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COMMENTARY

THE NEW BIOLOGY OF DRUG RECEPTORS

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The wide dissemination of recombinant DNA technology has transformed a number of the classical biomedical disciplines over the past decade. Nowhere is this more evident than in pharmacology. In the face of this dizzying progress, it is easy to lose sight of the fact that the fundamental questions of pharmacology have not changed all that much, only some of the approaches taken to answering them. For example, in the area of drug receptors the basic issues remain, such as: what are the receptors? What is their three-dimensional structure? How does their structure determine their function? Why do agonist and antagonist interactions with the receptors have different consequences? How is receptor function regulated? How can answers to these questions be used to design ever more selective and specific drugs? Here we will illustrate several examples of how the application of molecular biology techniques to understanding drug receptors may lead to fundamental changes in the way these questions are approached in the future. While comparable examples could as easily be drawn from the literature relating to several different types of receptors, e.g. ion channel receptors, growth factor tyrosine kinase receptors or steroid receptors, we will deal here with those that we have studied in our own research, the receptors coupled to guanine nucleotide regulatory proteins.

Receptors coupled to G-proteins

Perhaps the largest group of hormone and drug receptors consists of those which are functionally coupled via guanine nucleotide regulatory proteins or G-proteins [1] to a variety of cellular effectors such as adenylyl cyclase, phospholipase C, and several ion channels [2]. This family of receptors includes those for agents as diverse as small amines (e.g. catecholamines, serotonin, histamine, and acetylcholine), small peptides (e.g. vasopressin, oxytocin, and tachykinins), proteins (e.g. follicle-stimulating hormone, leuteinizing hormone, and glucagon), the visual "light receptor" rhodopsin, and perhaps even other sensory "receptors." In the past several years, the primary structures of several members of this family have been determined through molecular cloning of their genes and or cDNAs (reviewed in Refs 3 and 4). These include all the adrenergic receptor subtypes (α_1, α_2, β_1, β_2), several muscarinic cholinergic receptors, several serotonergic receptors, a tachykinin receptor (substance K), and rhodopsin. All of these receptors share a conserved structure and presumed membrane topography [5]. This is schematically illustrated in Fig. 1. A major feature of their amino acid sequences is that hydropathy analysis indicates the presence in all of these proteins of seven hydrophobic segments, each long enough (24–28 residues) to span the plasma membrane. The seven transmembrane spanning regions are connected by a series of extracellular and cytoplasmic loops. The only one of these regions which varies appreciably in length is the third cytoplasmic loop. The cytoplasmic carboxyl terminal tails and, to a lesser extent, the amino termini of these receptors are also of variable length. Sites for N-linked glycosylation are invariably present in the amino terminal segment. Conservation of amino acid sequence is greatest within the membrane spanning regions. This amounts to 40–50% among members of a subfamily (e.g. adrenergic receptors, muscarinic cholinergic receptors, and opsins), but appreciably less across "family lines," e.g. ~20–30% among adrenergic versus cholinergic receptors. The regions of variable lengths also contain the most divergent sequences, and this holds for members of a subfamily as well as across "family lines."

It must be underscored that the topography shown in Fig. 1 has not as yet been proven. Rather it rests largely on analogy with findings obtained by high-resolution electron diffraction for bacteriorhodopsin, the purple membrane protein of *Halobacterium halobium*, which demonstrates the presence of a bundle of transmembrane α-helices [6]. Such a structure has also been strongly supported for the mammalian rhodopsins by X-ray and neutron diffraction studies, circular dichroism and infrared dichroism [7]. Data obtained with the β_2AR using the technique of limited proteolysis are also consistent with such a model [8].

Receptor structure and function

What is the significance of the highly conserved structure of the "magnificent seven" superfamily of receptors? There must be features of this structural arrangement which make it uniquely suited to the task of transmitting extracellular signals to the internal surface of the cell membrane via conformational changes. Powerful molecular biological approaches are currently being used in an attempt to understand how receptor structure explains receptor function.

