Structural model of antagonist and agonist binding to the angiotensin II, AT_1 subtype, G protein coupled receptor

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Background: The family of G protein coupled receptors is the largest and perhaps most functionally diverse class of cell-surface receptors. Due to the difficulty of obtaining structural data on membrane proteins there is little information on which to base an understanding of ligand structure–activity relationships, the effects of receptor mutations and the mechanism(s) of signal transduction in this family. We therefore set out to develop a structural model for one such receptor, the human angiotensin II receptor.

Results: An alignment between the human angiotensin II (type 1; hAT_1), human β2 adrenergic, human neurokinin-1, and human bradykinin receptors, all of which are G protein coupled receptors, was used to generate a three-dimensional model of the hAT_1 receptor based on bacteriorhodopsin. We observed a region within the model that was congruent with the biogenic amine binding site of β2, and were thus able to dock a model of the hAT_1 antagonist L-158,282 (MK-996) into the transmembrane region of the receptor model. The antagonist was oriented within the helical domain by recognising that the essential acid functionality of this antagonist interacts with Lys199. The structural model is consistent with much of the information on structure–activity relationships for both non-peptide and peptide ligands.

Conclusions: Our model provides an explanation for the conversion of the antagonist L-158,282 (MK-996) to an agonist by the addition of an isobutyl group. It also suggests a model for domain motion during signal transduction. The approach of independently deriving three-dimensional receptor models and pharmacophore models of the ligands, then combining them, is a powerful technique which helps validate both models.

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Introduction

The G protein coupled receptors (GPCRs) are large transmembrane proteins, containing seven transmembrane domains. Their size and hydrophobic nature has made the experimental determination of their structures particularly difficult, and no detailed, high-resolution three-dimensional structure of a GPCR has yet been produced using X-ray or NMR methods. Even the generation of low-resolution structures of GPCRs using techniques such as electron cryo-microscopy [1,2] is not routine. Recent advances in isotope-edited, solid-state NMR experiments may provide some detail on the conformation of the ligand when bound to the receptor [3,4]; although these may answer some of the structural questions about GPCRs, other questions will remain.

Given these difficulties, one practical approach to understanding the function of a given GPCR may be to use homology modelling, taking as a starting point the postulated structural similarity between the GPCRs and bacteriorhodopsin. The structural models developed from homology modeling techniques should be viewed as attempts to integrate and summarize the existing experimental information about the receptor and its ligand. The available data include information on ligand structure–activity relationships, primary sequence comparisons with rhodopsin and other GPCRs and phylogenetic analyses based on these sequence comparisons, topological comparisons, mutation data, biological data and structural hypotheses. In the absence of detailed structural information our goal is to produce three-dimensional models of the GPCRs that will serve as a framework for developing new ligands and for understanding the physical processes of ligand binding and signal transduction. This approach was also used by Hibert [5].

The focus of our study is the human angiotensin II type 1 receptor (hAT_1). Its endogenous ligand, angiotensin II, is a short peptide whose sequence is Asp-Arg-Val-Tyr-Val-His-Pro-Phe. It is a potent vasoconstrictor [6] and blocking the interaction between angiotensin II and hAT_1 [7] promises to be important in the control of hypertension and congestive heart failure [8]. On the basis of the sequence homology between GPCRs, an alignment between angiotensin II type 1 receptor (AT_1) sequences from different species and the human β2 adrenergic, neurokinin and bradykinin receptors was developed. This alignment was used to generate a three-

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dimensional model of hAT_1, based on a common seven-helix transmembrane topology and the postulated structural similarity between GPCRs and bacteriorhodopsin. Docking of ligands into this model provides a structural framework for understanding the structure–activity relationships of ligands for this receptor and provides insights into the mechanisms of antagonism and agonism in this system.

Results and discussion

Fig. 1 shows an alignment of the currently known AT_1 sequences (rat, mouse, human, bovine, porcine and turkey). The degree of sequence homology between them ranges from 97.5 % for the rat and mouse AT_{1b} sequences to 86.9 % between mouse AT_{1b} and turkey AT_1. If the turkey receptor is excluded from the analysis, the overall homology between receptors is better than 94.7 %.

The aim of this study was to build a structural model of hAT_1. A number of recent reviews have discussed the structure and function of GPCRs [5,9–14]. There are clear sequence homologies in the putative transmembrane helical domains within the family of GPCRs whose ligands are biogenic amines, and the differences between the different members of the family can be understood in terms of the differences between their ligands. Thus it seemed reasonable that a structural model of the β2 adrenergic receptor ([15,16] and D. Donnelly *et al.* unpublished), based on the bacteriorhodopsin structure, could be used to build a model for hAT_1. There has been recent debate [12], however, about whether the bacteriorhodopsin structure [1] should be used for GPCR model-building or whether the recent 9 Å structure of rhodopsin [2], also from Henderson's group, should be used instead. It has been suggested [13] that the distinction between the structures is based on the interpretation of the electron diffraction maps and it is not certain which, if either, interpretation is correct. We believe that our GPCR structural model for hAT_1, which is based on the bacteriorhodopsin footprint, is consistent with the available data on the effects of mutations on receptor function, and the structure–activity relationships in ligands for this receptor.

Ligand binding sites

As our hAT_1 model relies heavily upon the theoretical and experimental models for the β2 adrenergic receptor, we begin with a brief review of this system. Within the biogenic amine family of GPCRs there is clear sequence homology in the putative transmembrane helical domains, and the affinity differences for various ligands can be understood in terms of the steric and electronic features of the endogenous ligands ([5,9–14] and C.D.S., unpublished). The functional roles of many of the conserved residues have been determined by measuring the changes in the potency and efficacy of agonists and antagonists using chimeric and single point mutations [17]. This has enabled the identification of a ligand-binding site for the biogenic amine receptors in the extracellular half of the transmembrane domain bounded by TM3 (transmembrane helix 3), TM4, TM5, TM6 and TM7.

The key residues for agonist binding to the β2-adrenergic receptor are Asp113 (TM3), Ser204 and Ser207 (both

```
      1                                                  50
mAT1b MILNSSIEDG IKRIQDDCPK AGRHNYIFVM IPTLYSIIFV VGIFGNSLVV
rAT1b .T....T... .......... .......... .......... ..........
mAT1a .A....T... .........R ....S..... .......... ..........
rAT1a .A....A... .......... ....S..... .......... ..........
 bAT1 .....T.... .......... ........I. .......... ..........
 pAT1 .....T..S. .......... .......... .......... ..........
 hAT1 .....T.... .......... .......... .......... ..........
 tAT1 .VP.Y.T.ET V...HV...V S...S..YI. V..V.....I I.........
