Mutations in the B₂ Bradykinin Receptor Reveal a Different Pattern of Contacts for Peptidic Agonists and Peptidic Antagonists**

(Received for publication, April 2, 1996, and in revised form, August 8, 1996)

Kurt Jarnagin‡§, Sunil Bhakta‡, Patty Zuppan‡, Calvin Yee‡, Teresa Ho‡, Thu Phan‡, Ram Tahliramanian‡, Joe H. B. Pease¶, Aaron Miller¶, and Richard Freedman‡

From ‡Molecular Pharmacology, ¶Medicinal Chemistry, §Molecular Structure Group, Inflammatory Diseases Unit, Roche Bioscience, Palo Alto, California 94304

The B₂ bradykinin receptor, a seven-helix transmembrane receptor, binds the inflammatory mediator bradykinin (BK) and the structurally related peptide antagonist HOE-140. The binding of HOE-140 and the binding of bradykinin are mutually exclusive and competitive. Fifty-four site-specific receptor mutations were made. BK's affinity is reduced about 2200-fold by F261A, 490-fold by F254A, F261A, and Q290A. In contrast, HOE-140 affinity is reduced less than 7-fold by F254A, F261A, Y297A, and Q292A. The almost complete discordanse of mutations that affect BK binding versus HOE-140 binding is surprising, but it was paralleled by the effect of single changes in BK and HOE-140. [Ala₈]BK and [Ala₈]BK are reduced in receptor binding affinity 27,000- and 150-fold, respectively, whereas [Ala₈]HOE-140 affinity is reduced 7-fold and [Ala₈]HOE-140 affinity is unchanged. NMR spectroscopy of all of the peptidic analogs of BK or HOE-140 revealed a β-turn at the C terminus. Models of the receptor-ligand complex suggested that bradykinin is bound partially inside the helical bundle of the receptor with the amino terminus emerging from the extracellular side of helical bundle. In these models a salt bridge occurs between Arg₉ and Asp₂₈₆; the models also place Phe₈ in a hydrophobic pocket midway through the transmembrane region. Models of HOE-140 binding to the receptor place its β-turn one α-helical turn deeper and closer to helix 7 and helix 1 as compared with bradykinin-receptor complex models.

Bradykinin is a central initiator of acute and chronic inflammation and the associated pain and edema. Most of the acute and many of the chronic responses to bradykinin are mediated by B₂ bradykinin receptors (1, 2), while some of the chronic responses to bradykinin are mediated by B₁ bradykinin receptors (3). B₁ and B₂ bradykinin receptors are members of the G-protein-coupled seven-transmembrane (GPC-7TM) receptor superfamily (4–7). For B₁ receptors, [des-Arg⁹]bradykinin is a more potent agonist than bradykinin, whereas B₂ receptors bind and respond to bradykinin about 10,000-fold more effectively than [des-Arg⁹]bradykinin.

Several peptidic B₂ bradykinin antagonists have been identified; these compounds reduce pain and inflammation (8–12). Peptidic antagonists also reduce death from experimental shock (13–15). Bradykinin receptor antagonists are potentially useful in the treatment of pain, acute and chronic inflammation, shock, allergic or infectious rhinitis, and asthma. The peptidic antagonists are useful tools; but, to date, these compounds have made poor human therapeutic agents because of their poor bioavailability and formulation difficulties (16, 17). The discovery of a nonpeptide antagonist of bradykinin would improve the prospects of treating bradykinin-instigated inflammation, pain, or edema. Thus, we have focused on a molecular understanding of the bradykinin receptor ligand binding site, believing that this information may help in the discovery and design of nonpeptidic antagonists.

We used the results from molecular modeling studies of B₂ bradykinin receptors and NMR studies of peptidic agonists and antagonists to generate a number of models for agonist binding to the B₂ BKR. These models were tested by site-directed mutagenesis of the receptor and by making single amino acid changes in agonist and antagonist peptides. The data reveal a disparity between the way peptidic agonists and antagonists bind to the BKR. We attempt to reconcile the disparities by proposing new models of the BKR-ligand complex.

EXPERIMENTAL PROCEDURES

Materials

[³H]Bradykinin was from DuPont NEN and had a specific activity of about 100 Ci/mm³. Catalytic hydrogenation with tritium gas of 2-[3,4-dehydro]propyl 5-[4-ido]phenylalananyl HOE-140 yielded [³H]Phe²⁻HOE-140 (18, 19). The product was HPLC-purified and then characterized by HPLC and mass spectrometry as to its chemical purity (>96%) and specific activity (56.5 Ci/mm³). Media and other cell culture additives were from Life Technologies, Inc. Biochemicals and enzymes were from Boehringer Mannheim. Common reagents were from Sigma.

Methods

Standard molecular biological and cell culture methods were used except as specified (20, 21).