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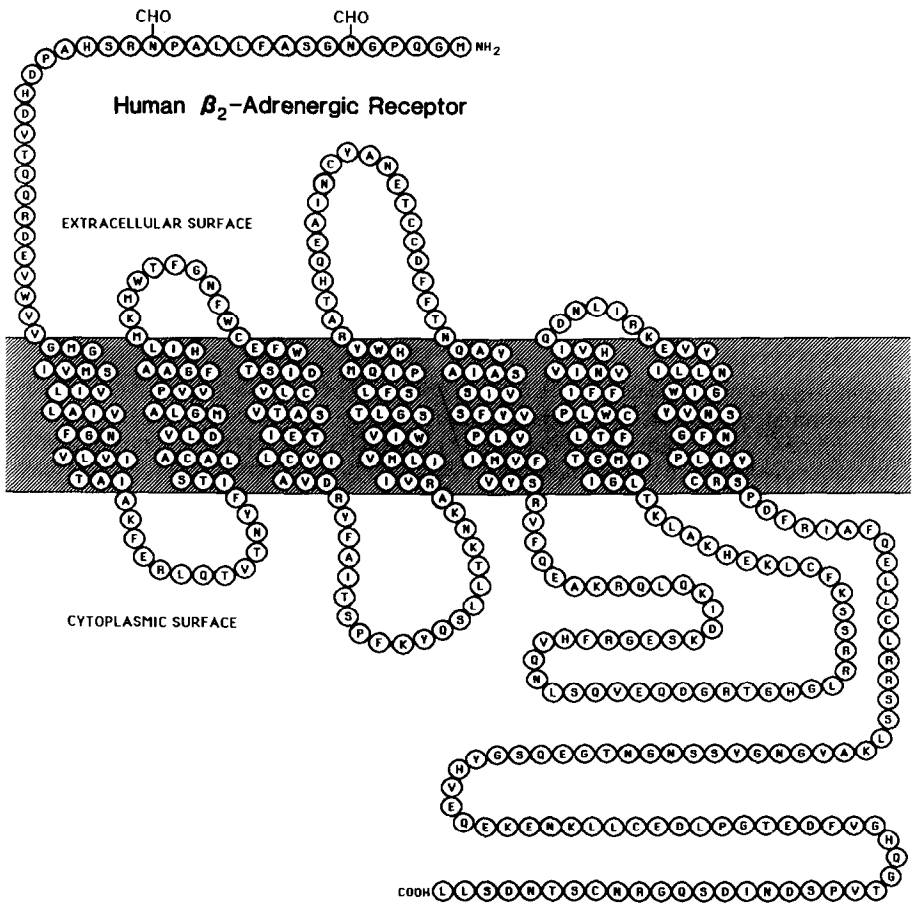

Fig. 1. Topographical representation of the primary sequence of the human β_2-adrenergic receptor. The receptor protein is illustrated as possessing seven hydrophobic regions each capable of spanning the plasma membrane, thus creating extracellular as well as intracellular loops as well as an extracellular amino terminus and a cytoplasmic carboxyl terminal region.

There are three primary functional attributes of the receptor which any comprehensive structure–function map needs to explain: ability to bind ligands with a characteristic specificity; ability to activate characteristic and specific effectors; and ability to undergo functional regulation, as might be the case in the phenomenon of desensitization.

Several distinct but complementary approaches are being utilized, each with specific advantages and disadvantages. The first involves creation of deletion mutants in which one or another segment of the receptor gene is deleted. When expressed in appropriate cellular systems, the mutant receptors which are produced will lack the corresponding region of the protein. A potential drawback to this approach is that it assumes that any loss of function associated with a particular deletion must be due to removal of the region in question. However, it is also possible that allosteric effects are operative such that changes in the conformation of distant regions of the molecule, which occur secondary to the remote deletion, are responsible for the observed functional changes. Notwithstanding this caveat, important results have been obtained with this approach [9].

A second approach involves site-directed mutagenesis in which the codons for individual amino acids, or for stretches of amino acids, are altered, thus leading to changes in the amino acid sequence of the expressed receptor [10, 11]. This very versatile method has also led to new insights about the relationship of receptor structure and function. Again, however, this approach is subject to the limitations imposed by the necessity of generally drawing conclusions from a loss or attenuation of a biological function which may or may not be due to the specific amino acid changes made (as opposed to changes occurring in a distant region of the molecule).

