

COMMENTARY

Crystallizing Thinking about the β 2-Adrenergic Receptor

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Received January 31, 2008; accepted January 31, 2008

ABSTRACT

Two recently determined crystal structures of the human β 2-adrenergic receptor (β 2AR) provide a long-awaited advance in the field of G protein-coupled receptor research. The β 2AR is only the second member of this, the largest family of receptors encoded in the human genome, whose structure has been

solved. It follows structures of rhodopsin that were determined previously. Here we set these developments in historical context, discuss the daunting challenges that have been overcome, and appraise what has and has not been learned.

For many years, the study of two prototypic members of the huge family of GPCRs, the visual light “receptor” rhodopsin and the β 2AR for adrenaline and noradrenaline, has guided research in this field (Lefkowitz, 2007). Now, the recent publication of two crystal structures of the human β 2AR once again permits new insights to be gained from comparison of the properties of these two model seven transmembrane-spanning receptors (7TMRs) (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). The remarkable and unique abundance of rhodopsin in rod outer segments (it constitutes ~90% of the protein in rod outer segment membranes) and its stability led to the determination of its complete amino acid sequence in 1982 by classic Edman degradation and to the appreciation of its seven transmembrane organization (Ovchinnikov, 1982; Hargrave et al., 1983). By comparison, the rarity of the β 2AR and essentially all other GPCRs (they need to be purified several hundred thousand-fold from naturally occurring sources to obtain homogeneous preparations) greatly hindered its biochemical study. Nonetheless, sufficient protein was purified so that, based on small stretches of amino acid sequence obtained from the receptor, its gene and cDNA were successfully cloned in 1986 (Dixon et al., 1986). It is remarkable that, in retrospect, it

was only then that its close structural relationship with rhodopsin was first appreciated. This, despite the general understanding at the time that both rhodopsin and the β 2AR signaled by activation of G proteins (transducin and G_s , respectively). The discovery of the homology between the β 2AR and rhodopsin followed rapidly by the cloning of additional adrenergic receptors and others in turn triggered the rapid realization that all GPCRs share a conserved seven transmembrane organization and are members of the same gene family (Dohlman et al., 1991).

The first crystal structure of rhodopsin in its inactive state was reported in 2000 (Palczewski et al., 2000), and the new β 2AR structures are the first of any other GPCR to appear. Given the close parallels and centrality of research on these two model 7TMRs, it seems somehow fitting that a comparison of their molecular structures should once again be in the spotlight of molecular pharmacologists.

Why Did It Take So Long?

Crystallization of membrane proteins, especially eukaryotic ones, remains a very difficult and time-consuming process. In contrast to several thousand Protein Data Bank entries for soluble proteins, only 148 unique structures of membrane proteins have been determined, of which only 40 are eukaryotic membrane proteins, and only 4 of these are of human origin (available at http://www.blanco.biomol.uci.edu/Membrane_Proteins_xtal). There are multiple problems as-

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Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.108.045849.

ABBREVIATIONS: GPCR, G protein-coupled receptor; β 2AR, β 2-adrenergic receptor; TM, transmembrane; 7TMR, seven transmembrane-spanning receptor.

sociated with the crystallization of membrane proteins and GPCRs in particular. As noted, unlike rhodopsin, which is present in abundant amounts in rod outer segments and which can be purified easily, other GPCRs, including the β 2AR, are expressed in only tiny amounts in tissues. Therefore, isolation of sufficient amounts for crystallization purposes requires heterologous overexpression of recombinant receptors, and even then, substantial purification is required.

Another problem associated with crystallization of these receptors is their intrinsic conformational flexibility. To interact with a set of diverse ligands and transmit signals through multiple signaling pathways, 7TMRs adopt ensembles of different conformations (or different active and inactive states), which leads to conformational heterogeneity. Moreover, approximately 50% of the residues in these receptors are buried in the membrane bilayer, which limits the polar surface area available for crystal contacts. As discussed below, the successful addressing of these problems, in addition to recent advances in crystallography, were the keys to obtaining diffraction-quality crystals, which finally led to the structure determination of the β 2AR.

What Made It Possible?

