ELSEVIER

European Journal of Pharmacology 375 (1999) 247–260

ejp

www.elsevier.nl/locate/ejphar

Review

The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm

Edward C. Hulme *, Zhi-Liang Lu, Stuart D.C. Ward, Karen Allman, Carol A.M. Curtis

Division of Physical Biochemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK

Accepted 30 April 1999

Abstract

The rhodopsin-like superfamily of 7-transmembrane receptors is the largest class of signalling molecules in the mammalian genome. Recently, a combination of mutagenesis, biophysical and modelling studies have suggested a credible model for the α-carbon backbone in the transmembrane region of the 7-transmembrane receptors, and have started to reveal the structural basis of the conformational switch from the inactive to the active state. A key feature may be the replacement of a network of radial constraints, centred on transmembrane helix three, which stabilise the inactive ground state of the receptor by a new set of axial interactions which help to stabilise the activated state. Transmembrane helix three may act as a rotary switch in the activation mechanism.

Keywords: 7-Transmembrane receptor; G-protein; G-protein coupling pocket; Muscarinic receptor; Acetylcholine

1. Introduction

The rhodopsin-like superfamily of 7-transmembrane (7-TM) receptors is the largest class of signalling molecules in the mammalian genome. It contains approximately 1000 distinct sequences (Horn et al., 1998). Interestingly, the superfamily is almost as well represented in the genome of *C. elegans* (Bargman, 1999), even though the nematode has only a total of 302 neurons in its simple nerve net. The 7-TM receptor family has undergone an enormous expansion during the evolutionary transition from the unicellular to the multicellular eukaryotes. Arguably, it represents the most elemental, and the most versatile, of the ligand-gated transmembrane signalling mechanisms.

Even 25 years ago, we had clearly grasped the concept that the ability of a neurotransmitter such as acetylcholine to induce a pharmacological response is quantitatively related to its ability to induce, or to stabilise, an activated conformational state of its cognate receptor (Birdsall and Hulme, 1976). The hypothesis of a two-state mechanism of receptor activation grew naturally out of the pioneering work of Monod, Wyman and Changeux on allosteric conformational changes in enzymes (Monod et al., 1965).

Recent results have started to reveal the structural basis of the conformational switch from the inactive to the active state in the rhodopsin-like 7-TM receptors. The switch mechanism may be well conserved throughout the superfamily. In this review, we discuss it with particular reference to recent findings on the muscarinic acetylcholine receptors.

2. The three-dimensional structures of the 7-TM receptors

As yet, it has not been possible to determine an atomic-resolution structure for any of the 7-TM receptors. However, the fact that several such structures have now been determined for the proton pump bacteriorhodopsin gives reasons for hope (Essen et al., 1998). Despite this, the availability of a multitude of related sequences, combined with a series of low-resolution structure determinations of rhodopsin, have rendered the 7-TM receptors a test-bed for the application of the emerging principles of protein structure prediction, and modelling. In some respects, this presents an easier problem for a transmembrane protein, in which the major structural elements are confined to two dimensions, than it is for a globular protein, in which the polypeptide chain has more degrees

* Corresponding author. Tel.: +44-181-959-3666; Fax: +44-181-906-4477; E-mail: e-hulme@nimr.mrc.ac.uk

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00297-6

of freedom. The modelling enterprise has been informed and tested by a large body of data derived from mutagenesis studies (reviewed in (Van Rhee and Jacobson, 1996; Pogozheva et al., 1997; Herzyk and Hubbard, 1998)). This dialectical process has helped to put mechanistic flesh on the bones of the models.

Sequence comparisons have shown that the consensus structure of the 7-TM receptors contains about 40 residues (out of a total of ca. 320 in a 'minimal' 7-TM sequence; Baldwin, 1993) which are highly conserved throughout the superfamily. These residues are entirely within the transmembrane region, or in probable extensions of the transmembrane domains (Unger et al., 1997), effectively making them part of the transmembrane fold itself.

Early on, circular dichroism and polarised infrared spectroscopic measurements showed rhodopsin to be largely α-helical. The helices are oriented more or less perpendicular to the plane of the membrane (Findlay and Pappin, 1986). There have been four rounds of structure determination by electron cryomicroscopy, and electron diffraction of a series of two-dimensional crystals of bovine and frog rhodopsins (Krebs et al., 1998 and references therein). These have yielded maps of the structure with a resolution of up to 5 Å in the plane of the membrane, and 16.5 Å perpendicular to it. The maps have proved adequate to support a model of the α-carbon backbone of rhodopsin based on an attempt to integrate a detailed analysis of sequence variation within the 7-TM superfamily with the low resolution structure (Unger et al., 1997). Particular attention was paid to the probable initiation and termination of the TM helices, and to the definition of the lipid-facing sectors of each helix. Further refinements have been based on distance-geometry calculations, incorporating restraints derived from experimental (mainly mutagenesis) data (Herzyk and Hubbard, 1998), or on the satisfaction of the hydrogen bonding potential of buried polar side-chains (Pogozheva et al., 1997). There are small differences in helix packing, position and tilt angles between these models.

The Schertler, Baldwin, Unger and Hargrave model (referred to hereafter as the Cambridge model) is consistent with most of the mutagenesis data which indicates helix–helix proximities (Unger et al., 1997), although some putative intramolecular interactions, such as that postulated to exist between the highly conserved aspartate residue in TM 2 ($Asp^{2.50}$, using the nomenclature of Van Rhee and Jacobson, 1996, which is used throughout this review) and the conserved $Asn^{7.49}$ in TM7 are in better agreement with subsequent models (Pogozheva et al., 1998). Nonetheless, the Cambridge model has proved to be a useful starting point for interpreting the results of mutagenesis studies, and it is used as a basis for further discussion here.

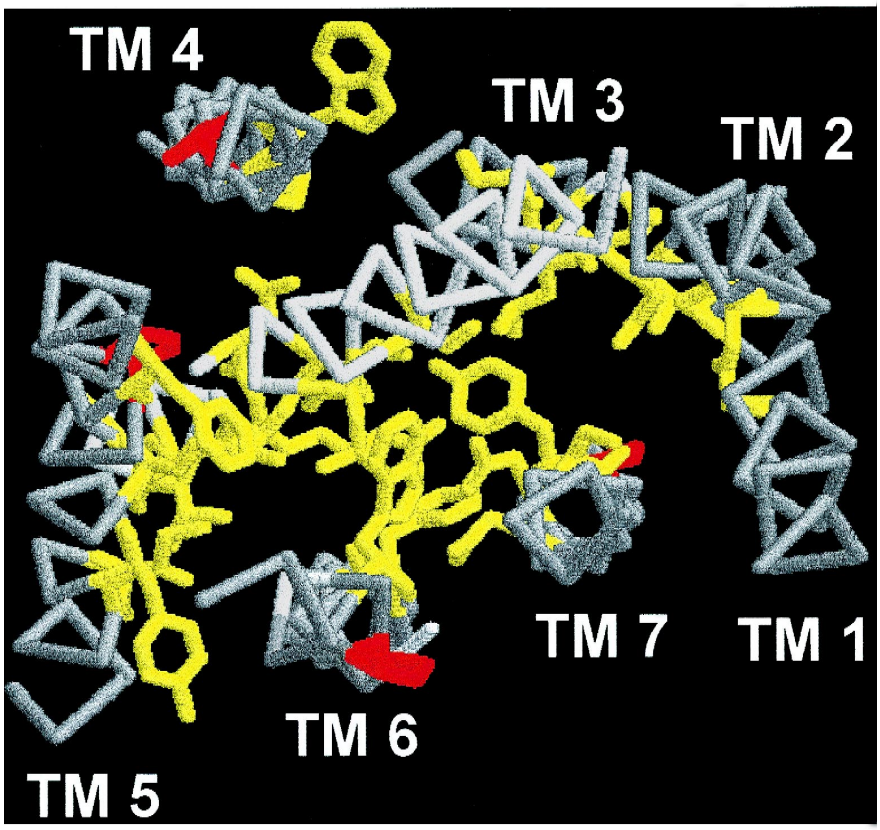
All of the models concur in showing an anti-clockwise arrangement of the 7 transmembrane helices when viewed from the extracellular side of the molecule (Fig. 1). TM 4, 6 and 7 are perpendicular to the plane of the membrane. TM 1, 2 and 5 have a moderate degree of counter-clockwise tilt. TM 3 is very highly tilted, and longer than the other TM helices. It passes through the structure to end between TM helices 4 and 5. Thus, TM 3 makes multiple inter-helical contacts, successively with TM 2 within the outer leaflet of the bilayer, with TM 6 and 7 within the inner leaflet, and with TM 4 and 5 near the intracellular surface. A view from the intracellular side, normal to the plane of the bilayer (Fig. 1) shows TM 2, 4, 5, 6, 7 to be clustered around TM 3, the arrangement being reinforced by TM 1. TM 3 is therefore ideally placed to mediate and to co-ordinate any rearrangements of the TM structure of the receptors which may accompany their activation. Spin-labelling studies on cysteine substitution mutants of rhodopsin indicate that TM 3, 5 and 6 extend beyond the plane of the lipid bilayer (Farahbakhsh et al., 1995; Altenbach et al., 1996). These findings have been supported by random saturation mutagenesis studies on the M_5 muscarinic acetylcholine receptor (Hill-Eubanks et al., 1996; Burstein et al., 1998a,b).