A third approach involves the construction and expression of chimeric receptor genes. The idea is to switch various structural domains between receptors and thereby accomplish a switch in function. As an example, let us consider the α_2- and the β_2-adrenergic receptors. These two receptors have distinguishable ligand binding functions even though both bind adrenaline with high affinity. Highly specific agonists and antagonists for each receptor subtype exist. Moreover, the two receptors have quite different effector functions. Thus, the α_2-receptor inhibits adenylyl cyclase through G_i, whereas the βAR activates the enzyme via G_s. By creating chimeric α_2-β_2-

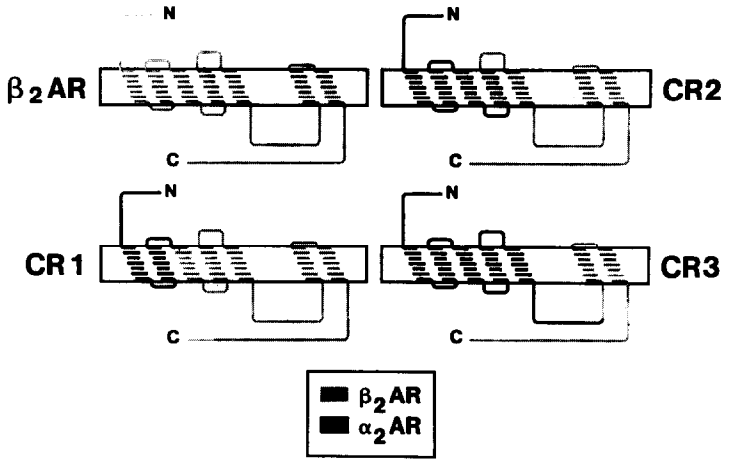

Fig. 2. Schematic representation of a series of chimeric α_2–β_2 receptors. In the diagrams α_2 receptor sequence is represented by dark lines, whereas β_2 receptor sequence is represented by clear lines. Chimeric receptors (CR) 1–3 were obtained by progressively substituting the amino terminal portion and the first two (CR1), four (CR2) or five (CR3) transmembrane segments of the α_2 receptor into the β_2-adrenergic receptor sequence. Constructs were obtained by methods described previously [12], and the ability of receptors to bind ligands and mediate stimulation of adenylyl cyclase was assessed after expression of mRNA in *Xenopus* oocytes [12].

receptor genes we can find out whether there is any structural domain of, for example, the β_2AR which might be inserted into the α_2AR, thus bestowing upon it the ability to activate rather than inhibit adenylyl cyclase. Any such domain would presumably be important in mediating coupling of the β_2AR to G_s.

Similarly, if one or another domain could be found to convert ligand binding specificity from α to β, then this domain likely functions in an important way in the native receptors to determine ligand binding specificity. The essential point of difference between this approach and those described above is that one is looking for the acquisition of a new function by the chimeric receptor which correlates with the *acquisition* of a new structural domain. Accordingly, it is much easier to draw secure inferences about a particular function being associated with a particular structural domain under such circumstances.

To illustrate the power of this approach, consider the receptor chimeras shown in Fig. 2 [12]. It can be observed that each of these chimeric receptors (CR1–3) contains progressively more and more α_2AR sequence. When such chimeras are expressed in *Xenopus laevis* oocytes or transiently in COS-7 cells, some expected and some very unexpected findings are obtained. Not particularly surprising is that, as α_2-receptor sequence replaces β_2-receptor sequence, progressive changes in the affinities for binding agonists occur. Intermediate patterns of agonist binding specificity conforming to neither α_2 nor β_2 are observed. The affinity of binding agonists, moreover, is quite low for all the chimeras, suggesting that none of them is able to form as stable a ligand binding structure as are either the native α_2ARs or β_2ARs. Much more surprising, however, are the results of antagonist binding studies. All of the chimeras shown in Fig. 2 bind β_2-adrenergic antagonists with essentially wild type affinity, and none of them bind α_2-adrenergic antagonists (cf. Fig. 3A). Given that chimera 3, for example, contains largely α_2AR sequence, this result is indeed a surprising one.