Mutagenesis—BKR mutants were made by a modification of the polymerase chain reaction mutagenesis method (22). The mutagenesis used the BglII/PvuII fragment of the rat cDNA (4) as the polymerase chain reaction template. The full mutant receptor was obtained by second l-Arg is numbered 1; thus, the 10 residues of HOE-140 are numbered 0–9, while the nine residues of BK are numbered 1–9; Boc, t-butyloxycarbonyl; TM, predicted transmembrane region (numbered 1–7 beginning at the amino terminus; thus, TM-7 designates transmembrane region 7); NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; CHO, Chinese hamster ovary; HPLC, high pressure liquid chromatography.
replacing the Yglu/Phe5 fragment of the wild type BKr in the expression vector of the SRF-159 vector (a derivative of the Srα2 promoter vector pCDLRSr296 (23)). The oligonucleotides used to create mutations incorporated the desired mutation and, when possible, an additional protein coding-silent mutation to yield a new restriction site. The “silent restriction sites” were used to rapidly screen candidate mutant cDNAs for the presence of the mutation. All mutagenesis cassettes were sequenced on an ABI-373A sequencer using the dterminator method; only results from mutants in which the desired sequence was confirmed are reported.

Cell Growth—COS-7 (ATCC number CRL-1651) cells and CHO-K1 (ATCC number CCL-61) cells were obtained from ATCC. CHO cells which the desired sequence was confirmed are reported. COS-7 cells, 95–100% confluent (1 x 10^6 cells/162 flask), were washed with phosphate-buffered saline and then transfected using 15 μg of DNA and 300 μg of Lipofectin. The Lipofectin and DNA were mixed prior to the addition to the cells in polystyrene tubes and added, dropwise, to the cells covered by 15 ml of Opti-MEM medium. After 6–7 h, the DNA/Lipofectin medium mixture was supplemented with 20 ml of growth medium. Twenty-four hours after transfection the cells were split, 1:3. Membranes were prepared 60–72 h after transfection; these membranes usually contained 0.9 fmol of receptors/μg of protein (31,000 receptors/cell).

Stable cell lines of the following receptor mutants were made: wild type, N200A, N204A, F261A, T265A, D286A, and Q290A. Transfection of the CHO cells began as above and used five parts of the appropriate cDNA in the pSRF-159 vector along with 1 part of a neomycin resistance plasmid, pSV2-neo (Stratagene). After 24 h, the cells split, 1:5, 1:10, 1:20, and 1:40, into medium containing 500 μg/ml G418. The cells were transferred to medium containing 250 μg/ml G418 after 5–7 days and cloned 6–10 days later. After clone expansion, 20–60 clones were assayed for [3H]Phe5-HOE-140 binding sites. A single clone was selected for all subsequent work. The clones ranged from 0.4 to 2.32 fmol of receptor/μg of membrane protein (16,000–87,000 receptors/cell).

Receptor Binding Assays—Competition assays were performed and analyzed as described in Krstenansky et al. (24). The assays used crude cell membranes from transfected cells. Competition binding assays contained the following components in a total volume of 550 μl: 50 μl of [3H]bradykinin (~20,000 dpm, ~0.15 nM) or [3H]Phe5-HOE-140 (~20,000 dpm, ~0.3 nM), 100 μl of an appropriate drug dilution, 200 μl of binding buffer, and 200 μl of membranes at 0.25 mg/ml. Nonspecific binding was determined by the addition of an excess of unlabeled bradykinin or analogue. Binding buffer had the following composition: 10 mM KH2PO4 (pH 6.8), 1 mM EDTA, 0.014 mg/ml bacitracin, 1 mM dithiothreitol, 10 μM captopril, 0.2 mg/ml 1,10-phenanthroline, 1 μM leupeptin, and 100 μM phenylmethylsulfonyl fluoride.

Saturation binding assays contained the following components in a total volume of 550 μl: 50 μl of [3H]bradykinin (final concentrations were 0.001–6 nM) or [3H]Phe5-HOE-140 (final concentrations were 0.001–6 nM), 200 μl of membranes at 0.25 mg/ml, and 300 μl of binding buffer. The nonspecific binding was measured in a separate mixture that contained 100 μl of 5.5 μM nonradioactive ligand dissolved in binding buffer, 1 μM final concentration, and 200 μl of additional binding buffer. Both competition and saturation assays were done in triplicate using 12 concentrations, and usually each assay was repeated at least twice. Incubation, filtration, and scintillation counting were as in Krstenansky et al. (24).

Saturation data were calculated by nonlinear curve fitting using a one site model, B = (Bmax × L/(Kd + L)) + (m × L) + b, where B is the amount of ligand bound, Bmax is the maximum specific ligand binding, L and Rb have the same meaning as in Krstenansky et al. (24), and (m × L) + b is a line describing the nonspecific binding component. Nonspecific binding calculated by the above method was found to agree well with the nonspecific binding measured by the addition of 1 μM nonradioactive ligand.