      51                                                 100
mAT1b IVIYFYMKLK TVASVFLLNL ALADLCFLLT LPLWAVYTAM EYQWPFGNHL
rAT1b .......... .......... .......... .......... ..R.......
mAT1a .......... .......... .......... .......... ..R.......
rAT1a .......... .......... .......... .......... ..R.......
 bAT1 .......... .......... .......... .......... ..R.....Y.
 pAT1 .......... .......... .......... .......... ..R.....Y.
 hAT1 .......... .......... .......... .......... ..R.....Y.
 tAT1 ....C..... ....I..... ........I. C....A.... ........C.
      101                                                150
mAT1b CKIASASVSF NLYASVFLLT CLSIDRYLAI VHPMKSRLRR TMLVAKVTCI
rAT1b .......... .......... .......... .......... ..........
mAT1a .......... .......... .......... .......... ..........
rAT1a .......... .......... .......... .......... ..........
 bAT1 .......... .......... .......... .......... ..........
 pAT1 .......... .......... .......... .......... ..........
 hAT1 .......... .......... .......... .......... ..........
 tAT1 ..L...GI.. .......... .......... ...V...I.. ..F..R....
      151                                                200
mAT1b IIWLMAGLAS LPAVIHRNVY FIENTNITVC AFHYESQNST LPIGLGLTKN
rAT1b .......... .....Y.... .......... .......... ..........
mAT1a .......... .......... .......... ......R... ..........
rAT1a .......... .......... .......... ......R... ..........
 bAT1 ....L..... ..TI.....F .......... .......... ..V.......
 pAT1 ....L..... ..TI.....F .......... .......... ..V.......
 hAT1 ....L..... ...I.....F .......... .......... ..........
 tAT1 V...L..V.. ..VI....IF .A..L.M... G.R.DNN.T. .RV....S..
      201                                                250
mAT1b ILGFVFPFVI ILTSYTLIWK ALKKAYKIQK NTPRNDDIFR IIMAIVLFFF
rAT1b .......L.. .......... .......... .......... ..........
mAT1a ....L...L. .......... ......E... .K........ ..........
rAT1a ....L...L. .......... ......E... .K........ ..........
 bAT1 ....L...L. .......... T.....E... .K..K....K ..L.......
 pAT1 ....L...L. .......... ......E... .K.......K ..........
 hAT1 ....L...L. .......... ......E... .K.......K ..........
 tAT1 ....LI..L. .......... T.....Q..R .KT......K M.V...F...
      251                                                300
mAT1b FSWVPHQIFS FLDVLIQLGV IHDCEIADVV DTAMPITICI AYFNNCLNPL
rAT1b .........T .........I .R......I. .......... ..........
mAT1a .........T .......... ....K...I. .......... ..........
rAT1a .........T .......... ....K.S.I. .......... ..........
 bAT1 .........T .M.......L .R..K.E.I. .........L ..........
 pAT1 .........T .........I ....K...I. .........L ..........
 hAT1 ...I.....T .........I .R..R...I. .......... ..........
 tAT1 ...I...V.T ........H. .T..K.T.I. .....F.... .........F
      301                                                350
mAT1b FYGFLGKKFK RYFLQLLKYI PPKARSHAGL STKMSTLSYR PSDNMSSSAR
rAT1b .......... K......... ..T.K..... .......... .........K
mAT1a .......... K......... ....K..SS. .......... .......A.K
rAT1a .......... K......... ....K..SS. .......... .........K
 bAT1 .......... K......... ....K..SN. .......... ..E.GN..TK
 pAT1 .......... K......... ....K..SS. .......... ..E.G...TK
 hAT1 .......... .......... ....K..SN. .......... ....V...TK
 tAT1 ..V.F..N.. K.....I... ..NVST.PS. T....S.... .PE.IRLPTK
      351
mAT1b KSAYCPEVE
rAT1b ...SF....
mAT1a .P.S.S...
rAT1a .P.S.....
 bAT1 .P.P.I...
 pAT1 ...P.T...
 hAT1 .P.P.....
 tAT1 .T.GS.DT.
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Fig. 1. Sequence alignment for human angiotensin II (A-II) type 1 receptor (hAT1), rat A-II/AT_{1a} (rAT1a), rat A-II/AT_{1b} (rAT1b), mouse A-II/AT_{1a} (mAT1a), mouse A-II/AT_{1b} (mAT1b), bovine A-II/AT_1 (bAT1), porcine A-II/AT_1 (pAT1) and turkey A-II/AT_1 (tAT1). The sequence for mAT1b is shown. In the other sequences a period indicates an exact match with mAT1b and only those residues which differ from mAT1b are shown.

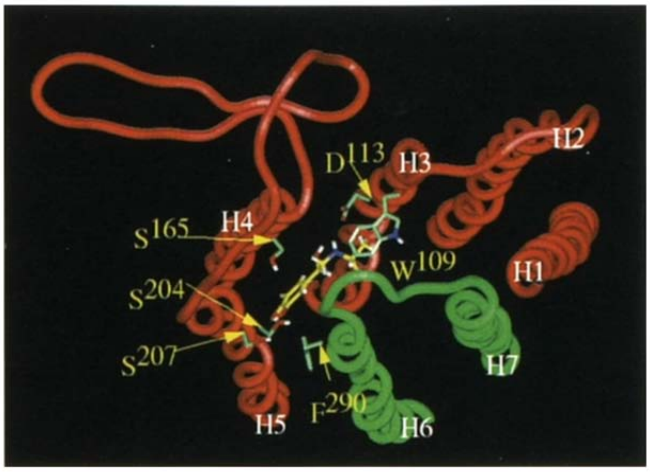

Fig. 2. Three-dimensional structural model of the hβ2 GPCR from the extracellular side of the membrane. The receptor is mostly represented by a Cα trace. Docked in the receptor is isoproterenol (carbons colored yellow). The docked orientation is consistent with the known mutation data for this receptor. The key residues in this interaction are Asp113, Ser204, Ser207 and Phe290.

found in TM5) and Phe290 (TM6). Fluorescence experiments with the β2 adrenergic receptor have localized the binding site of ligands such as carazolol to the transmembrane domain, more than 11 Å from the extracellular fluid [18]. Fig. 2 illustrates the important interaction sites between isoproterenol (carbons in yellow) and receptor in Donnelly's hβ2 receptor model ([15,16], and Donnelly *et al.*, unpublished). For simplicity only the Cα trace is shown. Trp109 lies above Asp113 in TM3; mutation studies suggest that the latter interacts directly with the amine of the ligand. Ser165 (TM4) has been postulated from modeling studies to interact with the benzylic (S)-hydroxyl of the catecholamine (required for potency) and Ser204 and Ser207, which are one turn apart on TM5, have been shown by mutagenesis studies to interact with the hydroxyls of the catechol. The aromatic sidechain of Phe290 (TM6) interacts with the catechol ring. The details of mapping of the human β2 adrenergic receptor and neurokinin 1 receptor can be found in recent reviews [14,18].