These findings reveal several interesting features about the structural basis of ligand binding in this class of molecules. First, the determinants of agonist and antagonist binding, while obviously overlapping, are not identical. Second, the most important determinants of antagonist binding affinity must reside in the 6th and 7th membrane spanning domains. Further confirming the importance of the 7th membrane spanning domain in determining antagonist and agonist binding specificity are the results obtained with chimeric receptor 8, the structure of which is shown in Fig. 3B. This chimera differs from chimeric receptor 2 (Fig. 3A) only in that the 7th membrane spanning domain and short carboxy terminal cytoplasmic tail have been converted from that of the β_2- to that of the α_2AR. Nonetheless, as shown by the ligand binding curves in Fig. 3B, the switch of this one domain converts the antagonist binding specificity from β to α_2. Thus, whereas chimera 2 binds the β-antagonist radioligand [^{125}I]cyanopindolol, chimera 8 binds the α_2-antagonist [^{3}H]yohimbine. Moreover, in chimeric receptor 8, *p*-aminoclonidine is quite potent and isoproterenol very weak, whereas the β-adrenergic blocker, alprenolol, is several orders of magnitude weaker than it is at chimeric receptor 2.

Chimeric receptor 8 is instructive in terms of the effector coupling specificity of the receptors as well. While binding ligands with classical α_2-adrenergic specificity, chimeric receptor 8 is, nonetheless, able to activate adenylyl cyclase. As shown in Fig. 4, however, it does so with a classical α_2-adrenergic receptor specificity. Para-aminoclonidine is much more potent than isoproterenol in activating the enzyme. Thus, chimeric receptor 8 is truly a chimeric receptor, binding ligands with the specificity of one

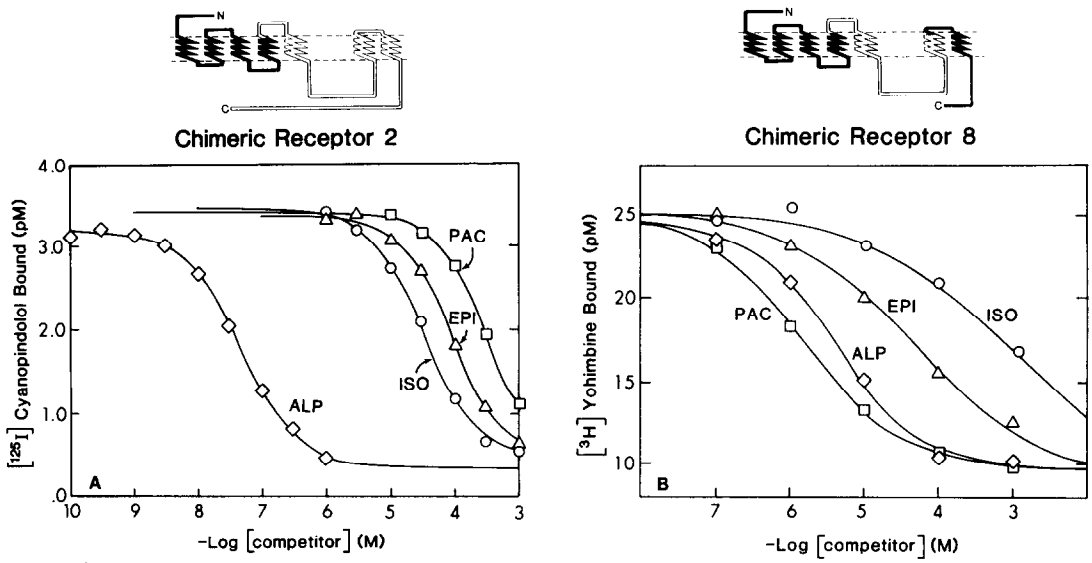

Fig. 3. Ligand binding properties of chimeric α_2–β_2 receptors expressed in *Xenopus* oocytes. As shown in the diagram (top), chimeric receptor 2 (A) contained the first four transmembrane segments of the α_2 and the last three transmembrane segments of the β_2 receptor. Chimeric receptor 8 (B) was obtained from CR2 by substituting the seventh membrane segment and the carboxyl terminal of the β_2 receptor with α_2 receptor sequence. After expression of mRNA in *Xenopus* oocytes, the ability of the expressed receptor to bind β- and α_2-adrenergic ligands was assessed in membrane fractions. Results are taken from Kobilka *et al.* [12]. Abbreviations: PAC, *p*-aminoclonidine; EPI, epinephrine; ISO, isoproterenol; and ALP, alprenolol.

