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COMMENTARY

MOLECULAR BIOLOGY OF ADRENERGIC AND MUSCARINIC CHOLINERGIC RECEPTORS

A PERSPECTIVE

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The cloning and sequence analysis of neurotransmitter receptor genes have already had an important impact on the approaches available to researchers studying receptor structure, function and evolution. It should be clear that the cloning and sequencing of receptor genes provide a new beginning point or a clear transition phase with respect to the biochemical and pharmacological studies of the past decade. Those studies were performed on extremely limited amounts of receptor protein, whereas the studies that can now be performed with site directed mutagenesis and over-expressed receptor proteins allow detailed structure/function analyses which were previously impractical.

Recent advances in receptor research have been due to steady progress in the purification and biochemical analysis of receptor proteins and to advances in technology that have permitted protein sequencing of minute quantities of receptor peptides. The cloning and sequencing of receptor genes have provided new information and approaches in a number of key areas.

Receptor evolution—multigene family

One area where primary sequence data can be of direct benefit is in the study of evolutionary relationships among proteins. In a comprehensive review of the evolution of adrenergic and cholinergic receptors [1], we discussed the physiological, pharmacological, immunological and biochemical evidence for the presence of these receptors throughout evolution. We concluded that the evidence to date supported the hypothesis that the adrenergic and cholinergic receptors existed for hundreds of millions of years and that the same receptor molecules have been put to a variety of uses in different species and tissues [1]. The extent of structural homology among these proteins became more apparent with the cloning and sequence analysis of genes encoding adrenergic and muscarinic cholinergic receptors from a variety of species [2–16]. While gene cloning and sequencing have provided data which have proven that a high degree of structural homology exists between a number of neurotransmitter receptors and the opsins, earlier studies were highly predictive of some of these relationships. We proposed in 1983 and 1984 [17–19] that an evolutionary relationship existed between the adrenergic and muscarinic cholinergic receptors and that they evolved from one common ancestor via gene duplication events. However, the extent of the multigene family, which includes the substance K receptor, bacteriorhodopsin, yeast mating factors, the opsins and the 5-HT receptor [20–29], was clearly not addressed in the earlier studies.

Secondary-structure similarities have been found by analysis of the protein sequences deduced from receptor gene sequences and by hydropathy calculations which predict potential membrane-spanning regions. We have found that, by the comparison of secondary structural features, the relationship among some of the members of this multigene family becomes more apparent. The beta-adrenergic and muscarinic receptor hydropathy profiles are strikingly similar and almost superimposable on the hydropathy profiles of the yeast alpha-factor receptor, bacteriorhodopsin, human rhodopsin, and human transforming protein (*mas*) (see Ref. 2) (Fig. 1). Similar models, consisting of seven membrane-spanning segments, have been proposed for each of these proteins (Fig. 2).

Recent sequencing of the genes encoding rat cardiac beta-adrenergic and muscarinic receptors provides further evidence for the extent of identity between these pharmacologically diverse receptors [2]. The cardiac muscarinic and beta-adrenergic receptor genes have been shown to have an overall 50% homology in the coding regions of these two genes with certain regions showing even higher homology when favored amino acid substitutions are considered [2] (Fig. 3). Extensive studies are underway on the cloning and sequencing of adrenergic and muscarinic cholinergic receptor genes from *Drosophila*, blue sharks and from *Caenorhabditis elegans*. It is already apparent that these receptors exist in the ancient species. Forthcoming studies from a number of laboratories will help complete the evolutionary picture and the full extent of this multigene family.

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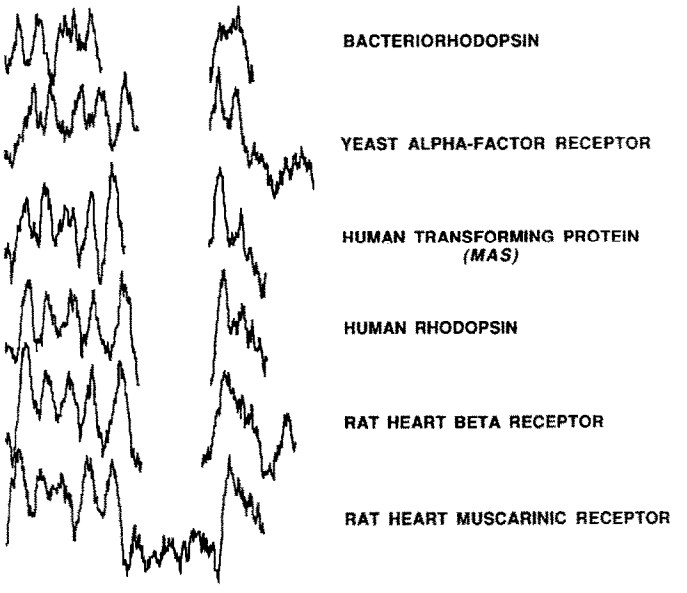

Fig. 1. Hydropathic analysis of members of a multigene family. Kyte–Doolittle plots [30] were arbitarily broken in the middle of the cytoplasmic loop connecting putative membrane-spanning regions V and VI to align all of the potential transmembrane regions. From [2].

Continuous expression and site-directed mutagenesis of receptors

One of the most exciting and potentially most informative aspects of receptor molecular biology is in the area of receptor gene expression and site-directed mutagenesis. The expression of adrenergic and muscarinic receptors in permanent cell lines has already begun to have a considerable impact on our knowledge of receptor properties and mechanisms of cell activation. Over-expression of these same neurotransmitter receptors provides a unique source of receptor protein for detailed biochemical and structural studies. For pharmacologists and the pharmaceutical industry, the permanent expression of specific receptors [30–35] provides a pure population of human receptors for drug development and testing, a substantial improvement over the use of animal tissues (Figs. 4 and 5).*

Site-directed mutagenesis has already provided considerable information concerning the fine structure of the $beta_2$-adrenergic receptor [36–38]. Our studies, described below, have provided the first structural information concerning agonist high- and low-affinity states and have shown conclusively that significantly different amino acid residues are involved in agonist versus antagonist binding [36, 37].

