt von Mentzer © 2007 John Wiley & Sons, Ltd. ISBN: 978-0-470-51647-8

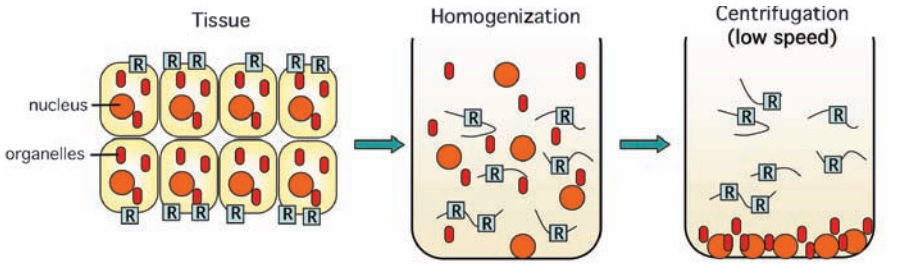


Figure 26 Preparation of cell membranes.

Separation of free and receptor-bound drug represents the most delicate step. This is commonly done by one of the three following techniques (Figure 27):

- *Filtration*: the free radioligand passes through the filter whereas the receptor-bound radioligand remains on the filter. Counting the radioactivity on the

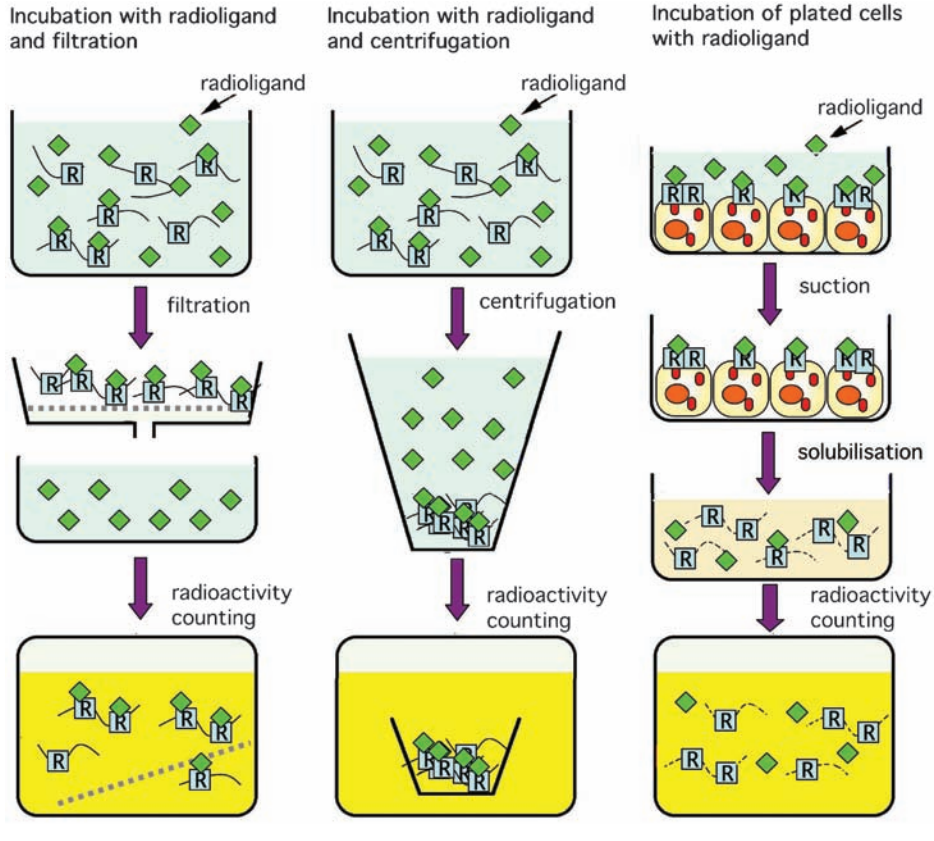


Figure 27 Measurement of radioligand binding by different methods involving the removal of free radioligand.

filter allows the amount of receptor-bound radioligand to be quantified. This technique is usually employed when using membrane preparations and when performing radioligand binding to intact cells in suspension. The popularity of this technique results from the ability to handle a large number of samples with relative ease as well as the commercial availability of a variety of filtration devices. Moreover, the filters can be washed thoroughly and rapidly with fresh buffer (preferentially ice-cold to prevent dissociation of the radioligand-receptor complex). This allows the removal of remaining traces of free radioligand. The filters are usually of glass fibre, but sometimes it is also necessary to coat them with polyethyleneimine or to siliconize them to prevent radioligand absorption to the filter. For 'high throughput screening', the radioligand binding may be performed in microtiter plates with 96, 384 or even more wells. After the incubation, the contents of the wells are filtered simultaneously with a cell harvester. For modern high-throughput screening, robots are used to handle screen compounds and buffers as well as to perform the filtration step.

- *Centrifugation*: membranes or cells precipitate, whereas the free radioligand remains in solution, and can be discarded. Quantitation of the amount of receptor-bound radioligand is done by counting the radioactivity of the pellet. Since no thorough washing is involved, this technique is especially useful when the radioligand–receptor complex dissociates rapidly. However, this technique results in high background radioactivity due to the trapping of radioligand in the pellet. Manual manipulations and the resulting risk of contamination constitute additional disadvantages of the technique.

- *Suction* binding to intact cells may be achieved by plating them on the bottom of each well in (e.g. 24 well) multiwell plates. After the incubation, the free radioligand is removed by suction, the cells may then be washed with fresh buffer (preferentially ice-cold to prevent dissociation of the radioligand–receptor complex), and the remaining receptor-bound radioligand in each well is counted. For this purpose, plated cells are often treated with a detergent solution to solubilize the membranes. The radioactivity moves into solution and can then be counted easily. Here again, many manual manipulations are required.

The scintillation proximity assay (SPA) technique demands even fewer manipulations since the separation between free and bound radioligand is avoided (Figure 28). For this technique, small scintillant-containing beads are already present in the incubation tube/well. When these beads are also coated with wheat germ agglutinin (WGA), they will attach intact cells or membranes. The principle of the technique is based on the assumption that the overwhelming majority of the free radioligand molecules are too far from the beads for the scintillant to be activated whereas the receptor-bound radioligand is in close proximity to the beads and, hence, capable of stimulating the scintillant. Therefore, the measured scintillation will mainly arise from bound radioligand molecules.

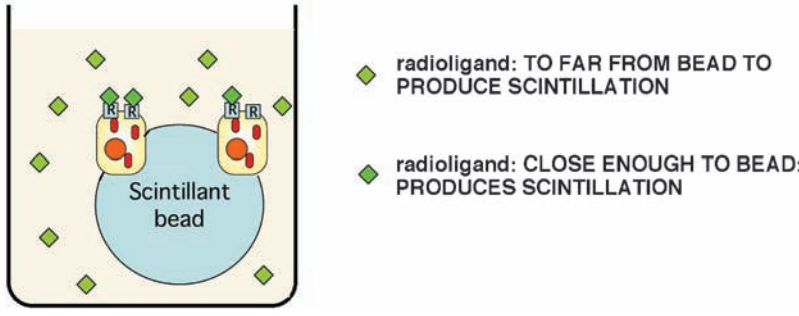


Figure 28 Measurement of radioligand binding by the SPA technique.

Binding of a radioligand to a physiologically relevant receptor (i.e. '*specific binding*') should at least obey the following criteria:

- The binding should be saturable, since a finite number of receptors are expected in a biological preparation.
- The potency of unlabelled ligands to compete with the radioligand for binding to the receptor should parallel their potency to provoke (for agonists) or block (for antagonists) receptor-mediated responses.

Radioligands not only bind to their receptor (Figure 29). They might also bind to other receptors and to non-receptor sites such as carrier proteins, enzymes, cell components recognizing certain chemical moieties of the radioligand (e.g. the catechol moiety for radiolabelled catecholamines). In addition, they might even

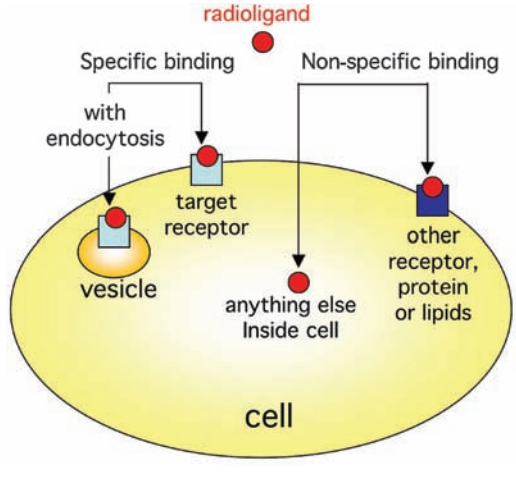


Figure 29 Possible interactions between radioligands and different cellular sites.

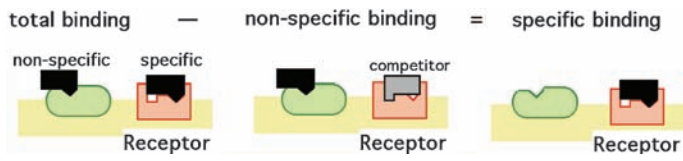


Figure 30 Determination of total and non-specific binding and the calculation of specific binding.

bind to separation materials such as filters or test tubes. This binding is called '*non-specific binding*'. One of the major problems in developing a suitable binding assay is the selection of a radioligand that shows enough specificity towards the receptor. In general, a hydrophilic (to avoid partitioning in the lipid bilayer of the membrane) radioligand with high affinity for the desired receptor may be a good candidate. However, some of the measured binding will always be non-specific. To deal with this problem, radioligand binding experiments always comprise two determinations: total binding and non-specific binding and the *non-specific binding* must be subtracted from the *total binding* to obtain the *specific binding*; i.e. binding to the receptor of interest (Figure 30).

Obtaining a correct non-specific binding value constitutes the most delicate aspect of a radioligand binding technique. In theory, non-specific binding can simply be obtained by adding an excess of competitor to the incubation mixture, so that binding of the radioligand to the receptors is completely displaced. In practice, care must be taken to choose a competitor that displaces the radioligand from the receptor only, and not from the other, non-specific sites. It is recommended to choose a potent competitor whose chemical structure is quite distinct from that of the radioligand.

Radioligand binding studies provide three main categories of information: saturation binding data, competition binding data and kinetic data.

2.2 Saturation binding

These experiments provide information about the concentration of a receptor. They are solicited to compare the concentrations of different receptors in a given tissue and to monitor variations in receptor concentration as a result of normal physiological regulation, medication and pathophysiological conditions.

For saturation binding experiments, constant amounts of membrane suspension are incubated with increasing concentrations of radioligand. Obviously, both total and non-specific binding should be measured at each concentration of radioligand (Figure 31). In the example shown, binding is expressed as a function of the free concentration of radioligand by a *saturation binding plot*. Obviously, only the specific binding is of interest.

To analyze these saturation binding data, it is necessary to advance a relevant molecular model for the radioligand–receptor interaction. In the simple (and fortunately the most common) situation, the interaction of the radioligand (L) with the

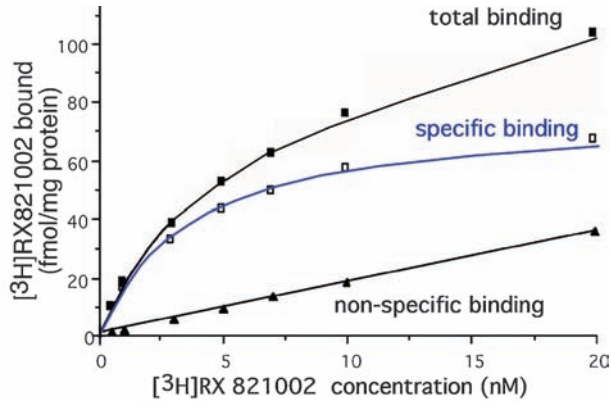


Figure 31 Saturation binding of the α_2-adrenergic antagonist [^{3}H]RX 821002 to α_2 adrenergic receptors in membranes from the human frontal cortex. Reprinted from *Neurochemistry International*, 17, Vauquelin G., De Vos H., De Backer J.-P. and Ebinger G., Identification of a2 adrenergic receptors in human frontal cortex membranes by binding of [3H]RX 821002, the 2-methoxy analog of [3H]idazoxan, 537–546. Copyright (1990), with permission from Elsevier.

receptor (R) can be expressed as a reversible bimolecular reaction that obeys the law of mass action: i.e.

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L\text{-}R \qquad (1)$$

Where k_1 and k_{-1} are the association and dissociation rate constants, respectively. The *equilibrium dissociation constant* (K_D) is given by:

$$K_D = k_{-1}/k_1 = [L] \times [R]/[L{-}R] \qquad (2)$$

Where [R] is the amount of free receptors, [L] the amount of free ligand and [L−R] the amount of bound ligand/receptors.

The relationship between the amount of occupied receptors and the free radioligand concentration (i.e. the saturation binding plot) is as follows:

$$[L{-}R] = [R_{tot}]/(1 + K_D/[L]) \qquad (3)$$

Where $[R_{tot}]$ is the total number of receptors.

'B' and 'B_{max}' (which stand for binding and maximum binding and are often expressed in fmol/mg protein) usually replace [L − R] and $[R_{tot}]$. Equation (3) then becomes:

$$B = B_{max}/(1 + K_D/[L]) \qquad (4)$$

This equation is analogous to the Michaelis–Menten equation of enzyme kinetics and describes a rectangular hyperbola. Initially, B increases almost linearly with L. Then

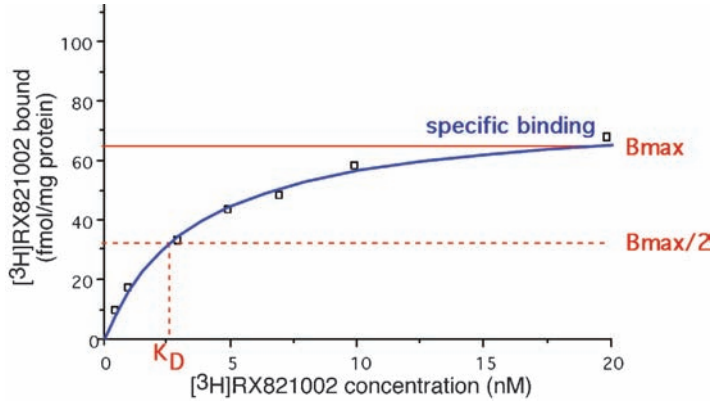


Figure 32 Graphical analysis of the saturation binding plot from Figure 31. Only specific binding is important here.

B tends to level off when L is further increased. The limit value is B_{max} (Figure 32). It is important to notice that this that B_{max} will be attained only at infinite concentrations of L. Thus, one will never observe B_{max} experimentally; B_{max} may be approached but never attained. Half-maximal binding is obtained when $L = K_D$ (since Equation (4) becomes $B = B_{max}/2$). In other words, the K_D of a radioligand corresponds to its concentration for which half of the receptors are occupied. The K_D value is thus an 'inverse' measure of the radioligand's affinity for the receptor: a low K_D corresponds to high affinity and a high K_D to low affinity.

B_{max} and K_D cannot be easily determined by graphical analysis of the saturation binding plot (Figure 32) since Equation (4) is a non-linear relationship and since B_{max} is only reached when $L = \infty$. This equation can, however, be transformed mathematically to yield a linear '*Scatchard plot*' (Figure 33) corresponding to the following equation:

$$B/[L] = -B/K_D + B_{max}/K_D \quad (5)$$

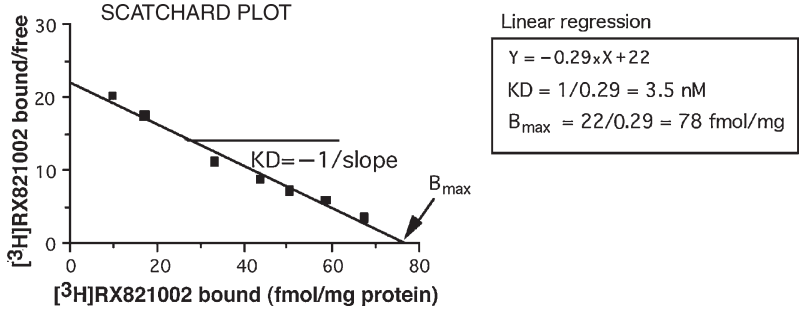


Figure 33 Scatchard analysis (Scatchard, 1949) of bound [^{3}H]- RX821002 to α-adrenergic receptors. Obtained from (Figure 31).

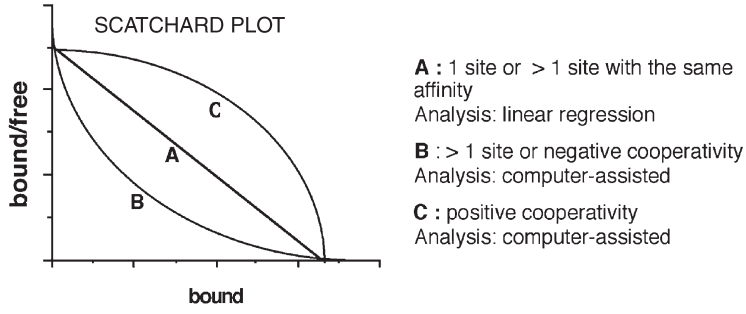


Figure 34 Scatchard plots: different possibilities.

The Scatchard plot of the above saturation binding data reveals a linear relationship between B/[L] (the ordinate) and B (the abscissa). K_D corresponds to the negative reciprocal of the line. The intercept of the line with the abscissa (i.e. when B/[L] = 0) is B_{max}. Thus, it is relatively easy to calculate K_D and B_{max} values by linear regression analysis of the Scatchard plot.

The relationship described by Equation (5) is for the simplest case; i.e. a single class of non-interacting receptor sites. However, it is possible that the radioligand binds to two different receptors with different affinities or even that one receptor is present in two or more (non-interconverting) affinity states for the radioligand. This situation will result in a non-linear Scatchard plot: i.e. showing downward concavity (Figure 34 curve B).

Moreover, certain receptors (e.g. ion channel-gating receptors which make part of a larger structure) possess multiple binding which influence each other's binding characteristics. This may result in either negative or positive co-operative interactions among the binding sites. In other words, binding of the radioligand to one site decreases (negative co-operativity) or increases (positive co-operativity) the affinity of the radioligand for other sites. This will also result in non-linear Scatchard plots with, respectively, downward concavity (negative co-operativity, Figure 34 curve B) or upward concavity (positive co-operativity, Figure 34 curve C).

A more sensitive method to ascertain whether radioligand binding obeys the law of mass action is to analyse the '*Hill plot*' of the saturation binding data (Figure 35). The Hill equation is, in fact, a logarithmic transformation of Equation (4).

$$\text{Log}(B/(B_{max} - B)) = nH \times \text{Log}([L]) - \text{Log}(K_D) \qquad (6)$$

$\text{Log}(B/(B_{max} - B))$ is the ordinate and $\text{Log}([L])$ is the abscissa of the Hill plot. The slope corresponds to the Hill coefficient: 'nH'. The law of mass action is obeyed if $nH = 1$ (in practice, values between 0.8 and 1.2 will do). This means that the radioligand binds with the same affinity to all the sites. $nH < 1$ is indicative of either negative co-operativity or of the existence of binding sites with different affinity. $nH > 1$ is indicative of positive co-operativity, i.e. where radioligand binding to one site increases the affinity of the radioligand for other sites.

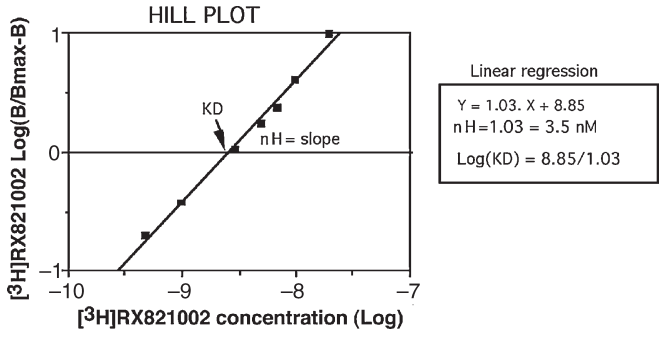


Figure 35 Hill plot of the saturation binding data from Figure 31 (B in fmol/mg protein, F in nM).

A disadvantage of a Scatchard plot is that the extrapolation of data obtained over an insufficient concentration range of L may give an artificial impression of the lack of complexity of the radioligand–receptor interactions. This may result in inaccurate Hill plots since they rely on a correct estimation of the B_{max} value. Although Scatchard and Hill plots are still sometimes shown in publications for the sake of clarity (e.g. it is easy to visualize differences in K_D and B_{max} values of one or more radioligands with a Scatchard plot), radioligand binding parameters are now almost always calculated by computer programmes which are based on non-linear regression analysis of the saturation binding data. In the case of two non-interconverting binding sites, these programmes even allow the calculation of the concentration of each site and its respective affinity for the radioligand.

Finally, certain important considerations need to be taken into account before correctly analyzing saturation binding data; they include:

- The data must represent an equilibrium situation. In practice, this means that the incubation must have occurred long enough for equilibrium to be reached. Investigating binding of a given concentration of radioligand as a function of the incubation time can check this. This binding will increase time-wise until a plateau value (corresponding to the equilibrium situation) is reached. Equilibrium binding is often obtained within minutes at usual incubation temperatures (20–37 °C), but that it may become considerably longer when the temperature is lowered to (0–4 °C).
- Binding is expressed as a function of the free concentration of radioligand. This concentration may be set equal to the concentration of radioligand added (i.e. $[L] = [L_{init}]$) if only a small fraction of the added radioligand is bound (in other words, if most of the added radioligand still remains free). If a more substantial amount of radioligand is bound (e.g. >5%), then [L] is smaller than $[L_{init}]$, and its correct value should be calculated by the equation: $[L] = [L_{init}] - [L - R]$.
- The ligand must not aggregate, at higher concentrations, to a dimer or multimer.

2.3 Competition binding

Radioligands are fairly expensive and only very few of them are specific enough for the purpose of receptor identification. Fortunately, radiolabelling of a drug is not strictly required for determining its affinity for a given receptor. This parameter can indeed be determined on basis of the drug's ability to compete with a (specific) radioligand for binding to that receptor. These competition binding experiments are now widely used by pharmacologists as a screening tool to evaluate the affinity of newly synthesized compounds (or of natural substances) for one or more receptors of interest. This approach has several advantages over the measurement of physiological responses. First, the same experimental set-up can be used to investigate the affinity of a drug for different receptors, whereas physiological responses may be very diverse and, hence, need to be monitored by different techniques. Second, the affinity of a drug for a specific receptor can be determined without ambiguity, whereas physiological responses are remote events, which may be triggered by different receptors or even be modulated at steps intermediate between receptor-stimulation and the final response.

It is important to note that the terms '*competition binding*' and '*competitor*' (for the non-radioactive substance) are commonly utilized irrespective of whether the 'competitor' is truly competitive or not. This semantic problem merits proper attention.

For competition binding experiments, constant amounts of membrane suspension are incubated with a fixed concentration of radioligand and increasing concentrations of the non-radioactive substance to be tested (the competitor), after which binding of the radioligand is measured. Binding of the radioligand is expressed as a function of the free concentration of the competitor by a *competition binding plot* (Figure 36). Competitor concentrations may span several orders in magnitude, so they are often expressed on a logarithmic scale. In the simple situation (in which the competitor is truly 'competitive') the radioligand and the competitor bind in a reversible fashion to the same site of the receptor. The radioligand (L)–receptor (R) and the competitor

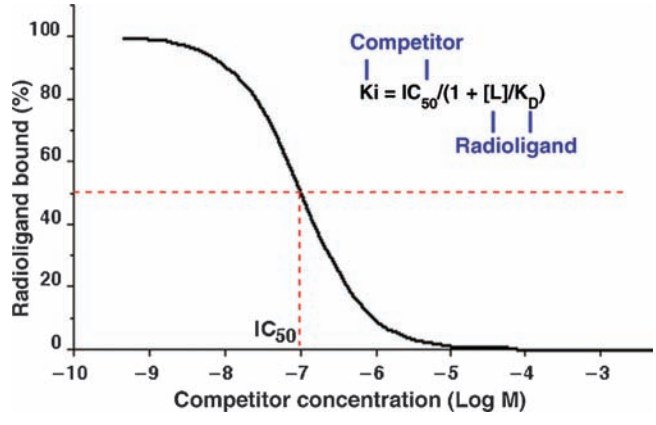


Figure 36 Competition binding curve (100% binding is binding in the absence of competitor) and determination of the competitor's K_i from the IC_{50}.

(I)–receptor (R) interactions can be expressed as reversible bimolecular reactions: i.e.

$$\begin{matrix} L \\ + R \\ I \end{matrix} \quad \begin{matrix} \rightleftharpoons \\ \rightleftharpoons \end{matrix} \quad \begin{matrix} L\text{-}R \\ I\text{-}R \end{matrix} \tag{7}$$

The equilibrium dissociation constants for these interactions are denoted as K_D for the radioligand and K_i (with i instead of D, to avoid confusion) for the competitor. The relationship between the amount of radioligand binding (B) and the competitor concentration (i.e. the competition binding plot) obeys the following equation:

$$B = B_{control} - B_{control}/(1 + K_i \times (1 + [L]/K_D)/[I]) \tag{8}$$

where control binding ($B_{control}$) represents radioligand binding in the absence of the competitor.

An interesting situation occurs when the competitor has decreased control binding by 50% (i.e. when $B = B_{control}/2$). This situation occurs when the concentration of competitor (usually denoted as IC_{50}) is equal to $K_I \times (1 + [L]/K_D)$. The competitor's K_i can thus be calculated from the experimental IC_{50} value by the following equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/(1 + [L]/K_D) \tag{9}$$

[L] is known and K_D is obtained from saturation binding experiments. Please note that K_i is a constant, but that the IC_{50} value is dependent on the concentration and the K_D of the radioligand used (Figure 37). Accordingly:

- K_i values represent affinity constants. They are the only valid parameters when comparing data from competition binding experiments which have not been performed under strictly the same conditions (e.g. with receptors from different

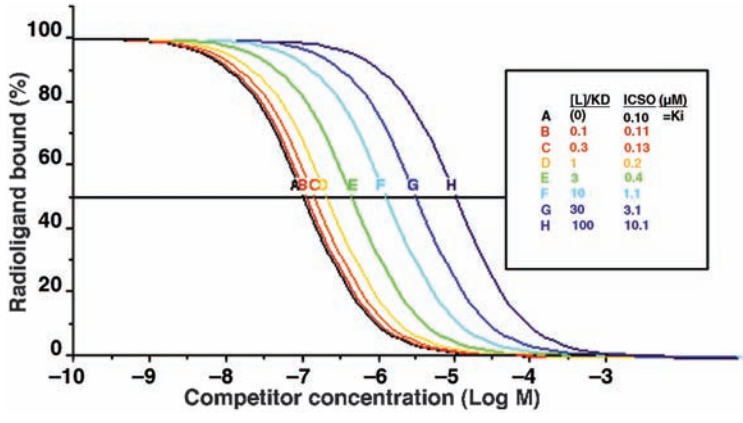


Figure 37 Effect of the $[L]/K_D$ ratio of the radioligand on the competition curve of a drug with K_i = 0.1 μM.

cell types, different radioligands). K_i values of different competitors may be compared with each other, e.g. to give a rank order of drug affinities. Yet it should be kept in mind that their value depends on experimental factors such as the incubation temperature and buffer composition.

- IC_{50} values may only be compared to one another when they are obtained under strictly identical conditions; i.e. when the same source of receptors (membranes or cells of the same origin) and the same concentration of the same radioligand are used for all the competition binding experiments. This provides information about the rank order of drug affinities and about drug affinity ratios without the need to know the proper K_i value of each drug.
- IC_{50} values approximate K_i values when the radioligand concentration is well below its K_D for the receptor. This is often the case in high-throughput screening.

A practical example of the utility of competition binding curves for finding out whether a radioligand truly binds to the desired receptor is shown in Figures 38 and 39. In these experiments, various unlabelled drugs compete with the tritiated β-adrenergic antagonist [^{3}H]-dihydroalprenolol for binding to turkey erythrocyte membranes. The experiments were performed under identical conditions (i.e. the radioligand concentration: 10 nM) so that the IC_{50} values of the curves can be compared with each other. The affinity of the agonists decreases as: (−)-isoproterenol > (−)-noradrenaline ⩾ (−)-adrenaline.

The non-selective α-adrenergic antagonist phentolamine has only very low affinity and no competition can be demonstrated for the non-bioactive compounds catechol

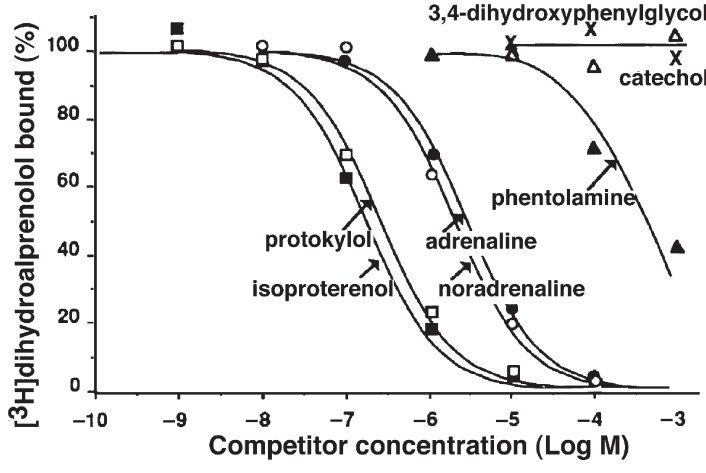


Figure 38 Competition binding curves for β_1-adrenergic receptors in turkey erythrocyte membranes. Reprinted from *Biochemical and Biophysical Research Communications*, **86**, Bottari S., Vauquelin G., Durieu O., Klutchko C. and Strosberg A.D., The beta-adrenergic receptor of turkey erythrocyte membranes: conformational modification by beta-adrenergic agonists, 1311–1318. Copyright (1979), with permission from Elsevier.

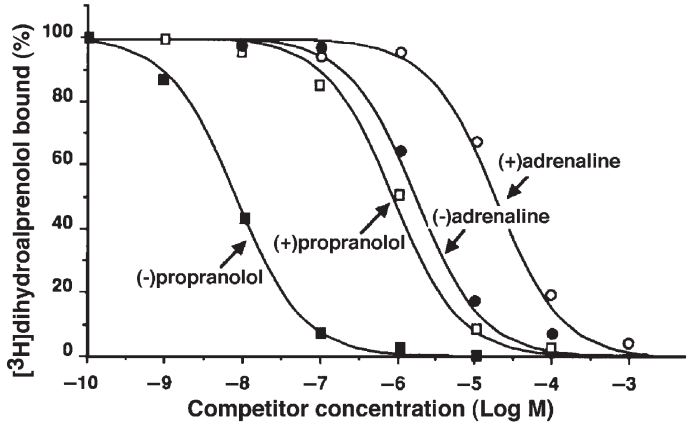


Figure 39 Stereoselective competition binding for β_1-adrenergic receptors (Strosberg, 1987).

and 3,4-dihydroxy phenylglycol. These characteristics fit with those obtained for β_1-adrenergic receptors by functional studies. Moreover, in agreement with the known stereoselectivity of β-adrenergic receptors for antagonists such as propranolol and agonists such as adrenaline, the dextrorotary isomers display lower affinity than the levorotary isomers. In this respect, adrenaline and noradrenaline possess an asymmetric carbon atom in the ethanolamine side chain. They can thus exist either as levorotary (prefix 'L' or '(−)') or as dextrorotary (prefix 'D' or '(+)') stereoisomers. Only the levorotary form of adrenaline is produced and released into the bloodstream. It had already been observed in 1926 that this natural messenger is about 10 times more active than its (synthetic) (+)-isomer in raising blood pressure. This difference was explained in 1933 by the three-point attachment model of Easson and Stedmann (Figure 40): i.e.

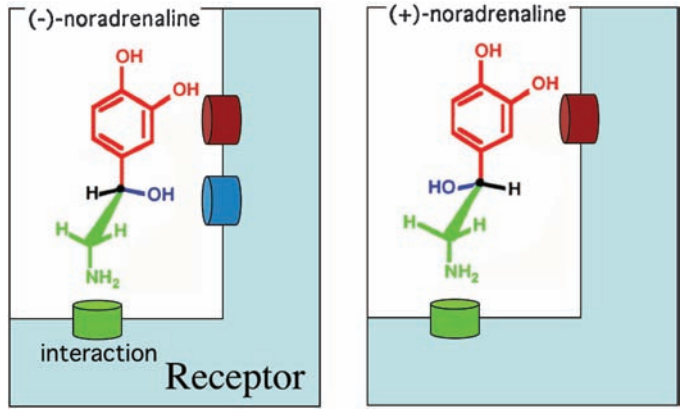


Figure 40 Three-point attachment model of Easson and Stedmann: three bonds for (−)-noradrenaline and only two bonds for (+)-noradrenaline.

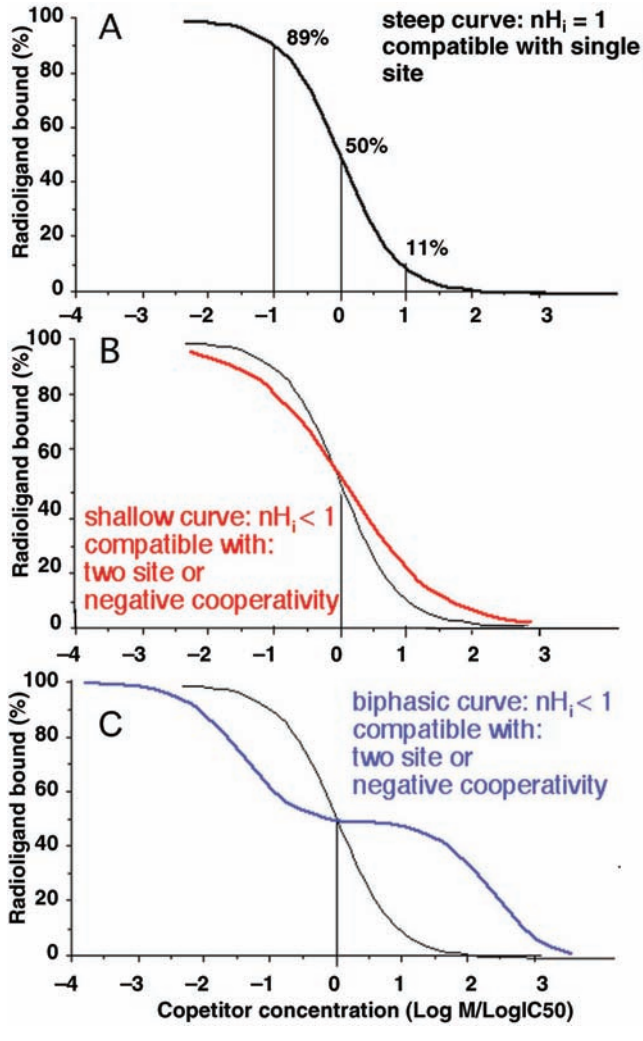


Figure 41 Sigmoidal, shallow and biphasic competition binding curves.

three of the four groups linked to the assymetric carbon of (−)-adrenaline (aromatic ring, OH and amino group) are involved in the interaction with the receptor whereas the OH group of (+)-adrenaline has the 'wrong' orientation. Such 'stereoselectivity' is very common for receptors.

When the radioligand and the competitor bind in a reversible fashion to a single population of non-interacting receptors (i.e. in the simple situation), the competition binding curve should have a steep sigmoidal shape (with 11, 50 and 89% decrease in radioligand binding when the competitor concentration is 1/10, equal or 10 times its IC_{50} value) (Figure 41A). The Hill coefficient of the competitor 'nH_i' can be calculated

from competition binding plots (Equation 10). In the case of a sigmoidal plot, nH_i equals 1.

$$\text{Log}((B_o - B_i)/B_i) = nH_i \times (\text{Log}([I]) - \text{Log}(IC_{50}) \quad (10)$$

where B_o and B_I are the binding of the radioligand in the absence and presence of competitor (I), respectively.

Radioligands may possess the same affinity for two (or more) receptors, receptor subtypes or even receptor subpopulations. When such different receptors co-exist in the same cells or membrane preparation, they will not be discriminated from each other by the radioligand. Indeed, the saturation binding curves appear as if the radioligand binds to a single class of non-co-operative sites. However, these different receptors (subtypes, subpopulations) may possess different affinities for certain unlabelled drugs, so that they can be detected and discriminated from each other by competition binding experiments with these drugs. In such cases, the nH_i values of such curves will be less than one. There are two situations:

- First, the competitor displays a large (>1000-fold) difference in affinity for the different receptors, subtypes or subpopulations (Figure 41C). In this situation, the competition binding curve will be biphasic (i.e. with a plateau) and the parameters of each component (% of binding, IC_{50}) are easy to measure. This is the case for [^{3}H]-rauwolscine, which binds with the same affinity to α_2-adrenergic receptors and serotonergic receptors of the 5-HT_{1A}- type (Figure 42). Serotonin possesses

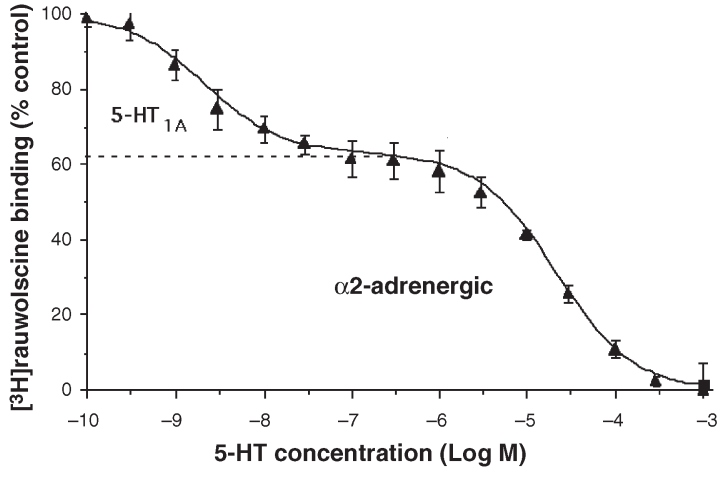


Figure 42 Competition binding curve of serotonin (5-HT) for α_2-adrenergic and 5-HT_{1A} serotonergic receptors in membranes from human frontal cortex. The radioligand, [^{3}H]rauwolscine, binds with equal affinity to both receptors and both receptors are present in human frontal cortex. Reprinted from *Journal of Neurochemistry*, **58**, De Vos H. Czerwiec E. De Backer J.-P. De Potter W. and Vauquelin G., Regional distribution of alpha2A and alpha2B adrenoceptor subtypes in post-mortem human brain, 1555–1560. Copyright Blackwell Publishing.

a much higher affinity for its own receptor than for the α_2-adrenergic receptors and can be used to distinguish both receptors from each other in, for example, human frontal cortex membranes. At low concentrations it will first occupy the 5-HT_{1A} receptors and only when its concentration gets high enough will it start to occupy the α_2-adrenergic receptors. 5-HT_{1A} receptors represent 40% of the binding and α_2-adrenergic receptors 60%. The K_i values of serotonin for these receptors can be calculated from the IC_{50} values according to the equation of Cheng and Prusoff.

- Second, the competitor only possesses a limited (<100 times) difference in affinities for the different receptors, subtypes or subpopulations (Figure 41B). Such competition curves are shallow ($nH_i < 1$) but, since both components are not separated by a distinct plateau, it is necessary to calculate the competition binding parameters of each component (% of binding, IC_{50}) by computer-assisted analysis. Analysis of shallow competition curves is illustrated by Figure 43 for α_{2A}- and α_{2B}-adrenergic receptors. A radioligand such as the antagonist [^{3}H]-RX821002 is unable to discriminate between them, but certain antagonists such as prazosin possesses a relatively weak selectivity for the α_{2B} receptors. For the nucleus caudatus, the competition curve is quite shallow ($nH_i = 0.48$). This indicates that α_{2A} and α_{2B} receptors are both present. The simplest way to describe such curve is to give its nH_i and IC_{50}. However, since K_is refer to individual competitor–receptor interactions, it is not possible to calculate any K_i from this IC_{50}. Computer-assisted analysis is necessary to calculate the proportion of α_{2A} and α_{2B} receptors and their IC_{50} (and K_i) for prazosin.

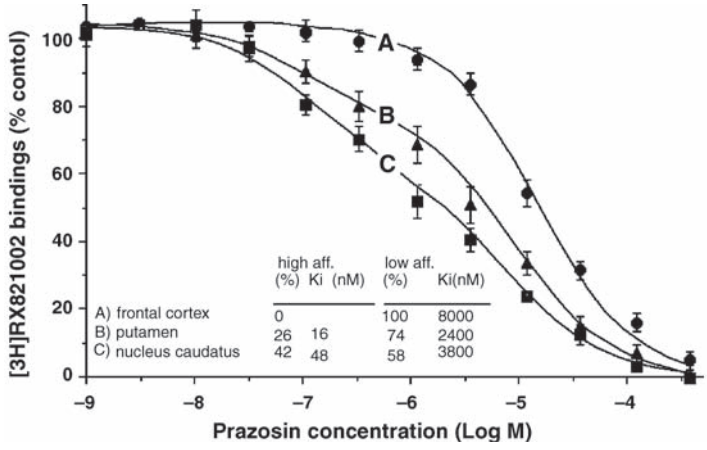


Figure 43 Competition binding curve of prazosin (α_{2B}- subtype- selective antagonist) for α_2-adrenergic receptors in membranes from different human brain regions. Reprinted from *European Journal of Pharmacology*, **207**, De Vos, H., Vauquelin, G., De Keyser, J., De Backer, J.-P. and Van Liefde, I., [3H]rauwolscine behaves as an agonist for the 5-HT1A receptors in human frontal cortex membranes, 1–8. Copyright (1991), with permission from Elsevier.

- The curve is also shallow for the putamen but it is steep (nH_i = 1.01) for the cortex. For this brain area, the curve can be analyzed according to a single-site model and the high K_i of prazosin indicates that only α_{2A} receptors are present in this brain region.

An interesting situation occurs for G protein-linked receptors (GPCRs) in broken cell preparations. These can often be divided into two subpopulations with different affinities for agonists, but with the same affinities for antagonists. This heterogeneity towards agonists is not related to differences in primary amino acid sequence, but rather to their capability to undergo functional coupling to the G proteins (see Section 4.9). The receptor population that undergoes functional coupling to G proteins (coupling-prone receptors) displays high agonist affinity. The receptor population that is unable to couple (non-coupled receptors) displays low agonist affinity. The competition curves will therefore depend on the nature of the radioligand and competitor used (Figure 44):

- If the radioligand is an antagonist, it will regard the receptors as a single class of non- co-operative sites. Antagonist competition binding curves will be steep, but agonist competition binding curves will be shallow.
- If the radioligand is an agonist, it will preferentially label the coupling-prone receptors (especially at low concentrations). Hence, the non-coupled receptors may not be detected in these assays.

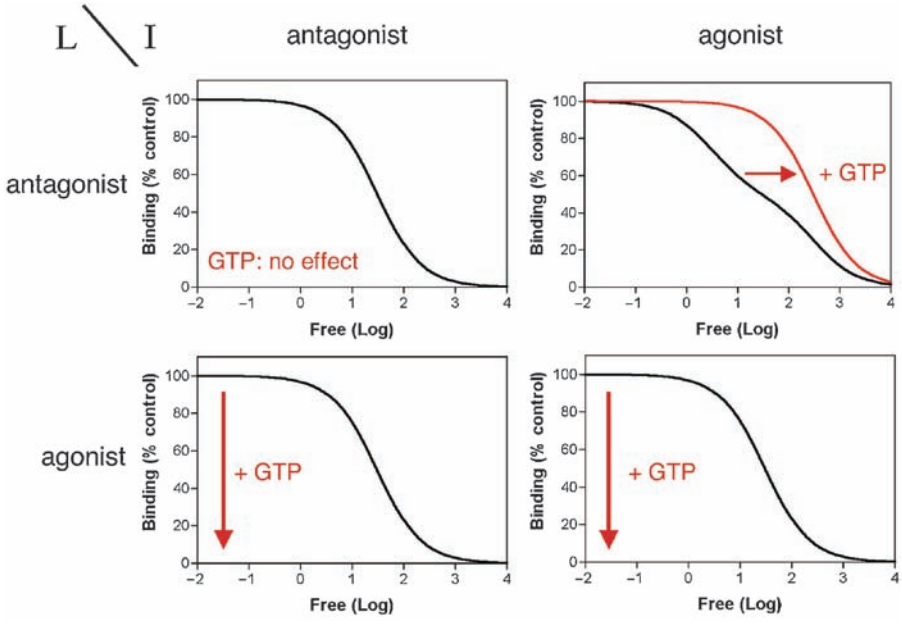


Figure 44 Effect of GTP and related guanine nucleotides on competition binding curves.

Shallow competition binding curves for agonists may thus reflect two distinct phenomena: the presence of different receptors or functional receptor heterogeneity. Fortunately, it is possible to distinguish between these two possibilities by using guanine nucleotides such as GTP. These compounds break up agonist–receptor–G protein complexes, so that the receptors return to the uncoupled, low agonist-affinity form (see Section 4.1). In practice, GTP is thus capable of producing a rightward shift and steepening of the agonist versus radiolabelled antagonist competition binding curve, at least if the high affinity is related to functional coupling of the receptor to the G proteins (Figure 44, Figure 157). When the radioligand is an agonist, its binding will be greatly reduced by GTP.

2.4 Kinetic experiments

Unlike the saturation and competition binding experiments, kinetic studies provide information about the time-course of the binding. These studies usually comprise two types of experiments (Figure 45):

- Determination of dissociation rate constant: in these experiments the radioligand is incubated with the receptor and the dissociation is initiated either by adding an excess of unlabelled ligand (so that free receptors are immediately occupied and no longer accessible to the radioligand) or by dilution (usually after washing away the free radioligand, so that its free concentration is too low to undergo noticeable re-association). Then, the amount of binding is measured after different periods of time (t). First-order reactions occur when the radioligand–receptor complex is a single bimolecular species (L−R). Binding decreases exponentially and the dissociation rate constant (k_{-1}) can be shown to be related to the time it takes for half of the L−R complexes to dissociate ($t_{1/2}$) by the equation $k_{-1} = 0.693/t_{1/2}$. Dissociation data can easily be calculated by plotting ln(binding at time t/binding at the start of the dissociation experiment) (i.e. $\ln(B_t/B_{t=0})$) versus the dissociation time. In the case of a first-order reaction, the plot will be linear and the slope corresponds to the negative value of k_{-1} (usually expressed in min^{-1}).
- Determination of the association rate constant: in these experiments the radioligand is incubated with the receptor and the amount of binding is measured after different periods of time (t). Binding will increase until equilibrium (equilibrium binding B_{eq}) is reached. Under circumstances where [L] is added at concentrations in considerable excess of [R] (as is most often the case), [L] can be assumed not to change throughout the incubation. In contrast, as only [R] decreases, the rate of association may be regarded as being a 'pseudo first-order' reaction. When plotting $\ln(B_{eq}/(B_{eq} - B))$ versus the association time, the slope of the plot gives the pseudo first-order rate constant (k_{obs}). Since the radioligand also undergoes dissociation from the receptor in this type of experiments, it ensues that k_{obs} reflects both the association and

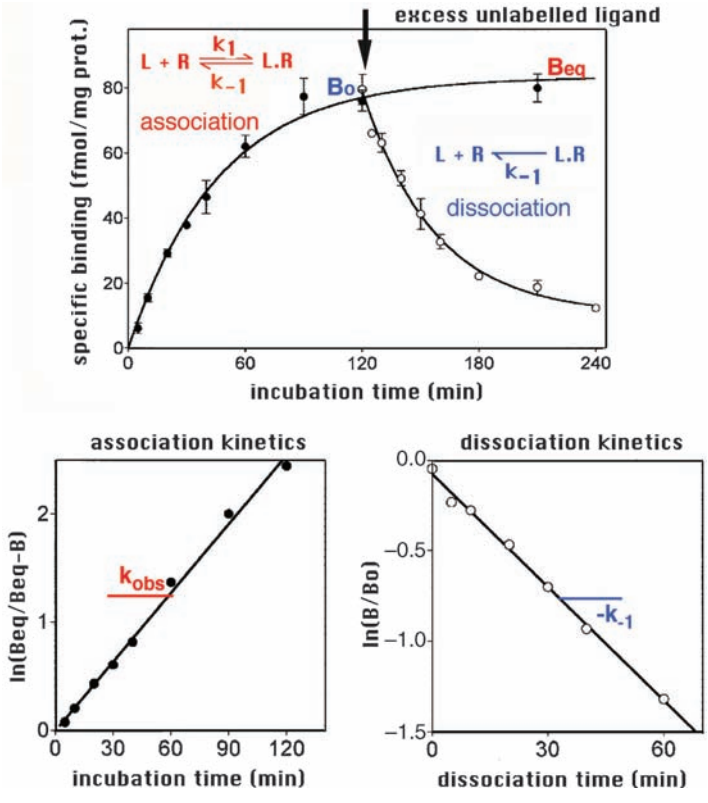


Figure 45 Association and dissociation binding of [^{3}H]NPY to the neuropeptide Y receptors of the Y_1-type in the human SK-N-MC cells. Reprinted from *European Journal of Pharmacology*, **346**, Van Liefde, I., Vanderheyden, P. M., Fraeyman, N., De Backer, J. P., Vauquelin, G., Human neuropeptide YY1 receptors exert unequal control of the extracellular acidification rate in different cell lines, 87–95. Copyright (1998), with permission from Elsevier.

the dissociation of the radioligand. The true, bimolecular association rate constant k_1 (usually expressed in $M^{-1} \times min^{-1}$) can be obtained by the following equation:

$$k_1 = (k_{obs} - k_{-1})/[L] \tag{11}$$

Kinetic data allow the discrimination between fast reversible, slowly reversible and irreversible ligands (dissociation kinetics). Both the association and dissociation constants provide an estimation of the equilibrium dissociation constant (K_D) independently of saturation binding experiments, i.e.:

$$K_D = k_{-1}/k_1 = [L] \times k_{-1}/(k_{obs} - k_{-1}) \tag{12}$$

When the k_{-1}/k_1 ratio is quite distinct from the K_D obtained from saturation binding experiments, the possibility arises that the ligand induces a time-dependent change in receptor conformation that goes along with an increase (or decrease) in receptor affinity. An alternative explanation is that the k_{-1} value is incorrect due to 'rebinding' of dissociated radioligand molecules to the receptors (see below) or to the fact that the unlabelled ligand used to prevent such 'rebinding' interacts with an allosteric site at the receptor (see Section 4.14).

Kinetic data also provide information about the time required for binding of a radioligand reaching equilibrium (association kinetics). This information is crucial for the set-up of saturation and competition binding experiments. Indeed, the K_D and K_i values which are derived from such experiments are only meaningful when, at any concentration of radioligand and competitor, binding is at or at least close to equilibrium. When the incubation time is to short, it could:

- Produce a false estimation of B_{max} and K_D values for saturation binding.
- Produce a false estimation of IC_{50} and K_i values for competition binding experiments.

Radioligands that are dissociated from the receptor will accumulate in the medium and, if they are not constantly removed, they may bind to the receptor again. This

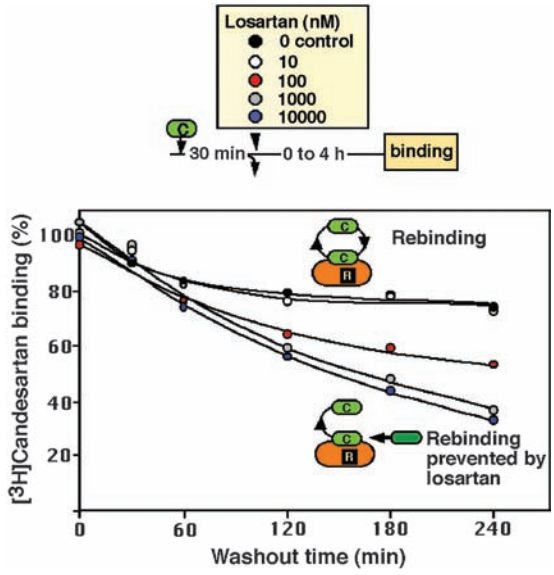


Figure 46 Cells expressing AT_1 receptors were incubated for 30 min at 37 °C with [^{3}H]candesartan, washed and incubated in fresh medium with no or different concentrations of the unlabelled AT_1-receptor antagonist losartan. The remaining binding of [^{3}H]candesartan was measured after the time intervals indicated. Reprinted from *European Journal of Pharmacology*, **367**, Fierens, F., Vanderheyden, P. M., De Backer, J. P., Vauquelin, G., Binding of the antagonist [3H]candesartan to angiotensin II AT1 receptor-transfected Chinese hamster ovary cells, 413–422. Copyright (1999), with permission from Elsevier.

'reassociation' or '*rebinding*' is particularly prominent when the radioligand displays high affinity for the receptor. Whereas rebinding might take place if the incubation medium is simply replaced by fresh medium, it will be effectively prevented when the same or another unlabelled ligand is present in sufficiently large excess in the fresh medium. This is because the receptors become immediately occupied by the unlabelled ligand as soon as the radioligand dissociates. This effectively prevents the 'rebinding' of the radioligand. It is only in the absence of such rebinding that the dissociation rate/half-life of a radioligand–receptor complex can be correctly measured.

This phenomenon has unequivocally been demonstrated by binding studies with the AT_1-receptor antagonist [^{3}H]candesartan (Figure 46). When AT_1-receptor expressing cells were incubated with [^{3}H]candesartan and the medium was replaced by fresh medium only, its dissociation was estimated to be half-maximal after 6–8 hours. Yet, when unlabelled candesartan or any other AT_1-receptor ligand was present in the replacing medium, they all produced a concentration-wise decrease in the apparent half-life of the [^{3}H]candesartan–receptor complex until a half-life of two hours was attained. This value reflects the actual dissociation of this radioligand.

Another factor that severely affects kinetic measurements is the temperature (Figure 47). In this respect, the association and dissociation of radioligands and their receptors are markedly accelerated upon increasing the temperature.

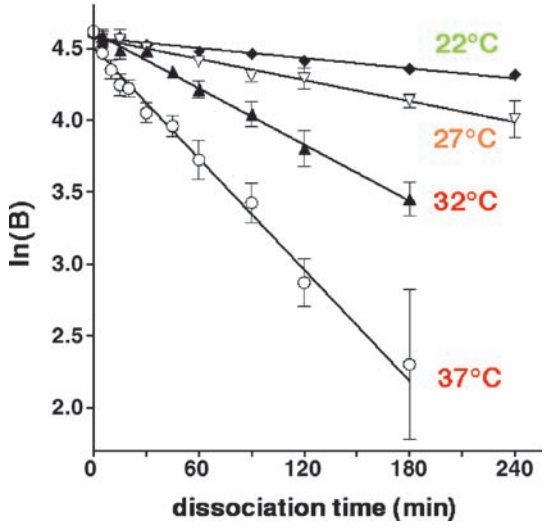


Figure 47 Dissociation of [^{3}H]candesartan from human AT_1 receptors on CHO cells. Cells were incubated for 30 min at 37 °C with [^{3}H]candesartan, washed and incubated in fresh medium (containing losartan) at the temperatures indicated. Remaining binding of [^{3}H]candesartan (B, expressed as a percentage of B_o) was measured after the time intervals indicated. Reprinted from *Biochemical Pharmacology*, **63**, Fierens, F., Vanderheyden, P.M. L., Roggeman, C., Vande Gucht, P., De Backer, J.-P. and Vauquelin, G., Distinct binding properties of the AT1 receptor antagonist [3H]candesartan to intact cells and membrane preparations, 1273–1279. Copyright (2002), with permission from Elsevier.

2.5 Regional distribution of receptors

Radioligand binding on membrane preparations provides information concerning the interaction between drugs and well-defined receptors, as well as about the occurrence of such receptors in certain tissues or organs. However, most tissues and organs are very complex and comprise a number of different cell types, each with their own receptor and response specificity. In this context, the brain is especially complex since it contains a great number of different neurones. Yet, neurones that are responsible for very specific brain functions are often confined to small regions (nuclei) of the brain. Information about the regional distribution of receptors in complex tissues such as the brain therefore contributes to our understanding of their physiological role and their implication in certain pathophysiological conditions. However, radioligand binding experiments on membrane preparations provide only a little information: the resolution is limited by the resolution of the dissection. *Autoradiography* of radioligand binding to thin sections of brain allows the localization of receptors with a much higher degree of resolution (i.e. to the light microscopic level) (Figure 48). However, the resolution is still insufficient to make a distinction between pre- and postsynaptic receptors.

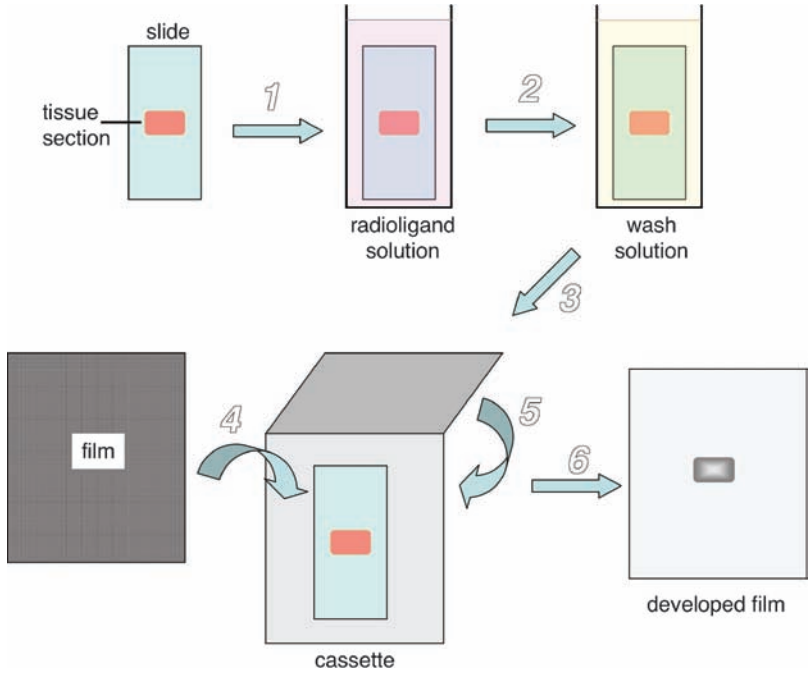


Figure 48 Classical technique for autoradiographic detection of receptors on thin tissue section. Steps: 1) incubation with radioligand, 2) wash, 3) put in casette, 4) overlay film, 5) close cover and expose, 6) remove film and develop.

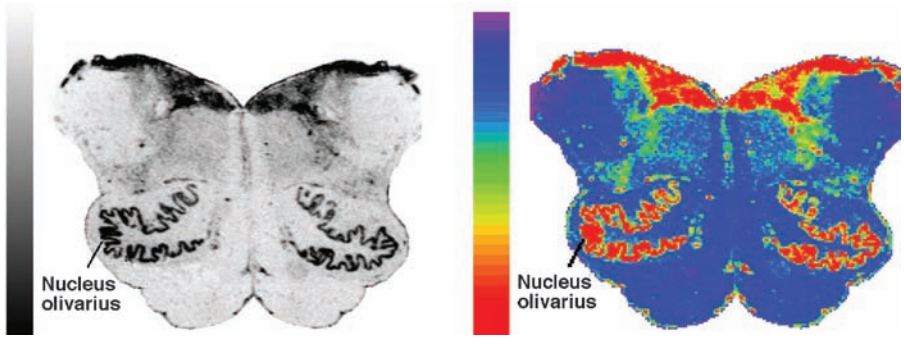


Figure 49 Autoradiography of [^{3}H]idazoxan binding to imidazoline binding sites (I_2-receptors) in a cross section of the human medulla: left: grey scale, right: adopted colours. Reprinted from *Progress in Histochemistry and Cytochemistry* **26**, De Vos, H., De Backer, J.-P., Convents, A., De Keyser, J. and Vauquelin, G., Identification of alpha2 adrenoceptors in the human nucleus olivarius by radioligand binding, 259–265. Copyright (1992), with permission from Elsevier.

For this approach, tissues are cut in thin (usually not exceeding 10 μM) slices and put on coverslips. The slices are subsequently incubated with radioligand and washed. The radioactivity on the slices can then be determined by apposition of a sensitive film or by dipping the slice in a photographic emulsion. After exposure (from a few hours for [^{125}I]-labelled ligands to a few weeks for [^{3}H]-labelled ligands), the film or emulsion is developed. Black corresponds to a high density of radioligand (Figure 49, left). Newer detection techniques are available for [^{3}H]-labelled ligands.

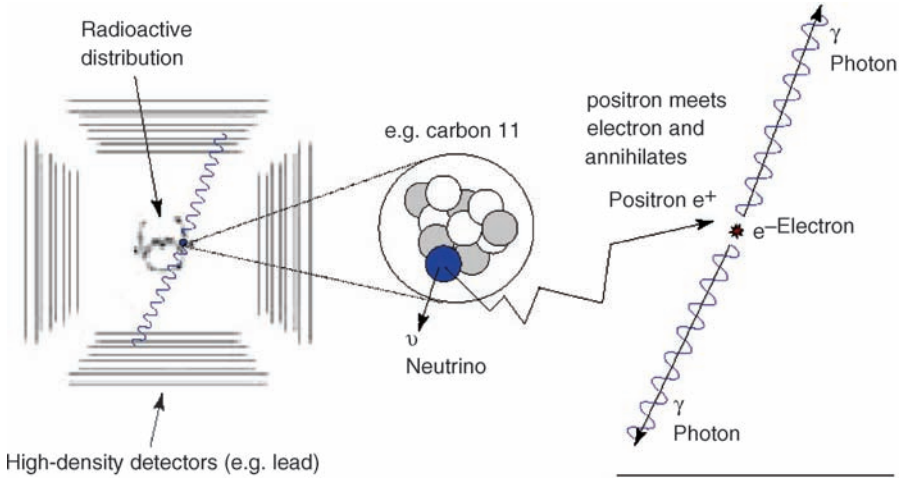


Figure 50 Principle of PET scan. Reprinted from *Trends in Pharmacological Sciences*, **22**, Reader, A.J. and Zweit, J., Developments in whole-body molecular imaging of live subjects, 604–607, © (2001), with permission from Elsevier.

They rely on the apposition of a solid scintillator sheet and real-time imaging of the emitted photons. This brings down the exposure time to hours instead of days and weeks. Commercially available radioactive standards can be exposed along with the tissue sections and this allows the conversion of the different grey levels into binding values. The amount of radioligand bound to each location of the slice can therefore be quantified by densitometry. Computer-assisted image analysis greatly facilitates this task and it also allows different grey levels to be replaced by specific colours (Figure 49).

Finally, the *in vivo* determination of the regional distribution of receptors in e.g. the brain can be obtained by the *PET scan* technique (Figure 50). 'PET' stands for positron emission tomography and involves the administration of a positron emitting radioligand to a patient. The radioligand will accumulate in receptor-rich areas and the emitted positrons will, upon encounter with electrons, annihilate to produce gamma rays, which can be detected. This technique is fairly safe for the patient since the radioactive half-life is very short (in the order of minutes) but, on average, the positrons will only meet electrons a few millimetres away from their point of departure so that the degree of resolution is rather poor (Figure 51).

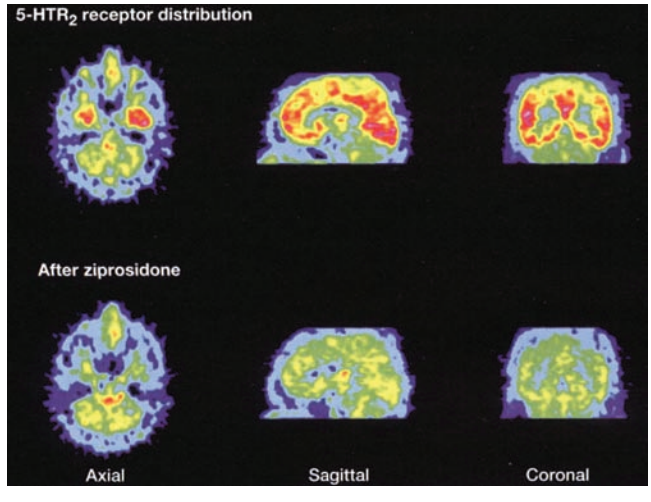


Figure 51 Distribution of 5-HT_2 receptors in the brain. Accumulation of positron emitting radioligand ([^{18}F]-setoperone) in brain 'sections' in untreated (total) and Ziprosidone-treated (non-specific accumulation) humans. Reproduced from Fischman, A. J., Bonab, A. A., Babich, J. W., Alpert, N. M., Rauch, S. L., Elmaleh, D. R., Shoup, T. M., Williams, S. A. and Rubin, R. H. (1996) Positron emission tomographic analysis of central 5-hydroxytryptamine2 receptor occupancy in healthy volunteers treated with the novel antipsychotic agent, ziprasidone. *Journal of Pharmacology and Experimental Therapeutics*, **279**, 939–947, with permission from the American Society for Pharmacology and Experimental Theraputics.

2 Radioligand binding studies

2.1 Technical aspects of radioligand binding

For a long time, hormone and neurotransmitter receptors remained abstract concepts whose existence was proposed only to explain pharmacological effects on target tissues. Since the mid-seventies, it has become possible to investigate of the receptor molecules themselves by the means of radioligand binding. This technique also allows the direct evaluation of the binding properties of any compound for a given membrane-bound receptor. Very often, radioligand binding experiments are performed with more-or-less purified cell membranes (Figure 26).

Radioligand binding is initiated by the incubation of cells, cell homogenates or purified plasma membrane preparations with an adequate radioactively labelled drug; the '*radioligand*'. Adequate radioligands can be selected out of the wide variety of commercially available agonists and antagonists. Obviously, these radioligands should display high affininty and selectivity towards the receptor of interest. If no such radioligands are available, ligands can be custom-labelled by the investigator (for radioiodination) or by specialized institutions:

- Tritium [^{3}H] and iodine [^{125}I] are the most frequently used isotopes. Because of the long half-life of tritium (12.3 years), the tritiated ligand does not have to be resynthesized or repurchased frequently. They can be stored for a rather long time but, nevertheless, care should be taken to check for radiation-induced ligand degradation. In addition, because of the relatively low specific radioactivity of tritium (29 Ci/mmol), tritiated ligands are only suitable when the biological material contains sufficient amounts of the desired receptor.
- If not, radioiodinated ligands are more suitable because of the relatively high specific radioactivity of ^{125}I (2125 Ci/mmol). However, the short half-life (60 days), the exposure of the investigator to gamma rays and the fact that the pharmacological and physicochemical properties of the iodinated ligand may deviate considerably from those of the original ligand constitute major drawbacks of this isotope.

G Protein-Coupled Receptors: Molecular Pharmacology From Academic Concept to Pharmaceutical Research
Georges Vauquelin and Bengt von Mentzer © 2007 John Wiley & Sons, Ltd. ISBN: 978-0-470-51647-8

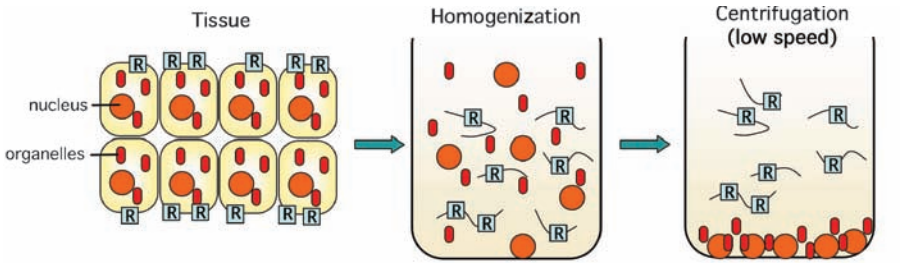


Figure 26 Preparation of cell membranes.

Separation of free and receptor-bound drug represents the most delicate step. This is commonly done by one of the three following techniques (Figure 27):

- *Filtration*: the free radioligand passes through the filter whereas the receptor-bound radioligand remains on the filter. Counting the radioactivity on the

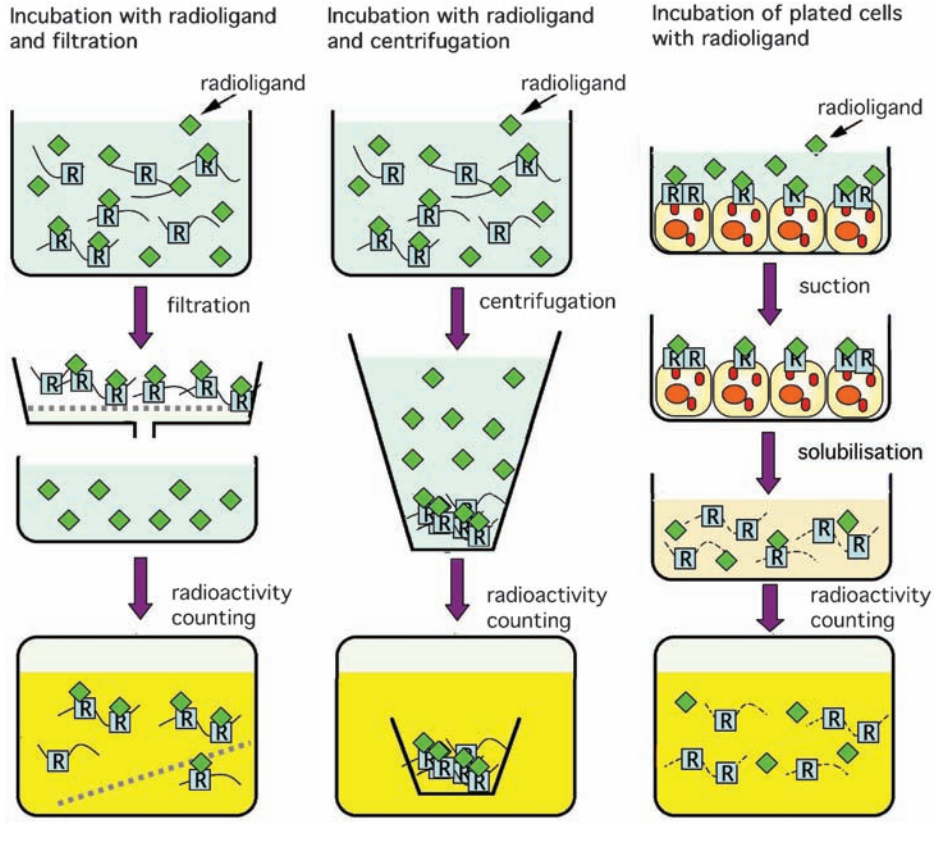


Figure 27 Measurement of radioligand binding by different methods involving the removal of free radioligand.

filter allows the amount of receptor-bound radioligand to be quantified. This technique is usually employed when using membrane preparations and when performing radioligand binding to intact cells in suspension. The popularity of this technique results from the ability to handle a large number of samples with relative ease as well as the commercial availability of a variety of filtration devices. Moreover, the filters can be washed thoroughly and rapidly with fresh buffer (preferentially ice-cold to prevent dissociation of the radioligand-receptor complex). This allows the removal of remaining traces of free radioligand. The filters are usually of glass fibre, but sometimes it is also necessary to coat them with polyethyleneimine or to siliconize them to prevent radioligand absorption to the filter. For 'high throughput screening', the radioligand binding may be performed in microtiter plates with 96, 384 or even more wells. After the incubation, the contents of the wells are filtered simultaneously with a cell harvester. For modern high-throughput screening, robots are used to handle screen compounds and buffers as well as to perform the filtration step.

- *Centrifugation*: membranes or cells precipitate, whereas the free radioligand remains in solution, and can be discarded. Quantitation of the amount of receptor-bound radioligand is done by counting the radioactivity of the pellet. Since no thorough washing is involved, this technique is especially useful when the radioligand–receptor complex dissociates rapidly. However, this technique results in high background radioactivity due to the trapping of radioligand in the pellet. Manual manipulations and the resulting risk of contamination constitute additional disadvantages of the technique.

- *Suction* binding to intact cells may be achieved by plating them on the bottom of each well in (e.g. 24 well) multiwell plates. After the incubation, the free radioligand is removed by suction, the cells may then be washed with fresh buffer (preferentially ice-cold to prevent dissociation of the radioligand–receptor complex), and the remaining receptor-bound radioligand in each well is counted. For this purpose, plated cells are often treated with a detergent solution to solubilize the membranes. The radioactivity moves into solution and can then be counted easily. Here again, many manual manipulations are required.

The scintillation proximity assay (SPA) technique demands even fewer manipulations since the separation between free and bound radioligand is avoided (Figure 28). For this technique, small scintillant-containing beads are already present in the incubation tube/well. When these beads are also coated with wheat germ agglutinin (WGA), they will attach intact cells or membranes. The principle of the technique is based on the assumption that the overwhelming majority of the free radioligand molecules are too far from the beads for the scintillant to be activated whereas the receptor-bound radioligand is in close proximity to the beads and, hence, capable of stimulating the scintillant. Therefore, the measured scintillation will mainly arise from bound radioligand molecules.

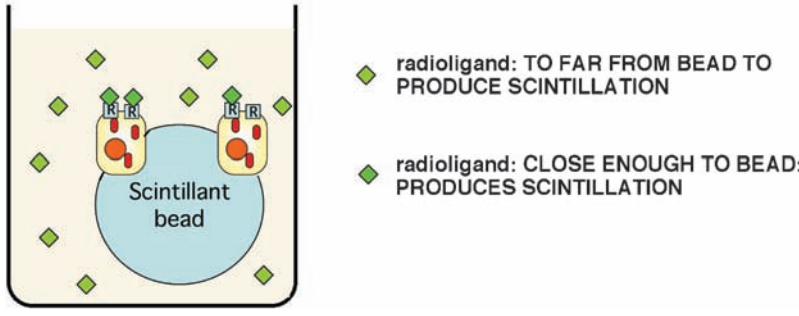


Figure 28 Measurement of radioligand binding by the SPA technique.

Binding of a radioligand to a physiologically relevant receptor (i.e. '*specific binding*') should at least obey the following criteria:

- The binding should be saturable, since a finite number of receptors are expected in a biological preparation.
- The potency of unlabelled ligands to compete with the radioligand for binding to the receptor should parallel their potency to provoke (for agonists) or block (for antagonists) receptor-mediated responses.

Radioligands not only bind to their receptor (Figure 29). They might also bind to other receptors and to non-receptor sites such as carrier proteins, enzymes, cell components recognizing certain chemical moieties of the radioligand (e.g. the catechol moiety for radiolabelled catecholamines). In addition, they might even

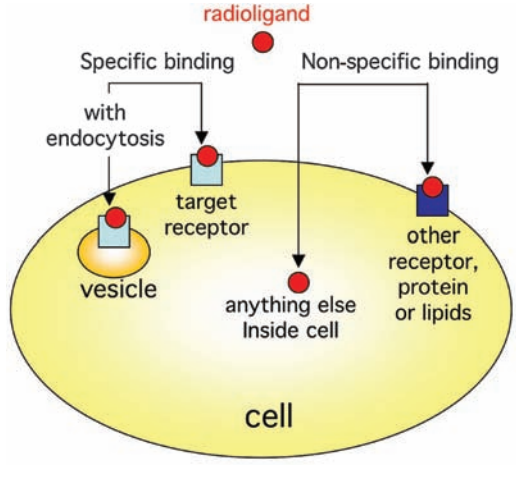


Figure 29 Possible interactions between radioligands and different cellular sites.

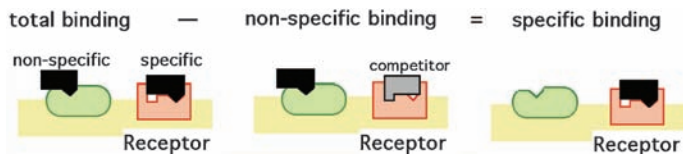


Figure 30 Determination of total and non-specific binding and the calculation of specific binding.

bind to separation materials such as filters or test tubes. This binding is called '*non-specific binding*'. One of the major problems in developing a suitable binding assay is the selection of a radioligand that shows enough specificity towards the receptor. In general, a hydrophilic (to avoid partitioning in the lipid bilayer of the membrane) radioligand with high affinity for the desired receptor may be a good candidate. However, some of the measured binding will always be non-specific. To deal with this problem, radioligand binding experiments always comprise two determinations: total binding and non-specific binding and the *non-specific binding* must be subtracted from the *total binding* to obtain the *specific binding*; i.e. binding to the receptor of interest (Figure 30).

Obtaining a correct non-specific binding value constitutes the most delicate aspect of a radioligand binding technique. In theory, non-specific binding can simply be obtained by adding an excess of competitor to the incubation mixture, so that binding of the radioligand to the receptors is completely displaced. In practice, care must be taken to choose a competitor that displaces the radioligand from the receptor only, and not from the other, non-specific sites. It is recommended to choose a potent competitor whose chemical structure is quite distinct from that of the radioligand.

Radioligand binding studies provide three main categories of information: saturation binding data, competition binding data and kinetic data.

2.2 Saturation binding

These experiments provide information about the concentration of a receptor. They are solicited to compare the concentrations of different receptors in a given tissue and to monitor variations in receptor concentration as a result of normal physiological regulation, medication and pathophysiological conditions.