Molecular Modeling—Models of the BKr were based on the electron-cryomicroscopic picture of bacteriorhodopsin developed by Henderson et al. (25). Each of the seven helices was built using the BUILDER module of INSIGHT. For all GPC-7TM receptors the exact termination point of each helix is not known; thus, an extra turn was added to the extracellular side of each helix in order to prevent missing potential interactions. Our assumption that the binding site lies at least in part in the helical regions was based on structural and mutagenic studies of rhodopsin and mutagenic studies of amine-transmitter GPC-7TM receptors. Each helix was superimposed using the bacteriorhodopsin structure, and strong overlaps were removed by manual manipulation of side chain torsional angles. The ensemble of helices was minimized using the amber force field in DISCOVER-2.7, using 100 steps of steepest descent followed by 500 steps of conjugate gradient minimization. To stabilize the ensemble, short loops (TM-2 to TM-3, TM-3 to TM-4, and TM-4 to TM-5) were added to the ensemble using the loop builder in SYBYL. Longer loops and termini (NH2 terminus, TM-1 to TM-2, and the COOH terminus) cannot be accurately modeled and thus were omitted. The TM-5 to TM-6 loop (19 residues) has been postulated to be two α-helices connected by a spacer; thus, this loop was initially arranged as two helices connected by a turn region with unconstrained regions between the ends of TM-5 and TM-6 and the loop. The ensemble was again energy-minimized, with the atoms in the backbone of the transmembrane helices fixed and all other atoms free. Subsequent to the energy minimization, the model was subjected to 30 ps of molecular dynamics at 100 K, 15 ps at 200 K, and 75 ps at 300 K. The dynamics were limited by distance constraints on backbone hydrogen bonds within transmembrane helices. The lowest energy conformer occurring during the last 50 ps of dynamics was selected and energy-minimized using 1000 iterations of conjugate gradient minimization.

Bradykinin-receptor complexes were modeled with bradykinin constrained in a COOH-terminal β-turn. This model was manually docked with the receptor using INSIGHT II. The torsional angles of the peptide were then manually adjusted to maximize contacts with receptor residues suggested by the mutagenesis experiments. Finally, the complex was energy-minimized using 100 steps of steepest descent followed by 500 steps of conjugate gradient minimization. The complex minimizations were constrained by a term that preserves the α-helix backbone hydrogen bonds; no other constraints were used.

NMR Spectroscopy—All of the peptide samples contained approximately 3 mM peptide. Bradykinin was studied in aqueous solution (90% H2O, 10% D2O, 20 mM acetate, pH = 4.6, 0°C), SDS solution (180 mM SDS in 90% H2O, 10% D2O (~1 peptide molecule/micelle), 30°C), and Me2SO solution (50 °C). HOE-140 was analyzed in both the aqueous solution and the SDS solution. All of the other peptides were analyzed in the aqueous solution only. The samples with SDS were sonicated for 5 min before use to homogenize the micelles.

Data recording conditions and methods were as described in Krstenansky et al. (24). Amide temperature coefficients were measured between 25 °C and 30 °C for the samples in a H2O/D2O mixture and 25 and 40 °C for the samples in Me2SO and SDS solutions. A combination of NOEs, amide temperature coefficients, and spin-spin coupling constants were used to analyze each peptide’s conformation.

Peptide Synthesis—The peptides were synthesized using standard Boc chemistry protocols. For HOE-140 and analogs, Boc-Arg(tosyl-amido)-(phenylacetamidomethyl) resin was used (0.58 mmol/g; Applied Biosystems, Inc.). Boc-Tic-Oh and Boc-Oic-Oh were purchased from Synthetech, Inc. After synthesis and cleavage of the linear peptides from the resin using liquid HF containing 5% anisole, the peptides were purified by reverse-phase HPLC. The composition of each amino acid in each peptide was consistent with the expected value, ±15%. The mass spectra were all consistent with the desired structure, ±450 ppm.

RESULTS

To begin our effort to understand the bradykinin receptor’s ligand binding site we addressed the question of the relationship of the bradykinin and HOE-140 binding sites. We asked whether BK and Phe5-HOE-140 were competitive at the BKr binding site. [3H]BK and [3H]Phe5-HOE-140 saturation curves were constructed at various concentrations of competitor (Fig. 1). It is important to note in these assays that both ligands and the receptor are incubated together for 1–1.5 h to allow for equilibrium. Equilibrium is attained in 45 min (data not

2 INSIGHT and DISCOVER are available from Biosym Technologies, 9865 Scraton Rd, San Diego, CA 92121.

3 SYBYL is available from Tripos Associates, Inc., 1699 S. Hanley Rd, Suite 303, St. Louis, Missouri 63114.
shown). The data clearly demonstrate that the maximum binding of each ligand is not depressed by the other ligand, which indicates that the interaction is competitive.