The Cambridge model gives a good account of the distribution of the highly conserved residues within the TM structure of the 7-TM receptors (Fig. 1). These are mostly clustered at the helix–helix boundaries, where they are well-positioned to mediate stabilising inter-helical contacts. Viewed from either the extracellular or intracellular aspect, an arc-shaped structure is visible, consisting of TM 1, TM 2, TM 3 and TM 5. This is buttressed by TM 4, and further stabilised by the second extracellular loop, which acts like a strut, disulfide-bonding the N-terminus of TM 5 to the N-terminus of TM 3 (Kurtenbach et al., 1990). The N-terminal domain enfolds TM 6 and TM 7, which are linked both to TM 1-5, and to one another, by a nexus of conserved amino acid side-chains spanning the cleft in the centre of the structure.

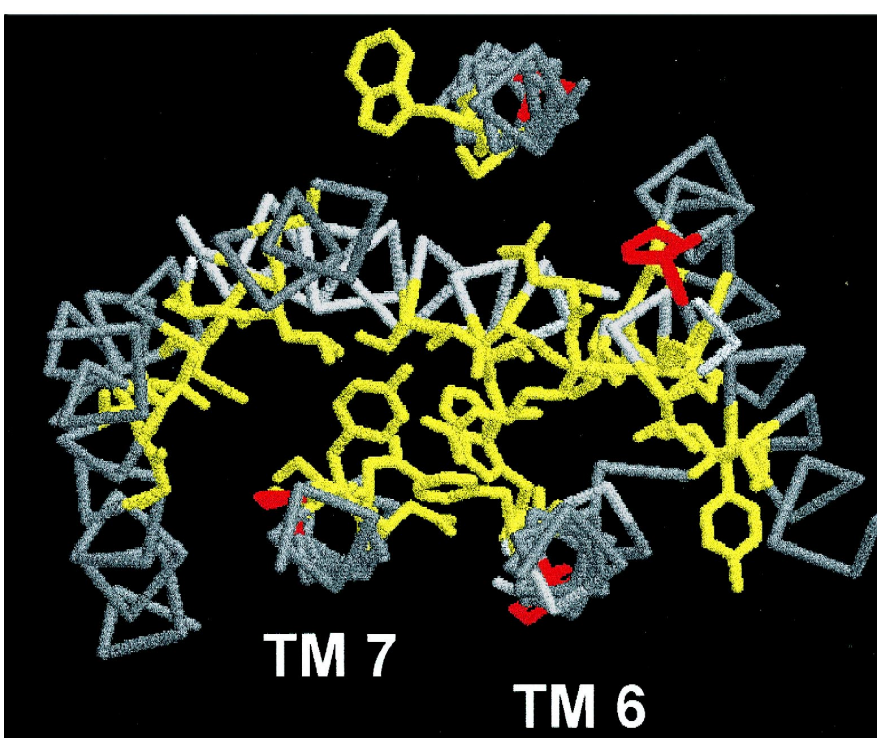
Peptides corresponding to the intracellular loops of rhodopsin have been subjected to structure determination by nuclear magnetic resonance spectroscopy, both individually, and in combination. The resulting distance constraints have been used to model the structure of the cytoplasmic domain of rhodopsin (Yeagle et al., 1997), assuming the cytoplasmic loops to be continuous with the TM helices defined by the Cambridge model. The results suggest the presence of helical structures, particularly in the third intracellular loop, the second intracellular loop, and the proximal part of the C-terminal domain, and of β-structure in the first and second intracellular loops, and the distal part of the C-terminal domain. The lysine and

Fig. 1. The positions of the conserved proline residues (red), and the other most highly conserved residues (yellow) in the 7-transmembrane receptors superimposed on the Cambridge version of the backbone of the M_1 muscarinic acetylcholine receptor.

Outside view



Inside view



arginine residues tend to map to the exposed parts of the loops, creating a positively charged rim which might be involved in G protein docking.

The extracellular domain of rhodopsin has also been postulated to have a compact, folded structure (Hwa et al., 1997). This may be important in dictating the disposition of the TM helices, although there is now much evidence to suggest that the TM helices also possess specific affinities for one another (Ridge et al., 1995; Wess et al., 1996). The extracellular domain is further stabilized by the presence of the conserved disulfide link between the top of TM 3 and the second extracellular loop (Karnik et al., 1988; Kurtenbach et al., 1990).

3. Mutagenesis studies

3.1. Mutations can be used to categorise the roles of amino acids in receptors

The extended ternary complex model represents the minimum scheme able to encompass the phenomenology of G protein activation by 7-TM receptors (Samama et al., 1993). In particular, it provides a useful framework for interpreting the effects of point mutations. A conformational equilibrium is postulated to exist between predefined ground and active states of the receptor. This is governed by an isomerisation constant K. Agonists bind to the ground state conformation with low affinity, K_A, but to the activated state with a higher affinity, αK_A. In the simplest case, the G protein binds selectively to the activated conformation, with an affinity constant K_G. The complex between the activated state of the receptor, and the G protein, is assumed to be the form which undergoes GDP–GTP exchange.

A point-substitution mutation in which a moiety is deleted, for instance, by replacement of the target residue by alanine, can have four basic outcomes. These are illustrated in Fig. 2.

(i) It may produce a null effect. Residues which tolerate multiple substitutions can be regarded as plugging functionally unimportant gaps in the receptor structure. In TM regions, such *filler* residues will usually be found to face towards the lipid bilayer.

(ii) It may induce a simple reduction of the structural stability of the receptor. This would be the expected result if the target residue makes equal contributions to the architecture of the receptor in the ground and the activated states. The mutation of such a *stabiliser* residue may reduce the expression level of the receptor by lessening its probability of folding successfully, and undergoing correct trafficking. However, it would not be expected to affect the intrinsic signalling ability of those receptor molecules which succeed in achieving the folded state, because it does not alter the isomerisation constant governing the equilibrium between the ground and activated states. The *ligand anchor* residues can be regarded as a particular subset of the stabiliser residues. These amino acids make inter-molecular contacts with the ligand in the ground state of the receptor. Their mutation reduces ligand affinity, by diminishing K_A, but does not affect signalling efficacy, because, once again, there is no effect on the isomerisation constant K.

(iii) A mutation may reduce the structural stability of the receptor, while simultaneously increasing its agonist affinity, basal activity and signalling efficacy. This portfolio of related effects suggests that the targeted residue contributes intra-molecular bonds which stabilise the ground state, but are weakened or broken in the activated state of the receptor. Thus, such amino acids participate in *constraints* that help to maintain the receptor in its inactive state.

(iv) The mutation may reduce the signalling efficacy of the receptor. This indicates that the target residue makes interactions that come into play in the activated conformation of the receptor. Usually, the alanine substitution of such a residue will not have dramatic effects on the stability, and expression level of the receptor, since its effect is exerted selectively on the activated state, not on the resting state of the receptor. The interactions made by such an *activator* residue may either be intramolecular, or intermolecular. In the latter case, it may be further categorised either as a *ligand-transducer*, or a *G protein-transducer* residue.

Combinations of stabilising, constraining, and activator properties are common. For instance, it is easy to envisage that residues may act as *double-agents*, having a constraining role in the ground state, but changing their affiliations to exercise a ligand or G protein binding function in the activated state of the receptor.

The functions of residues are most clearly categorised by alanine substitution mutations, which delete the side-chain of the amino acid beyond the beta-carbon, leaving a small ‘hole’ in the receptor structure. In contrast, the substitution of a side-chain by a bulkier variant can change the structural role of a residue, for instance, by converting a stabilising interaction into a destabilising one, through the introduction of steric hindrance at a sensitive point in the structure. It should be pointed out that negative interactions of this kind do not generally persist in proteins which have been subjected to the rigours of evolutionary natural selection, which tends to remove such ‘frustrated’ contacts. This has been formalised as the ‘principle of minimum frustration’ (Nymeyer et al., 1998).

3.2. The G protein binding site

Intensive mutagenesis studies have been performed within the intracellular loops of the 7-TM receptors, particularly in those parts of the sequences which lie adjacent to the TM domains. The field has been comprehensively reviewed by Wess (1996, 1998). These studies have shown