Recent experimental evidence on the neurokinin family of receptors, AT_1 and the bradykinin receptor indicates that the peptide-hormone-receptor class of GPCRs contain ligand-binding sites that are equivalent to the biogenic amine-binding site described above. Fig. 3 compares the sequences for the human β2-adrenergic receptor (hβ2) [19], hAT_1 [20], the human neurokinin 1 receptor (hNK1) [21] and the human bradykinin receptor (hBK) [22], all of which are GPCRs. This alignment was based on the observation that regions within the sequence were invariant across most GPCRs and that these regions are flanked by residues that are found to be involved in ligand binding. For TM5, the ΦXX-Pro-XXXXXXXΦ motif (where Φ is aromatic — either Tyr, Phe or Trp — and X is any residue) is observed in most GPCRs; a similar motif, ΦXXXTyrXProΦ, is found in TM6. Also shown in Fig. 3 (highlighted in yellow) are residues in TM5 and TM6 which have been mutated and found to affect ligand binding. The residues described are not the sole determinants of ligand recognition and binding, but there is a clear commonality between the peptide hormone and the biogenic amine receptors in this region; different GPCR families necessarily have additional characteristic ligand interactions, however.

An important link between the biogenic amine family of receptors and the peptide hormone receptors is evident in studies of AT_1. The top of TM5 and TM6 form part of the biogenic amine binding site and site-directed mutation studies have indicated that Lys199 (top of TM5) in rat AT_1 (conserved in hAT_1 and the rat angiotensin II type 2 (AT_2) receptor [23,24]) is important for binding angiotensin II. The mutation Lys199→Gln reduces the

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                                                         ______TM1______
hAT1      MILNSS     TEDGIKR IQDDCPKAGR HNYIFVMIPT LYSIIFVVGI
 hBK   MLNVTLQGP     TLNG.TF AQSKCPQVEW LGWLNTIQPP FLWVLFVLAT
hNK1  MDNVLPVDSD     LSPNIST NTSEPNQFVQ PAWQIVLWAA AYTVIVVTSV
 hβ2    MGQPGNGS AFLLAPNGSH APDHDVTQQR DEVWVVGMGI VMSLIVLAIV
      _______                      ____________TM2_____________
       46                                74
hAT1  FGNSLVVIVI YFYMKLKTVA SVFLLNLALA DLCFLLTLPL WAVYTAMEYR
 hBK  LENIFVLSVF CLHKSSCTVA EIYLGNLAAA DLILACGLPF WAITISNNFD
hNK1  VGNVVVMWII LAHKRMRTVT NYFLVNLAFA EASMAAFNTV VNFTYAVHNE
 hβ2  FGNVLVITAI AKFERLQTVT NYFITSLACA DLVMGLAVVP FGAAHILMKM
                 ___________TM3 __________            ______
         101                         125
hAT1  WPFGNYLCKI ASASVSFNLY ASVFLLTCLS IDRYLAIVHP MKSRLRRTML
 hBK  WLFGETLCRV VNAIISMNLY SSICFLMLVS IDRYLALVKT MSMGRMRGVR
hNK1  WYYGLFYCKF HNFFPIAAVF ASIYSMTAVA FDRYMAIIHP   LQPRLSAT
 hβ2  WTFGNFWCEF WTSIDVLCVT ASIETLCVIA VDRYFAITSP FKYQSLLTKN
      _________TM4_________
           153
hAT1  VAKVTCIIIW LLAGLASLPA IIHRNVFFIE NTNITVCAFH YESQNSTLPI
 hBK  WAKLYSLVIW GCTLLLSSPM LVFRTMKEYS DEGHNVTACV ISYPSLIWEV
hNK1  ATKVVICVIW VLALLLAFPQ GYYSTTETMP SRVVCMIEWP E HPNKIYEK
 hβ2  KARVIILMVW IVSGLTSFLP IQMHWYRATH QEAINCYANE T CCDFFTNQ
      ____________TM5____________
                 204        215
hAT1  GLGLTKNILG FLFPFLIILT SYTLIWKALK KAYEIQKNKP
 hBK  FTNMLLNVVG FLLPLSVITF CTMQIMQVLR NNEMQK          FKE
hNK1  VYHICVTVLI YFLPLLVIGY AYTVVGITLW ASEIPGDSSD  RYHEQ
 hβ2  AYAIASSIVS FYVPLVIMVF VYSRVFQEAK RQLQKIDKSE GRFHVQNLSQ
                                ____________TM6_____________
                                      249 253 256
hAT1                       RN DDIFKIIMAI VLFFFFSWIP HQIFTFLDVL
 hBK                     IQTE RRATVLVLVV LLLFIICWLP FQISTFLDTL
hNK1                     VSAK RKVVKMMIVV VCTFAICWLP FHIFFLLPYI
 hβ2  VEQDGRTGHG LRRSSKCLKE HKALKTLGII MGTFTLCWLP FFIVNIVHVI
                           ____________TM7____________
                                  294  298
hAT1  IQLGIIRDCR IADIVDTAMP ITICIAYFNN CLNPLFYGFL GKKFKRYFLQ
 hBK  HRLGILSSCQ DERIIDVITQ IASFMAYSNS CLNPLVYVIV GKRFRKKSWE
hNK1  N      PDLY LKKFIQQVYL AIMWLAMSST MYNPIIYCCL NDRFRLGFKH
 hβ2  Q           DNLIRKEVYI LLNWIGYVNS GFNPLIYC R SPDFRIAFQE

hAT1  LLKYIP                      PKAKSH SN    LSTKM STLSYRPSDN
 hBK  VYQGVC                      QKGGCR SEPIQMENSM GTL   RTSIS
hNK1  AFRCCPFISA GGDYELEMKS TRYLQTQGSV YKVSRLETTI STV     VGAH
 hβ2  LL CLRRSSL KAYGNGYSSN GNTGEQSGYH VEQEKENKLL CEDLPGTEDF

hAT1  VSSSTKKP    APCFEVE
 hBK  VERQIHKLQD WAGSRQ
hNK1  EEEPEDGPKA TPSSLDLTSN CSSRSDSKTM TESFSFSSNVL S
 hβ2  VGHQGTVPSD NIDSQGRNCS TNDSLL
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Fig. 3. Comparison of the sequences for the human β2-adrenergic receptor (hβ2) [19], the human angiotensin II type 1 receptor (hAT1) [20], the human neurokinin 1 receptor (hNK1) [21] and the human bradykinin receptor (hBK) [22]. The key residues used in the alignment are highlighted in green. Only the important hAT_1 residues have been numbered. The residues highlighted in yellow are those residues for which there is mutation data. Gaps are indicated by blank spaces.