In addition to high-level heterologous expression of the β 2AR in Sf9 cells to produce large amounts of functional receptors, two additional technical advances, one at the protein engineering level and the other at the crystallographic level, finally yielded well-ordered crystals that diffracted to

high resolution. Based on prior biophysical studies, the third intracellular loop and the C terminus of the β 2AR seem to be the most flexible regions (Granier et al., 2007). In addition to truncating part of the flexible C terminus of the β 2AR, two parallel approaches were taken to address this issue. First, an antibody fragment (Fab) was generated against a three-dimensional epitope corresponding to the third intracellular loop (Fig. 1A) using hybridoma technology (Day et al., 2007). This approach of cocrystallizing membrane proteins with an antibody fragment was first developed and used successfully in the cytochrome *c* oxidase structure determination and since then has been used for several other membrane proteins (Hunte and Michel, 2002). The second approach used to reduce the flexibility of the receptor and increase its polar surface area was to replace the third intracellular loop with T4 lysozyme, a highly crystallizable soluble protein (Fig. 1B).

In addition to these protein engineering and antibody approaches, recent developments in membrane protein crystallography were also crucial to the success. In contrast to traditional detergent crystallization of membrane proteins, the β 2AR was crystallized in either DMPC/CHAPSO bicelles or monolein lipidic cubic phase with cholesterol as the additive. Both of these methods, which essentially rely on the use of different lipids to present a more native environment to the protein, have been reported to yield well-diffracting crystals for several membrane proteins such as bacteriorhodopsin and the photosynthetic reaction center (Faham and Bowie, 2002). Moreover, because the crystals of the β 2AR