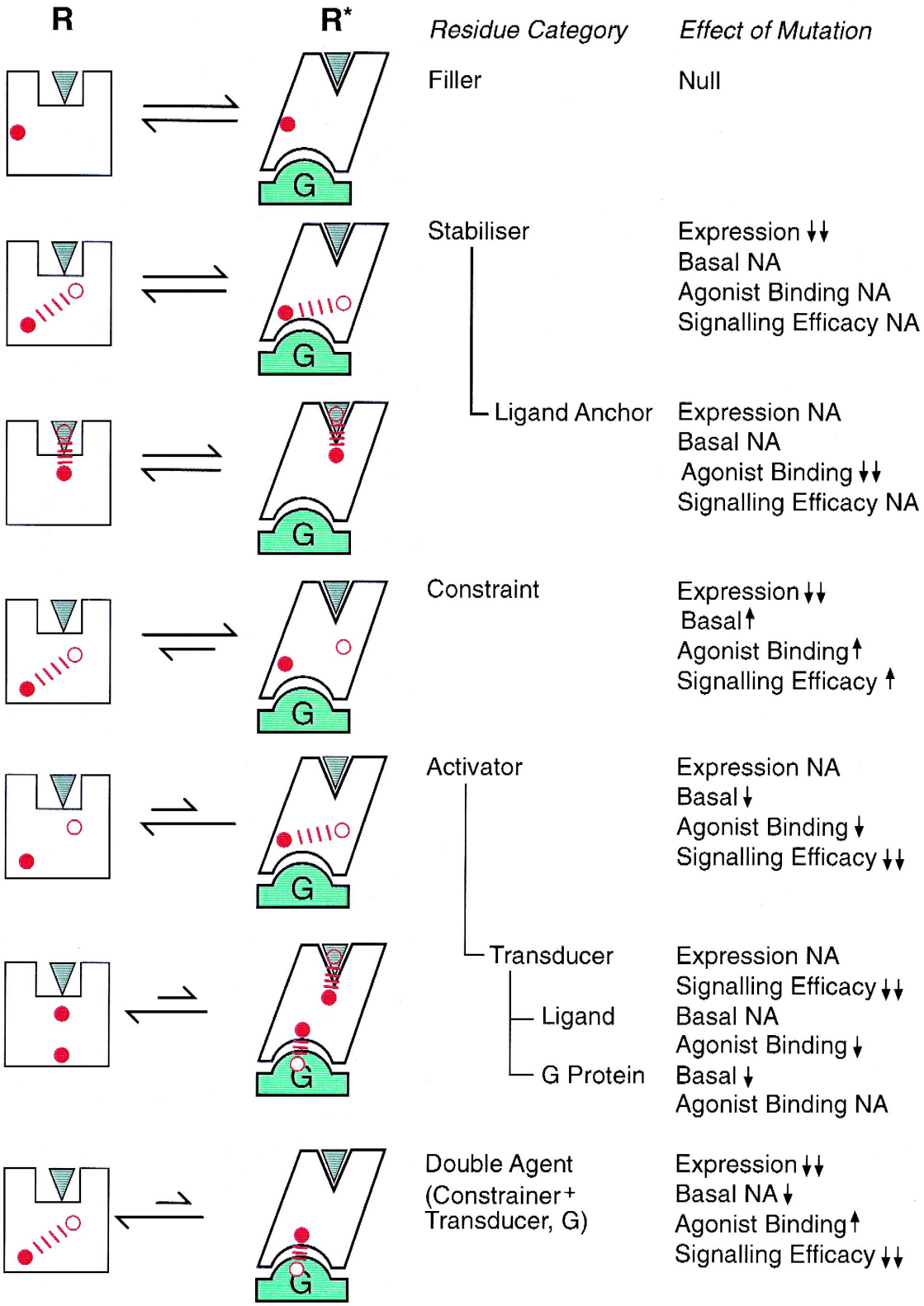


Fig. 2. The categorisation of the function of amino acid side chains in 7-transmembrane receptors by alanine scanning mutagenesis.

that residues in the N- and C-terminal regions of the third intracellular loop, supplemented by four polar residues in the second intracellular loop, are critical for the selective recognition of G proteins belonging to particular classes, for instance the G_q as opposed to the G_i family. Experiments involving rescue of the function of chimaeric receptors co-expressed with chimaeric G-proteins have indicated that a sequence at the C-terminus of the third intracellular

loop contacts a sequence at the C-terminus of the G protein α-subunit (Kostenis et al., 1997). A corresponding set of residues contributes to the binding of the C-terminal peptide from transducin to rhodopsin (Acharya et al., 1997). Random saturation experiments on the i3 loop of the M_5 muscarinic acetylcholine receptor (Burstein et al., 1996) are consistent with these findings, as well as with the earlier site-directed mutagenesis experiments.

In rhodopsin, hydrophobic residues in the second intracellular loop also contribute to the binding site for the transducin C-terminal peptide (Acharya et al., 1997). These findings are also consistent with studies on the muscarinic acetylcholine receptors (Moro et al., 1993; Blin et al., 1995). Studies using synthetic peptides based on the sequences of the cytoplasmic ends of TM 4, 5, 6 and 7 of the luteinizing hormone receptor have suggested that the G protein recognition site may extend part way into the transmembrane domain of the 7-TM receptors (Abell et al., 1998). In agreement with this, mutation of the highly conserved NPXXY sequence at the end of TM 7 is highly disruptive to signalling by the M_1 muscarinic acetylcholine receptor (Wess et al., 1993; Lu and Hulme, 1999), and by the β-adrenoceptor (Barak et al., 1995).

In sum, these findings suggest that the G-protein coupling pocket on the receptor may consist of a positively charged lip, encircling a hydrophobic core (Burstein et al., 1998a,b). This is a common design for protein–protein interfaces. Within the hydrophobic pocket, particular residues, such as Tyr^{254} in the N-terminal segment of the third intracellular loop of the M_3 muscarinic acetylcholine receptor, play a critical role in the selective recognition of particular G proteins, such as that of $G_{q/11}$ by the M_3 muscarinic acetylcholine receptor (Blüml et al., 1994).

These experiments are consistent with the hypothesis that particular sequences in the cytoplasmic domain of the 7-TM receptors become accessible to epitopes on the G protein as a consequence of ligand-induced activation. One crucial interaction is, evidently, with the C-terminus of the α-subunit of the G protein. This is important in dictating receptor specificity (see (Wess, 1998) and references therein). The N-terminus of the α-chain is another important source of specificity, acting by restricting the access of the G protein to particular receptor types. The N-terminal sequence, which is anchored near the membrane surface by myristoylation or palmitoylation, may fold to form a compact variable domain, which acts as a negative filter (Wess, 1998). The sites of other important interaction points in G-protein α-subunits may include the α4–β6 loop, which contributes to the binding site for the guanine ring (Onrust et al., 1997).

There are sequences in the C-terminus of the third intracellular loop of rhodopsin, and in the rhodopsin C-terminal domain which also interact with the transducin β-subunit (reviewed by Bourne, 1997). It may be suggestive that, in the crystal structure of the βγ complex of transducin, the amphiphilic α-helix from the N-terminus of an adjacent β-subunit is bound to the negatively charged 'mouth' of the β-propeller structure (Sondek et al., 1996). This could act as a model for the binding of the intracellular loops of receptors, which possess a very similar, amphiphilic nature. Indeed, it would be geometrically possible to envisage a match between the positively charged 'lip' of the receptor intracellular domain, and the negatively charged 'mouth' of the β-propeller. Finally, the prenylated C-terminus of the γ-subunit of transducin, which acts as another membrane attachment site, may also be capable of interacting with rhodopsin, particularly when the latter is in the activated state (Kisselev et al., 1995).

3.3. The ligand recognition domain

A large number of ligand recognition modalities are available to 7-TM receptors. These range from interactions primarily within the core of the receptor (the model for biogenic amines) to interactions with the N-terminus and extracellular loops (as for peptides and glycoprotein hormones; see Ji et al., 1998 for a recent review). In the case of the muscarinic acetylcholine receptors, acetylcholine appears to make an initial set of ligand anchoring interactions which exploit polar and Van der Waals interactions with moieties which are mostly in TM 3–7. Using the nomenclature of van Rhee and Jacobson, these are: TM 3:$Tyr^{3.33}$ OH group, $Ser^{3.36}$, $Asn^{3.37}$; TM 5:$Thr^{5.39}$, $Thr^{5.42}$, $Ala^{5.46}$; TM 6: $Tyr^{6.51}$ OH group, $Asn^{6.52}$ and TM 7:$Tyr^{7.39}$ OH group (Wess, 1993; Allman et al., 1997; Hulme and Lu, 1998; Lu and Hulme, 1999; Ward and Hulme, 1999; Ward et al., submitted). Amongst the specific bonds thought to be formed are a hydrogen bond between the OH group of $Tyr^{6.51}$ and the ester function of acetylcholine (Wess et al., 1992; Ward and Hulme, 1999), and a Van der Waals interaction between the acetyl methyl group of acetylcholine and $Thr^{5.42}$ (Allman et al., 1997). These interactions are initiated and stabilised by electrostatic steering of the quaternary ammonium headgroup by the negatively charged carboxylate group of $Asp^{3.32}$, which has been shown to be directly labelled by the alkylating headgroup of a nitrogen mustard derivative of acetylcholine (Spalding et al., 1994).

Following the initial binding step, a process of aromatic cage formation around the tetramethylammonium headgroup of acetylcholine by the phenyl rings of $Tyr^{3.33}$ (TM 3), $Tyr^{6.51}$ (TM 6) and $Tyr^{7.39}$ (TM 7) appears to provide the energy to close the binding site, and drive the activation step (Ward et al., submitted). This agrees with the proposal of Hibert et al. (1991), based on modelling studies of the 7-TM receptors, that aromatic as well as polar interactions are likely to be important in binding cholinergic ligands. This is also the case in acetylcholine esterase (Axelsen et al., 1994) and in the nicotinic acetylcholine receptor (Sine et al., 1994). In the muscarinic acetylcholine receptors, the key tyrosine residues which

participate in these processes are situated about 2 helical turns above the network of intra-molecular contacts between TM 6/7 and TM 1–5. Ligand-induced rearrangements of the binding site are, therefore, in an excellent position to modify the intra-molecular constraints.

3.4. Constitutively activating mutations imply the existence of a network of intramolecular constraints which stabilise the ground state of the 7-TM receptors

A large number of mutations of 7-TM receptors have been reported which raise their basal, agonist-independent, signalling activity (reviewed by (Gether and Kobilka; Rao and Oprian, 1996; Leurs et al., 1998). Most frequently, these involve changes in TM 6, and 3 (and their helical extensions), but mutations in TM 2, 5 and 7 which lead to constitutive activity have also been described.