To continue our study of the receptor-ligand complex we examined the structure of bradykinin and HOE-140 by NMR. In aqueous solution BK does not have a single conformation but instead undergoes rapid motion. The NOE cross-peaks from the NOESY spectrum are relatively weak at room temperature but significantly increase at lower temperatures (0 °C). There is a relatively strong NOE between the Arg9 and Phe8 amide protons, which provide supporting evidence for a type-II \( \beta \)-turn involving residues 6–9. In addition, the Arg9 amide temperature coefficient is small (it shifts the least with temperature for this peptide, \(-4.9\) ppb/degree kelvin), suggesting that the Arg9 amide proton is somewhat protected from solvent exchange. The NOE pattern and amide temperature coefficients for the rest of the peptide are inconclusive as to the preferred conformations for these regions. However, it is interesting to note that the Phe6 amide proton temperature coefficient is the next lowest (\(-5.7\) ppb/degree kelvin, reported by Lee et al. (26)), possibly due to a partial hydrogen bonding interaction. The Phe6 amide proton has been observed to be involved in a type II \( \beta \)-turn under other solvent conditions (see below).

In Me\(_2\)SO, BK adopts a fairly stable conformation. The NOE cross-peaks in the NOESY spectrum were significantly more intense than those observed in aqueous solution. The amide temperature coefficients for Gly4, Phe5, and Arg9 are all within the range where these protons are significantly protected from solvent exchange. The NOE pattern in combination with these exchange data indicates that there are two type-II \( \beta \)-turns involving residues 2–5 and 6–9. These two \( \beta \)-turns have also been observed by Mirmira and co-workers (27). The Arg9 amide proton hydrogen bonds to the Ser6 carbonyl oxygen, and the Phe5 amide proton hydrogen bonds to the Pro2 carbonyl oxygen. The orientation of these two \( \beta \)-turns relative to each other is uncertain. The Gly4 amide proton is protected from solvent exchange, but it is not clear to which carbonyl this amide proton is hydrogen bonding.

In SDS solution, BK adopts a similar conformation to that observed in Me\(_2\)SO. Again the NOE cross-peaks in the NOESY spectrum were significantly more intense than those observed in aqueous solution. Both of the \( \beta \)-turns observed in the Me\(_2\)SO solution are present in the SDS solution. As was observed in the Me\(_2\)SO study, the relative positioning of the two turns is still uncertain. In contrast to the Me\(_2\)SO study, however, the Gly4 amide proton is not protected from solvent exchange in this case.

In aqueous solution, HOE-140 undergoes rapid motion. The NOE cross-peaks were relatively weak at room temperature but became significantly more intense at 0 °C. At the lower temperature, there is good evidence for a COOH-terminal type II \( \beta \)-turn being present a significant amount of the time. The
value of the amide temperature coefficient for Arg9 (−1.0 ppb/degree kelvin, Table II, part A) and the NOE pattern confirm this assignment. The NOE pattern and amide temperature coefficients for the rest of the peptide are inconclusive as to the preferred conformations for the NH-terminal region.

As was found for bradykinin, the NOE intensities for HOE-140 increased significantly in the SDS solution. HOE-140 forms two $\beta$-turns: a type II $\beta$-turn between residues 2–5 and a type-II $\beta$-turn between residues 6 and 9 (type-II due to the Asp amino acid in position 7). This conformation is very similar to that reported by Guba and co-workers (28).

NMR was used to assess the effect of the single amino acid change on the COOH-terminal hydrogen bond (Table II, part A). As a base line for the NMR studies, we measured the exchange rate of a non-hydrogen-bonded amide, the amide A). As a base line for the NMR studies, we measured the change on the COOH-terminal hydrogen bond (Table II, part A) and the NOE pattern confirm this assignment. The NOE pattern and amide temperature coefficients for the rest of the peptide are inconclusive as to the preferred conformations for the NH-terminal region.

We asked whether TM-3 played a central role in ligand binding as it does for the amine hormone binding receptors and in rhodopsin. We mutated every hydrogen bond donor or acceptor in TM-3 and mutated the only charged residue, Arg106, to alanine. The mutations were made, expressed, and tested for their ability to bind bradykinin, an agonist, and Phe$^2$-HOE-140, an antagonist (Table I). The only charged residue in TM-3, Arg106, was changed to alanine with no alterations in bradykinin or Phe$^2$-HOE-140 affinity; thus, Arg106 does not contribute an ionic interaction to either ligand-receptor complex. The rest of the TM-3 mutants (Table I) show that TM-3 is not a major contributor of dipole interactions with bradykinin since every potential hydrogen bond donor or acceptor in TM-3 was changed to alanine with no alteration in agonist or antagonist binding affinity.