For saturation binding experiments, constant amounts of membrane suspension are incubated with increasing concentrations of radioligand. Obviously, both total and non-specific binding should be measured at each concentration of radioligand (Figure 31). In the example shown, binding is expressed as a function of the free concentration of radioligand by a *saturation binding plot*. Obviously, only the specific binding is of interest.

To analyze these saturation binding data, it is necessary to advance a relevant molecular model for the radioligand–receptor interaction. In the simple (and fortunately the most common) situation, the interaction of the radioligand (L) with the

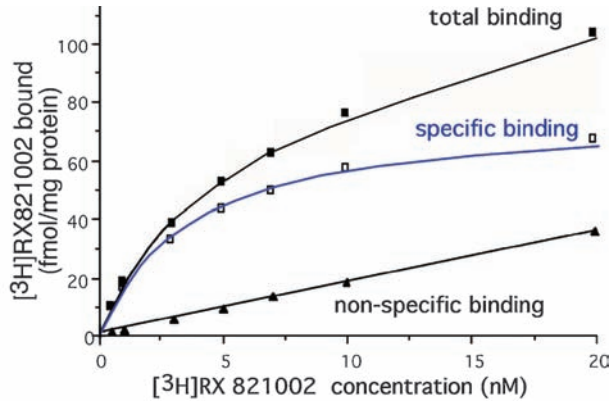


Figure 31 Saturation binding of the α_2-adrenergic antagonist [^{3}H]RX 821002 to α_2 adrenergic receptors in membranes from the human frontal cortex. Reprinted from *Neurochemistry International*, 17, Vauquelin G., De Vos H., De Backer J.-P. and Ebinger G., Identification of a2 adrenergic receptors in human frontal cortex membranes by binding of [3H]RX 821002, the 2-methoxy analog of [3H]idazoxan, 537–546. Copyright (1990), with permission from Elsevier.

receptor (R) can be expressed as a reversible bimolecular reaction that obeys the law of mass action: i.e.

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L\text{-}R \qquad (1)$$

Where k_1 and k_{-1} are the association and dissociation rate constants, respectively. The *equilibrium dissociation constant* (K_D) is given by:

$$K_D = k_{-1}/k_1 = [L] \times [R]/[L{-}R] \qquad (2)$$

Where [R] is the amount of free receptors, [L] the amount of free ligand and [L−R] the amount of bound ligand/receptors.

The relationship between the amount of occupied receptors and the free radioligand concentration (i.e. the saturation binding plot) is as follows:

$$[L{-}R] = [R_{tot}]/(1 + K_D/[L]) \qquad (3)$$

Where $[R_{tot}]$ is the total number of receptors.

'B' and 'B_{max}' (which stand for binding and maximum binding and are often expressed in fmol/mg protein) usually replace [L − R] and $[R_{tot}]$. Equation (3) then becomes:

$$B = B_{max}/(1 + K_D/[L]) \qquad (4)$$

This equation is analogous to the Michaelis–Menten equation of enzyme kinetics and describes a rectangular hyperbola. Initially, B increases almost linearly with L. Then

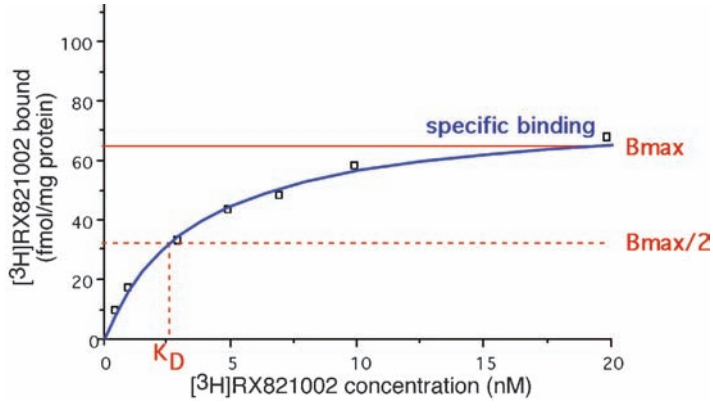


Figure 32 Graphical analysis of the saturation binding plot from Figure 31. Only specific binding is important here.

B tends to level off when L is further increased. The limit value is B_{max} (Figure 32). It is important to notice that this that B_{max} will be attained only at infinite concentrations of L. Thus, one will never observe B_{max} experimentally; B_{max} may be approached but never attained. Half-maximal binding is obtained when $L = K_D$ (since Equation (4) becomes $B = B_{max}/2$). In other words, the K_D of a radioligand corresponds to its concentration for which half of the receptors are occupied. The K_D value is thus an 'inverse' measure of the radioligand's affinity for the receptor: a low K_D corresponds to high affinity and a high K_D to low affinity.

B_{max} and K_D cannot be easily determined by graphical analysis of the saturation binding plot (Figure 32) since Equation (4) is a non-linear relationship and since B_{max} is only reached when $L = \infty$. This equation can, however, be transformed mathematically to yield a linear '*Scatchard plot*' (Figure 33) corresponding to the following equation:

$$B/[L] = -B/K_D + B_{max}/K_D \quad (5)$$

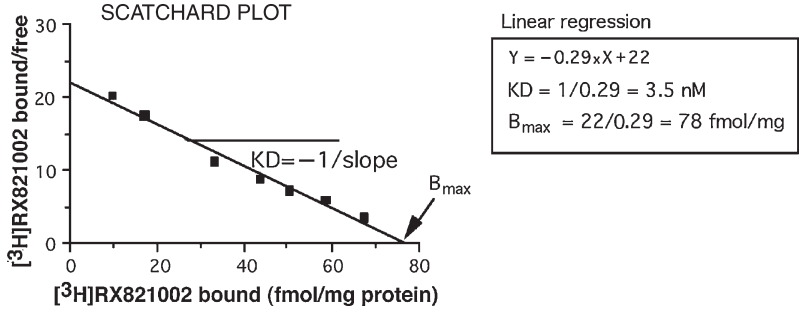


Figure 33 Scatchard analysis (Scatchard, 1949) of bound [^{3}H]- RX821002 to α-adrenergic receptors. Obtained from (Figure 31).

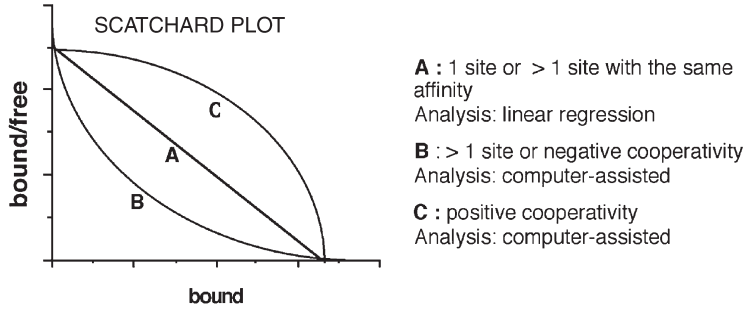


Figure 34 Scatchard plots: different possibilities.

The Scatchard plot of the above saturation binding data reveals a linear relationship between B/[L] (the ordinate) and B (the abscissa). K_D corresponds to the negative reciprocal of the line. The intercept of the line with the abscissa (i.e. when B/[L] = 0) is B_{max}. Thus, it is relatively easy to calculate K_D and B_{max} values by linear regression analysis of the Scatchard plot.

The relationship described by Equation (5) is for the simplest case; i.e. a single class of non-interacting receptor sites. However, it is possible that the radioligand binds to two different receptors with different affinities or even that one receptor is present in two or more (non-interconverting) affinity states for the radioligand. This situation will result in a non-linear Scatchard plot: i.e. showing downward concavity (Figure 34 curve B).

Moreover, certain receptors (e.g. ion channel-gating receptors which make part of a larger structure) possess multiple binding which influence each other's binding characteristics. This may result in either negative or positive co-operative interactions among the binding sites. In other words, binding of the radioligand to one site decreases (negative co-operativity) or increases (positive co-operativity) the affinity of the radioligand for other sites. This will also result in non-linear Scatchard plots with, respectively, downward concavity (negative co-operativity, Figure 34 curve B) or upward concavity (positive co-operativity, Figure 34 curve C).

A more sensitive method to ascertain whether radioligand binding obeys the law of mass action is to analyse the '*Hill plot*' of the saturation binding data (Figure 35). The Hill equation is, in fact, a logarithmic transformation of Equation (4).

$$\text{Log}(B/(B_{max} - B)) = nH \times \text{Log}([L]) - \text{Log}(K_D) \qquad (6)$$

$\text{Log}(B/(B_{max} - B))$ is the ordinate and $\text{Log}([L])$ is the abscissa of the Hill plot. The slope corresponds to the Hill coefficient: 'nH'. The law of mass action is obeyed if $nH = 1$ (in practice, values between 0.8 and 1.2 will do). This means that the radioligand binds with the same affinity to all the sites. $nH < 1$ is indicative of either negative co-operativity or of the existence of binding sites with different affinity. $nH > 1$ is indicative of positive co-operativity, i.e. where radioligand binding to one site increases the affinity of the radioligand for other sites.

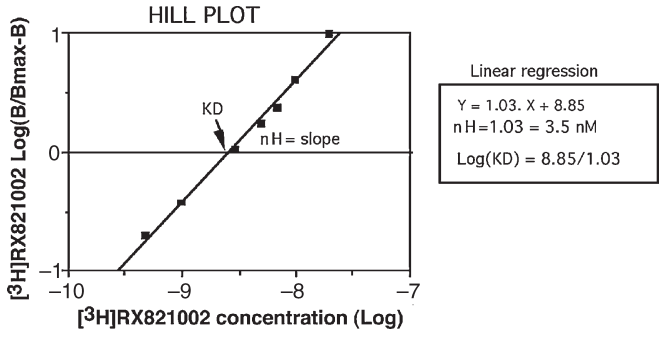


Figure 35 Hill plot of the saturation binding data from Figure 31 (B in fmol/mg protein, F in nM).

A disadvantage of a Scatchard plot is that the extrapolation of data obtained over an insufficient concentration range of L may give an artificial impression of the lack of complexity of the radioligand–receptor interactions. This may result in inaccurate Hill plots since they rely on a correct estimation of the B_{max} value. Although Scatchard and Hill plots are still sometimes shown in publications for the sake of clarity (e.g. it is easy to visualize differences in K_D and B_{max} values of one or more radioligands with a Scatchard plot), radioligand binding parameters are now almost always calculated by computer programmes which are based on non-linear regression analysis of the saturation binding data. In the case of two non-interconverting binding sites, these programmes even allow the calculation of the concentration of each site and its respective affinity for the radioligand.

Finally, certain important considerations need to be taken into account before correctly analyzing saturation binding data; they include:

- The data must represent an equilibrium situation. In practice, this means that the incubation must have occurred long enough for equilibrium to be reached. Investigating binding of a given concentration of radioligand as a function of the incubation time can check this. This binding will increase time-wise until a plateau value (corresponding to the equilibrium situation) is reached. Equilibrium binding is often obtained within minutes at usual incubation temperatures (20–37 °C), but that it may become considerably longer when the temperature is lowered to (0–4 °C).
- Binding is expressed as a function of the free concentration of radioligand. This concentration may be set equal to the concentration of radioligand added (i.e. $[L] = [L_{init}]$) if only a small fraction of the added radioligand is bound (in other words, if most of the added radioligand still remains free). If a more substantial amount of radioligand is bound (e.g. >5%), then [L] is smaller than $[L_{init}]$, and its correct value should be calculated by the equation: $[L] = [L_{init}] - [L - R]$.
- The ligand must not aggregate, at higher concentrations, to a dimer or multimer.

2.3 Competition binding

Radioligands are fairly expensive and only very few of them are specific enough for the purpose of receptor identification. Fortunately, radiolabelling of a drug is not strictly required for determining its affinity for a given receptor. This parameter can indeed be determined on basis of the drug's ability to compete with a (specific) radioligand for binding to that receptor. These competition binding experiments are now widely used by pharmacologists as a screening tool to evaluate the affinity of newly synthesized compounds (or of natural substances) for one or more receptors of interest. This approach has several advantages over the measurement of physiological responses. First, the same experimental set-up can be used to investigate the affinity of a drug for different receptors, whereas physiological responses may be very diverse and, hence, need to be monitored by different techniques. Second, the affinity of a drug for a specific receptor can be determined without ambiguity, whereas physiological responses are remote events, which may be triggered by different receptors or even be modulated at steps intermediate between receptor-stimulation and the final response.

It is important to note that the terms '*competition binding*' and '*competitor*' (for the non-radioactive substance) are commonly utilized irrespective of whether the 'competitor' is truly competitive or not. This semantic problem merits proper attention.

For competition binding experiments, constant amounts of membrane suspension are incubated with a fixed concentration of radioligand and increasing concentrations of the non-radioactive substance to be tested (the competitor), after which binding of the radioligand is measured. Binding of the radioligand is expressed as a function of the free concentration of the competitor by a *competition binding plot* (Figure 36). Competitor concentrations may span several orders in magnitude, so they are often expressed on a logarithmic scale. In the simple situation (in which the competitor is truly 'competitive') the radioligand and the competitor bind in a reversible fashion to the same site of the receptor. The radioligand (L)–receptor (R) and the competitor

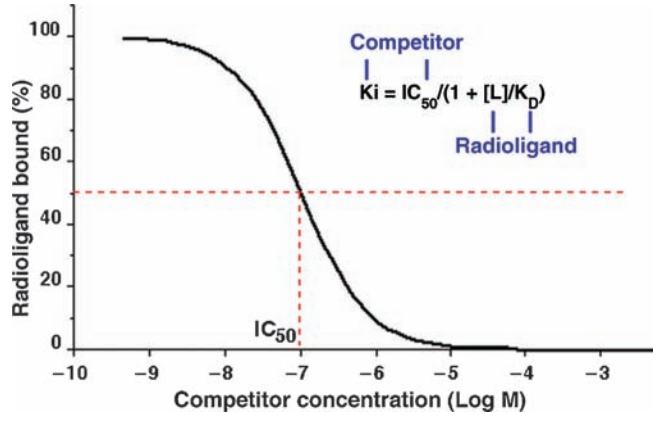


Figure 36 Competition binding curve (100% binding is binding in the absence of competitor) and determination of the competitor's K_i from the IC_{50}.

(I)–receptor (R) interactions can be expressed as reversible bimolecular reactions: i.e.

$$\begin{matrix} L \\ + R \\ I \end{matrix} \quad \begin{matrix} \rightleftharpoons \\ \rightleftharpoons \end{matrix} \quad \begin{matrix} L\text{-}R \\ I\text{-}R \end{matrix} \tag{7}$$

The equilibrium dissociation constants for these interactions are denoted as K_D for the radioligand and K_i (with i instead of D, to avoid confusion) for the competitor. The relationship between the amount of radioligand binding (B) and the competitor concentration (i.e. the competition binding plot) obeys the following equation:

$$B = B_{control} - B_{control}/(1 + K_i \times (1 + [L]/K_D)/[I]) \tag{8}$$

where control binding ($B_{control}$) represents radioligand binding in the absence of the competitor.

An interesting situation occurs when the competitor has decreased control binding by 50% (i.e. when $B = B_{control}/2$). This situation occurs when the concentration of competitor (usually denoted as IC_{50}) is equal to $K_I \times (1 + [L]/K_D)$. The competitor's K_i can thus be calculated from the experimental IC_{50} value by the following equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/(1 + [L]/K_D) \tag{9}$$

[L] is known and K_D is obtained from saturation binding experiments. Please note that K_i is a constant, but that the IC_{50} value is dependent on the concentration and the K_D of the radioligand used (Figure 37). Accordingly:

- K_i values represent affinity constants. They are the only valid parameters when comparing data from competition binding experiments which have not been performed under strictly the same conditions (e.g. with receptors from different

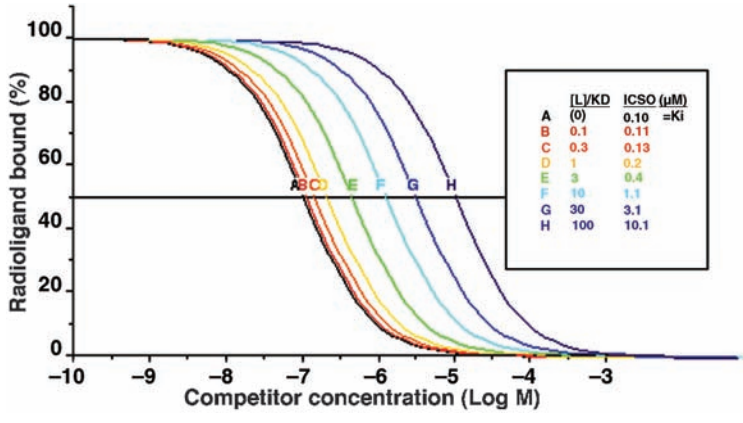


Figure 37 Effect of the $[L]/K_D$ ratio of the radioligand on the competition curve of a drug with K_i = 0.1 μM.

cell types, different radioligands). K_i values of different competitors may be compared with each other, e.g. to give a rank order of drug affinities. Yet it should be kept in mind that their value depends on experimental factors such as the incubation temperature and buffer composition.

- IC_{50} values may only be compared to one another when they are obtained under strictly identical conditions; i.e. when the same source of receptors (membranes or cells of the same origin) and the same concentration of the same radioligand are used for all the competition binding experiments. This provides information about the rank order of drug affinities and about drug affinity ratios without the need to know the proper K_i value of each drug.
- IC_{50} values approximate K_i values when the radioligand concentration is well below its K_D for the receptor. This is often the case in high-throughput screening.

A practical example of the utility of competition binding curves for finding out whether a radioligand truly binds to the desired receptor is shown in Figures 38 and 39. In these experiments, various unlabelled drugs compete with the tritiated β-adrenergic antagonist [^{3}H]-dihydroalprenolol for binding to turkey erythrocyte membranes. The experiments were performed under identical conditions (i.e. the radioligand concentration: 10 nM) so that the IC_{50} values of the curves can be compared with each other. The affinity of the agonists decreases as: (−)-isoproterenol > (−)-noradrenaline ⩾ (−)-adrenaline.

The non-selective α-adrenergic antagonist phentolamine has only very low affinity and no competition can be demonstrated for the non-bioactive compounds catechol

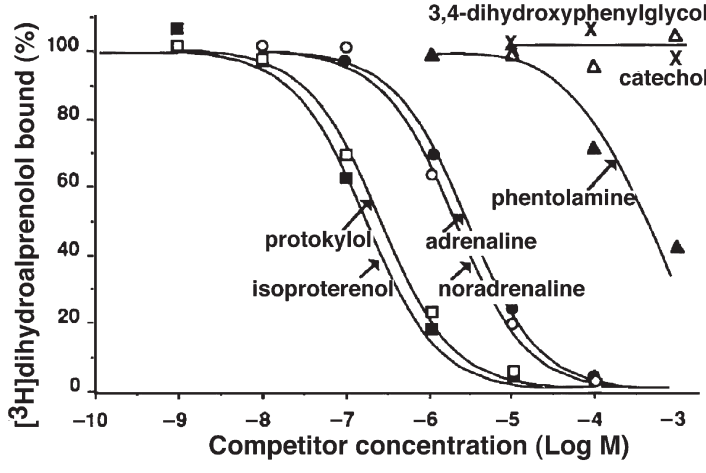


Figure 38 Competition binding curves for β_1-adrenergic receptors in turkey erythrocyte membranes. Reprinted from *Biochemical and Biophysical Research Communications*, **86**, Bottari S., Vauquelin G., Durieu O., Klutchko C. and Strosberg A.D., The beta-adrenergic receptor of turkey erythrocyte membranes: conformational modification by beta-adrenergic agonists, 1311–1318. Copyright (1979), with permission from Elsevier.

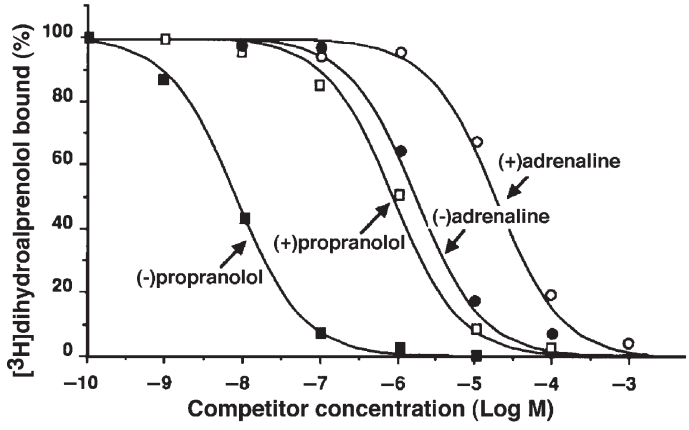


Figure 39 Stereoselective competition binding for β_1-adrenergic receptors (Strosberg, 1987).

and 3,4-dihydroxy phenylglycol. These characteristics fit with those obtained for β_1-adrenergic receptors by functional studies. Moreover, in agreement with the known stereoselectivity of β-adrenergic receptors for antagonists such as propranolol and agonists such as adrenaline, the dextrorotary isomers display lower affinity than the levorotary isomers. In this respect, adrenaline and noradrenaline possess an asymmetric carbon atom in the ethanolamine side chain. They can thus exist either as levorotary (prefix 'L' or '(−)') or as dextrorotary (prefix 'D' or '(+)') stereoisomers. Only the levorotary form of adrenaline is produced and released into the bloodstream. It had already been observed in 1926 that this natural messenger is about 10 times more active than its (synthetic) (+)-isomer in raising blood pressure. This difference was explained in 1933 by the three-point attachment model of Easson and Stedmann (Figure 40): i.e.

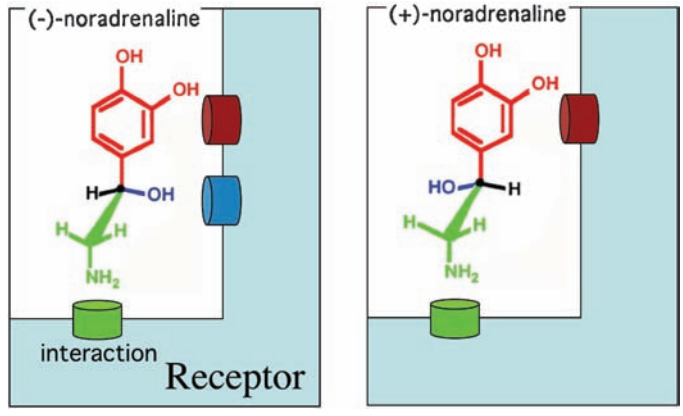


Figure 40 Three-point attachment model of Easson and Stedmann: three bonds for (−)-noradrenaline and only two bonds for (+)-noradrenaline.

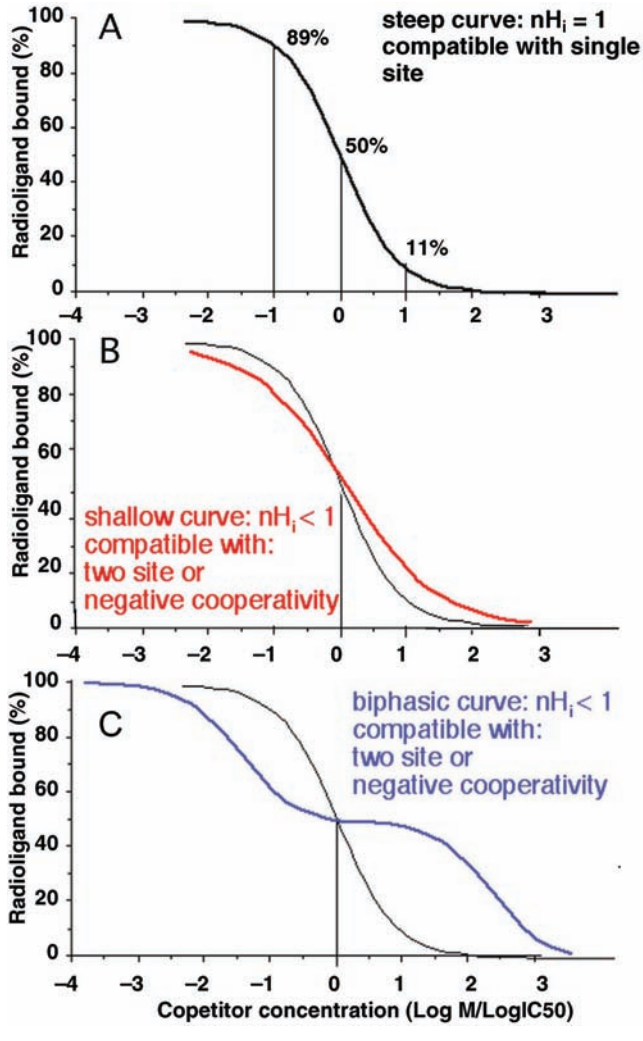


Figure 41 Sigmoidal, shallow and biphasic competition binding curves.

three of the four groups linked to the assymetric carbon of (−)-adrenaline (aromatic ring, OH and amino group) are involved in the interaction with the receptor whereas the OH group of (+)-adrenaline has the 'wrong' orientation. Such 'stereoselectivity' is very common for receptors.

When the radioligand and the competitor bind in a reversible fashion to a single population of non-interacting receptors (i.e. in the simple situation), the competition binding curve should have a steep sigmoidal shape (with 11, 50 and 89% decrease in radioligand binding when the competitor concentration is 1/10, equal or 10 times its IC_{50} value) (Figure 41A). The Hill coefficient of the competitor 'nH_i' can be calculated

from competition binding plots (Equation 10). In the case of a sigmoidal plot, nH_i equals 1.

$$\text{Log}((B_o - B_i)/B_i) = nH_i \times (\text{Log}([I]) - \text{Log}(IC_{50}) \quad (10)$$

where B_o and B_I are the binding of the radioligand in the absence and presence of competitor (I), respectively.

Radioligands may possess the same affinity for two (or more) receptors, receptor subtypes or even receptor subpopulations. When such different receptors co-exist in the same cells or membrane preparation, they will not be discriminated from each other by the radioligand. Indeed, the saturation binding curves appear as if the radioligand binds to a single class of non-co-operative sites. However, these different receptors (subtypes, subpopulations) may possess different affinities for certain unlabelled drugs, so that they can be detected and discriminated from each other by competition binding experiments with these drugs. In such cases, the nH_i values of such curves will be less than one. There are two situations:

- First, the competitor displays a large (>1000-fold) difference in affinity for the different receptors, subtypes or subpopulations (Figure 41C). In this situation, the competition binding curve will be biphasic (i.e. with a plateau) and the parameters of each component (% of binding, IC_{50}) are easy to measure. This is the case for [^{3}H]-rauwolscine, which binds with the same affinity to α_2-adrenergic receptors and serotonergic receptors of the 5-HT_{1A}- type (Figure 42). Serotonin possesses

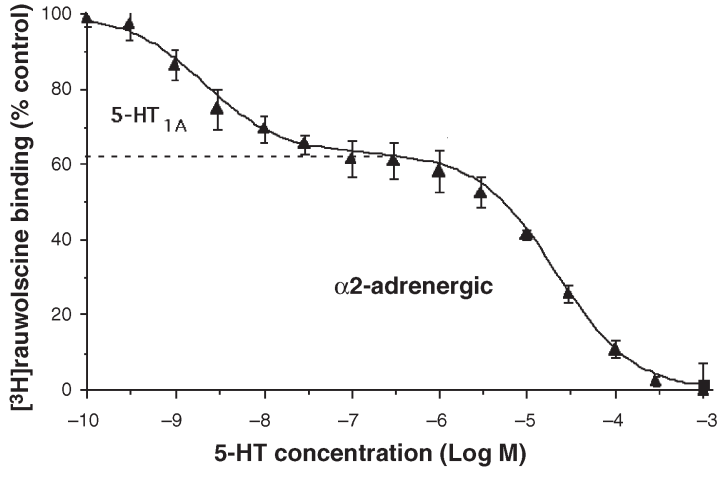


Figure 42 Competition binding curve of serotonin (5-HT) for α_2-adrenergic and 5-HT_{1A} serotonergic receptors in membranes from human frontal cortex. The radioligand, [^{3}H]rauwolscine, binds with equal affinity to both receptors and both receptors are present in human frontal cortex. Reprinted from *Journal of Neurochemistry*, **58**, De Vos H. Czerwiec E. De Backer J.-P. De Potter W. and Vauquelin G., Regional distribution of alpha2A and alpha2B adrenoceptor subtypes in post-mortem human brain, 1555–1560. Copyright Blackwell Publishing.

a much higher affinity for its own receptor than for the α_2-adrenergic receptors and can be used to distinguish both receptors from each other in, for example, human frontal cortex membranes. At low concentrations it will first occupy the 5-HT_{1A} receptors and only when its concentration gets high enough will it start to occupy the α_2-adrenergic receptors. 5-HT_{1A} receptors represent 40% of the binding and α_2-adrenergic receptors 60%. The K_i values of serotonin for these receptors can be calculated from the IC_{50} values according to the equation of Cheng and Prusoff.

- Second, the competitor only possesses a limited (<100 times) difference in affinities for the different receptors, subtypes or subpopulations (Figure 41B). Such competition curves are shallow ($nH_i < 1$) but, since both components are not separated by a distinct plateau, it is necessary to calculate the competition binding parameters of each component (% of binding, IC_{50}) by computer-assisted analysis. Analysis of shallow competition curves is illustrated by Figure 43 for α_{2A}- and α_{2B}-adrenergic receptors. A radioligand such as the antagonist [^{3}H]-RX821002 is unable to discriminate between them, but certain antagonists such as prazosin possesses a relatively weak selectivity for the α_{2B} receptors. For the nucleus caudatus, the competition curve is quite shallow ($nH_i = 0.48$). This indicates that α_{2A} and α_{2B} receptors are both present. The simplest way to describe such curve is to give its nH_i and IC_{50}. However, since K_is refer to individual competitor–receptor interactions, it is not possible to calculate any K_i from this IC_{50}. Computer-assisted analysis is necessary to calculate the proportion of α_{2A} and α_{2B} receptors and their IC_{50} (and K_i) for prazosin.

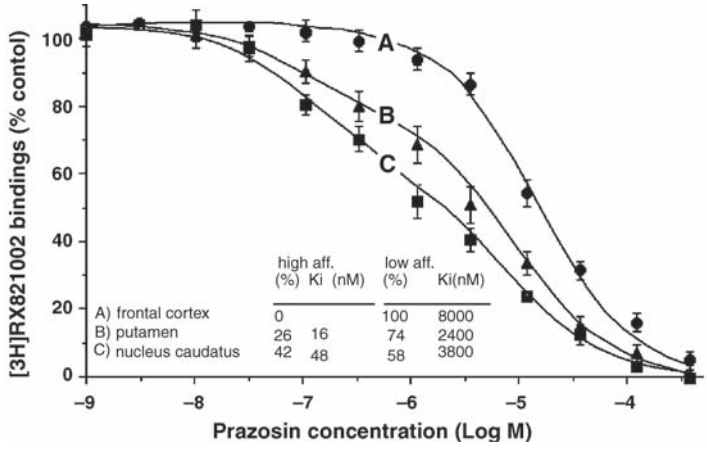


Figure 43 Competition binding curve of prazosin (α_{2B}- subtype- selective antagonist) for α_2-adrenergic receptors in membranes from different human brain regions. Reprinted from *European Journal of Pharmacology*, **207**, De Vos, H., Vauquelin, G., De Keyser, J., De Backer, J.-P. and Van Liefde, I., [3H]rauwolscine behaves as an agonist for the 5-HT1A receptors in human frontal cortex membranes, 1–8. Copyright (1991), with permission from Elsevier.

- The curve is also shallow for the putamen but it is steep (nH_i = 1.01) for the cortex. For this brain area, the curve can be analyzed according to a single-site model and the high K_i of prazosin indicates that only α_{2A} receptors are present in this brain region.

An interesting situation occurs for G protein-linked receptors (GPCRs) in broken cell preparations. These can often be divided into two subpopulations with different affinities for agonists, but with the same affinities for antagonists. This heterogeneity towards agonists is not related to differences in primary amino acid sequence, but rather to their capability to undergo functional coupling to the G proteins (see Section 4.9). The receptor population that undergoes functional coupling to G proteins (coupling-prone receptors) displays high agonist affinity. The receptor population that is unable to couple (non-coupled receptors) displays low agonist affinity. The competition curves will therefore depend on the nature of the radioligand and competitor used (Figure 44):

- If the radioligand is an antagonist, it will regard the receptors as a single class of non- co-operative sites. Antagonist competition binding curves will be steep, but agonist competition binding curves will be shallow.
- If the radioligand is an agonist, it will preferentially label the coupling-prone receptors (especially at low concentrations). Hence, the non-coupled receptors may not be detected in these assays.

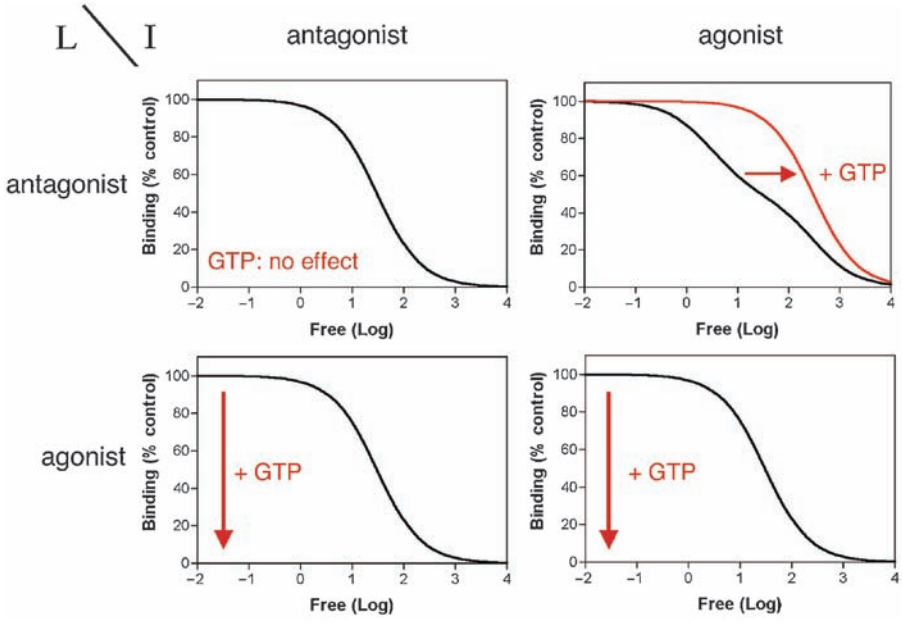


Figure 44 Effect of GTP and related guanine nucleotides on competition binding curves.

Shallow competition binding curves for agonists may thus reflect two distinct phenomena: the presence of different receptors or functional receptor heterogeneity. Fortunately, it is possible to distinguish between these two possibilities by using guanine nucleotides such as GTP. These compounds break up agonist–receptor–G protein complexes, so that the receptors return to the uncoupled, low agonist-affinity form (see Section 4.1). In practice, GTP is thus capable of producing a rightward shift and steepening of the agonist versus radiolabelled antagonist competition binding curve, at least if the high affinity is related to functional coupling of the receptor to the G proteins (Figure 44, Figure 157). When the radioligand is an agonist, its binding will be greatly reduced by GTP.

2.4 Kinetic experiments

Unlike the saturation and competition binding experiments, kinetic studies provide information about the time-course of the binding. These studies usually comprise two types of experiments (Figure 45):

- Determination of dissociation rate constant: in these experiments the radioligand is incubated with the receptor and the dissociation is initiated either by adding an excess of unlabelled ligand (so that free receptors are immediately occupied and no longer accessible to the radioligand) or by dilution (usually after washing away the free radioligand, so that its free concentration is too low to undergo noticeable re-association). Then, the amount of binding is measured after different periods of time (t). First-order reactions occur when the radioligand–receptor complex is a single bimolecular species (L−R). Binding decreases exponentially and the dissociation rate constant (k_{-1}) can be shown to be related to the time it takes for half of the L−R complexes to dissociate ($t_{1/2}$) by the equation $k_{-1} = 0.693/t_{1/2}$. Dissociation data can easily be calculated by plotting ln(binding at time t/binding at the start of the dissociation experiment) (i.e. $\ln(B_t/B_{t=0})$) versus the dissociation time. In the case of a first-order reaction, the plot will be linear and the slope corresponds to the negative value of k_{-1} (usually expressed in min^{-1}).
- Determination of the association rate constant: in these experiments the radioligand is incubated with the receptor and the amount of binding is measured after different periods of time (t). Binding will increase until equilibrium (equilibrium binding B_{eq}) is reached. Under circumstances where [L] is added at concentrations in considerable excess of [R] (as is most often the case), [L] can be assumed not to change throughout the incubation. In contrast, as only [R] decreases, the rate of association may be regarded as being a 'pseudo first-order' reaction. When plotting $\ln(B_{eq}/(B_{eq} - B))$ versus the association time, the slope of the plot gives the pseudo first-order rate constant (k_{obs}). Since the radioligand also undergoes dissociation from the receptor in this type of experiments, it ensues that k_{obs} reflects both the association and

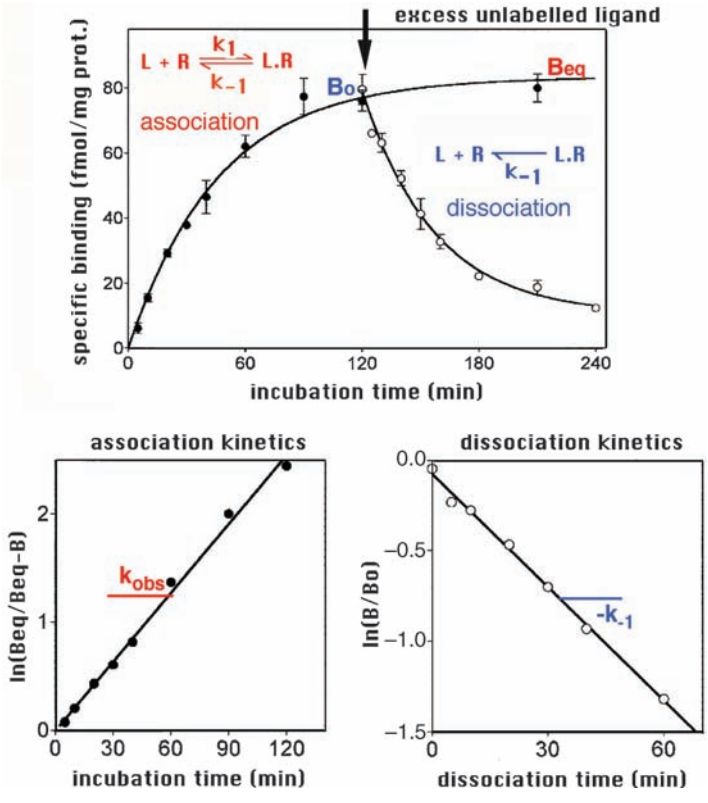


Figure 45 Association and dissociation binding of [^{3}H]NPY to the neuropeptide Y receptors of the Y_1-type in the human SK-N-MC cells. Reprinted from *European Journal of Pharmacology*, **346**, Van Liefde, I., Vanderheyden, P. M., Fraeyman, N., De Backer, J. P., Vauquelin, G., Human neuropeptide YY1 receptors exert unequal control of the extracellular acidification rate in different cell lines, 87–95. Copyright (1998), with permission from Elsevier.

the dissociation of the radioligand. The true, bimolecular association rate constant k_1 (usually expressed in $M^{-1} \times min^{-1}$) can be obtained by the following equation:

$$k_1 = (k_{obs} - k_{-1})/[L] \tag{11}$$

Kinetic data allow the discrimination between fast reversible, slowly reversible and irreversible ligands (dissociation kinetics). Both the association and dissociation constants provide an estimation of the equilibrium dissociation constant (K_D) independently of saturation binding experiments, i.e.:

$$K_D = k_{-1}/k_1 = [L] \times k_{-1}/(k_{obs} - k_{-1}) \tag{12}$$

When the k_{-1}/k_1 ratio is quite distinct from the K_D obtained from saturation binding experiments, the possibility arises that the ligand induces a time-dependent change in receptor conformation that goes along with an increase (or decrease) in receptor affinity. An alternative explanation is that the k_{-1} value is incorrect due to 'rebinding' of dissociated radioligand molecules to the receptors (see below) or to the fact that the unlabelled ligand used to prevent such 'rebinding' interacts with an allosteric site at the receptor (see Section 4.14).

Kinetic data also provide information about the time required for binding of a radioligand reaching equilibrium (association kinetics). This information is crucial for the set-up of saturation and competition binding experiments. Indeed, the K_D and K_i values which are derived from such experiments are only meaningful when, at any concentration of radioligand and competitor, binding is at or at least close to equilibrium. When the incubation time is to short, it could:

- Produce a false estimation of B_{max} and K_D values for saturation binding.
- Produce a false estimation of IC_{50} and K_i values for competition binding experiments.

Radioligands that are dissociated from the receptor will accumulate in the medium and, if they are not constantly removed, they may bind to the receptor again. This

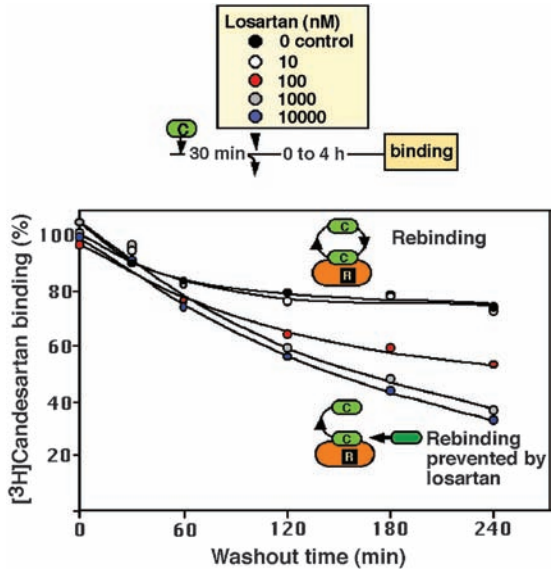


Figure 46 Cells expressing AT_1 receptors were incubated for 30 min at 37 °C with [^{3}H]candesartan, washed and incubated in fresh medium with no or different concentrations of the unlabelled AT_1-receptor antagonist losartan. The remaining binding of [^{3}H]candesartan was measured after the time intervals indicated. Reprinted from *European Journal of Pharmacology*, **367**, Fierens, F., Vanderheyden, P. M., De Backer, J. P., Vauquelin, G., Binding of the antagonist [3H]candesartan to angiotensin II AT1 receptor-transfected Chinese hamster ovary cells, 413–422. Copyright (1999), with permission from Elsevier.

'reassociation' or '*rebinding*' is particularly prominent when the radioligand displays high affinity for the receptor. Whereas rebinding might take place if the incubation medium is simply replaced by fresh medium, it will be effectively prevented when the same or another unlabelled ligand is present in sufficiently large excess in the fresh medium. This is because the receptors become immediately occupied by the unlabelled ligand as soon as the radioligand dissociates. This effectively prevents the 'rebinding' of the radioligand. It is only in the absence of such rebinding that the dissociation rate/half-life of a radioligand–receptor complex can be correctly measured.

This phenomenon has unequivocally been demonstrated by binding studies with the AT_1-receptor antagonist [^{3}H]candesartan (Figure 46). When AT_1-receptor expressing cells were incubated with [^{3}H]candesartan and the medium was replaced by fresh medium only, its dissociation was estimated to be half-maximal after 6–8 hours. Yet, when unlabelled candesartan or any other AT_1-receptor ligand was present in the replacing medium, they all produced a concentration-wise decrease in the apparent half-life of the [^{3}H]candesartan–receptor complex until a half-life of two hours was attained. This value reflects the actual dissociation of this radioligand.

Another factor that severely affects kinetic measurements is the temperature (Figure 47). In this respect, the association and dissociation of radioligands and their receptors are markedly accelerated upon increasing the temperature.

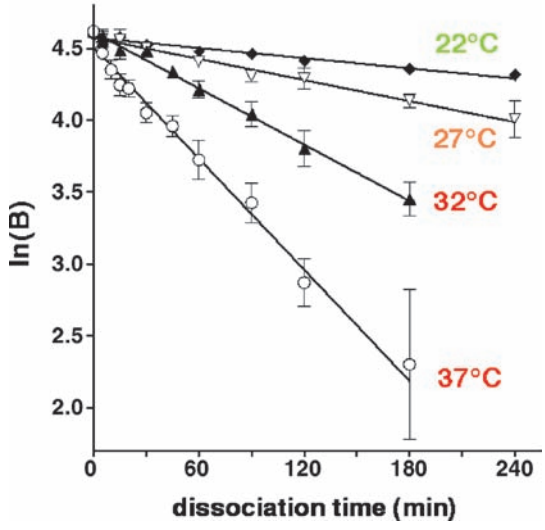


Figure 47 Dissociation of [^{3}H]candesartan from human AT_1 receptors on CHO cells. Cells were incubated for 30 min at 37 °C with [^{3}H]candesartan, washed and incubated in fresh medium (containing losartan) at the temperatures indicated. Remaining binding of [^{3}H]candesartan (B, expressed as a percentage of B_o) was measured after the time intervals indicated. Reprinted from *Biochemical Pharmacology*, **63**, Fierens, F., Vanderheyden, P.M. L., Roggeman, C., Vande Gucht, P., De Backer, J.-P. and Vauquelin, G., Distinct binding properties of the AT1 receptor antagonist [3H]candesartan to intact cells and membrane preparations, 1273–1279. Copyright (2002), with permission from Elsevier.

2.5 Regional distribution of receptors

Radioligand binding on membrane preparations provides information concerning the interaction between drugs and well-defined receptors, as well as about the occurrence of such receptors in certain tissues or organs. However, most tissues and organs are very complex and comprise a number of different cell types, each with their own receptor and response specificity. In this context, the brain is especially complex since it contains a great number of different neurones. Yet, neurones that are responsible for very specific brain functions are often confined to small regions (nuclei) of the brain. Information about the regional distribution of receptors in complex tissues such as the brain therefore contributes to our understanding of their physiological role and their implication in certain pathophysiological conditions. However, radioligand binding experiments on membrane preparations provide only a little information: the resolution is limited by the resolution of the dissection. *Autoradiography* of radioligand binding to thin sections of brain allows the localization of receptors with a much higher degree of resolution (i.e. to the light microscopic level) (Figure 48). However, the resolution is still insufficient to make a distinction between pre- and postsynaptic receptors.

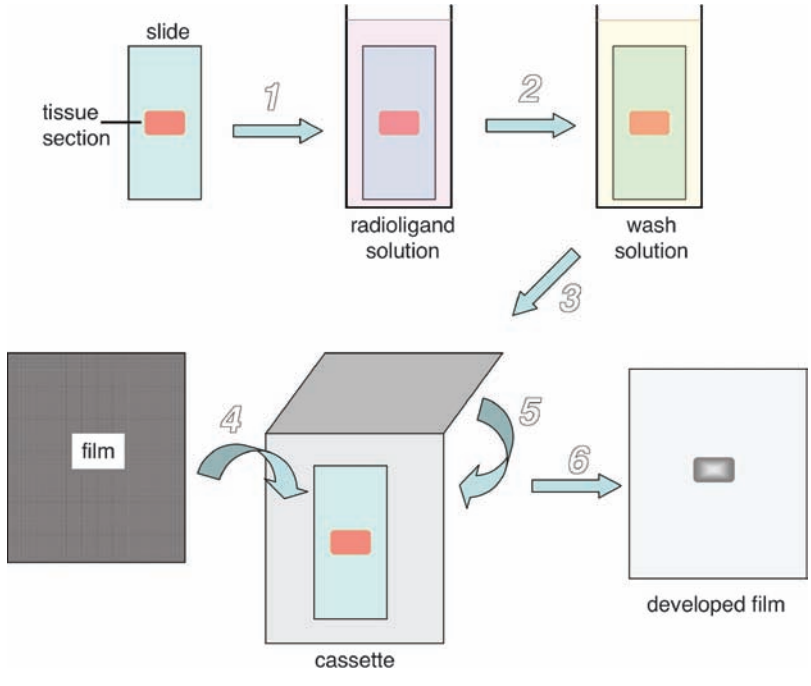


Figure 48 Classical technique for autoradiographic detection of receptors on thin tissue section. Steps: 1) incubation with radioligand, 2) wash, 3) put in casette, 4) overlay film, 5) close cover and expose, 6) remove film and develop.

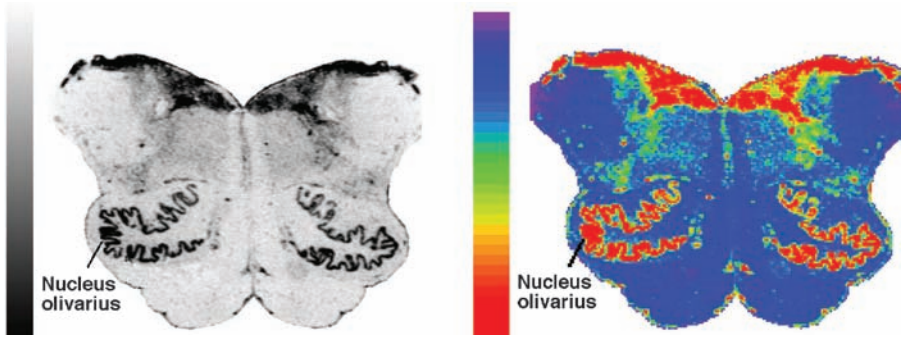


Figure 49 Autoradiography of [^{3}H]idazoxan binding to imidazoline binding sites (I_2-receptors) in a cross section of the human medulla: left: grey scale, right: adopted colours. Reprinted from *Progress in Histochemistry and Cytochemistry* **26**, De Vos, H., De Backer, J.-P., Convents, A., De Keyser, J. and Vauquelin, G., Identification of alpha2 adrenoceptors in the human nucleus olivarius by radioligand binding, 259–265. Copyright (1992), with permission from Elsevier.

For this approach, tissues are cut in thin (usually not exceeding 10 μM) slices and put on coverslips. The slices are subsequently incubated with radioligand and washed. The radioactivity on the slices can then be determined by apposition of a sensitive film or by dipping the slice in a photographic emulsion. After exposure (from a few hours for [^{125}I]-labelled ligands to a few weeks for [^{3}H]-labelled ligands), the film or emulsion is developed. Black corresponds to a high density of radioligand (Figure 49, left). Newer detection techniques are available for [^{3}H]-labelled ligands.

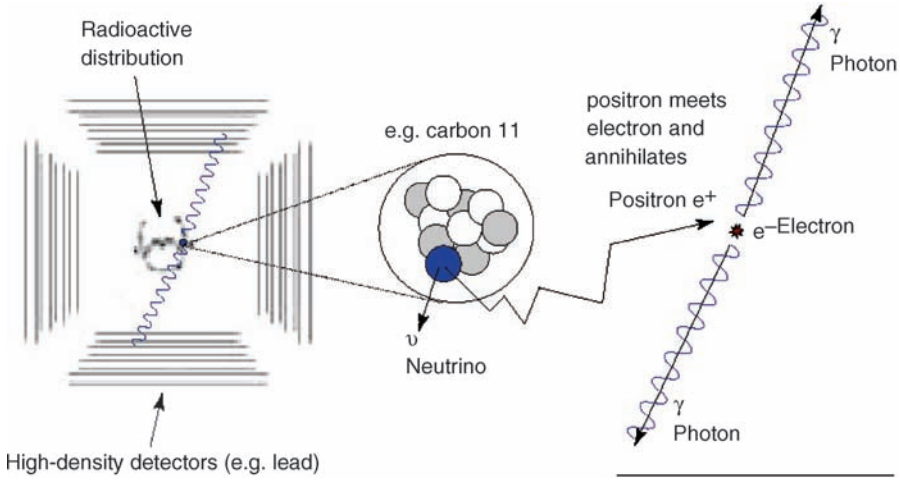


Figure 50 Principle of PET scan. Reprinted from *Trends in Pharmacological Sciences*, **22**, Reader, A.J. and Zweit, J., Developments in whole-body molecular imaging of live subjects, 604–607, © (2001), with permission from Elsevier.

They rely on the apposition of a solid scintillator sheet and real-time imaging of the emitted photons. This brings down the exposure time to hours instead of days and weeks. Commercially available radioactive standards can be exposed along with the tissue sections and this allows the conversion of the different grey levels into binding values. The amount of radioligand bound to each location of the slice can therefore be quantified by densitometry. Computer-assisted image analysis greatly facilitates this task and it also allows different grey levels to be replaced by specific colours (Figure 49).

Finally, the *in vivo* determination of the regional distribution of receptors in e.g. the brain can be obtained by the *PET scan* technique (Figure 50). 'PET' stands for positron emission tomography and involves the administration of a positron emitting radioligand to a patient. The radioligand will accumulate in receptor-rich areas and the emitted positrons will, upon encounter with electrons, annihilate to produce gamma rays, which can be detected. This technique is fairly safe for the patient since the radioactive half-life is very short (in the order of minutes) but, on average, the positrons will only meet electrons a few millimetres away from their point of departure so that the degree of resolution is rather poor (Figure 51).

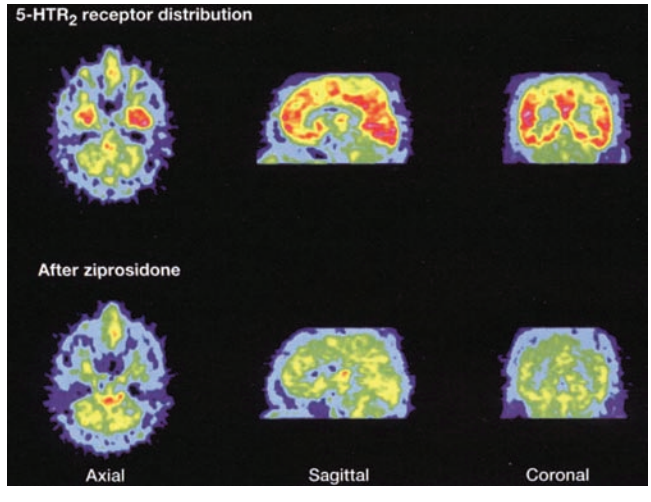


Figure 51 Distribution of 5-HT_2 receptors in the brain. Accumulation of positron emitting radioligand ([^{18}F]-setoperone) in brain 'sections' in untreated (total) and Ziprosidone-treated (non-specific accumulation) humans. Reproduced from Fischman, A. J., Bonab, A. A., Babich, J. W., Alpert, N. M., Rauch, S. L., Elmaleh, D. R., Shoup, T. M., Williams, S. A. and Rubin, R. H. (1996) Positron emission tomographic analysis of central 5-hydroxytryptamine2 receptor occupancy in healthy volunteers treated with the novel antipsychotic agent, ziprasidone. *Journal of Pharmacology and Experimental Therapeutics*, **279**, 939–947, with permission from the American Society for Pharmacology and Experimental Theraputics.

3 Functional studies

3.1 Dose-response curves and associated problems

Stimulation of a membrane-bound receptor by an agonist will provoke the onset of a whole chain of intracellular events. These events will ultimately lead to a 'physiological' response. This response, as well as intermediate intracellular events, can be measured to obtain (indirect) information about the receptor, for example, to investigate the effect of β-adrenergic drugs on the heart. One of the most proximate 'biochemical' responses is adenylate cyclase stimulation. The activity of this enzyme can be measured either in broken cell preparations or purified membranes (i.e. measurement of the conversion of [^{32}P]-ATP into [^{32}P]-cAMP) as well as in intact cell or whole organ preparations (measurement of the cAMP concentration). The more distant 'physiological' events comprise the positive inotropic (i.e. increased force of contraction) and positive chronotropic (i.e. increased rate of contraction) responses. The relationship between the drug-evoked response and receptor occupancy is often complex, especially when a long chain of intermediary events separates both phenomena from each other. In an attempt to define such relationships, pharmacologists have introduced concepts such as 'intrinsic activity', 'efficacy' and 'receptor reserve'.

Ligands may be roughly divided into agonists and antagonists. Figure 52 compares the ability of the β-adrenergic agonists like isoproterenol and of antagonists like propranolol to stimulate lipolysis in (i.e. glycerol release from) rat adipose cells. As expected, the antagonists produce no response, even at very high concentrations. In contrast, the response increases with the agonist concentration until a plateau value is reached. Such representation, wherein the response (ordinate) is expressed as a function of the ligand concentration (abscissa), is called a '*dose-response curve*' or '*concentration-response curve*' (the concentration is usually expressed in a logarithmic scale since it spans several orders in magnitude).

In 1926, Clark developed the '*occupation theory*', wherein he proposed that the agonist-mediated response should be proportional to the number of occupied receptors (Figure 53). The response (E) at a given concentration of agonist ([L]) is then related to the maximal response (E_{max}) by:

$$E = E_{max}/(1 + K_D/[L]) \quad (13)$$

G Protein-Coupled Receptors: Molecular Pharmacology From Academic Concept to Pharmaceutical Research
Georges Vauquelin and Bengt von Mentzer © 2007 John Wiley & Sons, Ltd. ISBN: 978-0-470-51647-8

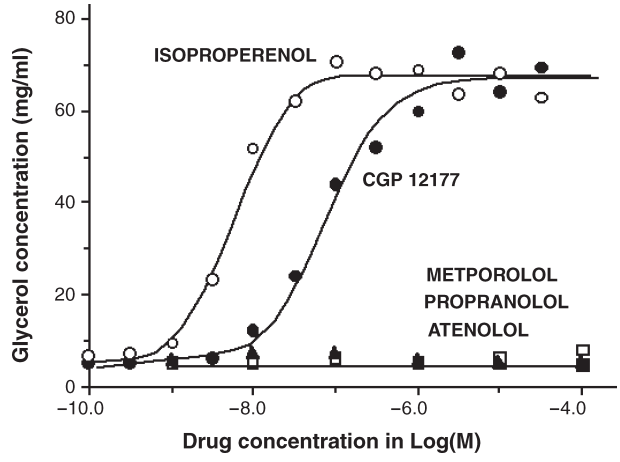


Figure 52 Dose-dependent effect of the β-adrenergic agonists isoproterenol and CGP12177 and of the β-adrenergic antagonists metoprolol, propranolol and atenolol on the lipolysis (measured by the amount of released glycerol) in rat fat cells. Reproduced from Van Liefde, I., Van Witzenburg, A. and Vauquelin, G. (1992) Multiple beta adrenergic receptor subclasses mediate the l-isoproterenol-induced lipolytic response in rat adipocytes. *Journal of Pharmacology and Experimental Therapeutics*, **262**, 552–558, with permission from the American Society for Pharmacology and Experimental Theraputics.

The concentration that causes a half-maximal response (denoted as 'EC_{50}') is therefore equal to the K_D of the agonist for the receptor. However, the occupation theory is unable to explain two major sets of findings:

- The first complication arises from the observation that, in some situations, the maximal response is already attained when only some of the receptors are occupied. In such situations, the occupation theory is no longer valid since EC_{50}

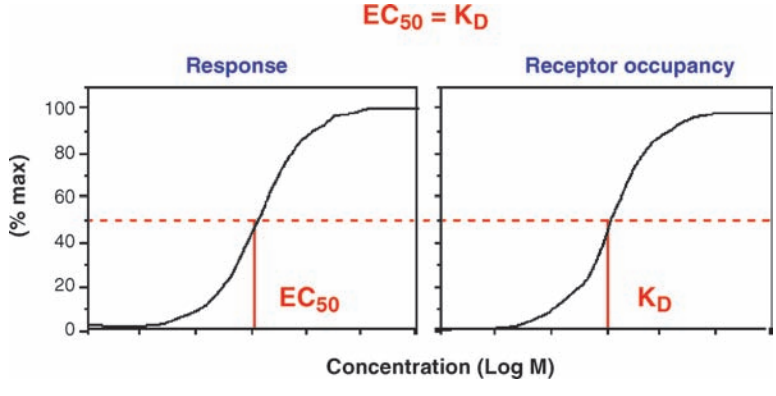


Figure 53 Essential characteristics of the occupation theory by Clark.

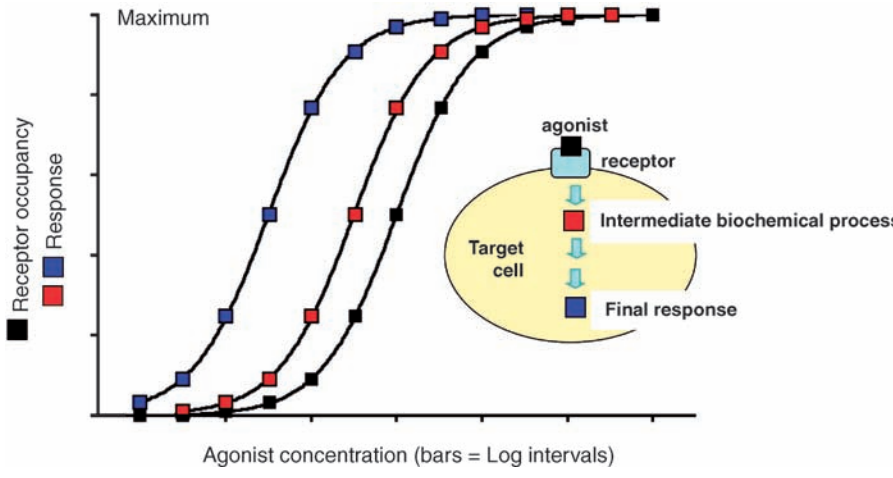


Figure 54 Comparison between receptor occupancy by isoproterenol (binding studies), intermediate biochemical events and the final response.

$< K_D$ (Figure 54). This means (at first glance) that the response may be maximal when only some of the receptors are occupied by the agonist. In other words, cellular amplification systems allow agonists (the natural messengers as well as synthetic drugs) to produce a maximal response at receptor subsaturating levels. The terms '*receptor reserve*' or '*spare receptor*' were introduced as an attempt to describe this phenomenon: i.e. the receptor reserve is the fraction of receptors greater than that required to produce the maximal tissue response by an agonist.

- A more subtle distinction between agonists also became necessary after the realization that agonists do not necessarily produce the same maximal response. As a typical example Figure 55, compares dose-response curves of different β-adrenergic agonists to produce adenylate cyclase stimulation in turkey erythrocyte membranes. The maximal degree of adenylate cyclase stimulation is clearly different from one agonist to another. To deal with this finding, Ariens (Ariens, 1954) introduced, in 1954, the term '*intrinsic activity*' (α) as an experimental parameter to indicate the maximal response of an agonist of interest compared to the most potent agonist known (E_{max}).

$$E = \alpha \times E_{max}/(1 + K_D/[L]) \qquad (14)$$

Depending on the value of α, ligands can be divided into three categories:

- $\alpha = 1$. This category of ligands produces the maximal response (E_{max}) at full receptor occupancy. They are called 'full agonists'. Isoproterenol is a full agonist for β-adrenergic receptors (and so are the natural messengers for this receptor: adrenaline and noradrenaline).

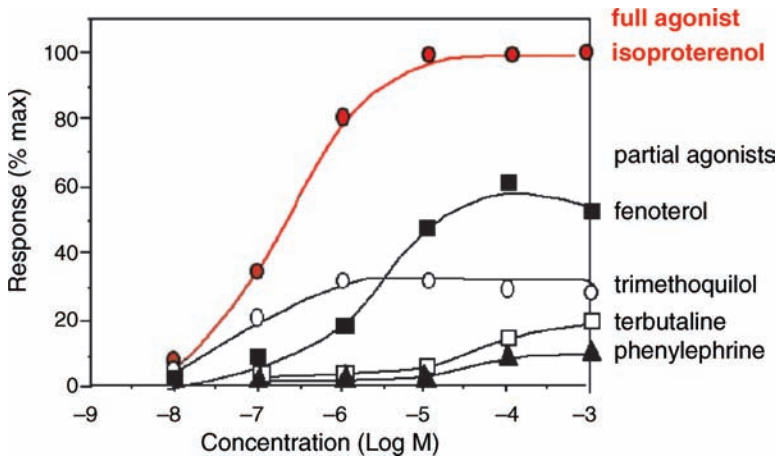


Figure 55 Dose-response (adenylate cyclase stimulation in turkey erythrocytes) curves of the full agonist isoproterenol and of partial agonists. Reproduced from Vauquelin, G., Bottari, S. and Strosberg, A. D. (1980) Inactivation of beta-adrenergic receptors by N-ethylmalmeimide: permissive role of beta-adrenergic agents in relation to adenylate cyclase activation. *Molecular Pharmacology*, **17**, 163–171, with permission from the American Society for Pharmacology and Experimental Theraputics.

- $0 < \alpha < 1$. These ligands are denoted as 'partial agonists'. At full receptor occupancy, they will produce a response equal to $\alpha \times E_{max}$. The rank order of the α values for the partial agonists presented (Figure 55) is: phenylephrine (0.09) < terbutaline (0.20) < trimethoquilol (0.33) < fenoterol (0.64). Figure 55 also clearly shows that there is no correlation between a drug's EC_{50} and its intrinsic activity.
- $\alpha = 0$. This situation occurs for antagonists. These ligands bind to the receptor without eliciting a response.

A close examination of the molecular events which link receptor occupation by the ligand and the final ligand-evoked response provides a better insight concerning the actual physical meaning of dose-response curves and concepts such as '*intrinsic activity*', '*efficacy*' and '*receptor reserve*'.

3.2 From receptor occupation to stimulus and response

From receptor occupation to stimulus

The distinction between agonists and antagonists has been explained by the ability of agonists, but not of antagonists, to initiate (or favour) a conformational modification of the receptor molecule (or molecular complex) and that this modification

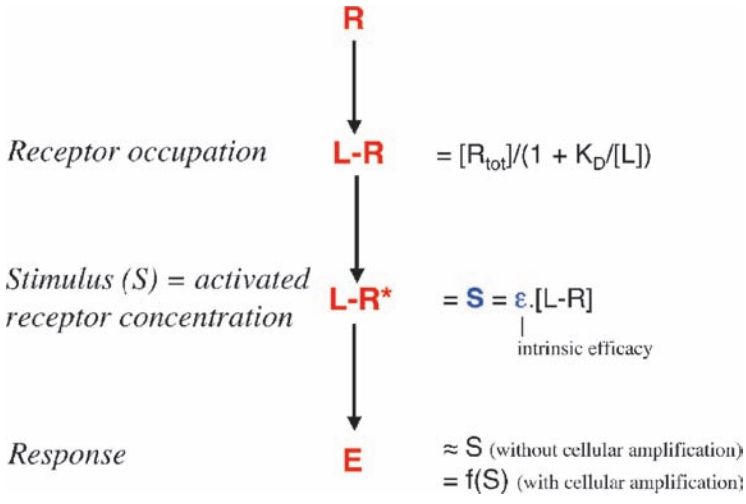


Figure 56 From receptor occupation '[L−R]' to stimulus 'S' to response 'E'.

is the first step in the initiation of the cellular response. This first step represents the *stimulus* (Figure 56). Major current models assume that receptors can only adopt one active conformation and that the stimulus of a ligand reflects the fraction of occupied receptors residing in this active conformation.

The simplest model to deal with such situation is the '*two-step model*'. In this model, the bound agonist induces a conformational change in the receptor by reducing the difference in free energy between both receptor conformations. The ligand (L) binds first to the non-active receptor (Rn) with the 'microscopic' equilibrium dissociation constant (K), and this non-active ligand–receptor complex (L−Rn) is in equilibrium with the active complex (L−Ra). This latter equilibrium represents a first-order reaction with the 'microscopic' equilibrium dissociation constant ($K' = [L-Rn]/[L-Ra]$).

$$\begin{array}{ccccc} & \overset{K}{} & & \overset{K'}{} & \\ L + Rn & \Leftrightarrow & L\text{-}Rn & \Leftrightarrow & L\text{-}Ra \end{array} \qquad (15)$$

The second equilibrium forms the key element for discriminating between agonists and antagonists:

- For antagonists, the second equilibrium is completely shifted to the left (i.e. $K' \gg 1$): all of the occupied receptors remain in the non-active conformation.
- For agonists, the second equilibrium is shifted more to the right for strong agonists than for weak agonists, so that more of the occupied receptors reside in the active conformation; i.e. K' (full agonist) $< K'$ (partial agonist).

The fraction of occupied receptors residing in the active conformation is related to K′ by the following equation:

$$[L{-}Ra]/([L{-}Ra] + [L{-}Rn]) = 1/(K' + 1) \quad (16)$$

Several authors have proposed an alternative 'allosteric model', which is derived from the Monod–Wyman–Changeux 'Plausible Model' (Monod *et al.*, 1965). In this model, both receptor conformations are in equilibrium, even in the absence of ligand. Here, the agonist 'favours' a conformational change of the receptor because of its higher affinity for the active conformation. The equilibrium constant for the transition between the two forms of the receptor (K′ = [Rn]/[Ra]) is very high since the great majority of receptors are inactive in the absence of drug. Nevertheless, this model allows unoccupied receptors to produce a small stimulus. Ligands are able to bind both to Rn and Ra with the 'microscopic' equilibrium dissociation constants Kn and Ka, respectively:

$$\begin{array}{ccc} L + Rn & \Leftrightarrow & L + Ra \\ \Uparrow & K' & \Uparrow \\ Kn & & Ka \\ \Downarrow & & \Downarrow \\ L\text{-}Rn & & L\text{-}Ra \end{array} \quad (17)$$

In this model, agonists can be discriminated from antagonists based on differences between their binding affinities for the active and non-active receptors. This model also provides an explanation for the existence of so-called '*inverse agonists*':

- *Antagonists* are supposed to bind with equal affinity to both receptor conformations (i.e. Kn = Ka); the [Ra]/[Rn] ratio remains the same as in the basal situation.
- *Agonists* bind with higher affinity to Ra as compared to Rn (i.e. Kn > Ka) so that the whole equilibrium will be pulled to the right, resulting in an increase in the [Ra]/[Rn] ratio. The Kn/Ka ratio is higher for full agonists than for partial agonists.
- *Inverse agonists* bind with higher affinity to Rn as compared to Ra (i.e. Ka > Kn) so that the whole equilibrium will be pulled to the left, resulting in a decrease of the [Ra]/[Rn] ratio. Some of the compounds that interact with benzodiazepine receptors are inverse agonists: they decrease the affinity of GABA for the $GABA_A$ receptor.

The fraction of occupied receptors residing in the active conformation is related to Ka, Kn and K′ by the following equation:

$$[L{-}Ra]/([L{-}Ra] + [L{-}Rn]) = 1/(1 + K' \times Ka/Kn) \quad (18)$$

Studies during the past few years have led to the introduction of even more complex models to explain the activation of G protein-coupled receptors. They will be developed in Sections 4.10 to 4.14.

The capability of the bound ligand to stimulate the receptor has been termed the '*intrinsic efficacy*' (ε) of the ligand by Furchgott in 1966 (Furchgott, 1966). ε is proportional to the fraction of occupied receptors residing in the active conformation in the two above models, i.e.:

$$\varepsilon \approx [L{-}Ra]/([L{-}Ra] + [L{-}Rn]) \tag{19}$$

The *stimulus* (*S*) is dependent on the amount of occupied receptors ([L−R]) and on the intrinsic efficacy (ε) of the ligand, i.e.:

$$S \sim \varepsilon \times [L{-}R] \tag{20}$$

Substitution of [L−R] by $[R_{tot}]/(1 + K_D/[L])$ yields:

$$S \sim \varepsilon \times [R_{tot}]/(1 + K_D/[L]) \tag{21}$$

S depends on properties of the ligand–receptor interaction: ε and K_D. S also depends on $[R_{tot}]$, a tissue-dependent property. Figure 57 compares the binding (upper panel) and the stimulus (mid panel) that can be obtained at different concentrations of an agonist with the highest intrinsic efficacy known (ε_{max}), one with only half of that intrinsic efficacy (a partial agonist) and an antagonist.

Equation 21 was first presented by Stephenson in a more simplified form (Stephenson, 1956): $\varepsilon \times [R_{tot}]$ was expressed as a single term, the 'efficacy' (e), which is dependent on the tissue (because of $[R_{tot}]$) as well as on the ligand–receptor interaction (because of ε).

From Stimulus to response: linear relationship

To deal with the many steps which might succeed this initial stimulus, the '*response*' (**E**) should considered to be an undefined function (F) of S, i.e.:

$$E = F(S) = F(\varepsilon \times [L{-}R]) = F(\varepsilon \times [R_{tot}]/(1 + K_D/[L])) \tag{22}$$

A special case of Equation 22 occurs when E is proportional to the stimulus. This equation can then be written as:

$$E \sim \varepsilon \times [L{-}R] = \varepsilon \times [R_{tot}]/(1 + K_D/[L]) \tag{23}$$

The maximal response of the most active agonist known (i.e. with ε_{max}) is:

$$E_{max} \sim \varepsilon_{max} \times [R_{tot}] \tag{24}$$

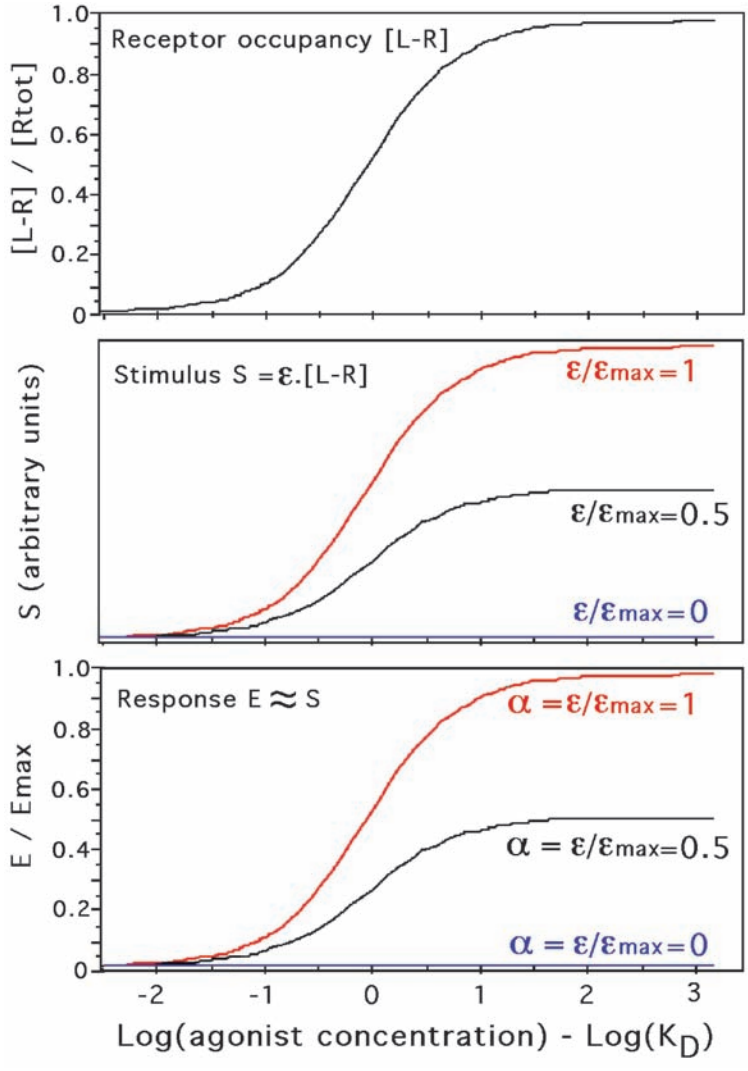


Figure 57 Linear relationship between receptor occupancy, stimulus and response. Curves are shown for an agonist with the highest intrinsic efficacy known (ε_{max}), one with only half of that intrinsic efficacy (a partial agonist) and an antagonist.

When the response (E) of any agonist is expressed relative to this maximum, we have:

$$E / E_{max} = (\varepsilon/\varepsilon_{max})/(1 + K_D/[L]) = \alpha/(1 + K_D/[L]) \qquad (25)$$

This equation is similar to the one originally proposed by Ariens (Equation 14, in red). Alpha is an experimental parameter but, under the particular condition of a linear

stimulus-response relationship, it corresponds to the ratio between the intrinsic efficacy of the agonist of interest and the intrinsic activity of the most active agonist known to date ($\alpha = \varepsilon/\varepsilon_{max}$) (Figure 57).

From Stimulus to Response: Non-linear relationship

The equation of Ariens represents only a special case of Equation 22: i.e. when E is proportional to S. Quite often, however, the number of activated receptors will exceed the maximal response capacity of the system. In other words, the maximal response is already attained when only some of the receptors are occupied. In such situations, α is no longer proportional to $\varepsilon/\varepsilon_{max}$.

In Equation 22, the response is an undefined function of the stimulus. F is, in principle, undefined for two potential reasons:

- The undefined nature of the cascade of cellular events following the initial stimulus.
- The undefined relationship between consecutive events.