In rhodopsin, disruption of the salt bridge between the retinal Schiffs base in TM 7 and the $Glu^{3.28}$ counter-ion in TM 3 causes constitutive activation of opsin in the absence of retinal (Cohen et al., 1992). In the thyroid releasing hormone receptor, such experiments imply that hydrophobic interactions between the highly-conserved $Trp^{6.49}$, and $Phe^{5.46}$ may mediate an inter-helical constraint (Colson et al., 1999). It is notable, however, that mutations which cause constitutive activation of one receptor type do not always do so when transferred to another. For instance, mutations of $Trp^{6.49}$ and $Phe^{5.46}$ do not promote constitutive activation of the M_1 and M_3 muscarinic acetylcholine receptors. Mutation of the highly conserved $Asp^{2.50}$ evokes constitutive activation of the B_2 bradykinin receptor, (Quitterer et al., 1996), but inhibits signalling by the muscarinic acetylcholine receptors, although it does enhance acetylcholine affinity (Fraser et al., 1989). Thus, the primary inter-helical contacts have been tailored to suit the energetics, and the G protein or ligand binding properties of individual receptors.

Constitutive activation often promotes receptor instability (Gether et al., 1997a; Lee et al., 1997). It is suggested, therefore, that mutations which increase basal signalling activity usually do so by destabilising particular helix-helix interactions which act as ground state constraints (Gether and Kobilka, 1998). The resulting conformation of the receptor may not always correspond to the form stabilised by the receptor's natural agonist. In rhodopsin, the replacement of the highly conserved acidic residue $Glu^{3.49}$ by a neutral residue only produces a partially activated conformation of the protein (Kim et al., 1997). Similarly, a mutation at position 3.35 in the α_{1B} receptor promotes activation of the phospholipase C but not the phospholipase A_2 signalling pathway (Perez et al., 1996).

In other cases, the effects of constitutively activating mutations can be explained by a simple increase in the isomerisation constant *K* between the inactive and active states of the receptor. This is true for a series of mutations of $Ser^{6.58}$ at the extracellular end of TM 6 of the M_5 muscarinic acetylcholine receptor (Spalding et al., 1997), and of $Met^{6.41}$ near the cytoplasmic end of TM 6 in rhodopsin (Han et al., 1998); interestingly, mutation of position 6.41 in the M_5 muscarinic acetylcholine receptor also causes constitutive activation (Spalding et al., 1998). The orientation of TM 6 is altered in a constitutively-activated mutant of the β-adrenoceptor (Javitch et al., 1997). A reorganisation of the intra-molecular contacts between TM 6 and surrounding TM helices is likely to follow the activation of rhodopsin, the β-adrenoceptor, and the muscarinic acetylcholine receptors, and is probably part of a more general conformational change accompanying the activation of the 7-TM receptors.

3.5. Scanning and random-saturation mutagenesis studies reveal the functional topography of transmembrane domain 3 in the muscarinic acetylcholine receptors

Recent studies from two laboratories have shed light on the possible role of TM 3 in controlling the activation of muscarinic acetylcholine receptors (Hulme and Lu, 1998; Burstein et al., 1998b; Lu and Hulme, 1999). These complementary studies, one conducted by random saturation mutagenesis, the other by alanine scanning mutagenesis have allowed us to categorise the amino acids making up TM 3 and its cytoplasmic helical extension.

The first group consists of amino acids whose mutation had little or no effect on receptor expression, ligand binding or signal transduction. These null 'filler' residues are concentrated in the outer, extracellular part of the domain. The second category contains residues such as $Asp^{3.49}$ and $Tyr^{3.51}$ whose mutation primarily seems to reduce receptor expression levels (Lu et al., 1997; Hulme and Lu, 1998; Lu and Hulme, 1999). These 'stabiliser' residues may contribute intramolecular interactions to both the ground state and the activated state of the receptor. The mutations that reduce receptor expression level are concentrated in the section of TM 3 located within the inner leaflet of the phospholipid bilayer. This is consistent with the view that the inner segment of TM 3 forms multiple intramolecular contacts with the surrounding TM domains.

The third category consists of residues which are important for fixing the inactive, ground state of the receptor. Mutation of these constraining residues selectively destabilises the ground state of the receptor, and favours its transition to the active state. In several cases, this leads to pronounced agonist-independent basal activity. In this category, the highly conserved residue $Leu^{3.43}$ is particularly important, but mutations of $Ser^{3.47}$ and the less-conserved residues at positions 3.40, 3.52, 3.56, 3.59, 3.60, 3.64 also cause constitutive activation. In some receptors, such as rhodopsin and the α_1 and β_2-adrenoceptors, the acidic residues homologous to $Asp^{3.49}$ may have more of a constraining and less of a general stabilising character than

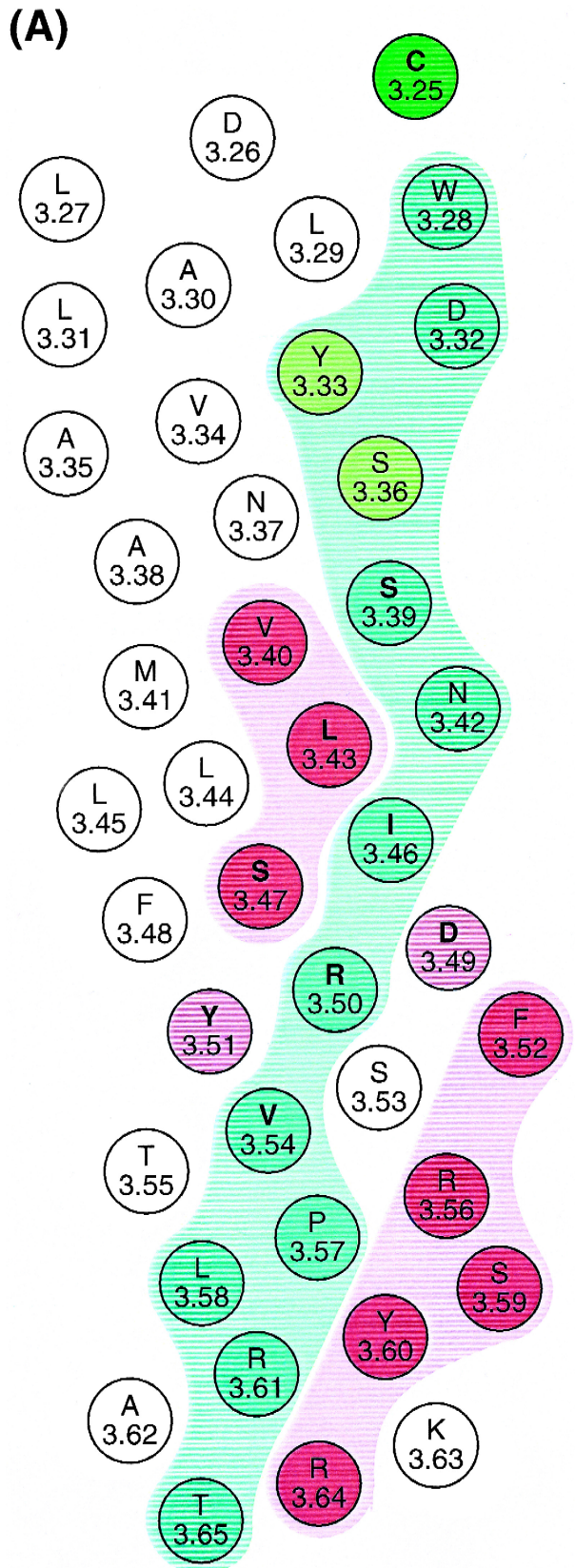
seems to be the case in the M_1 and M_5 muscarinic acetylcholine receptors (Scheer et al., 1997).

The fourth category consists of residues whose integrity is important for successful receptor activation. The sidechains of these amino acids appear to make intra-molecular (intra-receptor), or inter-molecular (receptor–ligand or receptor–G protein) bonds which are important for stabilising the conformationally rearranged activated state of the receptor. The binding site residues $Asp^{3.32}$, $Tyr^{3.33}$, and $Ser^{3.36}$, and the highly conserved $Ser^{3.39}$, $Ile^{3.46}$, $Arg^{3.50}$ and $Val^{3.54}$ belong to this category, as do the less conserved residues at positions 3.57, 3.58, 3.61 and 3.65.

The functionally important groups of residues, the constraints, and the activators, are segregated into adjacent domains on the surface of TM 3 (Fig. 3a). The activators form a continuous axial strip, extending throughout the TM domain, and winding around its cytoplasmic helical extension. Near the outer, extracellular end of the domain, they have an acetylcholine anchoring and ligand-transducing role. In the middle of the domain, they may form intramolecular contacts that selectively stabilise the activated state of the acetylcholine occupied receptor. Near the cytoplasmic end of the domain, they may also have a G protein binding function, or a role in the catalysis of GDP–GTP exchange, although there is no direct evidence for this. A number of these residues are among the most highly conserved in the 7-TM receptor superfamily, particularly $Ser^{3.39}$, $Ile^{3.46}$, $Arg^{3.50}$ and $Val^{3.54}$ (Baldwin, 1993).

The constraining residues are concentrated in the inner half of the domain, and extend into the intracellular projection of TM 3. They are segregated into two domains, one lying above the stabilising residues Asp^{122} and Tyr^{124}, and one below. The two domains are displaced by 100 degrees of arc clockwise to, or 100 degrees of arc anticlockwise to the strip of activator residues, when viewed from the N-terminus of the helix. The Cambridge model (Fig. 3b) suggests that the N-terminal patch of residues, including the highly-conserved $Leu^{3.43}$ and $Ser^{3.47}$ should be oriented primarily towards TM 6 and 7. The less-conserved C-terminal patch may be oriented towards TM 4 and 5. It is plausible that the resulting intramolecular contacts may be the origin of the apparent constraints on the ground state structure exercised by these residues, and of the ability of mutations of their sidechains to cause constitutive activation of the receptors.