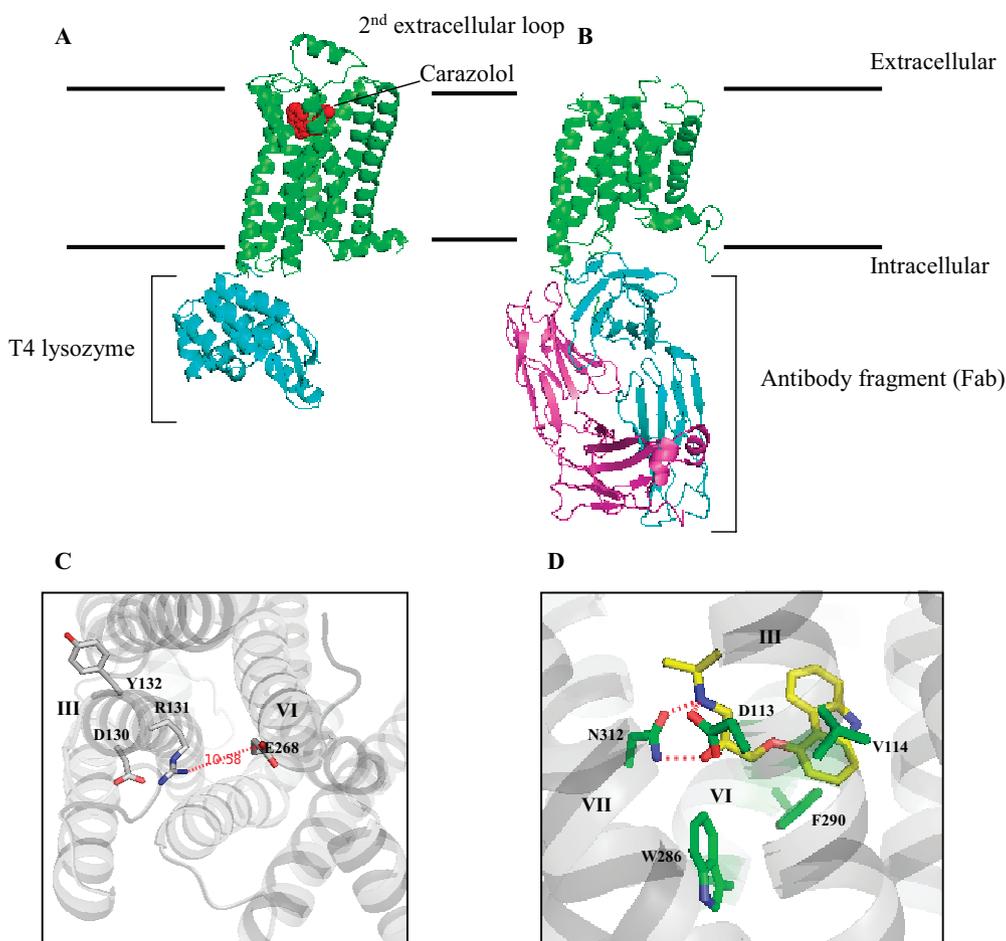


Fig. 1. 3-Dimensional crystal structure of the human β 2AR. A, an engineered β 2AR-T4 lysozyme chimera. B, the wild-type β 2AR (2–365) crystallized in complex with a Fab. C, region of the β 2AR around the conserved D/ERY motif showing the ionic lock. D, the ligand binding pocket of β 2AR with carazolol bound. Colors in the ligand (carazolol) are used to indicate oxygen (red), nitrogen (blue), and carbon (yellow). The distance between Arg131 and Glu268 (ionic lock) is shown for the β 2AR-T4 lysozyme chimera structure.

were small and very radiation-sensitive, microfocused beam lines were used instead of a conventional synchrotron source, for both screening and data collection.

Why the β 2AR Rather Than Others?

The large body of previously obtained biophysical and biochemical information available for the β 2AR definitely helped in the design of constructs that yielded protein samples suitable for crystallization. For example, the information on the flexibility of the third intracellular loop and the C terminus of the receptor led to strategies to stabilize and/or remove these regions. However, a feature of paramount importance and one almost unique to the β 2AR was the use of a ligand affinity purification column that was developed many years ago (Caron et al., 1979). As discussed above, one of the major obstacles in crystallizing proteins is obtaining functional and homogenous material, because any heterogeneity hampers the formation of well-ordered crystals. Heterologously produced receptors are often purified using affinity tags genetically fused to the protein. Although such purification schemes may result in reasonably pure receptors as judged by gel analysis, there generally still exists a mixture of both functional and nonfunctional (i.e., denatured) receptor populations, which substantially decreases the chances of crystallization. In this regard, the alprenolol (a β -adrenergic antagonist) affinity column developed for the β 2AR (Caron et al., 1979) offers a unique advantage and yields receptor samples with almost 100% specific ligand binding activity and, thus, a very homogenous receptor preparation. It should also be noted that carazolol, used as a ligand in both structures, has exceptionally high affinity for the receptor and very slow dissociation kinetics, thus stabilizing the receptor even under relatively harsh conditions.

How Similar Is the Structure to That of Rhodopsin?

Based on sequence conservation, GPCRs have been classified into five different classes. Rhodopsin and β 2AR belong to class A, the largest and most studied of all five classes. For rhodopsin, a high-resolution structure of the full polypeptide chain is available, whereas the β 2AR structures represent truncated versions, and therefore, it is difficult to make a complete head-to-tail comparison. However, based on the available information, the overall topology of rhodopsin and the β 2AR are quite similar. The root mean square deviation, a measure of structural similarity between protein structures, for the α -carbon backbone of the transmembrane region between rhodopsin and β 2AR is 1.56 Å, which indicates a very similar arrangement of the transmembrane helices. This feature also supports the previous notion of a conserved activation mechanism (i.e., agonist-induced conformational rearrangement) across this class of transmembrane receptors. Nonetheless, there are structural features in the β 2AR that differ significantly from rhodopsin and that thus highlight the existence of receptor-specific patterns in this class of GPCRs. For example, the second extracellular loop of the β 2AR contains a previously unanticipated short helix. This helix contains two disulfide bonds which seem to maintain this loop in a constrained state, presumably providing space for the diffusion of ligands to the binding pocket of the receptor (Fig. 1A). This is in contrast with rhodopsin, in which the

second extracellular loop contains a buried β -sheet which, together with the interactions of the N terminus with other extracellular loops, forms a cap-like structure that isolates the retinal-binding site in a hydrophobic pocket.