Although many of these cellular events are already known in great detail, the relationship between consecutive events appears, very often, not to be a linear one. A common reason for such a non-linear relationship is that cellular events are capable of amplifying the signal (stimulus) to an extent that exceeds the response capacity of the subsequent event (Figure 79). In other words, the response capacity of the second event becomes saturated even before the magnitude of the first event has reached its maximum. The stimulus-response relationship may thus be composed of any number of saturable and linear functions arranged in sequence. An overall saturable output will still be expected. The classical (and simplest) way to describe F is to represent it as a rectangular hyperbolic function: i.e. $E/E_{max} = S/(S + 1)$. However, F should also reflect the efficiency of the cellular events converting receptor stimulus into tissue response, as well as the number of events (i.e. the greater the number of saturable steps, the greater the global amplification). A fitting parameter (β), which deals with the number and efficiency of the intermediate cellular events, is therefore introduced in the stimulus-response relationship:

$$E/E_{max} = S/(S + \beta) \tag{26}$$

The relationship between E and ε can now be represented as:

$$E/E_{max} = \varepsilon \times [L-R]/(\varepsilon \times [L-R] + \beta) \tag{27}$$

$$= \varepsilon \times [R_{tot}]/(1 + K_D/[L])/(\varepsilon \times [R_{tot}]/(1 + K_D/[L]) + \beta)$$

$$= \varepsilon \times [R_{tot}]/(\varepsilon \times [R_{tot}] + \beta + \beta \times K_D/[L]) \tag{28}$$

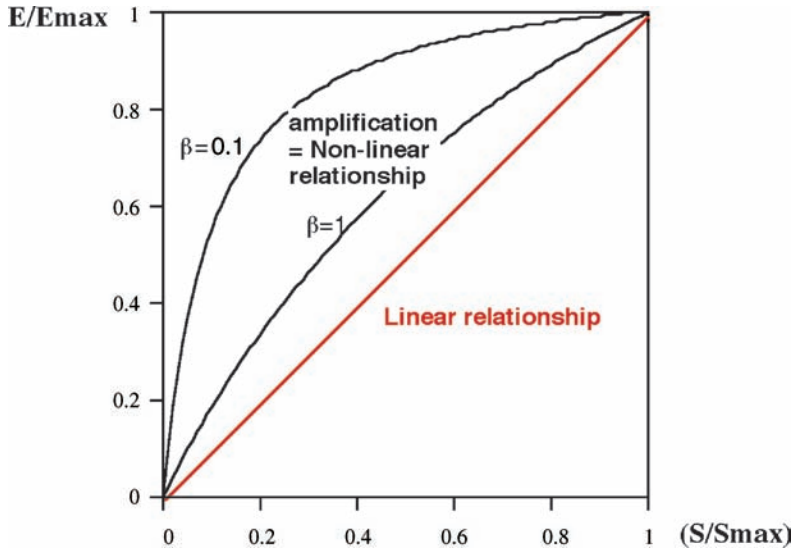


Figure 58 Linear and non-linear relationship between E/E_{max} and S.

Beta is a 'fitting parameter' which is inversely related to the global cellular amplification (Figure 58). It is dependent on the efficiency of intermediary cellular evens to amplify the stimulus. Since each successive event may produce a further amplification of the stimulus, β is also likely to decrease with the 'distance' between the stimulus and the measured response. Graphic representations of Equation 28 allow us to evaluate the consequences of varying β (Figure 59). For different types of agonists, a cellular amplification of the stimulus will produce distinct changes of the dose-response curve:

- Full agonists (ε is very high): Dose-response curves will be shifted to the left of the actual binding curves (i.e. $EC_{50} \ll K_D$). This effect will be even more pronounced as β decreases (Figure 59, top panel). In the heart, for example, the dose-response curve for the isoproterenol-mediated inotropic effect is shifted to the left by about one order in magnitude when compared to the less distant adenylate cyclase response (Figure 54).
- 'Strong' partial agonists (ε intermediate): Their maximal response will increase and dose-response curves will gradually shift to the left (Figure 59, mid panel).
- 'Weak' partial agonists (ε very low): The maximal response will increase but the EC_{50} will remain very similar to K_D (Figure 59, lower panel). This implies that compounds, which are almost not distinguishable from antagonists in test systems without amplification system, will show up as partial agonists in test systems with an amplification system.

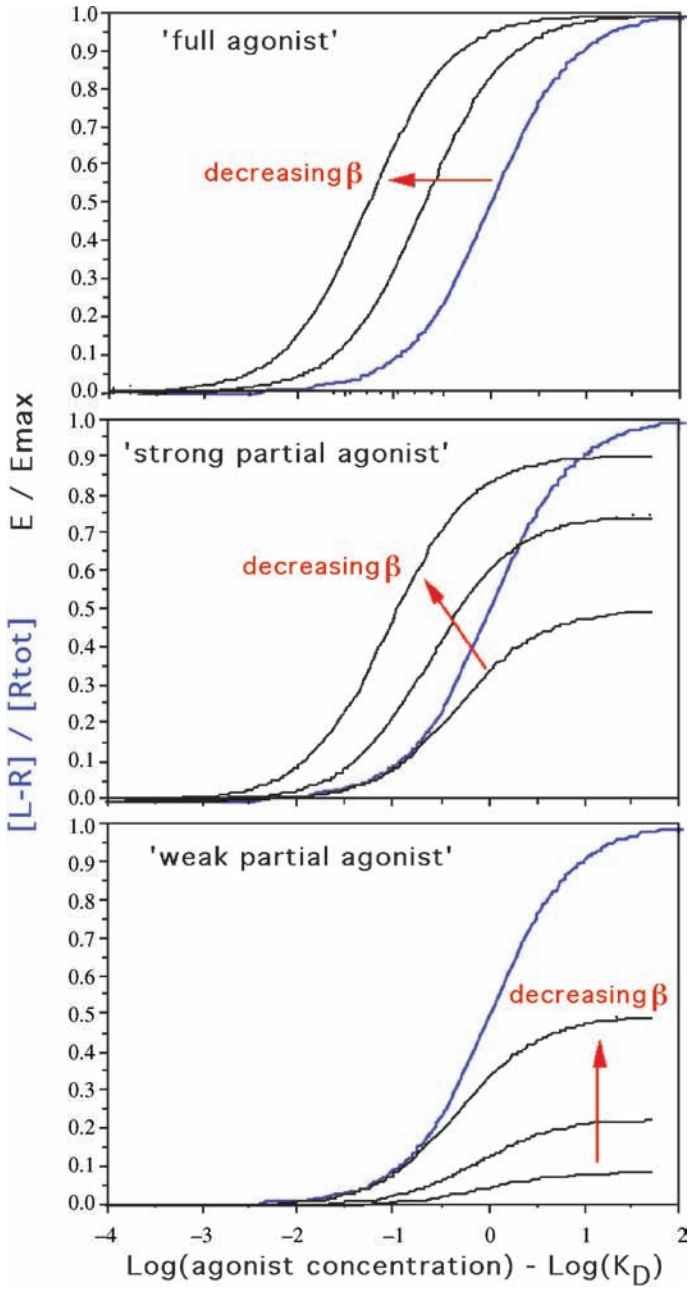


Figure 59 Theoretical dose-response curves of different types of agonists: effect of introducing cellular amplification of the signal and of decreasing the value of β (red arrow). The blue curve corresponds to actual receptor occupancy.

$EC_{50} \ll K_D$ means (at first glance) that the response may already be maximal when only some of the receptors are occupied by the agonist. The terms 'receptor reserve' or 'spare receptor' were introduced as an attempt to describe this phenomenon. However, one must be aware that this definition is ambiguous since:

- The cellular amplification of the stimulus that is responsible for $EC_{50} \ll K_D$ is likely to occur at different levels, involving biochemical events well beyond the initial process of receptor stimulation (see Figure 79).
- Strictly speaking, all of the receptors should be required to produce a maximal response. Obviously, this can never be attained experimentally since it should require [L] to be infinitely high.

$[R_{tot}]$ refers to the total concentration of functionally active receptors (i.e. coupled to a response mechanism). Their densitiy can vary dramatically from one tissue to another, and even within a given tissue. Receptor desensitization (see Section 4.8) constitutes a typical example wherein the cells defend themselves against prolonged stimulation by agonists by decreasing $[R_{tot}]$, both by decreasing the total receptor number and the fraction of functionally active receptors.

Decreasing $[R_{tot}]$ may have profound effects on different types of ligands (Figure 60):

- Apparently full agonists may undergo a large increase in EC_{50} or even become partial agonists.
- Partial agonists (i.e. with $\alpha < 1$ to start with) may become antagonist-like, with little variation of EC_{50}.

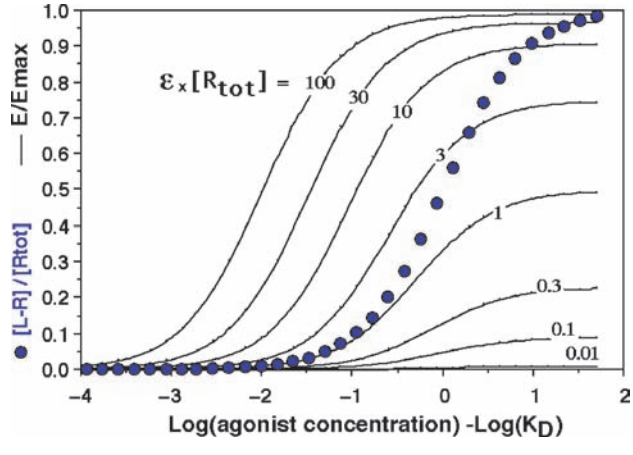


Figure 60 Theoretical dose-response curves of an agonist for different values of $\varepsilon \times [R_{tot}]$. Dots correspond to receptor occupancy and $\beta = 1$.

3.3 Receptor classification and antagonist affinity

Adrenergic receptors were initially classified in 1948 by Ahlquist into the α- and β-subtypes on basis of differences in the order of potencies of agonists (Figure 61):

- The α-adrenergic receptor is associated with most of the excitatory functions (e.g. vasoconstriction and contraction of the smooth muscle of the uterus). The potency of catecholamines to trigger these responses decreases in the order: adrenaline > noradrenaline ≫ isoproterenol.
- The β-adrenergic receptor is associated with most of the inhibitory functions (e.g. vasodilatation and relaxation of the uterine and bronchial smooth muscle) and an important excitatory function (myocardial stimulation). The potency of catecholamines to trigger these responses decreases in the order: isoproterenol ≫ adrenaline > noradrenaline.

However, because the EC_{50} values of agonist dose-response curves do not necessarily reflect their K_D, such classification could be hazardous in certain circumstances. This is well illustrated in the following example (Figure 62). Consider that agonists A and B have the same affinity, but that the intrinsic efficacy (ε) of B is well above than that of A. Whereas the competition binding curves with these two unlabelled agonists will overlap (because of their equal K_D), functional assays in systems with an outspoken 'receptor reserve' are likely to reveal a higher potency for B when compared to A.

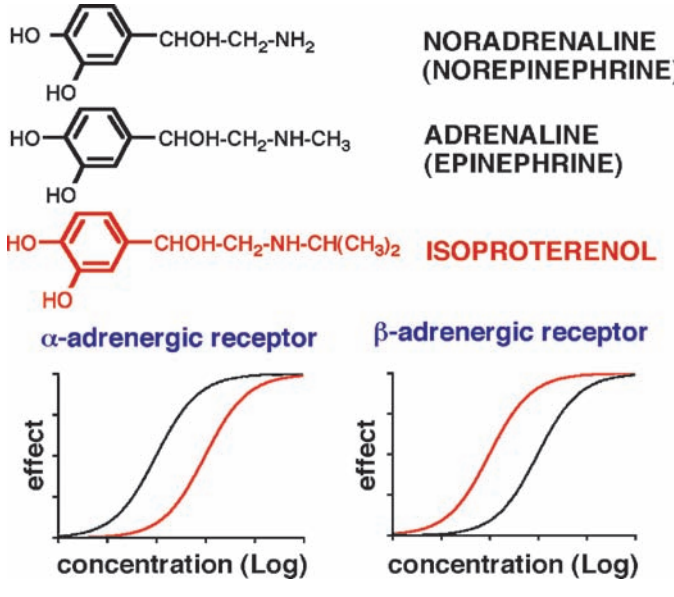


Figure 61 Distinction between α- and β-adrenergic receptors.

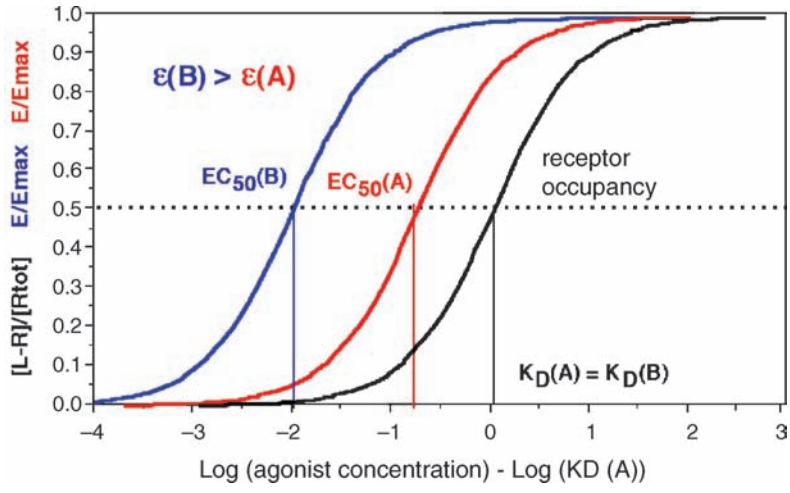


Figure 62 Different potency ratios for agonists may be obtained by binding studies and physiological experiments in systems with a large 'receptor reserve' (same K_D for A and B, but the intrinsic efficacy (ε) of B is well above that of A).

The distinct potency patterns might eventually lead to the conclusion that the labelled receptors (binding studies) are different from those which produce the agonist-mediated response.

An axiom of receptor pharmacology is that agonist potency ratios represent a unique identifier of receptors: i.e. the rank order of agonist potencies is dependent on the molecular properties of affinity and efficacy and, hence, a constant that should be independent of the experimental system. However, agonist EC_{50} values and intrinsic activities (α) constitute weak arguments for classifying receptors.

Antagonist K_i values could be calculated from their IC_{50} values from competition binding experiments by the Cheng and Prusoff (Cheng and Prusoff, 1973) formula (Section 2.3) provided that the radioligand's K_D value was known. However, because of the potential (but a priori unknown) involvement of cellular amplification phenomena in functional assays, agonist EC_{50} values from agonist dose- response curves could also reflect tissue-dependent factors besides the actual agonist–receptor interaction and, accordingly, they do not necessarily reflect the true K_D for the receptor. This handicap prevents the calculatation of antagonist K_i values from their IC_{50} values from inhibition experiments. Yet, IC_{50} values may be compared to one another when they are obtained under strictly identical conditions. This provides information about the rank order of antagonist affinities and about antagonist affinity ratios (Figure 63).

Fortunately, antagonist Ki values can be obtained with functional studies by the method developed by Schild and coworkers (Arunlakshana and Schild, 1959). This method is based on the fact that competitive antagonists produce parallel rightward

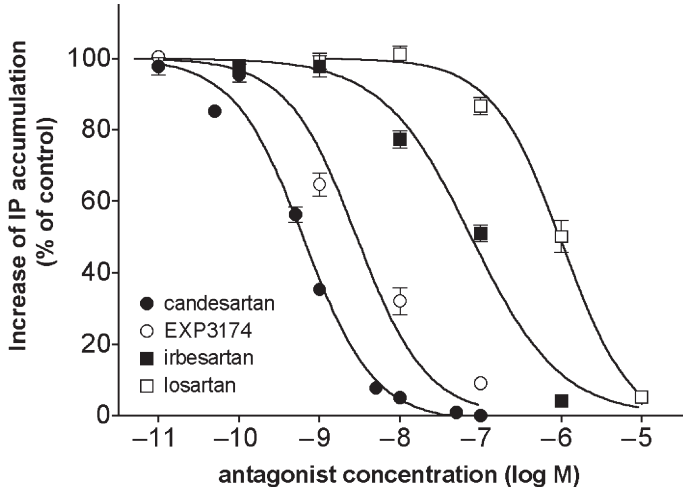


Figure 63 Ability of different antagonists to inhibit angiotensin II (0.1 μM) induced inositol phosphate accumulation in CHO cells expressing the human AT_1 receptor. Reprinted from *British Journal of Pharmacology*, **126**, Vanderheyden, P.M.L., Fierens, F.L.P., De Backer, J.-P., Frayman, N. and Vauquelin, G., Distinction between surmountable and insurmountable selective AT1 receptor antagonists by use of CHO-K1 cells expressing human angiotensin II AT1 receptors, 1057–1065, © (1999).

shifts of the agonist dose-response curve (Figure 64). In other words, an agonist may produce a certain response at concentration $[L_1]$ when present alone and in the presence of a competitive antagonist (at concentration [I]), the agonist concentration must be increased to $[L_2]$ to obtain the same response. The ratio of these equi-active agonist concentrations ($[L_2]/[L_1]$) is often referred to as 'dose ratio, DR' or 'concentration ratio, CR'.

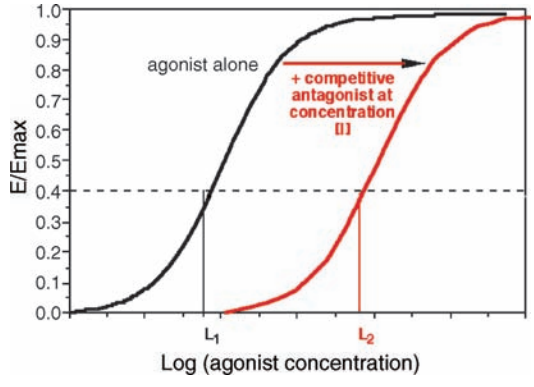


Figure 64 Agonist dose-response curve: effect of a fixed concentration of a competitive antagonist.

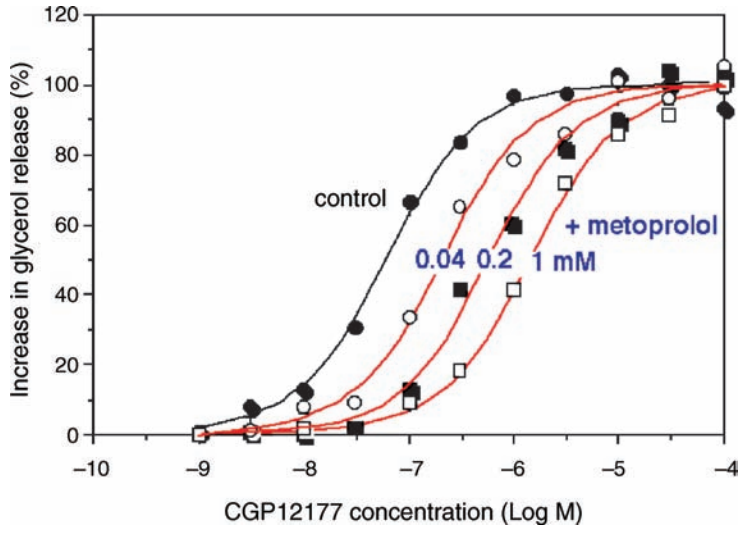


Figure 65 CGP12177 (β_3-selective agonist) dose response (lipolysis in rat fat cells) curve: effect of 0.04, 0.2 and 1 mM metoprolol (β_1-selective antagonist). Reproduced from Van Liefde, I., Van Witzenburg, A. and Vauquelin, G. (1992) Multiple beta adrenergic receptor subclasses mediate the l-isoproterenol-induced lipolytic response in rat adipocytes. *Journal of Pharmacology and Experimental Therapeutics*, **262**, 552–558, with permission from the American Society for Pharmacology and Experimental Theraputics.

When [I] equals the inhibitor's K_D, it will be twice as difficult for the agonist to produce the same response: i.e. $[L_2] = 2 \times [L_1]$. The method presented below allows us to calculate this particular value of [I] by linear regression analysis of dose-response data obtained in the presence of different antagonist concentrations.

As an example, Figure 65 shows the dose-response curve of CGP12177 to stimulate lipolysis in rat adipocytes, and the ability of increasing concentrations of the β-adrenergic antagonist metoprolol to produce rightward shifts of this curve. Figure 65 clearly shows that the dose ratio will be more pronounced when the antagonist concentration increases. The $[L_1]$ value for the control curve (i.e. without agonist) corresponds to the agonist concentration producing an arbitrarily chosen response. $[L_2]$ values of the agonist are then measured for the curves obtained in the presence of the different [I]. The next step is to plot $\text{Log}([L_2]/[L_1] - 1)$ versus Log([I]). This plot is referred to as a *Schild Plot* (Arunlakshana and Schild, 1959) and reflects the following equation:

$$\text{Log}([L_2]/[L_1] - 1) = \text{Log}([I]) - \text{Log}(K_i) \qquad (29)$$

$\text{Log}([L_2]/[L_1] - 1) = 0$ corresponds to the intercept of the plot with the abscissa and, in this situation, $\text{Log}(K_i)$ is equal to Log([I]). Hence, the antagonist's $\text{Log}(K_i)$ (often referred to in the literature as 'pA_2') can easily be calculated by linear regression of the *Schild Plot* (Figure 66). Schild regressions represent the most useful physiological tool

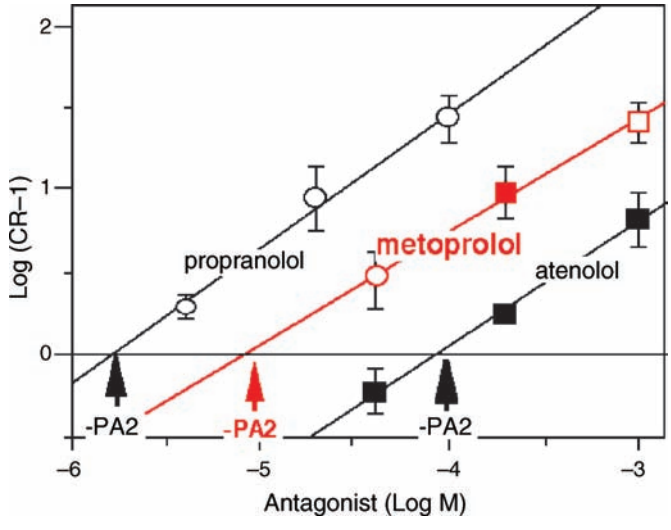


Figure 66 Schild plot of the shifted dose-response curves of metoprolol (and for similar experiments with the antagonists propranolol and atenolol). Reproduced from Van Liefde, I., Van Witzenburg, A. and Vauquelin, G. (1992) Multiple beta adrenergic receptor subclasses mediate the l-isoproterenol-induced lipolytic response in rat adipocytes. *Journal of Pharmacology and Experimental Therapeutics*, **262**, 552–558, with permission from the American Society for Pharmacology and Experimental Theraputics.

for pharmacological receptor classification. It is, for example, on basis of such studies that the β-adrenergic receptors in rat adipocytes were discovered to constitute a new subclass, possessing unusually low affinity for antagonists. Indeed, the K_i value of metoprolol (9.3 μM) is well above the values typical for β_1- and β_2-adrenergic receptors.

3.4 Pharmacological models

Nowadays, ligand and receptor interactions can be studied with a large variety of experimental systems and techniques (Figure 67). The information from each system is often complementary.

For a long time, physiological experiments constituted the sole approach for testing ligand–receptor interactions. Because of the indirect nature of the results (i.e. a 'distant' response is measured), information about the ligand–receptor interaction could be biased by nature of the experimental system. The positive side of this is that physiological experiments in intact organs or even *in vivo* are rather close to the clinical reality. The negative side of physiological experiments is that they do not provide clear-cut information about ligand–receptor interactions. Hence, they only constitute marginal tools for the purpose of receptor classification and identification. Indeed, EC_{50} values and intrinsic activities (α) of agonists are easy to measure, but tissue dependent. K_D values and intrinsic efficacies (ε) describe the

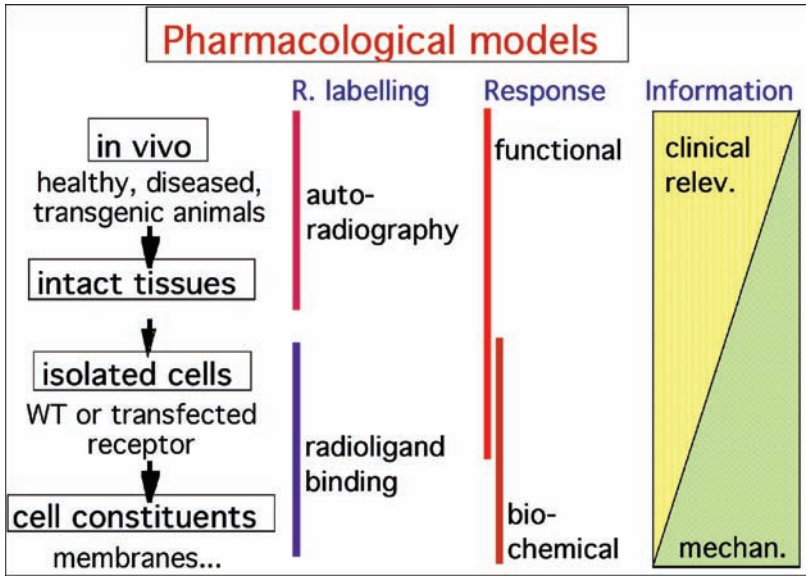


Figure 67 Pharmacological approaches and relevance of the provided information.

agonist–receptor interactions more accurately. Yet they are difficult to obtain (at least in the absence of radioligand binding studies). Schild regressions of shifted dose-response curves provide an accurate determination of antagonist K_i values, and they may represent the most useful physiological tool for pharmacological receptor classification.

Radioligand binding studies provide direct information about agonist and antagonist affinities for receptors. They also allow detection of the co-existence of receptor subclasses in a given tissue and, in certain instances (G protein-coupled receptors), even discrimination between agonists and antagonists (Figure 44). Because of its simplicity and accuracy, the radioligand binding approach is a very useful tool for the identification, classification and discovery of receptors, as well as for investigating the affinity and specificity of new potential drugs for receptors of interest. However, radioligand binding experiments provide only crude information about the physiological actions and the therapeutic benefit of the investigated drugs.

Receptors from animal sources have long been used as templates for predicting drug activity on human receptors, and, in general, they are sufficiently good for this purpose. However, it has also been found that slight differences between human and animal receptors can have profound effects on drug activity. It is known that there are differences in affinity that result from relatively small amino acid sequence differences (even a single amino acid) between human and animal receptors. This is especially true for non-peptide antagonists for peptide receptors where it appears that evolution has produced mutations that have not altered binding of natural peptides, but do produce differences for foreign non-peptide ligands.

Possible species-related differences in drug action could be avoided by using human blood cells or on post-mortem obtained tissues. However, this material is sometimes hard to obtain and, because of post-mortem delays, of unequal quality. It has now become possible to circumvent the use of human tissues by using tumor cell lines containing the desired receptor or by transfecting tumour cell lines (e.g. Chinese Hamster ovary cells) with human DNA coding for the desired receptor, and to use the expressed receptors for screening tests. Additional advantages of such systems include:

- Radioligand binding and functional experiments (i.e. the measurement of receptor-evoked responses) can be measured under the same experimental conditions so that the experimental data are directly comparable. The receptors can be investigated by the binding of both agonist and antagonist radioligands as well as by the measurement of various functional responses (e.g. angiotensin II-mediated inositol phospholipid hydrolysis, transient rise in the cytosolic calcium concentration and extracellular acidification by CHO cells expressing the human AT_1 receptor) (Figure 68).
- The non-transfected, wild type cells can be used as negative control.

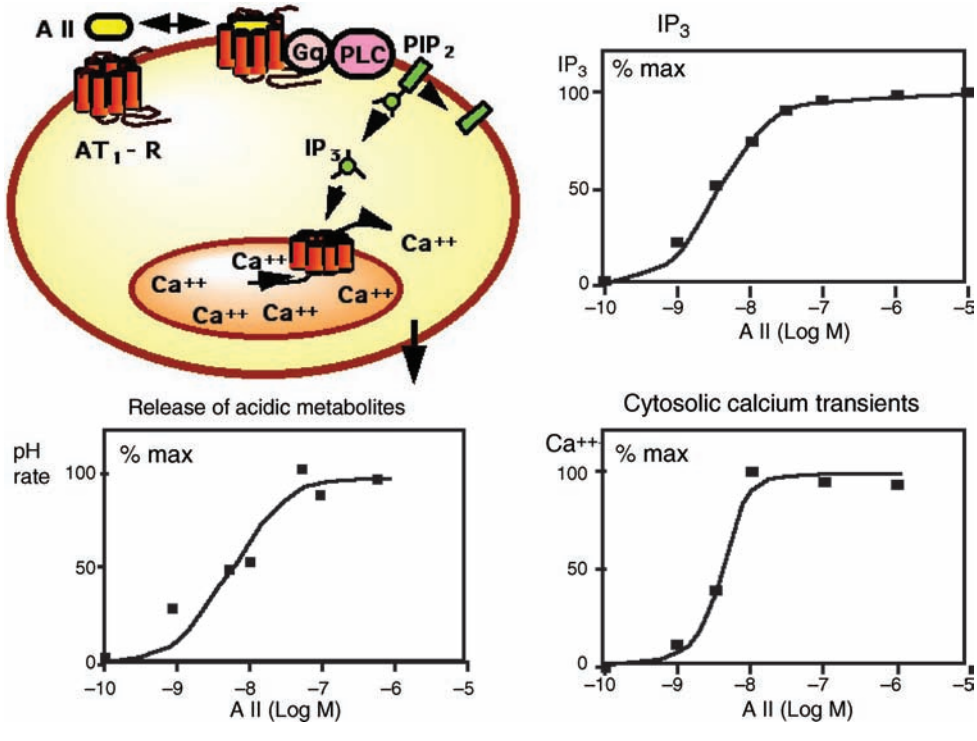


Figure 68 *In vitro*-measurable responses upon exposing angiotensin II (A II) to CHO cells expressing the human AT_1 receptor.

- Cells can be transfected with genes coding for mutated receptors to identify amino acids that are crucial, e.g. for the binding of a given agonist or antagonist.
- Cells can be transfected with genes coding for two or more receptors to investigate any synergism or opposing effects.

The choice of cell line into which a G protein-coupled receptor is best transfected depends on the subsequent studies to be performed. In general, it is best for a host cell line to have a reasonably rapid growth rate and high transfection efficiency. Obviously, it is important to ascertain that the appropriate G proteins and effectors are endogenously expressed in the host cell line. It must also be ascertained that the cDNA to be transfected is not already expressed endogenously in the host cell line.

The majority of cells in a typical transfection experiment will express exogenous DNA transiently (Figure 69). Hence, transfected DNA will be lost from the host cell after a number of cell divisions. In a small proportion of transfected cells, the exogenous DNA will be randomly integrated into the chromosomal DNA of the recipient. If this takes place, the exogenous DNA has become a stable element of the genome of the host cell and this cell is now stably transfected (Figure 69). The number of stable transfectants is dependent on the efficiency with which the cells initially take up the exogenous DNA as well as on the frequency at which stable integration of the exogenous DNA into the chromosomal DNA occurs. With the incorporation of a selectable marker in the exogenous DNA, it is possible to select for cells that have this DNA integrated into their own chromosomal DNA. Finally,

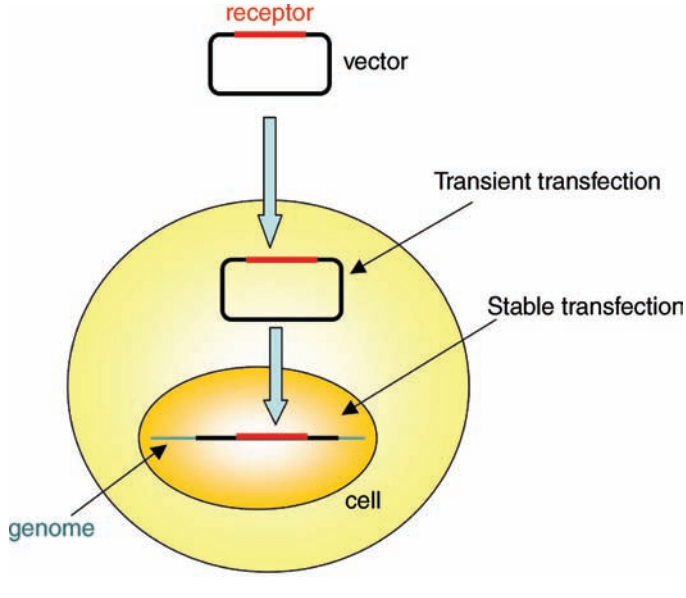


Figure 69 Stable and transient transfection of host cells with cDNA.

it is important to consider that the expression of integrated exogenous DNAs is subject to the local environment at the site of integration, e.g. strong expression can be obtained from even the weakest of promoters if integration occurs near a strong enhancer sequence.

It must be assumed that the genetic material introduced into the surrogate cell can find its way to the appropriate locus, be translated correctly and the resulting product processed as in native systems. In general, there is considerable evidence that non-standard translational events may affect the nature of expression products. Expression of multi-unit receptors can be especially difficult because of the potential for incorrect assembly. Although this is usually not a problem with GPCRs, there are cases in which alternative splicing of pre-mRNA or post-translational changes (glycosylation, palmitoylation, terminal amino acid acylation, carboxy-terminal amidation, sulfation, methylation) account for differences in G protein coupling and ligand affinity. Moreover, whereas the stoichiometry between receptors and G proteins is fixed in natural systems, recombinant systems deal with two new potential phenomena:

- Constitutive receptor activity (receptor-mediated increase in basal activity; i.e. without agonist present) (see also Section 4.11).
- Increased receptor promiscuity with respect to activation of different types of G proteins (see also Section 4.5).

Quantitative measurements have also been considerably developed during the past few years. Thanks to recent advances in chemical techniques (combinatorial chemistry) thousands of structurally related substances can theoretically be made in a single day. This demands high throughput screening techniques for their testing. At present, this is routinely done by radioligands, but this technique only provides information about whether and how well a drug is recognized by a given receptor, not about its ability to stimulate that receptor. The search for agonist activity is now greatly facilitated due to the development of receptor-independent assays (Figure 70):

- Binding of [^{35}S]GTPγS to cell membranes allows the detection of agonists for nearly all GPCRs by a single assay. This is based on the faculty of agonists to promote the exchange of G protein-bound GDP by GTP. [^{35}S]GTPγS acts like GTP and stimulates dissociation of the G protein into the [^{35}S]GTPγS-bound Gα subunit and the βγ complex (see Section 4.5). However, unlike GTP, [^{35}S]GTPγS is relatively resistant to hydrolysis by the endogenous GTP-ase activity of Gα so that it remains bound.
- Based on the observation that the α subunits of the mammalian G proteins G_{15} and G_{16} are able to be activated by a much wider range of receptors as compared to the other G proteins and that the C-terminus of the α subunit is most important for receptor recognition, attempts are now made to transfect cells with chimaeric G proteins. This strategy involves co-expression of the receptor with chimeric

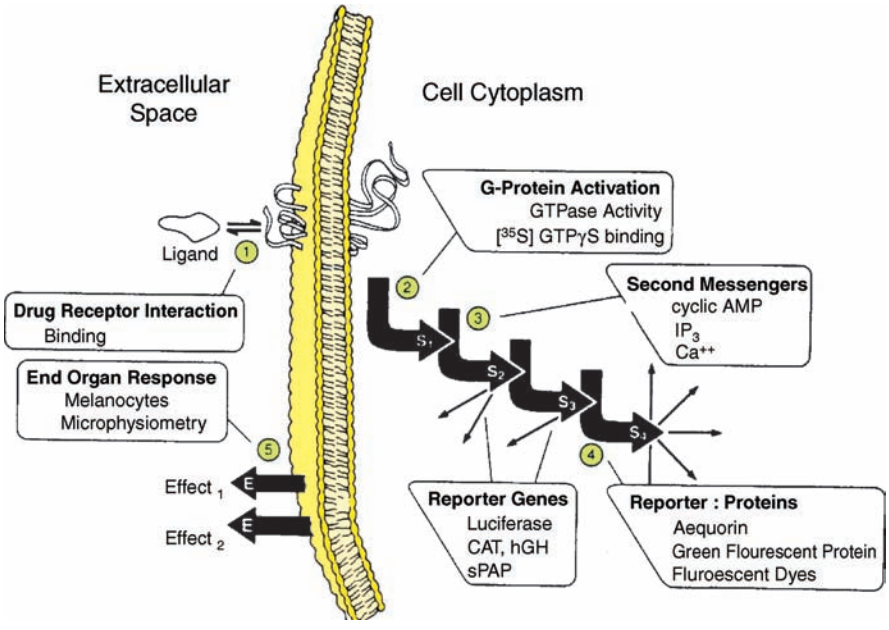


Figure 70 G protein-coupled receptor-triggered cascade of biochemical events in the cytosol. (1) Ligand–receptor binding, (2) G protein activation, (3) second messenger generation, (4) second messenger-triggered events (and detection with reporter systems), (5) the observable end-organ responses. Reproduced from Kenakin, T. (1996) The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacological Reviews*, **48**, 413–463, with permission from the American Society for Pharmacology and Experimental Theraputics.

G proteins that preserve the receptor-coupling domain for the GPCR of interest, but have been fused with a domain that interacts with a different effector protein. This should enable the detection of agonists for nearly all GPCRs by a single assay.

- A rapidly expanding technology is in the field of 'reporters' of cytosolic second messengers such as cAMP. There are several types of reporters.
  - Introduction of reporter genes whose expression is affected by the second messenger. Receptor activation should result in an increased transcription of the gene and expression of the gene product can be quantitated some hours later. For example, the luciferase gene can be set under transcriptional control of a regulatory DNA sequence responsive to cAMP. The intracellular level of the luciferase enzyme can be quantitated by measuring its activity (bioluminiscence reaction with luciferin as substrate).
  - Introduction of reporter proteins that signal the elevation of the second messenger directly in the cytosol. For example, cells can be transfected with the

gene coding for the calcium-binding protein, aequorin. The cytosolic calcium concentration can then be sensed by fluorescence measurements.

- The melanophore system allows a rapid evaluation of the effects of drugs on receptors that regulate cAMP. When melanophores respond to light or are stimulated by factors that elevate cAMP, they respond by dispersing their melanosomes throughout the cytoplasm and the cells appear dark. In contrast, signals that result in a decrease in cAMP levels, result in melanosome aggregation to the cell centre and the cells appear light.
- Finally, it has also been proposed that the measurement of extracellular acidification constitutes a universal assay system for receptor stimulation. The concept relates to the fact that the rate of cellular metabolism is directly linked to hydrogen ion extrusion by the cell, and this can be measured by an increase in the pH in the medium surrounding the cell.

4 G protein-coupled receptors

4.1 From receptor to response: introduction to GPCRs

All drugs that are presently on the market for clinical therapy are estimated to target less than 500 biomolecules, ranging from nucleic acids to enzymes, G protein-coupled receptors (GPCRs) and ion channels (Figure 71). Presently, GPCRs constitute one of the principal targets of drugs used in pharmacology and more than 1000 genes encoding GPCRs have been identified from human genome sequencing efforts. This represents a substantial part (± 3%) of the human genome.

A great deal of information concerning GPCRs has been acquired by investigating the β-adrenergic receptors.

Sutherland and coworkers discovered in the early sixties (Sutherland and Robison, 1966) that these receptors are able to stimulate the adenylate cyclase enzyme in isolated cell plasma membranes. Initially, it was speculated that the β-adrenergic receptor and the adenylate cyclase enzyme were parts of a single molecule. At the end of the seventies, it became clear that receptor and adenylate cyclase functions are carried by different membrane proteins and that a third, GTP-binding protein (i.e. a '*G protein*'), is required to transfer the information between the receptor and the enzyme. This model is based on the discovery that guanine nucleotides such as GTP are absolutely required for adenylate cyclase stimulation and that guanine nucleotides must bind to a regulatory component in the membrane.

G proteins refer to a family of closely related membrane-associated polypeptides. By acting as a 'shuttle', they form central elements for the signal transduction between receptors and effector components (enzymes or ion channels) in the membrane. At rest, they consist of a heterotrimer (Figure 72), possessing a guanine nucleotide binding α subunit (38–52 kDa), a β subunit (35 KDa) and a γ subunit (8–10 KDa). The β and γ subunits are always closely associated (i.e. β–γ), and the β–γ complexes are presumed to be interchangeable from one G protein to another. G proteins are not integral membrane proteins, but are anchored to the cytoplasmic face of the plasma membrane. The α subunits are predominantly hydrophilic. They are anchored to the plasma membrane due to their coupling to the β–γ complexes. In addition, some of the α subunits also have a 14-carbon myristic acid added to their N-terminal domain at Gly^2 (i.e. glycine located at position 2).

G Protein-Coupled Receptors: Molecular Pharmacology From Academic Concept to Pharmaceutical Research
Georges Vauquelin and Bengt von Mentzer © 2007 John Wiley & Sons, Ltd. ISBN: 978-0-470-51647-8

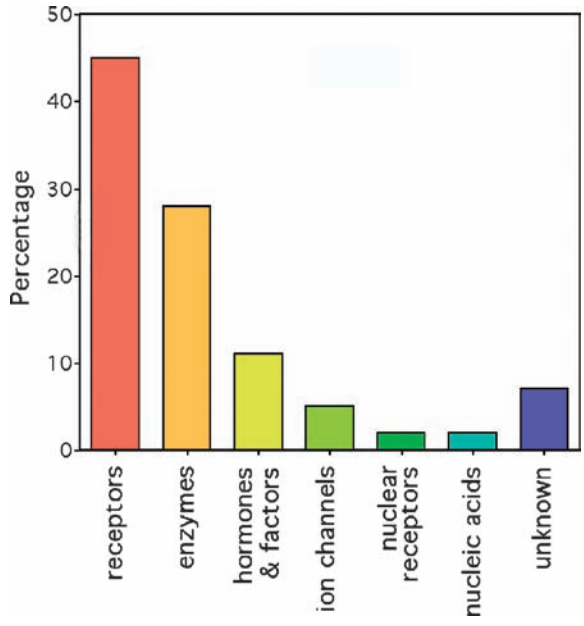


Figure 71 Therapeutic target classes (year 2000).

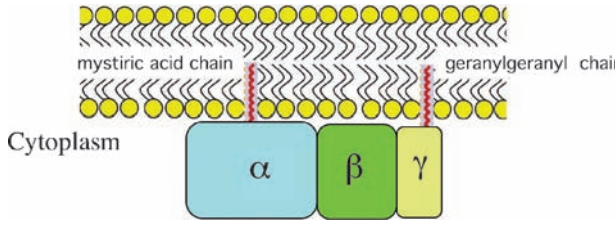


Figure 72 Association of an inactive, heterotrimeric G protein to the cytoplasmic face of the cell plasma membrane.

The *G protein* that is required to transfer the information between the receptor and the adenylate cyclase enzyme was the first to be purified and characterized (around 1980). This G protein is now called 'G_s' (previously 'Ns'), with 's' standing for stimulatory. Since then, several additional G proteins have been discovered; the latest ones by screening DNA libraries with oligonucleotide probes. The α subunits constitute the receptor-recognizing part of the G proteins. They are also largely involved in the recognition of 'effector components' like the adenylate cyclase enzyme. The β–γ complexes are not without signalling function (see later), but this is often only secondary to that of the α subunits. This explains why the identity of a G protein is determined by the identity of its α subunit.

Table 7 Principal G protein subunits and their primary effectors. Reprinted from *Pharmacology and Therapeutics*, **99**, Hermans E., Biochemical and pharmacological control of the multiplicity of coupling at G protein-coupled receptors, 25–44, © (2003), with permission from Elsevier.

Subunit	Family	Main subtypes	Primary effector
α	α_s	$G\alpha_s$, $G\alpha_{olf}$	Adenylate cyclase ↑
	$\alpha_{i/o}$	$G\alpha_{i\text{-}1}$, $G\alpha_{i\text{-}2}$, $G\alpha_{i\text{-}3}$	Adenylate cyclase ↓
		$G\alpha_{oA}$, $G\alpha_{oB}$	K^+ channels ↑
		$G\alpha_{t1}$, $G\alpha_{t2}$	Ca^{2+} channels ↓
		$G\alpha_z$	Cyclic GMP Phosphodiesterase ↑
	$\alpha_{q/11}$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ $G\alpha_{15}$, $G\alpha_{16}$	Phospholipase C ↓
	α_{12}	$G\alpha_{12}$, $G\alpha_{13}$	?
β	$\beta_{1\text{-}5\ (6?)}$	Different assemblies of β and γ subunits	Adenylate cyclase ↑/↓ Phospholipases ↑ Phosphatidylinositol 3-kinase ↑
γ	$\gamma_{1\text{-}11\ (12?)}$		Protein kinase C ↑ Protein kinase D ↑ GPCR kinases ↑ Ca^{2+}, K^+ (and N^+) channels

There is a striking homology between the amino acid sequences of the Gα subunits, suggesting that they have also evolved from a common ancestor. Based on the sequence of the α subunits, G proteins have been grouped into four families (Table 7):

- The G_s family includes several splice variants of α_s, as well as α_{olf} (which is specifically expressed in olfactory epithelia).
- The $G_{i/o}$ family consists of three distinct α_i species (α_{i1}, α_{i2} and α_{i3}, plus splice variants) α_o (which exists in two splice variants, α_{o1} and α_{o2}), the two retinal transducins (α_{t1} and α_{t1}), an α-subunit found in the gustatory epithelium (α_{gust}) and α_z.
- The $G_{q/11}$ family consists of α_q, α_{11}, α_{14}, α_{15} and α_{16} (α_{15} and α_{16} appear to be the murine and human versions of the same gene).
- The $G_{12/13}$ family consists of only two members, α_{12} and α_{13}.

Besides the adenylate cyclase, there are many more G protein-linked effector components. They are either enzymes (e.g. guanylate cyclase, phospholipase A_2 and C) or ion channels. The effector enzymes will produce second messengers including cAMP, cGMP, diacylglycerol and IP_3, which in turn cause downstream effects including the opening of Ca^{2+} or K^+ channels and the generation of other messengers, such as

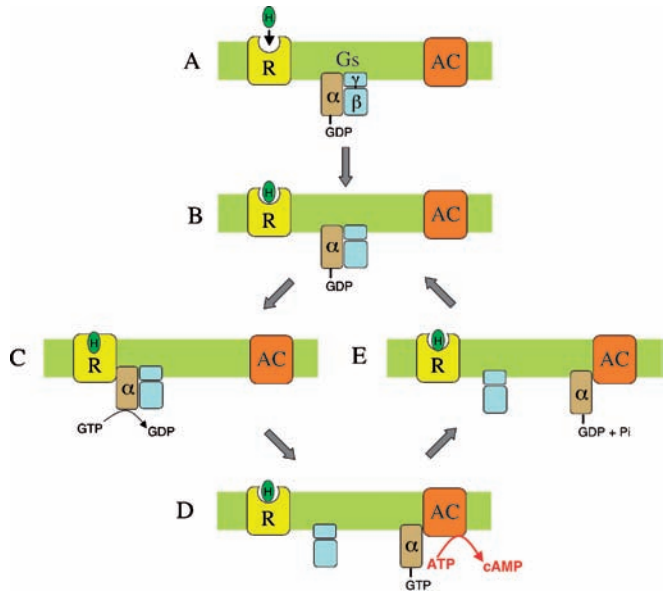


Figure 73 β-adrenergic stimulation of the adenylate cyclase system.

arachidonic and phosphatidic acid. In this respect, α subunits with similar sequences often regulate the activity of the same effector systems.

Adenylate cyclase stimulation by β-adrenergic receptors illustrates the most common molecular mechanism by which G proteins transfer information from receptor to effector components in the cell membrane (Figure 73):

- In the resting state, the receptor (R), G_s and the adenylate cyclase enzyme (AC) do not interact with each other. The α subunit of G_s (i.e. α_s) contains tightly bound GDP.
- A messenger molecule (H) binds to the receptor to form H–R.
- H-R can now associate with G_s to form H–R–G_s. An important property of H–R–G_s is that GDP is bound less tightly and that it can be exchanged with GTP from the cytosol. In fact, the major role of the β-adrenergic receptors consists in the facilitation of the GDP/GTP exchange at the level of α_s.
- GTP binding disassembles the H–R–G_s complex as well as G_s itself. The complex dissociates into three parts: H–R, β–γ and α_s which contains bound GTP (i.e. α_s–GTP).
- The free α_s–GTP (i.e. the active form of G_s) is able to associate with, and to stimulate, the adenylate cyclase enzyme. α_s possesses an endogenous GTP-ase activity, which is responsible for the hydrolysis of GTP into GDP (which remains

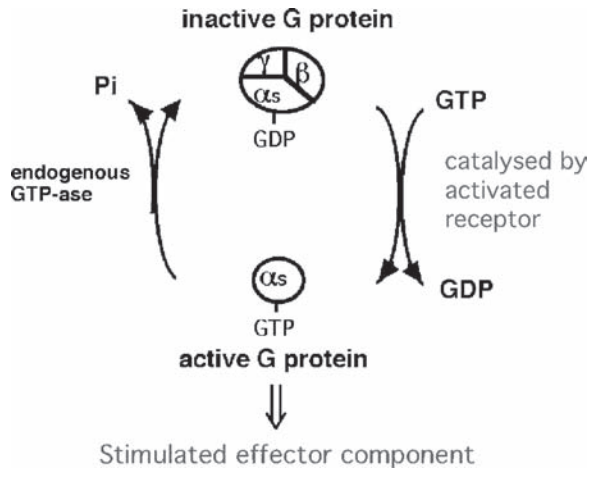


Figure 74 Central role of G proteins.

tightly bound). This process terminates the stimulation of the adenylate cyclase enzyme.

A more general representation of this model, which clearly evidences the central role of the G proteins, is presented in Figure 74. In this model, the messenger–receptor complex facilitates the activation of the G protein (by GDP/GTP exchange and disassembling) while the endogenous GTP-ase activity of the α subunit allows the G protein to return to the basal, inactive state. Although the central 'shuttling' role of the α subunits is well established, it has also become clear over the years that some signalling functions may also be ascribed to β–γ (Table 7).

Regulation (activation/inhibition) of the cyclic AMP concentration (Figure 75) and stimulation of inositol phospholipid hydrolysis (Figure 76) constitute two major mechanisms by which GPCRs affect the cell metabolism.

The *adenylate cyclase enzyme* was the first effector component to be discovered. It is an intrinsic protein that spans the membrane with no less than 12 hydrophobic α helixes. Nine isoforms of the mammalian adenylate cyclase have been cloned to date, and all of them are stimulated by G_s to catalyze the conversion of cytosolic ATP into the second messenger cyclic AMP and PPi. Cyclic AMP acts as an intracellular substitute for the chemical messenger, and it is therefore denoted as a '*second messenger*'. Indeed, increased levels of cyclic AMP form the initial step in a cascade of molecular events that will give rise to the final cellular response (Figure 75). The events comprise:

- Stimulation of a specific protein kinase (protein kinase A) by cyclic AMP.
- Protein kinase A-mediated phosphorylation of a specific proteins (including the receptor itself, see later).

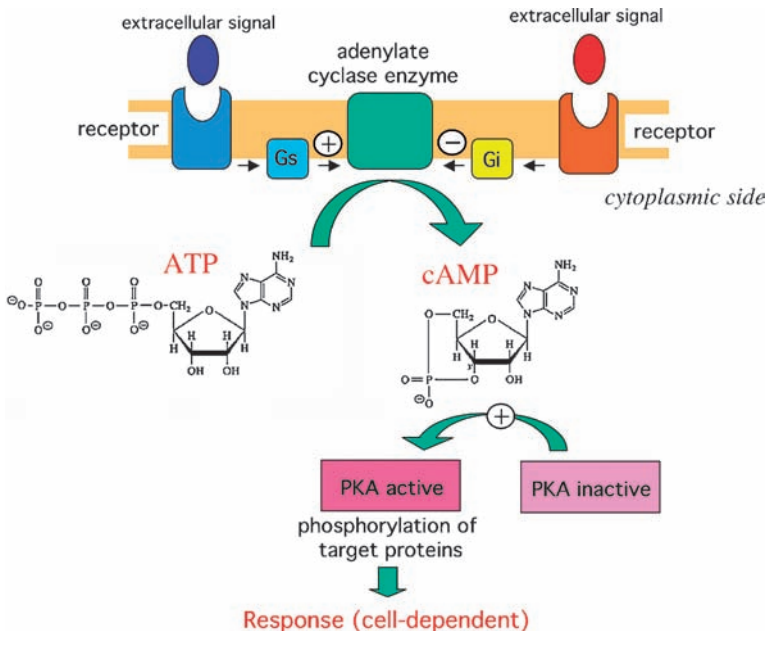


Figure 75 Opposite control of the adenylate cyclase enzyme activity by G_s- and G_i- coupled receptors. ATP: adenosine triphosphate, cAMP: 3′,5′ cyclic AMP, PKA: protein kinase A enzyme.

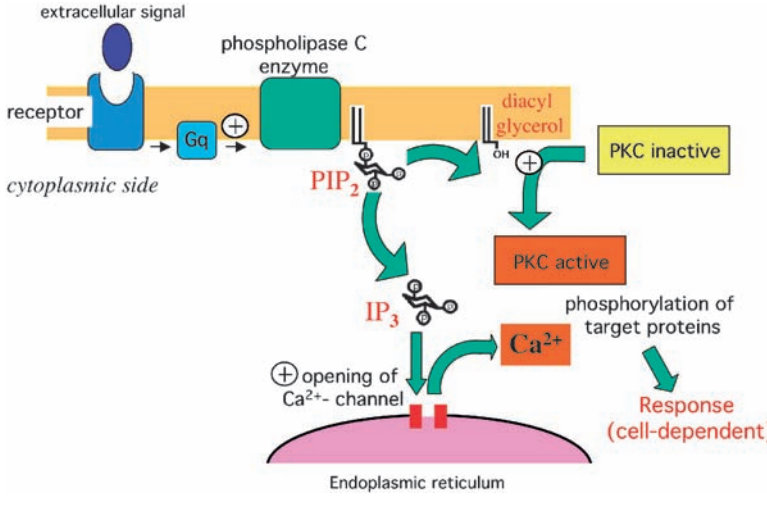


Figure 76 Inositol phospholipid hydrolysis and action of the hydrolysis products as second messengers. PIP_2: phosphatidyl inositol 4,5-bisphosphate, IP_3: inositol 1,4,5-trisphosphate, PKC: protein kinase C enzyme.

Table 8 Cellular responses mediated by cyclic AMP.

Target Tissue	Hormone	Major Response
Thyroid	Thyroid-stimulating hormone (TSH)	Thyroid hormone synthesisand secretion
Adrenal cortex	Adrenocorticotropic hormone (ACTH)	Cortisol secretion
Ovary	Luteinizing hormone (LH)	Progesterone secretion
Muscle, liver	Adrenaline	Glycogen breakdown
Bone	Parathormone	Bone resorption
Heart	Adrenaline	Increase in heart rate and force of contraction
Kidney	Vasopressin	Water resorption
Fat	Adrenaline, ACTH, glucagon, TSH	Triglyceride breakdown

- Altered activity/properties of the phosphorylated proteins. The nature of the phosphorylated proteins may differ from one cell type to another, so that the response will be cell-dependent (Table 8).

It has been noticed since the early eighties that several receptors do not stimulate, but rather inhibit the adenylate cyclase activity. This inhibition is also dependent on the presence of GTP and, hence, also mediated by a G protein. This inhibitory G protein has been designated as 'G_i'. In this respect, only certain isozymes of adenylate cyclase have been reported to be sensitive to α_i. Since the basal cyclic AMP production is usually low in a living cell, it is not possible to observe the inhibitory effect of receptors on the adenylate cyclase activity without prior stimulation of the enzyme. This can be achieved by the simultaneous stimulation of a G_s-coupled receptor or by direct stimulation of the enzyme by forskolin.

Inositol phospholipid hydrolysis constitutes a second major mechanism by which many G protein-coupled receptors affect the cell metabolism (Figure 76). The steps involved were elucidated to great extent in the mid-eighties. The receptors recruit $G_{q/11}$ proteins to stimulate a phospholipase C enzyme (whose active site is located at the cytoplasmic side of the membrane). There are four classes of phospholipase C enzymes, called PLC-β, -γ, -δ and -ε. From these, only the members of the PLC-β class are activated via $G_{q/11}$ proteins; the γ-class is stimulated by receptor tyrosine kinases.

PLC-β cleaves 'PIP_2' (phosphatidyl inositol 4,5-bisphosphate, a minor membrane phospholipid) into two compounds: diacyl glycerol and 'IP_3' (inositol 1,4,5-trisphosphate). Both compounds act as 'second messengers' inside the cell:

- Diacyl glycerol remains in the membrane (since it contains the two hydrophobic fatty acid chains), but it is able to activate a cytosolic protein: protein kinase C. The activated kinase can then phosphorylate various target proteins, resulting in a modification of their activity. The action of diacylglycerol is thus very similar

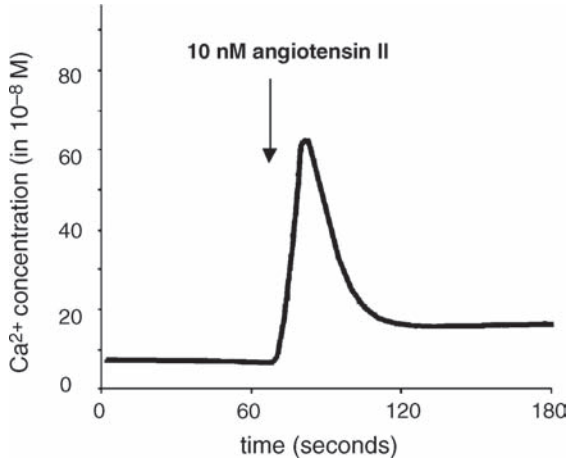


Figure 77 Time dependence of the cytosolic calcium concentration following the stimulation of G_q-coupled receptors (angiotensin II-stimulation in CHO cells expressing human AT_1 receptors).

to that of cyclic AMP, but the substrate specificity of both the kinases involved is quite different.

- On the other hand, cytosoluble IP_3 will interact with specific receptors at the endoplasmic reticulum and so trigger the release of calcium from this intracellular compartment into the cytosol. The resulting increase in the cytosolic calcium concentration is only transient and can be monitored by fluorescent techniques (Figure 77). At a later stage, IP_3 can be phosphorylated to IP_4, which promotes the influx of extracellular calcium into the cell by opening specific calcium channels in the plasma membrane. Calcium can affect the cell metabolism on its own or via calmodulin (a soluble protein with high affinity for calcium). Calcium–calmodulin complexes can associate to other proteins in the cell, and so alter their activity.

The calcium concentration is high in the extracellular fluid (10^{-3} M) and in certain intracellular compartments, such as the mitochondria and the endoplasmic reticulum (= sarcoplasmic reticulum in muscle cells), but normally very low in the cytoplasm (10^{-7} M) (Figure 78). This is because calcium is continually pumped out of the cytoplasm (to the extracellular medium and in the calcium-sequestering compartments) by specific ATP-ases. The opening of small calcium-selective channels in the membrane of the endoplasmic reticulum (by IP_3 receptors) as well as in the plasma membrane (by ligand- and voltage-gated channels) will allow calcium to rush down its concentration gradient, into the cytoplasm.

In general, G protein-mediated events permit an important amplification of the incoming signal (Figure 79). Indeed, a hormone- or neurotransmitter-bound receptor can stimulate many G proteins per second and a stimulated G protein may keep an effector component active for as long as 10–15 seconds. Hence, a single messenger molecule is capable of triggering the flux of a large amount of ions across the membrane

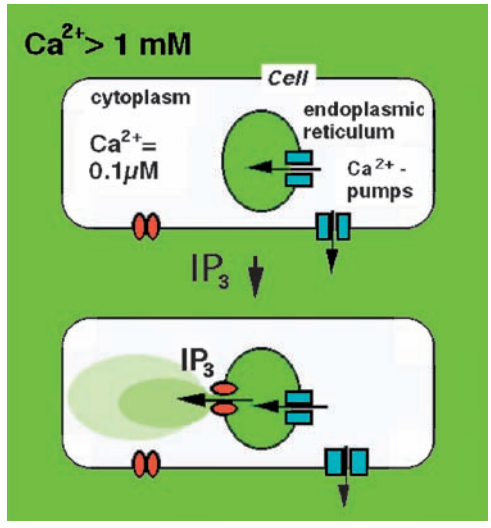


Figure 78 Ca^{2+} is removed from the cytoplasm by specific ATP-ases (in blue). An increase in cytoplasmic IP_3 concentrationm will produce the transient opening of Ca^{2+} channels at the surface of the endoplasmic reticulum.

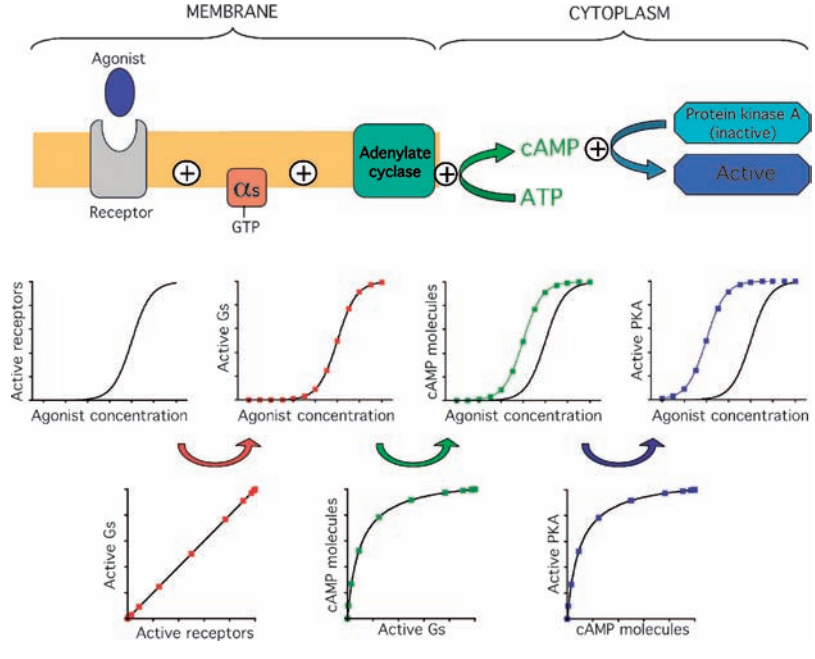


Figure 79 Signalling cascade amplification from receptor stimulation to protein kinase A activation. Black curves represent receptor occupancy.

(in the case of ion channels) or the production of a large amount of second messengers (in the case of enzymes). Such cascade-wise amplification of the signal explains the phenomenon of the 'receptor reserve', which is often encountered when comparing the degree of receptor occupation by an agonist with the evoked response.

4.2 GPCR structure

During the mid-seventies, it became possible to identify the β-adrenergic receptors directly (i.e. by radioligand binding) and soon afterwards the receptors could be purified by affinity chromatography. Amino acid sequences from small fractions of the purified receptors could be determined, and this opened new horizons for the molecular biologist.

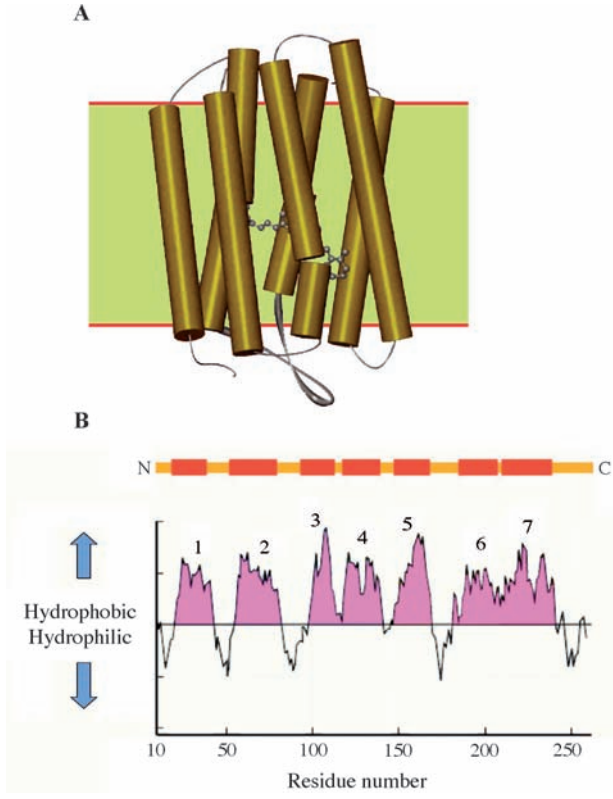


Figure 80 A: Three-dimensional structure of bacteriorhodopsin (reprinted from *Biochimica Biophysica Acta*, **1460**, Subramaniam S. and Henderson R. Crystallographic analysis of protein conformational changes in the bacteriorhodopsin photocycle, 157–165 Copyright (2000), with permission from Elsevier). B: Hydrophobicity pattern of bacteriorhodopsin (reprinted from *Journal of Molecular Biology*, **157**, Kyte J. and Doolittle R.F., A simple method for displaying the hydropathic character of a protein, 105–132. Copyright (1982), with permission from Elsevier).

These sequences allowed the synthesis of oligonucleotide probes with which DNA libraries could be screened for the presence of genes coding for the receptor molecules themselves as well as for closely related receptors. The complete amino acid sequence of the receptors could then be deduced from their DNA sequence. Using this approach, the first complete sequence of (hamster lung) β-adrenergic receptor was reported in the mid-eighties. Progress in this field has been very rapid since, and the sequences of more than 1000 different G protein-coupled receptors are already known. The GPCR 'superfamily' is a collection of proteins with structural and functional characteristics in common, but which lack obvious sequence similarity.

They are composed of a single peptide, usually 400–500 but also up to 1200 amino acids long. The major amino acid sequence similarity of GPCRs is the presence of seven hydrophobic segments, each of about 20–25 amino acids long, separated from each other by hydrophilic segments. Due to the inherent difficulties in crystallizing complex membrane proteins, high-resolution structural information has not been available for GPCRs for a long time.

Fortunately, the light-driven proton pump from *Halobacterium halobium* (i.e. bacteriorhodopsin) also possesses seven hydrophobic segments and, based on its X-ray diffraction pattern, a high-resolution structure of this enzyme has been available for several years (Figure 80). Bacteriorhodopsin has therefore been considered to be a bacterial homologue of vertebrate GPCRs, and its tertiary structure has been widely used as a template for GPCRs. It is now generally accepted that GPCRs possess seven transmembrane-spanning α helices (also called TM domains) connected by alternating intracellular and extracellular loops, with the amino terminus located on the extracellular side and the carboxy terminus on the intracellular side (Figure 81). Because of this characteristic, GPCRs are also often called seven transmembrane receptors (7TM receptors).

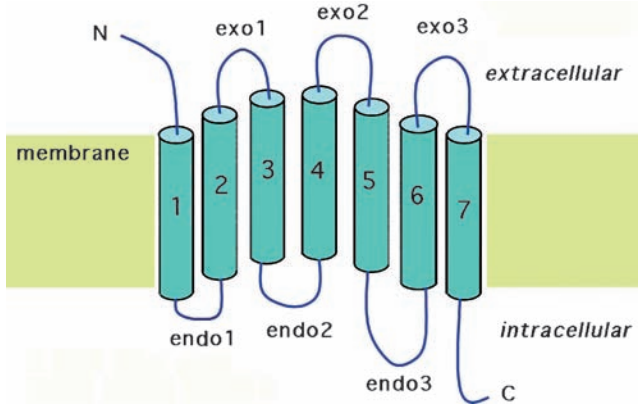


Figure 81 Structure of an archetypal GPCR. Transmembrane helices are numbered 1–7. Intracellular loops are marked endo1 to endo3 and the extracellular loops are exo1 to exo3. Reprinted from *Biochimica Biophysica Acta*, **1422**, Flower, D. R., Modelling G protein-coupled receptors for drug design, 207–234. Copyright (1999), with permission from Elsevier.

Bacteriorhodopsin remained for a long time the only protein with seven hydrophobic segments that could be crystallized successfully (and were therefore suited for X-ray diffraction studies). When viewed from above, its TM domains form a circle around a central pocket. However, bacteriorhodopsin is a proton pump, it is not linked to a G protein and it does not even display remote sequence homology with any GPCR. Low-resolution structures of both bovine and frog rhodopsin based on cryo-electron microscopy became available a few years ago. Recent X-ray crystallography of three-dimensional crystals of rhodopsin (actually a complex between the GPCR opsin and its ligand, retinal) offers for the first time a tertiary structure model of a GPCR at atomic resolution (2.8Å). Whereas the overall organization of these receptors is rather close to that of bacteriorhodopsin, (i.e. the presence of seven membrane-spanning domains) there are also clear differences. Therefore, the use of bacteriorhodopsin as a template for molecular models should now be considered obsolete. It is now believed that the helices of all GPCRs are organized sequentially in a counter-clockwise fashion (forming a flattened circle around a central pocket as seen from the extracellular side) with TM3 being tilted and almost in the centre of the molecule. On the extracellular side the helical arrangement opens up and forms a cavity that serves as a binding pocket for the ligand. The cavity of rhodopsin is lined by TM3 to TM7 and is closed toward the intracellular side by the tilted TM3 (Figure 82).

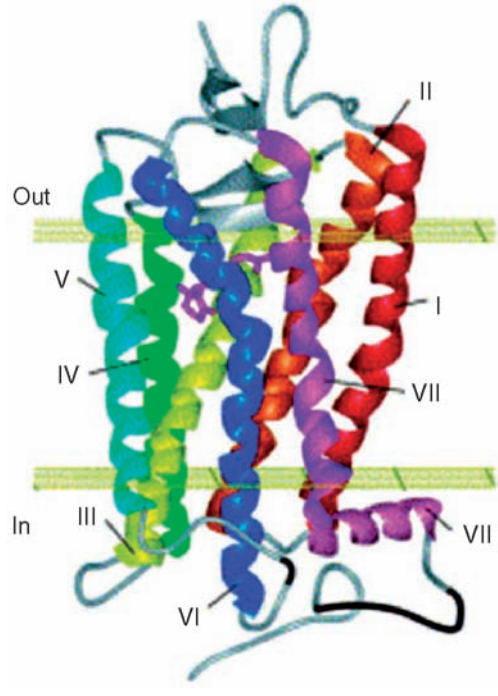


Figure 82 Three-dimensional structure of rhodopsin (side view). Reprinted from *Trends in Pharmacological Sciences*, **22**, Meng, E.C. and Bourne, H.R., Receptor activation: what does the rhodopsin structure tell us?, 587–593. © (2001), with permission from Elsevier.

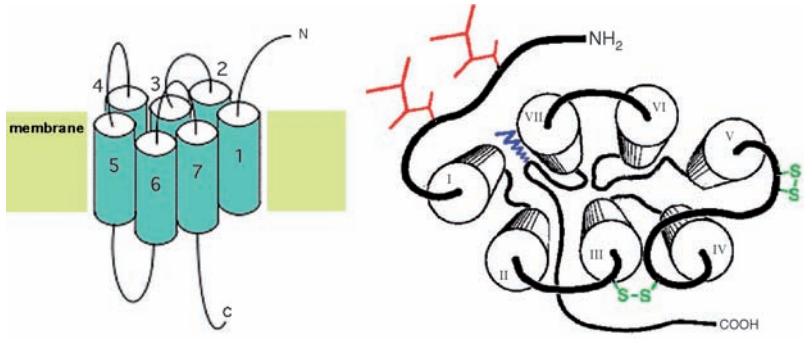


Figure 83 Overall three-dimensional schematic structure of GPCRs: (left) side view of an archetypal GPCR (reprinted from *Biochimica Biophysica Acta*, **1422**, Flower, D. R., Modelling G protein-coupled receptors for drug design, 207–234, Copyright (1999), with permission from Elsevier) and (right) more complete top view of a β-adrenergic receptor (Ostrowski *et al.*, 1992, reproduce by permission of Annual Reviews).

Besides these major structural features, G protein-coupled receptors may have (or undergo) (Figure 83):

- **Disulfide bonds** between cysteine residues present at the extracellular loops. This allows a circular arrangement of the α helices that is correct for messenger binding.
- Post-transcriptional **palmitoylation** of the C-terminal domain. The resulting additional 'anchoring' of the C-terminal domain to the cell membrane is important for the signalling efficacy of β-adrenergic receptors.
- **Glycosylation** sites at the N-terminal domain.
- Phosphorylation sites at the intracellular loops and at the C-terminal domain. These are important for modulating the receptor activity (e.g. desensitization, internalization).

GPCRs have been divided into several 'subfamilies' (also denoted as 'classes' or 'clans') whose protein sequences share greater than 20% sequence identity in their TM domains (Figure 84). They are presumed to have evolved from a common ancestor. Today there are three major families: family A (class I) is the rhodopsin-like receptor family with ligands such as neuropeptides, chemokines and prostaniods; family B (class II) are also called the secretin/glucagon/VIP family; family C (class III) receptors are metabotropic-glutamate-receptor-like. All GPCRs possess an integral membrane heptahelical domain (7TM) where the transmembrane helices (TMs) are linked by loops that extend outwards on both sides of the membrane. Compared to family A receptors, family B and C receptors have large extracelleular N-terminal domains.

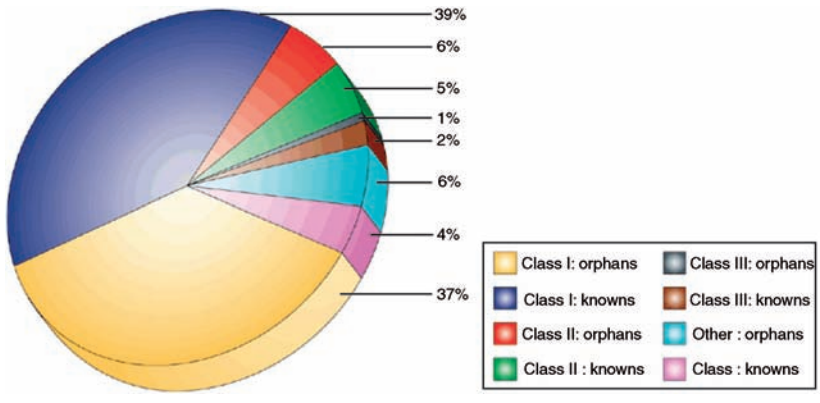


Figure 84 Percentage of known and orphan GPCRs of the various GPCR families. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Drug Discovery*, **1**, Chalmers, D.T. and Behan, D.P., The use of constitutively active GPCRs in drug discovery and functional genomics., 599–608, © (2002).

Family A: The subfamily of 'light receptor' rhodopsin/ β_2-adrenergic receptor-like receptors (Figure 85) is by far the largest and the most studied (Figure 84). Phylogenetically, family A receptors can be subdivided further into six major subgroups. In most family A receptors, a disulfide bridge connects the second and third extracellular loop (exo2 and exo3) (white letters in black circles). In addition, the majority of the receptors have a palmitoylated cysteine in the carboxy-terminal tail causing formation of a putative fourth intracellular loop. The membrane-proximal portion of the carboxy-terminal tail may also be α-helically arranged, giving rise to an 8th α helix.

The overall homology among all family A receptors is low and restricted to a number of highly conserved key residues. The high degree of conservation among these key residues suggests that they have an essential role for the structural and/or functional

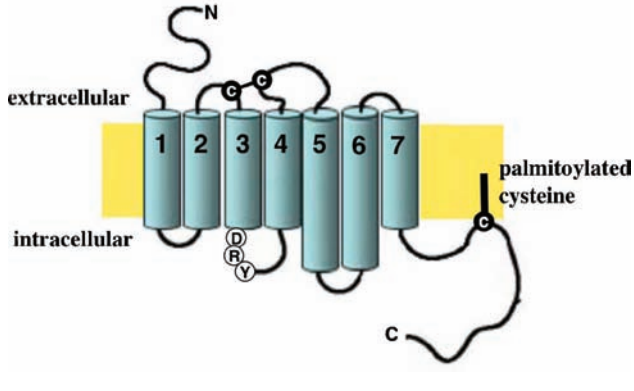


Figure 85 Two-dimensional structure of family A (Class 1) receptors and rhodopsin.

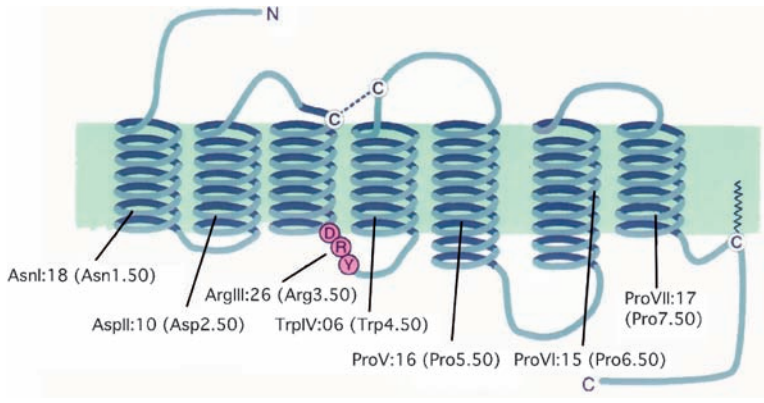


Figure 86 Denomination of the most conserved residues of the β_2-adrenergic receptor (Gether and Kobilka, 1998, reproduced by permission of the American Society for Biochemistry) using both the Ballesteros–Weinstein (Ballesteros and Weinstein, 1995) and Schwartz (Schwartz *et al.*, 1995) nomenclatures.

integrity of the receptors. The only residue that is conserved among all family A receptors is the arginine in the Asp/Glu-Arg-Tyr (D/ERY) motif at the cytoplasmic side of TM3.

To facilitate comparison of residues between the large number of different receptors belonging to family A there is an obvious need to formulate and use a common numbering scheme. Different numbering schemes have been suggested (Figure 86).

- In the Ballesteros–Weinstein numbering scheme, the most conserved residue in each helix has been given the number 50, and each residue is numbered according to its position relative to this conserved residue. For example, 6.55 indicates a residue located in TM6, five residues carboxy terminal to Pro6.50, the most conserved residue in TM6.
- In the Schwartz nomenclature the most conserved residue in each helix had been given a generic number according to its position in the helix.