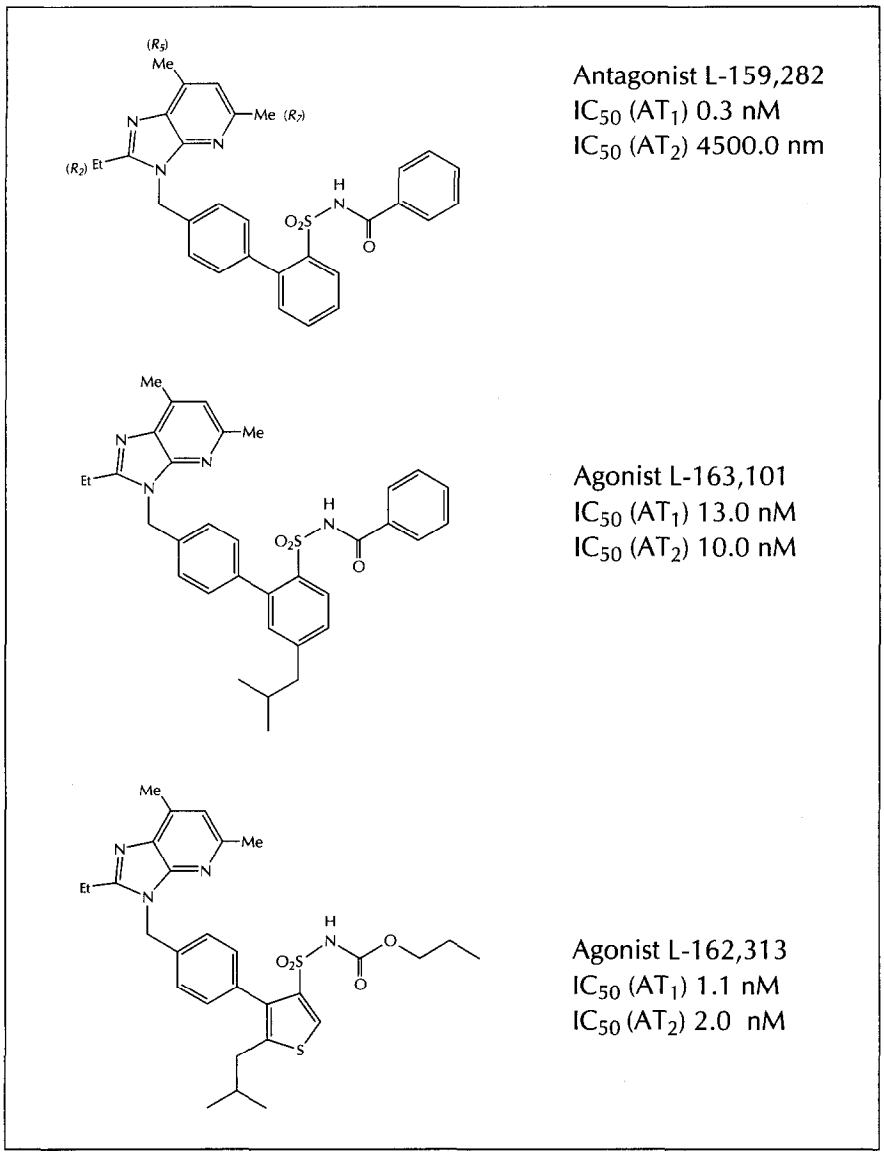

Fig. 4. Structures and agonist and antagonist activities of L-159,282, L-163,101 and L-162,313 measured against the rat angiotensin II receptors AT_1 and AT_2.

affinity of angiotensin II for the mutant receptor by ~30 fold [25]. This residue is in a similar position to Ser203 in hβ2 (see Fig. 3; note that the residue numbering in Fig. 3 refers to the hAT_1 sequence only). Several mutations in the TM5 and TM6 region of the rat bradykinin receptor (rB_2-BK) also have an effect on both agonist and peptidic antagonist binding [26,27]. The Asn202→Ala mutation in hBK (Asn200→Ala in rB_2-BK) shows an 11-fold reduction in affinity for bradykinin; this residue is at the top of TM5 in a similar region to Lys199 in hAT_1, His197 in hNK1 and Ser204, and Ser207 of hβ2. Bradykinin binding is reduced by 2000-fold for the mutation Phe264→Ala (Phe261→Ala in rB_2-BK) in TM6 (see Fig. 3) and 240-fold by the mutation Tyr267→Ala (Tyr265→Ala in rB_2-BK). Mutation of Asp284 to Arg in TM7 decreases bradykinin binding by 800-fold and reduces the binding of the antagonist HOE-140 [28] 10-fold.

Site-directed mutagenesis on the hNK1 receptor, which binds the endogenous peptide agonist Substance P, indicates that Gln165 (TM4), His197 (TM5) and His265 (TM6), among others, are important in the binding of the quinuclidine NK1 antagonists (for example, CP-96,345 and L-709,232) [21,29] and the N-acyl-D-tryptophan benzyl esters [30]. It appears that most of the residues involved in quinuclidine binding are not involved in peptide agonist binding [14,31–38]. In contrast, binding of the peptide agonist NKA to the hNK2 receptor is affected by the analogous mutations in the NK2 receptor. Both peptide agonists and perhydroisoindole antagonists are affected by mutation of Tyr287 (TM7) in NK1, indicating that some elements of the peptide binding site are also important for binding to at least one small-molecule antagonist [39].