Another interesting difference between rhodopsin and the β 2AR structures is the state of the “ionic lock”, defined as a network of hydrogen bonding and charge interactions between Arg131 in TM3 and Glu268 in TM6 (Arg135 and Glu247 in rhodopsin). The ionic lock is considered to maintain rhodopsin and presumably other class A GPCRs, including the β 2AR, in an inactive conformation (Ballesteros et al., 2001). Indeed the crystal structure of inactive rhodopsin displays an intact ionic lock with a distance of 2.9 Å between Glu247 and Arg135, whereas in the light-activated structure of rhodopsin, the lock is broken with a distance of 4.1 Å between Arg135 and Glu247 (Palczewski et al., 2000; Salom et al., 2006). Both β 2AR structures contain an inverse agonist, carazolol, and based on classic receptor theory, one would expect such a ligand to stabilize the receptor in an inactive state, much as covalently attached retinal does for rhodopsin. However, in both β 2AR structures, the ionic lock is broken with a distance of 6.2 Å between Arg131 and Glu268 in the β 2AR-Fab structure and 10.58 Å between Arg131 and Glu268 in the β 2AR-T4 chimera structure (Fig. 1C). Although one cannot exclude the possibility of alteration in some structural features of the β 2AR due to binding of Fab or the presence of T4 lysozyme, the fact that similar ionic lock features were seen in both structures speaks against this being an artifact. However, the increased affinity of the β 2AR-T4 chimera for agonists compared with the wild-type β 2AR also suggests that the receptor is in an “active-like state” and that the broken ionic lock is a genuine feature of the receptor in the conformation that has been crystallized.

Comparison of the ligand binding pockets and the orientation of ligands between rhodopsin and the β 2AR reveals another significant difference between these two receptors. Although carazolol in the β 2AR and *cis*-retinal in rhodopsin exhibit similar binding modes, their interaction with the highly conserved “rotamer toggle” (Trp265 in rhodopsin and Trp286 in β 2AR) differs. It has been suggested that a change in the rotameric state of this tryptophan in rhodopsin and other class A GPCRs serves as a second activation switch (Shi et al., 2002). Direct interaction of *cis*-retinal with Trp265 has been proposed to maintain the inactive state of rhodopsin. Although carazolol does not directly interact with Trp286 in the β 2AR, it seems to control the rotameric state of Trp286 indirectly via interacting with Phe289 and Phe290. Although these interactions are proposed based on a relatively high-resolution structure of the β 2AR, the only direct way to validate these mechanisms would be to determine the structure of the β 2AR in an active state.

Another distinctive feature is the different patterns of the two receptors with respect to their oligomeric state in the crystal structure. All of the structures of rhodopsin determined so far exhibit a receptor dimer. On the contrary, the β 2AR crystals show monomers or minimal inter-receptor contacts.

What Does the Structure Teach Us?

The crystal structures of the β 2AR allow a re-examination and reassessment of existing data obtained from mutagenesis and biochemical approaches. In fact, many data correlat-

ing receptor mutations and ligand binding can now be explained from a structural perspective. There are extensive interactions observed between the receptor and the ligand carazolol (Figs. 1D and 2A). For example, Asp113 was found previously to be crucial for ligand binding to the β 2AR. Mutation of Asp113 to asparagine leads not only to complete loss of antagonist binding but also decreases in the potency of agonists by several orders of magnitude (Strader et al., 1988). Likewise, mutations of Asn312 result in partial or complete loss of ligand affinities (Suryanarayana and Kobilka, 1993). Indeed, both Asp113 and Asn312 are seen to interact with O17 and N19 of carazolol, thus explaining why these mutations are detrimental to ligand binding. Along the same line, Val114 and Phe290 extensively interact with the C₈-to-C₁₃ ring of the carbazole moiety of carazolol and form a hydrophobic sandwich with this aryl group. These interactions might explain the loss of affinity for aryl moiety containing antagonists (e.g., alprenolol) and agonists (e.g., epinephrine) upon mutation of Val114 to alanine.