The identities of the intramolecular contacts made by $Leu^{3.43}$ and $Ser^{3.47}$ have been pursued by histidine-substitution mutagenesis, followed by probing with zinc ions. The introduction of His imidazole rings at the correct distances, and having the appropriate orientations to one another allows the creation of high-affinity Zn^{2+} binding sites (Schwartz, 1994). The preferred geometry of Zn^{2+} binding sites in proteins is tetrahedral. The apical nitrogens of the imidazole rings take up three of the coordination positions. The fourth is typically occupied by a water molecule (Alberts et al., 1998). The bond lengths are approximately 2 Å. Thus, the successful creation of a Zn^{2+} binding site imposes strong constraints on the relative orientations and distances of the participating residues.



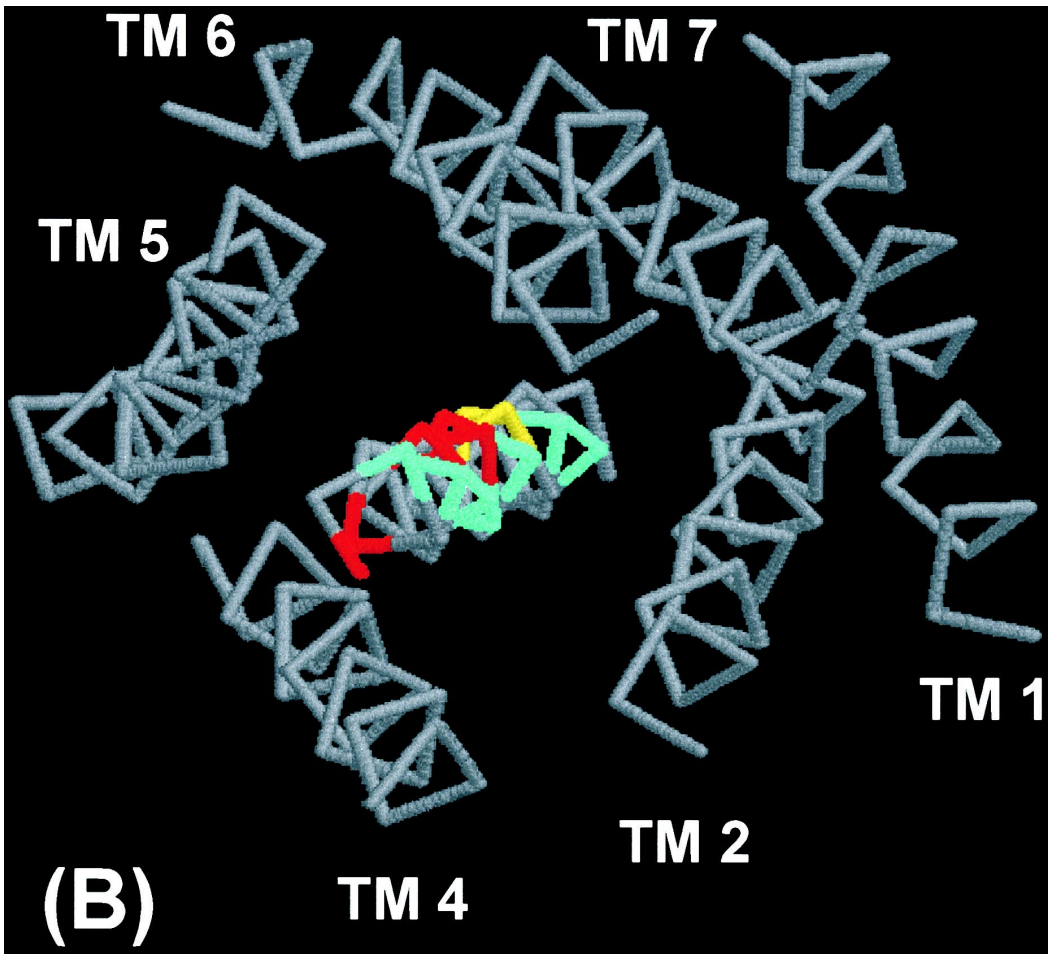


Fig. 3. Scanning mutagenesis of transmembrane domain 3 in the M_1 and M_5 muscarinic acetylcholine receptors. (a) A standard helical net plot of transmembrane domain 3 of the M_1 or M_5 muscarinic acetylcholine receptors showing the location of the constraining residues (red), activator residues (cyan) and stabiliser residues (pink). The ligand transducer residues $Tyr^{3.33}$ and $Ser^{3.36}$ are shown in yellow. The numbering convention is that of Van Rhee and Jacobson (1996). The disulfide-bonded cysteine is coloured green. Highly conserved residues are shown in bold. (b) The constraining, activator and ligand transducer residues superimposed on the Cambridge model. This model only extends to $Arg^{3.56}$ at the cytoplasmic end.

So far, using this approach, we have been able to suggest the existence of a network of interactions involving the side-chains of $Leu^{3.43}$ and $Ser^{3.47}$ in TM 3, $Phe^{6.44}$ in TM6 and $Asn^{7.49}$ and $Tyr^{7.53}$ in TM 7 (Lu and Hulme, unpublished data). In each case, the mutation of these residues to Ala causes increases in agonist binding affinity. The mutation of $Leu^{3.43}$, $Ser^{3.47}$ (Hulme and Lu, 1998; Lu and Hulme, 1999) and $Phe^{6.44}$ (Spalding et al., 1998) causes constitutive activation of the receptor. $Asn^{7.49}$ and $Tyr^{7.53}$ are particularly interesting in that they may act as 'double agents', imposing constraints on the receptor's ground state, but at the same time, participating directly or indirectly in stabilising the receptor–G protein interaction in the activated state. In the gonadotrophin releasing hormone and thyrotropin releasing hormone receptors, residue Asn (or Asp)$^{7.49}$ has been suggested to participate in a network of contacts with Asp (or Asn)$^{2.50}$ (TM 2) and $Asn^{1.50}$ (TM 1) (Zhou et al., 1994; Perlman and Gershengorn, 1997; Pogozheva et al., 1998). These interactions may be further modulated by the binding of a metal ion, either Na^+, or Mg^{++} (Quitterer et al., 1996). This so-called 'polar pocket' (Oliveira et al., 1994) makes a selective contribution to G-protein binding, or to maintaining the equilibrium between the active and inactive states of the receptor.

4. Transmembrane domain 3 may be the switch that controls receptor activation

The segregated distribution of the constraining and activating residues on the surface of TM 3 naturally suggests that the transition from the ground state to the activated state of the M_1 and M_5 muscarinic acetylcholine receptors results from the disruption of the set of constraints, and their replacement by the set of activator contacts. The highly conserved nature of many of these contacts, particularly of the constraining network centred on $Leu^{3.43}$, and the activating contacts made by $Ser^{3.39}$, $Ile^{3.46}$, $Arg^{3.50}$ and $Val^{3.54}$ suggests that this may form the

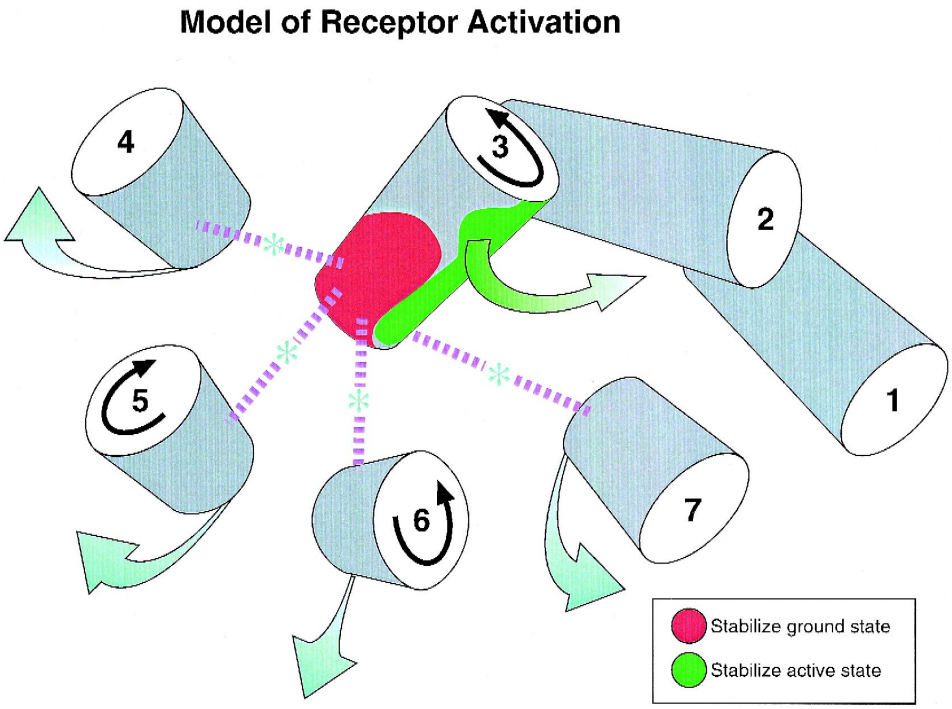


Fig. 4. Cartoon of the proposed activation mechanism of the 7-transmembrane receptors, showing the role of transmembrane helix 3 as a conformational switch.