Receptor expression. Characterization of the genes encoding neurotransmitter receptors necessitates their expression and assay in an appropriate system. A number of techniques are available for the transient or permanent expression of genes in eucaryotic cells; however, detailed pharmacological and biochemical studies of expressed receptors can be best accomplished using a system that allows for continuous expression of receptor genes. To this end, we have utilized plasmid vectors containing selectable markers for expression of beta-adrenergic and muscarinic cholinergic receptors in cultured mammalian cells lacking receptors [36]. This approach has allowed for a comparison of the properties of related receptor subtypes in a single cell and also for examination of the properties of a single receptor in different cell types.

Continuous expression of the genes encoding rat and human $beta_2$-adrenergic receptors in B-82 cells, murine L cells that lack beta-receptors but contain a PGE_1-stimulated adenylate cyclase, has revealed that species differences in the primary structure of the $beta_2$ receptor may be of little functional consequence.† Rat and human $beta_2$ receptors displayed essentially identical antagonist binding properties; the rat $beta_2$ receptor displayed significantly higher agonist affinities and a larger shift in agonist affinity in the presence of guanine nucleotides. However, in the presence of high concentrations of GTP, the agonist affinities were essentially the same.† Similarly, expression of the human $beta_2$-adrenergic receptor in B-82 cells and CHO (Chinese hamster ovary) cells has revealed that different cell environments can alter profoundly the extent of receptor coupling to G_s and the apparent affinity of the receptor for agonists in isolated membranes.† Human $beta_2$ receptors expressed in B-82 cells display an affinity for isoproterenol that is approximately 7-fold higher than that for the same receptor expressed in CHO cells at the same density.

* Mei L, Lai J, Roeske WR, Fraser CM, Venter JC and Yamamura HI, Pharmacological characterization of the M1 muscarinic receptors expressed in murine fibroblast B82 cells J Pharmacol Exp Ther, in press.

† Robinson DA and Fraser CM, Stable expression of rat and human $beta_2$-adrenergic receptors in B-82 and CHO cells: Pharmacological and biochemical comparisons (submitted).

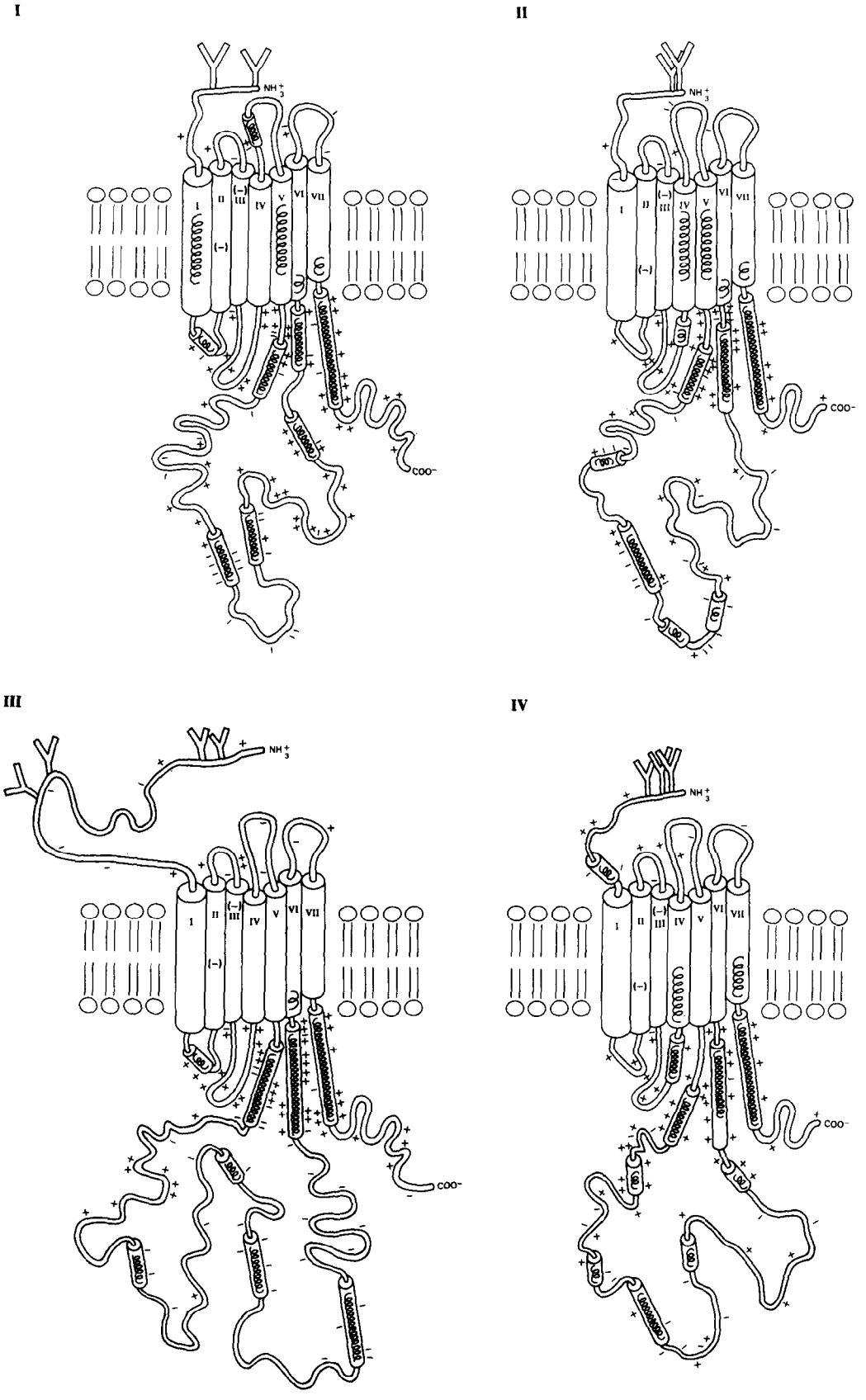

Fig. 2. Models of the rat M_1 (I), M_2 (II), M_3 (III) and M_4 (IV) muscarinic receptors. Peralta *et al.* [15] have reported the reverse classification of M_3 and M_4. From [16].