A feature of many GPCRs is that they can weakly signal even in the absence of ligand, a property referred to as constitutive activity. This has been presumed to be due to spontaneous, albeit scant, isomerization of inactive receptor into the active conformation. Agonists stabilize active conformations of the receptor, thus promoting cellular signaling. Some years ago, it was discovered that the mutation of certain residues in several adrenergic receptors greatly augmented constitutive activity of the receptors (Cotecchia et al., 1990). Spontaneous occurrence of such mutations in a variety of receptors is now known to cause several human diseases (Spiegel, 1998). Mutations leading to enhanced constitutive activity have been thought to abrogate crucial intramolecular interactions between amino acid residues, which normally constrain the receptor to its inactive state, thus somehow mimicking the effects of agonists. Mutagenesis studies had earlier suggested that residues at the cytoplasmic surface of TM helices 3 and 6 form the “ionic lock,” discussed above, which is crucially involved in maintaining the inactive state of the receptor. As noted, the crystal structures of the β 2AR accord well with these prior studies. For instance, Leu272 in TM6, which was the first residue identified to lead to the constitutively active phenotype of the β 2AR (Samama et al., 1993), exhibits interactions with residues toward the cytoplasmic ends of TM3 and TM5 and packing interactions with neighboring Glu268. Therefore, one can speculate that mu-

tation of Leu272 may relieve the constraints at the intracellular side of the helices and promote disruption of the “ionic lock,” thus resulting in an “active-like” state of the receptor. More importantly, several residues that lead to constitutively active mutants (e.g., Leu124 and Leu272) and others that lead to uncoupling (uncoupled mutant) (e.g., Asp79, Asn318, Asn322, and Tyr326) are linked through packing interactions (i.e., certain residues that pack against residues in TMs 3 and 6 responsible for constitutive activity) and are also involved in interactions with residues in TM 7, which lead to uncoupling from G proteins. Thus, rearrangement of the side chains of one of these residues can affect the packing and/or orientation of others. It is interesting that these residues are also in close proximity to Trp286, which constitutes the rotamer “toggle switch” in the receptor. Similar to rhodopsin, a cluster of water molecules in this region is also observed that can potentially promote an extended network of hydrogen bonding interactions. It is likely that the water-filled region will impose relatively low steric hindrance on the side chains of amino acids in this region, thus facilitating conformational transitions and repacking. This might be a general feature for the class A GPCRs that allows them to adopt multiple active conformations.

What Conformation Is the Receptor in?

Does This Shed Light on the Mechanisms of Activation?

As mentioned earlier, both structures of the β 2AR contain carazolol, an inverse agonist, which should stabilize the receptor in an inactive state much as covalently attached retinal does for rhodopsin. However, in contrast to the inactive structure of rhodopsin in which the ionic lock is intact, both β 2AR structures display a broken ionic lock (Cherezov et al., 2007; Rasmussen et al., 2007; Rosenbaum et al., 2007). There are several possible explanations for this unexpected feature. As Rasmussen et al. report, carazolol reduces the basal activity of the receptor only by ~50%, which makes it a partial inverse agonist. Therefore, the first possibility is that the broken ionic lock is due to the remaining basal activity of the receptor. In fact, the enhanced agonist affinity of the β 2AR-T4 chimera that was observed (Rasmussen et al., 2007) fits with this possibility. The second more intriguing possibility is that the current structures represent an alternative “active” signaling conformation. In this context, it was reported recently that carvedilol, an inverse agonist of the β 2AR very similar to carazolol (Fig. 2, A and B), has no detectable G protein signaling properties but is capable of inducing β -arrestin-mediated signaling (Wisler et al., 2007). This finding suggests that the current structures may represent a β -arrestin-coupled signaling state of the receptor. However, further structural and functional analysis will be required to support this interesting possibility.

What Do the Structures Not Tell Us?

Many GPCRs, including the β 2AR and rhodopsin, are known to exist as dimers (Milligan, 2004). Although the dimeric nature of rhodopsin has been well established both by biochemical and structural data, this issue remains controversial for the β 2AR. It has been suggested that dimers or higher-order oligomers of the β 2AR may regulate the export of the receptor to the plasma membrane (Salahpour et al., 2004), although receptor dimer-