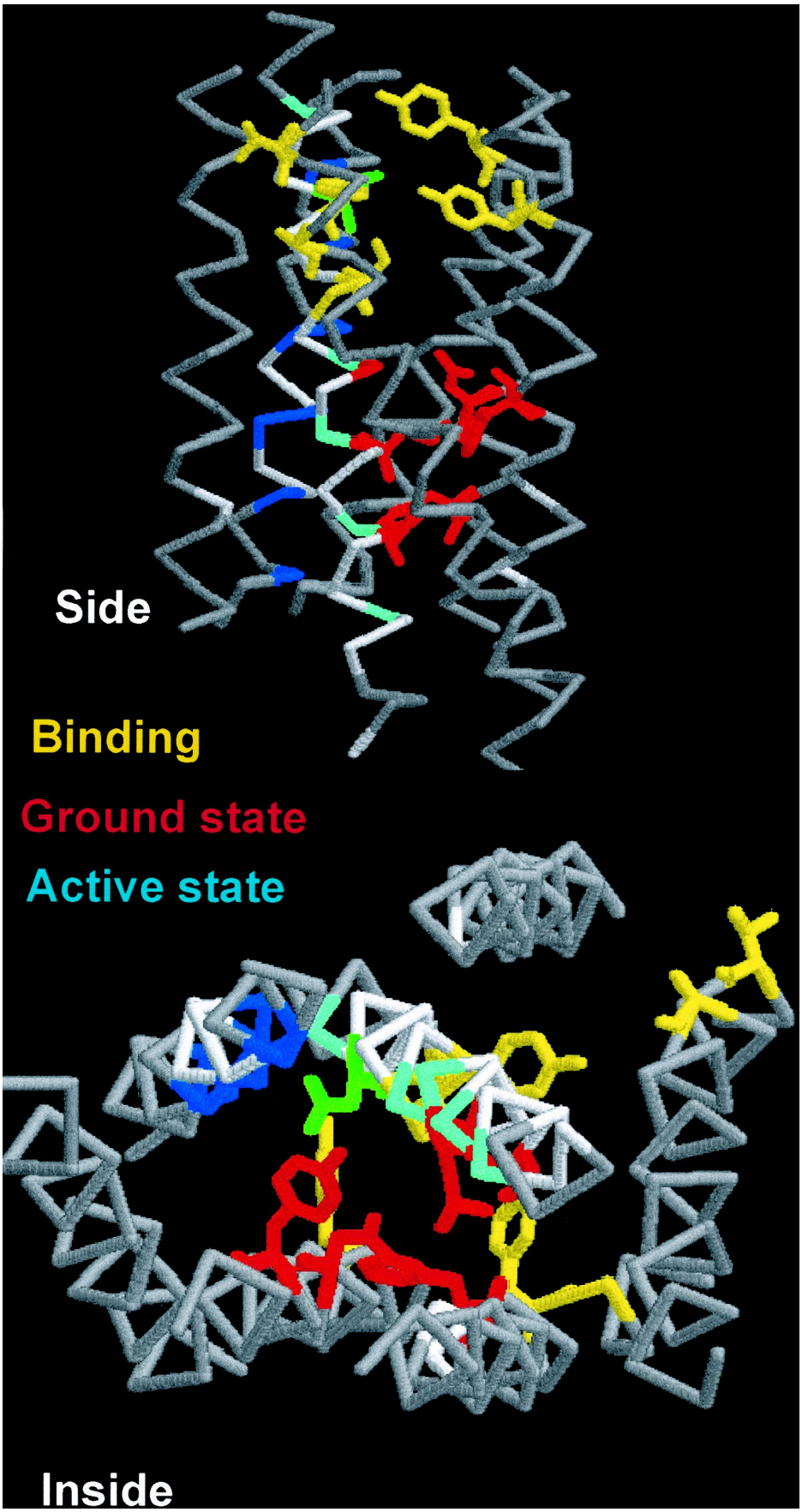
basis of a conserved switch mechanism which operates, as a theme with variations, throughout the entire superfamily of rhodopsin-like 7-TM receptors.

Several key biophysical observations have suggested that relative movements of the transmembrane helices of the 7-TM receptors accompany their activation. Spin-labelling studies on cysteine-substituted mutants of rhodopsin have indicated that its activation induces an outward movement of TM 3 (Farahbakhsh et al., 1995), as well as a larger outward movement, and a rotation by about 30° (anti-clockwise when viewed from the extracellular side of the structure) of the cytoplasmic end of TM 6 relative to TM 3 (Farrens et al., 1996). The substitution of histidines for residues at the cytoplasmic ends of TM 3 renders the activation of transducin by photoactivated rhodopsin sensitive to inhibition by Zn^{2+} ions (Sheikh et al., 1996). A residue on the inward-facing surface of TM 6 of rhodopsin becomes accessible to a fluorescent probe after light activation (Dunham and Farrens, 1999). Similarly, the reaction of cysteine residues in TM 3 and 6 of the β_2-adrenoceptor with an environmentally sensitive fluorescent probe has enabled the detection of agonist-induced changes in fluorescence emission intensity, which have been interpreted as indicating anticlockwise rotations of TM 3 and 6 (Gether et al., 1997b; Kobilka and Gether, 1998). A constitutively activating mutation of the β2-adrenoceptor, involving a modification of the sequence of the cytoplasmic helical extension of TM 6, increased the accessibility of a Cys residue in the centre of the transmembrane region to a polar sufhydryl reagent (Javitch et al., 1997). All of these studies support the hypothesis that rotations and translations of TM 3 and 6 accompany the activation of the 7-TM receptors.

In addition, an epitope at the end of TM 7, including $Tyr^{7.53}$ in the very highly-conserved NPXXY sequence, which as we have seen, is a signature motif of the rhodopsin-like 7-TM receptors, becomes exposed and selectively accessible to a monoclonal antibody when the photoreceptor is activated (Abdulaev and Ridge, 1998). This sequence is a candidate for mediating direct contact between the M_1 muscarinic acetylcholine receptor and G_q in the activated state of the receptor (Lu and Hulme, unpublished data). It has also been suggested to be important in the recognition of small G proteins, such as Arf and RhoA, by activated M_3 muscarinic acetylcholine receptors (Mitchell et al., 1998).

These findings, together with the mutagenesis studies indicating the presence of distinct sets of radially dis-

Fig. 5. Summary of the distribution of the key functional residues in the M_1 muscarinic acetylcholine receptor showing the acetylcholine binding residues (yellow), interhelical constraints (red), activator residues (cyan), and hypothetical intramolecular interaction sites for the activator residues in the activated state (blue). The ligand-transducer side chains of $Tyr^{3.33}$, $Tyr^{6.51}$, $Tyr^{7.39}$, the acetylcholine binding side chains of $Thr^{5.42}$ and $Ala^{5.46}$ and the constraining network of $Leu^{3.43}$, $Ser^{3.47}$, $Phe^{6.44}$, $Asn^{7.49}$ and $Tyr^{7.53}$ are shown. $Asp^{3.32}$ is shown in green. The upper panel shows the binding cleft viewed from between TM 5 and TM 6. The lower panel shows the receptor from the intracellular side, tilted to show all of the transmembrane helices.


Side
Binding
Ground state
Active state
Inside

tributed ground state constraints, and axially distributed activating interactions centred on TM 3 can be integrated into the model of receptor activation shown in Fig. 4.

It is proposed that the essence of the process of activation involves a rotation of TM 3, accompanied by a small translation of the C-terminal end of the helix. This leads to a concerted and co-operative disruption of the network of mainly radial inter-helical constraints centred on TM 3, and their replacement by a new set of axial interactions. These are postulated to be with an adjacent helix, which might be TM 2, according to the Cambridge model.

The disruption of the constraining interactions could, plausibly, result in mobilisation of the cytoplasmic ends of the TM helices surrounding TM 3. This may trigger outward movements, and rotations of these helices, such as that inferred for TM 6, leading to the exposure of previously concealed epitopes. These would then be available to participate in G protein binding and activation.

The proposition is that TM 3 acts as a rotational switch which integrates and propagates conformational changes induced by ligand binding through the transmembrane structure of the receptor. It is ideally placed to do this. Because of its high degree of tilt, it interacts, sequentially, with all of the other TM helices, with the exception of TM 1. TM 1 might come into its own in stabilising a new configuration of TM 2 and 3 in the activated state.

Such a mechanism can explain several of the key structural features of the 7-TM receptors.

First, the conservation of proline residues in TM 4, 5, 6 and 7. Because of their helix-breaking properties, these help to mobilise the cytoplasmic segments of these TM helices, allowing them to move independently of the extracellular segments. It is the cytoplasmic segments which carry the most important epitopes for G protein binding and activation.

Secondly, it accounts for the presence of the conserved disulfide bond which links the extracellular end of TM 3 to the second extracellular loop. In particular, it explains the observation that the disulfide bond plays a critical role in maintaining the stability of the activated, rather than the ground state of rhodopsin (Davidson et al., 1994). The disulfide bond maintains a stabilising linkage between TM 1, 2 and 3, and TM 4, 5, 6 and 7 which becomes vital in the absence of the normal constraining interactions, which are proposed to be broken by activation.

5. Ligand binding may activate the switch mechanism using two basic strategies

Two basic strategies may be hypothesised for triggering the postulated activation mechanism.

The first of these is allosteric destabilisation of the constraining interactions. This appears to be the mechanism used by acetylcholine to activate the muscarinic acetylcholine receptors. Here, we postulate that the closing of the binding site around the acetylcholine headgroup leads to the rupture of the network of constraining interactions which lies immediately beneath the acetylcholine binding site in the TM structure of the receptors (Fig. 5). In rhodopsin, the light-induced isomerisation of 11-*cis* to all trans retinal may introduce new steric interactions, which have the same effect (Shieh et al., 1997). A prediction would be that agonist activation by this mechanism should reduce the stability of the receptor. The natural target for the binding of allosteric destabilising ligands would be the cleft between TM 3, 4, 5, 6 and 7.

The second mechanism is direct or allosteric stabilisation of the axial activating interactions. The natural target for such ligands would be predicted to be TM 1, 2 and 3, and those parts of the N-terminal domain and the extracellular loops which undergo significant rearrangements during the transition to the activated state. This may be the mechanism by which many peptides activate the 7-TM receptors, including 'tethered ligand' receptors, such as the thrombin receptor. A prediction is that the agonist receptor complexes formed by such receptors might be more stable than those formed by ligands which operate primarily by destabilisation of the ground state.