Family B: Family B (class 2) receptors contain about 65 members, all of which share some amino acid sequence in common. This receptor family represents an ancient signalling system that appears to play an important role in many biological processes. Therefore, they constitute interesting therapeutic targets in pharmaceutical research. Based on their sequence, family B GPCRs can be divided into three subfamilies: those recognizing peptide hormones, those with a GPCR proteolytic site (GPS) domain and those with cysteine-rich domains.

The secretin/glucagon/VIP receptor family (peptide hormone receptor family/subfamily B1) includes approximately 20 different members for a variety of peptide hormones and neuropeptides with relatively high molecular weight. The origin of this receptor family comes from secretin, the first hormone to be discovered in intestinal

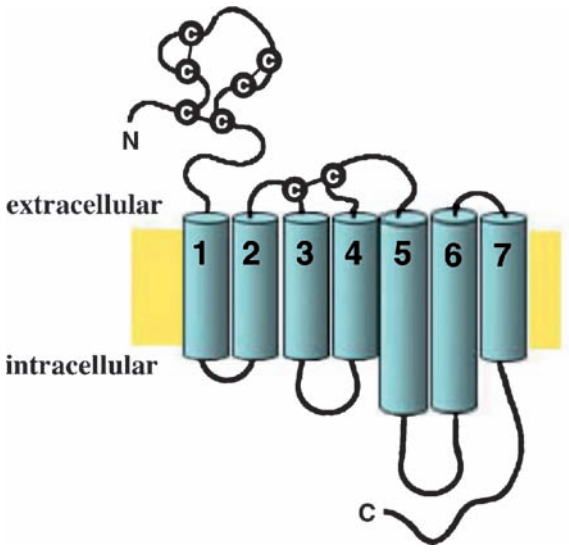


Figure 87 Two-dimensional structure of subfamily B1 receptors.

extracts. Secretin is released by acid from S-cells in the duodenum. It stimulates pancreatic fluid and bicarbonate secretion, leading to neutralization of acidic chyme in the intestine. It also inhibits gastric acid secretion and intestinal motility. The secretin receptor was also the first member of this family to be cloned in the early nineties and it has therefore been elected as the prototype.

The B1 receptor family regulates many important physiological processes, including somatic growth, energy intake, nutrient absorption and disposal, and cell proliferation and apoptosis. They mainly signal through Gs, resulting in increased adenylate cyclase enzyme activity and, hence, cyclic AMP production. However, they may also signal through Gq but this signalling pathway is triggered less efficiently. This has been clearly illustrated for the secretin receptors. To obtain a transient rise in the cytosolic calcium, the concentrations of secretin need to be more than 100-fold higher than those required for stimulating the Gs pathway. Moreover, some of the B1 receptors have been shown to interact with receptor activity modifying proteins (RAMPs). These proteins span the cell membrane with a single TM domain and, at least in the case of the CGRP receptors, they are essential for obtaining the correct pharmacological profile and transport to the cell membrane (see Section 4.8). Whether RAMPs also affect ligand selectivity and activity of other subfamily B1 receptors under normal physiological conditions remains to be established.

With a few exceptions, members of this subfamily have significant sequence similarities and are very uniform in length. The N-terminal part is typically 120 residues long and contains six highly conserved cysteine residues (Figure 87) and multiple potential glycosylation sites. These cysteines are likely to form a network of disulfide bridges critical for obtaining a functional receptor conformation. Moreover,

these receptors also possess a disulfide bond linking the first and second extracellular loops. Despite quite different amino acid sequences, it appears that the TM domains and intra- and extracellular loops of family A (rhodopsin-like receptors) and family B1 GPCRs have several properties in common (Frimurer and Bywater, 1999). The lengths and orientations of the TM helixes are quite similar; the most tilted helices are TM1, TM2, TM3 and TM5 for family A receptors and TM1, TM3 and TM5 for family B1 receptors. TM3 and TM5 seem to be the longest and most tilted in both families. Finally, the minimum loop lengths are also comparable for family A and family B1 receptors. These similarities suggest that rhodopsin may be a good template for family B1 receptors as well.

The second subclass of the B receptor family is most frequently termed LNB-7TM (>30 members). Although they represent the largest subclass, they are also the least well known. What distinguishes LNB-7TM receptors from the others is their unusual mode of processing in the endoplasmic reticulum. Indeed, they are cleaved at a defined region of the N-terminal part (i.e. the GPS domain) and a non-covalent linkage (presumably a disulfide bond) then rejoins both ends. This suggests that release of the extracellular (i.e. N-terminal) portion of these receptors may play some functional role.

Despite the sequence similarities between LNB-7TM receptors and other B receptor family members, it is not clear whether they are true GPCRs. First, they all appear to be orphan receptors (i.e. with no extracellular messenger known so far). Moreover, only a few LNB-7TM receptors have been associated with G protein signalling. CD97 is one of them; it is present in white blood cells and is induced in activated leucocytes. On the other hand, examination of LNB-7TM receptor structures suggests that they may be involved in alternative cellular functions:

- The extracellular domains of some LNB-7TM receptors suggest that they may be involved in cell adhesion, either by interacting with the cellular matrix or with other cells (Figure 88).
- Several members of this subfamily have extremely large intracellular tails (Figure 88), suggesting that they exert biological functions by interacting with intracellular proteins.

Based on these two structural characteristics, it has been suggested that LNB-7TM receptors induce cell signalling pathways in response to recognition of molecules at the surface of other cells and/or the extracellular matrix.

Frizzled and smoothened receptors (10 and 1 members, respectively) are related to each other and constitute the third subclass of the B receptor family. They show slight but significant sequence similarity to other B receptor family members and they are characterized by the presence of cysteine-rich domains in their N-terminal part. Their denomination arises from the fact that they were discovered by investigating the genetics of the fruit fly *Drosophila melanogaster*. They play an important role in the coordination of embryological development. Frizzled receptors are activated by secreted

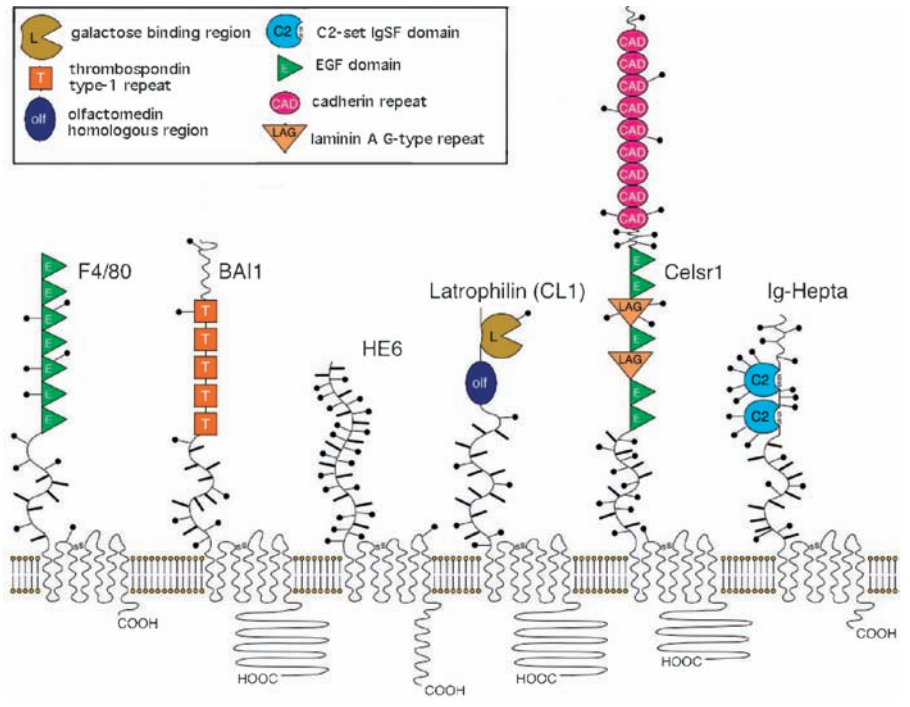


Figure 88 Schematic representation of LNB-TM7 receptor proteins. These membrane-spanning proteins have very long N-terminal domains with well-known protein modules. Reprinted from *Trends in Biochemical Science*, **25**, Stacey, M., Lin, H. H., Gordon, S. and McKnight, A. J., LNB-TM7, a group of seven-transmembrane proteins related to family-B G protein-coupled receptors, 284–289. Copyright (2000), with permission from Elsevier.

proteins of 350–360 amino acids (named Wnts) but it is not clear whether they are able to produce G protein signalling. On the other hand, smoothened receptors can clearly activate G proteins and this process has been found to take place without extracellular ligand (i.e. smoothened receptors are constitutively active – see Section 4.4).

Like the peptide hormone receptor family, LNB-7TM, frizzled and smoothened receptors also appear to be associated with, and controlled by, other integral membrane proteins (e.g. LRP for frizzled receptors) (Figure 89). However, these 'accessory proteins' are structurally dissimilar from the RAMPs.

Family C: Family C (class 3) receptors include the metabotropic glutamate receptors (eight subtypes), GABA receptors, calcium-sensing receptors and three receptors involved in taste perception. Metabotropic glutamate receptors constituted the first members of this family to be identified and cloning of the mGlu1a subtype in the early nineties revealed that this protein does not share obvious sequence similarity with the rhodopsin-like family A GPCRs. The metabotropic glutamate and related GPCRs are therefore regarded as constituting a new family (Figure 90).

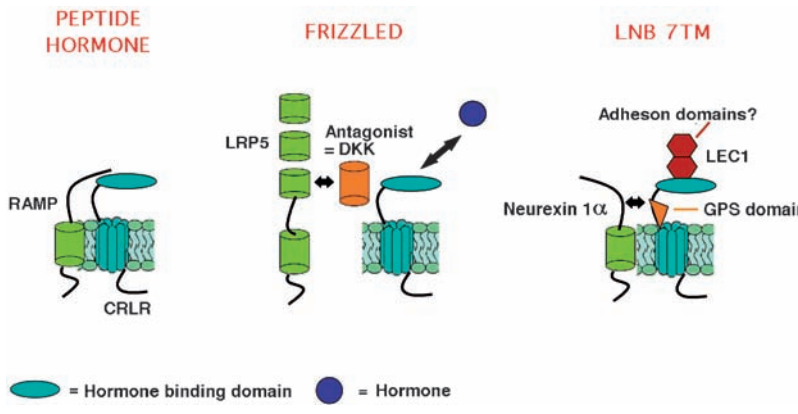


Figure 89 Family B GPCRs tend to associate with parner/accessory proteins. Peptide receptors are represented by CLCR-RAMP associations, frizzled receptors by FR7. LPR5: low-density lipoprotein receptor-related protein, DKK: Dikkopf1, GPS: GPCR proteolytic site. Adhesion domains may bind to components of the extracellular matrix or participate in cell–cell interactions. Reproduced with permission, from S.M. Foord, S. Jupe and J. Holbrook, (2002), *Biochemical Society Transactions*, **30**, 473–479. © The Biochemical Society.

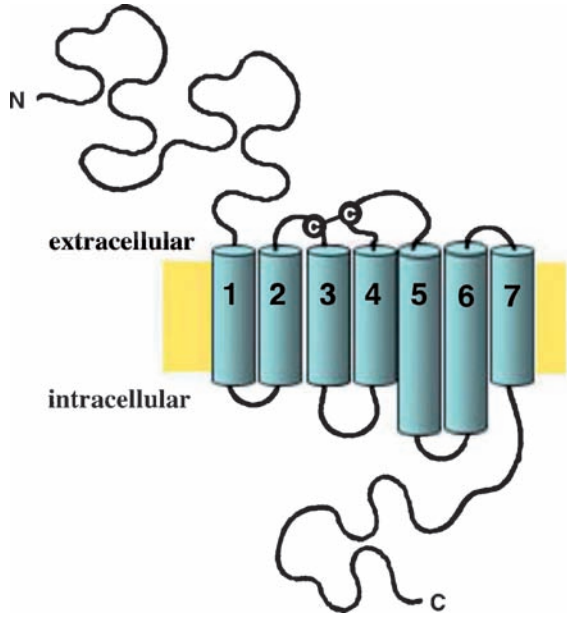


Figure 90 Two-dimensional structure of family C (Class 3) receptors.

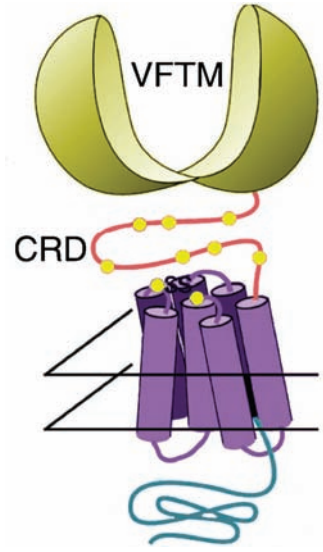


Figure 91 In family C GPCRs, the bilobal Venus Fly trap module (VFTM) is connected via a cysteine-rich domain (CRD) to the transmembrane domain. Yellow circles: conserved cysteine residues among this GPCR family. Reprinted from *Pharmacology and Theraputics*, **98**, Pin, J. P., Galvez, T., Prezeau, L., Evolution, structure, and activation mechanism of family 3/C G protein-coupled receptors, 325–354. Copyright (2003), with permission from Elsevier.

Family C GPCRs are characterized by a very long amino terminus (600 amino acids). This large extracellular segment comprises a N-terminal Venus Fly trap module (VFTM) that is linked via a cysteine-rich domain (CRD, containing nine conserved cysteines) to the 7TM spanning region (Figure 91). The VFTM contains the binding site for the natural messenger and of interest is its sequence similarity with bacterial periplasmic-binding proteins, which are involved in the transport of small molecules. The CRD domain is present in all family C GPCRs except for $GABA_B$ receptors and its function is presently ill understood. The seven TM helices from family C GPCRs are interconnected by short (<30 amino acids) intra- and extracellular loops. The short and highly conserved third intracellular loop (Figure 90), especially, contrasts with the sometimes long and variable one in family A GPCRs. Despite the low overall sequence similarity between the seven TM helices of family A and C GPCRs, it appears that both families share a number of highly conserved amino acid residues as well as a conserved disulfide bond between the top of TM3 and the second extracellular loop. Taken together, these findings suggest that both GPCR families originate from a common ancestral gene and that for family C GPCRs it has fused with the gene for a periplasmic-binding protein.

Many GPCRs have been found to form homo- and heterodimers, especially when over-expressed in recombinant cell systems and, in this respect, family C receptors have all been shown to form dimers (see Section 4.7). In this respect, the $GABA_B$ receptor was the first identified GPCR to function exclusively in a heterodimeric form

(involving the $GABA_BR1$ and $GABA_BR2$ proteins). Contacts between both proteins appear to be multiple, involving their VFTMs (an interaction that is stabilized by a disulfide bond) and their C-terminal tails. More recently, two additional family C GPCRs were also shown to act as heterodimers: a sweet taste receptor and an L-amino acid taste receptor.

Yeast pheromone receptors make up two minor unrelated subfamilies, *family D* (STE2 receptors) and *family E* (STE3 receptors). In *Dictyostelium discoideum* four different cAMP receptors constitute yet another minor, but unique, subfamily of GPCRs (*family F*).

GPCRs are often denominated according to their natural messenger. In some cases, such as for the hormone glucagon, only one receptor (a glucagon receptor) is known to exist. However, most messengers are capable of interacting with multiple receptor molecules. This offers the possibility for an additional subclassification of GPCRs based on their ability to interact with a given messenger. For example, angiotensin II interacts with AT_1 and AT_2 receptors. A somewhat more complicated situation arises with adrenaline and noradrenaline. These messengers interact with nine different 'adrenergic' receptors (Figure 111).

Finally, several cloned receptors possess the structural properties of GPCRs, but their natural messengers are still unknown. Pending the discovery of such messengers, they are denoted as '*orphan receptors*'. To complicate things even further, certain messengers (e.g. the neurotransmitter acetylcholine) are capable of interacting with G protein-coupled receptors (muscarinic acetylcholine receptors), as well as with receptors with a completely unrelated structure (nicotinic acetylcholine receptors).

4.3 Ligand interactions with family A, B and C receptors

The chemical diversity among the endogenous ligands is exceptional (Table 9). They include biogenic amines, peptides, glycoproteins, lipids, nucleotides, ions and proteases. Moreover, the sensation of exogenous stimuli, such as light, odour and taste, is mediated via GPCRs. Ligand size has a profound effect on the nature and location of binding. Large ligands, such as proteins and peptides, bind to the extracellular loop scaffold, while small molecules, including pharmacological agents, bind within the transmembrane region of the receptor. Peptides can exhibit a mixed binding mode whereby they bind primarily to the extracellular loops while part of the structure penetrates the transmembrane region.

Much information about the ligand binding sites has been acquired through mutation studies (Figure 92). The most prominent mutations in this respect comprise:

- Deletion of an amino acid or even a whole part of the amino acid sequence of the receptor.
- Substitution of single amino acids of the receptor by another amino acid (e.g. to change acidic or basic residues by neutral ones for investigating the role of electrostatic interactions).

Table 9 Examples of endogenous ligands for GPCRs. Reprinted from *Biochimica Biophysica Acta*, **1422**, Flower, D. R., Modelling G protein-coupled receptors for drug design, 207–234. Copyright (1999), with permission from Elsevier.

Type of messenger	Examples
Biogenic amines (and related compounds)	Adrenaline, dopamine, histamine, acetylcholine, noradrenaline, tyramine, serotonin, melatonin
Peptides and proteins	Angiotensin, bradykinin. bombesin, C3a, C5a, calcitonin, chemokines (MIP-Iα, -1β, -2, -3α. –3β; eotaxin; IP-IO; RANTES; MCP-l, -2, -3, -; 4, -5; interleukin 8; TARC; HCC-I; MDC; MIG; I-TAC; 1-309; TECK; SDF-I; fractalkine; GCP-2; PARC; DC-CKI; Iymphotactin; ENA-78; NAP-2; LIX; ELC EBII; LARC; SLC), cholecystokinin, conopressin, corticotropin-releasing factor, decay- accelerating factor, diuretic hormone, endothelin, enkephalins and endorphins, follitropin, fMLP and other formylated peptides, glycoprotein hormones, fungal mating pheromones, galanin, growth hormone-releasing hormone,growth hormone secretagogue, gastric inhibitory peptide, gastrin, glucagon, gonadotropin-releasing hormone, LH glycoprotein hormone, melanocortin, neuropeptide Y, neurotensin, opioids, oxytocin, thrombin, protease-activated pituitary adenylyl cyclase-activating peptide, PTHrP, secretin, somatostatin, tachykinin, thyrotropin-releasing hormone, glycoprotein hormones, vasopressin, vasotocin, vasoactive intestinal peptide
Lipids	Anandamide, cannabinoids, leukotrienes, Iysophosphatidic acid, platelet- activating factor
Eicosanoids	prostacyclins, prostaglandins, thromboxanes
Purines and nucleotides	Adenosine, cAMP, ATP, UTP, ADP, UDP
Excitatory amino acids & ions	Glutamate, calcium, GABA

- Production of chimaeric receptors, in which part of the amino acid sequence of a receptor is replaced by the corresponding sequence of another, often related receptor.

Ligand interaction with family A receptors

Rhodopsin is unique among the GPCRs in that its ligand is covalently attached to the receptor within a binding crevice formed by the transmembrane helices (Figure 93). This may be necessary to facilitate the very rapid response of rhodopsin to light.

The binding sites for the classical ‘small-molecule’ transmitters (biogenic amines like epinephrine, norepinephrine, dopamine, serotonin, histamine and acetylcholine)

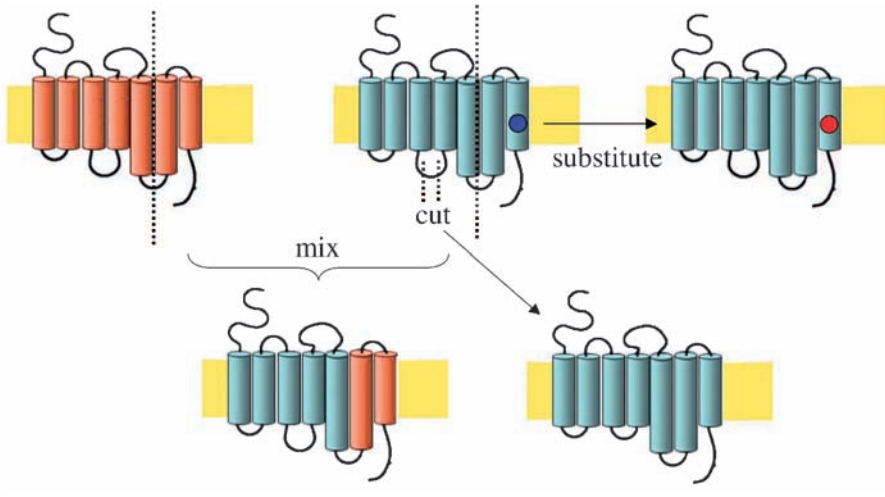


Figure 92 Commonly performed receptor mutations to investigate ligand binding.

are located in the central pocket of their GPCRs (Figure 93). Hence, these messengers interact with amino acid residues belonging to some of the membrane-spanning domains (especially TM3, TM5, TM6 and TM7). For example, mutation studies with the β_2-adrenergic receptor indicate that at least two of its membrane-spanning

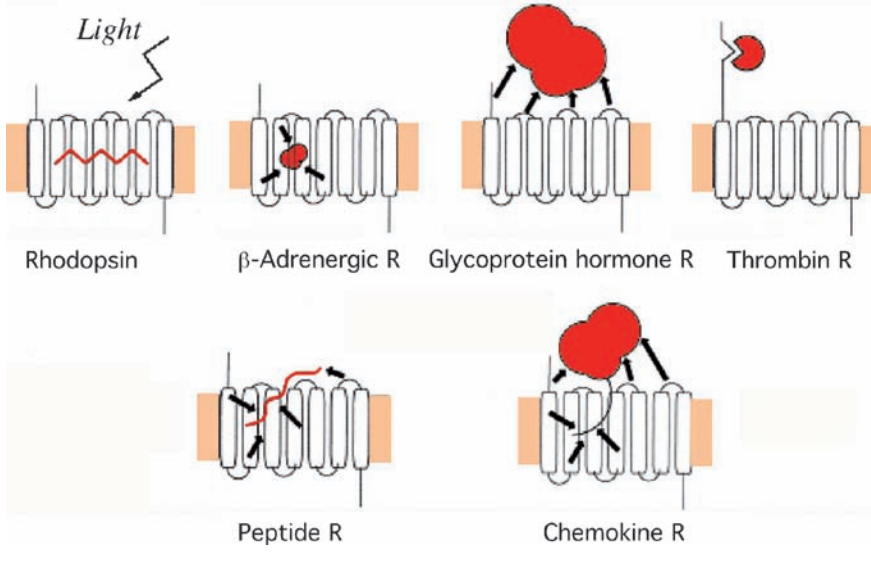


Figure 93 Ligands are able to interact with extracellular and transmembrane domains of family A GPCRs. Reprinted from *Biochimica Biophysica Acta*, **1422**, Flower, D. R., Modelling G protein-coupled receptors for drug design, 207–234. Copyright (1999), with permission from Elsevier.

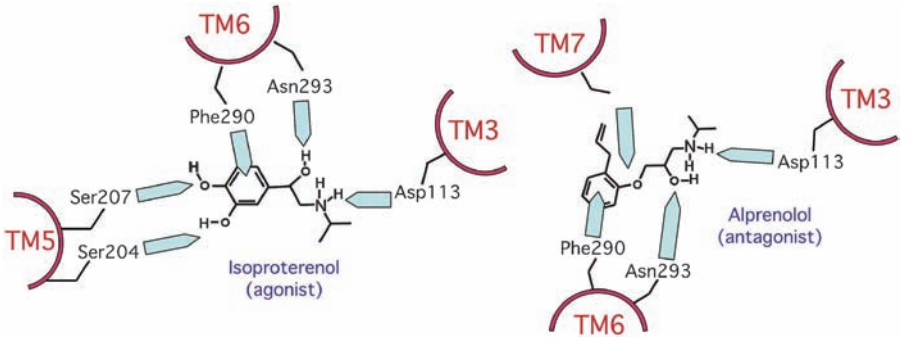


Figure 94 Ligand interactions with amino acid side chains of the β_2-adrenergic receptor. Reprinted from *Biochimica Biophysica Acta*, **1422**, Flower, D. R., Modelling G protein-coupled receptors for drug design, 207–234. Copyright (1999), with permission from Elsevier.

domains (i.e. TM3 and TM5) are involved in the binding of agonists like adrenaline and isoproterenol (Figure 94 and Figure 95). The hydroxyls on the aromatic ring interact with Ser^{204} and Ser^{207} in TM5 and the secondary amine interacts with Asp^{113} in TM3. Phe^{289} and Phe^{290} on TM6 probably make π-stacking interactions with the catechol ring. These results provide information about the proximity of TM3 and TM5 in the messenger/agonist-bound receptor.

The binding site for a representative antagonist, alprenolol, overlaps with that of isoproterenol, but this overlap is only partial and the nature of receptor–ligand interaction is different also (Figure 94). It is of interest that Asp^{113} in TM3 of the β_2-adrenergic receptor is conserved among the biogenic amine receptors ($Asp^{3.32}$ according to the Ballesteros–Weinstein numbering) and is also thought to interact with the positively charged head group of the monoamines and related antagonists.

On the other hand, because of the relatively large size of peptide messengers, the binding site for these molecules is more likely to comprise extracellular domains

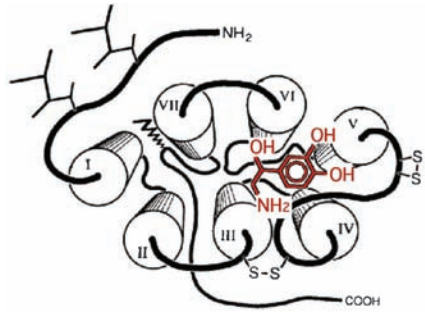


Figure 95 Molecular models of the adrenaline–β_2-adrenergic receptor complex (top view) (Ostrowski *et al.*, 1992, reproduced by permission of Annual Reviews).

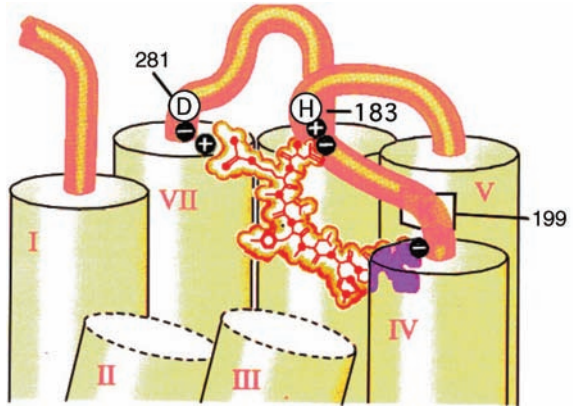


Figure 96 Interaction between angiotensin II and the AT_1 receptor (Feng *et al.*, 1995, reproduced by permission of The American Society for Biochemistry).

(i.e. the N-terminal part and the three extracellular loops) of GPCRs (Figure 93). As an example, mutation studies revealed that certain amino acids belonging to extracellular domains of the AT_1 receptor are crucial for the recognition of angiotensin II. A model for the binding of this peptide to the AT_1 receptor is given in Figure 96. In this model, His^{183} and Asp^{281}, both located in the extracellular domain of the AT_1 receptor, are involved in binding the N-terminal Asp^{1} and Arg^{2} residues from angiotensin II, respectively. In addition, Lys^{199} in the TM7 of the receptor binds the C-terminal carboxyl group of angiotensin II.

Some of the peptides may also have additional points of interaction in the TM domains and therefore, they may enter the central cleft to different degrees. Moreover, peptide receptors also recognize some small synthetic molecules. They usually behave as antagonists, but some of them also behave as agonists nothwithstanding the fact that their binding pocket may be topographically distinct from the peptide binding site. Mutation studies reveal that (similar to the binding of biogenic amines to their receptors) these small molecules rather bind within the central cleft.

For example, non-peptide antagonists of the NK-1 receptor (prototype: CP 96345) bind to residues clustering in a crevice formed by TM3 to TM6 and mutation of these residues does not affect peptide agonist binding (Figure 97). Hence, this binding pocket is most likely not occupied by substance P. Accordingly, an actual overlap in the binding sites is not required for a competitive mode of action of the non-peptide antagonists.

The protease-activated thrombin receptors also belong to family A GPCRs (Figure 98). The unique activation mechanism of the thrombin receptor involves cleavage of the N-terminal segment by thrombin. The resulting 33-amino acid N-terminus subsequently acts as tethered peptide ligand, which, through interactions with the extracellular loop regions of the receptor, is able to activate the receptor. In this vein, relatively short synthetic peptides (5–14 amino acid residues) based on the sequence of the unmasked N-terminal receptor sequence activate the receptor in the same way as thrombin.

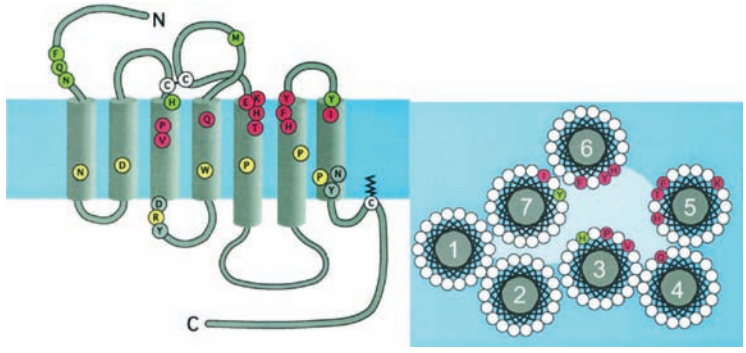


Figure 97 Snake diagram and (left) and wheel diagram (right) of the NK-1 receptor: Yellow: the most conserved residue in each helix, green: residues involved in substance P (= messenger) binding, red: residues involved in small-molecule antagonist binding. Reprinted from *Endocrine Review*, **21**, Gether, U., Uncovering molecular mechanisms involved in activation of G protein-coupled receptors, 90–113. Copyright 2000, The Endocrine Society.

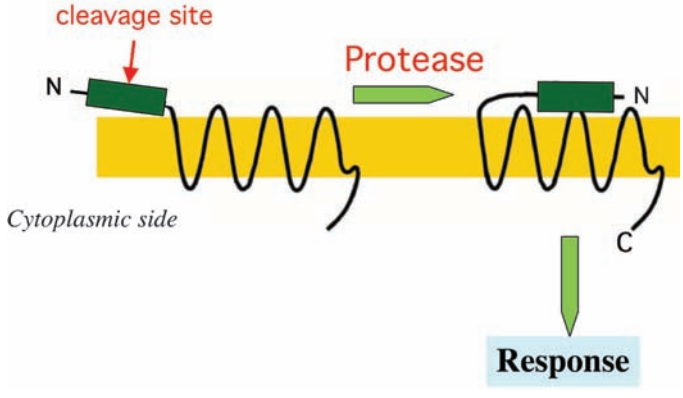


Figure 98 Thrombin clieves the N-terminal part of its receptor. The newly formed terminus behaves as a 'tethered' agonist to activate the receptor.

Ligand interaction with family B receptors

Except for the recognized activation of frizzled receptors by Wnts (350–360 amino acid proteins) soluble extracellular ligands for LNB-7TM and smoothened receptors appear to be more elusive or may even be inexistent. In contrast, all members of the secretin/glucagon/VIP receptor family are stimulated by known peptide hormones and neuropeptides. These have a relatively high molecular weight (e.g. 27 amino acids for human secretin, 29 for pancreatic glucagon, 37 for calcitonin gene-related peptide and 41 for corticotropin-releasing factor).

Mutation studies have provided considerable information concerning the interaction between the family B1 peptide hormone receptors and their ligands. For example, it was shown for the glucagon receptor that:

- All seven TM domains of the receptor are needed for its proper folding and processing.
- Receptor glycosylation is not essential for binding or activation.
- The extracellular amino terminus is required for ligand binding.
- The 206–219 segment of the first extracellular loop is important for ligand binding and activation but the terminal portion of the intracellular carboxyl terminus is not.

In general, as illustrated for the glucagon-like protein 1 (GLP1) receptor (Figure 99), it seems that the large amino terminus of these receptors plays a key role for the binding

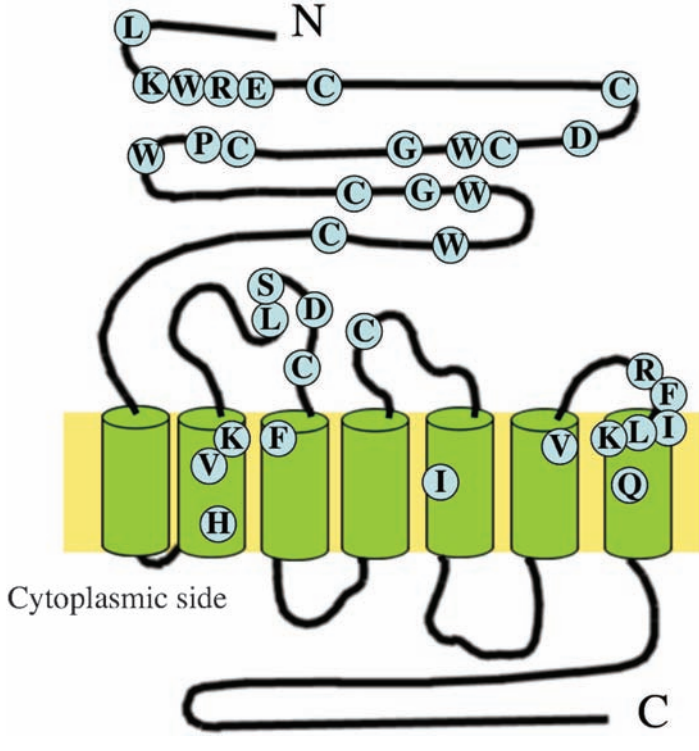


Figure 99 Schematic representation of the glucagon like protein 1 (GLP1) receptor. Residues that are important for binding are shown: they are present in the N-terminal portion, extracellular loops and TM helices. Reproduced from Frimurer, T. M. and Bywater, P., Structure of the integral membrane domain of the GLP1 receptor. *Proteins* **35**, 375–386 (1999), by permission of John Wiley & Sons Ltd.

of the peptide ligands. However, additional interactions involving the extracellular loops and specific TM domains appear also to be required for binding and activation.

The existence of topographically distinct binding domains at these receptors allows both ends of their cognate peptide ligands to display different, but complementary functions. For example, CRF receptors (CRF1R and CRF2R) use their amino terminus to anchor the N-terminal part of their different peptide ligands. This plays an important role with respect to their receptor subtype selectivity. On the other hand, binding of the C-terminal part of these ligands to specific receptor TM domains is necessary to produce receptor activation.

While the peptide hormone receptors have distinct binding domains for peptide ligands, small (i.e. synthetic) molecules are thought to mainly bind to the TM region. The occurrence of topographically distinct binding sites for the peptide agonists and small molecule antagonists paves the way to allosteric interactions and thereby associated 'non-competitive' antagonism (see Section 4.15). This may invalidate the classical methods of analyzing antagonist–receptor interactions.

Of note is that many family B GPCRs have been shown to interact with other membrane-associated proteins and for the calcitonin receptor-like receptor (CRLR), this has clearly been demonstrated to affect their migration to the cell plasma membrane as well as their pharmacological profile/identity (see Section 4.8). Whereas the heterodimer between CRLR and a type 1 RAMP (a receptor activity modifying protein with a single TM domain) generates a CGRP receptor, CRLR–RAMP2 or –RAMP3 complexes generate an adrenomedullin receptor (Figure 152). This example clearly illustrates that GPCRs do not necessarily act on their own. Instead, their ligand binding and other properties may be highly sensitive to the presence of certain proteins in their surroundings.

Ligand interaction with family C receptors

The VFTMs of family C GPCRs are responsible for agonist recognition. This is well illustrated by the ability of such modules to bind ligands even when produced as an isolated soluble protein. The crystal structure of the VFTM of the mGlu1 metabotropic glutamate receptor subtype has been solved with and without bound messenger (glutamate) and antagonist (α-methyl-4-carboxyphenylglycine). In fact, these receptors constitute the first GPCRs for which we have precise structural information about their binding domain. Similar to bacterial periplasmic-binding proteins, the VFTM of the mGlu1 receptor contains two lobes separated by a cleft where ligands bind. In the absence of ligand, the VFTM appears to oscillate between an 'open' and a 'closed' conformation, but the 'open' conformation predominates (Figure 100). Both conformations also exist in the presence of bound agonist, but now the 'closed' conformation is stabilized by the agonist. Hence, after binding of the messenger or related agonist, the VFTM is likely to 'close', trapping the messenger between both lobes in somewhat the same way the carnivorous Venus Fly trap plant

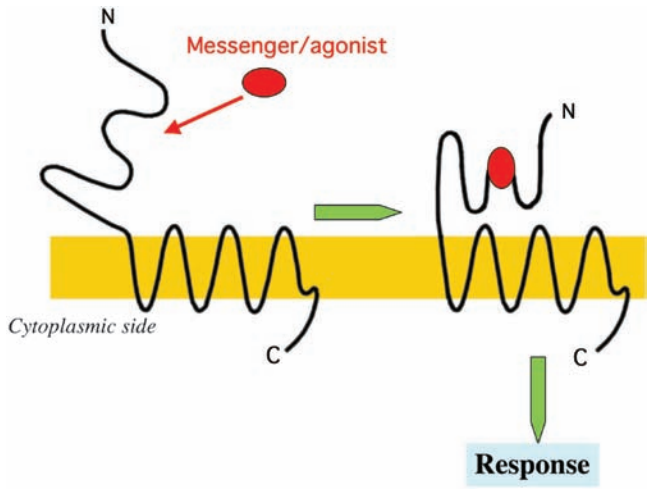


Figure 100 The 'Venus Flytrap' model for family C receptor activation. The agonist binds first to the N-terminal VFTM, the module 'closes' and is then presented to the extracellular loops of the receptor.

does to catch insects. Competitive mGluR antagonists appear to bind within the same binding pocket, but contrary to agonists, they are able to prevent the closure of the VFTM. In fact, its correct closure can only take place if the ligand 'fits' in the closed conformation. This explains why drugs that are structurally related to agonists, but which contain additional or misplaced substituents are able to act as antagonists.

Although the closure of the VFTM represents an important step, it is not sufficient to confer family C GPCR activation. Based on the observation that these receptors act as dimers, it has now been proposed that agonist binding to (and closure of) one VFTM provokes a change in the relative orientation of both VFTMs and that this somehow produces a conformational change in the 7TM domain, ultimately resulting in G protein binding and activation (see also Section 4.4). The participation of the 7TM domain in family C GPCR activation allows ligands that specifically bind to this domain either to potentiate or to inhibit (Figure 101) the action of VFTM-binding agonists. Such ligands are non-competitive and they are termed 'positive allosteric modulators' and 'negative allosteric modulators', respectively (see Section 4.15). These compounds do not need to activate the receptor by themselves. Instead, they may act by modulating the potency and/or efficacy of the VFTM-binding agonists. To become a positive allosteric modulator, the compound has to stabilize the VTFM–7TM domain interaction and, hence, the closed state of the VFTM. On the other hand, negative allosteric modulators may destabilize the VTFM–7TM domain interaction and/or prevent conformational changes within the 7TM domain that are necessary for receptor activation without affecting agonist binding to the VTFMs *per se* (Hulme *et al.*, 1999; Urwyler *et al.*, 2001).

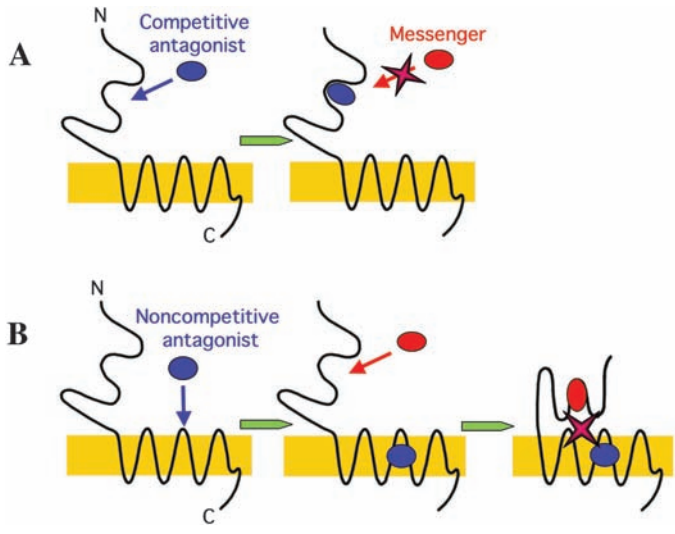


Figure 101 Family C receptor antagonists may (A) bind to the N-terminal domain of their receptors and so prevent the binding of agonists (competitive antagonism) or (B) bind within the central cleft formed by the 7TM domains (non-competitive antagonism). This will not necessarily affect agonist binding, but it will prevent conformational changes within the transmembrane region of the receptor that are necessary for its activation.

4.4 Receptor activation

The basal activity of a wild-type GPCR might vary from totally inactive (AT_{1A} receptor) to partially active, depending on the nature of the GPCR. Partially active receptors are referred to as 'constitutive active receptors' (constitutive activity being defined as agonist-independent activity). The β_2-adrenergic receptor is one of them; in transfected cell systems it triggers second messenger (cAMP) production in the absence of agonist.

The greatest insights into the molecular basis of GPCR activation have come from the analysis of mutant receptors. However, there are many issues to consider regarding its interpretation. Most useful are single or multiple point mutants. Such mutagenesis is generally characterized as either loss-of-function (i.e. decrease in binding affinity or functional response) mutants or gain-of-function mutants. All loss-of-function mutants are subject to problems of interpretation as they may result from the loss of critical interactions between receptor and agonist, from a mis-folded receptor structure or reduced receptor expression levels. Gain-of-function mutagenesis generally introduces or re-introduces binding and/or function of an 'inactive' receptor.

The functions of residues are most clearly categorized by alanine substitution mutations. These delete the side chain of the amino acid beyond the β-carbon,

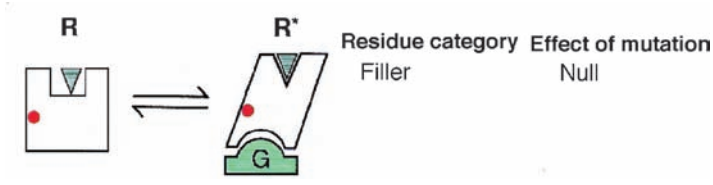


Figure 102 Filler residue mutations produce a null effect. Reprinted from *European Journal of Pharmacology*, **375**, Hulme, E. C., Lu, Z. L., Ward, S. D., Allman, K. and Curtis, C. A., The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm, 247–260. Copyright (1999), with permission from Elsevier.

leaving a small 'hole' in the receptor structure. With regard to receptor activation, such point-substitution mutation may have four basic outcomes (Figures 102 to 105):

- It may produce a null effect (Figure 102). Residues that tolerate multiple substitutions can be regarded as plugging functionally unimportant gaps in the receptor structure (i.e. filler residues).
- It may induce a simple reduction of the structural stability of the receptor (Figure 103). The mutation of such a stabilizer residue may reduce the expression level of the receptor by lessening its probability of folding successfully, and undergoing correct trafficking. However, it should not affect the signalling ability of those receptor molecules because it does not impair receptor activation.
- A mutation may increase the basal activity of the receptor (Figure 104). Such *constraining residues* are likely to contribute to maintain the receptor in its inactive state by forming intramolecular bonds. As for the mutant, these bonds are weakened or broken in the ligand-activated state of the receptor.

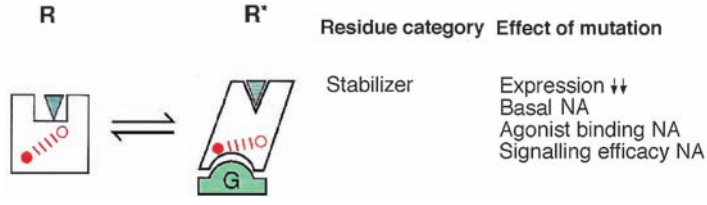


Figure 103 Stabilizer residue mutations tend to decrease receptor expression. Reprinted from *European Journal of Pharmacology*, **375**, Hulme, E. C., Lu, Z. L., Ward, S. D., Allman, K. and Curtis, C. A., The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm, 247–260. Copyright (1999), with permission from Elsevier.

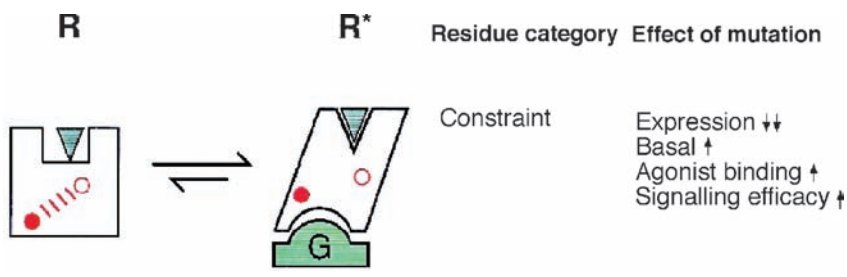


Figure 104 Constraining residue mutations produce constitutive receptor activity. Reprinted from *European Journal of Pharmacology*, **375**, Hulme, E. C., Lu, Z. L., Ward, S. D., Allman, K. and Curtis, C. A., The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm, 247–260. Copyright (1999), with permission from Elsevier.

- The mutation may reduce the signalling efficacy of the receptor (Figure 105). This indicates that the target residue makes interactions necessary for the activated conformation of the receptor. The interactions made by such an *activator residue* may either be intramolecular, or intermolecular (i.e. with the agonist or G protein).

There are major differences in the molecular mechanisms of activation between GPCRs: some receptors are easy to activate and many different single mutations activate them (adrenergic or TSH receptors), when others (AT_1 receptor) require more complex molecular changes. In general, it is believed that:

- In the ground state, the receptors are constrained in an inactive conformation by a network of intramolecular *constraining interactions*; and

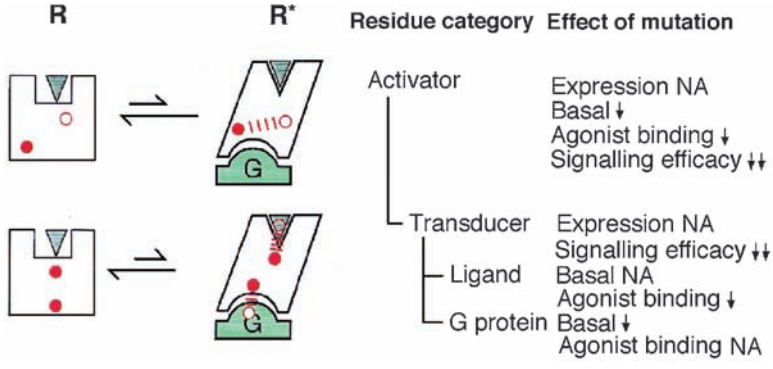


Figure 105 Activator residue mutations decrease signalling efficacy. Reprinted from *European Journal of Pharmacology*, **375**, Hulme, E. C., Lu, Z. L., Ward, S. D., Allman, K. and Curtis, C. A., The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm, 247–260. Copyright (1999), with permission from Elsevier.

- The presence of an agonist is responsible for the release of these inactivating constraints and the creation of a new network of *activating interactions* resulting in the formation and/or stabilization of the active state of the receptor.
- This activation process possibly occurs in a multistep sequence. In this respect, it has been proposed that small molecules should mainly act by destabilizing constraining interactions and that peptide ligands should mainly act by creating 'activator contacts'.

Compelling evidence for the existence of constraining intramolecular interactions that normally keep the ligand-free receptor inactive was obtained by mutation experiments involving the substitution of single amino acids. It was observed that certain substitutions might produce receptors that have higher basal activity as compared to the wild-type counterparts (these are termed 'constitutive active receptor mutants' or 'CAMs') (Figure 106). A dramatic example of this was provided by mutation of Ala^{293} ($Ala^{6.34}$)

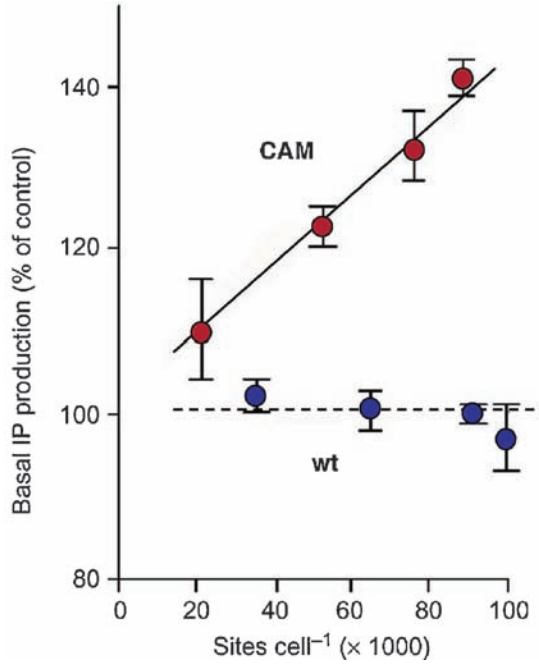


Figure 106 Basal inositol phosphate (IP) production in recombinant cells with wild-type (wt) AT_{1A} receptors and L305Q constitutively active mutant (CAM) receptors (production increases with receptor expression). Reprinted from *Proceedings of the National Acadademy of Science USA*, **97**, Parnot, C., Bardin, S., Miserey-Lenkei, S., Guedin, D., Corvol, P. and Clauser, E., Systematic identification of mutations that constitutively activate the angiotensin II type 1A receptor by screening a randomly mutated cDNA library with an original pharmacological bioassay, 7615–7620. Copyright (2000) National Academy of Sciences, USA.

in the C-terminal part of the third intracellular loop of the α_{1B} adrenergic receptor: any substitution by one of the 19 other amino acids resulted in full receptor activation (Kjelsberg *et al.*, 1992). This suggests that Ala^{293} is implicated in an intramolecular interaction (with Glu^{289} on TM6) that prevents the receptor from being active in the absence of agonist. The marked structural instability and enhanced conformational flexibility of constitutively activated β_2-adrenergic and histamine H_2-receptor mutants (compared to the wild-type receptors) add further support to the existence of stabilizing/constraining intramolecular interactions when a wild-type receptor is at rest.

Comparison of several CAMs in family A GPCRs suggests that the conserved D/ERY motif (at the cytoplasmic end of TM3) plays a major role in constraining their inactive conformation. Charge-neutralizing mutations, which mimic the protonated state of the aspartic acid/glutamic acid in this motif, cause dramatic constitutive activation of, for example, the α_{1B}- and the β_2-adrenergic receptors. It is therefore believed (i.e. the 'protonation hypothesis') that protonation of the aspartic acid/glutamic acid in this motif is at least one of the key events in the activation of this GPCR family.

Additional conformational constraints may also be operative for certain GPCRs (Figure 107). For example, random mutagenesis of the AT_{1A} receptor revealed that the substitution of several amino acids present on its TM regions are prone to produce CAMs and it is of notice that several of those amino acids are located on one side of TM3. Taken together, such mutation studies support the notion that TM3 and TM6 play a very general role in the conformational changes associated with GPCR activation. However, it should be mentioned that individual mutations that cause constitutive activation of one receptor type do not always do so when transferred to another. This indicates that the primary interhelical contacts have been tailored to suit the properties of individual receptors. Most CAMs are supposed to release the conformational constraints of the GPCR inactive state without creating new interactions. Such mutants help our understanding of the structure of the inactive state, but not about the structure of the ligand-induced active conformation.

The conformations of these CAMs are thus approximations of the real active conformation and this represents a severe limitation for the study of GPCR activation. In this respect, mutations mimicking activator interactions have been suggested to be better models of ligand-activated receptor conformations.

The occurrence of activator interactions is supported by the finding that certain GPCRs, like the AT_1 receptor, are difficult to activate constitutively by a single mutation. Most mutations only double the basal activity of the receptor, which is far below the maximal agonist stimulation. One possible explanation is that several constraining interactions need to be overcome to get full receptor activation. Another explanation is that the binding of an agonist generates a set of activator interactions, resulting in additional changes in the receptor conformation.

Still very little is known about the structural changes that go along with GPCR activation. The three-dimensional structure of a light-activated state of rhodopsin (metarhodopsin II) was recently obtained by X-ray crystallographic studies. However, it should be remembered that rhodopsin is unique among the GPCRs in that its ligand is covalently bound to the receptor and that, upon absorption of a photon, it isomerizes

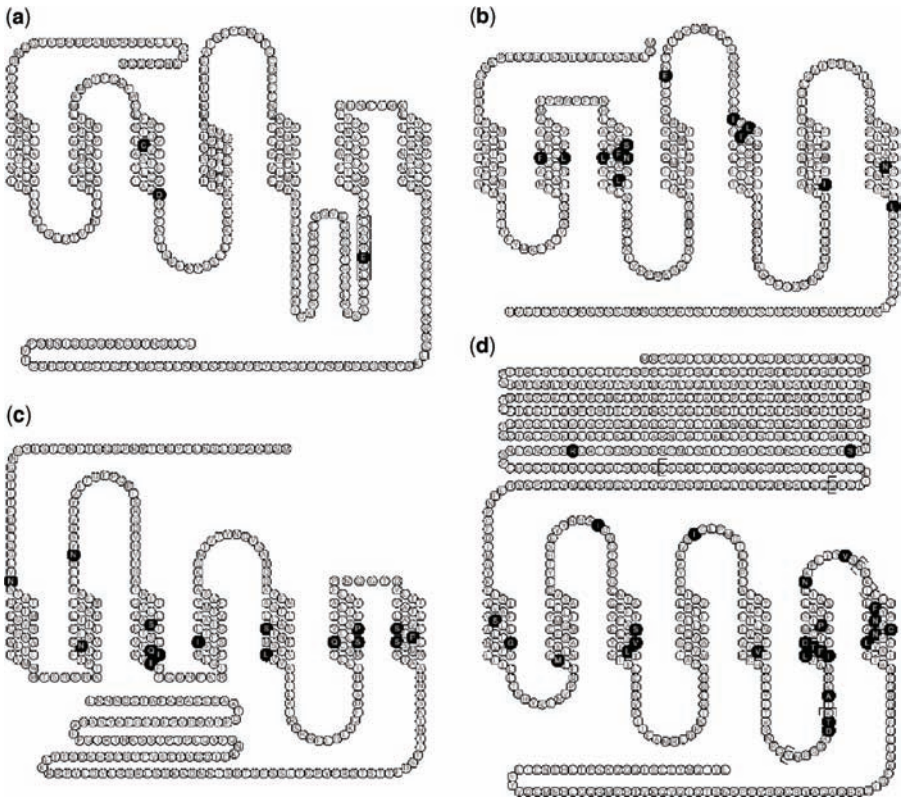


Figure 107 Amino acids (in black) whose mutations lead to strong constitutive activity in four representative GPCRs. (a) Human β_2-adrenergic receptor, (b) rat AT_{1A} receptor, (c) yeast α-factor receptor, (d) human TSH receptor. Reprinted from *Trends in Endocrinology and Metabolism*, **13**, Parnot, C., Miserey-Lenkei, S., Bardin, S., Corvol, P. and Clauser, E., Lessons from constitutively active mutants of G protein-coupled receptors, 336–343. Copyright (2002), with permission from Elsevier.

to an agonist within the binding pocket. Thus, the process of ligand binding is not an integrated part of the activation process. The three-dimensional structural determination of typical agonist-bound GPCRs has not yet been determined by X-ray crystallography, but mutation studies and biochemical and biophysical approaches (especially with β_2-adrenergic receptors and rhodopsin) have provided indirect information about the structural changes that go along with family A GPCR activation. Biochemical and spectroscopic approaches to study receptor activation include:

- The generation of artificial 'bridges' between two TM helices. These will prevent the separation and rotation of the involved TM domains and, hence, may prevent receptor activation, at least if such structural changes are required. In this respect, several cysteine–cysteine disulfide bridges have also been shown to prevent light activation of rhodopsin.

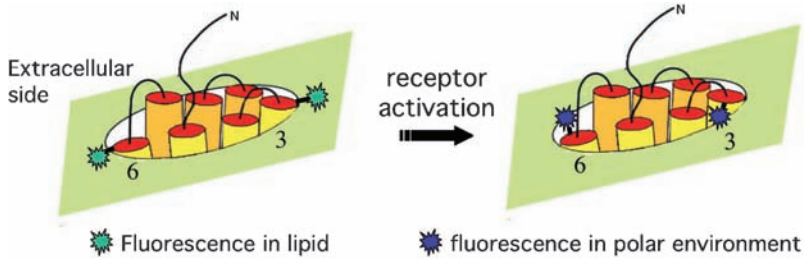


Figure 108 Orientation of the environment-sensitive fluorophores ^{125}Cys-NBD and ^{285}Cys-NBD at the extracellular side of the β_2-adrenergic receptor (top view). Left: inactive receptor, Right: receptor with rotated TM3 and TM6 in active conformation (according to Gether, 1998).

- Zinc-binding sites can be formed by introducing pairs of histidines in positions predicted to be in close proximity. Zinc binding to such engineered sites provides information about the proximity of the histidine residues and, hence, about the orientation of the involved TM domains. In this respect, a bridge joining TM3 and TM6 was found to prevent the activation of, for example, NK-1 receptors and rhodopsin.
- Spectroscopic approaches with wild-type or mutant receptors having single or a limited number of cysteines. This provides information about whether the environment of a side-chain is aqueous or hydrophobic and whether it is buried. They include:
  - Electron paramagnetic resonance spectroscopy with sulfhydryl-specific nitroxide spin labels (whose unpaired electrons can be probed spectroscopically). Such spin labels can indicate whether the environment of a side-chain is aqueous or hydrophobic and whether it is buried.
  - Covalent labelling of receptors with cysteine-reactive fluorescent probes whose fluorescence is highly sensitive to the polarity of their environment (Figure 108).

The current picture is as follows: TM3 is very highly tilted and longer than the other TM helices. Thus, TM3 makes multiple interhelical contacts and its movement is therefore ideally placed to propagate conformational changes induced by agonist binding through the transmembrane structure of the receptor. Studies with rhodopsin, β_2-adrenergic and muscarinic receptors suggest that TM3 and TM6 play critical roles for the transition of family A GPCRs to their fully activated state. This should involve a counter-clockwise rotation of these TM domains and an outward (away from the bundle) movement of their cytoplasmic ends (Figure 109). Other helices probably also adjust their positions upon activation. As a result, the helical bundle is thought to blossom open at its cytoplasmic end. This enables the G proteins to interact with previously inaccessible receptor residues located within the endo2 and endo3 loops and the C-terminal tail.

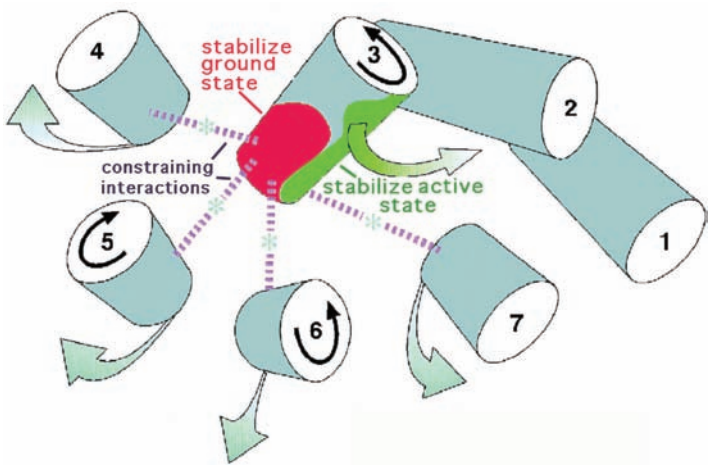


Figure 109 Model for muscarinic receptor activation. Reprinted from *European Journal of Pharmacology*, **375**, Hulme, E. C., Lu, Z. L., Ward, S. D., Allman, K. and Curtis, C. A., The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm, 247-260. Copyright (1999), with permission from Elsevier.

Based on the model of rhodopsin (family A GPCR), the three-dimensional structure of the GLP1 receptor (family B GPCR) in the absence or presence of its endogenous hormone GLP1 has recently been proposed. These molecular modelling studies suggest that both receptors may undergo the same kind of structural changes upon activation.

4.5 Activated GPCRs: interaction with G proteins

Recently, X-ray crystallography has provided substantial insight into the tertiary structure of the heterotrimeric G proteins. These studies reveal that the α subunits consist of a ras-like GTPase domain (six-stranded β sheets surrounded by six α helices) and a helical domain. The bound guanine nucleotide is deeply buried between these two domains. Whereas α and β subunits make extensive contacts, there appear to be no direct interactions between α and γ subunits (Figure 110).

The three-dimensional structure of a light-activated state of rhodopsin was recently obtained and modelling studies reveal that transducin must alter its conformation to bind to the activated receptor. Rather than a 'lock' and 'key' fit, interactions between receptors and G proteins should include an 'induced fit' (analogous to that proposed by Koshland to describe enzyme–substrate interactions) at the heart of the signal transduction mechanism (Yeagle and Albert, 2003).

Pending the resolution of the atomic structure of a receptor–G protein complex, still little is known about the actual points of interactions between the receptor and the G protein and, thus, how the two proteins are oriented relative to one another. This would

help us to understand how hundreds of different GPCRs with remarkably diverse amino acid sequences can all interact with a similar subset of G proteins. Presently, the model for GPCR–G protein interaction includes the following elements:

- GPCR activation enables the α subunit of a G protein (further denoted as Gα subunit) to interact with previously inaccessible receptor residues located within the endo2 and endo3 loops and the C-terminal tail.
- β–γ complexes increase the affinity of Gα for activated GPCRs but it is not clear whether the β–γ complexes also make specific contacts with the receptor proteins.
- GPCR binding induces changes in the Gα conformation. This will lead to a weakening of GDP binding and eventually GDP release. Because it is unlikely that the nucleotide can be contacted directly by the receptor protein (Figure 110), GPCRs are thought to trigger GDP release by an allosteric mechanism.

When activated by appropriate agonists, most GPCRs preferentially activate a limited set of G proteins (Table 10). Mutation studies show that multiple intracellular receptor regions act in a co-operative fashion to get correct G protein recognition and efficient G protein activation (Table 11). They are (Wess, 1998):

- The Asp/Glu-Arg-Tyr triplet at the N-terminal region of endo 2.
- Residues in the N- and C-terminal regions of endo 3.
- A few residues in the N-terminal region of the cytoplasmic C-terminal tail (including the highly conserved NPXXY sequence of family A GPCRs), but deletion mutagenesis studies have shown that most of the tail itself is not required for efficient G protein coupling.

Differences in G protein recognition pattern among two receptors can be attributed to subtle differences in those intracellular regions as well as in their co-operative behavior. Hence, the selectivity of G protein recognition may vary among different classes of GPCRs and even among structurally closely related members of the same receptor subfamily (adrenergic receptors represent a nice example of this) (Figure 111).

A large body of evidence indicates that certain amino acid residues in the C-terminal portions of Gα can directly contact the receptor protein and play a key role in dictating the specificity of coupling (Table 12). Since this region is fairly similar for Gα components of the same G protein family, receptors will often be capable of stimulating all the members of this family. Hence, based on their G protein-coupling preference, GPCRs can be broadly subclassified into $G_{i/o}$-, G_s- and $G_{q/11}$-coupled receptors. In this respect, it is of notice that replacing the last five amino acids of $G\alpha_q$ with the corresponding $G\alpha_i$ or $G\alpha_o$ sequences allows traditional $G_{i/o}$-coupled receptors to stimulate PLC-β (a typical $G_{q/11}$-mediated response).

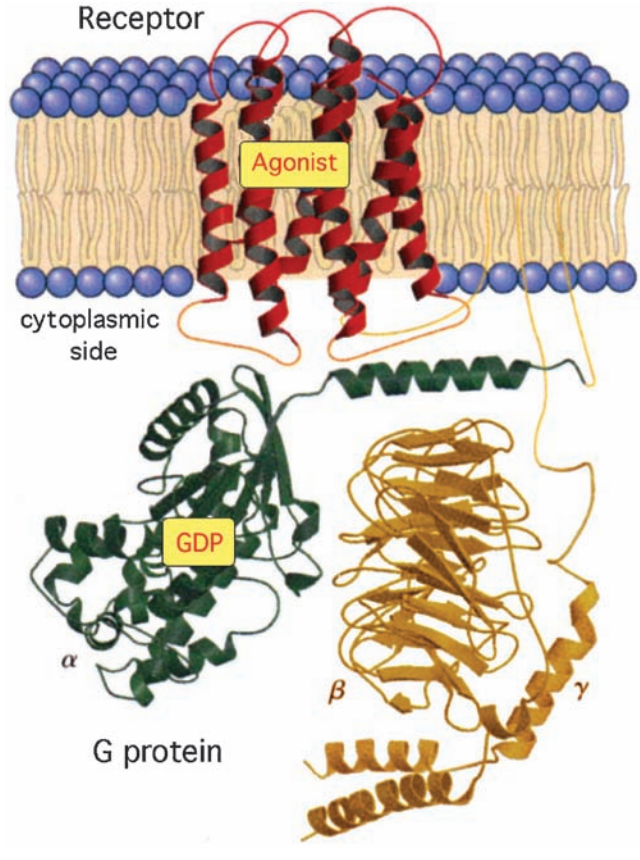


Figure 110 Proposed binding of a G protein heterotrimer to an activated GPCR. *Principles of Biochemistry* by Albert L. Lehninger, *et al.* © 2000, 1993, 1982 by Worth Publishers. Used with permission.

Studies performed with recombinant cell systems have clearly established that, although most GPCRs are preferentially coupled to a certain subfamily of G proteins, they can also activate other classes of G proteins, but with reduced efficiency. The first evidence for such multiplicity of G protein coupling arose from studies indicating that, in addition to inhibiting adenylate cyclase, some $G_{i/o}$-coupled receptors mediate inositol phosphate production through a pertussis toxin-insensitive pathway. In some cases, a single receptor was found to simultaneously activate members of three or even four unrelated classes of G proteins (Table 13). Many factors may affect the receptor–G protein interaction (Figure 112).

In general, the nature of the observed response not only depends on which G protein is preferentially recognized by the receptor, but also by which ones are present in the studied cell or tissue. If more than one type of G protein is activated in a single cell,

Table 10 Preferred GPCR–G protein interactions. Reprinted from *Pharmacology and Therapeutics*, **80**, Wess, J., Molecular basis of receptor/G protein-coupling selectivity, 231–264. Copyright (1998), with permission from Elsevier.

Peptides		Biogenic amines	
Angiotensin II ($AT_{1a, b}$)	Gq/11	Muscarinic receptors: m1, m3, m5)	Gq/11
Angiotensin II (AT_2)	Gi/o	muscarinic receptors: (m2, m4)	Gi/o
Bombesin (BB1–3)	Gq/11	Dopamine (D1, D5)	Gs
Bradykinin (B_1, B_2)	Gq/11	Dopamine (D2, D3, D4)	Gi/o
C5a Anaphylatoxin	Gq/11	adrenoceptors: (α_{1a}, α_{1b}, α_{1d})	Gq/11
Cannabinoids (CB1, CB2)	Gi/o	adrenoceptors: (α_{2a}, α_{2b}, α_{2c})	Gi/o
Chemokines (CCR1–CCR5)	Gi/o	adrenoceptors: (β_1, β_2, β_3)	Gs
CCK/Gastrin (CCK_A, CCK_B)	Gq/11	Histamine: H_1	Gq/11
ET (ET_A, ET_B)	Gq/11	Histamine: H_2	Gs
Galanin	Gi/o	5-HT ($5\text{-}HT_{1a–f}$)	Gi/o
Gonadotropin-releasing horm.	Gq/11	5-HT ($5\text{-}HT_{2a–c}$)	Gq/11
Melatonin ($MEL_{1A, 1B}$)	Gi/o	5-HT ($5\text{-}HT_{4, 6, 7}$)	Gs
Melanocortins (MC1–5)	Gs		
Neuropeptide Y ($Y_{1–5}$)	Gi/o		
Neurotensin	Gq/11		
Oxytocin	Gq/11		
Opioid peptides (μ, κ, δ)	Gi/o		
Somatostatin (SSTR1–5)	Gi/o		
Tachykinins (NK1–3)	Gq/11		
Thyrotropin-releasing hormone	Gq/11		
Vasopressin (V_{1a}, V_{1b})	Gq/11		
Vasopressin (V2)	Gs		

the magnitude of the different responses (and therefore the ratio of their magnitude) will be affected by the concentrations of these G proteins, their effectors, and the further downstream signalling components. In this respect, multiplicity in G protein coupling is frequently observed in artificial expression systems. However, because

Table 11 Mutagenesis of α_2-adrenergic receptors differently affect adenylate cyclase (AC) stimulation (via G_s) and inhibition (via $G_{i/o}$). (I = intracellular loop).

Region	Mutation	AC inhibition	AC stimulation
I2	substitution	+	+
I3 N-terminus	deletion	no effect	+
I3 C-terminus	substitution basic residues	+	no effect

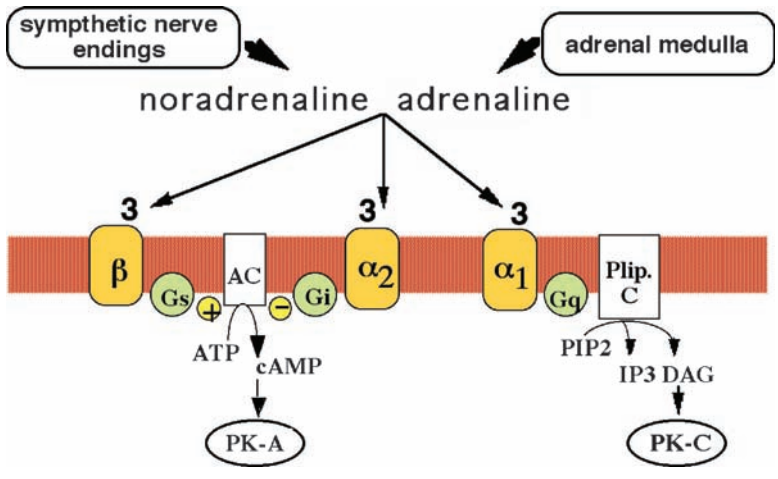


Figure 111 G protein-coupling preference of α_1-, α_2- and β-adrenergic receptors.

both receptors and G proteins are usually expressed at rather high levels, it has been argued that receptor/G protein combinations in these co-expression experiments are not necessarily of physiological relevance. This raises the question of whether such complex signalling reveals artefactual promiscuous coupling or whether it is a genuine property of GPCRs.

Table 12 C-terminal portions of Gα subunits. Reprinted from *Pharmacology and Therapeutics*, **80**, Wess, J., Molecular basis of receptor/G protein-coupling selectivity, 231–264. Copyright (1998), with permission from Elsevier.

		− 7	− 6	− 5	− 4	− 3	− 2	− 1
α_s	–	L	R	Q	Y	E	L	L
α_{olf}	–	L	K	Q	Y	E	L	L
$\alpha_{q,\ 11}$	–	L	K	E	Y	N	L	V
α_{14}	–	L	R	E	F	N	L	V
$\alpha_{15,\ 16}$	–	L	D	E	I	N	L	L
$\alpha_{i1,\ 2}$	–	L	K	D	C	G	L	F
α_{i3}	–	L	K	E	C	G	L	Y
$\alpha_{o1,\ 2}$	–	L	R	G	C	G	L	Y
$\alpha_{t1,\ 2}$	–	L	K	D	C	G	L	F
α_z	–	L	K	Y	I	G	L	C
α_{gust}	–	L	K	D	C	G	L	F
α_{12}	–	L	K	D	I	M	L	Q
α_{13}	–	L	K	Q	L	M	L	Q

Table 13 Typical examples of receptors showing multiplicity in G protein coupling. Reprinted from *Pharmacology and Therapeutics*, **99**, Hermans, E., Biochemical and pharmacological control of the multiplicity of coupling at G protein-coupled receptors, 25–44, © (2003), with permission from Elsevier.

Receptor	G_s	G_i	$G_{q/11}$	G_{12}
Adenosine (A_3)		×	×	
(α_2-Adrenergic	×	×		
β-Adrenergic	×	×		
Corticotropin- releasing hormone	×	×	×	
Dopaminc (D_1)	×	×		
Metabotropic glutamate (la)	×	×	×	
Endothelin (ET_B)		×	×	
Galanin		×	×	×
Glucagon	×	×		
Gonadotrophin releasing hormone	×	×	×	
Histamine H_2	×		×	
Luteinizing hormone	×	×	×	
Melatonin		×	×	
Muscarinic (ml and m3)		×	×	
Neurotensin		×	×	
Pancreastin		×	×	
Parathyroid hormone	×		×	
Platelet-activating factor		×	×	
Prostacyclin	×	×	×	
Prostaglandin (EP3D)	×	×		
Serotonin (5-HT_{2C})		×	×	
Sphingosine 1-phosphate (Edg3)	×		×	×
Substance P			×	×
Thyrotropin	×	×	×	×
Thrombin		×	×	×
Vasopressin V1a		×	×	
Vasoactive intestinal peptide	×	×		

Considerations in favour of artefactual coupling:

- Whereas several tachykinins are able to stimulate cAMP as well as IP_3 production in CHO cells expressing a recombinant NK-1 receptor, only the latter response is observed in cells that express the endogenous receptor (Torrens *et al.*, 2000).
- Distinct coupling properties of the thyrotropin receptor have been observed when comparing intact cells with cell membranes (Allgeier *et al.*, 1997) and this suggests that disruption of the cellular organisation may favour the promiscuous coupling of GPCRs. In this respect, it is worth mentioning that the signalling specificity at the G protein level is often studied on cell homogenates or membrane preparations.

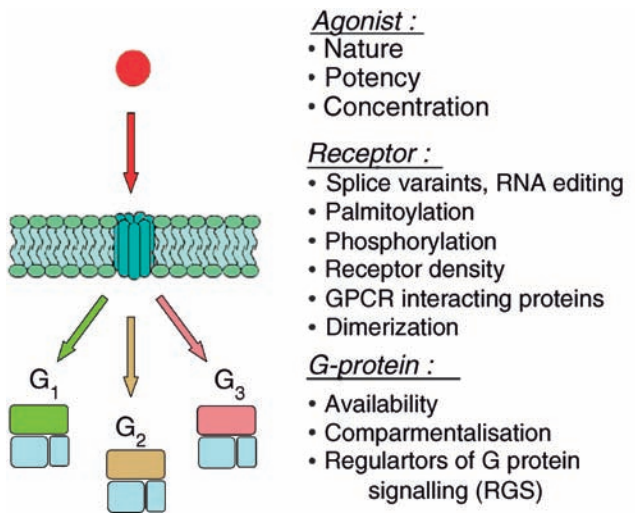


Figure 112 The coupling of GPCRs with multiple G proteins is selectively regulated at different levels. Reprinted from *Pharmacology and Therapeutics*, **99**, Hermans, E., Biochemical and pharmacological control of the multiplicity of coupling at G protein-coupled receptors, 25–44, © (2003), with permission from Elsevier.

Considerations in favour of a genuine property:

- Some GPCRs also interact with multiple G proteins in non-transfected cells. For example, activation of the thyrotropin receptor in dog thyroid membranes led to increased incorporation of [α^{32}P]GTP azidoanilide into $G\alpha_s$, $G\alpha_q$ and $G\alpha_i$. In addition, pretreatment of intact thyrocytes with pertussis toxin allows TSH to shift from ($G_{i/o}$-mediated) adenylate cyclase inhibition to (G_s-mediated) adenylate cyclase stimulation.
- Similarly, endogenously expressed α_{2B}-adrenergic receptors have been found to interact with $G\alpha_q$ in addition to $G\alpha_i$ in neuroblastoma × glioma cells (Holmberg *et al.*, 1998).

Techniques to study G protein-coupling preference

Several experimental strategies have been employed to analyze the selectivity of receptor–G protein interactions:

- Transient co-expression of individual GPCRs with different $G\alpha$ subunits in cultured mammalian cells.
- Reconstitution of purified receptors and G protein subunits in artificial lipid bilayers. This represents a very difficult and laborious task. Moreover, receptor–G protein interactions are studied in a highly artificial environment.