The pair of mutants N200A and N204A, located at the top of TM-5, were made because they are in positions identical to two serines implicated as hydrogen bond partners with the catechol hydroxyls of adrenergic agonists. These two mutations reduced bradykinin affinity by 4.8- and 2.7-fold, respectively. These very modest reductions in affinity are much smaller than the 10–50-fold reductions in affinity seen by loss of the catechol hydroxyl hydrogen bonds in the adrenergic receptor (33). Neither mutation affected the binding of Phe$^2$-HOE-140 to the receptor. Thus, the participation of Asn$^{200}$ and Asn$^{204}$ in a strong receptor ligand hydrogen bond seems unlikely; however, weaker polar interactions remain a distinct possibility.

Several of the model receptor-ligand complexes suggested that residues in TM-6 and TM-7 might play important roles in ligand binding. These residues, particularly those in TM-6, included residues in positions analogous to residues implicated by mutagenesis and by biophysical and cross-linking studies in amine hormone receptors and in rhodopsin (32, 34, 35). Thus, we choose several of the sites in TM-5, -6, or -7 for further mutagenesis studies (Table I).

Each mutation in TM-5, -6, or -7 that altered bradykinin affinity was paired with another mutation three or four residues (one helical turn) away that also caused an alteration in bradykinin binding affinity. Thus, altered affinities were observed for N200A and N204A, F261A, T265A, and D268A, and D286A and Q290A (Table I and Fig. 3A). Furthermore, Q262A and S246A, mutations between Phe$^{261}$ and Thr$^{265}$ and thus on the opposite side of a helix, caused no alterations in bradykinin affinity. These results suggest that the prediction of a helical conformation in these regions is warranted.

Phe$^{261}$ and Thr$^{265}$ caused the largest reductions in bradykinin affinity, 2000- and 240-fold, respectively, and may reflect the reflections of these two residues in the agonist-ligand interaction (Table I). These two residues are one and two helical turns above and on the same helical face of a conserved tryptophan, Trp$^{258}$, which is part of the FXCXCWPX motif found in TM-6 of most G-protein-coupled receptors (36). Aromatic residues in positions analogous to Phe$^{261}$ and Phe$^{258}$ have been shown to interact with the ligand by forming a hydrophobic pocket around the organic amine hormones or retinal in rhodopsin in their cognate receptors (32, 34, 35).

D268A and D268N caused modest reductions, 3.5- and 3.6-fold respectively, in bradykinin binding affinity (Table I). Asp$^{268}$ was one turn above and on the same helical face of TM-6.
as Thr\textsuperscript{265} and Phe\textsuperscript{261}. Thus, the combined results of these mutations suggest strongly that this part of TM-6 is indeed helical and composes part of a ligand binding site. Asp\textsuperscript{268} could be participating in a salt bridge between the receptor and ligand. Such an interaction may occur, since both arginines at positions 1 and 9 of bradykinin are important for ligand-receptor interactions (Table II). However, a 3.5-fold reduction in affinity corresponds to a free energy of interaction of only 0.8 kcal/mol, which is small for a full ionic interaction. Furthermore, changing Asn\textsuperscript{268} to asparagine causes a 3.6-fold reduction in affinity. Since neither alanine nor asparagine can form anionic bond, the data do not support an ionic interaction with Asp\textsuperscript{268}.

D286A and Q290A caused reductions in bradykinin binding affinity of 60- and 11-fold, respectively. The 60-fold reduced affinity caused by D286A corresponds to 2.5 kcal/mol, which may reflect a moderately strong ionic interaction with the ligand. The smaller effect of the Q290A mutation may reflect a simple hydrogen bond. Several bradykinin structure activity studies, including those shown in Table II, had suggested that the guanido functions of bradykinin, Arg\textsuperscript{1} and Arg\textsuperscript{9}, might be important for receptor-bradykinin interaction, presumably by forming a salt bridge or bridges. In two cases, Asp\textsuperscript{286} was changed to a residue other than the simple loss of side chain change, D286A; these were D286R and D286K. These changes decrease the affinity of BK with D286R by 2000-fold and with D286K by 5000-fold; however, our ability to interpret these findings is limited by the large change in side chain volume and charge of these mutations.

**DISCUSSION**

The observed competition of bradykinin and HOE-140 on the B\textsubscript{2} receptor is in accord with other reports of competitive behavior (37, 38) but in contrast to several reports that in certain biological assays HOE-140 appears to be noncompetitive (38, 39). The finding of noncompetitive behavior for HOE-140 was surprising in view of the structural relationship of HOE-140 and earlier peptidic antagonists that are competitive (39). In the functional assays, the rapid response of the biological systems precludes attainment of equilibrium, thus making it difficult to determine the competitive nature of a ligand. The binding assay is not confounded by regulation of the receptor or postreceptor biological events. Thus, HOE-140 and bradykinin bind to the B\textsubscript{2} BKR in a competitive, mutually exclusive manner, and we speculate that the previous reports of noncompetitive behavior may have resulted from slow equilibrium of HOE-140 or other postbinding regulatory events.