Ligand structure–activity relationships

Our model uses a region congruent with the biogenic amine binding site of hβ2 to dock the hAT_1 antagonist L-159,282 (MK-996) into the helical domain of the GPCR model. Given the proven importance of TM5 for angiotensin II binding, we oriented the acidic NH of the acylsulfonamide of L-159,282 toward the (presumably

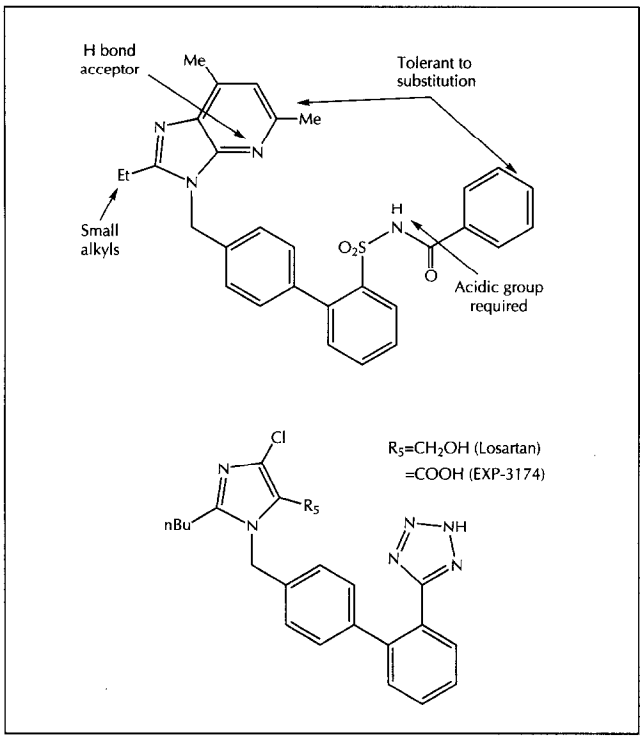

Fig. 5. Summary of the structure–activity relationships for hAT_1 antagonists of the L-159,282 type. The structures of Losartan and EXP-3174 are also shown.

positively charged) Lys199 in TM5. The biphenyl group of the antagonist was positioned in such a way as to stack with the aromatic sidechains which provide a 'floor' to the site (Tyr113 and Phe117 from TM3, Trp153 from TM4; Phe204 from TM5, Trp253 from TM6 and Tyr292 and Phe293, both from TM7). The R_5 position of the imidazopyridine (Fig. 4) was directed toward the extracellular loops and (as a consequence of the previous requirements and the ligand conformation) the arylsulfonamide was also directed toward the loops. This orientation is consistent with the structure–activity relationships observed for the heterocycle-biphenyl acid class of antagonists, which permits considerable latitude in substitution at R_5 and in the sulfonamide moiety (Fig. 5). Fig. 6 is an illustration of L-159,282 docked in the putative binding site. Residues Lys199, Trp153, Trp253, Ser107, His256 and Asp263 of the hAT_1 model are shown in Fig. 6b, with the remaining residues represented by a Cα trace.

Experimental tests such as site-directed mutagenesis, cross-linking, NMR and fluorescence are necessary to validate or invalidate this model. In the absence of such data, however, the structure–activity relationships of ligands that bind to the receptor can provide a sensitive probe of the kinds of interactions that are involved in ligand binding. We summarize the available data on structure–activity relationships for the small molecules known to bind to hAT_1 [40,41] in the context of our receptor model below.

An acid (carboxylic, tetrazole or aryl and acylsulfonamides) is required for potent binding to hAT_1 [42, 43]. In our model, Lys199 interacts with the C-terminus of angiotensin II as well as the acid moiety of the small molecules. This follows from the importance of TM5 to binding of ligands in other receptors and the requirement to satisfy the charge on Lys199, which is found in TM5. Ionic interactions have been seen before in the biogenic amine receptors in which the base is present in the ligand and the acid is present in the receptor. The β2-adrenergic receptor uses Asp113 on TM3, surrounded by Trp109 and other aromatic residues from other helices, to help stabilize the binding of the protonated amine of the endogenous ligand. Similarly, there are a

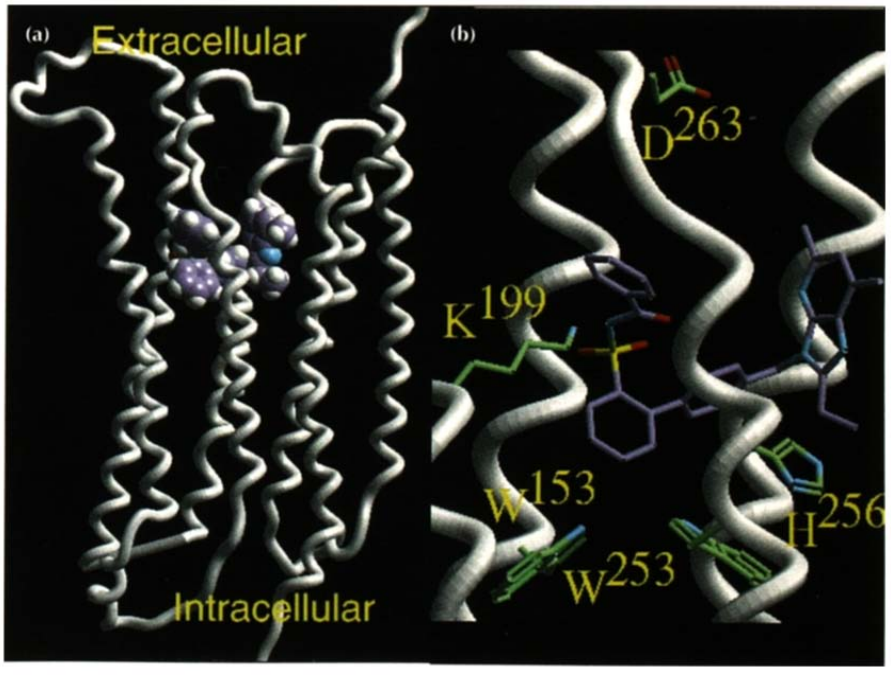

Fig. 6. The putative antagonist-binding site of the hAT_1 receptor. **(a)** Side (membrane-spanning) view of the three-dimensional structural model of the hAT_1 GPCR with L-159,282 (carbons colored purple) docked into the putative antagonist binding site. The conformation of L-159,282 was determined previously [50]. **(b)** Close-up of the putative antagonist-binding site. The receptor is represented by a Cα trace with the sidechains of Lys199 (TM5), Trp153 (TM4), Trp253 (TM6) and His256 (TM6) shown.

number of aromatic residues in the vicinity of Lys199 in hAT_1 which might serve to stabilize the protonated amino sidechain in a similar way in the absence of an appropriate ligand; these are Tyr113 and Phe117 from TM3, Trp153 from TM4, Phe204 from TM5, Trp253 from TM6 and Tyr292 and Phe293 from TM7. In each case the aromatic environment can potentiate the acid–base interaction; aromatics can stabilize a positive charge and accept hydrogen bonds from protonated amines (found on the ligand in the case of the β2-adrenergic receptor and on Lys199 in the case of the AT_1 receptor) [34,44–48]. The hydrophobic environment created by the aromatic sidechains also effectively stabilizes the acid–base interaction by diminishing the dielectric effects of water.