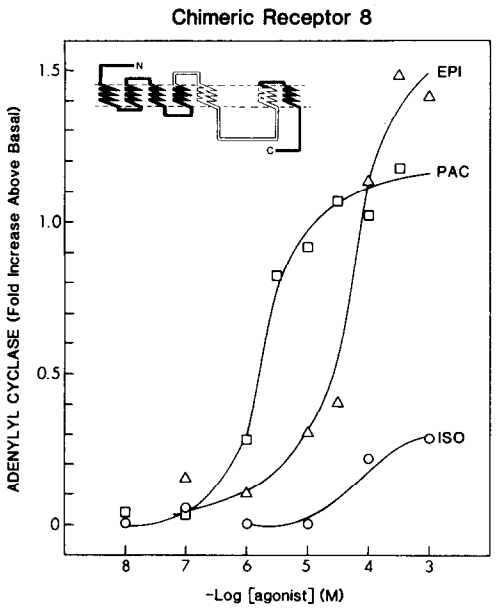

Fig. 4. Ability of the α_2–β_2 chimeric receptor 8 to mediate agonist-promoted stimulation of adenylyl cyclase. CR8 (shown in the diagram) was expressed by injecting mRNA into *Xenopus* oocytes and assessing membranes for adenylyl cyclase activity in response to various concentrations of agonist ligands. Abbreviations: PAC, *p*-aminoclonidine; EPI, epinephrine; and ISO isoproterenol. Results are taken from Kobilka *et al.* [12].

receptor but activating a biological effector (adenylyl cyclase) with the specificity of another. Moreover, the findings with this chimera indicate that sequences contained within the cassette of β-receptor sequence, which is present in chimeric receptor 8 (from amino acid residues 174 to 295 encompassing the 5th and 6th transmembrane spanning domains as well as the second extracellular and third intracellular cytoplasmic loops), are involved in determining the specificity of G-protein coupling of the receptors.

Our best current information about the structure–function relationships of these receptors comes from a synthesis of information obtained from the various experimental approaches described above. Binding of ligands appears to be a function largely of the membrane spanning domains. These are envisaged as being alpha-helical, with the seven membrane-spanning alpha-helices forming a cluster within which a ligand binding pocket is created. Such a pocket would be analogous to that in rhodopsin into which the chromophore retinal appears to fit. The hydrophilic regions in the cytoplasm and the extracellular space appear, in general, not to contribute importantly to the ligand binding function since a deletion analysis of these regions carried out by Dixon *et al.* [9] led to essentially no changes in ligand binding. An exception, however, is the second extracellular loop which contains several highly conserved cysteine residues that are likely involved in disulfide bonding (cysteines No. 106, 184, 190 and 191) [13, 14]. Since site-directed mutagenesis of these cysteines leads to a dramatic loss of ligand binding, it is presumed that disulfide bonding involving these residues is involved in somehow maintaining a crucial

ligand binding structure of the receptor molecule. Mutagenesis studies have also indicated that highly conserved aspartate residues within the membrane spanning domains at positions 79, 113 and 130 are essential for normal ligand binding [15, 16]. In particular, those at positions 113 and 130 may form counter ions for interaction with the positively charged protonated amine of the catecholamines [15, 16].

The localization of specific residues within the transmembrane domains which may be involved in the ligand binding site of these receptors agrees well with data obtained with a more biochemical approach. In this approach, affinity and photoaffinity ligands have been shown to covalently incorporate into peptides contained within several of the putative transmembrane segments of purified receptor proteins [17–20]. β_1- and β_2-Adrenergic receptors have been labeled, respectively, within the 7th and 2nd transmembrane segments [17, 18]. An α_2-adrenergic selective photoaffinity probe has been found to covalently label a peptide represented in the 4th transmembrane domain of the human platelet α_2-adrenergic receptor [19]. Finally, an alkylating propylbenzilylcholine mustard has been shown to incorporate into a peptide from the 3rd transmembrane domain of the purified rat brain M_1 muscarinic receptor [20].

Interaction of the receptor molecule with G-protein appears to be mediated by cytoplasmic regions of the receptor protein and especially those portions of the third cytoplasmic loop which lie in close apposition to the plasma membrane [9, 11, 12, 21] as well as a short stretch of amino acids on the amino terminal side of the carboxy terminus of the protein [11]. These regions are in general highly conserved amongst members of a receptor family coupled to the same G-protein. Some of these features are highlighted in Fig. 1. Much additional work will be required to determine the generality of these structure–activity relationships, the precise sequences involved, and the way in which the receptor structure is organized in three dimensions to interct with G-proteins to cause biological effects.