The solution conformation of bradykinin has been extensively studied by NMR spectroscopy (26, 27, 40–49). Here we systematically studied the conformation of BK, HOE-140, and several single residue-altered analogs in aqueous solution and aqueous solution with SDS micelles. In both aqueous solution...
and an aqueous solution containing SDS micelles there is considerable evidence that bradykinin and HOE-140 have a type-II β-turn between residues 6 and 9. Both molecules may also have a weaker tendency to form a type II β-turn between residues 2 and 5. Thus, we believe that the 6-9 β-turn is important in the receptor bond conformation of the peptides. Indeed the addition of the dTic-Oic pair was designed to strengthen this β-turn of HOE-140 (50, 51). The NMR results provide evidence for this structural model.

### Table I

**Affinity of bradykinin, an agonist, and Phe5-HOE-140, an antagonist**

| Residue | Mutant | Affinity (Kd) | Region |
|---------|--------|---------------|--------|
| 3       | R106A  | 1.27 ± 0.12   | TH-3   |
| 5       | R106Q  | 1.03 ± 0.12   | TH-3   |
| 6       | T110A  | 0.84 ± 0.10   | TH-3   |
| 7       | N109A  | 0.85 ± 0.09   | TH-3   |
| 8       | M111W  | 1.00 ± 0.20   | TH-3   |
| 9       | Y113F  | 0.67 ± 0.20   | TH-3   |
| 10      | Y113T  | 0.95 ± 1.44   | TH-3   |
| 11      | L116W  | 1.23 ± 0.67   | TH-3   |
| 12      | L116W/F118W | 1.08 ± 0.20 | TH-3 |
| 13      | Y117A  | 0.22 ± 0.10   | TH-3   |
| 14      | Y117D  | 2.36 ± 1.56   | TH-3   |
| 15      | Y117A/Y115T | 0.80 ± 0.21 | TH-3 |
| 16      | S118A  | 0.77 ± 0.47   | TH-3   |
| 17      | S119A  | 0.77 ± 0.47   | TH-3   |
| 18      | C123S  | 1.16 ± 0.36   | TH-3   |

**Binding Site of Agonists/Antagonists on Bradykinin Receptor**

**TABLE I**

| N | Kd (nM) | s.d. | Ratio (M/WT) |
|---|---------|------|--------------|
| 3 | 1.27    | 0.12 | 3.0          |
| 5 | 1.03    | 0.12 | 2.4          |
| 6 | 0.84    | 0.10 | 1.5          |
| 7 | 0.85    | 0.09 | 2.0          |
| 8 | 1.00    | 0.20 | 2.4          |
| 9 | 0.67    | 0.20 | 1.6          |
| 10| 0.95    | 1.44 | 2.2          |
| 11| 1.23    | 0.67 | 2.9          |
| 12| 1.08    | 0.20 | 2.9          |
| 13| 0.22    | 0.10 | 0.5          |
| 14| 2.36    | 1.56 | 5.6          |
| 15| 0.80    | 0.21 | 1.9          |
| 16| 0.77    | 0.47 | 1.8          |
| 17| 0.77    | 0.47 | 1.8          |
| 18| 1.16    | 0.36 | 2.7          |

**Comparison of wild type and mutant receptors**

**Table I**

| Residue | Mutation | Region |
|---------|----------|--------|
| 3       | R106A    | TH-3   |
| 5       | R106Q    | TH-3   |
| 6       | T110A    | TH-3   |
| 7       | N109A    | TH-3   |
| 8       | M111W    | TH-3   |
| 9       | Y113F    | TH-3   |
| 10      | Y113T    | TH-3   |
| 11      | L116W    | TH-3   |
| 12      | L116W/F118W | TH-3 |
| 13      | Y117A    | TH-3   |
| 14      | Y117D    | TH-3   |
| 15      | S118A    | TH-3   |
| 16      | S119A    | TH-3   |
| 17      | C123S    | TH-3   |

**N.M.T.**

- **Because of their lowered affinity, the competition method was used to determine the affinity of the designated mutants.**
- **Mixtures of data from transient transfection of COS-7 cells and stable CHO cell lines. No difference in the affinity of the receptor-expressed COS-7 cell or CHO cells was detected for wild type or mutant receptors, not shown.**
Fig. 3. A, summary diagram showing the positions and magnitudes of mutations that affect bradykinin binding affinity (circles) and HOE-140 binding affinity (triangles). B, stereoimage of one model of bradykinin binding to the BKR incorporating data from these studies. A two-dimensional representation of this model is shown in model 3 of Fig. 2. The identity of the helices are shown for the right image. The plane at the top shows the approximate extracellular boundary of the membrane.
confirm the presence of the β-turn, and the micelle results suggest that hydrophobic-hydrophobic interactions, such as those that may occur when the peptides interact with helical regions of the bradykinin receptor, will stabilize the turn.