A second proton acceptor site in ligands (hydroxyl, heterocyclic N or carboxylic acid) contributes to their ability to bind the hAT_1 receptor. In L-159,282 this site is provided by the pyridine N, and in Losartan and in its more potent metabolite EXP3174 (Fig. 5) it is the primary alcohol and the carboxylic acid, respectively [40]. Ser107 on TM3 is well positioned to donate a proton toward this functionality. Similarly, Asp113 in TM3 of hβ2 is involved in ligand binding and aligns near Ser107 in hAT_1. The proton acceptor site would probably map to a peptide bond in angiotensin II itself, however, and the current view of receptor-peptide binding is that it is predominantly driven by specific side chain recognition rather than hydrogen bonding to the peptide backbone. Thus it seems most likely that this interaction does not contribute to affinity for peptide ligands.

For the small molecules, hydrophobic alkyl chains on the heterocyclic ring (at the R_2 position of the imidazopyridine; see Figs 4 and 5) appear to be essential for high potency. There is a preference for small groups at this position; the alkyl group is optimally ethyl in the imidazopyridine series [43]. In the model, this group is positioned toward a hydrophobic region that lies between TM2 and TM7 in the interior of the receptor. Since this position is directed downwards, toward the center of the helical bundle, it is likely that there will be steric restrictions on substitution. The model is consistent with the observation that small hydrophobic groups are preferred at this position. Comparison of the modeled structure of peptidyl ligands with the modeled structure of the non-peptide antagonists suggests that the alkyl substituent on the heterocycle in the imidazopyridines maps to position 5 of angiotensin II and of peptide analogs; in peptide agonists, this position also requires a residue with a small alkyl side chain. Thus these regions of TM2 and TM7 may interact with both peptide and non-peptide ligands.

The R_5 position in the imidazopyridine series of antagonists can be functionalized with a wide variety of groups including acids and bases, and large hydrophobic groups such as aryl sulfonamides and di-*n*-pentylureas ([49] and D. Ondeyka *et al.*, personal communication) without sacrificing potency. Superposition of antagonists with models of peptide agonists (for example the angiotensin II analog Asp-Arg-Val-Thr-Ile-His-Pro-Ile in Fig. 7) suggests that the R_5 position on the heterocycle is directed toward the N-terminal residues which, in angiotensin II, bear acidic (Asp_1) and basic (Arg_2) side chains. In the receptor model this position is directed toward the extracellular loops and substitutions at this position would therefore be expected to be well tolerated. Again the structure–activity relationships for the peptide appear to be consistent with this hypothesis, and the receptor model has both basic and acidic side chains in this region (Lys102, Arg167, Asp263, and Asp281).

Potency in the small molecules can be enhanced by increasing the size of the acid (carboxylic acid < tetrazole=acylsulfonamide) at the position occupied by a sulphonamide in L-159,282 [43]. This is consistent with our suggested orientation of this ligand in the receptor model, which places the acid proton toward Asn198-Lys199-Asn200. These additional sites may provide potential loci for additional hydrogen bonding to the ligand which is preferentially offered by sulfonamides and tetrazoles over the localized and directional charge of a COOH. The proposed conformation of the peptide [50] contains a γ-turn at Pro_7, placing the C-terminal acid toward the Tyr_4 side chain.

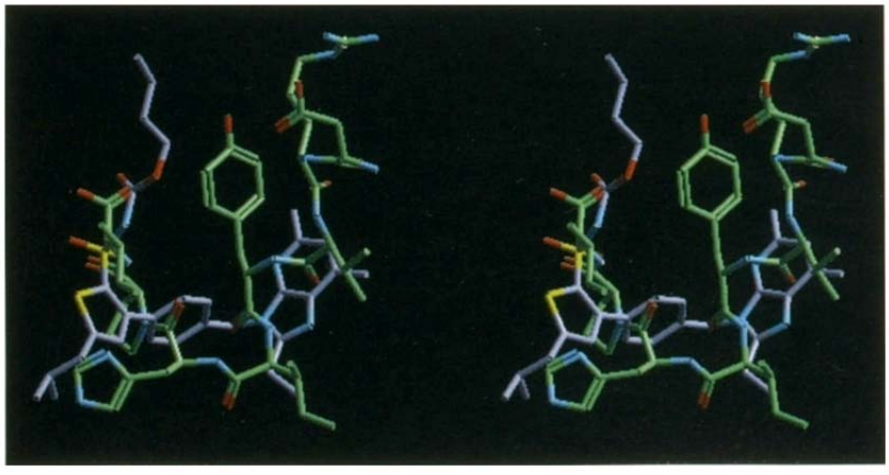

Fig. 7. Stereo view of L-162,313 (carbons colored purple) superimposed on a model of the angiotensin II peptide (carbons colored green). The conformation of L-162,313 was determined using pharmacophoric mapping techniques [50]. The conformational model of angiotensin II was based on literature data and has been described more fully previously [50].

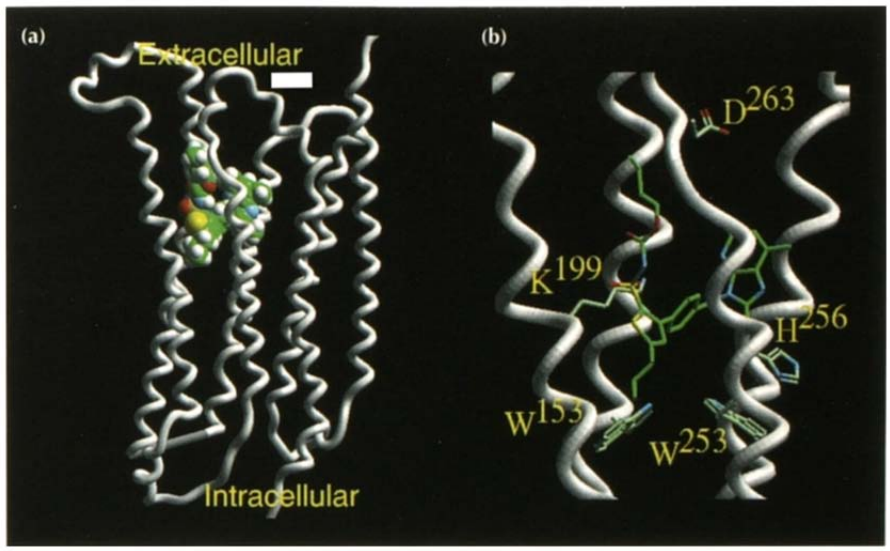

Fig. 8. The putative agonist-binding site of the hAT_1 receptor. **(a)** Side (membrane-spanning) view of the three-dimensional structural model of the hAT_1 GPCR. Most of the receptor is represented by a Cα trace. Docked in the receptor is L-162,313 (carbons are green) in the conformation determined from independent pharmacophore mapping techniques. The docked orientation is consistent with the known mutation data for this receptor. **(b)** Close-up of the putative agonist-binding site. The key residues (carbons in green) in this interaction are Lys199 (left), Trp153 and Trp253 (bottom), His256 (front) and Asp263 (top).