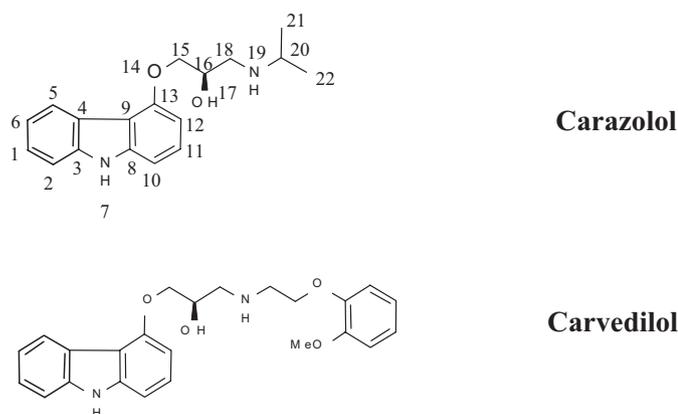


Fig. 2. Structure and numbering scheme of two β 2AR ligands: carazolol (a partial inverse agonist), and carvedilol (a β -arrestin-biased ligand).

ization seems not to be essential for G protein activation (Whorton et al., 2007). Although the crystals of β 2AR-Fab complex show no inter-receptor contacts, suggesting a receptor monomer, the β 2AR-T4 chimera crystals do show lipid-mediated contacts between helices 1 and 8. However, studies in native membranes have suggested helix 6 as the dimerization interface for the β 2AR (Hebert et al., 1996). Again, the possibility that the protein engineering or the Fab interferes with receptor dimerization cannot be ruled out at this stage, and further structural analysis will be required to draw definite conclusions. Furthermore, as discussed above, most of the C terminus of the receptor was truncated to reduce flexibility and heterogeneity. The C terminus is known to be a site of post-translational modification and for interactions with proteins such as β -arrestins, and therefore, the structural organization of this region is likely to carry important information. To visualize this domain, a complex of the receptor with an interacting partner stabilizing this region will probably be necessary. In addition, because the electron density of the N terminus of the receptor is not apparent in the current structures, this region of the molecule is absent from the model, and therefore features of this region remain unknown. Another important piece of information missing from these structures is the structural organization of the third intracellular loop. This loop is crucial for determining the G protein-coupling specificity of the receptors and for forming the binding interface with G proteins and other signaling partners. However in the β 2AR-T4 chimera, this loop is replaced by T4 lysozyme, whereas the β 2AR-Fab complex contains the Fab bound to this region.

Future Directions?

The most fundamental question one can hope to answer with the receptor crystal structures is how a signal (or stimulus) recognized at the extracellular surface is transmitted to the interior of the cell, leading to a specific functional outcome. However, each crystal structure represents a snapshot of only one of many conformational ensembles or states that the receptor can adopt, thus limiting the information that can be extracted from any individual structure. Thus, a major future goal is to obtain crystal structures of the receptor in its various active states. However, considering the conformational plasticity of the activated receptor and the relatively low affinity and chemical instability of typical agonist ligands, this may take more than protein engineering approaches.

In this context, a paradigm shift in the field of GPCR research in the last few years has been the discovery that the receptors can signal via G protein-independent mechanisms (e.g., via β -arrestins) (DeWire et al., 2007). Moreover, "biased" ligands have been identified that can direct receptor signaling exclusively through either β -arrestins or G proteins. β -Arrestin-biased ligands may ultimately represent a novel class of drugs which, like conventional blockers, shut off G protein signaling, but which also initiate the potentially beneficial effects of β -arrestin-mediated signaling. Moreover, mutant β 2AR and angiotensin II receptors have been generated, which are totally uncoupled from cognate G proteins but which still lead to extracellular signal-regulated kinase activation via β -arrestins. Determination of the structures of such mutant receptors bound to biased or unbiased ligands should help to clarify

the determinants on the receptor and on the ligands that are responsible for such conformational and functional selectivity. This information will be of great value in the design of ever-more selective therapeutic agents.

Another exciting and challenging goal will be the structure determination of GPCRs in complex with other signaling partners such as G proteins, β -arrestins, G protein-coupled receptor kinases, and others. Structural information derived from these signaling complexes will greatly aid the understanding of the interaction interfaces of the receptors and the conformational changes in both receptor and its interacting partners that follow these interactions. Moreover, despite the fact that the signaling mechanisms of GPCRs seem to be highly conserved, receptor-specific features are also likely to exist. Thus, structural information about other class A GPCRs and those of other classes will be necessary to obtain a full understanding of these mechanisms.

Acknowledgments

We thank Donna Addison and Elizabeth Hall for secretarial assistance. We also thank Dr. Erin J. Whalen for critically reading the manuscript.

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