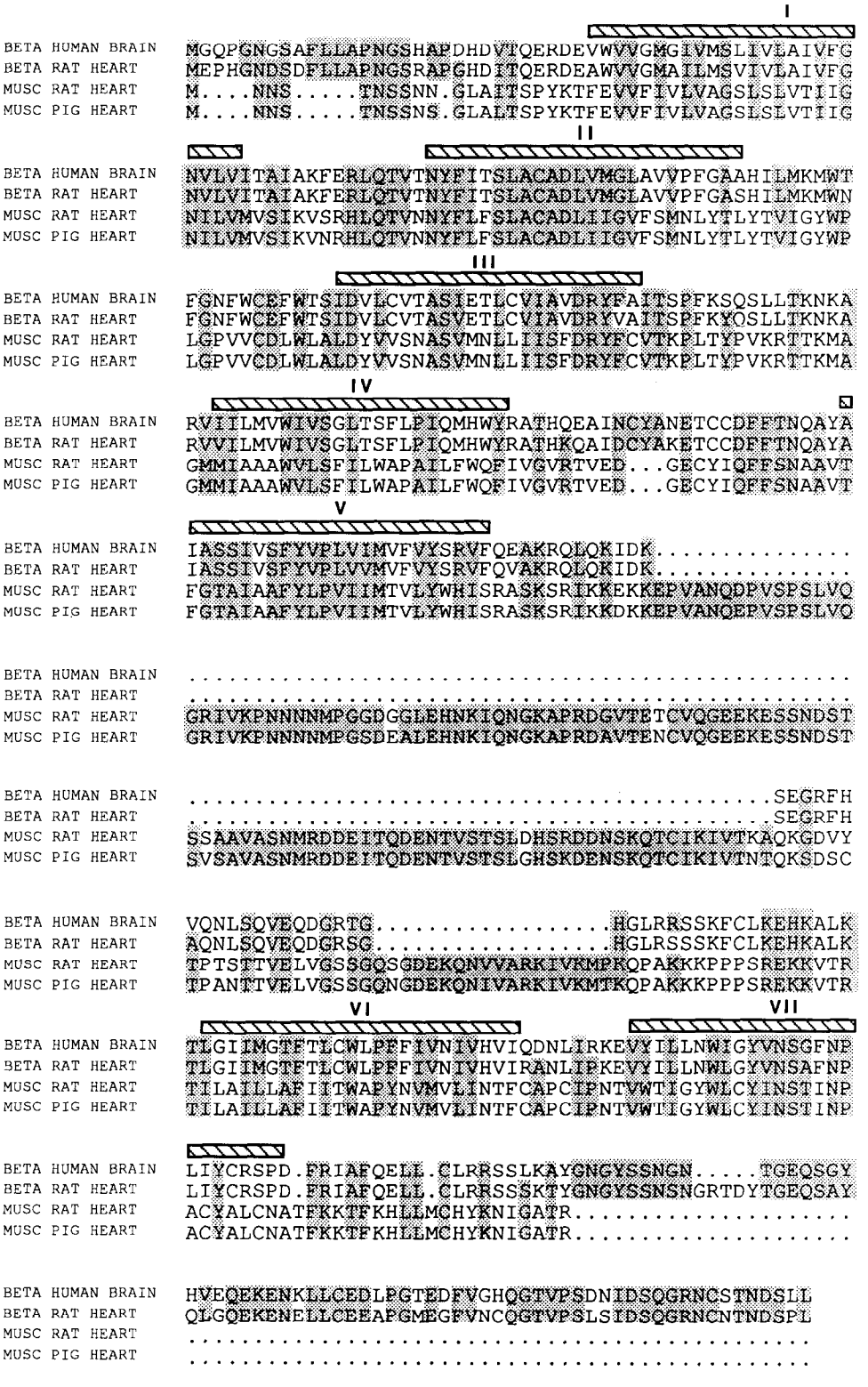

Fig. 3. Conserved structure of muscarinic cholinergic and beta-adrenergic receptors. Positions which contain identical residues or favored amino acid substitutions are boxed. Putative membrane spanning regions are shown by hatched bars above the sequences. From [16].