Acylsulfonamide AT_1 antagonists can be derivatized on the acyl group with a range of functionalities (acids and amines) with little effect on potency [51]. This suggests that the imidazopyridine R_5 substituent and the acylsulfonamide are close to each other, and from the model they both point toward the extracellular loops (see above). Samanen and coworkers [52] have observed that the ability of peptides to bind to AT_1 receptors and to act as agonists is sensitive to changes at positions 4 (Tyr_4) and 8 (Phe_8), consistent with our hypothesis that specific interactions with the receptor in the acylsulfonamide region enhance both binding and receptor activation by peptide agonists. There appears to be cooperativity between these postions; for example, good antagonists are obtained from changes at position 8 only when the residue at position 4 is a residue typically required for an agonist response. Overlay of the acylsulfonamide antagonist L-159,282 and angiotensin II places the C=O of the acylsulfonamide in a similar position to the OH of Tyr_4 of angiotensin II, providing further support for this orientation of the peptide and non-peptide ligands. The interaction site for this functionality might be the highly conserved Ser160 on TM4, which might be important for ligand binding as well as receptor activation.

Addition of an *iso*-butyl group to the terminal phenyl of the biphenyl of L-163,101 or the thiophene of L-162,313 [53] produces ligands that have *in vivo* agonist activity in rats. The peptide to non-peptide overlay shown in Fig. 7 provides a possible explanation for this behavior; the *iso*-butyl group of the antagonist and Phe_8 of angiotensin II are in similar positions. Specific changes at position 8 of angiotensin are known to switch these peptides from agonists to antagonists. For example, [N-Me-Gly_1]-angiotensin II is an agonist, [N-Me-Gly_1, cyclohexylalanine$_8$]-angiotensin II is a partial agonist and [N-Me-Gly_1, D-Phe_8]-angiotensin II and [N-Me-Gly_1, D-Ile_8]-angiotensin II are antagonists. Hence, angiotensin II tolerates substitution at position 1 but is sensitive to changes at position 8. Thus it is apparent that the correspondence between the peptides and the non-peptides occurs not only at a functional group level as described above but also at the level of interaction with the receptor to produce a functional response. The overlay shown in Fig. 7 highlights the interaction points [51] that are necessary and sufficient to cause an agonist response by non-peptides [52]. Our model is not sufficiently refined to predict potential differences in agonism based on the sequence differences between the rat and human receptors which are, in general, remote from the putative ligand-binding site.

Signal transduction

The addition of the *iso*-butyl substituent to the terminal aromatic ring in conjunction with the presence of an acylsulfonamide appears to provide the minimal necessary interactions for an agonist response in the imidazopyridines. The three-dimensional model suggests that the interaction between the *iso*-butyl functionality of L-163,101 or L-162,313 and the highly conserved aromatic residues that provide the floor of the binding site may be important in this process. Remarkably, the aromaticity of the sidechain in positions such as Tyr153 (TM4), Phe204 (TM5) and Phe249 and Trp253 (both in TM6) is conserved throughout almost all GPCRs. This strict conservation implies that these residues are crucial for receptor function. Their function may be to maintain receptor structure, provide a common binding site for the endogenous ligands, and/or couple the agonist binding site, presumably via a conformational change, to the G-protein binding site, thereby transducing the signal.

One can imagine many ways in which an antagonist might block the effect of an agonist at its receptor. Antagonists might compete for the same binding site or subsite as agonists, affect agonist binding allosterically, or prevent the transduction of the signal to the G protein. In contrast, an agonist must induce specific, directed interactions to initiate the signal transduction cascade. Based on the parallel between the structure–activity relationships of the peptide and non-

peptide agonists, we propose that the aromatic stacking interactions provided by residues such as Trp153, Phe204, Phe249 and Trp253 and interactions of Ser160 with the agonist are critical to the mechanism of receptor activation.

The importance of these conserved residues has been recognized previously. Studies by Wess and coworkers [54] indicate that mutation of the tryptophans in TM4 and TM6 of the muscarinic M3 receptor to phenylalanine results in a modest change in agonist and antagonist binding but little change in receptor efficacy. It should be recognized, however, that these mutations involve conservative changes and the effect of changing the aromatic character in this location of the receptor has not been investigated. The involvement of the conserved tryptophans in ligand binding has also been recognized in bacteriorhodopsin, using chemical cross-linking and site directed mutagenesis [55,56]. Fig. 8 shows L-162,313 (green) in the putative binding site of hAT_1. The *iso*-butyl chain on the thiophene is in a position to interact with the tryptophans and the other aromatics at this latitude of the receptor. As noted above, there appear to be regular motifs composed of aromatic residues in this region of most GPCRs, and aromatic–aromatic stacking interactions within the receptor itself and between the ligand and the receptor are critical. Our suggestion that the highly conserved Trp153 and Trp253 pair is the binding site for the *iso*-butyl moiety on the L-162,313 agonist strongly implicates this region as the site of signal transduction (and perhaps an alteration in the aromatic–aromatic interactions within the receptor as the mechanism of transduction).

Little is known about the signal transduction process for membrane-bound receptors. X-ray studies of growth hormone bound to its extracellular domain [57] and the trimeric TNF-receptor complex [58] suggested a surprisingly simple mechanism for transmission of the information that the hormone is present in the extracellular milieu to cytoplasmic messenger systems. The structure revealed that the extracellular domains (cleaved from the intact receptor for crystallization) were dimerized by interacting with a single growth hormone molecule. When the growth hormone receptor is intact, crosslinking the extracellular domains must also bring together the intracellular domains. This presumably is the signal to the cell that there is growth hormone in the extracellular environment. The data summarized in this paper suggest that a similarly simple event may also be at the heart of signal transduction in the GPCRs. Others have previously suggested the possibility of domain motion in GPCRs but few details were given (T.W. Schwartz, S. Hjorth and colleagues, unpublished). Topologically GPCRs can be described as two domains, one involving transmembrane domains TM1 to TM5 (the interconnecting loops are short to medium in length and contain a disulfide linkage between external loops 2 and 3, connecting helices 3 and 4), the other involving TM6 and TM7. The third cytoplasmic loop connecting TM5 and TM6 is variable in length but is in general long, providing a mechanism for dissociation of the domains. Furthermore, the junction between these domains (cytoplasmic loop 3) appears to serve as the primary binding site for the G protein. In an analogous manner to the growth hormone receptor one can imagine an agonist providing enough specific interactions with the receptor to bind the domains together, altering the G-protein binding site. In other words, one could consider the agonist binding site as an allosteric site to the G-protein binding site, and *vice versa*, according to the principle of micro-reversibility. This hypothesis is supported by the observation that it is the interaction of the catechol ring of the adrenergic agonists with Ser204 and Ser207 in TM5 and Phe290 in TM6 that is the critical event for agonist activation of the β2 adrenergic receptor. Although only a hypothesis, it is worth noting that van't Hoff plots suggest that agonism is entropy driven whereas antagonism is enthalpy driven, consistent with the large losses in entropy which would be expected from mechanisms requiring domain motion for signal transmission [59–61]. The loss of entropy on agonist binding is compensated for by the enthalpy of binding, so that the overall binding free energy remains favorable.