- 'Immuno-neutralization' studies have been performed with membrane preparations, as well as intact (following microinjection of the antibody) and permeabilized cells. A limitation of the use of such C-terminal Gα antibodies is their inability to discriminate between certain pairs of Gα subunits.
- Receptor–G protein interactions can also be studied via co-immunoprecipitation of receptor–G protein complexes using specific receptor or G protein-recognizing antibodies.
- Incorporation/expression of antisense oligonucleotides complementary to the mRNA for the Gα subunit of interest in cultured cells and transgenic animals. Based on the rules of complementary Watson–Crick base-pairing, an antisense oligonucleotide should bind to the mRNA in question, thereby selectively inhibiting the expression of the Gα subunit. Proper interpretation of such experiments requires demonstration that the expression of the Gα subunit of interest is truly abolished (or reduced) and that the antisense oligonucleotides do not exert non-specific effects on the expression and function of other Gα subunits.
- Traditionally, G protein-mediated responses have been classified into pertussis toxin-sensitive and pertussis toxin-insensitive responses, because of the ability of this bacterial toxin to selectively inactivate G proteins of the $G_{i/o}$ family (Figure 113). This property is widely exploited to check for the involvement of G_i and G_o in cellular responses. At the molecular level, pertussis toxin can catalyze the covalent binding of the ADP-ribose moiety of NAD to a conserved cysteine residue located near the C-terminus (position 4) of $G\alpha_i$ and $G\alpha_o$ (Figure 113). This will only occur when the G proteins are in the inactive, trimeric state. The ADP-ribosylated $G\alpha_{i/o}$ subunits can no longer interact with receptors and, hence, they can no longer become activated. To determine whether a given GPCR can discriminate between individual $G\alpha_{i/o}$ subunits, cultured cells can be transfected with mutant $G\alpha_{i/o}$ subunits lacking the pertussis toxin-sensitive cysteine residues and then treated with pertussis toxin

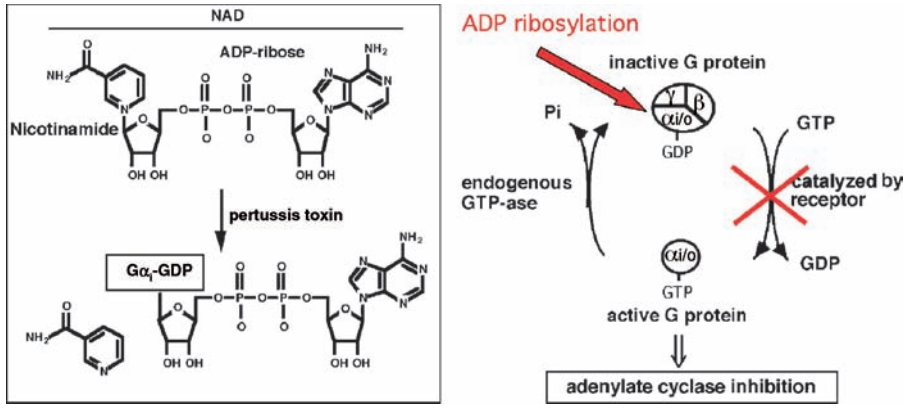


Figure 113 Pertussis toxin-mediated ADP ribosylation of the trimeric $G\alpha_{i/o}$-GDP.β.γ complex.

to inactivate endogenous $G\alpha_{i/o}$ subunits. Only the transfected $G\alpha_{i/o}$ subunits will remain active. However, as the pertussis-toxin-sensitive cysteine is located within a key contact region for GPCRs, the detailed pharmacology of the GPCR response could potentially be altered by this mutation.

In addition, it is not even necessary to monitor receptor–G protein interaction by measuring a response at the level of the concerned effector component (e.g. adenylate cyclase stimulation in the case of G_s) or even downstream to it. Indeed, techniques have been developed to measure the receptor–Gα coupling and the activation of Gα subunits directly:

- Some (but not all) GPCRs acquire high agonist affinity upon coupling to a G protein. In radioligand binding studies on membranes, this results in the appearance of high-affinity sites for radiolabelled agonists and of shallow agonist/radiolabelled antagonist binding curves.
- Two binding assays on membrane preparations are based on the ability of the activated receptor to promote the exchange of tightly bound GDP by GTP. The same exchange takes place when GTP is replaced by radioactively labelled analogs like [^{35}S]GTPγS or [α^{32}P]GTP azidoanilide (Figures 114 and 115). Accordingly, activation of Gα by a receptor will result in an increased binding rate of these GTP analogs. Since [^{35}S]GTPγS is resistant to the GTP-ase activity of Gα, its binding will be essentially irreversible. Upon photoactivation, [α^{32}P]GTP azidoanilide is even capable of covalently tagging receptor-activated Gα subunits. It should be

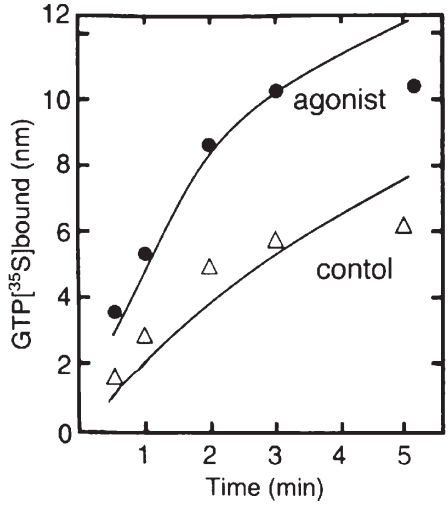


Figure 114 Time course of [^{35}S]GTPγS binding to bovine brain in absence or presence of adenosine A1 receptor agonist. Reproduced with permission, from Freissmuth, M., Selzer, E., Schutz, W., 1991, *Biochemical Journal*, **275**, 651–656. © The Biochemical Society.

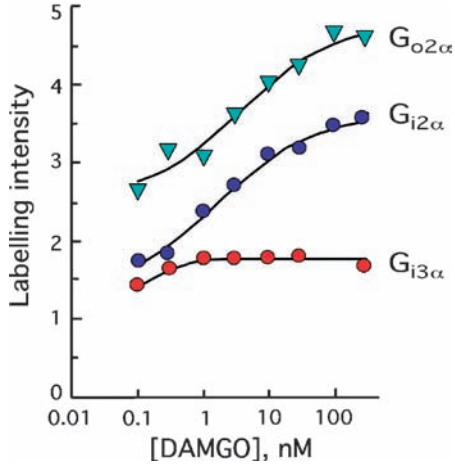


Figure 115 In CHO cells stably expressing the μ-opioid receptor, DAMGO (= agonist) increases the binding of α-azidianiolino[^{32}P]GTP to multiple G_α subunits. Reprinted from *Journal of Neurochemistry*, **64**, Chakrabarti, S., Prather, P. L., Yu, L., Law, P. Y. and Loh, H. H., Expression of the mu-opioid receptor in CHO cells: ability of mu-opioid ligands to promote alpha-azidoanilido[32P]GTP labeling of multiple G protein alpha subunits., 2534–2543. Copyright (1995) Blackwell Publishing.

noted that an agonist-stimulated receptor does not increase the maximal binding of these GTP analogs, but that they only enhance their association rate.

In practice, the [^{35}S]GTPγS binding assay cannot be used to evaluate receptor-driven G protein activation in intact cells because [^{35}S]GTPγS is unable to cross cell membranes. However, the [^{35}S]GTPγS assay has been used successfully in digitonin-permeabilized cells. Digitonin binds to cholesterol in eukaryotic plasma membranes, creating pores that are permeable to ions and proteins. [^{35}S]GTPγS binding has also been often restricted to the pertussis toxin-sensitive $G_{i/o}$ family. When compared to the other G proteins, these are usually expressed at higher levels and (together with unrelated cell components like tubulin) they provide a substantial contribution to [^{35}S]GTPγS binding under basal conditions (Milligan, 2003). However, as illustrated in Figure 116, a signal can be easily measured even with a small (e.g. fourfold) stimulation of [^{35}S]GTPγS binding to $G\alpha_{i/o}$ (green). By contrast, considerable (e.g. 20-fold) stimulation of [^{35}S]GTPγS binding to other Gα subunits might be expected to result in only a small signal above basal levels. Recent strategies to overcome these limitations consist in:

- Expression of GPCRs and G proteins of interest in various insect cell lines that express low levels of endogenous G proteins.
- Standard [^{35}S]GTPγS binding assays performed on membrane preparations, coupled with a selective immunocapture step to isolate the G protein(s) of interest.

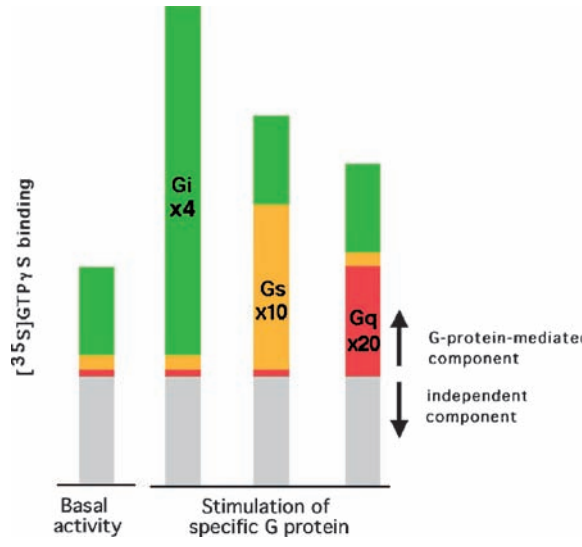


Figure 116 Effect of selective G protein stimulation (Gi: fourfold, Gs: 10-fold, Gq: 20-fold) on total [^{32}P]GTPγS binding to cell membranes. Binding under unstimulated (basal) conditions to Gi≫Gs>Gq.

A complex picture may arise when a GPCR is able to recognize two G proteins that mediate opposite effects such as adenylate cyclase stimulation by $G\alpha_s$ and adenylate cyclase inhibition by $G\alpha_i$. Such a situation arises for, for example, α_2-adrenergic receptors. As shown in Figure 117, the full agonist UK-14,304 inhibits the cAMP production in intact CHO cells expressing α_2-adrenergic receptors at high (α_{2A}-H) as well as low (α_{2A}-L) concentration. This process is $G_{i/o}$-mediated and when this is prevented (pretreatment of the cells with pertussis toxin), UK-14,304 starts to stimulate cAMP production (i.e. a G_s-mediated process). In fact this process is not very efficient, as demonstrated by the very low response in α_{2A}-L cells. A much higher response is seen in α_{2A}-H cells because of the operating 'receptor reserve'. This suggests that the α_2-adrenergic receptor can couple to both G_s and G_i under natural conditions but that coupling to the 'traditional' G_i prevails. In this respect, data with mutated α_2-adrenergic receptors suggest that quite different intracellular receptor regions are implicated in the recognition of $G\alpha_i$ and $G\alpha_s$ (Table 11).

Divergence of intracellular signalling

GPCR coupling to multiple G proteins is only one of the potential mechanisms accounting for the divergence of intracellular signalling in response to a single agonist. Taken together, these mechanisms (Figure 118) include:

- (A), agonist binding to different receptor subtypes, showing distinct G protein coupling specificities. Many monoamine-recognizing receptor subtypes have been

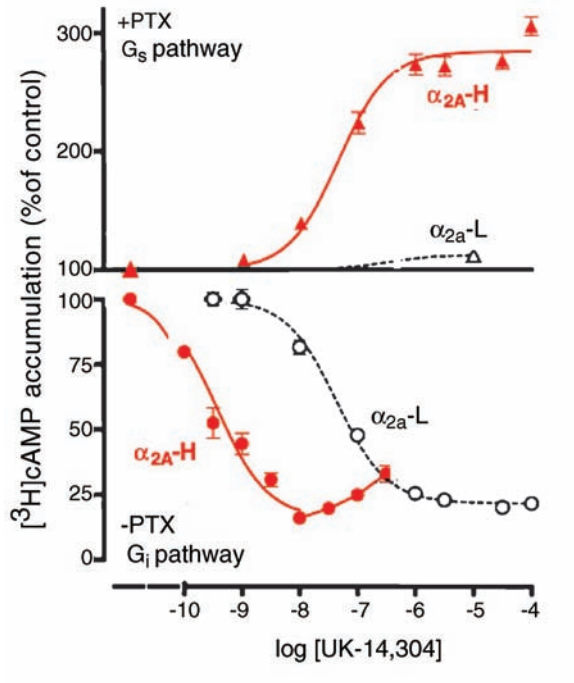


Figure 117 Effect of pertussis toxin (PTX) on UK-14,304-mediated stimulation of porcine α_2-adrenergic receptors stably expressed in CHO cells at low (α_2 -L) and high (α_2 –H) densities. Reproduced from Brink, C. B., Wade, S. M. and Neubig, R. R. (2000) *Journal of Pharmacology and Experimental Therapeutics*, **294**, 539–547, with permission from the American Society for Pharmacology and Experimental Theraputics.

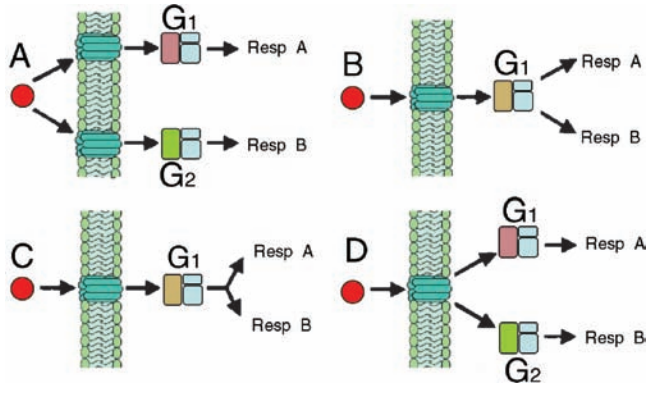


Figure 118 Different mechanisms accounting for the divergence of intracellular signalling in response to a single agonist. Reprinted from *Pharmacology and Therapeutics*, **99**, Hermans, E., Biochemical and pharmacological control of the multiplicity of coupling at G protein-coupled receptors, 25–44, © (2003), with permission from Elsevier.

identified showing distinct G protein coupling specificities (e.g., 12 mammalian serotonin receptors, nine adrenergic receptors, etc.).

- (B), agonist binding to a single receptor, triggering the direct activation of distinct intracellular effectors through a single G protein. For example, once activated, both α and β–γ subunits contribute to the modulation (in a synergistic or antagonistic fashion) of either same or unrelated effectors, resulting in dual intracellular signalling. The best-characterized example of such dual signalling through a single G protein is the $G_{i/o}$-mediated inhibition of adenylate cyclase (depending on the α subunit) and the stimulation of particular phospholipase C isoforms (depending on the β–γ subunits). It is noteworthy that in this case β–γ-related signals are frequently observed at higher agonist concentrations, probably reflecting the lower potency of these subunits in activating the effectors.
- (C), agonist binding to a single receptor that shows selectivity for a single intracellular effector through a single G protein, but divergence occurs at downstream levels in the signalling cascade.
- (D), agonist binding to a single receptor that mediates distinct signalling through direct interaction with multiple G proteins.

4.6 Activated GPCRs: phosphorylation and internalization

Receptor phosphorylation

Free β–γ subunits are only found in the plasma membrane at sites of receptor activation. They are able to recognize the C-terminus of 'G protein-receptor kinases' GRK2 and GRK3. This allows the rapid translocation of these kinases from the cytosol to the plasma membrane and provides an extremely precise mechanism for targeting GRK2 and GRK3 to activated GPCRs.

GRK2 and GRK3 were initially termed 'β-adrenergic-receptor kinases' βARK1 and βARK2. Other members of the GRK family have now also been discovered (Figure 119). The family comprises seven family members that share significant sequence homology. Each of the GRKs shares a similar functional organization with a central catalytic domain, an amino-terminal domain that is thought to be important for substrate recognition, and a carboxyl-terminal domain that contributes to the plasma membrane targeting of the kinase. Only GRK2 and GRK3 are attracted by free β–γ subunits. Other members may be palmitoylated on carboxyl-terminal cysteine residues (i.e. GRK4 and GRK6) and exhibit substantial membrane localization even in the absence of GPCR activation by agonist. Many factors have been found to regulate GRK activity (Table 14, Figure 120)

GRKs preferentially phosphorylate receptors that are in the agonist-occupied conformation. This phosphorylation occurs at both serine and threonine residues localized within either the endo3 loop or carboxyl-terminal tail domains (Figure 121). No distinct GRK phosphorylation consensus motifs have been identified, but localization of acidic amino acid residues proximal to the site of phosphorylation seems to

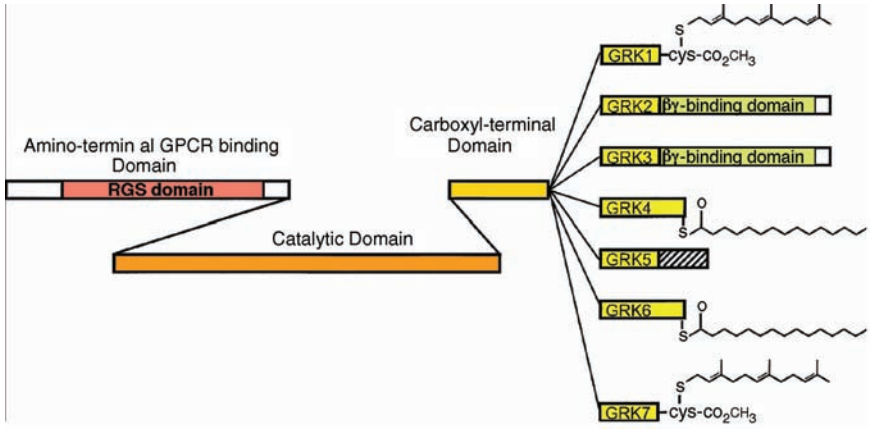


Figure 119 Functional domains of the different GRK family members. Reproduced from Ferguson, S. S. G. (2001), *Pharmacological Reviews*, **53**, 1–24, with permission from the American Society for Pharmacology and Experimental Theraputics.

favour GRK2-mediated phosphorylation. Furthermore, GRK phosphorylation alone has little effect on receptor–G protein coupling. However, it stabilizes a conformational state required to promote the interaction of GPCRs with β-*arrestins* (β-arrestin 1 or β-arrestin 2) (Figure 122). In other words, GRK phosphorylation will increase the

Table 14 GRK activity seems to be regulated by inositol phosphate binding as well as by a complex series of protein phosphorylation events. Reproduced from Ferguson, S. S. G. (2001), *Pharmacological Reviews*, **53**, 1–24, with permission from the American Society for Pharmacology and Experimental Theraputics.

Characteristics of GRK family members

Family Name	Size (kDa)	Covalent Modification	Activators	Inactivators
GRK1 (rhodopsin kinase)	63	Farnesylation	Polycations	Recoverin
GRK2 (βARKl)	79	N.D.	Gβγ, PIP_2, PKC, c-Src	MAPK
GRK3 (βARK2)	80	N.D.	Gβγ, PIP_2	N.D
GRK4	66	Palmitoylation	N.D.	N.D
GRK5	68	N.D	Polycations, PIP_2	PKC, calmodulin
GRK6	66	Palmitoylation	Polycations	N.D.
GRK7	62	N.D. (Farnesylation?)	N.D.	N.D.

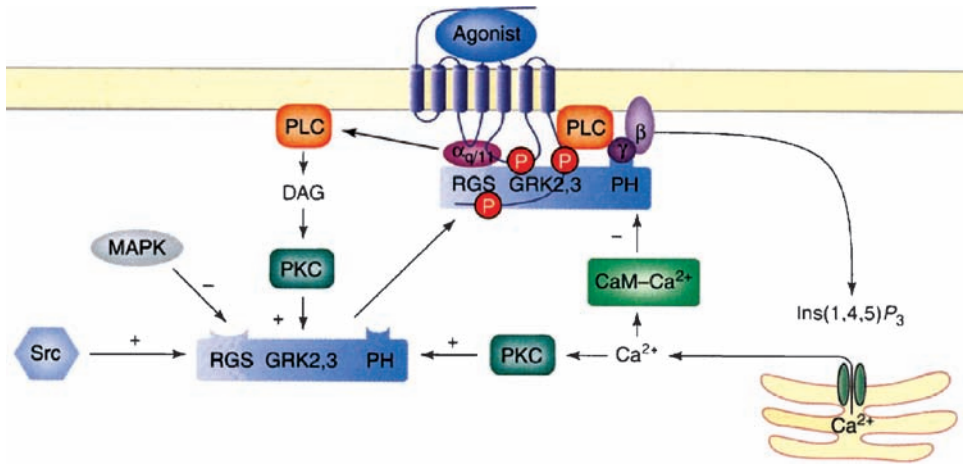


Figure 120 Complex regulation of GRK2/3 activity. Reprinted from *Trends in Pharmacological Science*, **24**, Willets, J. M., Challiss, R. A. and Nahorski, S. R., Non-visual GRKs: are we seeing the whole picture?, 626–633. Copyright (2003), with permission from Elsevier.

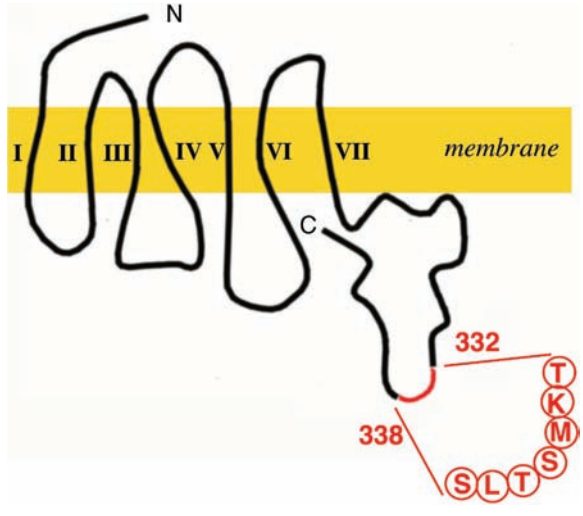


Figure 121 The central terminus of the AT_1 receptor (with residues T^{332}-S^{338}) is the major site for Ang II-mediated phosphorylation and is the binding site for β-arrestin. Reprinted from *Trends in Endocrinology and Metabolism*, **14**, Thomas, W. G. and Qian, H., Arresting angiotensin type 1 receptors, 130–136. Copyright (2003), with permission from Elsevier.

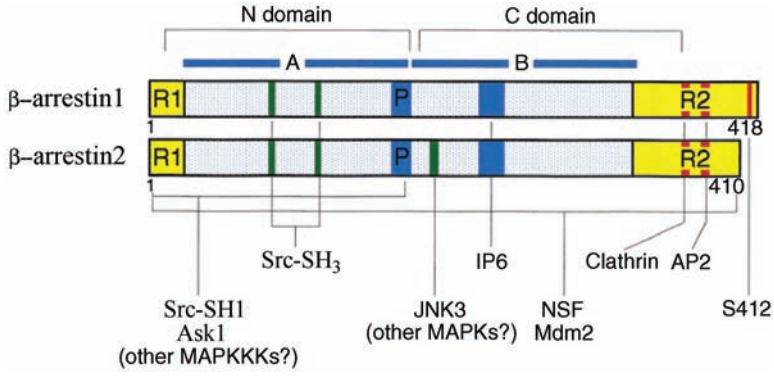


Figure 122 Putative domain architecture of the β-arrestins. Reproduced by permission of the Company of Biologists, *Journal of Cell Science*, **115**, Luttrell, L. M. and Lefkowitz, R. J., The role of beta-arrestins in the termination and transduction of G protein-coupled receptor signals, Fig. 2, 455–465, Copyright (2002).

affinity of the receptor for β-arrestins (e.g. 10-fold increase in affinity for the GRK2-phosphorylated β_2-adrenergic receptor). β-Arrestin binding will only be substantial after GRK phosphorylation for most of the GPCRs. However, depending on the β-arrestin concentration and its binding affinity for the GPCR of interest, substantial β-arrestin binding to the agonist-activated receptor may occur even in the absence of GRK-mediated phosphorylation. Alternatively, certain receptors (e.g. the AT_2 angiotensin receptor and β_3-adrenergic receptor) do not serve as substrates for GRK and do not bind β-arrestins.

β-Arrestin binding sterically precludes coupling between the receptor and heterotrimeric G proteins. This leads to the termination of the G protein-mediated signalling (Figure 123). The receptor becomes '*desensitized*' (i.e. it can no longer relay an external

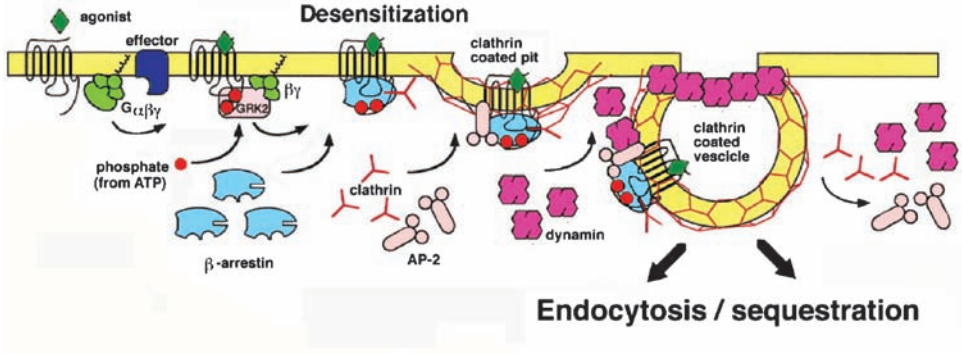


Figure 123 Molecular mechanism of (homologous) GPCR regulation: desensitization and internalization. Reproduced by permission of the Company of Biologists, *Journal of Cell Science*, **115**, Luttrell, L. M. and Lefkowitz, R. J., The role of beta-arrestins in the termination and transduction of G protein-coupled receptor signals, Fig. 1, 455–465, Copyright (2002).

signal). In this respect, it was noted in several cases that mutant, C-terminally truncated receptors couple to G proteins with improved efficacy. A possible explanation for this hypersensitivity is due to the removal of threonine and/or serine residues, which are involved in mediating GPCR desensitization by serving as substrates for GRKs.

Finally, it should be noted that the nature of the cellular host in recombinant systems strongly influences the ability of the transfected receptors to desensitize. This has been well documented for the β_3-adrenergic receptor (which does not serve as substrate for GRK and does not bind β-arrestins):

- The human β_3-adrenergic receptor desensitizes to isoproterenol in SK-N-MC and HEK293 cells, but not in CHO cells.
- The rat β_3-adrenergic receptor desensitizes in HEK293 cells, but not in rat adipocytes.

Interestingly, β-arrestin binding does not necessarily represent 'the end' of a GPCR. Instead, it may even endow the receptor with a 'new life'. This is because β-arrestins have the ability to bind to several proteins, which play a role either in endocytosis or in signalling (Figure 122). Regions involved in receptor or membrane recognition are shown in blue, those involved in controlling β-arrestin interaction with the endocytotic machinery are shown in red, while proposed interactions between β-arrestins and signalling proteins are shown in green:

- The A functional domain is responsible for recognition of activated GPCRs and the B domain is responsible for secondary receptor recognition. Both domains are separated by a phosphate sensor domain (P).
- The regulatory R2 domain contains the primary site of phosphorylation as well as the LIEF binding motif for *clathrin* and the RXR binding motif for the β2-adaptin subunit of the heterotetrameric *AP-2 adaptor complex*. (This complex is involved in the initiation of clathrin-coated pit formation.)
- Binding to the *c-Src*-SH3 domain occurs to one or more PXXP motifs within the A domain of β-arrestin 1.
- Binding to *MAP kinases* occurs to a recognition sequence, RRSLHL, within the B domain of β-arrestin 2.

β-*Arrestin binding-mediated GPCR endocytosis*

β-Arrestins act as endocytotic adaptor proteins targeting GPCRs to *clathrin-coated pits* (Figure 123, Figure 124). To this end, they bind with high affinity to clathrin as well as to the β2-adaptin subunit of the heterotetrameric AP-2 adaptor complex. When present in the *clathrin-coated pits*, the GPCRs may be internalized (also called sequestered) within the cell by an endocytotic process. This process is dependent on *dynamin*, a large GTPase that is involved in the pinching off of clathrin-coated

vesicles from the plasma membrane. In this respect, it seems that GPCR endocytosis is mediated by the same molecular intermediates as those involved in the endocytosis of receptor tyrosine kinases, except that GPCRs seems to use a common proximal intermediate, β-arrestin.

Advances in receptor visualization techniques have greatly contributed to our understanding of the cellular trafficking of GPCRs and other receptors. Several approaches are possible, such as:

- Transmission electron microscopy with an agonist-colloidal gold complex (Figure 124).
- Transfecting cells with the DNA coding for epitope-tagged receptors (e.g. flag epitope DYKDDDD at the extracellular N-terminus) in combination with immunofluorescence microscopy using anti-flag antibodies and fluorescently labelled secondary antibodies.
- Transfecting cells with the DNA coding for an amino- or carboxyl-terminal receptor/GFP fusion protein in combination with fluorescence microscopy (Figure 125).
- The internalization of GPCRs via clathrin-coated pits appears to be effectively inhibited by hypertonic concentrations of sucrose, by low temperatures, by concanavalin A and by the depletion of intracellular ATP and K^+.

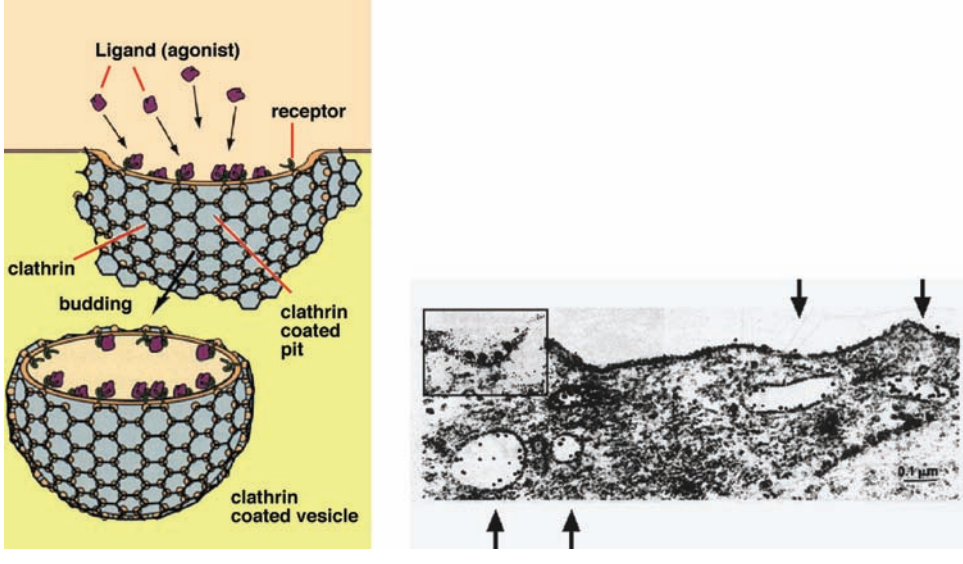


Figure 124 Left: Graphic representation of clathrin coated pits and vesicle. Reprinted from Geoffrey M.Cooper, *The Cell: A Molecular Approach* 4th edn., © (2007), with permission from ASM Press, Washington DC. Right: Presence of angiotensin II-colloidal gold complexes on coated pits and (insert) within large endosomes (arrows) at variable depths within the cytoplasm of rat aortic vascular smooth muscle cells (Anderson *et al.*, 1993, reproduced by permission of the American Physiological Society).

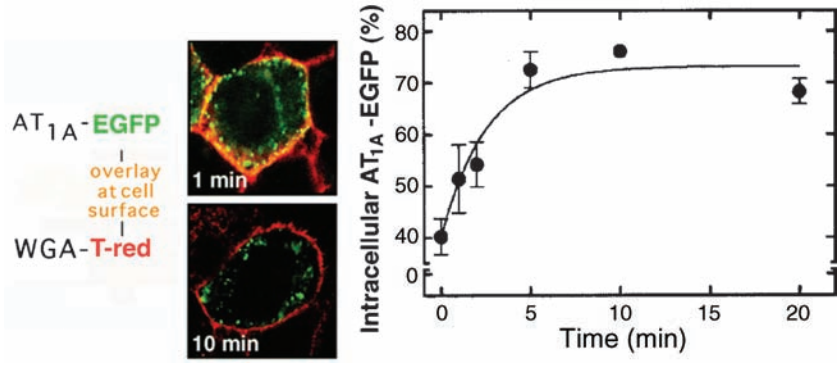


Figure 125 Confocal imaging (fluorescence microscopy) and time-dependence (right) of internalization of enhanced green fluoresccent protein (EGFP)-coupled AT_1 receptor in CHO cells: receptor fluorescence is green when inside the cell and provides a yellow pseudocolor when it colocalizes with the red fluorescence of wheatgerm agglutinin-Texas Red at the cell surface. In non-stimulated cells, the majority of the receptors are at the surface but 30–40% is already cytoplasmic. After 10 min angiotensin II stimulation, 70–75% of the receptors are cytoplasmic. Reproduced from Holloway *et al.*, 2002, *Molecular Pharmacology*, **61**, 768–777.

- The rapid dissociation of certain agonist- receptor complexes (e.g. AT_1 receptors) in an acidic environment has been exploited to differentiate agonists which are bound to cell surface receptors, from internalized agonist molecules. Whereas the former readily dissociate upon mild acid treatment of the cells, the latter will remain in the cell (Figure 126).

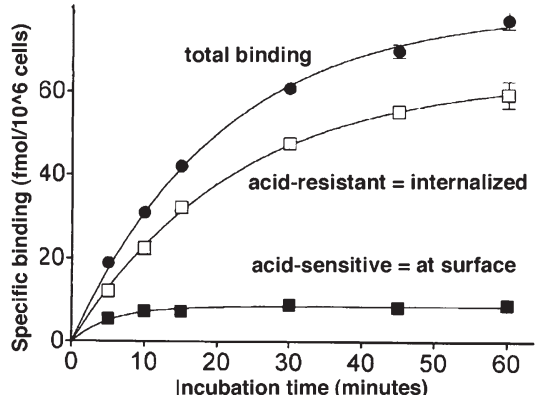


Figure 126 Incubation of AT_1-receptor-transfected CHO cells at 37 °C with [^{3}H]angiotensin II: evolution of acid-sensitive and -resistant binding with time. Reprinted from *British Journal of Pharmacology*, **126**, Vanderheyden, P.M.L., Fierens, F.L.P., De Backer, J.-P., Frayman, N. and Vauquelin, G., Distinction between surmountable and insurmountable selective AT1 receptor antagonists by use of CHO-K1 cells expressing human angiotensin II AT1 receptors, 1057–1065, © (1999).

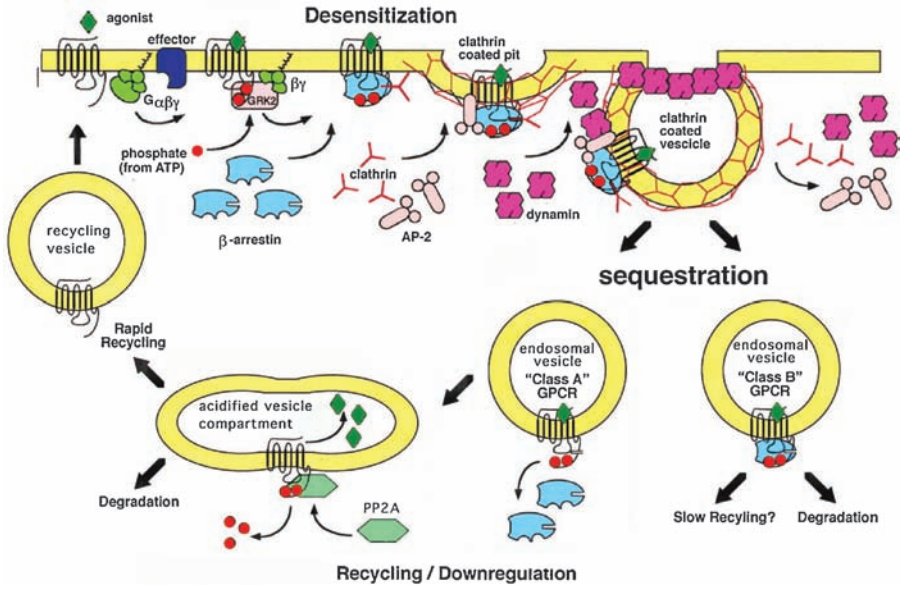


Figure 127 Molecular mechanism of (homologous) GPCR regulation: recycling or downregulation of internalized receptors. Reproduced by permission of the Company of Biologists, *Journal of Cell Science*, **115**, Luttrell, L. M. and Lefkowitz, R. J., The role of beta-arrestins in the termination and transduction of G protein-coupled receptor signals, Fig. 1, 455–465. Copyright (2002).

Internalization of GPCRs occurs more slowly than desensitization (within seconds), happening over a period of several minutes after agonist exposure. This process is important for receptor resensitization (i.e. recycling to the membrane) and downregulation (receptor degradation) (Figure 127). The 'sorting' between these two processes will depend on the stability of the GPCR–β-arrestin complex:

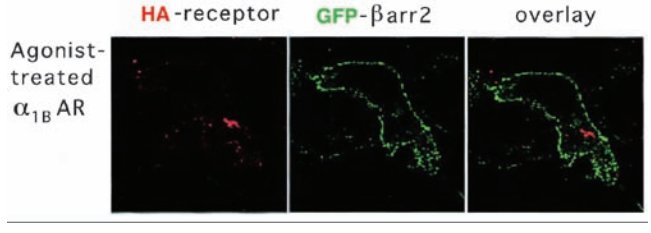


Figure 128 Confocal imaging (fluorescence microscopy) of HA-epitope-tagged α_{1B}-adrenergic receptors (revealed with a rhodamine-coupled antibody against the HA-epitope) and green fluoresccent protein (GFP)-coupled β-arrestin 1 in HEK-293 cells after 15 min agonist exposure (Tohgo *et al.*, 2003, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

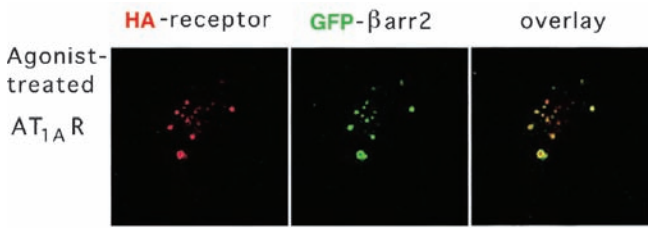


Figure 129 Confocal imaging (fluorescence microscopy) of AT_1 receptors and β-arrestin 2 in HEK-293 cells after 15 min agonist exposure (Tohgo *et al.*, 2003, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

- *Class A* GPCRs (e.g. β_2- and α_{1B}-adrenergic receptors, μ-opioid receptors…) recruite β-arrestin 2 more efficiently than β-arrestin 1. They rapidly dissociate from β-arrestin upon internalization. Whereas β-arrestin recycles to the cytosol, the receptors are trafficked to an *acidified endosomal compartment* (Figure 128) wherein the ligand is dissociated and the receptor dephosphorylated. The receptors are subsequently recycled to the plasma membrane. Receptor recycling is a constitutive process. This implies that, whereas the rate of endocytosis is dependent on the agonist concentration, the rate of recycling is not. Typical recycling takes place with a half-life in the range of 6 to 12 min. However, GPCR recycling to the cell surface can be slowed down by substances, like monensin and nigericin, which raise the pH within endosomes.
- *Class B* GPCRs (e.g. AT_1 and neurokinin NK-1 receptors) recruite both β-arrestins equally well and form stable complexes (Figure 129). These receptors accumulate in endocytic vesicles and are either slowly recycled to the membrane via as yet poorly defined routes or targeted for degradation in *lysosomes*. This latter process occurs over a period of hours to days. The thrombin receptor and proteinase-activated receptor-2 represent an interesting case since their extracellular N-termini are cleaved during their activation by thrombin and trypsin, respectively. This activation is permanent, which means that the cleaved receptors cannot be recycled to the plasma membrane in their native state. Hence, they need to be sorted to lysosomes for degradation.

Most studies do suggest that, under physiological conditions, the predominant pathway for GPCR endocytosis is β-arrestin- and dynamin-dependent. However, the precise mechanism(s) by which all GPCRs internalize remains a controversial topic and some findings suggest that alternative routes of GPCR internalization could take place. Some internalization could be caveolin-dependent (see Section 4.10).

The physiological importance of receptor resensitization in the maintenance of normal tissue homeostasis is obvious since prolonged or irreversible receptor desensitization would leave a cell unable to respond appropriately to extracellular stimuli. Just as GPCR desensitization provides a mechanism for protecting cells against receptor overstimulation, GPCR resensitization protects cells against prolonged receptor

desensitization. It has therefore been suggested that receptors that do not recycle will mediate transient responses to agonists, whereas those that are efficiently recycled mediate persistent responses. In this respect, the degree of receptor internalization depends on the ratio between the rate constant for its endocytosis (ke) and the rate constant for its exocytosis/recycling (kr): i.e.% internalized = 100 × ke/ (ke + kr):

$$\text{R(cell surface)} \underset{k_r}{\overset{k_e}{\rightleftharpoons}} \text{R(endosome)}$$

The term *down regulation* refers to a persistent loss of receptors after long-term exposure to agonists. From a physiological viewpoint, cells are rarely exposed continuously to hormones or neurotransmitters, since efficient mechanisms exist to remove them from the extracellular fluid. However, down regulation may occur under pathological circumstances, e.g. when there is continuous secretion of hormones and neurotransmitters from tumours. Down regulation is also important during long-term administration of receptor agonists for therapeutic reasons, since it may be responsible for tolerance. GPCR down regulation occurs as a result of:

- Targeting of internalized receptors to lysosomes. In this respect, continuous agonist exposure may cause multiple rounds of endocytosis and recycling. Even if only a minor portion if the internalized receptors is targeted to the lysosomes during each cycle, this process will finally deplete the cell of a substantial amount of its receptors.
- The second component of receptor down regulation is decreased receptor synthesis. This may be a result of reduced gene transcription or of a post-transcriptional event, such as mRNA destabilization. Receptor mRNA destabilization is the prevailing mechanism after long-term agonist stimulation of, for example, β_2-adrenergic receptors.

Finally, it has been demonstrated that three different CAMs of the AT_1 receptor are constitutively internalized and recycled. This constitutive internalization should be clearly distinguished from the accelerated degradation (down regulation) of misfolded CAMs.

Mechanisms to terminate signalling

Cellular responses to agonists of the GPCRs are usually rapidly attenuated. Signals may be attenuated by mechanisms that operate at the level of the agonist, the receptor, the G proteins and at numerous downstream steps of the signalling pathway.

Several processes contribute to the removal of hormones and neurotransmitters from the extracellular fluid, but their relative importance depends on the nature of the agonist (Figure 130):

- For all agonists, dilution in the extracellular fluid and subsequent excretion reduces concentrations to levels that are too low to produce detectable responses in target cells.

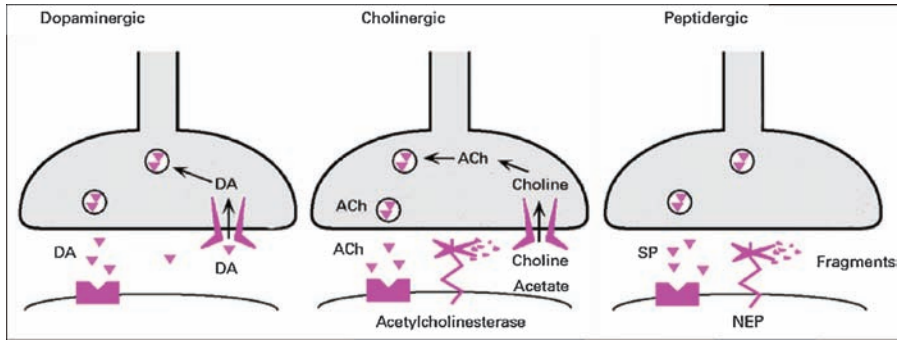


Figure 130 Release, reuptake and degradation of the biogenic amines dopamine (DA), acetylcholine (Ach) and the neuropeptide substance (SP). Reproduced with permission, from Bohm, S. K., Grady, E. F. and Bunnett, N. W., 1997, *Biochemical Journal*, **322**, 1–18. © The Biochemical Society.

- Uptake by high-affinity transporters is one of the most widespread mechanisms for removal of agonists of GPCRs and ligand-gated ion channels from the synapse. The importance of transporters in terminating neurotransmission has been well documented. Certain powerful psychoactive drugs exert their effects by inhibiting transporters and thereby prolonging the effects of the neurotransmitters: cocaine blocks the uptake of noradrenaline and dopamine.
- Extracellular degradation is the major mechanism for removing acetylcholine and peptides from the extracellular fluid. Acetylcholine released from nerve terminals is not taken up, but is degraded by acetylcholinesterase. Inhibition of this enzyme prolongs synaptic transmission, which ultimately desensitizes cholinergic receptors and results in paralysis and death due to respiratory failure. Indeed, nerve gases such as *Sarin* are acetylcholinesterase inhibitors. Peptides are removed from the extracellular fluid by enzymatic degradation, mainly by cell-surface peptidases.

Receptors are not static entities. The most extensively studied, and probably the most common form of regulation involves the reduction in responsiveness of a receptor upon prolonged stimulation by an agonist. This process, denominated as '*desensitization*', 'tolerance', 'refractoriness' or 'tachyphylaxis', probably occurs as a measure to prevent cell damage:

- If an agonist only affects the activity of its own receptor, we may speak about '*homologous desensitization*'. This form of desensitization takes place with β-arrestin binding since it only precludes coupling between the activated receptor and G proteins. This process affects no other receptor types (Figure 131).
- On the other hand, many examples are known where the activity of a receptor is either increased or decreased by unrelated drugs (e.g. drugs that interact with other receptors) and this form of regulation is termed '*heterologous desensitization*'.

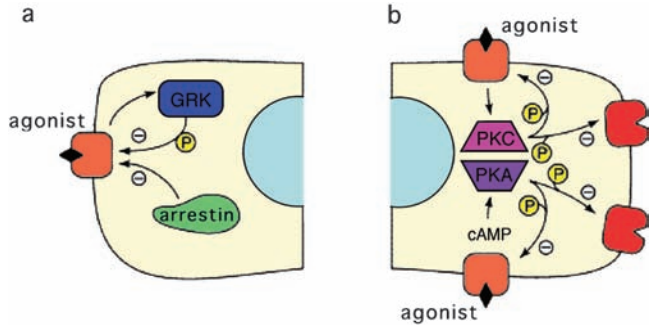


Figure 131 a) Homologous desensitization: only the agonist-occupied receptor is desensitized by a GRK/arrestin mechanism. b) Heterologous desensitization: activated PKA or PKC phosphorylates and desensitizes different types of receptors. Reprinted from *Trends in Pharmacological Science*, **17**, Chuang, T. T., Iacovelli, L., Sallese, M. and De Blasi, A., G protein-coupled receptors: heterologous regulation of homologous desensitization and its implications, 416–421. Copyright (1996), with permission from Elsevier.

This form of desensitization takes place with *second-messenger-dependent protein kinases*, including cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) (Figure 131). These kinases are also able to phosphorylate serine and threonine residues within the cytoplasmic loops and C-terminal tail domains of many GPCRs. This phosphorylation process is sufficient to impair receptor–G protein coupling, but agonist occupancy of the target GPCR is not required. This implies that the activation of second-messenger-dependent protein kinases by one receptor is able to desensitize receptors for other ligands as well. In this respect, PKC activation leads to the phosphorylation and desensitization of many G_i- and G_q-linked GPCRs.

There are also additional differences between second-messenger kinase- and GRK-mediated GPCR phosphorylation:

- Whereas no consensus sites were found for phosphorylation by the GRKs, such sites clearly exist for the second-messenger kinases.
- Whereas β-arrestins display increased affinity for GRK-phosphorylated receptors, their affinity is unaffected by PKA- and PKC-induced phosphorylation. This is in line with the view that β-arrestin contributes to homologous, but not heterologous, desensitization.
- PKA and PKC are thought to represent the predominant mechanisms by which GPCR desensitization is achieved at low agonist concentrations whereas GRK/β-arrestin- mediated receptor phosphorylation is thought to represent GPCR desensitization at high agonist concentrations. Indeed, because of the existence of a 'receptor reserve' in many cells, second-messenger kinase activity will be

more pronounced than the receptor occupancy at low agonist concentrations. Concentrations of agonists with high efficiency giving only 1–2% receptor occupancy, may therefore be sufficient to fully stimulate PKA- and PKC-mediated phosphorylation in most cells. In contrast, the EC_{50} for GRK-mediated phosphorylation is much higher, approaching the K_D for agonist binding. Thus, as the concentration of an agonist with high efficacy increases, GPCR desensitization gradually swings from second-messenger kinase-mediated to β-ARK-mediated.

Finally, GPCR signalling can also be terminated at the level of the G protein. For example, a family of proteins, termed regulators of G protein signalling (RGS) act to increase the rate of hydrolysis of GTP bound to both $G\alpha_i$ and $G\alpha_q$ subunits, thereby dampening signalling via G_i- and G_q- regulated signalling pathways.

4.7 β-Arrestin-binding and MAP kinase activation

The β-arrestins appear to play a dual role in GPCR signalling. They serve both to terminate G protein-dependent signals by hampering receptor–G protein coupling, and to induce novel signalling properties of the receptor by acting as adapters or scaffolds for signalling proteins that make up part of the ERK/mitogen-activated protein kinase (MAP kinase) signalling pathway.

MAP kinases are a family of evolutionarily conserved serine/threonine kinases that are involved in the transduction of externally derived signals regulating cell growth, division, differentiation and apoptosis. ERK1 and ERK2 were the initial MAP kinases to be discovered. They are important for the control of the G0–G1 cell cycle transition and the passage of cells through mitosis or meiosis. They perform the last step in a specific phosphorylation cascade in the cytosol, with the following sequence (Figure 132):

- Inactive Raf (a 74 kDa serine/threonine kinase) is in a complex with '14-3-3' proteins and, when activated, it will phosphorylate two serine residues on MEK.
- Phosphorylated MEK is active and will phosphorylate the tyrosine and threonine residues on ERK.
- Once phosphorylated/activated, ERK will phosphorylate a variety of membrane, cytoplasmic and cytoskeletal substrates as well as nuclear transcription factors involved in DNA synthesis and cell division. This last process implies the relocation of active ERK to the nucleus.

What keeps the signal on track appears to be the assembly of the cascade components onto the scaffold composed of '14-3-3' and MP1.

Several other kinase pathways follow the same pattern in mammalian cells (Figure 133). Each cascade consists of three protein kinases: a terminal MAP kinase

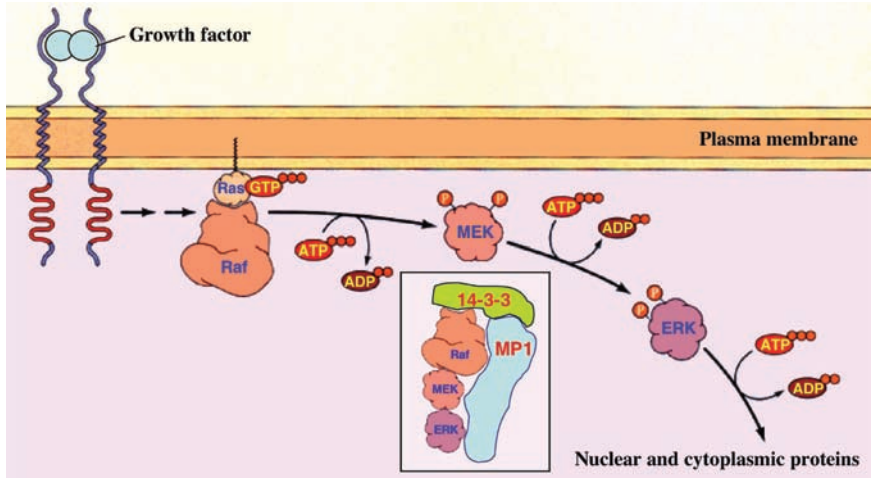


Figure 132 Stimulated growth factor receptors initiate signal transduction via the MAP kinase pathway. Reprinted from Geoffrey M.Cooper, *The Cell: A Molecular Approach*, 4th edn., © (2007), with permission from ASM Press, Washington DC.

and two upstream kinases (analogous to Raf and MEK) that regulate its activity. Additional MAP kinases are JNKs/SAPK and p38/HOG1. They are involved in regulation of growth arrest, apoptosis and activation of immune cells in response to stresses. In many cases, binding of the component kinases to a scaffolding protein controls

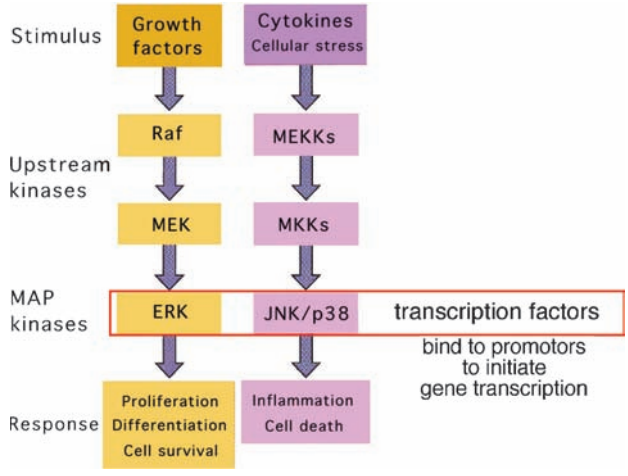


Figure 133 MAPK pathways consist of three protein kinases. The third kinase is a nuclear transcription factor. Reprinted from Geoffrey M.Cooper, *The Cell: A Molecular Approach*, 4th edn., © (2007), with permission from ASM Press, Washington DC.

activation of MAP kinase cascades. These scaffolds serve at least three functions in cells:

- To increase the efficiency of signalling between successive kinases in the phosphorylation cascade.
- To dampen cross talk between parallel MAP kinase cascades.
- To target MAP kinases to specific subcellular locations.

The ERK/MAP kinase pathway is well known for its predilection to be activated by growth factor receptors such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors. Auto phosphorylation of these receptor-tyrosine kinases recruits several adapter proteins, resulting in the activation of Ras (a small G protein that is attached to the plasma membrane by farnesyl and palmitoyl lipid groups). As for the GPCR-associated G proteins, inactive Ras contains tightly bound GDP and, during its activation, this nucleotide will dissociate and be replaced by GTP (Figure 134). Ras is a central player in signal transduction. Perhaps the best-characterized downstream effector of Ras is Raf, which leads to the ERK/MAP kinase pathway.

The link between GPCR–β-arrestin complexes and ERK/MAP kinase activation has been brought to light by data from yeast-2 hybrid screens (see Section 4.8) using β-arrestins, and from the biochemical characterization of receptor–β-arrestin complexes:

- AT_{1A} receptor activation results in the formation of complexes between the receptor, β-arrestin 2 and the component kinases of the ERK cascade (Raf-1, MEK1 and ERK2). β-Arrestins appear to act as scaffolds for ERK/MAP kinase cascade as well as for the JNK3 MAP kinase cascade.

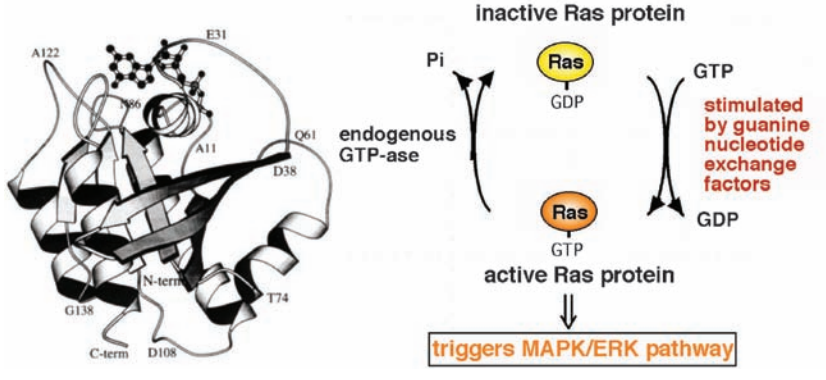


Figure 134 Three-dimensional structure (Reprinted with permission from *Biochemistry*, **33**, Kraulis, P. J., Domaille, P. J., Campbell-Burk, S. L., Van Aken, T.and Laue, E.D., Solution structure and dynamics of ras p21.GDP determined by heteronuclear three- and four-dimensional NMR spectroscopy, 3515–3531. Copyright (1994) American Chemical Society) and activation mechanism of the small G protein, Ras.

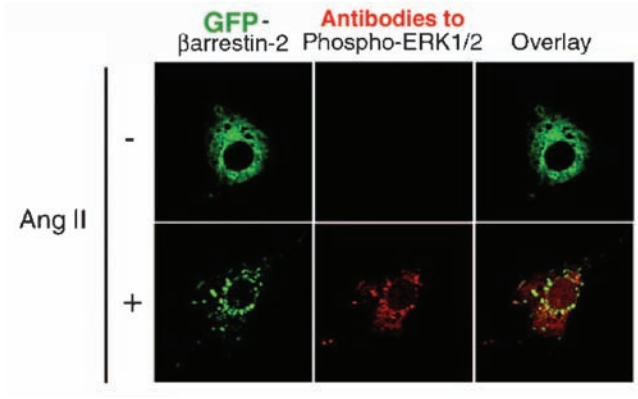


Figure 135 Fluorescence microscopy: GFP-β-arrestin 2 is in the cytosol of COS-7 cells and fluorescence of antibodies against active ERK (phospho-ERK1/2) is minimal. Angiotensin II redistributes its receptors along with GFP-β-arrestin 2 into large endosomal vesicles. Now, anti-phospho-ERK1/2 fluorescence partially co-localizes with GFP-β-arrestin 2. Note that there is only a little phospho-ERK1/2 in the nucleus (Tohgo *et al.*, 2002, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

- Receptor–β-arrestin complexes are also associated with the activation and cytosolic retention of ERK (Figure 135, Figure 136). In this respect, activated ERK1/2 does not appear to undergo nuclear translocation. Instead bound ERK1/2 may phosphorylate plasma membrane, cytosolic or cytoskeletal substrates, or it may lead to transcriptional activation through the activation of other kinases.
- Certain receptor–β-arrestin complexes also induce cell mitogenesis by stimulating the ERK/ MAP kinase cascade. This signalling cascade is initiated by the interaction between β-arrestin and the SH3 domain of c-Src, a protein kinase that is capable of activating EGF receptors by phosphorylating some of their intracellular tyrosine residues (Figure 137). This 'cross talk' between GPCRs and EGF receptors results in a Ras-dependent activation of the ERK/MAP kinase pathway and, hence, to a mitogenic response.

Many GPCRs simultaneously employ multiple mechanisms to activate MAP kinases. For example, the AT_{1A} receptor can activate ERK1/2 not only via β-arrestin-dependent pathways but also through G protein-dependent signals. G protein-dependent mechanisms (Figure 138) include:

- Protein kinase A-dependent phosphorylation of the small G protein, Rap1.
- Protein kinase C-dependent activation of Raf (a small G protein like Ras).
- 'Transactivation' of receptor tyrosine kinases, such as the EGF and PDGF receptors.

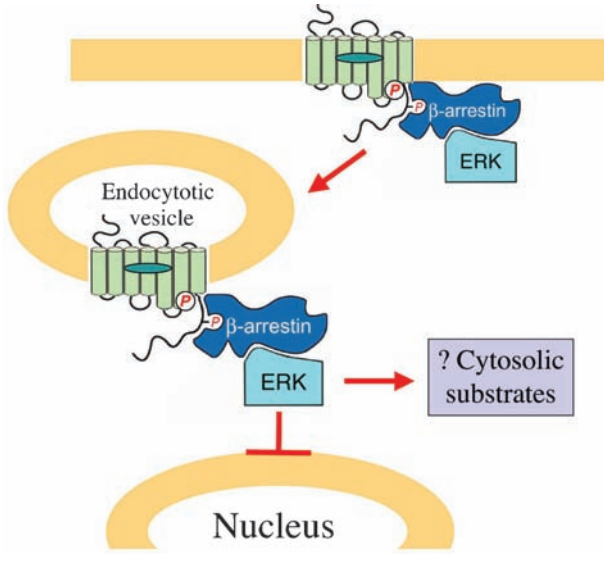


Figure 136 AT_{1A} receptor stimulation triggers the consecutive binding of β-arrestin and ERK1/2. However, such activated ERK1/2 is unable to undergo nuclear translocation (i.e. to act as a nuclear transcription factor). Reproduced by permission of the Company of Biologists, *Journal of Cell Science*, **115**, Luttrell, L. M. and Lefkowitz, R. J., The role of beta-arrestins in the termination and transduction of G protein-coupled receptor signals, 455–465, Copyright (2002).

- ERK activation by G_i -activating receptors is likely to result from the liberation of β–γ subunits from G_i that can directly activate PI3-kinase, leading to the activation of Ras and the Raf/MEK/ERK cascade.

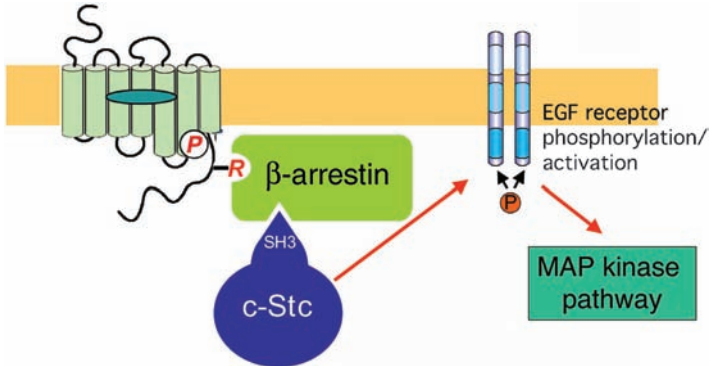


Figure 137 GPCR induces cell mitogenesis via EGF receptor activation. To start the signalling casade, GPCR-β-arrestin complexes bind to the SH3 domain of c-Src. c-Src is a protein kinase that is capable of phosphorylating EGF receptors. This triggers Ras-dependent activation of the ERK/MAP kinase pathway.

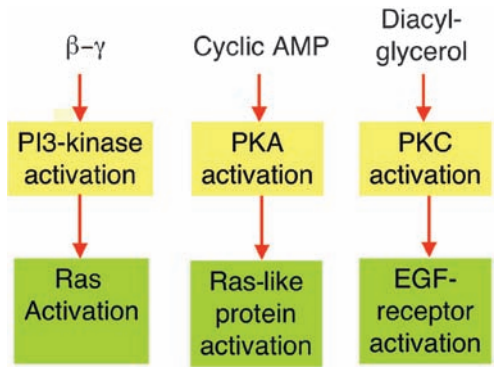


Figure 138 G protein-dependent pathways for GPCR-mediated MAP kinase stimulation. Note that GPCRs can simultaneously employ multiple mechanisms.

4.8 GPCR dimerization and association with other proteins

Introductory comments

GPCRs are traditionally known to behave as monomers. Yet, there is increasing evidence that they form dimers and physically interact with a variety of other membrane proteins. These include other receptors as well as non-receptor membrane proteins that affect GPCR cell surface expression, binding and functional properties (Figure 139). In certain situations, this has clearly been shown to alter the pharmacological profile of the GPCR.

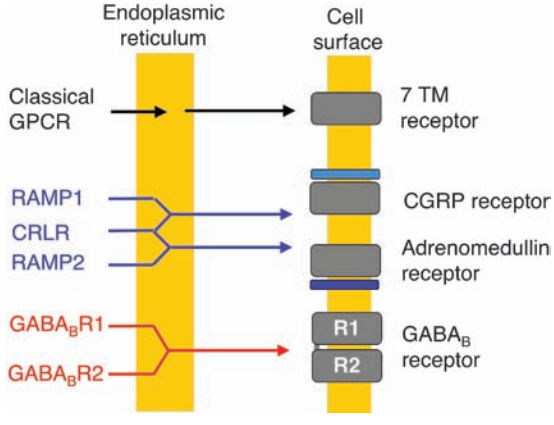


Figure 139 GPCR dimerization and association with chaperones may alter the pharmacological profile. Reprinted from *Trends in Pharmacological Science*, **20**, Mohler, H. and Fritschy, J. M., GABAB receptors make it to the top - as dimers, 87–89. Copyright (1999), with permission from Elsevier.

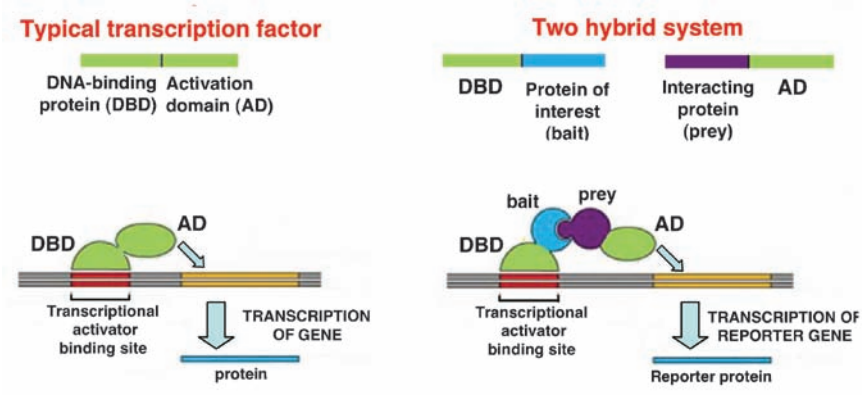


Figure 140 Principle of the yeast-2 hybrid technique.

Many GPCRs contain sequence motifs that direct protein–protein interactions and, therefore, have the theoretical capacity to interact with a wide range of other proteins. The yeast-2 hybrid technique (Figure 140) is well suited to monitor interactions between cytoplasmic GPCR regions and proteins within the cell. For this purpose, intracellular loops and the C-terminal tails of GPCRs can be isolated and used as 'bait' for cytosolic proteins in yeast-2 hybrid–protein interaction screens.

Using this approach, a considerable range of interactions (summarized in Figure 141) has been reported. Yet the full functional and physiological significance of some of them is not completely understood. However, some appear to affect the localization, signalling specificity, and in some cases, the pharmacological profile of GPCRs.

GPCR dimerization

GPCRs are traditionally regarded to exist and to be fully functional as monomers. Yet, recent findings suggest that they also exist as homo- as well as heterodimers (i.e. dimers between, respectively, the same and different receptor molecules). As dimerization is often observed in recombinant cell systems, there is a major concern that this process could be due to receptor overexpression and, hence, about the physiological relevance of this process. However, dimerization has also been observed when the receptors are expressed at endogenous levels and many other proteins exist and function as dimers (e.g. tyrosine kinase receptors, transcription factors and steroid receptor). GPCR dimerization also shows some specificity: i.e. homodimers and heterodimers containing closely related GPCRs appear to be more easily formed than heterodimers between distantly related GPCRs. The importance of heterodimerization in ligand recognition and signalling is further exemplified by the γ-aminobutyric acid-binding $GABA_B$ receptor (Figure 139). It is only functional as a heterodimer between $GABA_BR1$ and $GABA_BR2$ (two naturally occurring

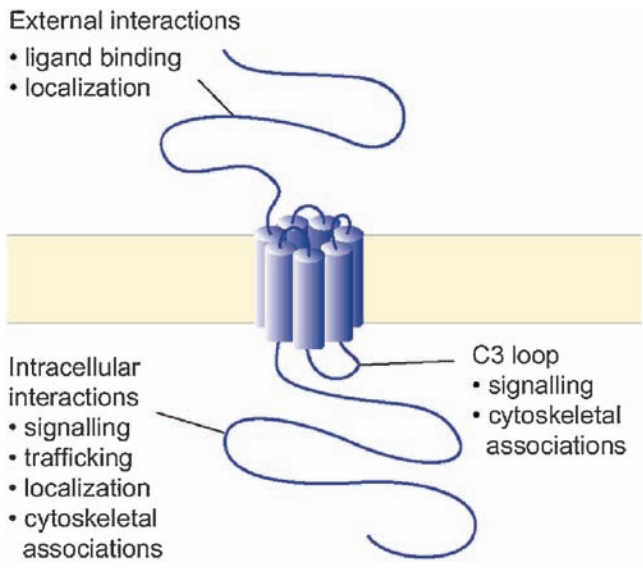


Figure 141 Interactions involving distinct GPCR domains that affect localization, signalling and pharmacological properties of the receptor. Reprinted from *Trends in Pharmacological Sciences*, **22**, Milligan, G. and White, J. H., Protein–protein interactions at G protein-coupled receptors, 513–518, © (2001), with permission from Elsevier.

non-functional GPCRs). $GABA_BR1$ alone displays low agonist affinity and does not traffic to the cell surface because it possesses an endoplasmic reticulum retention motif. This motif gets masked upon heterodimerization with $GABA_BR2$ (upon co-expression). The heterodimer now reaches the cell surface and is functionally active. In the same line, native tissue metabotropic glutamate mGlu5 receptors exist almost exclusively as homodimers.

Biochemical and biophysical techniques have been used to identify GPCR dimers:

- Radiation inactivation (target size analysis) was used in the eighties. This technique is based on the inverse relationship between the size of a macromolecule and the dose-dependent inactivation of that macromolecule by ionizing radiation. These studies suggested that, for example, β_2-adrenergic receptors had a higher molecular mass than expected and hence, could be dimers.
- The use of cross-linking agents and/or photoaffinity labelling reagents followed by purification of the radiolabelled complex using gel exclusion chromatography.
- Differential epitope tagging and selective immunoprecipitation have been an invaluable tool to provide biochemical evidence for the presence of GPCR dimers. Using this technique, the two GPCRs under investigation are each tagged with a distinct epitope and expressed in heterologous cells (that do not normally express these receptors). Antibodies to one epitope are used to immuno-isolate

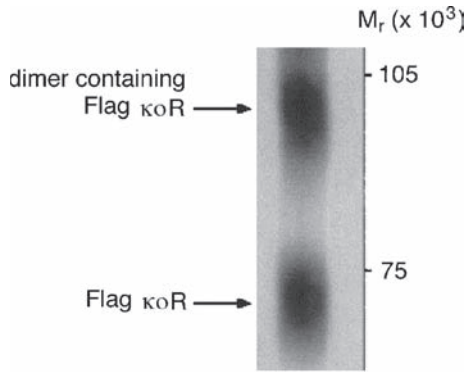


Figure 142 Cells were co-transfected with β_2-adrenergic and FLAG-tagged κ-opioid receptors. Samples were immunoprecipitated with anti-β_2-adrenergic receptor antibody, resolved by SDS/PAGE and immunoblotted with anti-FLAG antibody. Reproduced with permission, from Ramsay, D., Kellett, E., McVey, M., Rees, S. and Milligan, G., 2002, *Biochemical Journal*, **365**, 429–440. © The Biochemical Society.

the receptor-containing complex, and the associating receptor in the complex is visualized using antibodies to the second epitope (Figure 142). A major concern with this technique is the possibility of artifactual aggregation (due to the inherent hydrophobic nature of GPCRs) under the solubilization/immunoprecipitation conditions. To ensure that dimers are not induced, cells individually expressing differently tagged receptors can be mixed prior to solubilization and immunoprecipitation. Under these conditions, dimers should be observed only in cells co-expressing the two receptors and not in the mixture of cells.

- The bioluminescence resonance energy transfer (BRET) (Figure 143) technique involves the emission of light upon the catalytic degradation of a suitable substrate by a (receptor-coupled) enzyme. This light can activate an energy acceptor (such as green fluorescent protein (GFP) coupled to a second receptor), resulting in its fluorescence. Fluorescence can only be measured if the energy donor and acceptor are located within relatively small distances (i.e. 50 Å = one receptor radius) of each other. However, this does not necessarily imply a physical interaction. Whereas BRET uses the oxidation of coelenterazine by *Renilla* luciferase to excite the fluorescence of yellow fluorescent protein (YFP), a recent variant ($BRET^2$) (Figure 143) has been introduced in which *Renilla* luciferase oxidizes a modified form of coelenterazine to excite the fluorescence of a GFP mutant (designated GFP^2). The bioluminiscence and the fluorescence spectra are more effectively resolved in this newer assay so that the fluorescence signal becomes more distinct.
- The fluorescence resonance energy transfer (FRET) (Figure 144) technique is similar to BRET, with the exception that the energy donor molecule, a variant of GFP (generally, cyan fluorescent protein (CFP)) is excited by an external

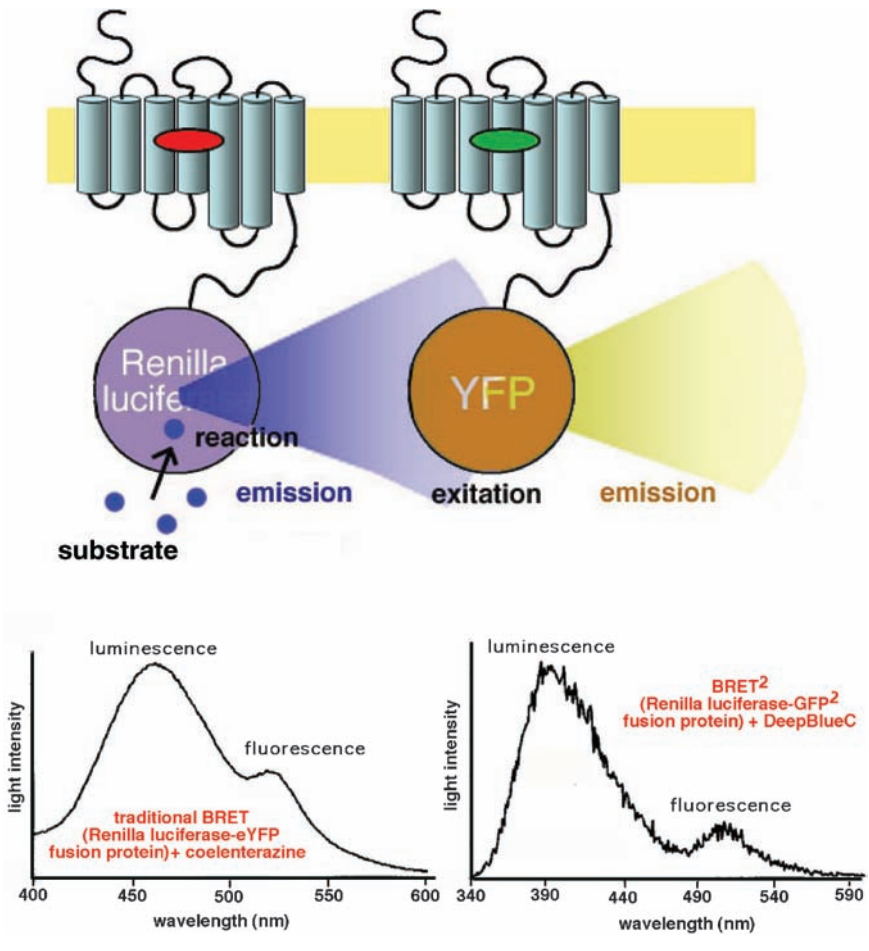


Figure 143 Top: Principle of the BRET^2 technique. Bottom: Luminescence and fluorescence spectra for the traditional BRET and the more recent BRET^2 approach. Reproduced with permission, from Ramsay, D., Kellett, E., McVey, M., Rees, S. and Milligan, G., 2002, *Biochemical Journal*, **365**, 429–440. © The Biochemical Society.

light source. The energy emitted by CFP is used to excite an acceptor molecule, another variant of GFP (generally, yellow fluorescent protein (YFP)). Here again, fluorescence can only be measured if the energy donor and acceptor are located within relatively small distances (i.e. 100 Å $= 2 \times$ receptor radius) of each other.

From the evidence gathered thus far, it appears that some GPCRs are assembled as dimers in the endoplasmic reticulum whereas others assemble to dimers (or even oligomers) at the cell surface. Also the effect of agonists on receptor di-/oligomers is quite variable. There are at least two possible scenarios for the assembly and maturation of the GPCR dimer (Figure 145):

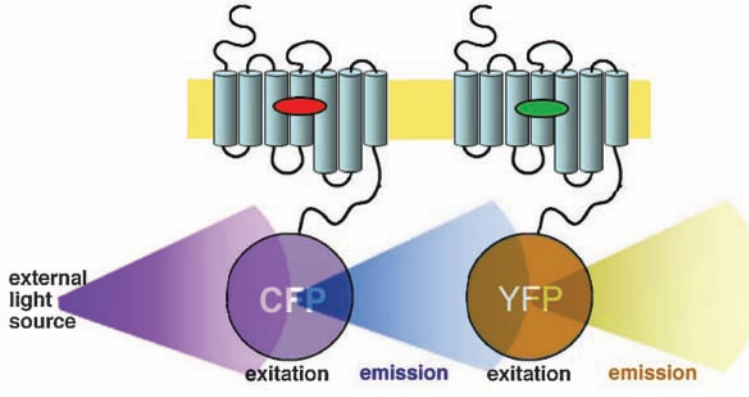


Figure 144 Principle of the FRET technique.

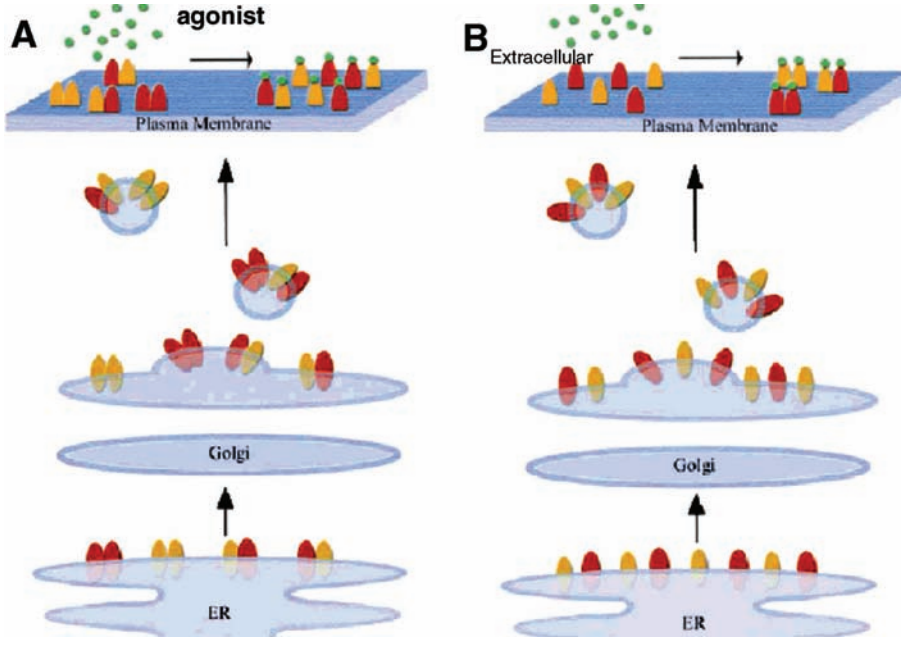


Figure 145 Subcellular locations where GPCR dimers are thought to form. (A) Receptor dimerization in the endoplasmic reticulum and transport as dimers to the cell surface. (B) Receptor di- or multimerization at the cell surface under the influence of agonists. Reprinted from *Pharmacology and Theraputics*, **92**, Rios, C. D., Jordan, B. A., Gomes, I. and Devi, L. A., G protein-coupled receptor dimerization: modulation of receptor function, 71–87. Copyright (2001), with permission from Elsevier.