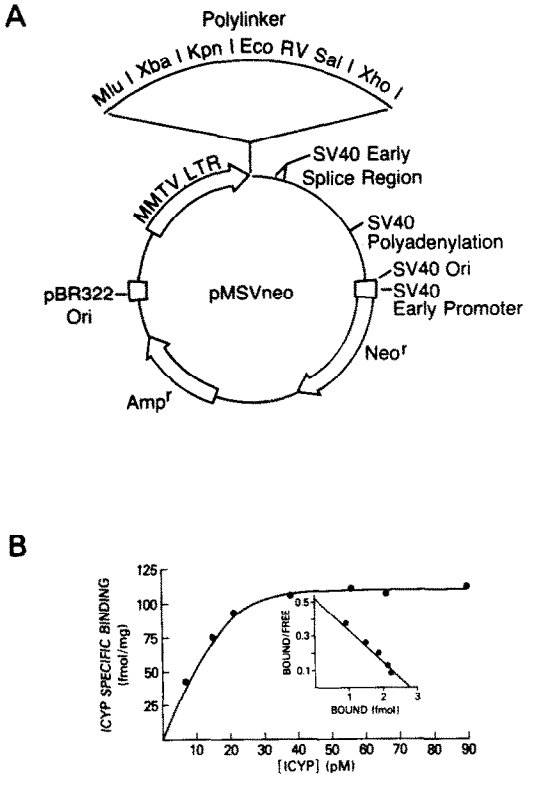

Fig. 4. Expression of human beta$_2$-adrenergic receptor gene in transfected B-82 cells. (A) Expression vector pMSVneo. (B) Saturation isotherms of ICYP binding to membranes from cells expressing the wild type human beta$_2$-adrenergic receptor. Inset is Scatchard analysis of ICYP binding. from [36].

In the presence of GTP, the affinity of the human beta receptor for isoproterenol is identical in both cell types, indicating that the observed affinity differences in B-82 cells and CHO cells reflect differences in receptor coupling to G_s in isolated membranes.* This observation may, in part, explain the diversity in agonist affinities of a single receptor subtype expressed in different tissues; however, it is unlikely that the differences seen in isolated membranes have any functional consequences in intact tissues.

Site-directed mutagenesis of beta-adrenergic receptors. Perhaps the most direct means of examining the relationship between receptor structure and function is by construction and expression of mutant receptor proteins. In general, two different types of mutations may be constructed, namely, deletion mutations and point mutations. The difference between these types of mutations is that large stretches of nucleotides encoding specific amino acids are removed from the gene sequence for deletion mutants, whereas only a single nucleotide, that converts one amino acid to another, is altered for point mutants. In theory, deletion mutations can provide information as to the importance of a defined receptor domain; however, interpretation of the results from deletion mutations can be difficult if the mutation alters receptor processing or membrane insertion. For this reason, the information obtained from point mutations may be of greatest utility in elucidation of important functional domains of a receptor [36, 37].

The relative merits of permanent as opposed to transient expression in the characterization of mutant receptor proteins are obvious. Establishment of permanent cell lines expressing mutant receptors allows for an in-depth characterization of the pharmacological and biochemical properties of these proteins by a number of laboratories and provides a continuous source of material for studies on the structure of the mutant receptors.

We have taken several approaches to the selection of amino acids in the sequence of the human beta$_2$-adrenergic receptor for study by point mutation. There are a number of highly conserved aspartate residues in the second and third transmembrane segments of all members of the neurotransmitter receptor gene family (Fig. 6), implying that these residues may be important in receptor function. To examine the role of these residues in detail, we constructed three mutant beta$_2$-adrenergic receptors in which the aspartate residues at positions 79, 113 and 130 were substituted with asparagine [36, 37]. This amino acid substitution replaces a negatively charged side group on aspartate with a neutral group of similar size in asparagine. Substitution of aspartate 113 with asparagine in the human beta receptor produced a mutant receptor with properties similar to those described by Strader *et al.* [38] for a mutant hamster beta$_2$ receptor containing the same amino acid substitution. The Asn 113 mutant beta receptor displayed markedly attenuated affinities for both adrenergic agonists and antagonists (K_d 10^{-6} M) and was unable to mediate isoproterenol stimulation of adenylate cyclase activity. These data suggest that Asp 113 may be involved in the binding of adrenergic ligands, possibly serving as a counterion for the amine substitutes of the catecholamines.

Substitution of Asp 79 with Asn produced a mutant receptor that displayed normal antagonist binding but a 40-, 140- and 240-fold loss in affinity for isoproterenol, epinephrine and norepinephrine respectively [36]. Hill coefficients for agonists binding to the mutant receptor indicated the presence of a single class of low-affinity binding sites. Consistent with this observation was the finding that Asn 79 mutant beta receptors were apparently uncoupled from G_s as evidenced by a loss in guanine nucleotide sensitive agonist binding to the mutant receptor. The ability of the Asn 79 mutant receptors to stimulate adenylate cyclase was also markedly attenuated, presumably due to the uncoupling of the mutant receptor and G_s [36].

In contrast to the findings with Asn 79 mutant receptors were the data obtained from characterization of Asn 130 receptors [37]. Like Asn 79 mutant receptors, Asn 130 receptors also displayed normal antagonist binding. However, Asn 130 mutant receptors displayed an approximately 10-fold

* Robinson DA and Fraser CM, Stable expression of rat and human beta$_2$-adrenergic receptors in B-82 and CHO cells: Pharmacological and biochemical comparisons (submitted).