Other mechanisms could explain the thermodynamic difference resulting from the binding of agonists and antagonists, for example a scissor motion in the transmembrane domains pivoted about the conserved prolines midway along the helices, or a more generalized conformational effect. Nonetheless it is interesting that when two fragments of the muscarinic M3 receptor, TM1–TM5 and TM6–TM7, are expressed in the same cell, they can produce a wildtype functional response in the presence of the agonist carbachol [62,63]. These experiments are consistent with domain clustering as part of an agonist mechanism. Our receptor model with angiotensin II docked into the putative binding site indicates that several conserved charged residues at the tops of transmembrane domains 3, 4, and 6 (Lys102, Arg167, Asp263 and Asp281) could bind to the N terminus of angiotensin II and nucleate such clustering. We would anticipate that mutation of these charged groups would affect peptide binding and perhaps interrupt signal transduction, but would not be important for the binding of the small molecule antagonist ligands discussed in this work.

Significance

G protein coupled receptors (GPCRs) mediate cellular responses to a diverse set of external stimuli ranging from the biogenic amines, such as epinephrine, dopamine and 5-hydroxytryptamine, to light (which activates retinal), and from alkaloids, such as cannabinoids, to large proteins such as C5a and glycoproteins. Their mechanism of action is not completely understood, but they are important targets for drug therapy in several diseases. GPCRs are functionally homologous; all

pass a message from the extracellular milieu to an intracellular second messenger system via the activation of one or more members of the family of heterotrimeric G proteins, initiating a number of signal transduction pathways [64,65]. The homology in the primary sequences of GPCRs presumably reflects a structural homology, which may provide a clue to the requirements for the common function of G protein binding. Sequence homology among GCPRs is greatest in the transmembrane region of the protein and the extra- and intra-cellular loops show the greatest divergence even within a GPCR subtype. Binding of the agonist to the receptor presumably causes a conformational change in the receptor, thereby causing one or more of the cytoplasmic loops of the receptor to interact with the G protein.

Sequence identity between bacteriorhodopsin (which is not a GPCR) and GPCRs is ~11 %, although slightly higher homology can be identified if different helical alignments between bacteriorhodopsin and GPCRs are postulated [66]; these would presumably have arisen either from exon shuffling or gene duplication [67]. The availability of a high-resolution structure of bacteriorhodopsin allowed others to build a model of the β2-adrenergic receptor, on which we have based a model of the angiotensin II receptor. An independently-derived model for the structure of an angiotensin II antagonists was docked into the receptor model, and was shown to be consistent with much of the available data on structure–activity relationships for agonists and antagonists of this receptor. The model suggests an explanation for the observation that substitution on the terminal aromatic (biphenyl or thiophene) of sulfonamide antagonists with branched alkyls or aryls switches the *in vivo* response from antagonism to agonism. The model also suggests that motion of the transmembrane helices relative to one another, bringing transmembrane helices 1–5 closer to helices 6 and 7, may be a useful model for signal transduction. This work is an attempt to unify into a single model much of the information that is known about these receptors and provides a framework within which to test ideas, and an hypothesis for testing and refinement.

Methods

Sequence alignments were performed by pair-wise hand alignments of hAT_1, hβ2, hNK1 and hBK using the graphical tools available in QUANTA 3.3 in the Protein Design utilities (Molecular Simulations, Inc., Boston, MA). Portions of the alignment were produced using the Needleman and Wunch algorithm [68] but, in general, the automatic methods gave less than satisfactory results, due to the low primary sequence homology between the receptors and the variability in length and sequence of the loop regions. A three-dimensional model of the human β2-adrenergic receptor (Donnelly, D., MacLeod, A. & Blundell, T., unpublished) was used initially as the basis for construction of the hAT_1 model shown in Fig. 1. Each of the side chain positions was placed according to the following considerations: all atoms of the side chain in common with atoms in the template (hβ2) were placed accordingly, and those side chain atoms that were not in common with the template were built according to known side chain preferences. Both receptor models were minimized using the CHARMm force-field [69].

The loop regions were added to the helices by searching the library of protein structures generated from the Brookhaven Protein Data Bank [70] for protein fragments that most closely match the geometric requirement of bridging from one helix to the next. This was done with the Search Fragment Database function within Protein Design of QUANTA 3.3. The loop regions, which are by definition lacking in secondary structure, are well solvated and are the least well-defined region of the GPCR model. It is difficult to define the end of the helices and the beginning of the more variable, hydrophilic loop regions to complete the structural model of the receptor. The presence of charged and polar residues appears to herald the transition from membrane buried helices to hydrophilic loop structure, however. Clearly, in a dynamic sense, the end of a helix and the beginning of a loop need not be distinct boundaries, since helices will tend to ravel and unravel in response to local environmental changes. With the exception of the third intracellular loop, the hydrophilic loops of the receptors are relatively short (~10 amino acids), consistent with a seven-helix bundle forming the transmembrane core of the receptor. The addition of a disulfide bridge between the conserved Cys residues on the second and third extracellular domains further constrains the flexibility of the loops. These receptor models provide a framework from which to explore the functional domains of the receptors, as described herein. The coordinates for the hAT_1 model have been deposited with the Brookhaven Protein Data Bank [70].

The conformation of the antagonist L-159,282 (MK-996) [71] (Fig. 4) shown in Fig. 6 was generated from a pharmacophore map that was derived previously [50]. The pharmacophore points and the distances that were used in the search procedure are highlighted. QUANTA 3.3 was used to dock L-159,282 into the binding site of hAT_1. The conformation of the agonists L-163,101 and L-162,313 [53] followed from re-minimizing L-159,282 after the new functionality was added.

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