The modeling of GPC-7TM receptor structure is a qualitative effort due to the small amount of structural data available about this class of receptors. Our models were designed to give ideas for further mutagenesis experiments and possible binding modes of agonists and antagonists. Our models differ somewhat from those previously described (52, 53). The major difference centers on each group’s choice for the ends of the α-helices, particularly TM-6 and TM-7. We chose to include one extra turn in the α-helices because of the uncertainties inherent in current methods of assigning helix start and end points; as a consequence of the differences in helical end points, our models suggest that Asp 286 is at the end of TM-7, while Kyle et al. predicted it is in a loop. We believe that the Gln 290 mutation four residues or one helical turn from Asp 286 suggests a helical conformation for this region. Current computation algorithms tend to preserve secondary structure present in the starting structure. This sensitivity of the current computational methods to initial starting conditions contributes greatly to the qualitative nature of the models.

Our results in combination with those of Novotny et al. (54) and Maradone and Hogan (55) provide mutations of all of the acidic residues in extracellular domains 3 and 4 and the extracellular halves of TM-4, TM-5, TM-6, and TM-7 of the B2 receptor. Only residues Glu 199 (4.7-fold reduced in bradykinin affinity (54)), Asp 286 (3.5-fold reduced in affinity, Table I) and Asp 286 (60-fold reduced in affinity, Table I) are potentially interacting with the receptor. Table I shows the affinity comparisons of BK and HOE-140 analogs, with each analog containing a single change from the parent molecule. The affinities were measured on membranes of CHO cells expressing the rat or human BKR. The ratio of analog parent is shown along with the amide-9 proton exchange rate measured by NMR. Part B, pairwise comparison of the affinity of [Asp-1]Bk, [Asp-9]Bk, and [Glu-9]Bk with the wild type receptor and a D286R mutant receptor.

### Table II

**Affinity comparisons**

| Compound          | Rat BKR-B2 Kd(nM) | Ratio-Arg<sup>9</sup>-Amide Parent | Human BKR-B2 Kd(nM) | Ratio-Arg<sup>9</sup>-Amide Parent | Arg<sup>9</sup>-Amide Proton Exchange (ppb/K) |
|-------------------|-------------------|-----------------------------------|-------------------|-----------------------------------|----------------------------------|
| BK-amide          | 64                | 0.42                              | 150               | 0.42                              | 360                              | -6.6                             |
| BK                |                   |                                   |                   |                                   |                                  | -4.9                             |
| HOE-140-amide     | 0.14              | 0.15                              |                   |                                   | 14                               | -2.0                             |
| HOE-140           |                   |                                   |                   |                                   |                                  | -1.0                             |
| des Arg<sup>9</sup>-BK | 15500             | 37,000                            | 63000             | 150,000                           |                                  | -                               |
| BK                | 0.42              | 0.42                              | 0.42              | 0.42                              |                                  | -4.9                             |
| HOE-140           | 4.5               | 41                                | 67                | 450                               |                                  | -1.0                             |
| des Arg<sup>9</sup>-HOE-140 | 0.14              |                                   | 0.15*             |                                   |                                  | -1.0                             |
| Ala<sup>3</sup>-BK |                   |                                   |                   |                                   |                                  |                                  |
| BK                | 0.42              |                                   | 11200             | 27,000                            | -4.6                             |
| HOE-140           | 0.11              |                                   | 1.1               | 7                                 | -2.9                             |
| Ala<sup>6</sup>-BK | 16                | 150                               | 43                | 100                               | -3.9                             |
| BK(Ser<sup>9</sup>) | 0.42              |                                   | 0.42              |                                   | -4.9                             |
| Ala<sup>6</sup>-HOE-140 | -                 | 0.12*                             | 1                 |                                   | -6.1                             |
| HOE-140           | 0.11              |                                   | 0.15*             |                                   | -1.0                             |
| Ala<sup>1</sup>-BK | 1650              | 3900                              |                   |                                   | -4.4                             |
| BK                | 0.42              |                                   |                   |                                   | -4.9                             |
| Des-Arg<sup>1</sup>-HOE-140 | 0.15              | 1                                 |                   |                                   | NM                               |
| HOE-140           | 0.11              |                                   |                   |                                   | -1.0                             |
| Ala<sup>1</sup>-Des-Arg<sup>1</sup>-HOE-140 | 19                | 170                               |                   |                                   | -5.9                             |
| HOE-140           | 0.11              |                                   |                   |                                   | -1.0                             |

**Gly 5 Amide Proton exchange (Average of 12 compounds)** (Mean ± S.D.) -8.7 ± 0.8

| Compound          | Wild Type Kd(nM) | Mutant D286R Kd(nM) | Arg<sup>9</sup>-Amide Proton Exchange (ppb/K) |
|-------------------|------------------|---------------------|----------------------------------|
| Asp<sup>1</sup>-BK | 24,000           | NM                  | NM                              |
| BK (Arg<sup>1</sup>) | 0.42             | 840                 | -4.9                            |
| Asp<sup>9</sup>-BK | >1 mM            | >1 mM               | -5.0                            |
| BK (Arg<sup>9</sup>) | 0.42             | 840                 | -4.9                            |
| Glu<sup>9</sup>-BK | >1 mM            | >1 mM               | -6.3                            |
| BK (Arg<sup>9</sup>) | 0.42             | 840                 | -4.9                            |
by an ionic bond. Of these potential interactions, only the D286A mutation seems strong enough (2.5 kcal/mol) to warrant clear assignment as an ionic interaction.