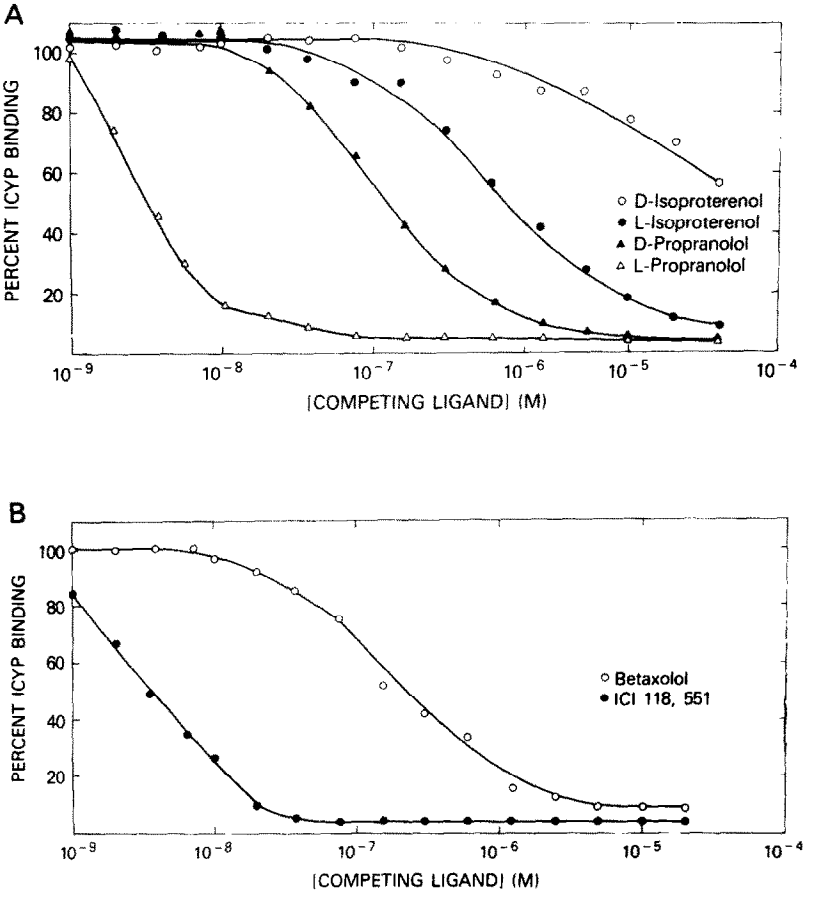

Fig. 5. Adrenergic agonist and antagonist competition for ICYP binding to expressed human beta$_2$-adrenergic receptors. (A) Stereoenantiomers of isoproterenol and propranolol. (B) Beta$_2$-adrenergic versus beta$_1$-adrenergic selective antagonists. From [31].

higher affinity for agonists as compared to wild type receptors (Table 1). Hill coefficients for agonist binding to Asn 130 receptors were consistent with a single class of high-affinity binding sites [37]. Asn 130 mutant receptors displayed a small, but significant, shift in agonist affinity in the presence of guanine nucleotides. This shift was considerably smaller than that seen with the wild type receptor, and the potency of various guanine nucleotide analogues to affect a change in agonist affinity was reduced, suggesting an altered receptor–G_s interaction (Table 1). Asn 130 mutant receptors were unable to mediate isoproterenol stimulation of adenylate cyclase, supporting the notion that this mutant receptor was also uncoupled from G_s [37].

The data obtained from characterization of the

Table 1. Human beta$_2$-adrenergic receptors: Affinities for adrenergic agents

	k_d (nM)		
	Wild type receptor	Asn 130 receptor	Asn 79 receptor
(−)Isoproterenol	17.6 ± 4	2.5 ± 0.2	697 ± 26
(−)Isoproterenol+Gpp(NH)p	120 ± 18	9.3 ± 3.2	650 ± 110
(+)Isoproterenol	200 ± 33	32.5 ± 15	12167 ± 2105
(−)Epinephrine	56.5 ± 6	4.3 ± 0.7	7000 ± 1800
(+)Epinephrine	500	75	
(−)Norepinephrine	219 ± 27	55 ± 4.8	52500 ± 2500
(−)Propranolol	0.22 ± 0.1	0.36 ± 0.15	0.22 ± 0.1
(+)Propranolol	36.8 ± 3.0	65 ± 15	36.8 ± 0.1
(±)Iodocyanopindolol	21 ± 5.0	32 ± 6.0	22.0 ± 5.0

The affinity constant (K_d) for the radioligand ICYP was determined directly in equilibrium binding studies. Data are from Chung *et al.* [36] and Fraser *et al.* [37]. Values are means ± SE.