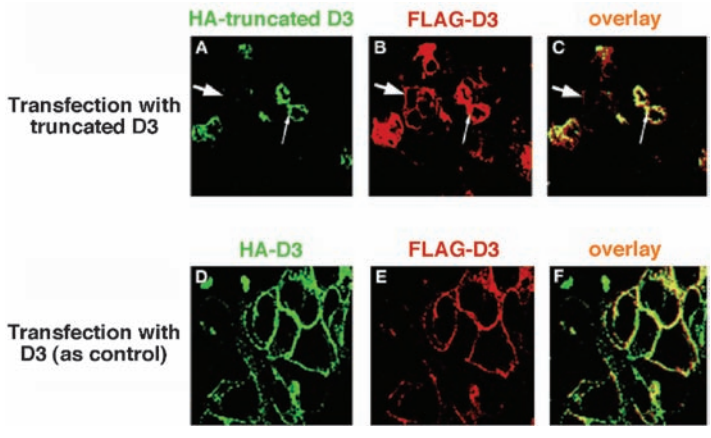


Figure 146 Top: Co-expression of HA-tagged D_3 dopamine receptor mutants (lacking TM6 and TM7) and FLAG-tagged wild-type D_3 receptors in the same cells. Both form heterodimers (co-immunoprecipitation experiments) and show a similar cytosolic localization (confocal laser microscopy shown here). Bottom: Control experiment. All receptors reside at the cell surface when HA- and FLAG-tagged wild-type D_3 receptors are introduced into the same cell line. Reproduced from Karpa, K. D., Lin, R., Kabbani, N. and Levenson, R. (2000) The dopamine D3 receptor interacts with itself and the truncated D3 splice variant D3nf: D3-D3nf interaction causes mislocalization of D3 receptors. *Molecular Pharmacology*, **58**, 677–683, with permission from the American Society for Pharmacology and Experimental Theraputics.

- (A) GPCRs assemble in an intracellular compartment and are shuttled to the cell surface as dimers (e.g. $GABA_BR1$ and $GABA_BR2$). In this respect, it has also been found for several GPCRs that co-expression of a mutant truncated at the N- and C-termini with wild-type receptors may inhibit the trafficking of the latter to the cell surface (Figure 146).
- (B) GPCRs are synthesized in the endoplasmic reticulum (ER) and shuttled to the cell surface as monomers, where they may assemble as dimers in response to agonists.

Also, the interactions that hold a dimer together seem to differ considerably among the receptors. It can be mediated either by covalent (disulfide) and/or non-covalent (hydrophobic) interactions, and can involve associations of different receptor domains. e.g.:

- Dimerization of the metabotropic glutamate receptors was found to be dependent on the formation of disulfide bonds between cysteines in their large amino-terminal domains.
- The involvement of TM6 and TM7 has been implicated for D_2 dopamine receptor dimerization since peptides encoding these regions inhibit dimer formation.

- For the δ-opioid receptor, dimerization was eliminated by deletion of 15 amino acids at the C-terminus, indicating the involvement of this part of the receptor in dimerization. C-terminal regions also participate in $GABA_BR1$–$GABA_BR2$ receptor heterodimerization.

Some receptors appear to swap some of their TM domains during the heterodimerization process. Evidence of this 'domain swapping' is provided by functional rescue experiments:

- When TM6 and TM7 are exchanged between α_{2C} adrenergic and muscarinic receptors, the receptor mutants are not able to bind their initial radioligands when expressed separately. However, both α_{2C} adrenergic and muscarinic radioligands are able to exhibit significant binding to cells where both mutants are co-expressed.
- Co-expression of truncated β-adrenergic receptors (one with TM 1 to TM5 and the other with TM 6 to TM7) restores its functional (i.e. adenylate cyclase stimulation) and binding activities.

To explain how binding pockets are recovered in such experiments, a model was proposed (Gouldson *et al.*, 1997) in which, upon receptor dimerization, the original binding pockets of the two subunit monomers are replaced by two binding pockets with similar structures except that they are formed from regions donated by both monomers (Figure 147).

Dimerization may alter how a receptor binds and functionally responds to a ligand but there is no evidence supporting a universal role of dimerization for GPCR activation (Table 15):

- In some cases the physical interaction between GPCRs leads to functional activation or enhanced functional activity. In other cases such an interaction appears to lead to functional receptor inactivation.

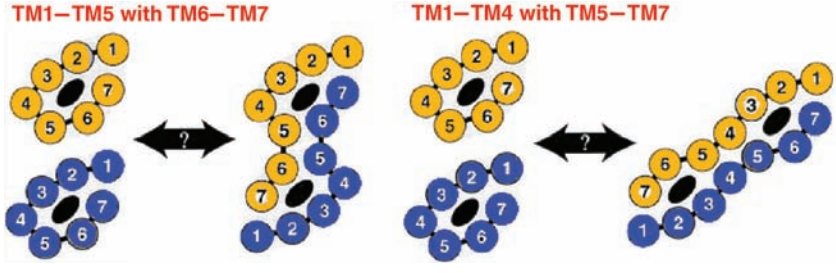


Figure 147 Possible formation of new binding pockets by GPCR transmembrane domains upon dimerization. Reproduced from Lee, S. P., O'dowd, B. F., Ng, G. Y. K., Varghese, G., Akil, H., Mansour, A., Nguyen, T. and George, S. R. (2000) Inhibition of cell surface expression by mutant receptors demonstrates that D2 dopamine receptors exist as oligomers in the cell. *Molecular Pharmacology*, **58**, 120–128, with permission from the American Society for Pharmacology and Experimental Theraputics.

Table 15 Interacting GPCRs. Reprinted from *Cellular Signalling*, **14**, Brady, A. E. and Limbird, L. E., G protein-coupled receptor interacting proteins: Emerging roles in localization and signal transduction, 297–309. Copyright (2002), with permission from Elsevier.

GPCR heterodimerization		Functional role(s) implicated
CCR2	CCR5	Required for activation of receptor associated JAK kinase, presumably due to transphosphorylation synergistic activation of Ca2+ responses by MCP-1 (CCR2) and RANTES (CCR5) ligands
Heterodimerization also recruits dissimilar signalling pathways		
GABAB R1	GABAB R2	Cell surface expression; pharmacologically appropriate ligand binding; coupling to GIRK/Kir3 currents
κ-opioid receptor	δ-opioid receptor	Modified pharmacological profile
μ-opioid receptor	δ–opioid receptor	Potentiation of m agonist signals by low concentrations of δ -selective agonist
A1 adenosine receptor	D1 receptor	Uncoupling of D1 receptor from activation of adenylate cyclase when A1 and D1 receptors are simultaneously activated
SSTR1	SSTR5	Dimerization induced by agonist; required for receptor activation; promotes internalization
D2 receptor	SSTR5	Creates novel receptor with enhanced affinity for dopamine and SST agonists; enhanced coupling to inhibition of adenylate cyclase
AT1 receptor	Bradykinin B2 receptor	Increases action of Gq and Gi receptor activation in heterologous and smooth muscle cells; modulates endocytic trafficking
GPCR Homodimerization		**Functional role(s) implicated**
κ–opioid receptor		Function unknown
δ-opioid receptor		Increasing levels of agonist causes dimer to monomer transition, which appears to induce receptor internalization
β-adrenergic receptor		BRET signal is constitutive and not regulated by agonist Agonist-facilitated; wild-type receptors have dominant positive effect on some mutant β2-adrenegic receptors
mGlu receptor		Occurs in ER and does not require glycosylation
M3 muscarinic receptor		Function unknown

Table 15 (*Continued*)

GPCR Homodimerization	Functional role(s) implicated
Ca2 +-sensing receptor	Increases affinity for extracellular Ca^{2+}; accelerates rate of agonist-elicited response
D2 receptor	Dimers are targets for nemonapride; monomers are targets for spiperone; disrupted by peptides derived from D2 R transmembrane regions
STE2 (yeast a -mating factor receptor)	Exist as dimers constitutively; dimers also exist during endocytosis TRH receptor
	Agonist stimulates BRET signal; constitutive oligomerization
LH receptor	Receptor oligomerization increases to a greater extent with hCG occupancy than with LH occupancy

- In the same line, agonists can stabilize the dimeric form (e.g. for β_2-adrenergic receptors) or decrease the level of dimer formation (e.g. for δ-opioid receptors).
- Finally, such interaction may result either in an increased or decreased (e.g. for the β_2-adrenergic–κ-opioid receptor combination) desensitization and internalization of the receptors.

Of special pharmacological interest is that the heterodimerization of some GPCRs may result in the formation of novel recognition sites with a completely distinct pharmacological profile. For example, heterodimers with κ- and δ-opioid receptors have no significant affinity for any of the subtype-selective ligands, but possess high affinity for ligands that are less subtype-selective. Moreover, a specific ligand for one receptor in a heterodimer can alter the binding of a specific ligand for the other receptor. For example, the affinity of an SSTR5-selective agonist for its receptor is affected by dopaminergic ligands when D_2 dopamine receptors are co-expressed (Figure 148).

GPCR interaction with other membrane receptors

One of the most dramatic examples to date of a GPCR-interacting protein is the ligand-gated ion channel $GABA_A$ receptor. The second intracellular loop of the $GABA_A$ receptor γ_2-subunit interacts specifically with the C-terminal tail of the dopamine D_5 receptors. When co-expressed, both receptors can be co-immunoprecipitated. Their interactions depend on the presence of agonists for both receptors:

- GABA reduces the capacity of dopamine to stimulate adenylate cyclase without a reduction in the affinity of dopamine for its receptor.

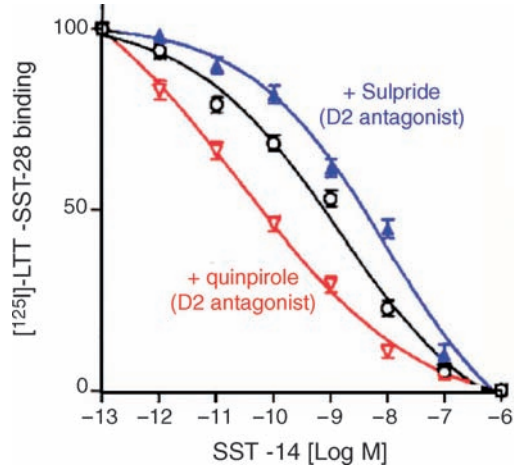


Figure 148 Co-transfection of SSTR5 and D_2 receptors in CHO cells: effect of a D_2-agonist on the competition binding characteristics of the SSTR5 agonist SST14. From Rocheville M. *et al.*, *Science*, **288**, 154–157, © (2000). Reprinted with permission from AAAS.

- The presence of the D_5 receptor also alters the function of the $GABA_A$ receptor: i.e. dopamine reduces $GABA_A$ receptor signalling.

GPCR interaction with other membrane proteins

RAMPs (receptor activity modifying proteins) constitute a well-known example. RAMP1 is a 148 amino acid protein that is predicted to have a single membrane-spanning domain, a large extracellular domain and a short cytoplasmic domain (Figure 149). RAMP2 and RAMP3 share ± 30% sequence identity with RAMP1. They form a family of chaperone proteins which interact with the calcitonin receptor-like receptor (CRLR), the related the calcitonin receptor and presumably with several other family B GPCRs.

The most striking consequence of RAMP-GPCR associations is the altered pharmacological profile/ phenotype of those receptors (Figure 152):

- The heterodimer between CRLR and RAMP1 behaves pharmacologically like a CGRP (calcitonin gene related peptide) receptor, which is characterized by a higher affinity for the 37 amino acid vasodilatory peptide CGRP than for adrenomedullin (Figure 150). CRLR itself does not display high affinity for CGRP and this explains why transfection with CRLR-coding DNA was unable to yield CGRP-responsive cells if they did not contain sufficient amounts of endogenous RAMP1.
- CRLR interaction with RAMP2 or RAMP3 generates an adrenomedullin receptor.
- The calcitonin receptor also interacts with individual RAMPs to generate different amylin receptors.

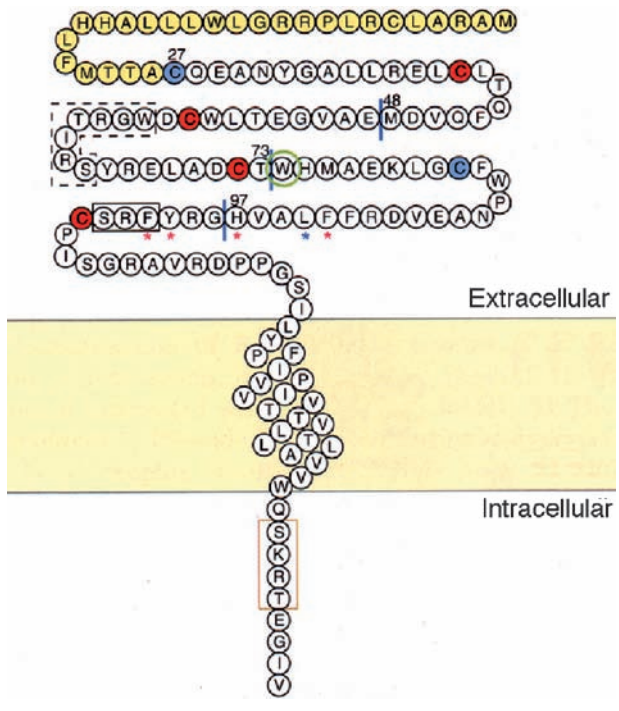
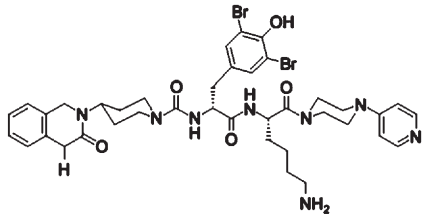


Figure 149 Structure of RAMP1. Reprinted from *Trends in Pharmacological Science*, **24**, Morfis, M., Christopoulos, A. and Sexton, P, M., RAMPs: 5 years on, where to now?, 596-601. Copyright (2003), with permission from Elsevier.

The pharmacological profile of the RAMP–GPCR heterodimers is determined by specific interactions. Extracellular domains of both proteins appear to play a primordial role in this process. CGRP, adrenomedullin and related peptide ligands are supposed to bind within the central cleft of the RAMP-associated GPCR. Recently, non-peptide antagonists such as BIBN4096BS have been found to display high affinity for the CGRP receptor when human RAMP1 makes up part of it, but not in the case of rat or porcine RAMP1. Mutation studies revealed that this difference could be attributed to the presence of Trp at location 74 in human RAMP1 as compared to a basic amino acid in rat or porcine RAMP1 (Figure 151). Because BIBN4096BS is much more sensitive

```
ADM       YRQSMNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY
CGRP1                 ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF
CGRP2                 ACNTATCVTHRLAGLLSRSGGMVKSNFVPTNVGSKAF
AMYLIN                KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY
CALCITONIN            CGNLSTCMLGTYTQD-----FNKFHTFPQTAIGVGAP
```

Figure 150 The amino acid sequences of the calcitonin family peptides (aligned for comparison).



	K_i BIBN4096BS
	nM
hCRLR/hRAMP1	0.018
rCRLR/rRAMP1	2.1
rCRLR/hRAMP1	0.032
hCRLR/rRAMP1	1.8
SK-N-MC	0.004
Rat brain	1.5

Figure 151 Chemical structure of BIBN4096BS (reprinted from *Trends in Pharmacological Science*, **21**, C. Juaneda, Y. Dumont and R. Quirion, The molecular pharmacology of CGRP and related peptide receptor subtypes, 432–438. Copyright (2000), with permission from Elsevier) and affinity for heterodimers between CRLR and different RAMPs (r = rat, h = human) (Mallee *et al.*, 2002, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

to the nature of the amino acids at location 74, it is currently believed that this and related antagonists act at the interface between RAMP 1 and CRLR.

RAMPs also assist/chaperone in the transport of CRLR to the cell surface (Figure 152). In this respect, CGRPs and their RAMP partners already form stable

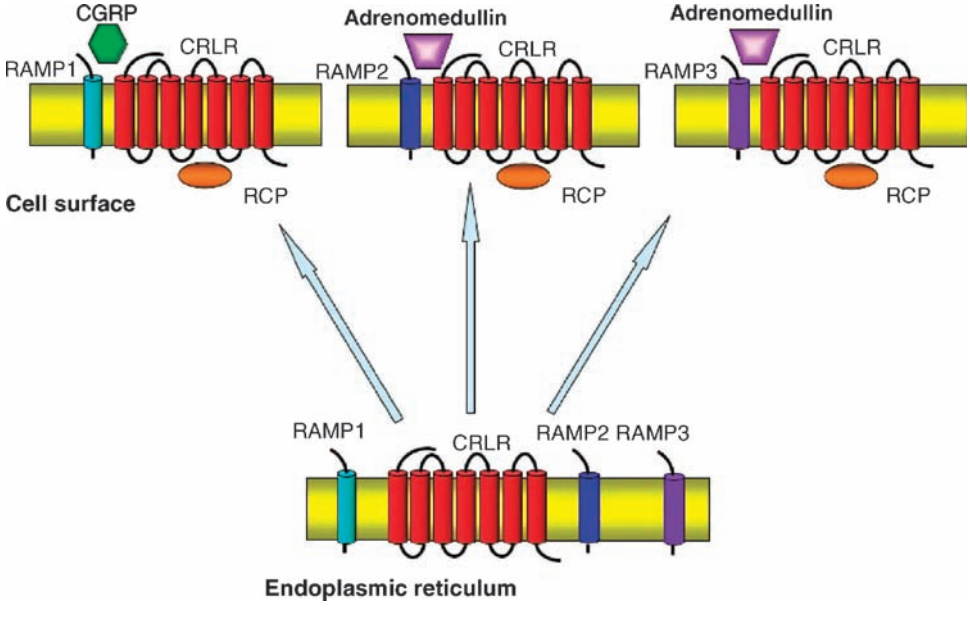


Figure 152 Assembly, transport and schematic view of the CGRP receptor complexes. RCP: receptor component protein.

Table 16 Effect of chaperone proteins on the cell surface expression and pharmacological profile of GPCRs. Reprinted from *Cellular Signalling*, **14**, Brady, A. E. and Limbird, L. E., G protein-coupled receptor interacting proteins: Emerging roles in localization and signal transduction, 297–309. Copyright (2002), with permission from Elsevier.

GPCR–chaperone interactions		Functional role(s) implicated
Rhodopsin (Rh1)	nina A (*Drosophila*)	Rh1 transport to surface of R1-6 photoreceptor cells
Odr10 (C. elegans)	Odr4 (*C. elegans*)	Transport Odr 10 to surface of *C. elegans* olfactory cilia
rFSH, rLH receptor	Calnexin	Detected in endoplasmic reticulum
V2 receptor	Calnexin	Overexpression of calnexin slows delivery of WT-V2 receptor to surface; interaction appears to account for intracellular accumulation of V2 receptor alleles responsible for X-linked nephrogenic diabetes
CRLR	RAMP1	Creates CGRP receptor at cell surface capable of stimulating cAMP generation
CRLR	RAMP2	Creates adrenomedullin receptor at cell surface capable of stimulating cAMP generation

heterodimers in the endoplasmic reticulum and golgi apparatus. These complexes are maintained during the process of translocation to the cell surface, agonist activation, internalization and lysosomal degradation. Olfactory receptors are another example of GPCR requiring a chaperone for proper cell surface expression (Table 16). Considerable biological diversity may arise when cells and tissues can exhibit differential pharmacological profiles based on the expression pattern of accessory proteins such as RAMPs.

Finally, RAMPs are also essential for terminal CRLR glycosylation, but this process does not seem to affect the recognition properties of the receptor.

Recent studies (McLatchie *et al.*, 1998) revealed the occurrence of four additional family B GPCRs that also bring RAMPs to the cell surface. VPAC1, the vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor, interacts with all three RAMPs. The glucagon receptor and the PTH1 parathyroid hormone receptor interact with RAMP2 and the PTH2 receptor interacts with RAMP3. Accordingly, a number of family B receptors may represent 'core subunits' that interact with RAMPs to produce physiologically relevant receptors. On the other hand, there are receptors within this family (such as CRF receptors) that have not been linked to a RAMP protein.

Other membrane proteins may affect the GPCR–G protein interaction. An interesting example is the D_1 dopamine receptor, which is routinely considered to stimulate cAMP production by activating G_s. However, a large increase in Ca^{2+} concentration is also obtained in the presence of calcyon (a glycosylated 24 kDa single TM protein that

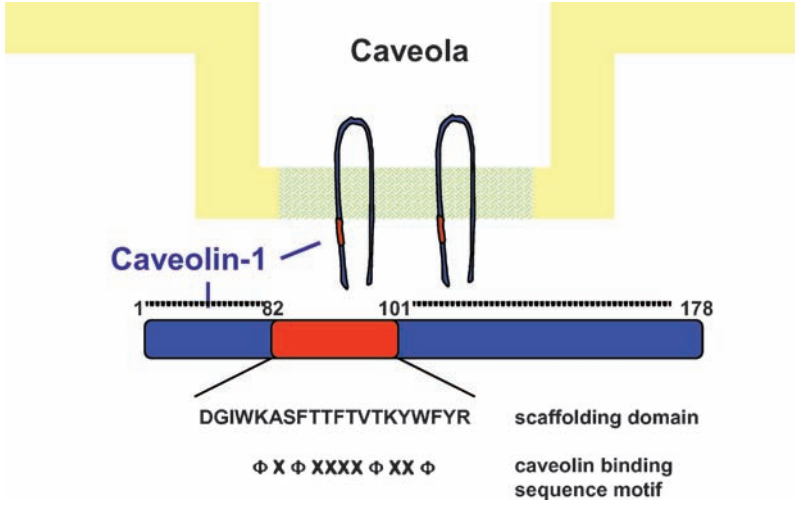


Figure 153 Structure of caveolae, caveolin and the caveolin binding sequence motif of other proteins (X = any amino acid; Φ = Phe, Trp or Tyr) (Okamoto *et al.*, 1998, reproduced by permission for the American Society for Biochemistry and Molecular Biology).

interacts within a 16 amino acid segment of the C-terminal tail of the D_1 receptor). Calcyon is believed to increase the affinity of the D_1 receptor for $G_{q/11}$.

The integral membrane protein caveolin forms an oligomeric coat structure around the bulb of caveolae (i.e. cholesterol-containing 50–100 nm 'flask-shaped' invaginations of the plasma membrane) (Figure 153). It spans the membrane twice and is capable of binding GPCRs (via a consensus site such as Tyr^{302}–Tyr^{312} of the AT_1 angiotensin receptor) and other different proteins that are involved in cell signalling. Therefore, it can serve as a scaffold and bring GPCRs in close proximity to other signalling pathways. This topic will be further discussed in Section 4.10.

GPCR interactions with cytoplasmic proteins

GPCRs have been found to interact with increasing numbers of cytoplasmic proteins (Table 17). The physiological significance of these interactions is often still unclear.

RCP is a small (20 kDa) intracellular peripheral membrane protein. Unlike the RAMP proteins, RCP does not act as a chaperone, but rather plays a role in coupling these receptors to downstream signalling molecules. In this respect, the new model for CGRP/adrenomedullin signalling requires a complex of three proteins to confer full receptor-mediated function: CRLR, RAMP1 or 2 and RCP (Figure 152).

Whereas G proteins are widely recognized to play a central role in the transfer of information between GPCRs and cellular effectors, several recent findings suggest that non-G proteins can also convey this information. To deal with these new findings, it has been suggested that 'G protein-coupled receptors' should no longer be called such,

Table 17 GPCR interaction with cytoplasmic proteins. Reprinted from *Cellular Signalling*, **14**, Brady, A. E. and Limbird, L. E., G protein-coupled receptor interacting proteins: Emerging roles in localization and signal transduction, 297–309. Copyright (2002), with permission from Elsevier.

GPCR interacting proteins		Functional role(s) implicated
β2 receptor	NHERF/EBP50	Regulation of brush border NHE3 activity; sorting selection of surface recycling versus lysosomal targeting after agonist-evoked endocytosis
5HT2C receptor	MUPP1	Not known, although co-localized in native brain
SSTR2	SSTRIP (member of Shank/Spank/ synamon family)	At postsynaptic densities; critical for PSD localization and/or SSTR2 signalling
D2 receptor	spinophilin	Function unknown
α2 receptor (A,B,C)	spinophilin	Agonist-enhanced co-IP in heterologous cells; function in native cells unknown
AT1a receptor	ATRAP	Interaction via AT1a receptor C-terminus; ATRAP overexpression inhibits angiotensin-stimulated PLC, smooth muscle cell growth; increases AT1a receptor internalization
CRLR/RAMP1 or 2	RCP	Trimer permits CGRP receptor (CLR/ RAMP1) and adrenomedullin receptor (CCRLR/RAMP2) to couple to activation of adenylyl cyclase
mGluR5 mGluR1α	Homer-1a	Regulation of mGluR availability for interaction with Homer-2 and -3 and their interacting proteins at postsynaptic densities; Homer-1a expression is upregulated by seizure-induced activation of the hippocampus
mGluR5, mGluR1α	Homer-1b	Retention of receptors in ER
mGluR5, mGluR1α	Homer-1c	Clustering of receptors at cell surface; enriched dendritic localization in rat hippocampal neurons
β2 receptor	AKAP250 (gravin)	Regulates receptor (gravin) sequestration and resensitization; associates with β2 receptor during agonist-elicited endocytosis
β2 receptor	AKAP79 (rat AKAP150)	Enhances β2 receptor phosphorylation by PKA
D2 R (long, short forms)	ABP-280 (Filamin A)	Clustering of receptors at cell surface; increased efficiency of coupling of receptor to inhibition of adenylyl cyclase
α2 receptor	14-3-3 ξ	Function unknown; competed for by phosphorylated Raf peptide
GABAB R1	14-3-3 ξ and η	Not known
α2 /β2 receptors	EIF2Bα	Subtle (15%) decrease in β2 receptor-stimulated adenylyl cyclase
EIF2Bα		Activity; colocalisation in surface blebs created by overexpression, but not at points of cell–cell contact

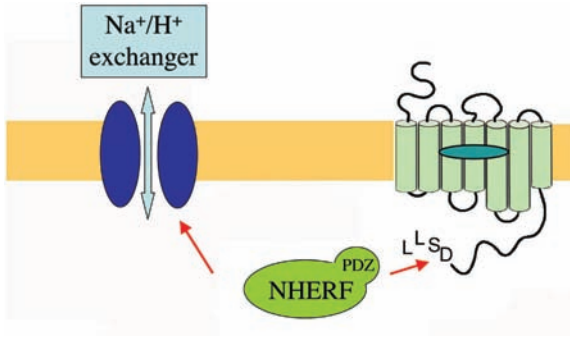


Figure 154 β_2-adrenergic receptor-mediated stimulation of the Na^+/H^+ exchanger type 3 in the plasma membrane. Reprinted from *Trends in Pharmacological Science*, **19**, Schwartz, T. W., IJzerman, A. P., Principles of agonism: undressing efficacy, 433–436. Copyright (1998) with permission from Elsevier.

but that their denomination as 'heptahelical receptors' or '*7TM receptors*' would be more appropriate.

Examples of alternative signalling pathways are:

- β_2-adrenergic receptors also activate a Na^+/H^+ exchanger type 3 (NHE3) in the cell plasma membrane independently of G_s (Figure 154). To this end, the activated receptor directly associates with the Na^+/H^+ exchanger regulatory factor (NHERF). Activated NHERF then activates the Na^+/H^+ exchanger, resulting in increased sodium reabsorption in the proximal tubule of the kidney. NHERF interacts through its 'PDZ domain' with the C-terminal tail of the β_2 receptor. The affected receptor region is not involved in the recognition of G proteins since a Leu to Ala mutation of the final residue of the receptor abolishes its interaction with NHERF without altering its ability to activate the adenyl cyclase system. Besides NHERF, GPCRs have been shown to interact with a number of PDZ domain-containing proteins, such as spinophilin.
- Recently, some GPCRs, including M_3 muscarinic acetylcholine receptor and H_1 histamine receptor, have been suggested to stimulate the membrane-associated phospholipase D enzyme in an Arf- and RhoA-dependent manner, without the involvement of G proteins. Receptors of this type contain an AsnProXXTyr motif in their TM7 domain that is able to form complexes with Arf and RhoA, leading to the activation of phospholipase D.
- Agonist-stimulated β_3-adrenergic receptors stimulate the ERK/MAP kinase cascade by directly binding to c-Src. This interaction requires proline-rich motifs (PXXP) in the endo3 loop and the C-terminal end of the receptors and the SH3 domain of c-Src. By contrast, the interaction between c-Src and the β_2-adrenoceptor requires β-arrestin as a scaffold.

GPCRs also interact with cytoplasmic proteins with a 'scaffolding' function (Wang and Limbird, 2002):

- Actin-binding protein-280 (ABP-280), also known as filamin A, is an abundant cytoplasmic protein that may act as a scaffold to anchor a number of different molecules at the cytoskeleton. ABP-280 helps D_2 receptor clustering (micro-compartmentalization) at the plasma membrane and, by enhancing the efficiency of coupling to effectors, it facilitates D_2 receptor signalling. This may explain the increased sensitivity of presynaptic D_2 to agonist stimulation (when compared to postsynaptic D_2 receptors).
- Spinophilin (120 kDa), endo3 (PDZ domain-containing) F-actin-binding protein, has been found to interact with the loop of both D_2 and α_2-adrenergic receptors. It may act as a scaffolding protein that links signalling proteins to microdomains at the cell surface (e.g. α_2 receptor retention at the basolateral surface of polarized epithelial cells).
- 14-3-3 proteins (31 kDa) are predominantly cytosolic and expressed as seven known mammalian isoforms. They recognize a phosphorylated serine/threonine motif (RSxSxP) of molecules like α_2-adrenergic receptors and modulate their function. 14-3-3 proteins exist as dimers, thus allowing them to act as a scaffold for coordinating signal transduction.

The interactions of the α_2-adrenergic receptor endo3 loop with spinophilin, 14-3-3ζ, and arrestin 3 are capable of competing with each other (Figure 155). Relative affinities of these peptides for the endo 3 loop of the receptor can be tested with a pull-down assay where a glutathione-S-transferase (GST)-spinophilin fusion protein is present at the surface of (glutathione) GSH-agarose. This is incubated with the ^{35}S-labelled endo3 loop of the α_{2A}-adrenergic receptor and increasing concentrations of competing peptides. At the end, the amount of ^{35}S present on the beads is measured and the relative binding affinity of the competing peptides can be evaluated.

4.9 Early models for GPCR activation

Earlier mechanistic hypotheses largely emphasised the fluid-mosaic model of plasma membrane structure (Singer and Nicolson, 1972) and the notion of 'collision coupling' of the components. Implicit in this explanation was the idea that GPCRs, G proteins and effector components were physically separate entities that were free to diffuse in the membrane. Models that gathered widespread acceptance in the late seventies and eighties are the *Collision Coupling Model* (Tolkovsky and Levitzki, 1978) and the *Ternary Complex Model* for GPCR activation (De Lean *et al.*, 1980) (Figure 156). Please note that, in this early models, R was inactive and that RG and ARG complexes were active (and represented the stimulus S); i.e. no attention was paid to the need of the receptor to adopt an active conformation before G protein coupling.

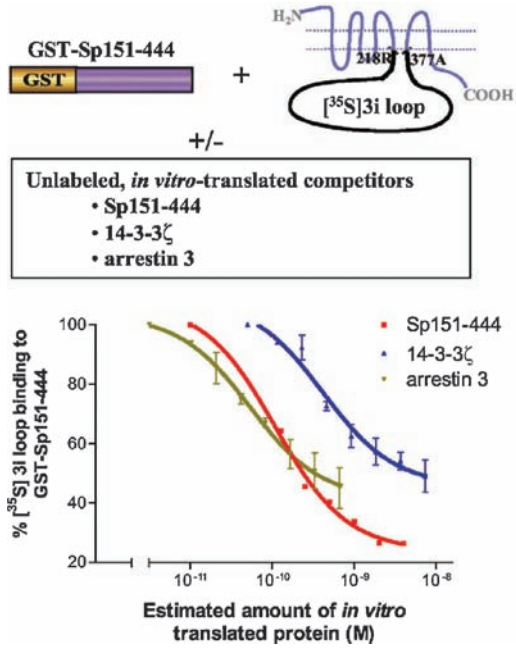


Figure 155 Pull-down assay to compare the relative affinities of different cytoplasmic proteins for the endo3 loop of α_{2A}-adrenergic receptors (Wang and Limbird, 2002, reproduced by permission for the American Society for Biochemistry and Molecular Biology).

A $\quad A + R + G \overset{K}{\Leftrightarrow} AR + G \overset{L}{\Leftrightarrow} ARG \overset{k_{act}}{\rightarrow} AR + G\alpha + \beta\gamma$

B

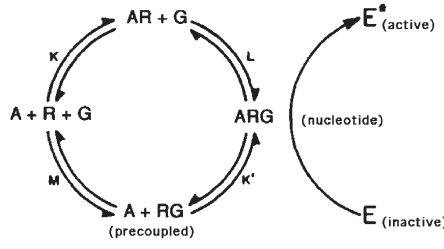


Figure 156 A: The Collision Coupling Model for GPCR activation by Tolkovski and Levitzki (Tolkovsky and Levitzki, 1978) and B: the Ternary Complex Model (De Lean *et al.*, 1980, reproduced by permission for the American Society for Biochemistry and Molecular Biology).

Key features of this model are:

- GPCRs and effector components do not come into contact with one another but rather communicate via shuttling G proteins.
- Receptors have access to all cognate G proteins present at the cell surface. The rate of G protein activation is proportional to the collision frequency between the agonist–receptor complex (AR) and the trimeric G protein; i.e. it is proportional to [AR] and [G]. The formation of the ternary ARG complex is slow compared to G protein activation (i.e. the step with k_{act} as rate constant). This means that the ARG complex is only transient in the presence of guanine nucleotides like GTP.

ARG complexes can be formed with agonists and these complexes display higher agonist affinity (i.e. Ki_H) as compared to AR (i.e. Ki_L). In fact, the presence of at least two distinct binding sites on the same receptor protein, one for the agonist and one for the G protein represents a simple example of an allosteric interaction: the agonist increases the receptor's affinity for the G protein and the G protein increases the affinity of the receptor for the agonist. G protein activation cannot take place in the absence of guanine nucleotides so that the ternary ARG complex is allowed to accumulate. High-affinity binding of radiolabelled agonists to cell membranes is therefore largely due to the formation of ternary ARG complexes.

In contrast, antagonists (I) will bind to the receptor without producing ternary IRG complexes. Hence, all receptors of the same kind will behave as a single class of antagonist binding sites. Agonist versus labelled antagonist competition binding curves on cell membrane preparations are often shallow in the absence of guanine nucleotides (Figure 157). This can be explained by:

- The high affinity component of the competition binding curve corresponds to ARG while the low affinity component corresponds to AR.
- Not all receptors can form an ARG complex at the same time. The original explanation (De Lean *et al.*, 1980) was a stoichiometric limitation in the amount of available G proteins.

It seems that the K_{iL}/K_{iH}-ratio in agonist/labelled antagonist competition binding curves on membranes can be used to predict agonist intrinsic efficacy. When comparing such data with agonist concentration–response curves, please remember that the receptor reserve can mask partial agonist activity, and so result in a misleading classification of ligands.

When an excess of GTP (or analogues such as the non-hydrolyzable analogues Gpp(NH)p or GTPγS) is added, GTP versus GDP exchange can adequately take place so that the ARG complex falls apart in AR, GTP-bound-Gα and β–γ. This process

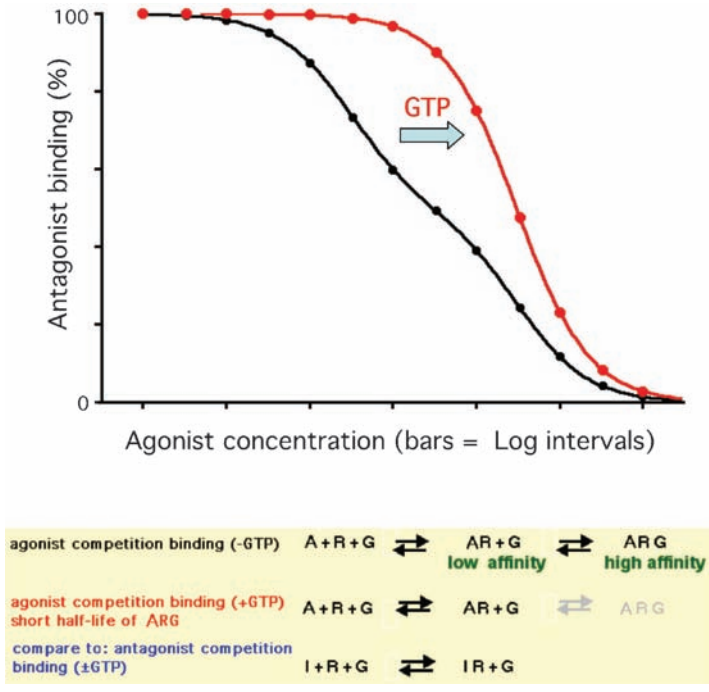


Figure 157 Receptor–G protein coupling: effect of GTP on agonist versus radiolabelled antagonist competition binding, when GTP is present only low agonist affinity binding sites are detected.

is likely to proceed swiftly so that the concentration of ARG remains virtually nil (Figure 157). This adequately explains why:

- Binding of radiolabelled agonists to membrane preparations is greatly reduced in the presence of GTP.
- The shallow agonist competition binding curves become steep and only display the low affinity component in the presence of GTP.

High affinity agonist saturation and/or competition binding in transfected cell systems can only be expected when the appropriate G protein is present in sufficient quantities to produce observable ternary complexation.

The simplicity of the ternary complex model makes it easy to verify its validity. To deal with new experimental findings, the model has been adapted over the years. Major modifications deal with:

- Restricted GPCR–G protein coupling.
- Constitutive receptor activity.

- Receptor coupling to different G proteins.
- Multiple affinity states of the receptor.

4.10 Restricted GPCR mobility and G protein coupling

Membrane compartimentalization

Controversy has arisen over why agonist competition curves are shallow in the first place. The initial explanation was based on the assumption that there is a stoichiometric limitation to the amount of G proteins. However, direct methods of measurement have subsequently shown that G proteins may be present in large excess over the number of cognate receptor proteins. For example, there is a substantial excess of G_s relative to both β-adrenergic receptors and adenylate cyclase in S49 murine lymphoma cells (R:G_s:effector ratio ~ 1:100:3). For $G\alpha_i$, it is believed that levels of expression are somewhat greater than those of $G\alpha_s$. Based on the assumed uniform accessibility of individual G proteins, agonist competition curves are expected to display a uniform population of high affinity sites in competition binding studies. To date, this has never been shown to take place. On the contrary, several experiments suggest that receptors may only have limited access to the total pool of G proteins.

- If different receptors couple to the same G protein pool and if the G proteins therein would be limiting and freely mobile, the ternary complex model predicts that binding of an agonist at one receptor should compete with other receptors for the same set of G proteins. Accordingly, an unlabelled agonist for one receptor should be able to decrease binding of a radiolabelled agonist to another receptor. Experimentally, even when G_i proteins in NG108-15 neuroblastoma-glioma cells are made limiting by a partial pertussis toxin treatment, δ-opiate or muscarinic receptors still fail to inhibit radiolabelled agonist binding to the α_{2B}-adrenergic receptors in the membranes thereof. Hence, there is no indication of cross talk with these G_i-preferring receptors (Graeser and Neubig, 1993).
- Agonist–β-adrenergic receptor–G_s complexes may be rendered permanent in the presence of the alkylating reagent N-ethylmaleimide (this effect is due to the reagent's ability to alkylate a crucial sulfhydryl group present on G_s) (Figure 158). In membrane preparations from several cell types, this produces a time-wise leftward shift of agonist competition curves and they even become biphasic at the

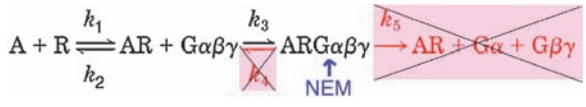


Figure 158 Affinity states for agonists and antagonists. These states are observed in competition binding experiments with radiolabelled antagonists.

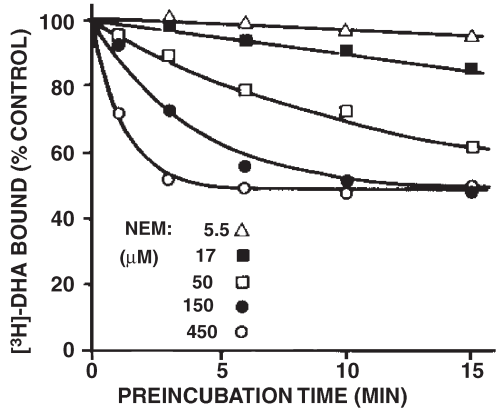


Figure 159 Only part of the β-adrenergic receptors undergo functional coupling to Gs. Turkey eythrocyte membranes were pre-incubated with isoproterenol and N-ethylmaleimide, washed and the remaining (i.e. non-coupled) β_1-adrenergic receptors were quantified by binding of the antagonist [^{3}H]dihydroalprenolol. Reprinted from *Recent Advances in Receptor Chemistry*, (C. Melchiorre and M. Giannella, Eds.), Vauquelin G., Severne Y., Convents A., Nerme V. and Abrahamsson T., Agonist-mediated activation of adrenergic receptors, pp. 43–61. Copyright (1988), with permission from Elsevier.

end. Yet, there is no time-wise increase in the amount of receptor sites with high agonist affinity (Figure 159). This amount appears to be fixed for each cell type (Table 18) and suggests that the β-adrenergic receptors have only limited accessibility to G_s. This may point to limitations of the mobility of the receptors and G proteins.

Table 18 Amount of coupling-prone (i.e. agonist/N-ethylmaleimide sensitive) β-adrenergic receptors in membranes from various tissues. Reprinted from Agonist-mediated activation of adrenergic receptors. In *Recent Advances in Receptor Chemistry*, Vauquelin, G., Severne, Y., Convents, A., Nerme, V. and Abrahamsson, T., Copyright (1988), with permission from Elsevier.

Membrane source	Receptor	Coupling
Turkey erythrocyte	β_1	50%
Human adipose	β_1	46%
Friend erythroleukemia	β_1	69%
S49 Lymphoma (WT)	β_1	65%
(unc, cyc$^-$)	β_1	0%
Human uterus	β_1	50%
Calf trapezius muscle	β_1	45%
Rat brain	$\beta_1 > \beta_2$	36%
Rat lung	$\beta_2 > \beta_1$	50%
Calf lung	β_2	25%

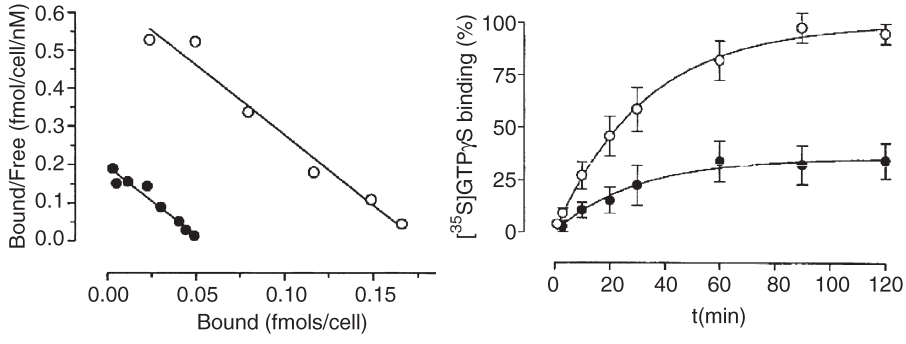


Figure 160 A reduction in the μ-opioid receptor concentration in C6 rat glioma cells produces a similar reduction in μ-agonist-mediated [^{35}S]GTPγS binding. Left: Scatchard plot for cells with different radioligand B_{max} values. Right: [^{35}S]GPPγS binding versus the incubation time, same symbols). Reproduced from Alt, A., Mcfadyen, I. J., Fan, C. D., Woods, J. H. and Traynor, J. R. (2001) *Journal of Pharmacology & Experimental Therapeutics*, **298**, 116–121, with permission from the American Society for Pharmacology and Experimental Theraputics.

- The ternary complex model predicts a reduction in receptor concentration would decrease the likelihood of a random encounter. This should decrease the rate of G protein activation, but should not affect the maximum number of G proteins activated. Yet, a reduction of the μ-opioid receptor concentration in C6 rat glioma cell membranes produced a similar decrease in the maximum μ-agonist-mediated G protein activation (maximal [^{35}S]GTPγS binding) but did not affect the rate of [^{35}S]GTPγS binding (Figure 160). Similar data were found for digitonin-permeabilized cells (digitonin is a cholesterol-binding detergent that makes pores in the membrane so that [^{35}S]GTPγS can get within the cell). These findings suggest that the receptors do not have access to the whole pool of G proteins, but that each receptor is only surrounded by a fixed, limited number of G proteins.
- Cells typically have multiple types of receptors, G proteins and effectors, and it is difficult to understand how specific receptor–effector communication would result from a multitude of promiscuous protein interactions. Still, receptor–effector communication does appear to be quite specific in living cells. Moreover, it is difficult to reconcile the low absolute concentration of GPCR, G protein and effector (ranging from femtomole to low picomole per milligram of protein) with the observed rapid, highly selective interaction of components required for signal transduction in cell membranes.

To account for these considerations, the current dogma is that GPCR signalling components are held in close association with one another as ‘prearranged signalling complexes’: i.e. they are not freely floating or dependent on random collision to interact. Several mechanisms imply some form of organization:

- One explanation is that only some of the receptors in cell membrane preparations are actually present at the cell surface. Indeed, only 5% of the membranes of eukaryotic cells are present at the cell surface. The other 95% are intracellular and make part of, for example, the endoplasmic reticulum, the golgi apparatus and small vesicles. Receptors may be present in the membranes of such intracellular compartments on their way to their translocation to the membrane or after their endocytosis and display only limited access to G proteins (e.g. G proteins not present, post-transcriptional processing of the receptor not yet complete, desensitization) when compared to the receptors at the cell surface.
- Another explanation is that receptors and G proteins are compartmentalized by cytoskeletal divisions of the cellular membrane. In this respect, cell plasma membranes are indeed compartmentalized in large specialized domains such as luminal and basolateral surfaces in epithelial cells. These have a distinct segregation of proteins, including receptors and other signalling molecules. The postsynaptic regions of neuronal target cells also, typically, have high concentrations of certain receptors, transporters and enzymes.
- Another explanation is that G proteins shuttle between receptors and effectors within restricted microdomains in the membrane. In this respect, evidence has been gathered for many signalling molecules to be enriched in lipid rafts/caveolae in the membrane (Figure 161).
- An even more provoking explanation is that receptors and G proteins may be held in constant physical proximity of each other.

Of particular note is that a variety of G proteins and a large number of G protein-coupled receptors have been shown to be enriched in lipid rafts or caveolae. These membrane structures may therefore serve as scaffolding centres for components involved in GPCR signalling:

- Lipid rafts are specialized membrane domains enriched in certain lipids like cholesterol and proteins. Due to the presence of cholesterol, a lipid raft forms a domain that exhibits less fluidity than the surrounding plasma membrane

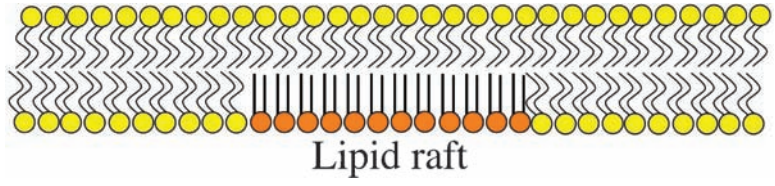


Figure 161 Lipid rafts: Glycosphingolipids and other lipids with long, straight acyl chains are depicted in orange.

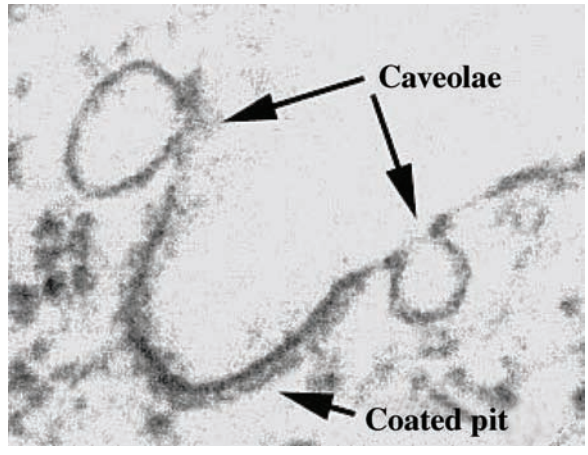


Figure 162 Caveolae and coated pit structures in the osteoblast plasma membrane. Shown: transmission electron micrograph of a murine MC3T3-E1 osteoblast. Reproduced from *Journal of Bone and Mineral Research* 2000, **15**, 2391–2401, with permission of the American Society for Bone and Mineral Research.

(Figure 161). Glycosphingolipids and other lipids with long, straight acyl chains are preferentially incorporated into the rafts. The fatty-acid chains of lipids within the rafts tend to be extended and so more tightly packed, creating domains with higher order. It is therefore thought that rafts exist in a separate ordered phase that 'floats' in a sea of poorly ordered lipids. Lipid rafts have an average diameter in the range of 100 to 200 nm and produce a rather extensive coverage of the plasma membrane surface.

- Caveolae are lipid rafts, which contain the cholesterol-binding protein caveolin-1. Caveolae are identified as 50–100 nm 'flask-shaped' invaginations of the plasma membrane (Figure 162). They are found in a variety of cell types, especially endothelial cells, but none exist as classical invaginated caveolae in neuronal tissues. Caveolin-1 is palmitoylated and forms an oligomeric coat structure around the bulb of caveolae. It binds cholesterol. This appears to be required for its role in maintaining caveolar structure.

Several techniques have been employed to investigate the presence of proteins and protein complexes in lipid rafts/caveolae:

- Fluorescence microscopy: the small size and apparently even distribution of lipid rafts/caveolae might result in an apparently even distribution of the constituent proteins, as visualized by this technique. Nevertheless, this approach has been used to reveal the co-localization of certain proteins with caveolin-1 (Figure 163).

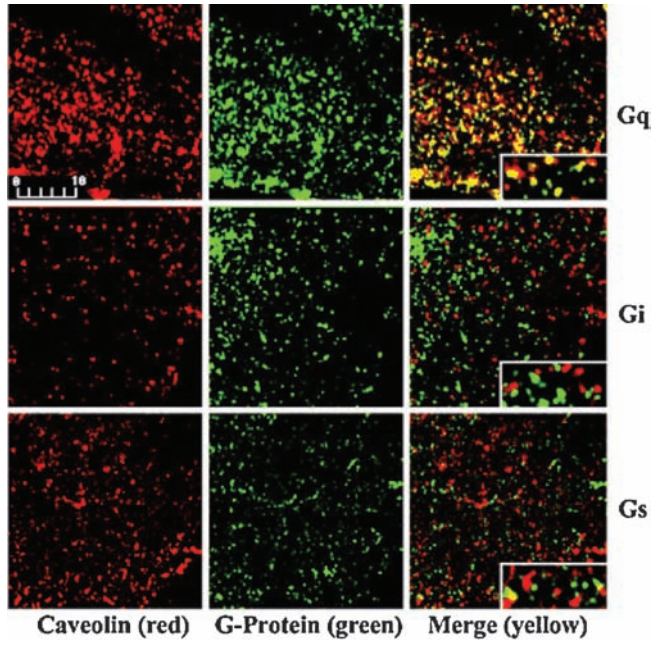


Figure 163 Immunofluorescence microscopy of G protein and caveolin-1 in isolated luminal endothelial cell plasma membranes from rat lung. All G proteins are present in discrete microdomains (i.e. punctate staining). Gq shows the greatest degree of co-localization with caveolin (Oh and Schnitzer, 2001, reproduced by permission of the American Society for Cell Biology).

- The traditional method of preparation of detergent-resistant lipid rafts and caveolae involves scraping cells into cold buffer containing 1% of the detergent Triton X-100, and homogenizing the lysate. Rafts are isolated by flotation in a 5 to 30% linear sucrose density gradient where they distribute in the top few fractions of the gradient. The caveolin-containing lipid rafts can be further separated from non-invaginated rafts by anti-caveolin immunoaffinity purification.
- An indirect approach for studying the function of lipid rafts involves depleting cells of cholesterol with agents (such as filipin or methyl-β-cyclodextrin) that sequester or remove cholesterol. Lipid rafts and caveolae are disassembled and the constituent molecules are dispersed to a more random distribution over the cell surface.

Many proteins and lipids are known to be enriched in caveolae (Table 19). This may, at least in part, be related to the ability of caveolin to recruit proteins bearing caveolin-binding motifs in these structures (see also Figure 153). Caveolin-1 and caveolin-2 are most prevalent in endothelial cells, smooth muscle cells, skeletal myoblasts, fibroblasts and adipocytes. Caveolin-3 is exclusively present in muscle cells, including cardiac myocytes and cells of the arterial vasculature. Caveolin-binding proteins comprise

Table 19 Signalling molecules expressed in caveola. Reproduced from Ostrom, R. S., Post, R. and Insel, P. (2000) *Journal of Pharmacology and Experimental Therapeutics*, **294**, 407–412, with permission from the American Society for Pharmacology and Experimental Therapeutics.

Receptors	Postreceptor components	
β-Adrenergic	G proteins (α and β–γ)	Raf-1
Bradykinin	Endothelial nitric-oxide synthase	Rac-l
Endothe1in	Mitogen-activated protein kinase	RhoA
M_2 muscarinic acetylcho1ine	Adenylyl cyclase	Src kinases
Adenosine A1	PKA (catalytic subunit)	Shc
Cholecystokinin	PKC (α)	Calmodulin
Platelet-derived growth factor	Diacylglycerol	IP_3 receptor
Epidermal growth factor	GRKs	
Insulin	Ras	

GPCRs and other receptors, including many growth factor receptors (EGF receptor, PDGF receptor, insulin receptor, etc.), as well as signal molecules like heterotrimeric G proteins, protein kinase C, Shc, SOS, Raf1 and Src family tyrosine kinases. Thus, the enrichment of receptors and signal molecules in lipid rafts/caveolae enables them to be in close contact with each other and, hence, to facilitate their interaction.

The enrichment of certain GPCRs and G proteins in lipid rafts/caveolae tend to limit the utility of analyzing the total cellular expression (and stoichiometry) of such proteins.

Instead, our conceptual models should take account of:

- The potential compartmentation of molecules *in vivo*. This may be necessary to provide rapid, efficient and specific propagation of extracellular stimuli to intracellular targets. As an example (Figure 164): β_1-adrenergic receptors are significantly enriched in caveolae in cardiac myocytes while PGE_2 receptors are excluded. This explains why overexpression of the type 6 adenyl cyclase enzyme (AC6), which is almost exclusively expressed in caveolae, enhances the maximal cAMP production to β_1-receptor activation, but not to PGE_2 receptor activation (not shown).

- The potential movement (or translocation) of receptors between cellular compartments (Table 20). In this respect, certain GPCRs reportedly translocate out of (e.g. cardiac β_2-adrenergic receptors) or into caveolae (e.g. bradykinin B_1 receptors) upon activation by an agonist.

  In the first example, β_2-adrenergic receptors are enriched in caveolae of myocytes but, upon stimulation, they translocate out of these structures (Figure 165). This may be attributed to receptor desensitization by GRK2. Indeed, when this desensitization is blunted (with βARKct, the C-terminal peptide of GRK2, which blocks activation of endogenous GRK2 by sequestering β–γ), they no longer translocate out of caveolae upon agonist exposure.

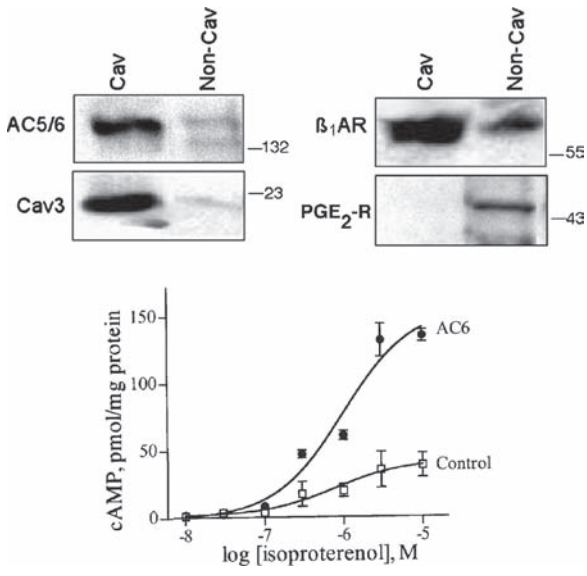


Figure 164 Top: Immunoblot after SDS-PAGE of caveolin-enriched and depleted cardiac myocyte membranes. Bottom: Effect of isoproterenol on cAMP production in membranes of control and in AC6-overexpressed cells (Ostrom *et al.*, 2001, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

Table 20 Movement of receptors between compartments (Pike, 2003, reproduce by permission of the American Society for Biochemistry).

Receptor	Moves into Rafts	Moves out of Rafts	Unaffected by Agonist
Tyrosine kinases			
EGF		X	
ErbB2			X
Insulin	X		X
NGF			X
PDGF			X
G protein-coupled			
Adenosine A1		X	
Angiotensin II type 1	X		
β_2-Adrenergic		X	
β_1-Adrenergic			X
M2 Muscarinic cholinergic	X		
Bradykinin 1,2	X		
Endothelin			X
Rhodopsin			X

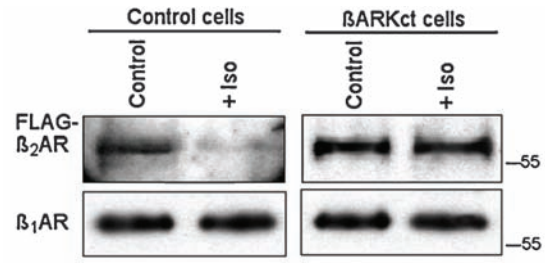


Figure 165 Immunoblot after SDS-PAGE of caveolin-enriched cardiac myocyte membranes. Effect of agonist (isoprotenorol, ISO) exposure on the presence of β_1- and β_2-adrenergic receptors in caveolae with (control cells) or without (cells transfected with βARKct) endogenous GRK2 activity (Ostrom *et al.*, 2001, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

- In the second example (Ostrom, 2002) (Figure 166), bradykinin B_1 receptors are predominantly localized in non-caveolar domains in unstimulated cells (A). Upon agonist exposure they generate a response (increase in $[Ca^{2+}]_i$ and stimulation of intracellular phospholipase A_2) via $G\alpha_q$ and PLC-β *and* translocate to caveolin-rich domains (B). Since PLC-β and its substrate (PIP_2)

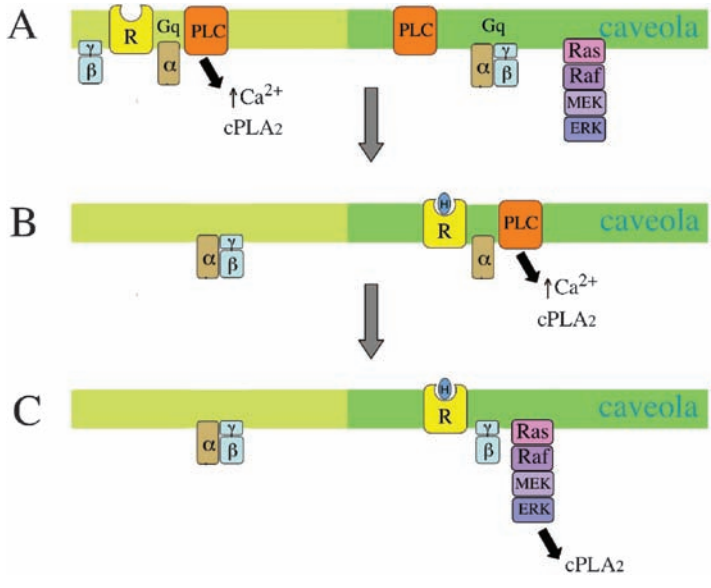


Figure 166 Schematic diagram illustrating the localization and translocation of bradykinin B_1 receptors and the potential effect on their signalling. Reproduced from Ostrom, R. S. (2002) *Molecular Pharmacology*, **61**, 473–476, with permission from the American Society for Pharmacology and Experimental Therapeutics.

are enriched in caveolae, the response is facilitated. The increase in $[Ca^{2+}]_i$ is only transient but, as the receptors do not internalize, they become able to activate ERK/MAP kinase signalling (again resulting in the stimulation of intracellular phospholipase A_2 enzymes) (C). This signalling is again facilitated by the fact that caveolae are enriched in upstream components of the ERK/MAP kinase pathway.

The overall picture is that lipid rafts/caveolae may exert both positive and negative control on signal transduction:

- In their positive role, receptors, coupling factors, effector enzymes and substrates would be co-localized in (or recruited to) single lipid rafts/caveolae. Signal transduction would occur rapidly and efficiently because of the spatial proximity of the interacting components. In this respect, it is of interest that cholesterol depletion generally impairs G protein-mediated signalling, suggesting that these signalling events require intact lipid rafts/caveolae and proceed within these membrane domains. Hence, it is believed that the spatial proximity of signalling components might be the rule rather than the exception for GPCR-mediated signalling as well as for the cross talk between GPCRs and other signalling systems, such as the ERK/MAP kinase system.
- In their negative role, rafts may spatially segregate interacting components to block non-specific pathways of activation. They may also directly suppress the activity of signalling proteins present in rafts (and explain why cholesterol depletion may increase signalling in a limited number of cases), favour the exit of activated receptors or even favour their desensitization and internalization. In this respect, it has been anticipated that caveolae are sites of endocytosis. This is due to their similarity in appearance to clathrin coated pits as they pinch off the plasma membrane. Various stimuli can lead to internalization of caveolae but, under normal conditions, they represent a largely immobile plasma membrane compartment not involved in constitutive endocytosis. It should be emphasized that caveola-mediated endocytosis is distinct from that of coated pits. Coated pit inhibitors do not affect caveolae internalization whereas the cholesterol-binding agent fillipin inhibits caveolae internalization without affecting coated pits. In the example (shown in Figure 167), fillipin was unable to inhibit the agonist-mediated internalization of AT_{1A} receptors. This suggests that the AT_{1A} receptor internalization process predominantly occurs via clathrin-coated vesicles. Additional evidence for such mechanism is produced by:

  - The requirement of β-arrestins as well as dynamin.
  - The inhibition by sucrose (which inhibits clathrin-coated vesicle-mediated endocytosis).

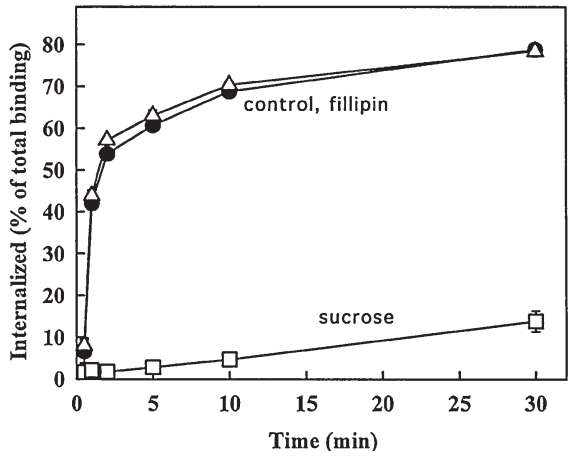


Figure 167 Treatment of AT_{1A} receptor-expressing CHO cells with filipin does not affect Ang II-mediated endocytosis of the receptor (i.e. acid resistant [^{125}I]-Ang II binding). Reproduced from Gaborik, Z., Szaszak, M., Szidonya, L., Balla, B., Paku, S., Catt, K. J., Clark, A. J. L. and Hunyady, L. (2001) *Molecular Pharmacology*, **59**, 239–247, with permission from the American Society for Pharmacology and Experimental Therapeutics.

Restricted GPCR–G protein coupling: effector activity

The ternary complex model by De Lean *et al.* (1980) is a *shuttling model*. The ligand-activated receptor activates G proteins, which freely diffuse to the effector enzymes allowing more G proteins to be activated. This should result in 'unlimited' G protein activation. When the numbers of receptors and effector proteins are similar, the shuttling model predicts that, upon increasing the amount of G proteins present, the maximal response (e.g. cAMP production) should increase first (Figure 168). When the amount of G proteins is increased further, it will produce a leftward shift of the agonist concentration response curves.

The two next models both comprise restricted mobility of GPCRs and G proteins:

- In the *complexing model*, the activated G protein remains bound to the receptor during its interaction with the effector. This blocks the interaction of the receptor with more G proteins (Figure 169). When the numbers of receptors and effector proteins are similar, the G protein activation (and therefore even the activity of the effector) is limited by the amount of receptors available, and no marked leftward shift of the response curve is observed. However, shifts may be observed if receptor levels are slightly increased.
- In the *pre-coupled mode*, the G protein is bound to the receptor even in the absence of the ligand and remains bound to the receptor during its interaction with the effector enzyme. This also blocks the interaction of the receptor with more

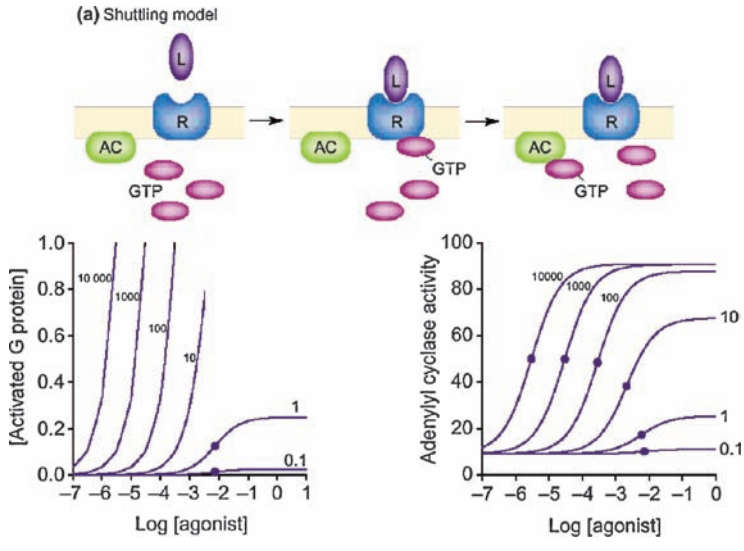


Figure 168 Top: Schematic representation of the classical 'shuttling model'. Bottom: Simulated agonist concentration versus (left) G protein activation and (right) cAMP production curves for different (indicated) G protein:receptor ratios. Reprinted from *Trends in Pharmacological Sciences*, **22**, Kukkonen, J. P., Nasman, J. and Akerman, K. E., Modelling of promiscuous receptor-Gi/Gs-protein coupling and effector response, 616–622, © (2001), with permission from Elsevier.

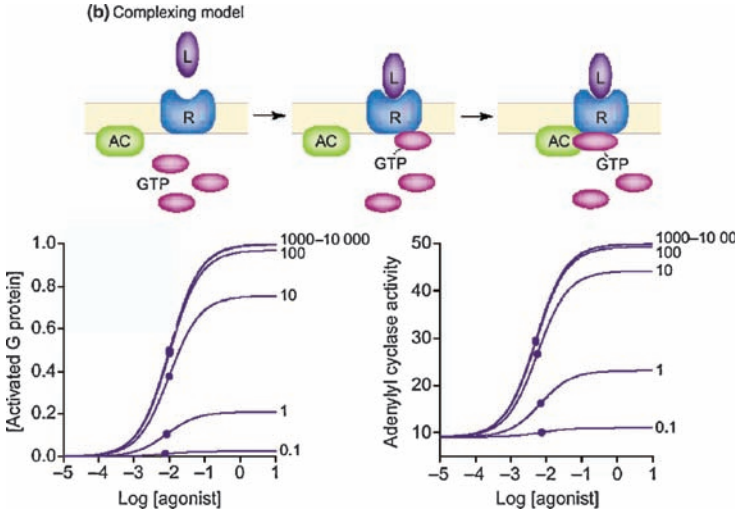


Figure 169 Top: Schematic representation of the 'complexing model'. Bottom: Simulated agonist concentration versus (left) G protein activation and (right) cAMP production curves for different (indicated) G protein/receptor ratios. Reprinted from *Trends in Pharmacological Sciences*, **22**, Kukkonen, J. P., Nasman, J. and Akerman, K. E., Modelling of promiscuous receptor-Gi/Gs-protein coupling and effector response, 616–622, © (2001), with permission from Elsevier.

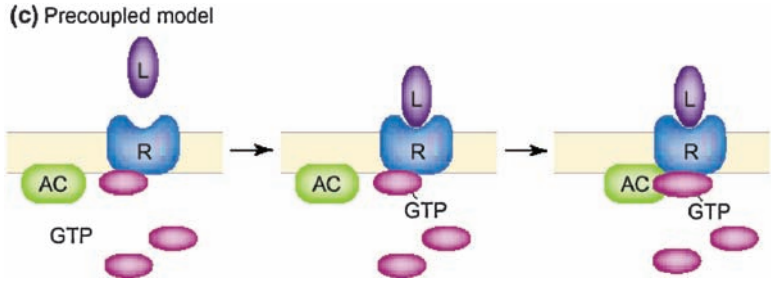


Figure 170 Schematic representation of the 'pre-coupled model'. Simulated agonist concentration versus G protein activation and cAMP production curves are similar to those for the 'complexing model'. Reprinted from *Trends in Pharmacological Sciences*, **22**, Kukkonen, J. P., Nasman, J. and Akerman, K. E., Modelling of promiscuous receptor-Gi/Gs-protein coupling and effector response, 616–622, © (2001), with permission from Elsevier.

G proteins (Figure 170). The simulated data according to this model are very similar to those obtained for the complexing model.

4.11 Spontaneous receptor–G protein coupling

Models

The models in this chapter are based on the assumption that receptors can only adopt an inactive (R) and an active (R*) conformation in the absence of G proteins and that only the latter is capable of G protein coupling. One view is to consider that agonists are necessary for receptor activation and that it is only the active agonist–receptor (AR*) complex that induces G protein activation (Figures 171 and 172). This model is, in fact, derived from Koshland's famous '*induced fit model*' (Koshland *et al.*, 1966). For such coupling to take place, the bound agonist is obviously supposed to induce a conformational change in the receptor.

'Ligand induction' predicts that transition from the inactive (R) to the active (R*) state is extremely rare in the absence of agonist because of the energy barriers between R and R*. The free energy of agonist binding to R is used to overcome the energy barrier and facilitates (or induces) the transition to R*. If the process of receptor activation is explicitly stipulated, the collision coupling model (Tolkovsky and Levitzki, 1978) can be represented by Figure 171.

$$A + R + G \quad \Leftrightarrow \quad AR + G \quad \overset{L}{\Leftrightarrow} \quad AR^* + G \quad \Leftrightarrow \quad AR^*G$$

Figure 171 Agonist-induced transition from the inactive (R) to the active (R*) receptor state is followed by G protein coupling to the active agonist–receptor complex (AR*).

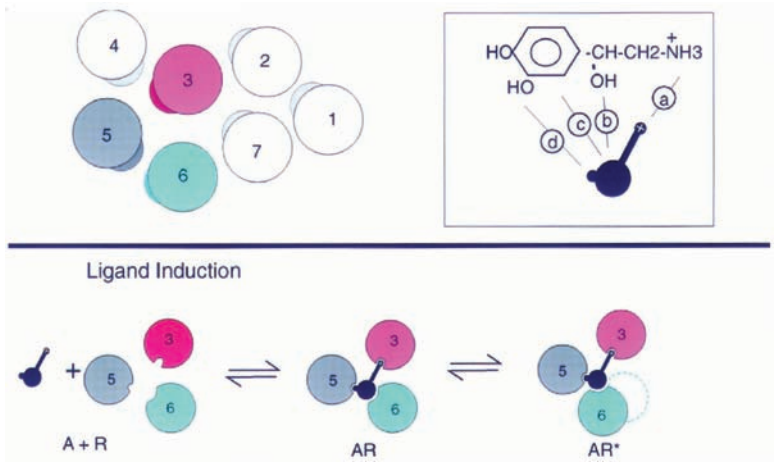


Figure 172 Ligand induction model for noradrenaline-induced β_2-adrenergic receptor activation (Gether and Kobilka, 1998, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

The second equilibrium (defined by equilibrium constant L) forms the key element for discriminating between agonists and antagonists:

- For antagonists, the second equilibrium is completely shifted to the left; all of the occupied receptors remain in the inactive conformation.
- For agonists, the second equilibrium is shifted more to the right for strong agonists as for weak agonists, so that more of the occupied receptors reside in the active conformation.

The concept of agonist-induced receptor conformation has been the subject of many debates, especially since the discovery of constitutive GPCR activity in recombinant receptor systems. In this respect, spontaneous receptor–G protein complex formation can be demonstrated by, for example:

- Co-immunoprecipitation of receptors and G proteins under basal conditions.
- The fact that basal levels of cellular activity in many systems is directly dependent on the receptor density.

A situation in where receptors acquire the active conformation and couple to G proteins even in the absence of ligands can be described by an alternative '*conformational selection model*' (Figures 173 and 174), which is derived from the 'plausible model' (Monod *et al*., 1965). In this model, the active and inactive receptor conformations (i.e. R* and R) are in equilibrium, even in the absence of ligand and the agonist 'favours' a conformational change of the receptor because of its

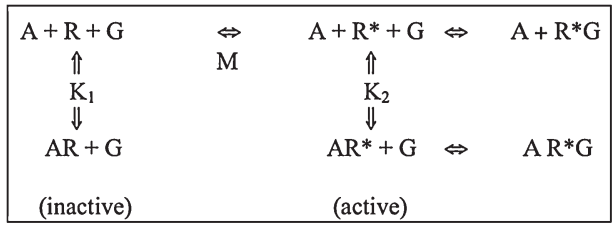


Figure 173 In the 'conformational selection model' ligands may have different affinities for the inactive (R) and the active (R*) receptor states.

higher affinity for R*. The equilibrium dissociation constant for the transition between the two forms of the receptor (M) is very high since the great majority of receptors are inactive in the absence of ligand. Nevertheless, this model allows unoccupied receptors to produce a small stimulus. Ligands are able to bind both to R and R* with the 'microscopic' equilibrium dissociation constants K_1 and K_2, respectively:

In this model, agonists can be discriminated from antagonists on the basis of differences between their binding affinities for the active and non-active receptors. This model also provides an explanation for the existence of so-called '*inverse agonists*':

- Antagonists are supposed to bind with equal affinity to both receptor conformations (i.e. $K_1 = K_2$); the [R*]/[R] ratio remains the same as in the basal situation.
- Agonists bind with higher affinity to R* as compared to R (i.e. $K_1 > K_2$) so that the whole equilibrium will be pulled to the right, resulting in an increase in the [R*]/[R] ratio. The K_1/K_2 ratio is higher for full agonists than for partial agonists.

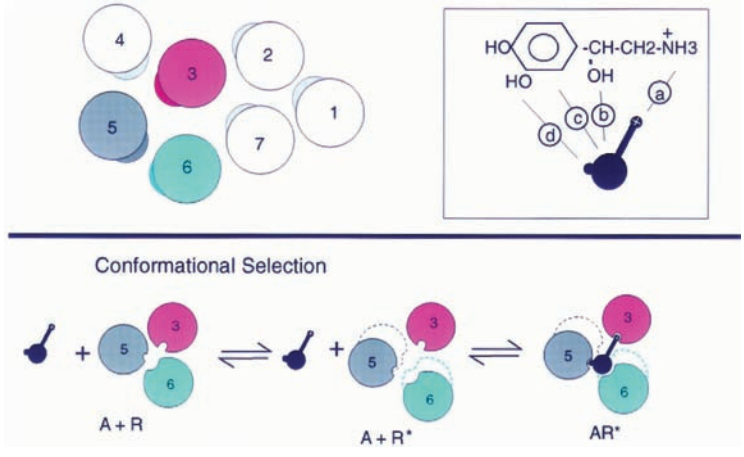


Figure 174 Conformational selection model for noradrenaline-induced β_2-adrenergic receptor activation (Gether and Kobilka, 1998, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

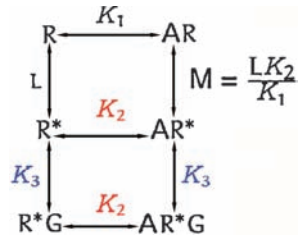


Figure 175 A strict receptor two-state model that integrates 'induced fit' and 'conformational fit'. Reproduced from Krumins, A. M. and Barber, R. (1997) *Molecular Pharmacology*, **52**, 144–154, with permission from the American Society for Pharmacology and Experimental Therapeutics.