Multi-gene receptor families: new paradigm for drug discovery

One of the more interesting and potentially more important consequences of recently developed molecular genetic approaches to the study of receptors is the emergence of a potentially new paradigm for drug development. The classical paradigm for understanding the biology of receptors has been as follows:

Physiology → Pharmacology
→ Biochemistry → Molecular genetics.

Study of the physiology of various transmitters, as for example epinephrine or acetylcholine, leads to an initial concept of receptor classification. Pharmacological probing with various agonists and antagonists then leads to refinement and a pharmacological definition of receptor subtypes using classical whole organ or tissue preparations. Availability of selective receptor subtype-specific agents has generally been followed by radioligand binding studies leading to the development of techniques for solubilization and purification of the receptors. This, in turn, has paved the way for the cloning of their genes and the elucidation of their primary structures. The major point at which the drug discovery process has been focussed is at the early points in this sequence of steps, namely the pharmacological identification and characterization of the various receptor subtypes.

A new paradigm, however, is emerging from the recent recombinant DNA work on receptors. This new paradigm completely reverses the flow of information. It is based on the fact that the genes for many members of a particular receptor family, e.g. the adrenergic receptors or the serotonin receptors, are sufficiently close in structure at the protein level and consequently at the DNA level that they can be used to isolate each other by techniques of cross-hybridization. In this method, radioactively labeled forms of a gene are used to screen an appropriate cDNA or genomic library, under appropriate stringency conditions, to isolate structurally closely related genes. These can then be sequenced, expressed in one or another cellular system, and their ligand binding and effector coupling properties identified.

One consequence of this approach has been the isolation of genes for closely related subtypes of receptors, the existence of which either had not been suspected previously or which had been only poorly appreciated. For example, in the case of the muscarinic cholinergic receptors, two subtypes had been defined by pharmacological work the so-called M_1 and M_2 muscarinic acetylcholine receptors [22, 23]. And, in fact, molecular cloning efforts initially demonstrated that these two receptor subtypes were encoded by distinct genes with closely related but distinct structures [24–26]. Soon thereafter, however, two more muscarinic receptor genes were isolated [27, 28] and then yet another [29]. Currently, the count stands at five subtypes of muscarinic receptors designated M_1–M_5. Although all have closely related structures, their binding properties and effector coupling specificities are distinguishable.

Another example is provided by the adrenergic receptors, in particular the α_2 subfamily. We have isolated the gene and cDNA for two distinct α_2-receptors [30, 31]. Within the membrane spanning domains these receptor proteins are approximately 80% identical, whereas in other regions they are much more divergent. While ligand binding specificities of these receptors are clearly distinguishable, their biochemical effector and physiological functions are only now being defined. Southern genomic blots suggest that there may be at least one more α_2-adrenergic receptor-like gene in the human genome [30], and recent radioligand binding results suggest the same conclusion [32]. Thus, there is the possibility of even greater heterogeneity.

What emerges from all this, is that the potential exists in such cross-hybridization studies for the isolation of genes for receptor subtypes, the existence of which has been only hinted at or not even predicted based on classical physiological and pharmacological studies. When this happens the flow of information pictured above is reversed. The isolation of the gene for the receptors is then actually the first step rather