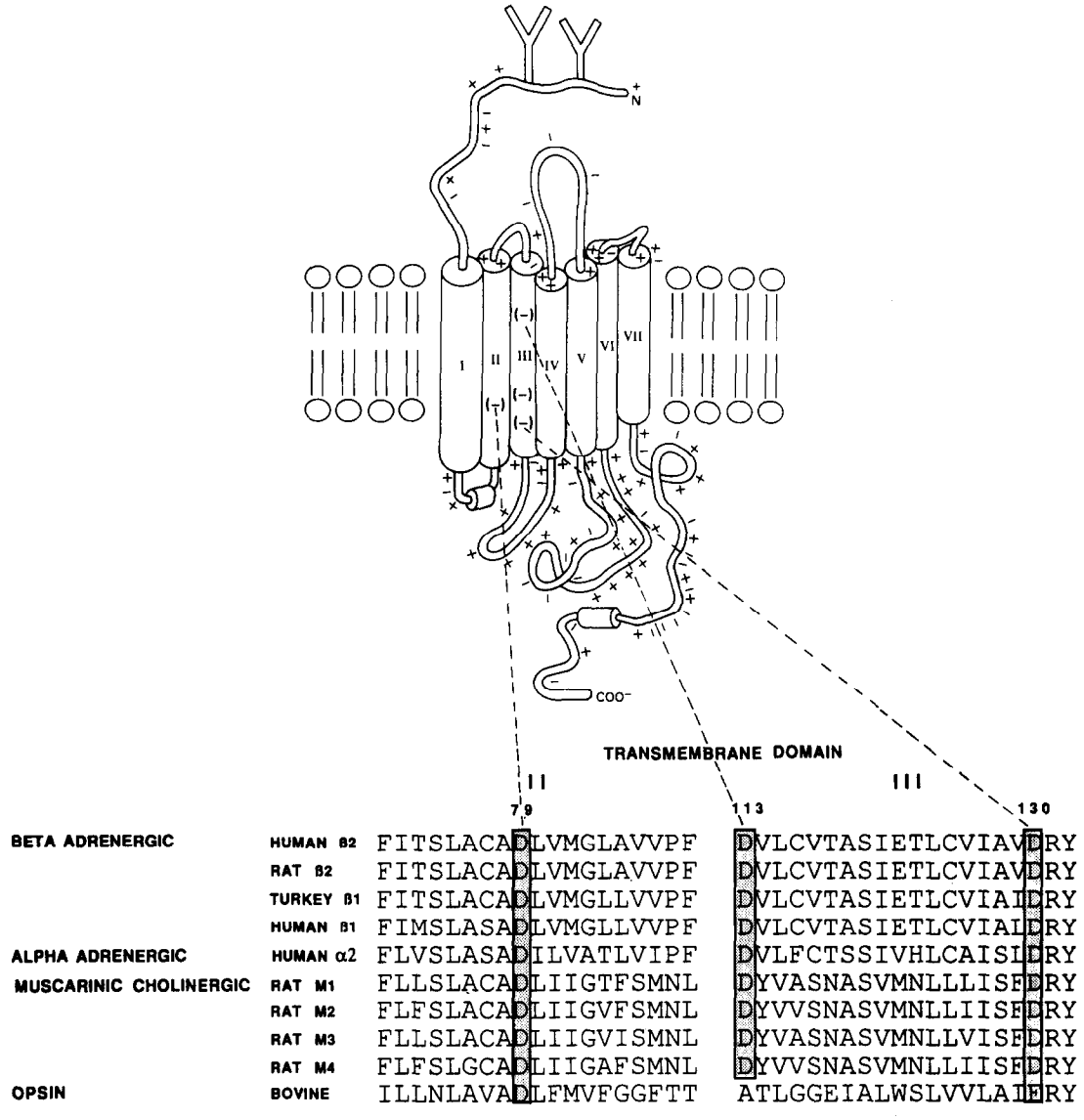

Fig. 6. Location of site-directed mutants and sequence homology of putative transmembrane regions II and III from a family of neurotransmitter receptors. Upper, schematic model of the human beta$_2$-adrenergic receptor. Lower, comparison of the amino acid sequences of transmembrane segments II and III from the indicated receptors. The shaded areas are the loci of site-directed mutagenesis for conserved aspartic acid residues 79, 113 and 130 in the human beta$_2$ receptor. From [37].

aspartate mutant receptors suggest that these residues are involved in agonist binding to the receptor and, perhaps more importantly, in the transduction of ligand binding to activation of G_s. Conservation of these residues may serve similar functions in all receptors that mediate their actions via guanine nucleotide regulatory proteins [37].

Three-dimensional structure of receptors

A major goal of receptor research is to correlate the function of a receptor with its three-dimensional structure. This goal has been achieved with a number of soluble proteins; however, a major stumbling block to determining the three-dimensional structure of integral membrane proteins is the difficulty in generating crystals of sufficient quality to refract for X-ray analysis. The extremely small amounts of receptor recoverable from tissues makes this a difficult task which may soon be remedied by the high density expression of cloned receptor genes. Even without these techniques, some progress has been made with bacteriorhodopsin, the structure of which has been determined to 3.5 Å resolution by a combination of electron microscopy and low dose electron diffraction analysis [39, 40]. These results were only possible because of the existence of bacteriorhodopsin in high density in a periodic protein lattice in the bacterial purple membrane and, thus, these techniques are not currently applicable to the neurotransmitter receptors. Since bacteriorhodopsin appears to be closely related structurally to several neurotransmitter receptors, it may be possible to