- Inverse agonists bind with higher affinity to R compared to R* (i.e. $K_1 < K_2$) so that the whole equilibrium will be pulled to the left, resulting in a decrease in the [R*]/[R] ratio.

In general, it is useful to think of conformational induction and conformational selection, but it is unclear which is the predominant one for agonism. The terenary complex model (De Lean *et al.*, 1980) integrates 'induced fit' and 'conformational selection'. The slightly more complex '*strict two-state model*' (Krumins *et al.*, 1997) (Figure 175) also refers to the receptor being in an active conformation (R*) in order to couple to the G protein. In fact, conformational selection and conformational induction represent two extremes of this model. This model makes it possible for the receptor to:

- Be in the active conformation even when not coupled to a G protein.
- Couple to a G protein even in the absence of agonist.

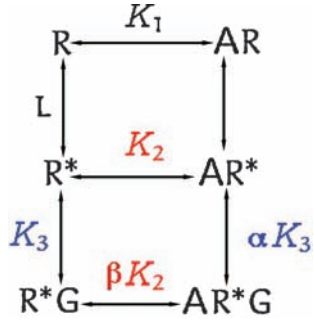


Figure 176 The 'extended ternary complex model' as initially proposed by Samama *et al.* (1993) implies the existence of three or more receptor states (unless α and $\beta = 1$) (Samama *et al.*, 1993, with permission from the American Society for Biochemistry and Molecular Biology).

By increasing the number of receptors present in the system, the number of spontaneously active receptors can be increased until a threshold is attained where the resulting response from the spontaneously formed R*G species can be observed. This explains why constitutive receptor activity is often observed in recombinant systems in where receptors are overexpressed.

The currently most widely accepted model for GPCR activation is the '*extended ternary complex model*' (Samama *et al.*, 1993) (Figure 176). This model has often been referred to as the '*two-state model*'. However, it must be emphasised that in a strict two-state model (Figure 175) R and R* represent uniquely defined conformational states. This means that the affinity of G for R is identical to that for HR (K_2) and its affinity for R* is identical to that for HR* (K_3) (Krumins and Barber, 1997). In the '*extended ternary complex model*' different affinities are used for the binding of H to the R* and the R*G states. This means that a different conformation is assumed for R* when bound and when not bound to a G protein. Since there are at least three states, the '*extended ternary complex model*' is actually a multistate model.

The 'extended ternary complex model' model only allows for the active receptor to interact with a G protein. However, some sparse experimental data also suggest that the inactive receptor is also capable of coupling to G proteins, giving rise to inactive RG and ARG complexes. This has culminated in the development of the even more complex, cubic ternary complex model (Weiss *et al.*, 1996) (Figure 177). Obviously, this model carries so many parameters that it is no longer possible to estimate them based on experimental observations.

Inverse agonism

In contrast to agonists that produce receptor activation and neutral antagonists that do not affect basal receptor activity, inverse agonists are able to decrease basal receptor

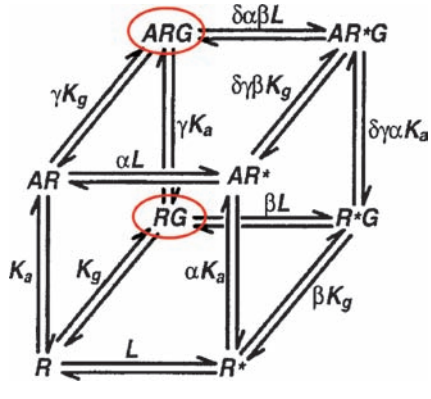


Figure 177 The cubic ternary complex model (Weiss *et al.*, 1996) allows the inactive receptor state to interact with a G protein (circled). Reprinted from *Journal of Theoretical Biology*, **181**, Weiss, J. M., Morgan, P. H., Lutz, M. W. & Kenakin, T. P., The cubic ternary complex receptor-occupancy model III. Resurrecting efficacy, 381–397. Copyright (1997), with permission from Elsevier.

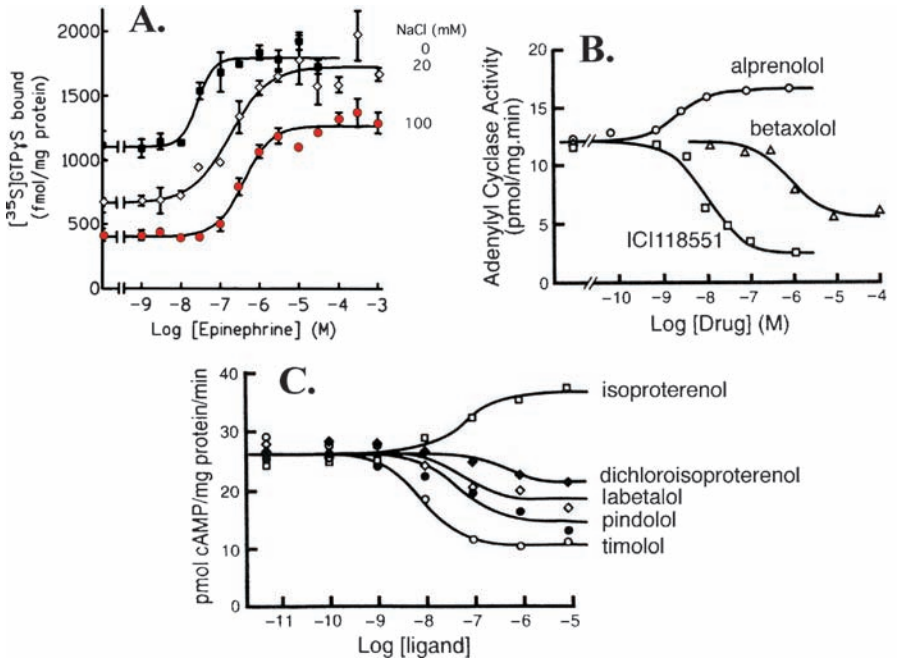


Figure 178 Increase in basal receptor activity to detect inverse agonism: A) increased basal α_{2D}-adrenergic receptor activity in recombinant PC12 cells by reducing the Na^+ concentration, B) effect of different ligands on constitutively active β-adrenergic receptor mutant, C) effect of different ligands on Sf9 cells with over-expressed β-adrenergic receptor. This figure has been adapted from Tian, W. N., Duzic, E., Lanier, S. M. and Deth, R. C. (1994) *Molecular Pharmacology*, **45**, 524–531; Samama, P., Pei, G., Costa, T., Cotecchia, S. and Lefkowitz, R. J. (1994) *Molecular Pharmacology*, **45**, 390–394; and Chidiac, P., Hebert, T., Valiquette, M., Dennis, M. and Bouvier, M. (1994) *Molecular Pharmacology*, **45**, 490–499, with permission from the American Society for Pharmacology and Experimental Therapeutics.

activity. This implies that the receptor possesses some constitutive activity (i.e. that it activity is not completely zero in the resting state). Inverse agonism is well known for benzodiazepine receptors, but this phenomenon has also been shown to take place for many GPCRs (Figure 178). To observe inverse agonism, constitutive GPCR activity can be obtained by:

- Over-expressing the receptor in recombinant systems (Table 21).
- Altering the ionic milieu of the assay system. This can often be achieved by substituting Na^+ by K^+.
- Using constitutively active receptor mutants. Yet, one criticism to this approach is that it generally requires mutagenesis of the GPCR and that this might alter the

Table 21 Inverse agonists for over-expressed wild-type adrenergic receptors. Reproduced from Kenakin, T. (1996) *Pharmacological Reviews*, **48**, 413–463, with permission from the American Society for Pharmacology and Experimental Therapeutics.

Receptor	System	Drug
β_2-Adrenergic	Sf9 membranes	DCI
		pindolol
		labetolol
		timolol
	CHW membranes	labetolol
		pindolol
		alprenolol
		propranolol
		timolol
	turkey erythrocytes	propranolol
		pindolol
	TG-4 murine atria	ICI 118,551
β_1-Adrenergic	cardiomyocytes	atenolol
		propranolol
α_2-Adrenergic	PC-12 cells	rauwolscine
		yohimbine
		WB 4101
		idazoxan
		phentolamine
		yohimbine
	bovine aorta	rauwolscine
	PC-12 cells	rauwolscine

details of receptor pharmacology. A second feature of many GPCR mutations with enhanced constitutive activity is their reduced stability in the absence of a ligand.

The physiological relevance of inverse agonists has been questioned because it is often only observed in artificial systems. In this respect, overexpression of wild-type receptors is thought to provide the most reliable information. Whereas experiments with constitutively active receptor mutants are most subject to caution, there is also evidence that GPCR mutation may sometimes lead to pathologically relevant constitutive activity. For example, transgenic mice that express a constitutively active mutant of the β_2-adrenergic receptor have been shown to display cardiac abnormalities and only inverse agonists were shown to correct these abnormal responses. Hence, the therapeutic potential of inverse agonists is proposed in human diseases ascribed to constitutively active mutant receptors as well as in diseases involving non-mutated receptors, which either have high basal activity or have constitutive activity due to over-expression.

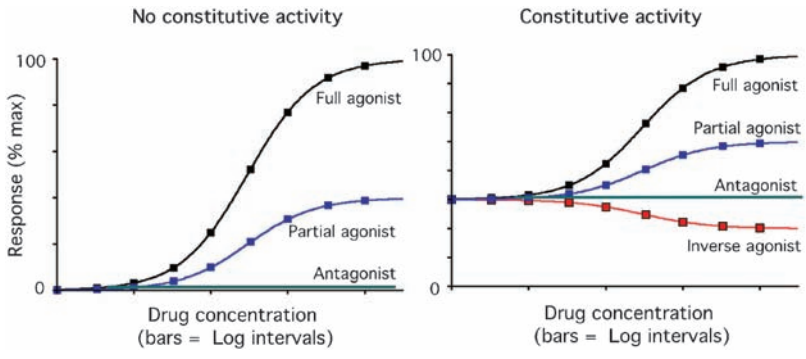


Figure 179 Difference in ligand efficacy in a quiescent (i.e. with no basal activity) and a constitutively active GPCR signalling system (with respect to the full agonist = 1).

Inverse agonists may also contribute to the pharmacological characterization of orphan GPCRs. Traditionally, drug candidates are tested for their ability to mimic or inhibit ligand binding to the targeted receptor. However, as there are no previously known ligands for orphan GPCRs, competition ligand-binding studies cannot be performed and thus functional assays are at the core of all such screening programmes. High levels of activity can be obtained by over-expressing or mutating the orphan GPCR

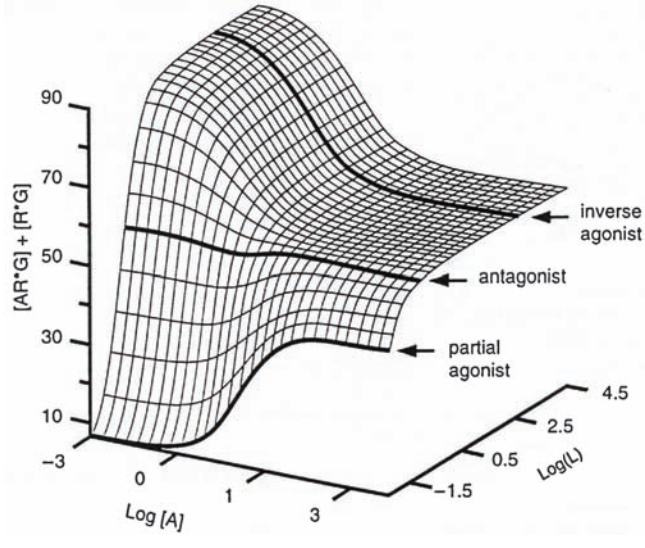


Figure 180 Simulations according to the cubic ternary complex model. Upon increasing L, partial agonists may become (neutral) antagonists and even inverse agonists. The number of active receptors is defined by [AR*G] + [R*G]. Reprinted from *Trends in Pharmacological Science*, **16**, Kenakin, T., Pharmacological proteus?, 256–258. Copyright (1995), with permission from Elsevier.

of interest. This allows the discovery of 'inverse agonists' and provides a more sensitive assay for the discovery of receptor agonists (since constitutively activated GPCRs often exhibit high affinity for agonists). It should be noted that neutral antagonists and inverse agonists behave in an identical manner in the absence of constitutive receptor activity and, in fact, it is only with the advent of constitutive active receptor systems that many ligands thought to be neutral antagonists were found to be inverse agonists (Figure 179).

The β-adrenergic receptor ligand dichloroisoproterenol is a well-known partial agonist in many systems, but it exhibits inverse agonism in β_2-receptors over-expressing Sf9 cells. Due to this duality in behaviour, such ligands have been termed protean ligands. The cubic ternary complex model permits weak partial agonists in systems with low basal activity to become inverse agonists in constitutively active systems (Figure 180). However, this is only possible if the receptor is permitted to adopt more than one activated state (see also Section 4.13).

4.12 Interaction of two G proteins with one activated receptor state

Studies with $\alpha_2$adrenergic, and many other GPCRs, indicate that they are capable of activating different types of G proteins. According to a two-state receptor model (i.e. where the receptor can only reside in an inactive or an active conformation), the role of agonists is to increase the number of activated receptors. Each agonist will have its own intrinsic efficacy (ε) for a given type of receptor (Figure 181). Hence, the amount of activated receptors (which may be regarded to represent the 'stimulus, S') is a unique property of the agonist–receptor combination and this also implies that a given agonist increases the likelihood of the receptor encountering and activating distinct types of G proteins in the same way. Hence, according to a strict two-state model for receptor activation, and in the absence of a 'receptor reserve', the same pharmacological profile (i.e. ligand potency and intrinsic efficacy orders) will be obtained irrespective of the G protein-mediated response pathway.

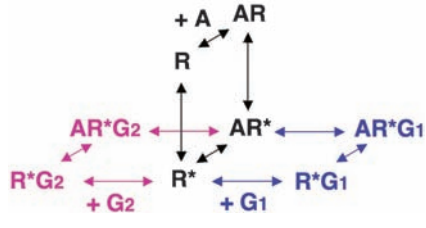


Figure 181 One receptor–two G proteins interaction model (Kenakin, 1995a). It must be emphasized that in a strict two-state model, R* and AR* will bind with the same affinities to a given G protein (i.e. R and AR*/R* represent uniquely defined conformational states). Reprinted from *Trends in Pharmacological Science*, **16**, Kenakin, T., Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity, 188–192. Copyright (1995), with permission from Elsevier.

In models dealing with the interaction between an activated receptor and distinct G proteins (i.e. G_1 and G_2) (Figure 181), the former is often regarded as acting as a 'ligand' and the latter as distinct 'receptors' (Kenakin, 1995; Kukkonen *et al.*, 2001). According to such models, the amount of R^*G_1 and R^*G_2 complexes will depend on:

- The ratio between the concentrations of the receptor and each type of G protein (and, hence, also on the ratio between the concentrations of each type of G protein).
- The affinity of the activated receptor for each type of G protein.

Simulations according to such models suggest that increased receptor expression may lead to receptor–G protein promiscuity (Figure 182). When two G proteins have different affinities for the activated receptor, the receptor–G protein selectivity is strictly preserved when levels of receptor are low. However, when the receptor expression level exceeds that of the G protein with the highest affinity, all of them may be solicited to form AR*G complexes. In the same vein, provided that the receptor concentration is sufficiently elevated, high efficacy agonists will produce sufficiently activated receptors to couple to both G proteins while partial agonists (i.e. with low efficacy) will just produce enough activated receptors to couple to the G protein with the highest affinity.

Although the accumulation of AR*G complexes provides a fair view of what is going on between receptors and distinct G proteins, such a process is only allowed to take place in the absence of GTP, such as in membrane preparations. In intact cells, these complexes will almost immediately fall apart because of the ongoing GDP/GTP exchange. Moreover, when more distant effects/responses are measured, agonist dose-response curves will also depend on how the different G proteins interact with their effector, as well as on the solicited response pathways. In this respect, two situations can be distinguished, i.e.:

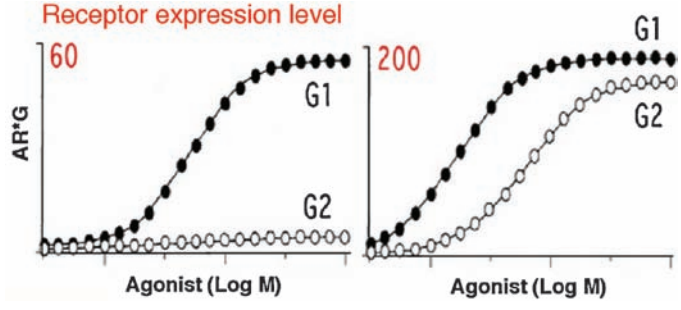


Figure 182 Computer-simulated AR^*G_1 and AR^*G_2 concentrations for increasing agonist concentrations. The receptor expression level is 60% (A) or 200% (B) of the G protein expression level. The activated receptor has higher affinity for G_1. Reprinted from *Trends in Pharmacological Science*, **16**, Kenakin, T., Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity, 188–192. Copyright (1995), with permission from Elsevier.

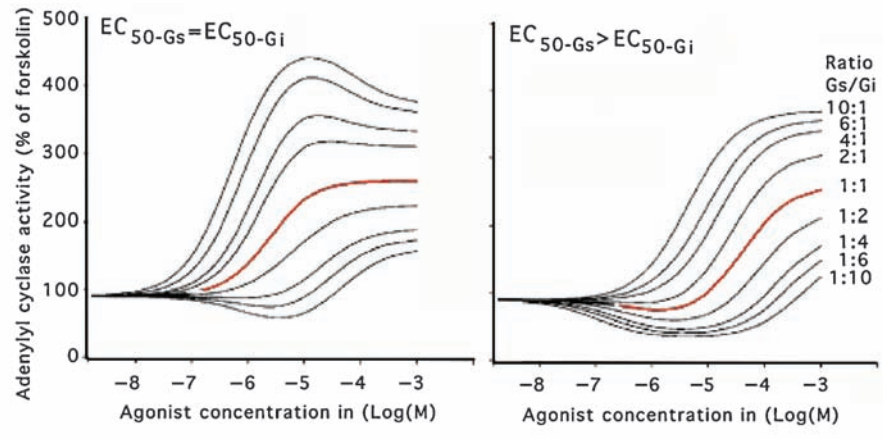


Figure 183 Computer-simulated adenyl cyclase activities for increasing agonist concentrations. Left panel, the stimulated receptor displays the same affinities for G_i and G_s. Right panel, the stimulated receptor displays 10-fold higher affinity for G_i. Reprinted from *Biochemistry and Pharmacology*, **62**, Nasman, J., Kukkonen, J. P., Ammoun, S. and Akerman, K. E., Role of G protein availability in differential signalling by alpha 2-adrenoceptors, 913–922. Copyright (2001), with permission from Elsevier.

- The pathways produce two independently measurable responses.
- The pathways can recombine to modulate one measurable response (Figure 183). An obvious example is the opposite regulation of the adenyl cyclase activity by both $G\alpha_s$ and $G\alpha_i$. In practice, this appears to happen for α_{2B} receptors: they produce pertussis toxin-sensitive adenyl cyclase inhibition (a G_i-mediated effect) as well as pertussis toxin-insensitive adenyl cyclase stimulation (a $G\alpha_s$-mediated effect). Biphasic/bell-shaped agonist dose-response curves can even be seen in certain α_{2B} receptor-containing cell types. Computer-assisted simulations reveal that such a situation may take place when oppositely acting G proteins are involved. In general, the shape of such curves will depend on the G protein concentration ratio and on their relative affinities for the activated receptor.

Fusion proteins between GPCRs and G proteins

Wild-type and mutant GPCRs may show constitutive activity, and the extended ternary complex model (Samama *et al.*, 1993) reveals that this activity is influenced by the GPCR:G protein ratio. As shown above, the GPCR:G protein ratio may also influence the cellular response to a given agonist. Yet, GPCR and $G\alpha$ protein densities may differ considerably from one cell type to another and (especially in the case of transient transfections) even from one day to another. In theory, this may lead to quite some variation in the agonist dose-response curves and in the receptor basal activities when comparing different experiments.

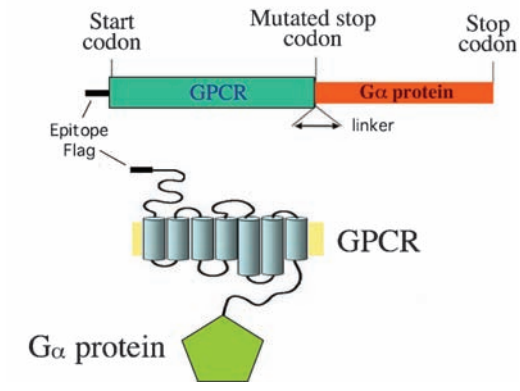


Figure 184 Schematic presentation of a GPCR–Gα fusion protein. Reprinted from *Journal of Pharmacology and Toxicological Methods*, **45**, Wurch, T. and Pauwels, P. J., Analytical pharmacology of G protein-coupled receptors by stoichiometric expression of the receptor and G(alpha) protein subunits, 3–16. Copyright (2001), with permission from Elsevier.

One strategy to overcome this potential cause of variability is to create fusion proteins by covalently linking the C-terminal portion of a GPCR to the N-terminal portion of a Gα protein subunit (Figure 184). By this way, a fixed 1:1 stoichiometry between receptor and Gα is achieved. Although this stoichiometry might not be the same as in physiological systems, it remains the same irrespective of the cell system in which the fusion protein is expressed and of the absolute level of expression.

According to its proponents, the major benefit of the GPCR–Gα fusion protein approach is that it should allow an accurate comparison of experimental data obtained in different laboratories. This is obviously quite a difficult task when dealing with traditional cellular systems in which receptors and G proteins are expressed individually. In favour of this claim, it has been shown that the α_{2A}-adrenergic receptor activation profile by a series of full and partial agonists was not affected over a 30-fold range of expression of the receptor–$G\alpha_{15}$ fusion protein, whereas an enhancement of the maximal response of partial agonists was observed when the free receptor was co-expressed with increasing amounts of $G\alpha_{15}$ (Figure 185).

The 1:1 stoichiometry should even allow the comparison of agonist *intrinsic efficacies*, provided that the measured response is measured at the point of GPCR–G protein interaction (e.g. by [^{35}S]GTPγS binding) rather than at some downstream point. Under these conditions, the intrinsic activity of a ligand (α) should not yet be corrupted by non-linear stimulus-effect coupling (i.e. 'receptor reserve') so that it should reflect the intrinsic efficacy (ε) of that ligand.

A major premise of the GPCR–Gα fusion protein approach is that fusion promotes efficient coupling without altering the fundamental properties of the signalling partners. From a structural point of view, several native Gα proteins have been found to bear a myristyl or palmitoyl fatty acid side chain. This post-translational modification allows the Gα protein to be attached to the plasma membrane and, hence, to reside in the

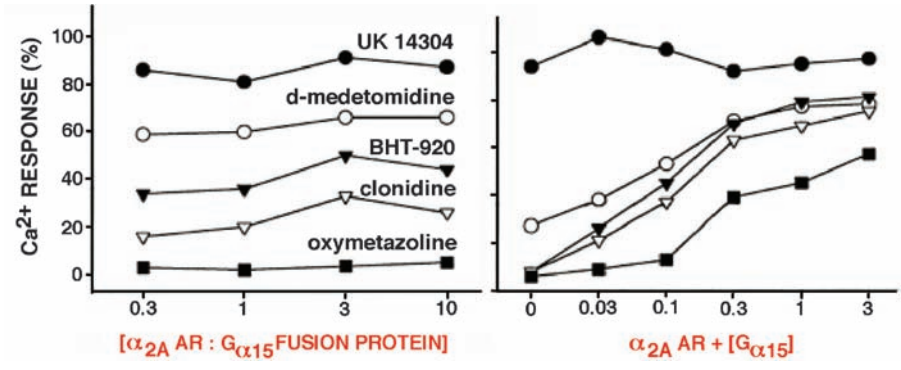


Figure 185 Intrinsic activity of α_2-adrenergic receptor ligands in transfected CHO cells as a function of (left) the receptor–$G\alpha_{15}$ fusion protein concentration or (right) Ga_{15} protein plus a fixed concentration of receptor. Reprinted from *Journal of Pharmacology and Toxicological Methods*, **45**, Wurch, T. and Pauwels, P. J., Analytical pharmacology of G protein-coupled receptors by stoichiometric expression of the receptor and G(alpha) protein subunits, 3–16. Copyright (2001), with permission from Elsevier.

proximity of GPCRs. In the same vein, fusion to a GPCR also produces a membrane anchor for Gα and, in theory, fusion proteins can increase the efficiency of Gα activation by a GPCR because they are even closer to each other, as in the case of the individual proteins. In support of his assertion, fusion has been shown to rescue functional interactions of a myristoylation-deficient $G\alpha_{i1}$ mutant with the α_{2A}-adrenergic receptor.

In practice, however, there are several points of concern with regard to this approach:

- GPCR–Gα fusion proteins often generate signals that resemble those of the free receptors. However, upon close comparison of ligand potencies or maximal responses between a fusion protein and the corresponding co-expression system, it turns out that the fusion protein response can be either enhanced, decreased or equal to the co-expression experiment (Table 22).
- The Gα–βγ interaction may be attenuated by the fusion process. Whereas βγ contributes to the interaction of Gα with the receptor under normal circumstances, the effects of βγ on the responsiveness of a fusion protein are usually of low magnitude. In this respect, GPCRs interacting with $G\alpha_i$ are known to stimulate the MAPK signalling pathway upon agonist activation via release of βγ subunits. The fusion constructs could be unable to signal down the ERK–MAPK cascade because the $G\alpha_i$ protein is tethered to the receptor and is therefore no longer able to interact with endogenous βγ subunits.
- Special care should be taken with PTX-resistant GPCR–$G\alpha_i Cys^{351}Gly$ fusion proteins since the mutation could produce a suboptimal GPCR–Gα interface. Whereas full agonists can produce sufficient conformational alterations in a GPCR to overcome this handicap, partial agonists might be less effective in doing so. This might result in a lower efficacy of partial agonists (Figure 186).

Table 22 Comparison between R–G_α fusion proteins and co-expressed R and G_α. Reprinted from *Journal of Pharmacology and Toxicological Methods*, **45**, Wurch, T. and Pauwels, P. J., Analytical pharmacology of G protein-coupled receptors by stoichiometric expression of the receptor and G(alpha) protein subunits, 3–16. Copyright (2001), with permission from Elsevier.

GPCR and G_α protein combination	Evaluation test	Ligand	EC_{50}	Maximal response
Fusion protein > coexpression				
β_2 AR and $G_{\alpha s}$S	cAMP formation	isoproterenol	Fusion 45 nM β_2 AR + $G_{\alpha s}$; 650 nM	100% 58%
Coexpression > fusion protein				
α_{2A} AR and $G_{\alpha 15}$	Ca^{2+} mobilisaiion	clonidine	Fusion 58 nM α_{2A} AR + $G_{\alpha 15}$; 6.0 nM	100% 170%
Fusion protein = coexpression				
5-HT_{1A} and $G_{\alpha i1}Cys^{351}Gly$	GTPase	5-HT	Fusion 100 nM 5-HT_{1A}; 80 nM	100% 100%

- GPCRs that are fused via their C-terminus to other proteins, such as green fluorescent protein, still appear to interact with and activate cellular G proteins. In the same way, agonist occupancy of a GPCR–Gα fusion protein can still cause the activation of endogenous G proteins. Hence, due attention must be given to which

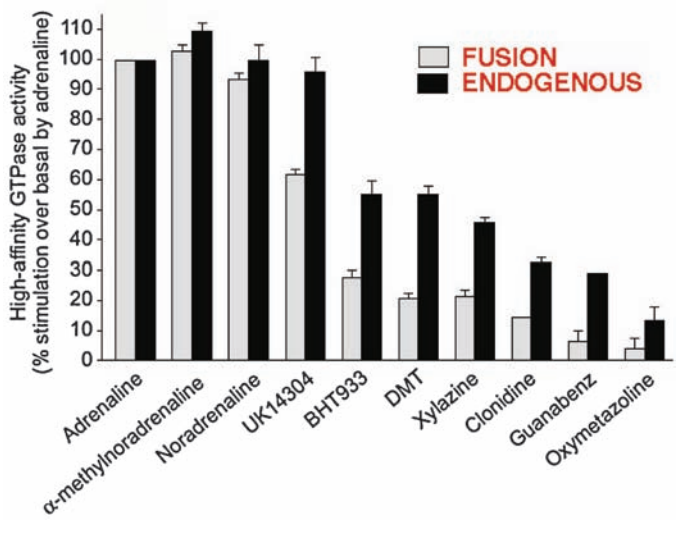


Figure 186 Capacity of different α_{2A}-adrenergic agonists to stimulate the GTPase activity of endogenous G_i-proteins and α_{2A} receptor–$G\alpha_iCys^{351}Gly$ fusion proteins in RAGI 77 cell membranes (Burt *et al.*, 1998, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

G protein is responsible for the observed activity. In this respect, it has also been reported that the Gα selectivity of fusion proteins could be different as compared to the free GPCR. For example, whereas isolated α_{2A}-adrenergic receptors were shown to activate endogenous G_s (i.e. following pertussis toxin treatment, adrenaline stimulated the adenylate cyclase activity), the receptor–$G\alpha_i Cys^{351}Gly$ fusion proteins were unable to do so. Several strategies have been adopted to limit the activation of endogenous Gα of the host cells; these include:

- Careful selection of the model system. Based on the observation that mammalian GPCRs couple poorly to endogenous insect Gα proteins, Sf9 insect cells are routinely used as a host for mammalian GPCRs and GPCR–Gα fusion proteins. In the same spirit, S49 cyc^- mouse lymphoma T cells do not express functional $G\alpha_s$. Therefore, they constitute a host of choice for GPCR–$G\alpha_s$ fusion proteins.
- Transfection with GPCR–Gα fusion proteins, which are naturally resistant to PTX, and treatment of the host cells with PTX to eliminate any potential interactions with endogenously expressed $G\alpha_i$. In this respect, certain Gα proteins ($G\alpha_{15}$, $G\alpha_{16}$) are naturally resistant to PTX since they do not possess the required ADP-ribosylation site. Alternatively, $G\alpha_i$ proteins may be mutated to become PTX-resistant (e.g. $G\alpha_i Cys^{351}Gly$).

Contrary to the belief that short-range interactions between GPRC and Gα may favour constitutive receptor activation, this phenomenon is not necessarily observed with GPCR–Gα fusion proteins. If observed, the constitutive activity of a receptor is likely to depend on the nature of the Gα involved. For example (Figure 187):

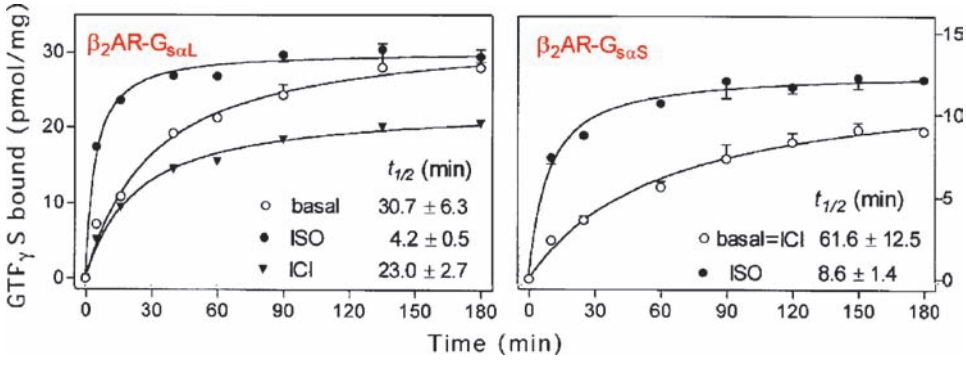


Figure 187 Effect of isoproterenol (ISO, β-adrenergic agonist) and ICI 118,551 (inverse agonist) on [^{35}S]GTPγS binding to Sf9 cell membranes bearing β_2-adrenergic receptor–$G\alpha_s$ fusion proteins. $G\alpha_{sL}$ is the long form of $G\alpha_s$ while $G\alpha_{sS}$ is the short form. Reproduced from Wenzel-Seifert, K. and Seifert, R. (2000) *Molecular Pharmacology*, **58**, 954–966, with permission from the American Society for Pharmacology and Experimental Therapeutics.

- When expressed in insect Sf9 cells, β_2-adrenergic receptor–$G\alpha_{sL}$ fusion proteins have been shown to display constitutive activity: [^{35}S]GTPγS binding to the G protein took place in the absence of agonist and this event was inhibited by the inverse agonist ICI 118,551.
- When expressed in the same cells, β_2-adrenergic receptor–$G\alpha_{sS}$ fusion proteins were devoid of constitutive activity since ICI 118,551 did not affect [^{35}S]GTPγS binding under basal conditions.

Because of the inherent 1:1 stoichiometry of GPCR–Gα fusion proteins, one could expect them to display a single high-affinity state for agonist binding. Yet this is not the case in practice. GPCR–G protein fusion proteins maintain both high and low agonist affinity states and as such behave like the co-expressed individual proteins. In this respect, it was shown that the β_2-adrenergic receptor couples much more efficiently to the $G\alpha_s$-fusion partner than to $G\alpha_i$ and $G\alpha_q$ partners in insect Sf9 membranes (Figure 188). Although further experimental confirmation may be necessary, these findings suggest

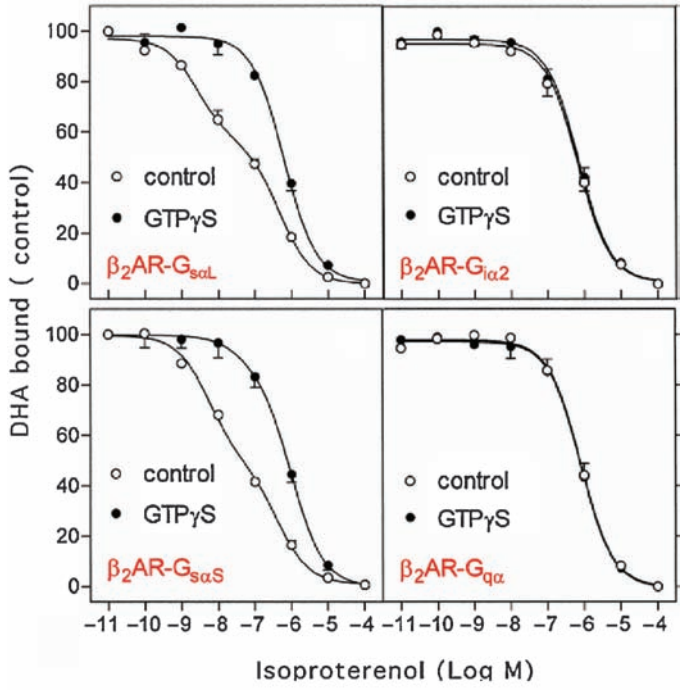


Figure 188 Isoproterenol competition binding curves with insect Sf9 membranes expressing β_2 receptor–$G\alpha_{sL}$ and –$G\alpha_{sS}$ fusion proteins are biphasic. GTPγS converts the receptors into a single population with low agonist affinity. The agonist displays only low affinity in membranes expressing β_2 receptor–$G\alpha_{i2}$ and –$G\alpha_q$ fusion proteins. Reproduced from Wenzel-Seifert, K. and Seifert, R. (2000) *Molecular Pharmacology*, **58**, 954–966, with permission from the American Society for Pharmacology and Experimental Therapeutics.

that membrane compartmentalization and stoichiometric limitation in the amount of available Gα proteins (Section 4.10) do not constitute the sole explanations for the presence on membranes of receptor sites with high and low agonist affinities.

4.13 Multiple receptor conformations

It is becoming increasingly clear that the two-state model cannot sufficiently explain the complex behaviour of GPCRs. In physicochemical terms, molecules are considered to exist in a very large number of conformational states, and trying to describe the properties of a receptor with just two states must be an approximation. Different receptor states may be regarded to constitute minima of an 'energy landscape'. At rest, most of the receptors should be in the 'inactive' state. A spectrum of 'active' states is supposed to exist and, upon ligand binding, the population of some of these 'active' states might increase in a ligand-specific manner (Figure 189).

The ability of receptors to dimerize, to internalize, to be phosphorylated, to be desensitized and to interact with other membrane proteins can sometimes be dissociated from the activation of G proteins. For example (Figure 190), some Tyr^4- and Phe^8-substituted Ang II analogues promote AT_{1A} receptor internalization (confocal microscopy of enhanced green fluorescent protein-tagged receptor) without phospholipase C signalling in CHO cells. It is difficult to explain within a simple

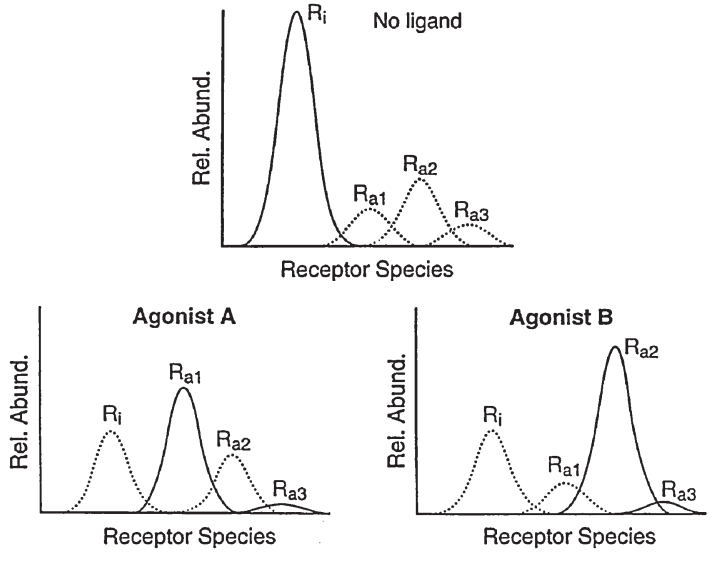


Figure 189 Relative abundance of 'inactive' (Ri) and 'active' (Ra) receptor states at rest and in the presence of two different agonists. Reproduced from Kenakin, T. (1996) *Pharmacological Reviews*, **48**, 413–463, with permission from the American Society for Pharmacology and Experimental Therapeutics.

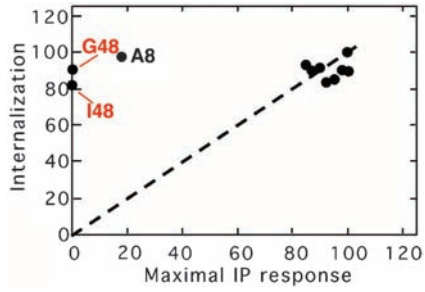


Figure 190 Comparison of AT_1 receptor activity and internalization in the presence of different Sar^1-Ang II analogues (denomination: e.g. G48 = substitution with glycine at positions 4 and 8, A = alanine, I = isoleucine). Reproduced from Holloway, A. C., Qian, H., Pipolo, L., Ziogas, J., Miura, S.-I., Karnik, S., Southwell, B. R., Lew, M. J. and Thomas, W. G. (2002) *Molecular Pharmacology*, **61**, 768–777, with permission from the American Society for Pharmacology and Experimental Therapeutics.

two-state model that these events do not coincide. To accommodate these findings, it might be necessary to consider that:

- Each function of the receptor is triggered by a broadly defined continuum of conformations instead of only one well-defined conformation.
- Conformations allowing GPCR phosphorylation, internalization and desensitization processes only partly overlap those activating G protein.
- Each ligand might only stabilize a certain subset of conformations.

Multistate models in which distinct conformations of the receptor are involved in coupling with distinct G proteins have been proposed. These models arose, in the first place, from the failure of the 'classical' two-state models to explain the reversals of ligand potency and/or intrinsic efficacy orders that can sometimes be observed when comparing two types of responses (involving distinct types of G proteins) that are generated by a single receptor. Some of the representative experiments are presented below:

- Human 5-HT_{2C} receptors expressed in CHO cells were found to trigger inositol phosphate accumulation and phospholipase A_2-mediated arachidonic acid release (Holloway *et al.*, 2002). Different agonists displayed the same potency for triggering both responses, but their relative efficacies differed depending on the response (Figure 191). Some agonists (e.g., 3-trifluoromethylphenyl-piperazine) preferentially activated inositol phosphate accumulation, whereas others (e.g. LSD) favoured arachidonic acid release. These data reflect true differences at the level of the agonist–receptor interaction if the stimulus–response relationship is linear for both signal transduction pathways. This was ascertained by experiments where some of the receptors were irreversibly inactivated with

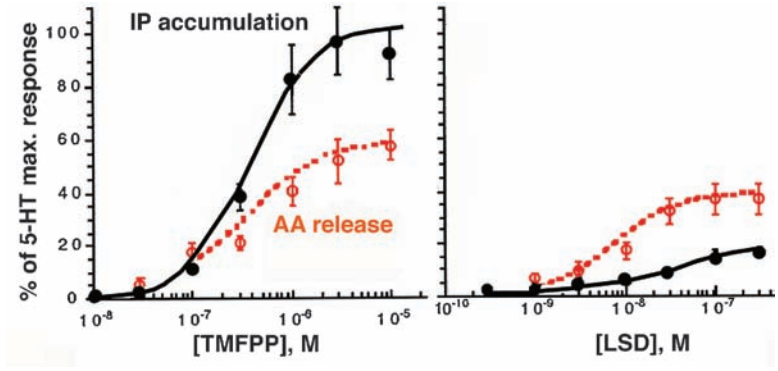


Figure 191 Effect of different agonists on the inositol phosphate accumulation in, and arachidonic acid release by, human 5-HT_{2C} receptor-expressing CHO cells. Reproduced from Berg, K. A., Maayani, S., Goldfarb, J., Scaramellini, C., Leff, P. and Clarke, W. P. (1998) *Molecular Pharmacology*, **54**, 94–104, with permission from the American Society for Pharmacology and Experimental Therapeutics.

the covalently binding drug, phenoxybenzamine (Figure 192). Under the same experimental conditions, this treatment produced a similar reduction in the maximal 5-HT-induced inositol phosphate accumulation and arachidonic acid release without affecting the EC_{50} of 5-HT for either pathway.

- Transfected PACAP receptors were found to trigger G_s-mediated cAMP accumulation and $G_{q/11}$-mediated inositol phosphate accumulation. Whereas $PACAP_{1\text{-}27}$ was more potent than its analogue $PACAP_{1\text{-}38}$ with regard to their ability to stimulate

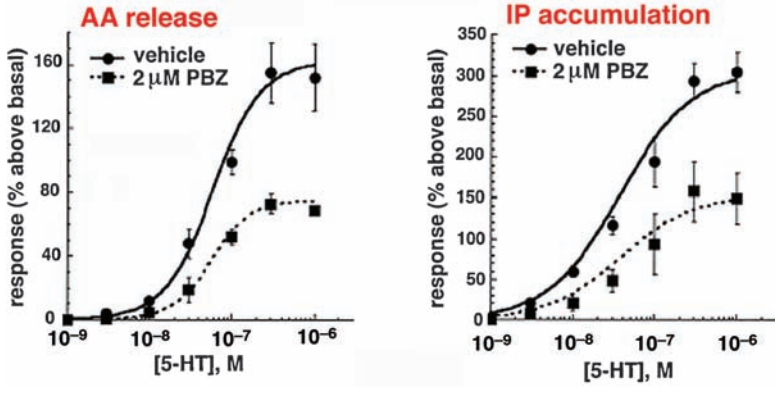


Figure 192 Irreversible inactivation of some of the 5-HT_{2C} receptors in CHO cells with phenoxybenzamine (PBZ) decreases the 5-HT-mediated inositol phosphate accumulation and arachidonic acid release to the same extent. Reproduced from Berg, K. A., Maayani, S., Goldfarb, J., Scaramellini, C., Leff, P. and Clarke, W. P. (1998) Molecular Pharmacology, **54**, 94–104, with permission from the American Society for Pharmacology and Experimental Therapeutics.

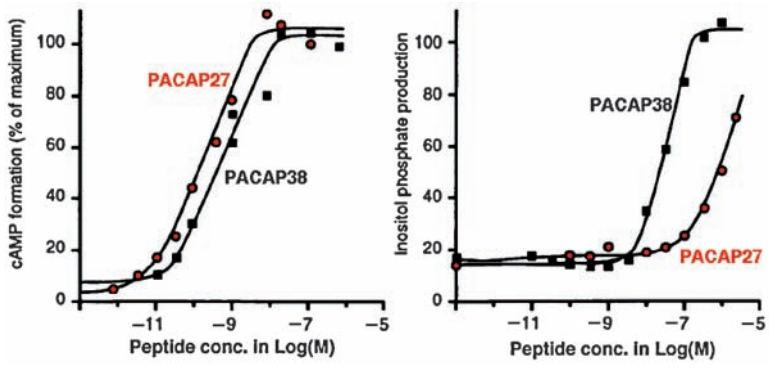


Figure 193 Effect of the agonists PACAP27 and PACAP38 on inositol phophate and cAMP accumulation in pituitary adenylate cyclase-activating polypeptide (PACAP) receptor-expressing LLC PK1 cells. Reprinted by permission from Macmillan Publishers Ltd: *Nature*, **365**, Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H. and Journot, L., Differential signal transduction by five splice variants of the PACAP receptor, 170–175, © (1993).

cAMP accumulation, the potency order of these agonists was reversed when measuring their ability to trigger inositol phosphate accumulation (Figure 193).

- 'Antagonists' have also been found to display distinct potencies for blocking the diverse signals that may elicited by the same agonist. Such behaviour has been observed for, for example, the CCK2 receptor in CHO cells (Figure 194). In these experiments, inositol phosphate formation and arachidonic acid release in response to the same agonist were inhibited with the same potency by L365260, but with different potencies by RB213.

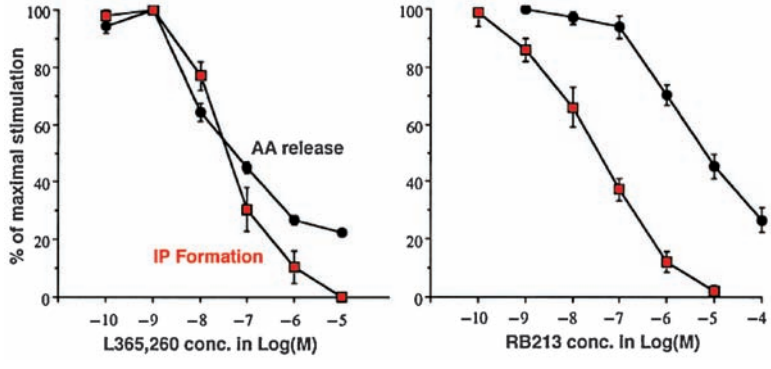


Figure 194 Effect of specific CCKB receptor antagonists on CCK8-mediated inositol phosphate accumulation and arachidonic acid release. Reprinted from *Journal of Neurochemistry*, **73**, Pommier, B., Da Nascimento, S., Dumont, S., Bellier, B., Million, E., Garbay, C., Roques, B. P. and Noble, F., The cholecystokinin B receptor is coupled to two effector pathways through pertussis toxin-sensitive and -insensitive G proteins, 281–288. Copyright (1999) Blackwell Publishing.

Table 23 Receptors with alluded ligand-selective conformations. Reprinted from *Trends in Pharmacological Sciences*, **24**, Kenakin, T., Ligand-selective receptor conformations revisited: the promise and the problem, 346–354. Copyright (2003), with permission from Elsevier.

Agonist-induced stimulus trafficking	**Agonist-selective antagonist potency**
PACAP receptor	CCK_2 receptor
Dopamine D2 receptor	$5\text{-}HT_{1A}$ receptor
Drosophila tyramine receptor	β_1-Adrenoceptor
NK_1 receptor	**Receptor internalization**
β_2-Adrenoceptor	CCK receptor
Muscarinic acetylcholine receptor	Opioid peptide receptor
Calcitonin receptor	Angiotensin II receptor
Adenosine A_1 receptor	Chemokine CCR5
α_{2A}-Adrenoceptor	PTH-1 receptor
Bombesin receptor	Opioid peptide receptor
Parathyroid hormone receptor	**Receptor phosphorylation and desensitization**
$5\text{-}HT_{2A}$ receptor	Angiotensin II receptor
Cannabinoid receptor	Opioid peptide receptor
Chemokine CXCR2	$5\text{-}HT_3$ receptor
Delta opioid peptide receptor	

To explain such findings, it has been assumed that agonists may trigger/stabilize specific receptor conformations (Table 23) and, in this way, modulate the receptor's preference for certain G proteins. The initial models by Kenakin (1995b) and ensuing models are largely based on the following premises:

- Different active conformations of the receptor do exist.
- Each agonist should promote its own specific active receptor conformation, a phenomenon that is commonly referred to as 'signalling-selective agonism', 'biased agonism', 'agonist-specific trafficking of receptor signalling' or simply '*agonist trafficking*'.
- Each active conformation has its own G protein preference. This notion is supported by experiments with mutated β_2-adrenergic receptors, which suggest that distinct intracellular receptor domains interact with each type of G protein.

'Agonist trafficking' implies that pharmacological diversity may be achieved through a single receptor by compounds that trigger distinct effector pathways (Figure 195). Obviously, this may have major positive consequences for the development of signalling-specific therapeutics (Figure 196). On the negative side, predictions of the clinical efficacy of drug constituents become more difficult when simply based on assays with recombinant cell systems. Moreover, whereas receptor subtypes might show up as single 'species' when investigating their antagonist binding properties, they might

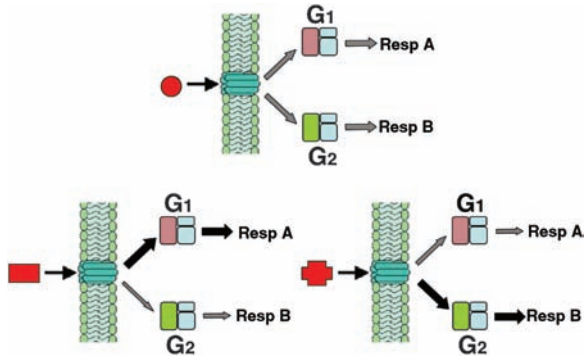


Figure 195 Agonist trafficking. Situation for two active receptor conformations, each with a specific G protein signalling. Top: non-selective agonists stabilize both active conformations equally well. Bottom: signalling selective agonists preferentially stabilize one of the active conformations. Reprinted from *Pharmacology and Therapeutics*, **99**, Hermans, E., Biochemical and pharmacological control of the multiplicity of coupling at G protein-coupled receptors, 25–44. © (2003), with permission from Elsevier.

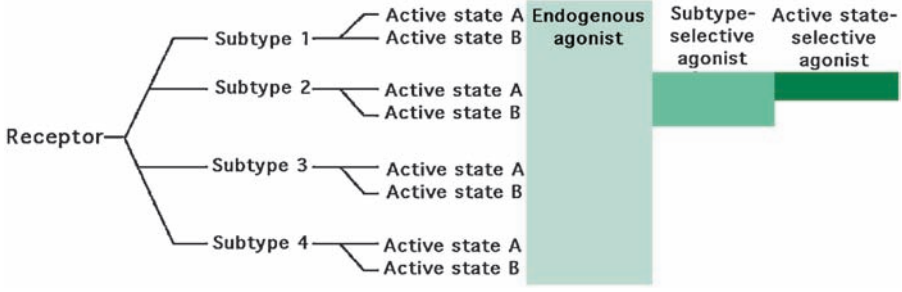


Figure 196 Ligand-selective receptor conformations: drugs could be designed to modify specific physiological effects of a given receptor. Reprinted from *Trends in Pharmacological Science*, **24**, T. Kenakin, Ligand-selective receptor conformations revisited: the promise and the problem, 346–354. Copyright (2003), with permission from Elsevier.

be split into many more pharmacologically distinct 'species' when investigating their interaction with agonists (Figure 197).

'Agonist trafficking': what do models predict?

In the initial model by Kenakin, there is a limitless number of active receptor conformations. For simulation studies, this was simplified by Leff *et al.* (1997) to three receptor conformations with one inactive (R) and two active conformations (R* and R**). In the *intact three-state model* (Figure 198), it was further assumed that:

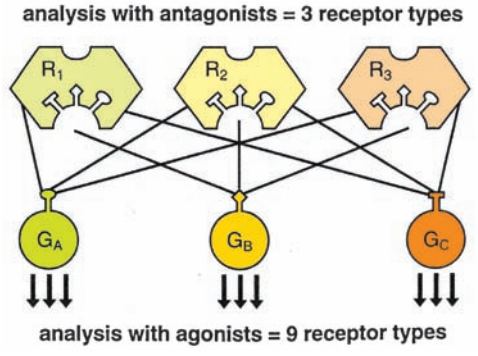


Figure 197 Schematic diagram of three receptors interacting with three G proteins. Antagonists would recognize three 'receptor types', while agonists would recognize up to nine 'receptor types'. Reproduced from Kenakin, T. (1996) *Pharmacological Reviews*, **48**, 413–463, with permission from the American Society for Pharmacology and Experimental Therapeutics.

- The AR* and AR** formations are linked to each other. Enrichment of one active conformation will be at the expense of the other.
- The amount of receptors in each active conformation (i.e. [R*] + [AR*] on one hand and [R**] + [AR**] on the other hand) represent a '*stimulus*'. Obviously, these stimuli do not represent the ultimate effects/responses of the receptor. To 'transpose' stimuli into responses, one may have to take account of potential pathway-related differences in 'receptor reserve'.
- In each active conformation, the receptor is able to couple to a distinct type of G protein.
- The receptors and G proteins have free access to one another (i.e. no membrane compartmentalization).

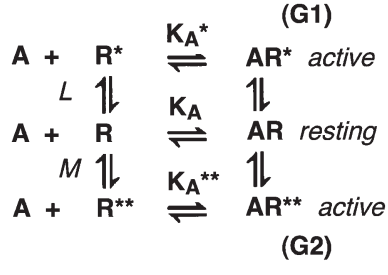


Figure 198 Intact three-state model describing the linked formation of AR* and AR**. Reprinted from *Trends in Pharmacological Science*, **18**, Leff, P., Scaramellini, C., Law, C. and McKechnie, K., A three-state receptor model of agonist action, 355–362. Copyright (1997), with permission from Elsevier.

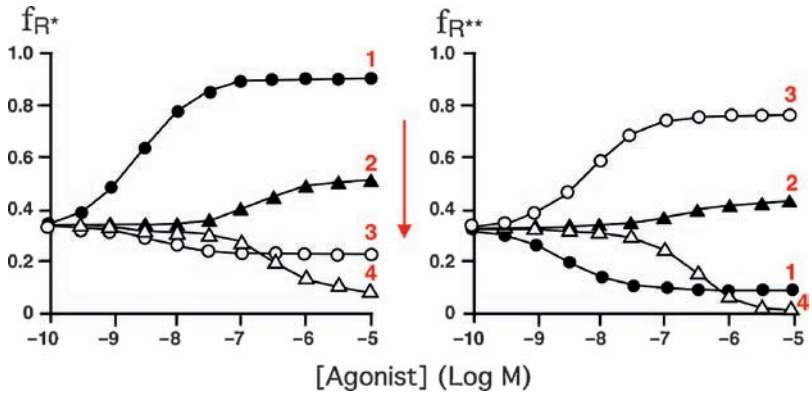


Figure 199 Simulations of agonist behaviour according to the intact three-state model. f_{R^*} or $f_{R^{**}}$ = fraction of receptors in active conformation(s). Reprinted from *Trends in Pharmacological Science*, **18**, Leff, P., Scaramellini, C., Law, C. and McKechnie, K., A three-state receptor model of agonist action, 355–362. Copyright (1997), with permission from Elsevier.

Simulations according to this intact three-state model (Figure 199) reveal that, while agonist trafficking may produce differences in ligand intrinsic efficacy orders and even that agonists for one type of response may behave as inverse agonists for the other response, each ligand should increase the AR* and AR** concentrations with the same potency. Hence, these simulations suggest that the rank order of agonist efficacies at the same receptor can differ from one response pathway to another. This constitutes a major break with the traditional receptor theory in which only a single active receptor conformation is allowed to exist.

Yet the rank order of agonist potencies may vary among response pathways if one considers that AR* and AR** are formed independently of one another (Figure 200). Under these conditions, the three-state model is actually composed of two pathways which operate in an isolated manner, one dealing with the R* conformation and the other dealing with the R** conformation.

Obviously, splitting of the intact three-state model into two independent two-state models implies that the AR* and AR** formations are no longer linked to one another. How can a receptor molecule be prevented from adopting a particular active conformation even before coupling is allowed to take place? One way to overcome this caveat is to change the premise of the three-state model by assuming that receptors are pre-coupled to G proteins and that pre-coupling influences the 'energy landscape' of the receptor. According to this view, the *stimulus* no longer reflects the concentration of activated receptors, but rather the *concentration of activated receptor–G protein complexes.*

Experimental 'evidence' for agonist trafficking: potential pitfalls

Much of the difficulty in correctly interpreting agonist dose-response curves arises from the lack of knowledge of the underlying molecular events. Indeed, interpretations

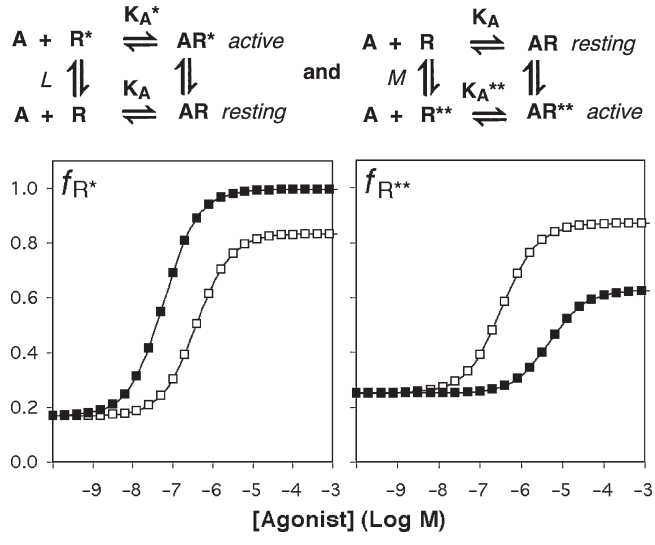


Figure 200 Separated two-state models describing the completely independent formation of AR* and AR** (reprinted from *Trends in Pharmacological Science*, **18**, Leff, P., Scaramellini, C., Law, C. and McKechnie, K., A three-state receptor model of agonist action, 355–362. Copyright (1997), with permission from Elsevier). Simulations according to this model with L = 5, M = 3. For ligand 1: KA = 2 μM, KA* = 0.08 μM, KA** = 0.1 μM. For ligand 2: KA = 10 μM, KA* = 0.01 μM, KA** = 2 μM.

could be biased by the fact that, although generated by a single type of receptor, the different response pathways are likely to display differences in 'receptor reserve'.

In addition, each agonist dose-response curve also depends on the strength of its stimulus. When defined as the concentration of activated receptor–G protein complexes, this parameter can be affected by the affinity of the activated receptors for their cognate G proteins as well as by the concentration ratios between each of these participants (see Section 4.12). In this respect, a single activated state of the receptor can still explain why one ligand is able to activate two pathways while another ligand is only able to activate one pathway. Indeed, when the activated receptor has a high affinity for G_1 and a low affinity for G_2, an agonist of high efficacy may produce enough activated receptors to produce significant coupling to both G proteins while an agonist of low efficacy may only produce enough activated receptors to produce significant coupling to G_1. Hence, there is no strict necessity to invoke '*agonist trafficking*' to explain the observation that oxymetazoline only stimulates G_i in α_2-adrenergic receptor-transfected CHO cells while adrenaline stimulates both G_i and G_s in these cells (Figure 201).

Also, before evoking '*agonist trafficking*', great care must be taken that no other receptor or receptor subtype than the receptor of interest is involved. This potential source of artefact is clearly illustrated in a recent study by on porcine α_{2A}-receptor-expressing CHO cell lines (Figure 202). Even when care was taken to avoid differences in 'receptor reserves' between Gs and Gi-mediated effects (see coinciding curves for noradrenaline and adrenaline); oxymetazoline showed selectivity for inducing signalling through the Gi pathway. However, this effect is an artefact since it is mediated

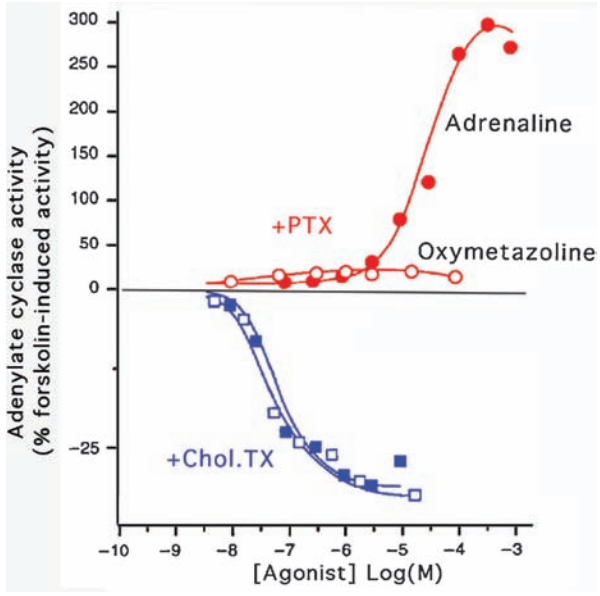


Figure 201 Gi- (in presence of cholera toxin) and Gs- (in presence of pertussis toxin) mediated effects of two agonists on α_2-adrenergic receptor transfected CHO cells. Reproduced from Eason, M., Jacinto, M. and Liggett, S. (1994) *Molecular Pharmacology*, **45**, 696–702, with permission from the American Society for Pharmacology and Experimental Therapeutics.

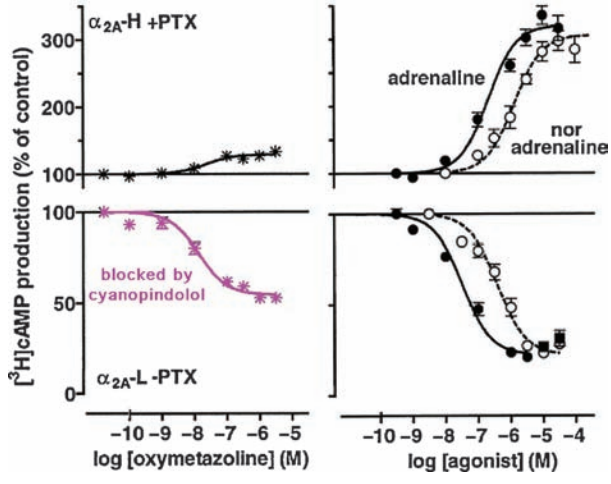


Figure 202 Effect of 'α_2-adrenergic agonists' in CHO cells with porcine α_{2A}-receptors at high (α_{2A}-H cells) and low (α_{2A}-L cells) concentrations, respectively. Adenylate cyclase stimulation is measured in the presence of pertussis toxin and is therefore Gs-mediated. Adenylate cyclase inhibition is Gi-mediated. Reproduced from Brink, C. B., Wade, S. M. and Neubig, R. R. (2000) *Journal of Pharmacology and Experimental Therapeutics*, **294**, 539–547, with permission from the American Society for Pharmacology and Experimental Therapeutics.

by endogenous 5-HT_{1B} receptors (i.e. it can be blocked by the 5-HT_1 antagonist (−)-cyanopindolol). This result strongly emphasises the importance of non-transfected control cells when studying the pharmacological properties of recombinant systems.

Multistate receptors: ligand-mediated sequential changes in receptor conformation

Real-time fluorescence spectroscopy of purified β_2-adrenergic receptors labelled with a conformationally sensitive fluorophore (i.e. with a cysteine-reactive, fluorescent probe whose fluorescence is highly sensitive to the polarity of its environment) revealed agonist-induced conformational changes with a $t_{1/2}$ of 2–3 min. This suggests that the rapid association of agonists is followed by a slower conformational change of the receptor. These findings led to the 'sequential binding and conformational selection' model by Gether and Kobilka (1998). It is assumed that the receptor spontaneously alternates between different active and inactive conformations (Figure 203) and that receptor activation by an agonist occurs sequentially, resulting in a series of intermediate conformational states (**Ra′** and **Ra″**) between **R** and **Ra***:

- Agonist binding may involve an initial interaction between receptor and one structural group of the agonist. After this initial event, binding of the remaining groups occurs in a sequential manner. Each interaction between the receptor and the agonist stabilizes one or more transmembrane domain until the agonist finally stabilizes the receptor in the active **Ra*** state.
- **Ri'** can be stabilized by inverse agonists in a similar way to **Ra*** stabilization by agonists.

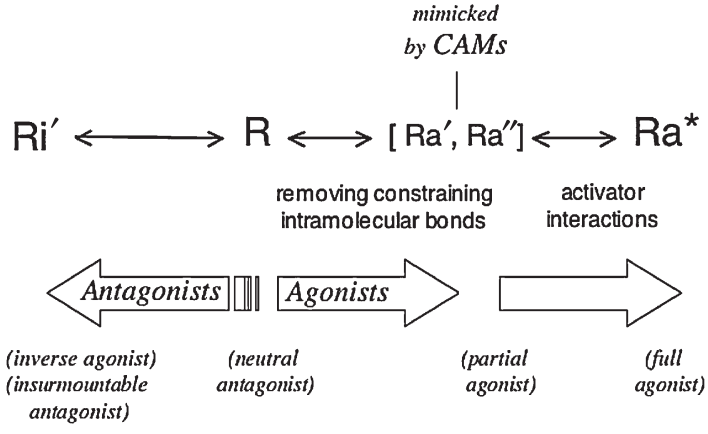


Figure 203 Sequential binding and conformational stabilization model for the molecular mechanisms of ligand action in GPCRs. Reprinted from *Fundamental and Clinical Pharmacology*, **19**, Vauquelin G. and Van Liefde I. G protein-coupled receptors: a count of 1001 conformations, 45–46, Copyright (2005) Blackwell Publishing.

- Partial agonists may stabilize one of the intermediate states, thereby increasing the chance of spontaneous isomerization to **Ra***. Alternatively, they may stabilize unique conformational states having lower affinities for the G protein.

The model is strongly supported by recent studies with angiotensin II (Ang II) analogues. These studies suggest that at least two steps take place to obtain full receptor activation (Figure 204):

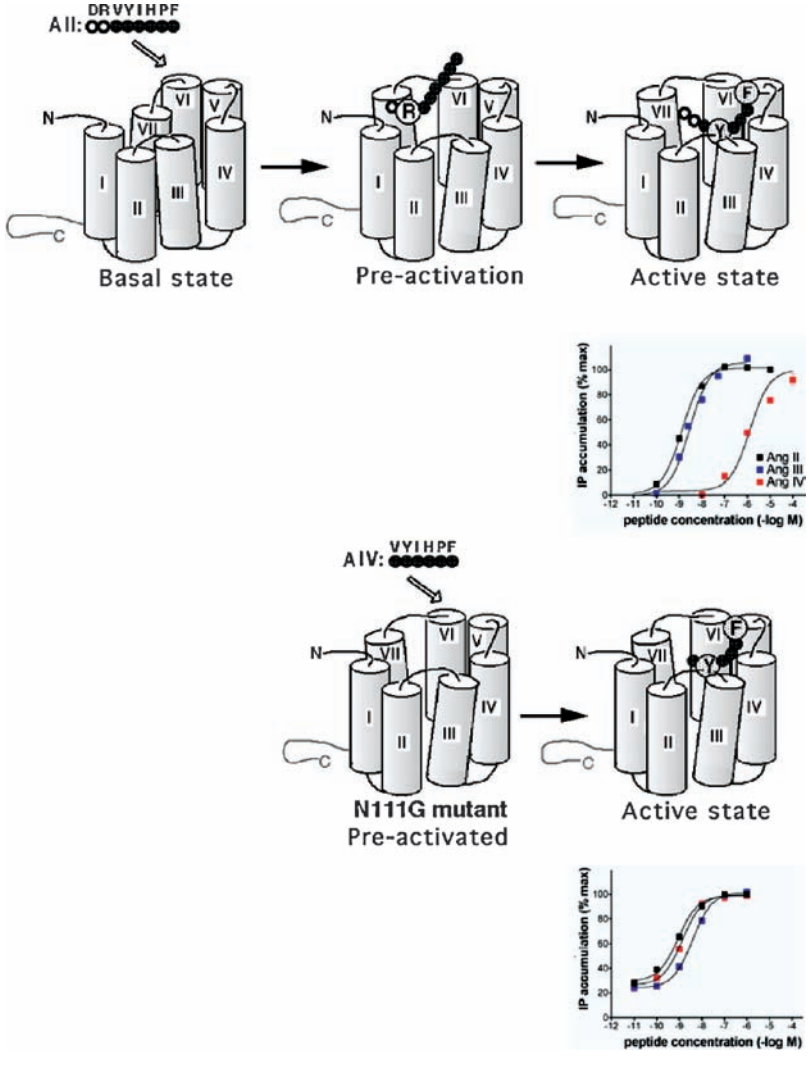


Figure 204 Activation of the wild-type AT_1 receptors (top) and of the $Asn^{111}Glu$ AT_1 receptor CAM by Ang II, Ang III and Ang IV (Le *et al.*, 2002, reproduced by permission of the American Society for Biochemistry and Molecular Biology).

- A pre-activation step, in which intramolecular interactions constrained within the receptor are broken by Arg^2 of angiotensin II. This explains why the potency of Ang IV (i.e. an Ang II fragment without the N-terminal Asp^1 and Arg^2) is 1000 times lower than the potency of Ang II and Ang III (i.e. an Ang II without Asp^1).
- A subsequent activation step in which the C-terminal side of Ang II plays an essential role. Here, Arg^2 is no longer needed and this explains why both Ang II and Ang IV have high potency for the $Asn^{111}Glu$ constitutively active AT_1 receptor mutant (which is assumed to mimic the pre-activated wild-type receptor).

A particularly striking observation with AT_1 receptors and many other GPCRs is that the apparent affinity and efficacy of agonists is better for CAMs than for the wild-type receptor. When the WT receptor has very low basal activity, certain CAMs may show hardly detectable constitutive activity in the absence of agonist. Yet, such CAMs could still be detected based on their increased affinity and efficacy for certain agonists. For example, only one AT_1 receptor CAM was identified by site-directed mutagenesis ($N^{111}A$). This CAM showed increased apparent affinity and efficacy for CGP42112A and, based on the same criterion, several other AT_1 receptor CAMs were identified following random mutagenesis of this receptor (Figure 205).

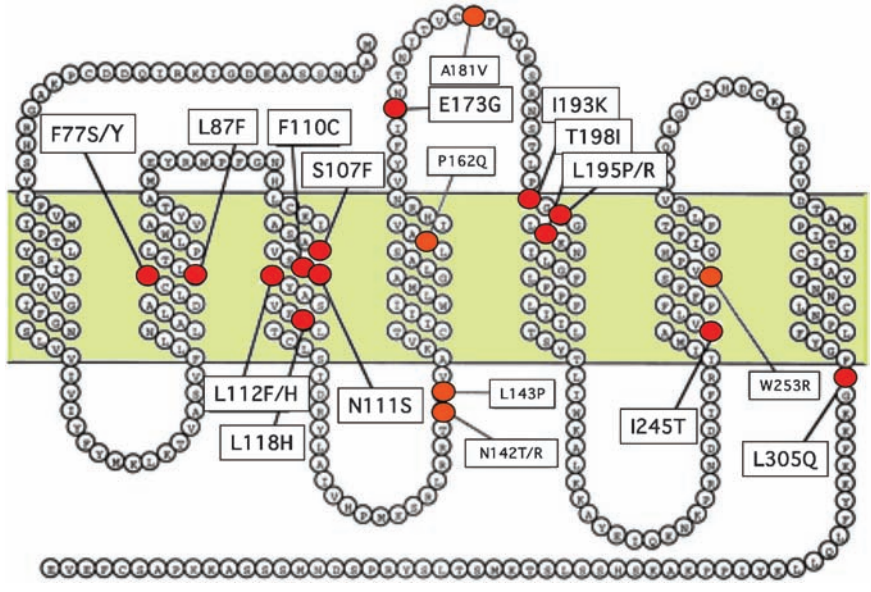


Figure 205 Amino acid substitutions resulting in a large (large captions) or small (small captions) increase in sensitivity of AT_1 receptors to CGP42112A. Reprinted from *Proceedings of the National Academy of Science USA*, **97**, Parnot, C., Bardin, S., Miserey-Lenkei, S., Guedin, D., Corvol, P. and Clauser, E., Systematic identification of mutations that constitutively activate the angiotensin II type 1A receptor by screening a randomly mutated cDNA library with an original pharmacological bioassay, 7615–7620. Copyright (2000) National Academy of Sciences, USA.

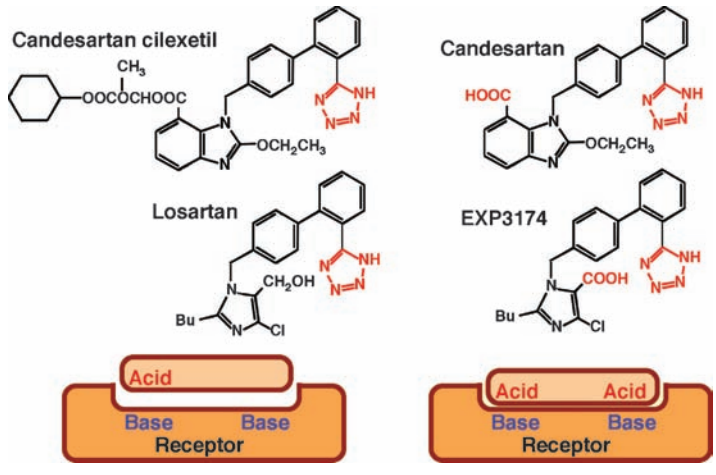


Figure 206 Biphenyltetrazole AT_1 receptor antagonists. Left: Pro-drugs with masked carboxyl group only undergo fast reversible binding. Right: the active drugs possess an exposed carboxyl group, which is likely to be involved in tight antagonist binding.

Antagonist–AT_1 receptor complexes have also been shown to adopt at least two distinct states (Figure 208). One state is formed swiftly and binding of the antagonist is fast and reversible, while the other state is formed more slowly (presumably by isomerization of the quickly reversible complex) and antagonist binding is much tighter. At equilibrium, both states co-exist and their ratio depends on chemical properties of the bound antagonist:

- Antagonist structure–activity relationship studies indicate that those with a carboxyl group in addition to their acidic tetrazole group (like candesartan and EXP3174) are most prone to forming tight binding complexes (Figure 206).
- Receptor mutation studies in which basic amino acids are replaced by neutral ones indicate that Lys^{199} at TM5 of the receptor is important for the recognition of the carboxyl group of these antagonists (i.e. replacing Lys^{199} by the polar, but non-charged Gln produces a much larger drop in affinity for candesartan and EXP3174 as compared to losartan) (Figure 207).

Multiple receptor states related to truncation, covalent modification and mutation

In addition to the ligand-mediated changes in receptor conformation, processes like covalent modification, truncation and mutation are also prone to affect the way in which receptors interact with other molecules.

As an example of covalent modification, the phosphorylated receptor clearly represents another ‘state’ to the ‘activated’ receptor. Accordingly, ‘activated’ and

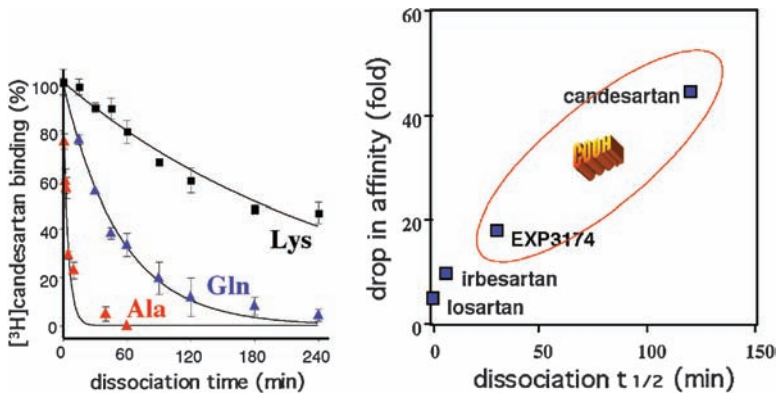


Figure 207 Left: Dissociation of [^{3}H]candesartan from wild-type AT_1 receptors (negative charge, polar Lys^{199}) or mutated receptors (neutral, polar Gln^{199} or neutral non-polar Ala^{199}) (Vauquelin *et al.*, 2002, reproduced by permission of Jraas Ltd.). Right: Lys^{199} to Gln mutation: drop in affinity of AT_1 receptor antagonists versus their dissociation half-life for the wild-type receptor.