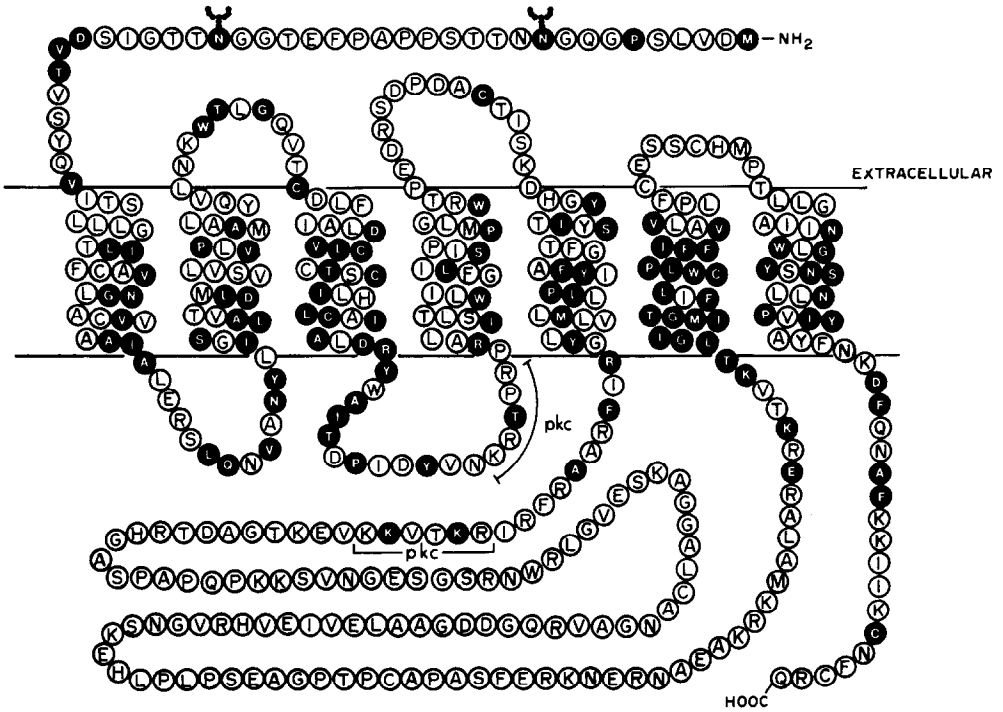

Fig. 5. Schematic representation of the primary sequence of the $5HT_{1A}$ receptor. The sequence is arranged according to the paradigm outlined in Fig. 1. Darkened circles with clear letters represent amino acid residues that are identical with the human β_2-adrenergic receptor [34, 35]. Brackets indicate potential consensus sites for protein kinase C phosphorylation.

than the last in characterizing the receptor. Identification of the new receptor gene must be followed by delineation of its tissue distribution (e.g. RNA blots, Western blots with antipeptide antibodies), isolation of the receptor protein, biochemical characterization, and ultimately characterization of its pharmacology and role in normal physiology. Examples of this have also been noted recently in the steroid receptor field where new members of this extended family of receptors have been isolated but for which no ligand is presently known [33]. With the discovery of such new receptor genes comes the possibility for development of ever more specific pharmacological agents. The history of pharmacology over the past 100 years is that, as receptor subtypes are delineated, newer and more selective pharmacological agents which are useful in therapy are developed. With the power of current recombinant DNA techniques, and the existence of much greater heterogeneity of receptors than anyone had suspected just a few years ago, the possibility exists for ever greater specificity of drug action. The expression in cells of genes for individual receptor subtypes provides an extremely useful technology for screening drugs by radioligand binding or other biochemical assays. This provides an ideal approach to development of highly selective pharmacological agents.

Receptor structure and pharmacological specificity

It should be pointed out that, while cross-hybridization studies using receptor genes as probes may often yield a closely related subtype, it is also possible that the yield may include more distantly related receptors. An interesting and instructive example is the isolation of one of the serotonin receptors, the so-called $5HT_{1A}$ receptor, by cross-hybridization with the β_2-adrenergic receptor gene [34, 35]. This gene was isolated because it is quite similar in its structure to that of the β_2-adrenergic receptor. In fact, in those regions of the genes which encode the sixth transmembrane spanning domain, almost all amino acid residues are identical (Fig. 5). It is this stretch of very concentrated homology which presumably leads to the cross-hybridization between the two genes. As noted above, chimeric receptor data [12] have suggested that this region of the molecule is particularly important in determining antagonist binding specificity. Interestingly, the $5HT_{1A}$ receptor is able to bind classical β-adrenergic antagonists with relatively high affinity. Thus, the structures of these two receptor genes illuminate their overlapping ligand binding specificities. In fact, the well known pharmacological "crossover" of antagonist binding to all sorts of receptors, including adrenergic, dopaminergic, histaminergic and serotonergic, amongst others, may now be clearly explainable in terms of the structural similarities present especially within the membrane spanning domains of these receptors.

The delineation of the precise structural features involved in ligand binding as well as effector coupling for these receptors may give clues to a predictable strategy in the choice of suitable probes for cross-hybridization experiments. The cloning of the genes

and/or cDNAs for several receptors, including a novel subtype of α_2-receptor [31], the $5HT_{1A}$ [34, 35], the $5HT_2$ [36] and a rat brain D_2-dopamine receptor [37] illustrate both the power and, to some extent, the unpredictability of this approach.

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