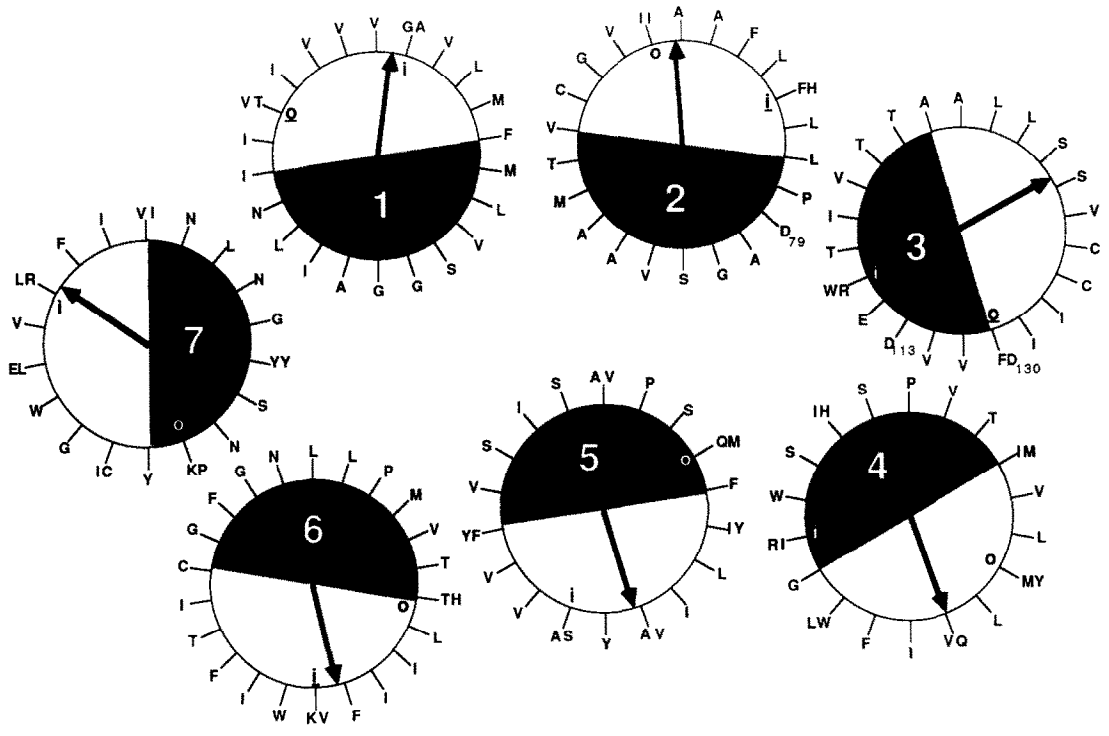

Fig. 7. Helical-wheel representation of the human beta$_2$-adrenergic receptor transmembrane segments. Numbered amino acids are those which have been altered by site-directed mutagenesis (see text). Transmembrane segments 1, 2, 3, and 6 are modeled as pi helices and segments 4, 5, and 7 are modeled as alpha helices. Shaded regions indicate the most hydrophilic face of each helix. Arrows indicate the direction of the Eisenberg hydrophobic moment vector [41].

infer the general structural organization of these receptors from that of bacteriorhodopsin. Electron diffraction studies of bacteriorhodopsin suggest that the protein contains seven closely packed helices arranged in a bundle perpendicular to the plane of the membrane. Hydrophobicity analyses of bacteriorhodopsin, rhodopsin, and the adrenergic and muscarinic cholinergic receptors show a strikingly similar pattern of seven strongly hydrophobic regions which presumably correspond to the seven helices observed in the bacteriorhodopsin diffraction maps. All of these proteins exhibit significant primary sequence homology in these putative membrane-spanning regions, simplifying analysis of their conserved structural motifs.

Several predictive techniques have been developed which attempt to derive secondary structures of proteins from the intrinsic properties of their constituent amino acids. One of the most commonly measured properties is hydrophobicity, and a number of scales have been used to quantitate this parameter. We have applied measures of hydrophobicity to the beta-adrenergic and muscarinic cholinergic receptors to define the end points of the membrane-spanning segments of these proteins. We have also used a helical-wheel analysis to predict the phasing of these helical segments, that is, which portion of each helix faces the interior of the protein and which portion interacts with the lipid bilayer. Independently, we have applied the hydrophobic moment analysis of Eisenberg *et al.* [41] to confirm the phasing assigments. The involvement of highly conserved amino acids in receptor function, as measured by site-directed mutagenesis (see above), was used as a guide in refining the proposed structure and selecting among alternative arrangements of the helices. The helical wheel model that we have derived for the human beta$_2$ receptor is shown in Fig. 7. The helices are arranged in a clockwise fashion when viewed from outside the cell membrane. The model comprises both alpha and pi helices.* The salient feature of the model is the alignment of four highly conserved negatively charged amino acids in transmembrane segments II and III. Three of these groups have been shown by site-directed mutagenesis to be involved in ligand binding and adenylate cyclase activation. The alignment of these groups in close proximity on one side of the receptor strongly suggests that this region forms an integral part of the binding pocket for agonists and antagonists (see below). Structural analyses such as these have enabled us to construct three-dimensional models of adrenergic and muscarinic cholinergic receptors (Fig. 8). These models have been instrumental in designing future experiments and in understanding the

* Kerlavage AR, Feldmann RJ and Venter JC, Secondary structure and three-dimensional modeling of the transmembrane segments of the beta-adrenergic receptor (submitted).

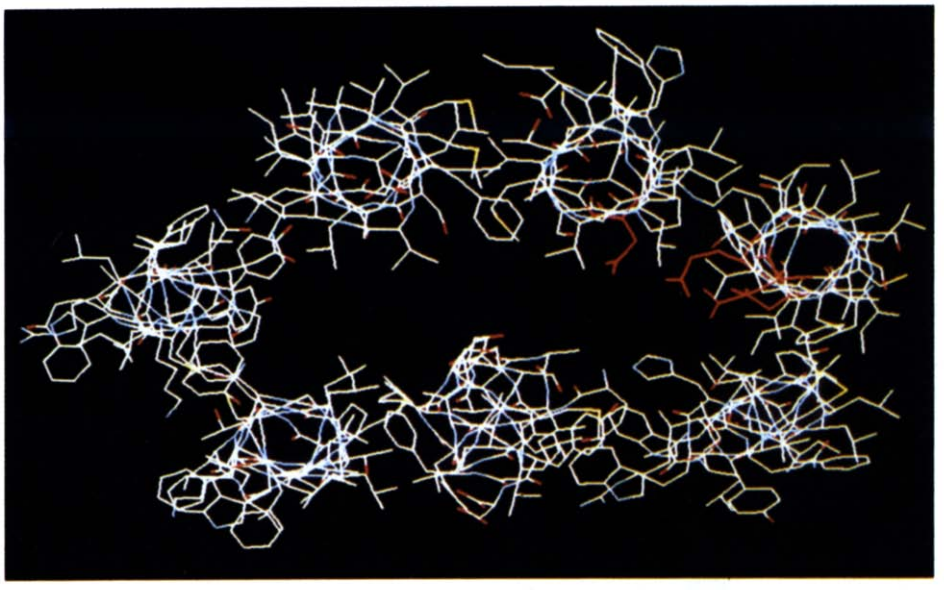

Fig. 8. Three-dimensional molecular model of the human beta$_2$-adrenergic receptor. The stick model shows the same orientation as in Fig. 7. Negatively charged side chains in transmembrane helices II and III are shown in red.