In summary, the key proposition of this review is that TM 3 of the rhodopsin-like 7-TM receptors acts as a ligand-activated switch. It mediates the transition from an inactive ground state of the receptors, characterised by a primary network of radial constraining interhelical interactions between TM 4, 5, 6 and 7 and the cytoplasmic section of TM 3 to an activated state in which these constraints are broken, and replaced by a new set of axial interactions of one face of TM 3 with an adjoining TM domain, possibly TM 2 (Fig. 5). This proposal can be tested by mutagenesis studies, and, more directly, by suitable biophysical studies on purified receptors.

Acknowledgements

This work was supported by the Medical Research Council (UK).

References

Abdulaev, N.G., Ridge, K.D., 1998. Light-induced exposure of the cytoplasmic end of transmembrane helix seven in rhodopsin. Proc. Natl. Acad. Sci. USA 95, 12854–12859.

Abell, A.N., Mccormick, D.J., Segaloff, D.L., 1998. Certain activating mutations within helix 6 of the human luteinizing hormone may be explained by alterations that allow transmembrane regions to activate G_s. Mol. Endocrinol. 12, 1857–1869.

Acharya, S., Saad, Y., Karnik, S.V., 1997. Transducin α-C-terminal peptide binding site consists of C–D and E–F loops of rhodopsin. J. Biol. Chem. 272, 6519–6524.

Alberts, I.L., Nadassy, K., Wodak, S.J., 1998. Analysis of zinc binding sites in protein crystal structures. Protein Sci. 7, 1700–1716.

Allman, K., Page, K.M., Hulme, E.C., 1997. Cysteine scanning mutagenesis of transmembrane domain V of the m1 muscarinic receptor. Life Sci. 60, 1177.

Altenbacetylcholine, C., Yang, K., Farrens, D.L., Farahbakhsh, Z.T., Khorana, H.G., Hubbell, W.L., 1996. Structural features and light-dependent changes in the cytoplasmic interhelical E–F loop region of rhodopsin: a site-directed spin-labelling study. Biochemistry 35, 12470–12478.

Axelsen, P.H., Harel, M., Silman, I., Sussman, J.L., 1994. Structure and dynamics of the active site gorge of acetylcholinesterase: synergistic use of molecular dynamics simulation and X-ray crystallography. Protein Sci. 3, 188–197.

Baldwin, J.M., 1993. The probable arrangement of the helices in G protein-coupled receptors. EMBO J. 12, 1693–1703.

Barak, L.S., Menard, L., Ferguson, S.S., Colapietro, A.M., Caron, M.G., 1995. The conserved seven-transmembrane sequence NP(X)2,3Y of the G protein-coupled receptor superfamily regulates multiple properties of the β2-adrenergic receptor. Biochemistry 34, 15407–15414.

Bargman, C.I., 1999. Neurobiology of the *Caenorhabditis elegans* genome. Science 282, 2028–2032.

Birdsall, N.J., Hulme, E.C., 1976. Biochemical studies on muscarinic acetylcholine receptors. J. Neurochem. 27, 7–16.

Blin, N., Yun, J., Wess, J., 1995. Mapping of single amino acid residues required for selective activation of $G_{q/11}$ by the m3 muscarinic acetylcholine receptor. J. Biol. Chem. 270, 17741–17748.

Blüml, K., Mutschler, E., Wess, J., 1994. Identification of an intracellular tyrosine residue critical for muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. J. Biol. Chem. 269, 402–405.

Bourne, H.R., 1997. How receptors talk to trimeric G proteins. Curr. Opin. Cell Biol. 9, 134–142.

Burstein, E.S., Spalding, T.A., Brann, M.R., 1996. Amino acid side chains that define muscarinic receptor/G-protein coupling. J. Biol. Chem. 271, 2882–2885.

Burstein, E.S., Spalding, T.A., Brann, M.R., 1998a. Structure/function relationships of a G-protein coupling pocket formed by the third intracellular loop of the m5 muscarinic receptor. Biochemistry 37, 4052–4058.

Burstein, E.S., Spalding, T.A., Brann, M.R., 1998b. The second intracellular loop of the m5 muscarinic receptor is the switch which enables G protein coupling. J. Biol. Chem. 273, 24322–24327.

Cohen, G.B., Oprian, D.D., Robinson, P.R., 1992. Mechanism of activation and inactivation of opsin: role of Glu^{113} and Lys^{296}. Biochemistry 31, 12592–12601.

Colson, A., Perlman, J.H., Jinsi-Parimoo, A., Nussenzveig, D.R., Osman, R., Gershengorn, M.C., 1999. A hydrophobic cluster between transmembrane helices 5 and 6 constrains the thyrotropin-releasing hormone receptor in an inactive conformation. Mol. Pharmacol. 54, 968–978.

Davidson, F.F., Loewen, P.C., Khorana, H.G., 1994. Structure and function in rhodopsin: replacement by alanine of cysteine residues 110 and 187, components of a conserved disulfide bond in rhodopsin, affects the light-activated metarhodopsin II state. Proc. Natl. Acad. Sci. U.S.A. 91, 4029–4033.

Dunham, T.D., Farrens, D.L., 1999. Conformational changes in rhodopsin: movement of helix F detected by site-specific chemical labeling and fluorescence. J. Biol. Chem. 274, 1683–1690.

Essen, L.-O., Siegert, R., Lehman, W.D., Oesterhelt, D., 1998. Lipid patches in membrane protein oligomers: crystal structure of the bacteriorhodopsin–lipid complex. Proc. Natl. Acad. Sci. U.S.A. 95, 11673–11678.

Farahbakhsh, Z.T., Ridge, K.D., Khorana, H.G., Hubbell, W.L., 1995. Mapping light-dependent structural changes in the cytoplasmic loop connecting helices C and D in rhodopsin: A site-directed spin labeling study. Biochemistry 34, 8812–8819.

Farrens, D.L., Altenbacetylcholine, C., Yang, K., Hubbell, W., Khorana, H.G., 1996. Requirement of rigid-body motion of transmembrane helices for light-activation of rhodopsin. Science 274, 768–770.

Findlay, J.B.C., Pappin, D.J.C., 1986. The opsin family of proteins. Biochem. J. 238, 625–642.

Fraser, C.M., Wang, C.-D., Robinson, D.A., Gocayne, J.D., Venter, J.C., 1989. Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved amino acids play important roles in receptor function. Mol. Pharmacol. 36, 840–847.

Gether, U., Kobilka, B.K., 1998. G protein-coupled receptors: II. Mechanism of agonist activation. J. Biol. Chem. 273, 17979–17982.

Gether, U., Ballesteros, J.A., Seifert, R., Sanders-Bush, E., Weinstein, H., Kobilka, B.K., 1997a. Structural instability of a constitutively active G protein-coupled receptor. Biol. Chem. 272, 2587–2590.

Gether, U., Lin, S., Ghanouni, P., Ballesteros, J.A., Weinstein, H., Kobilka, B.K., 1997b. Agonists induce conformational changes in transmembrane domains 3 and 6 of the β_2-adrenoceptor. EMBO J. 16, 6737–6747.

Han, M., Smith, S.O., Sakmar, T.P., 1998. Constitutive activation of opsin by mutation of methionine 257 on transmembrane helix 6. Biochemistry 37, 8253–8261.

Herzyk, P., Hubbard, R.E., 1998. Combined biophysical and biochemical information confirms arrangement of transmembrane helices visible from the three-dimensional map of frog rhodopsin. J. Mol. Biol. 281, 741–754.

Hibert, M., Trumpp-Kallmeyer, S., Bruinvels, A., Hoflack, J., 1991. Three-dimensional models of neurotransmitter G-binding protein-coupled receptors. Mol. Pharmacol. 40, 8–15.

Hill-Eubanks, D., Burstein, E.S., Spalding, T.A., Bräuner-Osborne, H., Brann, M.R., 1996. Structure of a G-protein-coupling domain of a muscarinic receptor predicted by random saturation mutagenesis. J. Biol. Chem. 271, 3058–3065.

Horn, F., Weare, J., Beukers, M.W., Horsh, S., Bairoch, A., Chen, W., Edvardsen, Ø., Campagne, F., Vriend, G., 1998. GPCRDB: an information system for G protein-coupled receptors. Nucleic Acids Res. 26, 275–279.

Hulme, E.C., Lu, Z.-L., 1998. Scanning mutagenesis of transmembrane domain 3 of the M_1 muscarinic acetylcholine receptor. J. Physiol. (Paris) 92, 269–274.

Hwa, J., Garriga, P., Liu, X., Khorana, H.G., 1997. Structure and function in rhodopsin: packing of the helices in the transmembrane domain and folding to a tertiary structure in the intradiscal domain are coupled. Proc. Natl. Acad. Sci. U.S.A. 94, 10571–10576.

Javitch, J.A., Fu, D., Liapakis, G., Chen, J., 1997. Constitutive activation of the β_2 adrenergic receptor alters the orientation of its sixth membrane-spanning segment. J. Biol. Chem. 272, 18546–18549.

Ji, T.H., Grossmann, M., Ji, I., 1998. G protein-coupled receptors: I. Diversity of receptor–ligand interactions. J. Biol. Chem. 273, 17299–17302.

Karnik, S.S., Sakmar, T.P., Chen, H.-B., Khorana, H.G., 1988. Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. Proc. Natl. Acad. Sci. U.S.A. 85, 8459–8463.