'phosphorylated' receptors are able to interact with distinct proteins and this provides a molecular basis to explain switches in cellular signalling during the sustained stimulation of a receptor. Whereas the 'activated' receptor prefers one or more specific G proteins, the phosphorylated receptor may:

- Trigger signal transduction pathways by interacting with non-G proteins (e.g. by forming β-arrestin–receptor complexes).
- Switch the signal between distinct G proteins. In this respect, work with the β_2-adrenergic receptor indicates that the selectivity of receptor–G protein coupling

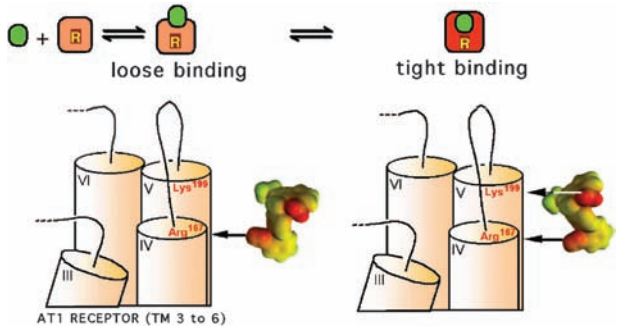


Figure 208 Graphical representation of the proposed interaction of Arg^{167} with the tetrazole moiety and of Lys^{199} with the carboxyl group of candesartan (Vauquelin *et al.*, 2002, reproduced by permission of Jraas Ltd.).

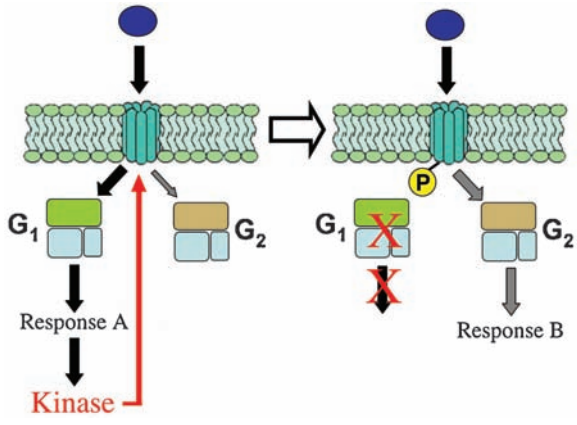


Figure 209 Possible mechanism underlying the 'switch' of the functional coupling of a given receptor with distinct G proteins. Reprinted from *Pharmacology and Therapeutics*, **99**, Hermans, E., Biochemical and pharmacological control of the multiplicity of coupling at G protein-coupled receptors, 25–44. © (2003), with permission from Elsevier.

may be regulated by receptor phosphorylation (Figure 209). Whereas the PKA-phosphorylated receptor shows reduced ability to couple to G_s, it gains the ability to interact with G_i. It is likely that this switch mechanism serves to attenuate the initial G_s-mediated increase in cAMP accumulation by a G_i-dependent feedback inhibition. The receptor–G_i protein interaction will also initiate the activation of MAPK (via βγ-mediated activation of a Src family tyrosine kinase).

In fact, any structural modification of a GPCR may affect its interaction with other molecules and it is not always easy to know whether the affected amino acids directly participated in the interaction or whether their modification produced a conformational change of the receptor. Examples of such altered interactions are widespread:

- Truncation of the AT_{1A} carboxyl terminus produces a receptor mutant that couples well to G_1 and signals in response to Ang II, but exhibits vastly reduced internalization.
- Deletion of part of TM7 of the calcitonin receptor favors G_s coupling over G_q.
- Mutations in the thyrotropin receptor uncouple from G_q while maintaining coupling to G_s.
- Related isoforms of some receptors (derived from alternative splicing of a single gene or generated after RNA editing) show different abilities to activate distinct G proteins.

CAMs represent a special class of structurally modified receptors. The conformations of CAMs are often regarded to reflect intermediate or even fully activated states

of the wild-type receptor. However, it is quite possible that their conformation only approximates one of the active conformations of the wild-type receptor and that, when further activated by an agonist, their final conformation is also quite different from that of the fully activated wild-type receptor. Hence, the study of GPCR activation through the analysis of such CAMs presents severe limitations. A safe standpoint is that CAMs help our understanding of the structure of the inactive state, but give no clue to the interactions resulting in the ligand-induced active conformation(s).

Certain CAMs only activate a single signalling pathway among those ordinarily activated by the agonist. This may explain why:

- For the α_{1B}-adrenergic receptor, it was found that a Cys-to-Phe mutation in TM3 constitutively activates the receptor when measuring the phospholipase C activity, but not when measuring the phospholipase A_2 activity. Thus, such a mutation is likely to stabilize the receptor in a conformation that approximates one of the active conformations of the wild-type receptor.
- [Sar^1,Ile^4,Ile^8]AngII can produce maximal inositol phosphate signalling through the CAM AT_{1A} receptors ($N^{111}A$ and $N^{111}G$). However, no internalization of these CAMs takes place, even in the presence of saturating concentrations of this ligand.

Different CAMs may display different conformations. This may explain why:

- They may be differentially phosphorylated and internalized although they convey a similar agonist-independent activity to the receptor. This has been observed for different CAMs of the α_{1B}-receptor: phosphorylation and internalization still proceeds with $A^{293}E$ mutation but not with $D^{142}A$ mutation (Mhaouty-Kodja *et al.*, 1999).
- [Bpa(2)]PTHrP(1-36) was a partial agonist for the wild-type parathyroid hormone/parathyroid hormone-related peptide receptor and its $T^{410}P$ CAM, but it acted as an inverse agonist for the $H^{223}R$ CAM (Carter *et al.*, 2001).

4.14 Multistate receptors and multiple ligand binding sites

In the multistate model (Schwartz *et al.*, 1995), the receptor is proposed to alternate spontaneously between multiple active and inactive conformations. The key elements in this model are:

- The biological response to a given ligand is determined by the receptor conformation to which this ligand binds with highest affinity. If the preferred conformation is recognized by the G protein as active, the ligand will behave like an agonist. If the preferred conformation is inactive, the ligand will behave like an inverse agonist.
- Two agonists acting at the same receptor do not necessarily have to share an overlapping binding site; they both must stabilize an active conformation.

- An overlap in the binding site of the agonist and a competitive antagonist is not required. The agonist and antagonist simply stabilize distinct receptor conformations to which they bind in a mutually exclusive fashion.

This model implies that there are multiple ways of propagating activation of GPCRs or, in other words, there is no common 'lock' for all agonist 'keys'. Support for the existence of non-overlapping binding sites is provided by a number of observations:

- In contrast to the rhodopsin-like family A receptors, the family C metabotropic glutamate receptors contain two domains that act synergistically to produce receptor activation: a very large extracellular N-terminal agonist binding domain and the 7-TM helices involved in receptor activation and G protein coupling. Interestingly, mutagenesis experiments have identified crucial amino acids required for binding of (allosteric) antagonists in TM3, TM6 and TM7 of these receptors, whereas no extracellular N-terminal regions appear necessary (Figure 210).
- Adrenergic, muscarinic and AT_1 receptor-activating antibodies are present in serum from patients with different pathologies (Table 24). These antibodies are directed against the extracellular loop regions of these receptors; antibodies directed against synthetic peptides mimicking such loop regions have also

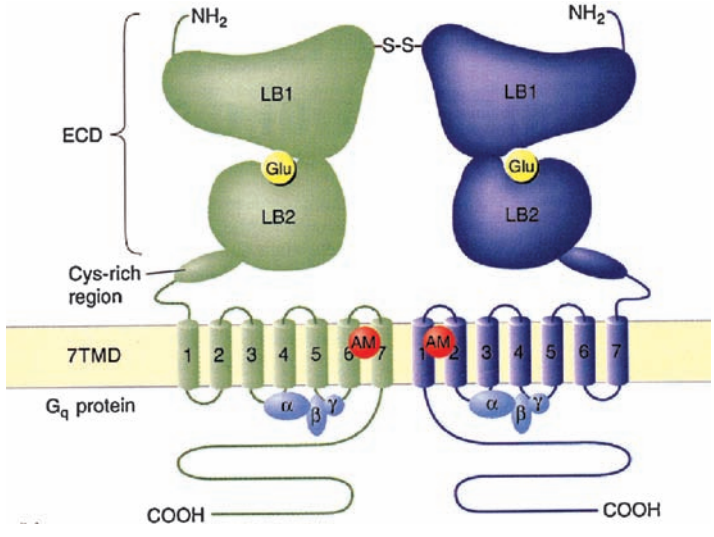


Figure 210 Proposed secondary structure of metabotropic glutamate receptor homodimers. Glutamate is bound between two globular lobes in the N-terminal extracellular region whereas allosteric modulators (AM) bind at TM3 (not shown) TM6 and TM7. Reprinted from *Trends in Pharmacological Science*, **24**, Pellegrini-Giampietro, D. E., The distinct role of mGlu1 receptors in post-ischemic neuronal death, 461–470. Copyright (2003), with permission from Elsevier.

Table 24 Examples of functional GPCR antibodies.

Receptor	Disease	Effect of AAB.	Epitope localization
α_1-R	hypertension	agonist-like	loop 1, 2
β_1-R	dilated cardiomyopathy	agonist-like	loop 1,2
β_1-R	myocarditis	agonist-like	loop 1,2
β_1-R	Chagas' disease	agonist-like	loop 2
β_2-R	Chagas' disease	agonist-like	loop 2
β_2-R	allergic asthma	inhibitory	loop 3
AT1-R	preeclampsia	agonist-like	loop 2
AT1-R	malignant hypertension	agonist-like	loop 2
AT1-R	vascular renal rejection	agonist-like	loop 2
muscarinic M_2-R	Chagas' disease	agonist-like	loop 2
muscarinic M_2-R	dilated cardiomyopathy	agonist-like	loop 2
5HT4-R	systemic lupus erythematosus	agonist-like	loop 2*

been shown to display agonistic properties (Figure 211). This implies that some GPCRs can be activated by the penetration of their small natural agonist into the central cleft as well as by the interaction of bulky antibodies with extracellular loop regions. It is still not clear whether the activated receptor displays the same conformation in both cases and, in fact, little is still known about the molecular

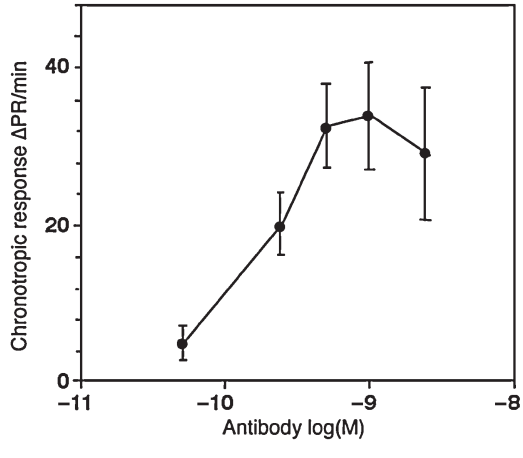


Figure 211 Dose-response curve of affinity-purified β_1-adrenergic receptor autoantibodies from patients with dilated cardiomyopathy. Reprinted from *Journal of Molecular and Cellular Cardiology*, **27**, Wallukat, G., Wollenberger, A., Morwinski, R., Pitschner, H. F., Anti-beta1-adrenoceptor antibodies with chronotropic activity from the serum of patients with dilatated cardiomyopathy: localization of two epitopes in the first and second extracellular loops, 397–406. Copyright (1995) with permission from Elsevier.

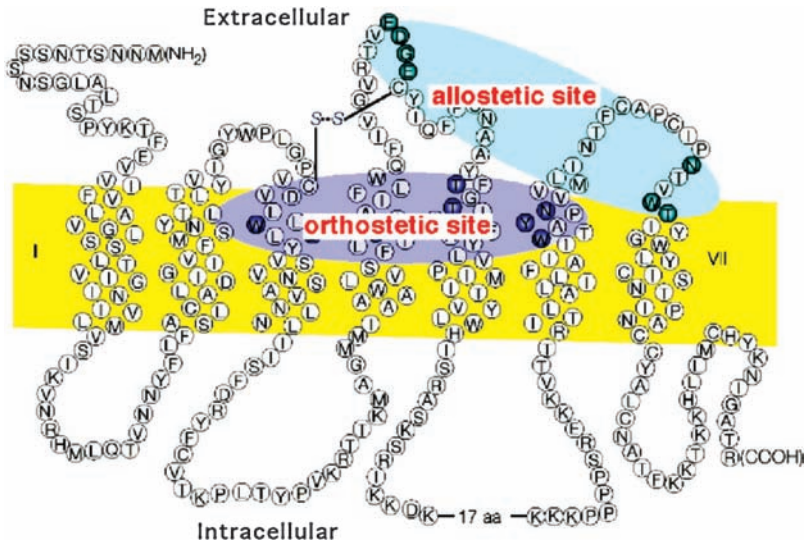


Figure 212 Structure of the M_2 muscarinic receptor with indicating the natural 'orthosteric' ligand-binding site and the binding site for allosteric modulators. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Drug Discovery*, **1**, Christopoulos, A., Allosteric binding sites on cell-surface receptors: novel targets for drug discovery, 198–210, © (2002).

mechanism of antibody activation in general. As a typical example, it was initially assumed that antibodies against the second extracellular loop of the β_2-adrenergic receptor could stabilize the receptor in its 'active' conformation. It is now thought that they act by dimerizing the receptor and that the conformation they induce is different from the (monomeric) resting state and the fully active conformation that is induced by small agonist molecules (Mijares *et al.*, 2000).

- Even though the majority of ligands seem to bind deep within the central cleft of biogenic amine receptors, mutation studies reveal that certain small antagonist molecules may partly interact with residues closer to the surface of the membrane (Figure 212). This has been most extensively studied for muscarinic and adrenergic receptors. As may be expected, such antagonists show no structural relationship with the natural agonists of these receptors.

The general allosteric ternary complex model

The term 'allosteric' (from the Greek meaning 'other site') was introduced by Monod *et al.* (1965) to define binding of ligands to sites on enzymes that were topographically distinct from the substrate-binding site. These accessory binding sites were called 'allosteric sites', in contrast to the substrate-binding (active) site, which was defined as the 'isosteric site'. For GPCRs, the *orthosteric site* refers to the agonist binding

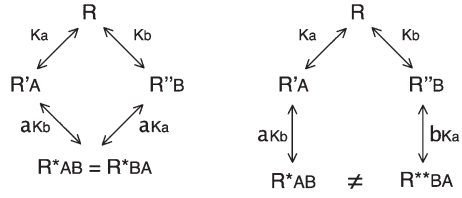


Figure 213 Allosteric models for the binding of two ligands (A and B) to one receptor (R, R′, R″, R* and R** represent different conformations). Left: the simplest 'allosteric terenary complex model' assumes that the conformation of the ternary complex is the same regardless of which ligand bound first (Christopoulos and Kenakin, 2002). Right: the more complete sequential 'KNF' model (Koshland *et al.*, 1966) allows the conformation of the ternary complex to depend on whether A or B bound first (reprinted from *Fundamental and Clinical Pharmacology*, **19**, Vauquelin, G. and Van Liefde, I., G protein-coupled receptors: a count of 1001 conformations, 45–56, Copyright (2005) Blackwell Publishing).

site on a receptor. *Allosteric sites* are additional binding sites on a receptor that are topographically distinct (i.e. not overlapping) from the orthosteric site, but that can modulate receptor activity. *Allosteric interactions* are interactions between two distinct binding sites on the same receptor complex. These interactions involve the transmission of a conformational change across the GPCR from one binding site to another. Hence allosteric interactions are reciprocal in nature: for GPCRs the binding of an *allosteric ligand* modulates the binding/function of the *orthosteric ligand* and vice versa. As discussed below, this allosteric modulation can be either negative or positive.

The general allosteric ternary complex model (Figure 213) is the simplest model to describe the binding of two ligands A and B to one receptor to form a ternary ARB complex. A binds to the orthosteric site whereas B, the allosteric modulator, binds to the allosteric site and whatever ligand A does to B, B does to A (Christopoulos and Kenakin, 2002). K-values are equilibrium dissociation constants for binding and α represents a co-operativity factor. It represents the magnitude by which the affinity of one ligand is changed by the other ligand when both are bound to the receptor (Figure 214).

- $\alpha < 1$ reflects positive co-operativity (i.e. the affinity of a ligand increases when the receptor is occupied by the other ligand). Saturation binding curves for A (where receptor occupation is defined as ([AR] + [ARB])/[R])) will shift to the left in the presence of B and a maximal shift will be reached when all receptors are occupied by B. In other words, as B increases, the apparent K_D of A will gradually decrease from K_a to αK_a.
- $\alpha = 1$ reflects no co-operativity (i.e. the affinity of a ligand is the same for the free receptor and when the receptor is already occupied by the other ligand).
- $\alpha > 1$ reflects negative co-operativity (i.e. the affinity of a ligand decreases when the receptor is occupied by the other ligand). Negative co-operativity between A and B will be manifested as a rightward shift of the binding curve for A.

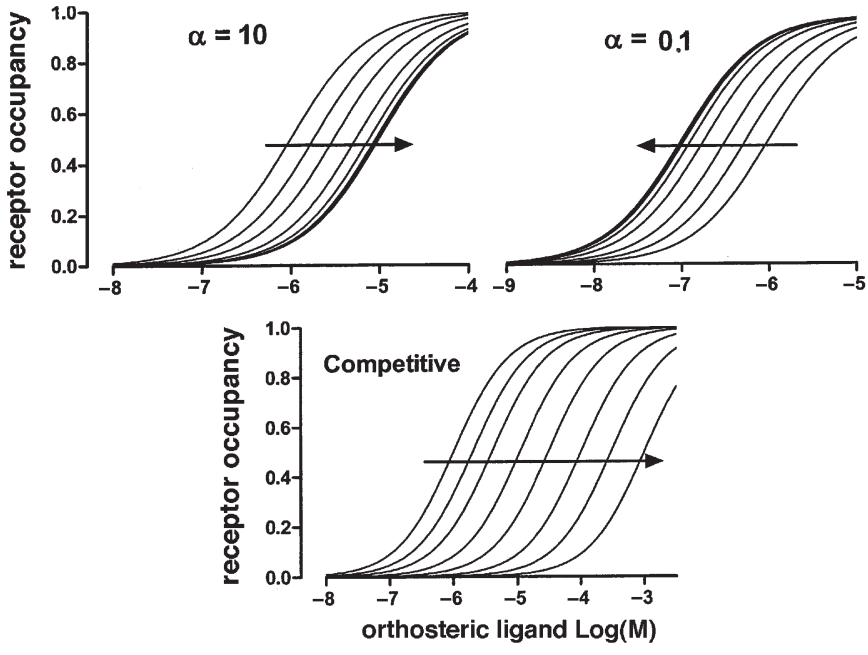


Figure 214 Saturation binding curves for an orthosteric ligand. The arrow represents the effect of increasing concentrations of a negative allosteric modulator ($\alpha = 10$), positive allosteric modulator ($\alpha = 0.1$), or a competitive ligand. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Drug Discovery*, **1**, Christopoulos, A., Allosteric binding sites on cell-surface receptors: novel targets for drug discovery, 198–210, © (2002).

Whereas the allosteric terenary complex model implies a strict reciprocality between the mandatory effects of orthosteric or allosteric ligands (to reach the same end state R*), the more complete sequential 'KNF' model (Figure 213) allows non-reciprocal modulations. The end state of the receptor will then depend on which ligand bound first.

Exogenous and endogenous allosteric modulators

An increasing number of rather small molecules have been identified as behaving as allosteric modulators at GPCRs (Table 25). However, they do not constitute the only ones.

The formation of a bond between two proteins also causes their conformational change and hence alters their properties. In this vein, G proteins may be regarded as behaving like allosteric GPCR 'ligands' since they affect the agonist–receptor interaction, but do not couple to the same site of the receptor as the agonist. The interaction between agonist binding and G protein coupling is positively co-operative in nature. Agonist binding to the receptor increases its affinity

Table 25 Small molecule allosteric modulators at GPCRs.

GPCR	Allosteric modulators
Adenosine A1	Thieno[2,3-c]pyridine derivatives, 2-amino-3-heteroaroylthiophenes, 2-aminothiophene-3-carboxylates, amilorides
Adenosine A3	1H-imidazo[4,5-c]quinolin-4-amine and 3-(2-pyridinyl)isoquinoline derivatives, amilorides
α_1-adrenergic	Conopeptide rho-TIA
α_2-adrenergic	SCH-202676, amilorides
β_2-adrenergic	Zinc
D1 dopamine	Methylisobutylamiloride, zinc
D2 dopamine	Homocysteine, L-prolyl-L-leucyl-glycinamide and analogues, methylisobutylamiloride, zinc
5-HT2C serotonin	L-threo-alpha-D-galacto-octopyranoside, PNU-69176E
5-HT3 serotonin	Verapamil, ifenprodi, GYKI-46903, 5-hydroxyindole and analogues
5-HT7 serotonin	Oleamide
M1 muscarinic	MT7 toxin, KT5720, AC-42
M2 muscarinic	Alkane-bisammonio-type and bispyridinium-type compounds, NGD-3366, W-84, gallamine
M3 muscarinic	Rapacuronium
M4 muscarinic	WIN 62,577, alcuronium, brucine
GABAB	CGP7930, GS39783
Calcium-sensing	Calindol, NPS R-568
M5 metabotropic glutamate	CDPPB, VU-29, DFB, CPPHA

for the G protein and, reciprocally, agonists display increased affinity for the G protein-coupled receptor.

From the perspective of the GPCR, the orthosteric site is the agonist binding site, whereas for the G protein, the orthosteric site may be defined as the guanine nucleotide binding site on the G subunit. The binding interface of the two proteins constitutes their allosteric site. The binding of GTP to the orthosteric site of the G protein will weaken the affinity of its allosteric site for the receptor. Following the dissociation of the complex, the receptor no longer senses the positive allosteric interaction of the G protein so that its orthosteric site again displays low agonist affinity. This explains the GTP-mediated rightward shift of agonist/labelled antagonist competition binding curves (Figure 44 and 157).

As addressed in previous sections, the classic picture of isolated monomeric GPCRs has given way to models in which they can form dimers and even combine with ‘accessory proteins’ that may act as partners in signalling events. In all of these instances, the possibility exists for allosterism as a consequence of protein–protein interactions. It is even possible that accessory proteins are required to unmask the

pharmacology of specific orphan receptors (i.e. receptors for which the gene product has been identified but not yet the endogenous ligand):

- GPCR homo- and heterodimers have been shown to generate receptor subtypes with a pharmacological profile that is distinct from that of either monomer alone. For the $GABA_B$ receptor, heterodimerization between its constituents $GABA_BR1$ and $GABA_BR2$ has even been shown to be essential for its ligand recognition and signalling functions. In other instances, homo- or heterodimerization may merely be the consequence of receptor overexpression in recombinant systems and, hence, have no or little physiological significance.
- A particularly well characterized case of GPCR interaction with accessory proteins is the association of the calcitonin receptor-like receptor (CRLR) with single TM-spanning receptor activity modifying proteins (RAMPs). These accessory proteins clearly change the phenotype of the receptor: association with RAMP1 produces a high-affinity CGRP receptor while association with RAMP2 or RAMP3 produces a receptor for adrenomedullin.
- Calcyon, another single TM-spanning protein, has been shown to physically associate with D_1 dopamine receptors in neurones and to potentiate their ability to increase the cytosolic Ca^{2+} concentration, a typical $G_{q/11}$-mediated response. Whereas calcyon does not seem to affect the affinity of agonists, it significantly enhances the proportion of the high-affinity state. This finding suggests a complex allosteric interaction involving calcyon, the receptor and the G protein.

Other endogenous allosteric modulators include cations. By interacting with a highly conserved aspartic acid located in TM2, sodium ions can exert an allosteric effect on the binding properties of both agonists and G proteins. Indeed, mutagenesis studies have shown that altering the charge of this single amino acid exerts a global alteration in GPCR conformational states. Other cations have been suggested to allosterically modulate GPCR binding properties by interacting with extracellular amino acid contact points.

Allosteric phenomena at GPCR: detection by radioligand binding

Allosteric phenomena at GPCRs can be evidenced using radioligand binding and functional assays. Usually, they are first detected when experimental data deviate from the expectations of simple (competitive) mass-action kinetics. Yet such findings may also reflect experimental artefacts, including inappropriate drug equilibration times, drug solubility problems, exceedingly high receptor concentrations or perturbation of the surrounding lipid bilayer. This latter mechanism allows many types of sufficiently hydrophobic compounds to non-specifically alter receptor conformation so that they could be mistakenly labelled as ‘allosteric modulators’. Hence, control experiments need to be performed to check for such potential sources of interference.

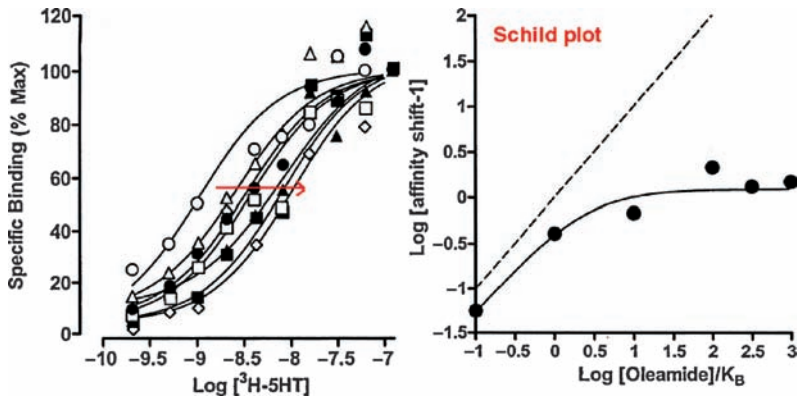


Figure 215 Allosteric modulation of [^{3}H]5-HT binding to 5-HT$_7$ receptors by oleamide. Arrow: shift upon increasing the concentration of oleamide. Right: Schild plot of the same data; the dashed line is the predicted behaviour of a competitive antagonist. Reprinted from *Biochemistry and Pharmacology*, **58**, Hedlund, P. B., Carson, M. J., Sutcliffe, J. G. and Thomas, E. A., Allosteric regulation by oleamide of the binding properties of 5-hydroxytryptamine7 receptors, 1807–1813. Copyright (1999), with permission from Elsevier.

Allosteric phenomena have repercussions on the three standard radioligand binding assays:

- *Saturation binding*. A negative allosteric modulator may produce a dose- dependent increase in the radioligand's K_D in the same way as a competitive antagonist would do. However, whereas this increase has no limit in the case of a competitive antagonist, it will reach a limit in the case of an allosteric modulator (Figure 215). When the saturation binding curve is plotted on a logarithmic scale, the modulator will produce a dose-dependent rightward shift until a maximum is reached. The corresponding Schild plot will be curvilinear and level off at high concentrations of the allosteric modulator. Importantly, allosteric phenomena depend on the nature of the orthosteric ligand, binding assays with different radioligands can yield different results even when the modulator and the receptor are the same.
- *Competition binding.* A negative allosteric modulator may produce a dose-dependent decrease in the binding of a fixed concentration of radioligand and the competitive ligand will decrease the binding down to non-specific binding levels. The maximal inhibition produced by a negative allosteric modulator will depend upon the magnitude of the co-operativity factor α as well as on the radioligand concentration (Figure 216): i.e. the maximal inhibition is highest at low radioligand concentrations and for modulators with a high degree of negativity (i.e. $\alpha \gg 1$). In extreme cases, when a negative allosteric modulator decreases the binding to close to the level of non-specific binding, it may be mistaken for a competitive ligand. Hence a 'complete' displacement to non-specific binding levels does not necessarily implicate competitive antagonism.

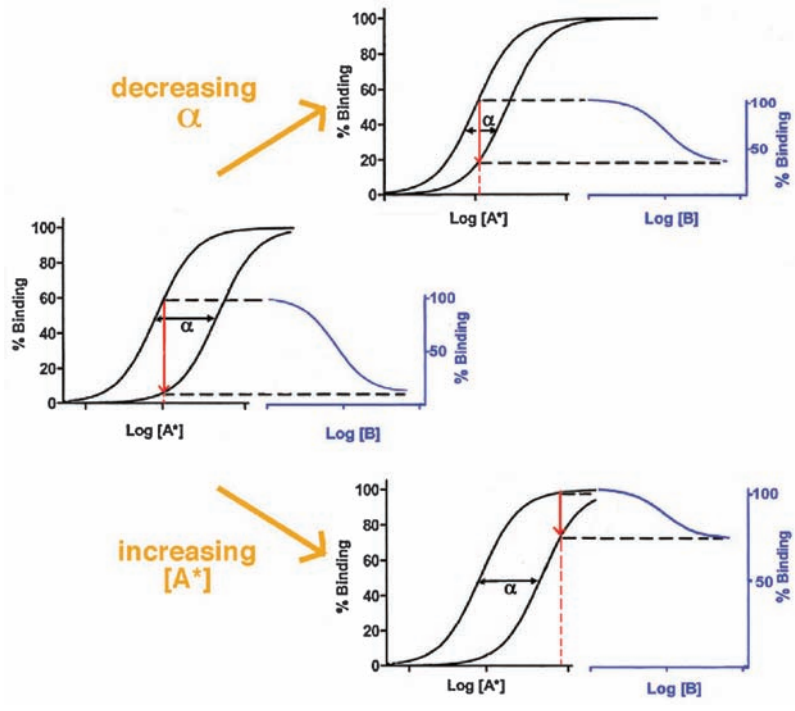


Figure 216 Inhibition of radioligand (A*) binding by a negative allosteric modulator (B): effect of decreasing the co-operativity factor and increasing the radioligand concentration. Left side of each figure: saturation binding of A* (on a logarithmic scale!) in the absence or presence of a saturating concentration of B. Right side of each figure: 'competition' binding by B with a constant concentration of [A*] indicated by the red dotted lines. Reproduced from Christopoulos, A. and Kenakin, T. (2002) *Pharmacological Reviews*, **54**, 323–374, with permission from the American Society for Pharmacology and Experimental Therapeutics.

A convenient way to differentiate both types of interactions is to compare the IC_{50} values of unlabelled compounds at different radioligand concentrations (Figure 217). Whereas the IC_{50} values increase proportionally with the radioligand concentration for true competitors, the IC_{50} values will level off for negative allosteric modulators.
On the other hand, a positive allosteric modulator may produce a dose-dependent increase in the binding of a fixed concentration of radioligand (Figure 218). Here again, the maximal increase will depend upon the co-operativity factor α, as well as on the radioligand concentration. Importantly, allosteric interactions are unique for each pair of orthosteric and allosteric ligands involved. A positive allosteric modulator of one particular orthosteric ligand is not necessarily a positive modulator of another orthosteric ligand. For example, alcuronium is a positive modulator for a variety of orthosteric ligands at the M_2 muscarinic receptor and a negative modulator for others.

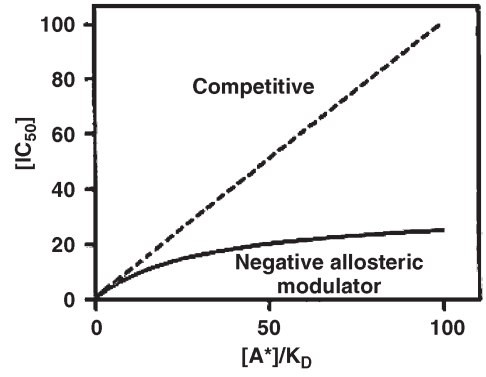


Figure 217 Unlabelled competitors can be discriminated from allosteric modulators in 'competition' binding studies when their IC_{50} values are plotted as a function of the radiolabelled orthosteric ligand (A*) concentration. Reproduced from Christopoulos, A. and Kenakin, T. (2002) *Pharmacological Reviews*, **54**, 323–374, with permission from the American Society for Pharmacology and Experimental Therapeutics.

- *Kinetic studies.* A change in receptor conformation induced by an allosteric agent is likely to alter the orthosteric ligand association and/or dissociation rate constants (Figure 219). This alteration is responsible for the effects of allosteric modulators on orthosteric ligand affinity at equilibrium. In practice, the study of the kinetic properties of a radioligand often allows very sensitive detection of allosteric interactions at GPCRs. Positive allosteric modulation can increase the association rate and/or decrease the dissociation rate. Negative allosteric modulators act the opposite way; they may decrease the association rate and/or increase the dissociation rate. Compared to association experiments, dissociation experiments are easier to interpret, as they only reflect the dissociation of a preformed

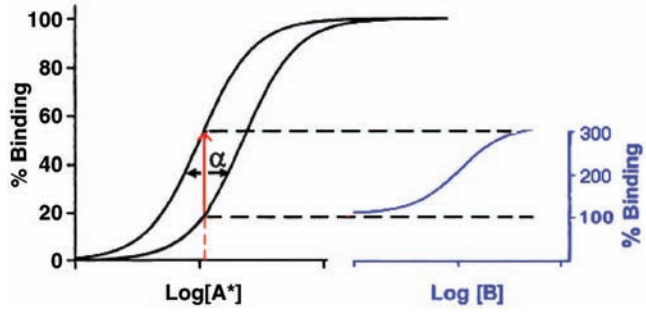


Figure 218 Increase of radioligand (A*) binding by a positive allosteric modulator (B). Left: saturation binding of A* (on a logarithmic scale!) in the absence or presence of a saturating concentration of B. Right: 'competition' binding by B with constant concentration of [A*] indicated by the red dotted line.

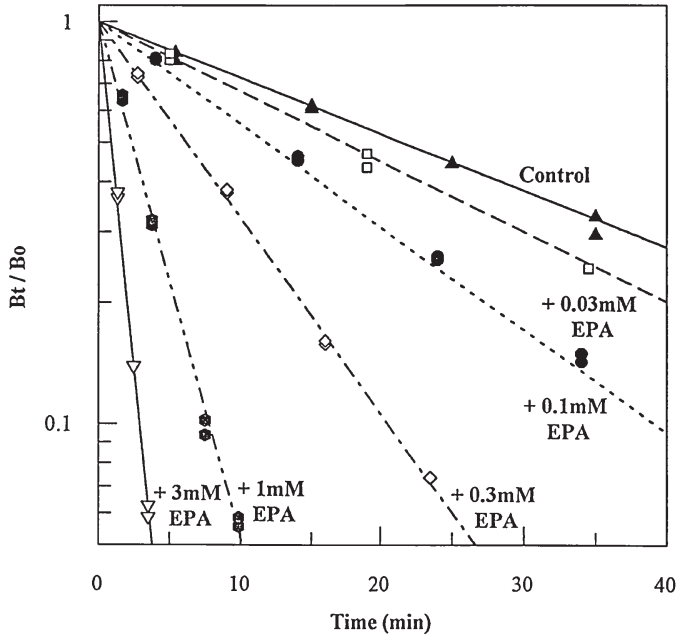


Figure 219 Increased dissociation of [^{3}H]yohimbine from the human α_{2A}-adrenergic receptor in the presence of the allosteric modulator 5-(N-ethyl-N-isopropyl)-amiloride (EPA). Reproduced from Leppik, R., Lazareno, S., Mynett, A. and Birdsall, N. (1998) *Molecular Pharmacology*, **53**, 916–925, with permission from the American Society for Pharmacology and Experimental Theraputics.

orthosteric ligand–receptor complex. Hence, they provide the simplest and most reliable means to detect allosterism at GPCRs.

Although dissociation experiments are usually straightforward to interpret, they could also give rise to over-interpretation or even false interpretations:

- Claims of co-operative binding based on dissociation kinetics using high-affinity radioligands and/or highly concentrated receptor preparations need to be viewed with caution due to the increased likelihood of 'rebinding' phenomena (see Figure 46).
- Finally, the dissociation rate of an orthostatic drug can also be affected by drugs that interfere with receptor–G protein coupling (in agonist dissociation experiments) or affect the receptor conformation indirectly by perturbing the surrounding lipid bilayer. For example, the AT_1 receptor antagonist candesartan dissociates much faster from its receptors in cell membrane preparations than from the same receptor in intact cells (Figure 220). The same increase in dissociation rate can be observed by treating the cells with minute amounts of filipin (a cholesterol-binding pore-forming agent) and saponin (a detergent).

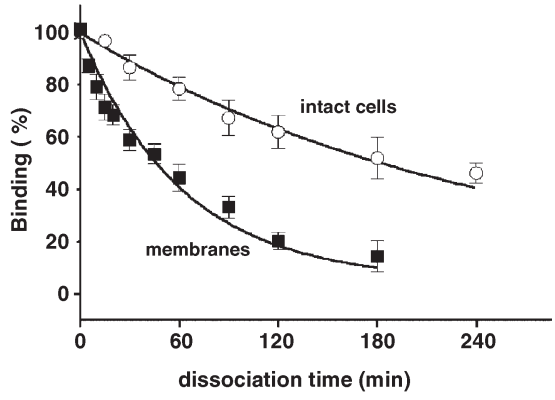


Figure 220 Dissociation of the antagonist [^{3}H]candesartan from AT_1 receptor-expressing CHO cells and derived cell membranes. Reprinted from *Biochemistry and Pharmacology*, **63**, Fierens, F., Vanderheyden, P.M.L., Roggeman, C., Vande Gucht, P., De Backer, J.-P. and Vauquelin, G., Distinct binding properties of the AT1 receptor antagonist [3H]candesartan to intact cells and membrane preparations, 1273–1279, Copyright (2002), with permission from Elsevier.

Detection of allosteric phenomena at GPCRs by functional assays

The cubic '*allosteric two-state model*' by Hall (2000) describes the interaction of an allosteric modulator and an orthosteric ligand on a receptor that can adopt active (R*) and inactive (R) conformations (Figure 221). This model allows an allosteric ligand to modulate the orthosteric ligand's affinity as well as its intrinsic efficacy. This model applies to any allosteric modulator. In the case of G proteins and allosteric modulators, the model is formally identical with the 'cubic ternary complex model' by Weiss (Weiss *et al.*, 1996).

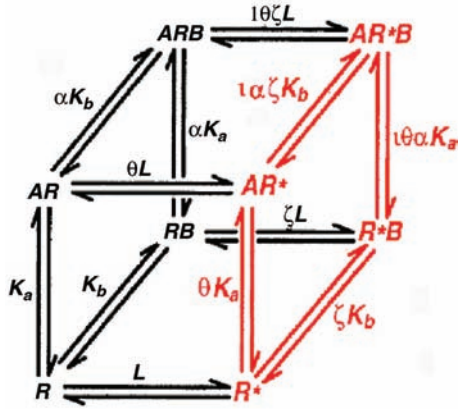


Figure 221 The cubic 'allosteric two-state model'. The active receptor conformation (R*) is in red. In practice, allosteric interactions between multiple ligands (usually the orthosteric ligand, (A), the G protein and another allosteric ligand) on the same GPCR may be even more complex. Reproduced from Hall, D. (2000) *Molecular Pharmacology*, **58**, 1412–1423, with permission from the American Society for Pharmacology and Experimental Theraputics.

Functional assays may allow the detection of specific receptor conformations promoted by allosteric modulators that may have escaped detection in radioligand binding assays. Indeed the affinity and efficacy of an agonist are independent parameters and an allosteric modulator may differentially affect them. Here again it is important to notice that allosterism may differ from one orthosteric ligand to another. Some specific situations are presented below:

- *Unchanged efficacy.* The allosteric modulator will produce parallel shifts of the concentration-response curves of an orthosteric agonist with no change in basal and maximal responses. The shift is to the left in the case of positive co-operativity and to the right in the case of negative co-operativity. Similar to radioligand binding assays, the shift will attain a limit (defined by α) as the concentration of allosteric modulator increases. However, modulators with high degrees of negative co-operativity ($\alpha \gg 1$) may be mistaken for competitive antagonists. Detection and quantification of negative allosteric modulation is, here again, improved by investigating the effects of as large a range of modulator concentrations as possible. Indeed, it may only be at high concentrations of modulator that the Schild plot deviates for linearity.
- *Unchanged potency.* Some allosteric modulators will either increase or decrease the maximal response of the orthosteric agonist without changing its potency (Figure 222). The change in maximal response will increase upon increasing the concentration of the allosteric modulator until a limit is attained. It is likely that such modulators also fail to perturb the binding of the radiolabelled agonist to membrane preparations.

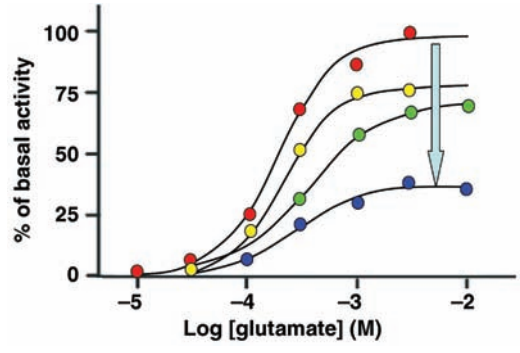


Figure 222 Glutamate-mediated inositol phosphate production in CHO cells expressing the metabotropic glutamate receptor 1. Effect of increasing concentrations of the allosteric modulator CPCCOEt. Reproduced from Litschig, S., Gasparini, F., Rueegg, D., Stoehr, N., Flor, P. J., Vranesic, I., Prezeau, L., Pin, J.-P., Thomsen, C. and Kuhn, R. (1999) *Molecular Pharmacology*, **55**, 453–461, with permission from the American Society for Pharmacology and Experimental Theraputics.

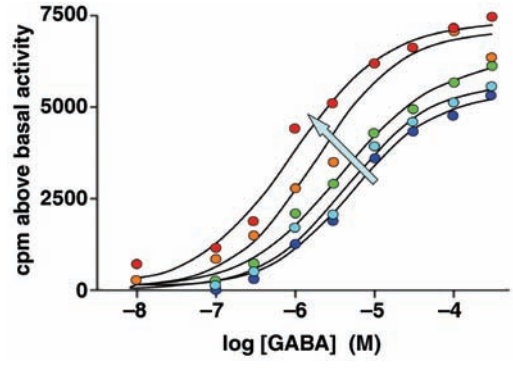


Figure 223 GABA-mediated [^{35}S]GTPγS binding in membranes from human $GABA_B$-receptor - expressing CHO cells. Arrow: effect of increasing concentrations of CGP7930. Reproduced from Urwyler, S., Mosbacher, J., Lingenhoehl, K., Heid, J., Hofstetter, K., Froestl, W., Bettler, B. and Kaupmann, K. (2001) *Molecular Pharmacology*, **60**, 963–971, with permission from the American Society for Pharmacology and Experimental Theraputics.

- *Changed potency and efficacy.* For example, CGP7930 increases the potency of the endogenous agonist, GABA, as well as its maximal response (Figure 223).
- *Receptor activation by the allosteric modulator* (Figure 224). The cubic allosteric two-state model allows the receptor to be activated by an allosteric modulator, even in the absence of an agonist. This may go along with negative, positive or no co-operativity with respect to agonist binding.

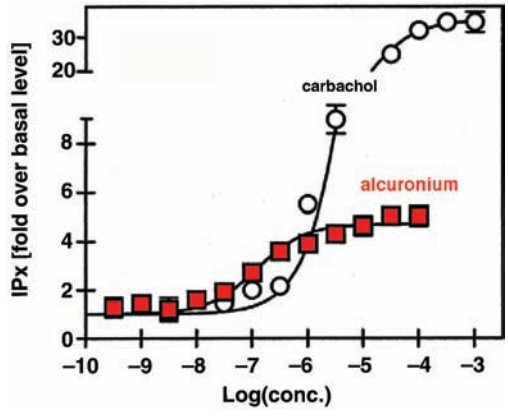


Figure 224 Alcuronium is an allosteric modulator of M_1 muscarinic receptors. It elicits significant receptor stimulation in the absence of orthosteric agonists. Reprinted from *Proceedings of the National Academy of Science USA*, **97**, Parnot, C., Bardin, S., Miserey-Lenkei, S., Guedin, D., Corvol, P. and Clauser, E., Systematic identification of mutations that constitutively activate the angiotensin II type 1A receptor by screening a randomly mutated cDNA library with an original pharmacological bioassay, 7615–7620. Copyright (2000) National Academy of Sciences, USA.

Usefulness of allosteric modulators

There are distinct advantages to producing physiological responses with allosteric ligands:

- First, there is often a 'ceiling' to the effects of an allosteric modulator. As a consequence, allosteric modulators would be generally much safer in overdosage than orthosteric ligands.
- Second, positive allosteric modulators could selectively 'sensitize' tissues where the endogenous agonist exerts its physiological effects. This will result in an increased response for the same concentration of endogenous messenger present.
- Finally, small molecules can effectively inhibit the interaction of large proteins through allosteric mechanisms (e.g. antagonists of metabotropic glutamate receptors) without the need to compete with those large proteins for binding to the orthosteric site.

4.15 'Competitive', 'non-competitive' and 'insurmountable' antagonism

The elaboration of agonist dose-response curves in the presence of increasing antagonist concentrations is an ancient, but still routine, approach to investigate antagonism in functional studies. Information can be gathered about the molecular mechanism of the antagonist–receptor interaction, but the relevance of this information is highly dependent on the experimental conditions (Figure 227) as well as on the extent of 'receptor reserve'.

Co-incubation, no receptor reserve

A rightward shift of the dose-response curves (Figure 224) without decrease in the maximal response can be observed for *competitive antagonists* (Figure 226). They compete for binding to the same or to partially overlapping sites at the receptor so that the binding of the one excludes the binding of the other. They produce a rightward shift of the agonist dose-response curve without affecting the maximal response. The shift increases with the antagonist concentration and is, in principle, unlimited. Based on these shifts, the antagonist affinity can be calculated by the Schild method.

A decrease in the maximal response (Figure 225) can be observed for different classes of *non-competitive* antagonists (Figures 226 and 227):

- *Allosteric antagonists* (Figure 226) bind to a site of the receptor that is topographically different from the orthosteric binding site. They are *non-competitive* since they do not compete with the agonist for binding to the orthosteric site. *Allosteric antagonists* may decrease the efficacy (and eventually also the potency) of the agonist (see Section 4.14).

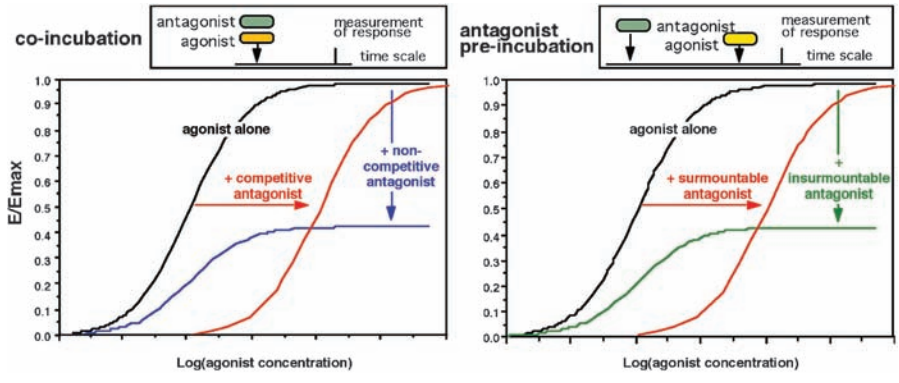


Figure 225 Antagonists are denoted as (non)-competitive or (in)surmountable depending on the incubation protocol. Co-incubation experiments: antagonists are non-competitive if the maximal response decreases and are usually competitive if the agonist dose-response curve is shifted to the right without limit.

- *Functional antagonists* (Figure 226) block an intracellular event that is triggered by the agonist–receptor interaction and thereby impair the chain of events linking the stimulus to the measured response. These antagonists do not bind to the receptor (and thus do not block agonist binding and receptor activation). Since a particular response may be triggered by a variety of different receptors in the same tissue (e.g. α-adrenergic, angiotensin II, Neuropeptide Y, serotonin, prostaglandin and endothelin receptors trigger vascular smooth muscle contraction), functional antagonists are likely to block the responses of all these receptors. Therefore, if an antagonist is found to be non-competitive for a given receptor, its ability to affect the action of related receptors (i.e. giving the same response) is usually checked to find out whether it is a functional antagonist or not. In radioligand binding experiments, functional antagonists are unlikely to affect the binding of orthosteric ligands, as they do not directly interact with the receptor. However, an

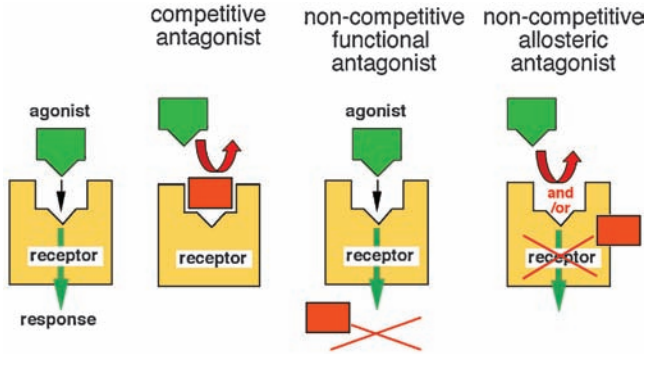


Figure 226 Molecular mechanisms resulting in competitive versus non-competitive antagonism.

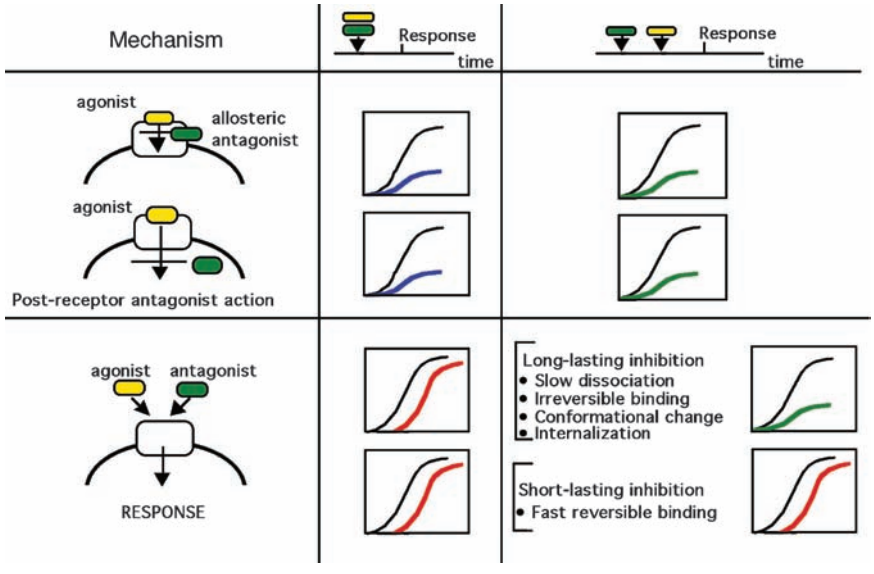


Figure 227 Relationship between (non)-competitive and (in)surmountable antagonists. Reprinted from *Angiotensin II Receptor Antagonists* (M. Epstein and H.R. Brunner, Eds.), Vauquelin G., Fierens F.L.P. and Vanderheyden P.M.L., Mechanisms of Angiotensin II Antagonism. Competitive versus Non-Competitive Inhibition, pp. 105–118. Copyright (2002), with permission from Elsevier.

exception should be made for those antagonists that bind to G proteins and impair their coupling to the receptor.

It is important to note that a decrease in the maximal response of an agonist only refers to non-competitive antagonism when both ligands are added together to the receptor (i.e. co-incubation, Figure 227). It is only under these conditions that agonists and antagonists are given an equal chance to compete with each other for binding to the receptor.

Antagonist pre-incubation, no receptor reserve

Functional studies very often include a pre-incubation step, in which the receptors are pre- equilibrated with the antagonist. Subsequently, the agonist will be added and the response measured. This implies that the antagonist had the opportunity to interact with the receptor for some time without any interference from the agonist. Therefore, one prefers to speak in terms of *surmountable* (parallel shifts of the dose-response curves) and *insurmountable* antagonism (depression of the maximal response) rather than in terms of competitive/non-competitive antagonism (Figure 225).

Surmountable antagonists (Figure 227, red curve on right panel only) are *competitive* antagonists that dissociate sufficiently fast from the receptor. This allows the subsequently added agonist to occupy all receptor sites, at least when its concentration is high enough.

Insurmountable antagonists (Figure 227, green curves) are either:

- *Non-competitive* antagonists (both allosteric and functional).
- *Competitive* antagonists, but with such long-lasting action that the subsequently added agonist does not get the opportunity to occupy (stimulate) all the receptor sites at the time the response is measured. In other words: the agonist is not long enough in contact with the receptors to surmount the antagonist's action. This type of antagonist is thus likely to display irreversible (i.e. covalent) or slowly reversible binding characteristics in radioligand binding studies. However, alternative theories have also been elaborated to explain insurmountable antagonism. According to some, the antagonists do not need to remain bound to the receptor to produce a long-lasting effect. They could induce a conformational change in the receptor, dissociate and leave the receptor in a conformation that cannot be recognized by agonist molecules. Alternatively, some antagonists could induce receptor internalization, so that it becomes inaccessible to the agonist molecules.

When functional studies include an antagonist pre-incubation step, surmountable inhibition can only be obtained for competitive antagonists. For insurmountable inhibition, it is not possible to find out whether the antagonist is truly non-competitive or not. However, this distinction can be made based on experiments in which the receptors are co-incubated with agonist and antagonist. The AT_1-receptor blocker, candesartan, represents a typical example of an insurmountable, yet competitive antagonist (Figure 228).

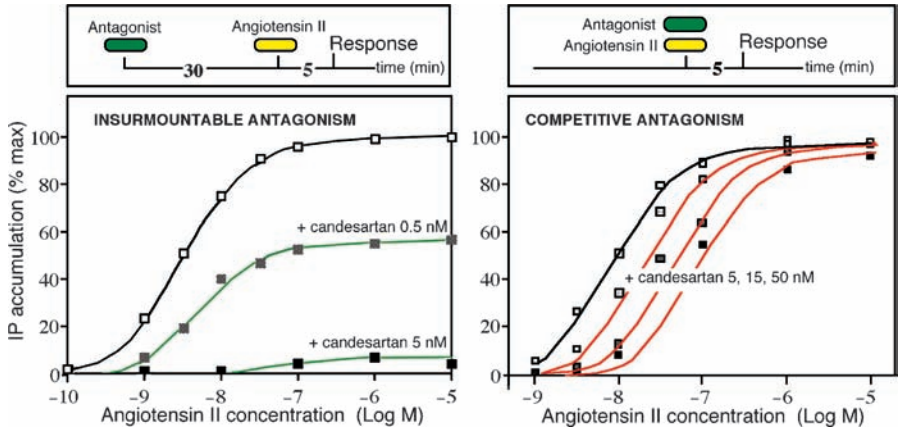


Figure 228 The AT_1-receptor blocker, candesartan, is an insurmountable, yet competitive antagonist (response is production of inositol phosphates in CHO-AT_1 cells). Reprinted with permission from Vanderheyden P.M.L., Fierens F.L.P., De Backer J.-P., Frayman N. and Vauquelin G.(1999) Distinction between surmountable and insurmountable selective AT1 receptor antagonists by use of CHO-K1 cells expressing human angiotensin II AT1 receptors, *British Journal of. Pharmacology*, **126**, 1057–1065; Reprinted from *European Journal of Pharmacology*, **372**, Fierens, F.L.P., Vanderheyden, P.M.L., De Backer, J.-P. and Vauquelin, G., Insurmountable angiotensin II AT1 receptor antagonists: the role of tight antagonist binding, 199–206. Copyright (1999), with permission from Elsevier.

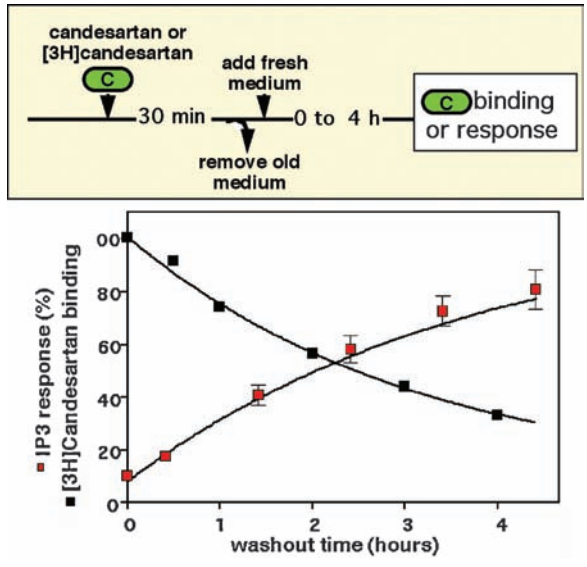


Figure 229 [^{3}H]Candesartan dissociates only slowly from the AT_1 receptor. Its dissociation coincides with the recovery of functional receptors. Reprinted from *European Journal of Pharmacology*, **367**, Fierens, F., Vanderheyden, P. M., De Backer, J. P. and Vauquelin, G., Binding of the antagonist [3H]candesartan to angiotensin II AT1 receptor-transfected Chinese hamster ovary cells, 413–422. Copyright (1999), with permission from Elsevier.

As expected for this situation, [^{3}H]candesartan dissociates only slowly from its receptor (dissociation half-life = 120 min under the same experimental conditions as in the functional assay). The dissociation of this radioligand corresponds to the recovery of functional receptors in washout experiments (i.e. experiments in which the receptors are incubated with antagonist, washed and incubated with fresh medium for various time intervals before adding agonist and measuring the response) (Figure 229). This indicates that the receptor can be activated as soon as the antagonist has dissociated.

Slow dissociation of an antagonist–receptor complex will delay the attainment of a mass action-type competition with the natural agonist/messenger each time the concentration of the latter increases. As recently proposed by Swinney (2004), this constitutes a mechanism to improve the 'biochemical efficiency' of the antagonist: i.e. the relationship between its IC_{50} to inhibit the agonist's functional response and its binding affinity (K_i). Drugs with high biochemical efficiency are therefore likely to achieve the required clinical effect at low concentrations and therefore to possess a high therapeutic window.

4.16 Naturally occurring mutations of GPCRs

A number of diseases have already been attributed to mutational defects of GPCRs and with the techniques now available to isolate and sequence genes many more are likely to be found. Genetic polymorphisms are frequently occurring genetic variants within

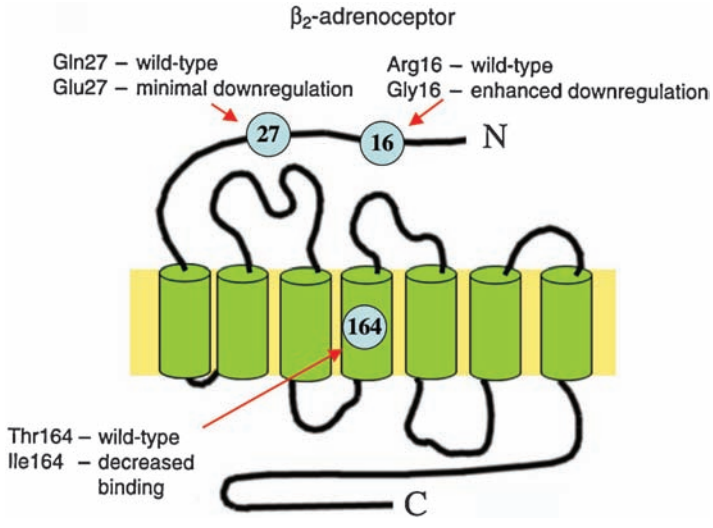


Figure 230 Polymorphisms of human β_2-adrenergic receptors. Circles indicate positions of most common polymorphisms and their functional significance. Reprinted from *Trends in Pharmacological Science*, **20**, Buscher, R., Herrmann, V. and Insel, P. A., Human adrenoceptor polymorphisms: evolving recognition of clinical importance, 94–99. Copyright (1999), with permission from Elsevier.

a population and, in this respect, β_2-adrenergic receptors are known to be highly polymorphic (Figure 230) and at least four polymorphisms are known in which an individual amino acid is different to that of the wild-type receptor. Further *in vitro* and *in vivo* studies are likely to extend the range of known mutations/genetic polymorphisms and provide a better insight concerning those which might predispose an individual to the onset of a disease, alter the clinical course of a disease or the response to clinical treatment.

GPCR mutations can be grouped according to whether they:

- Do not affect receptor function: for example, $Val^{34}Met$ has not yet been associated with changes in β_2-adrenergic receptor function.
- Cause a loss of function: they may be autosomal recessive so that the lack of receptor function can be compensated in heterozygotes by the normal gene product. Hence, they are only apparent in homozygotes. Alternatively, they may be autosomal dominant and cannot be compensated. For example, $Thr^{164}Ile$ for the β_2-adrenergic receptor leads to several functional effects, including lower binding affinities of the messenger and to a deficient coupling of the receptors to the adenylate cyclase system (Figure 230).
- Cause constitutive activation of the receptors: the role of CAMs in human disease was first demonstrated in 1993 for the thyrotropin (TSH) receptor in hyperfunctioning thyroid adenoma. Several dozen mutations affecting the TM or extracellular domains of this GPCR constitutively activate the

cAMP signalling pathway. This in turn activates thyroid hormone secretion, resulting in hyperfunctioning thyroid adenoma (somatic mutations) or familial hyperthyroidism (germinal mutations). This example was followed by many others (Table 26). There is growing evidence that somatic GPCR-activating mutations are also involved in cell growth, and probably also in the development

Table 26 Hereditary diseases linked to naturally occurring mutations in different GPCRs Reprinted from *Proceedings of the National Academy of Sciences*, **97**, Parnot, C., Bardin, S., Miserey-Lenkei, S., Guedin, D., Corvol, P. and Clauser, E., Systematic identification of mutations that constitutively activate the angiotensin II type 1A receptor by screening a randomly mutated cDNA library with an original pharmacological bioassay, 7615–7620. Copyright (2000), with permission from Elsevier.

Receptor	Mutations	Phenotype	Disease
TSH receptor	50 mutations: $S^{281}N/IT$, $R^{310}C$, $\triangle^{339\text{-}367}$, $S^{425}L$, $G^{431}S$, $M^{453}T$, $I^{486}F/M$, $I^{586}T$, $V^{597}L$, $S^{505}R/N$, $V^{509}A$, $L^{512}E/R$, $I^{568}M/T$, $\triangle^{613\text{-}621}$, $\triangle D^{619}$, $D^{619}G$, $T^{620}S$, $A^{623}I/S/V$, $L^{629}F$, $I^{630}L$, $F^{631}L/C/I$, $T^{632}I/A$, $D^{633}A/Y/E/H$, $P^{639}A$, $N^{650}Y$, $\triangle^{658\text{-}661}$, $V^{656}F$, $F^{666}S$, $N^{670}S$, $C^{672}Y$, $N^{674}D$, $L^{677}V$	Hyperthyroidism	Somatic: thyroid toxic adenoma Germinal: familial hyperthyroidism
LH receptor	14 mutations: $M^{398}T$, $L^{457}R$, $I^{542}L$, $D^{564}G$, $A^{568}V$, $M^{571}I$, $A^{572}V$, $I^{574}L$, $I^{575}L$, $T^{577}I$, $D^{578}H/G/Y$, $C^{581}R$	Male precocious puberty	Somatic: Leydig tumor and precocious puberty Germinal: sporadic and familial precocious puberty
FSH receptor	1 mutation: $D^{567}G$	Male fertility after hypophysectomy	
Rhodopsin and Opsins	4 mutations: $G^{90}D$, $E^{113}Q$, $A^{292}E$, $K^{296}N$	Blindness	Germinal: stationary night blindness, retinitis pigmentosa
PTH receptor	3 mutations: $H^{223}R$, $T^{410}P$, $I^{458}R$	Short-limb dwarfism, skeletal deformities, hypercalcemia and low PTH	Germinal: Jansen chondrodysplasia
Ca^{2+} sensing receptor	23 mutations: $K^{47}N$, $P^{55}L$, $R^{68}C$, $N^{118}K$, $F^{128}L/A$, $T^{151}M$, $N^{178}D$, $E^{191}K$, $Y^{218}S$, $P^{221}S/L$, $P^{227}L$, $E^{228}Q$, $Q^{245}R$, $F^{612}S$, $P^{747}L$, $L^{773}R$, $F^{788}C$, $V^{817}I$, $A^{835}T$, $\triangle^{895\text{-}1075}$	Hypocalcemia and hypercalciuria	Germinal: autosomal dominant hypocalcemia

of cancer. In this respect, several orphan GPCRs, such as the mas oncogene, have been identified by their tumorigenic properties.

- Affect receptor downregulation: for example, $Gln^{27}Glu$ is responsible for a decreased downregulation of the β_2-adrenergic receptor while $Arg^{16}Gly$ leads to enhanced downregulation of this receptor (Figure 230). This latter mutation occurs more frequently in patients with nocturnal asthma. Clinical studies also unveil that, for African- Caribbeans, the frequency of this mutation is significantly higher in those who are essentially hypertensive than in those which have a normal blood pressure.

Variation in splicing is another important mechanism leading to physiological diversity among GPCRs. Many GPCR genes contain multiple exons. Normally, the introns are removed at the level of processing of pre-mRNAs in the cell nucleus. Nevertheless, GPCR variants may be obtained due to alternative splicing, exon skipping and intron retention (Figure 231). There are now over 30 GPCRs with identified splice variants:

- The largest number of splice variants is at the C-terminus of the receptors, but some receptors have more than one site for variation in splicing.

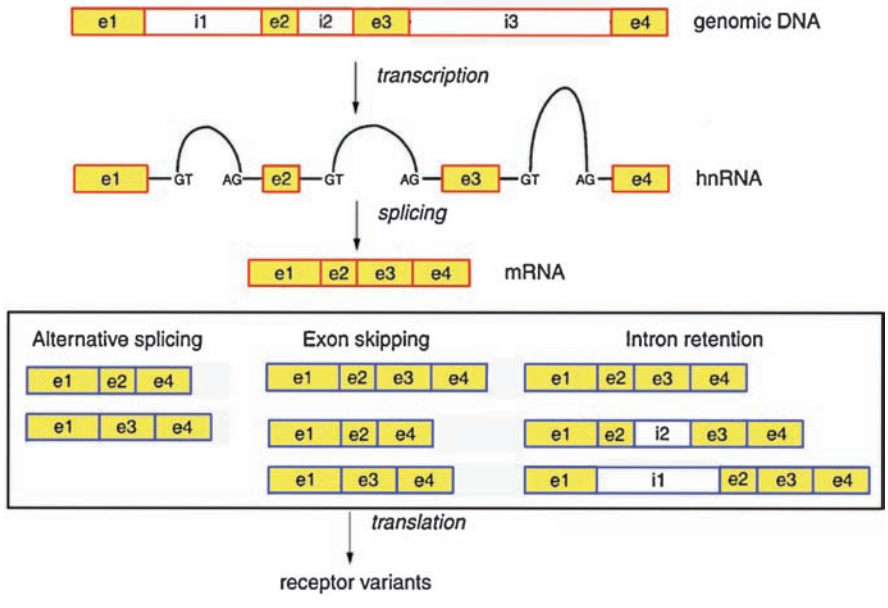


Figure 231 The process by which splice variants can be produced from a hypothetical gene (e = exons, i = introns). Reprinted from *Trends in Pharmacological Science*, **20**, Kilpatrick, G. J., Dautzenberg, F. M., Martin, G. R. and Eglen, R. M., 7TM receptors: the splicing on the cake, 294–301. Copyright (1999), with permission from Elsevier.

- The extent of splice variants depends to some extent on the complexity of gene structure (i.e. the amount of introns) and the nature of the variation might be species-specific.
- Few splice variants affect the messenger-binding domain of the receptors, but splice variants can have profound effects on the signalling pathway (e.g. adenylate cyclase stimulation versus inhibition for two variants of the thromboxane A_2 receptor) as well as on the coupling efficiency (especially when the variation affects intracellular domains of the receptor).

Splice variants of GPCRs are often dismissed as the consequence of leaky transcription and, hence, physiologically irrelevant. However, there are several reports linking splice variants with disease. The D_3 dopamine receptor is reported to be associated with schizophrenia, and abnormal processing of the CCK-B receptor has been associated with gallstones and obesity.

5 Concluding remarks

In summary, the investigation of GPCRs first relied on physiological approaches, then on biochemical approaches and nowadays on genetic approaches. The introduction of each new approach has always provided an impulse for the discovery of new receptors or receptor subtypes. These discoveries are very beneficial for the medical treatment of diseases, since it authorizes the use of more and more selective drugs with, hence, fewer possible side effects.

Our understanding of GPCR function has substantially improved during the last decade. It changed the way we look at GPCRs (Figure 232). They are no longer simple 'on/off' switches, but highly dynamic structures that exist in equilibrium between active and inactive conformations. An agonist is recognized as a molecule that can stabilize an active conformation, while an inverse agonist (i.e. an antagonist with negative intrinsic activity) is a molecule that can stabilize an inactive conformation. Thus, it has become clear that not only agonists, but also antagonists, are capable of actively modulating receptor function. Moreover, it has become evident that neither agonists nor antagonists necessarily have to share an overlapping binding site, even if they act at the same receptor.

Moreover, GPCRs no longer exclusively act as monomers, nor do they have to exclusively activate G proteins to produce cell signalling.

Genes coding for GPCR-like proteins have been discovered for some time and the recent sequencing of the human genome unveiled an even greater number of them. Based on the assumption that these proteins function as receptors, much effort is being spent to find their natural messengers as well as other ligands. Traditionally, orphan GPCR ligand identification relies on their expression in an appropriate cell line followed by their exposure to libraries of naturally occuring compounds. This task appears to be notoriously difficult, especially since it does not permit the discovery of antagonist molecules. Constitutively active orphan receptors may be obtained by mutagenesis. They are likely to be more sensitive to agonists, including their natural messenger(s). As they evoke a signal in the absence of agonist, they may respond to inverse agonists as well (Figure 233).

Whereas agonist and antagonist drugs have mainly been discussed in terms of efficacy and potency, there is now a growing tendency to pay attention to their kinetic properties as well. The kinetic properties of the drug–receptor complex, along with pharmacokinetic issues, will determine the drug residence time at its

G Protein-Coupled Receptors: Molecular Pharmacology From Academic Concept to Pharmaceutical Research
Georges Vauquelin and Bengt von Mentzer © 2007 John Wiley & Sons, Ltd. ISBN: 978-0-470-51647-8

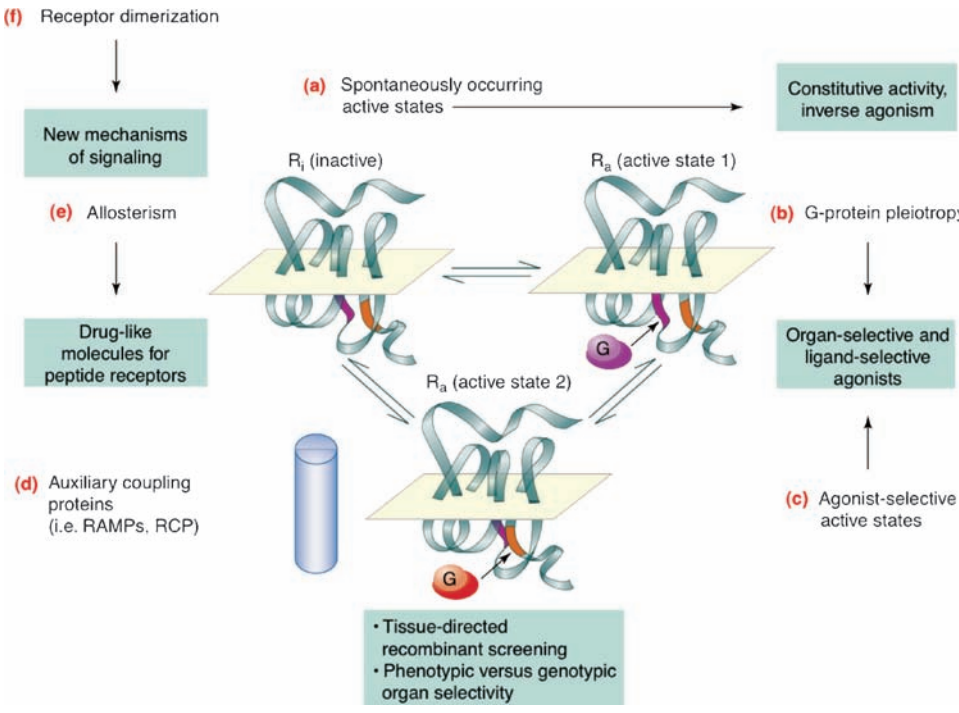


Figure 232 Molecular cloning, biochemical, immunological and spectroscopic techniques have led to dramatic advancements of our knowledge about G protein-coupled receptor behaviour. Specific issues (a to f) have been outlined in the preceding chapters. Reprinted from *Trends in Pharmacological Science*, **25**, Kenakin, T., Principles: receptor theory in pharmacology, 186–192. Copyright (2004), with permission from Elsevier.

target/receptor and this will have profound consequences on its *in vivo* efficacy and effect duration (Copeland *et al.*, 2006). Whereas drug structure–activity relationship (SAR) studies often merely rely on determinations of their binding affinity and functional effect, these authors recommend an additional screen for the kinetic properties of the drug–receptor interaction. For certain pathologies, such as hypertension, it is now widely accepted that a permanent reduction in blood pressure is beneficial for the patient. Among the drugs which are currently used to this end, the AT_1 receptor antagonist, candesartan, has been shown to dissociate quite slowly from its receptor in *in vitro* studies (Figure 229) and this process is likely to contribute to its long-lasting clinical effect. Conversely, so-called 'atypical antipsychotics' like clozapine and quetiapine produce fewer extrapyramidal side effects (i.e. symptoms that are similar to those that occur in the disease state of Parkinsonism) as the classical neuroleptics. This is thought to be related to the ability to dissociate rapidly from the D_2 receptors so that, in case of a surge of endogenous dopamine in the striatum, physiological dopamine transmission can still take place, to some extent.

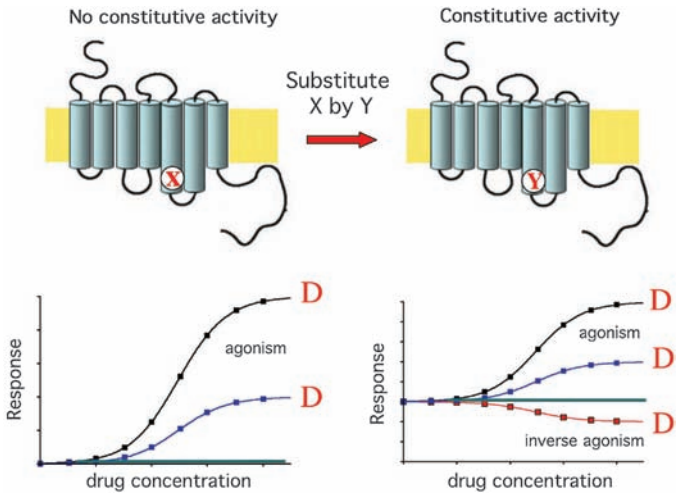


Figure 233 Production of constitutively activated orphan receptors for the discovery of agonist and inverse agonist ligands (D = detectable in functional studies).

Another emerging issue is receptor polymorphism. The etiology of many diseases remains unknown, but in major psychiatric conditions such as depression, bipolar disorder and schizophrenia, there is a higher concordance rate for the disease in monozygotic versus dizygotic twins. This suggests that genetic factors are involved. Among the potential causative genes, GPCRs are likely to play a primordial role. In support of this allegation, pharmacogenomic studies suggest that the 5-HT_{2A} receptor might be involved in the pathophysiology of hallucinations in humans. Interestingly, meta-analysis of several clinical studies (including 373 patients who responded to the treatment and 360 non-responders) revealed that patients with His^{452}Tyr-5-HT_{2A} receptors were less likely to respond satisfactorily to clozapine (Table 27). This suggests that GPCR polymorphism may also play a significant role in determining drug response.

Table 27 Meta-analysis of studies on genetic variation in 5-HT_{2A} receptors and clozapine response. R is responder, NR is non-responder. Reprinted from *Schizophrenia Research*, **32**, Arranz, M. J., Munro, J., Sham, P., Kirov, G., Murray, R. M., Collier, D. A. and Kerwin, R. W., Meta-analysis of studies on genetic variation in 5-HT2A receptors and clozapine response, 93–99. Copyright (1998), with permission from Elsevier.

His452Tyr	R (%)	NR (%)
Genotype		
His452/His452	320 (85)	238 (79)
His452/Tyr452	52 (14)	56 (18)
Tyr452/Tyr452	2 (1)	8 (3)
Total	374	302

Table 28 Pharmacological testing systems. Reproduced from Kenakin, T. (1996) *Pharmacological Reviews*, **48**, 413–463, with permission from the American Society for Pharmacology and Experimental Theraputics.

Evolution in pharmacological research
Pharmacological receptor testing systems
Animal receptors-animal tissues ↓ Animal genetic receptor material-animal surrogate cells ↓ Human genetic receptor material-animal surrogate cells ↓ Human genetic receptor material-human surrogate cells ↓ Human genetic receptor material-human target cells ↓ Human genetic receptor material-human target cells with appropriate pathology

Pharmacological research at the cellular and molecular levels is still likely to be different in five or ten years from now. Some of the developments pharmacologists dream of are:

- Testing of drugs on the human receptor in exactly the correct tissue under the appropriate pathology (Table 28). Currently, the state of the art mainly resides in systems where human receptor material (i.e., cDNA) coding for receptors is introduced into surrogate cells. This only constitutes a step toward the desired total correspondence between drug and disease.
- The development of the ideally fitting drug based on molecular modelling data without needing long and expensive structure–activity relationship studies. For this purpose, much effort is nowadays devoted towards the determination of the exact molecular structure of the receptors and especially of the binding sites of agonists and antagonists.
- The elucidation of the physiological role of orphan receptors and their potential implication into pathophysiological situations. They might constitute targets for new classes of drugs and, hence, allow new avenues in clinical therapy.

We hope that this book has given a foundation for a deeper understanding of current pharmaceutical research.

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