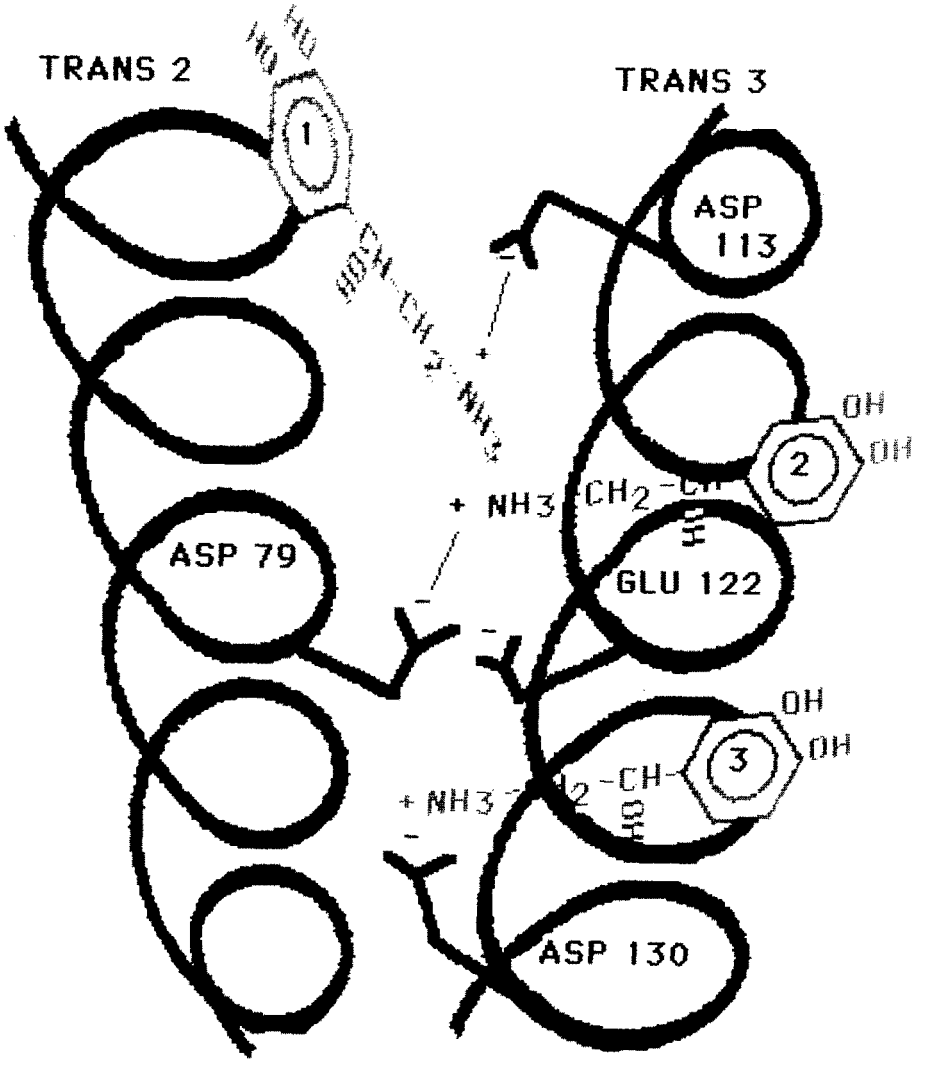

Fig. 9. Proposed model of receptor activation. The side chains of the negatively charged residues on transmembrane segments II and III of the beta-adrenergic receptor are represented in red. Norepinephrine molecules 1, 2 and 3 represent proposed positions of initial recognition, high-affinity binding site, and low-affinity binding site, respectively.

structure/function relationships of these receptor molecules. Studies such as these will become a major factor in new drug development.

Models of receptor activation

We have attempted to develop a working model for receptor activation based upon, and consistent with, the data obtained from our site-directed mutagenesis studies and molecular modeling. While we expect that portions of our "working model" may need modification, it is extremely useful to have a basis for hypothesis building and testing. It has become clear from our studies as well as those of other groups that the aspartate residues in transmembrane domains II and III are essential to the function of the adrenergic receptors [36–38]. It is also clear from the tremendous degree of amino acid identity in these transmembrane domains that these aspartate residues may be important to all members of this family of receptors. From studies on the beta receptor it appears that Asp 113 is an important residue that apparently functions as a counterion to both adrenergic agonists and antagonists. In our model (Fig. 9), we have suggested that the negative charge on Asp 113 may be important in initial ligand attraction into the receptor binding pocket. We also think it possible that the negatively charged residues in transmembrane segments II and III may form a charge network which comprises both high- and low-affinity binding sites for the amine group of catecholamines. In the absence of bound ligand, these negatively charged residues may be complexed with water or possibly monovalent or divalent cations. Displacement of these by agonists may result in the movement of one transmembrane segment relative to the others. As a result of this movement the hydrophilic loops of the receptor, located in the cytoplasm, may shift relative to each other and to the "G" proteins. This is consistent with the data which show that Asp 79 is responsible for high affinity agonist binding of catecholamines as an amine counterion [36] but is not involved with adrenergic antagonist binding [36]. Our data also indicate that Asp 130 is associated with agonist low affinity binding, perhaps as a second amine counterion [37]. It is possible that Asp 130 moves as a result of the receptor conformational changes following agonist binding. Also, there is the potential for the existence of a charge relay system from the outside of the cell to the inside, along transmembrane segments II and III. One might speculate that the catecholamine or positively charged species such as protons or cations may move along this network during receptor activation. While these receptors may not be ion channels in the manner of the bacteriorhodopsin proton pump [27], the movement/displacement of ions could be a key part of receptor activation mechanisms.

Conclusions

The details of receptor structure and function are now readily obtainable through the application of the wide range of new technologies. The approach being taken by our laboratory includes a good mixture of molecular biology with gene cloning, expression and mutagenesis, together with computer-assisted molecular modeling and detailed biochemical analysis. None of these studies would be complete, however, without asking significant questions relevant to physiology and pharmacology.

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