Kim, J.-M., Altenbach, C., Thurmond, R.L., Khorana, H.G., Hubbell, W.L., 1997. Structure and function in rhodopsin: rhodopsin mutants with neutral amino acids at E^{134} have a partially activated conformation in the dark state. Proc. Natl. Acad. Sci. U.S.A. 94, 14273–14278.

Kisselev, O., Ermolaeva, M., Gautam, N., 1995. Efficient interaction with a receptor requires a specific type of prenyl group on the G protein γ-subunit. J. Biol. Chem. 270, 25356–25358.

Kobilka, B.K., Gether, U., 1998. Examination of ligand-induced conformational changes in the β_2-adrenergic receptor by fluorescence spectroscopy. Adv. Pharmacol. 42, 470–473.

Kostenis, E., Conklin, B.R., Wess, J., 1997. Molecular basis of receptor/G protein coupling selectivity studied by co-expression of wild type and mutant m2 muscarinic receptors with mutant $G\alpha_q$ subunits. Biochemistry 36, 1487–1495.

Krebs, A., Villa, C., Edwards, P., Schertler, G.F.X., 1998. Characterisation of an improved two-dimensional $p22_12_1$ crystal from bovine rhodopsin. J. Mol. Biol. 282, 991–1003.

Kurtenbach, E., Curtis, C.A.M., Pedder, E.K., Aitken, A., Harris, A.C.M., Hulme, E.C., 1990. Muscarinic acetylcholine receptors: peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation. J. Biol. Chem. 265, 13702–13708.

Lee, T.W., Cotecchia, S., Milligan, G., 1997. Up-regulation of the levels of expression and function of a constitutively active mutant of the hamster α_{1b}-adrenoceptor by ligands that act as inverse agonists. Biochem. J. 325, 733–739.

Leurs, R., Smit, M.J., Alewjinse, A.E., Timmerman, H., 1998. Agonist-independent regulation of constitutively active G-protein coupled receptors. Trends Biochem. Sci. 23, 418–422.

Lu, Z.-L., Hulme, E.C., 1999. The functional topography of transmembrane domain 3 of the M_1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. J. Biol. Chem. 274, 7309–7315.

Lu, Z.-L., Curtis, C.A.M., Jones, P.G., Pavia, J., Hulme, E.C., 1997. The role of the aspartate–arginine–tyrosine triad in the m1 muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signalling. Mol. Pharmacol. 51, 234–241.

Mitchell, R., McCulloch, D., Lutz, E., Johnson, M., MacKenzie, C., Fennell, M., Fink, G., Zhou, W., Sealfon, S.C., 1998. Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. Nature 392, 411–414.

Monod, J., Wyman, J., Changeux, J.-P., 1965. On the nature of allosteric transitions. J. Mol. Biol. 12, 88–118.

Moro, O., Lameh, J., Högger, P., Sadee, W., 1993. Hydrophobic amino acid in the second intracellular loop plays a key role in receptor–G protein coupling. J. Biol. Chem. 268, 22273–22276.

Nymeyer, H., Garcia, A.E., Onuchic, J.N., 1998. Folding funnels and frustration in off-lattice minimalist protein landscapes. Proc. Natl. Acad. Sci. U.S.A. 95, 5921–5928.

Oliveira, L., Paiva, A.C.M., Vriend, G., 1994. A common motif in G protein-coupled seven transmembrane helix receptors. 7TM 1, 1.

Onrust, R., Herzmark, P., Chi, P., Garcia, P.D., Lichtarge, O., Kingsley, C., Bourne, H.R., 1997. Receptor and $\beta\gamma$ binding sites in the α subunit of the retinal G protein transducin. Science 275, 381–384.

Perez, D.M., Hwa, J., Gaivin, R., Mathur, M., Brown, F.G.R.M., 1996. Constitutive activation of a single effector pathway: evidence for multiple activation states of a G protein-coupled receptor. Mol. Pharmacol. 49, 112–122.

Perlman, J.H., Gershengorn, M.C., 1997. Interactions between conserved residues in transmembrane helices 1, 2, and 7 of the thyrotropin releasing hormone receptor. J. Biol. Chem. 272, 11937–11942.

Pogozheva, I.D., Lomize, A.L., Mosberg, H.I., 1997. The transmembrane 7-α-bundle of rhodopsin: distance geometry calculations with hydrogen bonding constraints. Biophys. J. 72, 1963–1985.

Pogozheva, I.D., Lomize, A.L., Mosberg, H.I., 1998. Opioid receptor three-dimensional structures from distance geometry calculations with hydrogen bonding constraints. Biophys. J. 75, 612–634.

Quitterer, U., AbdAlla, S., Jarnagin, K., Muller-Ester, W., 1996. Na^+ ions binding to the bradykinin B_2 receptor suppress agonist-independent receptor activation. Biochemistry 35, 13368–13377.

Rao, V.R., Oprian, D.D., 1996. Activating mutations of rhodopsin and other G protein-coupled receptors. Annu. Rev. Biophys. Biomol. Struct. 25, 287–314.

Ridge, K.D., Lee, S.S.J., Yao, L.L., 1995. In vivo assembly of rhodopsin from expressed polypeptide fragments. Proc. Natl. Acad. Sci. U.S.A. 92, 3204–3208.

Samama, P., Cotecchia, S., Costa, T., Lefkowitz, R.J., 1993. A mutation-induced activated state of the β2-adrenergic receptor. J. Biol. Chem. 268, 4625–4636.

Scheer, A., Fanelli, F., Costa, T., De Benedetti, P.G., Cotecchia, S., 1997. The activation process of the α_{1B}-adrenergic receptor: potential role of protonation and hydrophobicity of a highly conserved aspartate. Proc. Natl. Acad. Sci. U.S.A. 94, 808–813.

Schwartz, T.W., 1994. Locating ligand-binding sites in 7-TM receptors by protein engineering. Curr. Opin. Biotechnol. 5, 434–444.

Sheikh, S.P., Zvyaga, T.A., Lichtarge, O., Sakmar, T.P., Bourne, H.R., 1996. Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. Nature 383, 347–350.

Shieh, T., Han, M., Sakmar, T.P., Smith, S.O., 1997. The steric trigger in rhodopsin activation. J. Mol. Biol. 269, 373–384.

Sine, S.M., Quiram, P., Papanikolou, F., Kreienkamp, H.-J., Taylor, P., 1994. Conserved tyrosines in the α-subunit of the nicotinic acetylcholine receptor stabilise quaternary ammonium groups of agonists and curariform antagonists. J. Biol. Chem. 269, 8808–8816.

Sondek, J., Bohm, A., Lambright, D.G., Hamm, H.E., Sigler, P.B., 1996. Crystal structure of a G protein $\beta\gamma$ dimer at 2.1 Å resolution. Nature 379, 369–374.

Spalding, T.A., Birdsall, N.J., Curtis, C.A., Hulme, E.C., 1994. Acetylcholine mustard labels the binding site aspartate in muscarinic acetylchloine receptors. J. Biol. Chem. 269, 4092–4097.

Spalding, T.A., Burstein, E.S., Wells, J.W., Brann, M.R., 1997. Constitutive activation of the m5 muscarinic receptor by a series of mutations at the extracellular end of transmembrane 6. Biochemistry 36, 10109–10116.

Spalding, T.A., Burstein, E.S., Henderson, S.C., Ducote, K.R., Brann, M.R., 1998. Identification of a ligand-dependent switch within a muscarinic receptor. J. Biol. Chem. 273, 21563–21568.

Unger, V.M., Hargrave, P.A., Baldwin, J.M., Schertler, G.F.X., 1997. Arrangement of rhodopsin transmembrane alpha-helices. Nature 389, 203–206.

van Rhee, A.M., Jacobson, K.A., 1996. Molecular architecture of G protein-coupled receptors. Drug Dev. Res. 37, 1–38.

Ward, S.D.C., Hulme, E.C., 1999. Tyr381 in transmembrane domain six of the rat M_1-muscarinic acetylcholine receptor plays a dual role in receptor function. Life Sci. 64, 564.

Wess, J., 1993. Molecular basis of muscarinic acetylcholine receptor function. Trends Pharmacol. Sci. 14, 308–313.

Wess, J., 1996. Molecular biology of muscarinic acetylcholine receptors. Crit. Rev. Neurobiol. 10, 69–99.

Wess, J., 1998. Molecular basis of receptor/G-protein-coupling selectivity. Pharmacol. Ther. 80, 231–264.

Wess, J., Maggio, R., Palmer, J.R., Vogel, Z., 1992. Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. J. Biol. Chem. 267, 19313–19319.

Wess, J., Nanavati, S., Vogel, Z., Maggio, R., 1993. Functional role of proline and tryptophan residues highly conserved among G protein-coupled receptors studied by mutational analysis of the m3 muscarinic receptor. EMBO J. 12, 331–338.

Wess, J., Blin, N., Yun, J., Schöneberg, T., Liu, J., 1996. Molecular aspects of muscarinic receptor assembly and function. In: Klein, J., Löffelholz, K. (Eds.), Progress in Brain Research. Elsevier, Amsterdam, p. 153.

Yeagle, P.L., Alderfer, J.L., Salloum, A.C., Ali, L., Albert, A.D., 1997. The first and second cytoplasmic loops of the GPCR rhodopsin independently form beta turns. Biochemistry 36, 3864–3869.

Zhou, W., Flanagan, C., Ballasteros, J.A., Konvicka, K., Davidon, J.S., Weinstein, H., Millar, R.P., Sealfon, S.C., 1994. A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotrophin-releasing hormone receptor. Mol. Pharmacol. 45, 155–170.