To further test the idea that Asp286 provides an ionic interaction with bradykinin, we attempted a double switch, wherein Asp286 was changed to arginine, D286R, and the Arg of bradykinin was changed to aspartic acid, [Asp9]BK, or glutamic acid, [Glu9]BK (Table II, part B). [Asp9]BK and [Glu9]BK have no measurable affinity with wild type or D286R bradykinin receptors. We checked [Asp9]BK and [Glu9]BK for the presence of a COOH-terminal hydrogen bond by measuring the proton exchange rate of the residue-9 amide hydrogen. The COOH-terminal $\beta$-turn is apparently still intact, since the exchange rates for [Asp9]BK and [Glu9]BK were $-5.0$ ppb/degree kelvin and $-6.3$ ppb/degree kelvin, respectively; thus, the poor affinity of [Asp9]BK is not due to the loss of the COOH-terminal turn (Table II, part B). These data do not confirm the Asp286Arg ionic interaction hypothesis. However, other alterations in receptor-bound peptide structure or receptor structure could prevent Arg286 from a successful interaction with [Asp9]BK; thus, a residue 286 ionic interaction is still possible.

One of the most notable results of our mutagenesis study is the lack of effect of most of these mutations on antagonist, Phe5-HOE-140, binding (summarized in Fig. 3A). The simple elimination of side chain mutations, changes to alanine, which affected bradykinin affinity had no effect on Phe5-HOE-140 affinity (Table I) For example T265A, D286A, and Q290A caused large decreases in BK binding affinity, while Phe5-HOE-140 affinity was unaltered. F261A did cause a small 5.8-fold effect on Phe5-HOE-140 affinity, but the magnitude of this effect is much less than its effect on BK affinity, 2200-fold.

The apparent separation between mutations that affect bradykinin binding from mutations that affect HOE-140 binding led us to make a series of single site changes in bradykinin and HOE-140 (Table II, part A). As in the receptor mutation studies, bradykinin and HOE-140 did not change in parallel. For example, removal of the arginine 9 side chain from bradykinin, alanine 9 bradykinin, caused a 27,000-fold decrease in affinity toward the receptor; in contrast, removal of the arginine side chain from HOE-140 caused only a 7-fold decrease in receptor affinity. All of the peptides were found to have NOEs and Arg9 amide exchange times consistent with a COOH-terminal $\beta$-turn. These results suggest that the affinity changes observed for the mutant ligands are not due a gross ligand conformation change due to the change in peptide sequence. Other mutation pairs that illustrate the different behavior of agonist and antagonists include [Ala9]BK and [Ala9]HOE-140 or [Ala9]BK and [des-Arg9-Ala1]HOE-140. Possibly the most surprising result is the effect of the removal of the ninth residue, [des-Arg9]BK and [des-Arg9]HOE-140. These molecules cannot form a 6–9 $\beta$-turn, since residue 9 is missing; yet, [des-Arg9]HOE-140'a affinity is only reduced 41-fold on the receptor and 450-fold on the human receptor. In contrast, [des-Arg9]BK is reduced in affinity 37,000-fold on the receptor and 150,000-fold on the human receptor.

A model was built incorporating the receptor mutagenesis data and the ligand analog and NMR data; this model summarizes our best hypotheses of how BK and HOE-140 interact with the receptor and is shown in Fig. 3. This model is very similar to two-dimensional model 3 in Fig. 2. The main features of the model include a Asp286Arg salt bridge, a hydrophobic pocket composed of Tyr177, Trp179, Phe261, Trp261 and Trp286, these residues surround phenylalanine 8. The amino terminus of bradykinin emerges from the top of the receptor in the TM-4 and TM-5 region (Fig. 3B). Involvement of the top of TM-4 and TM-7 in agonist (BK) binding is also supported by the observed inhibition of bradykinin binding by antibodies directed to the amino-terminal half of extracellular domain 3 and the carboxy-terminal half of extracellular domain 4 (56). We are still uncertain of the relationship of the NH2 and COOH termini of bradykinin but tend to favor molecules with the termini close together because of the observation that e-amino cyclolalkadinin is an agonist, albeit with 1000-fold reduced affinity (24, 57).

Comparisons of the mutations that affect HOE-140 binding with those that affect BK binding suggest the binding pocket for HOE-140 might be one turn deeper in the transmembrane regions and closer to TM-7 than is the bradykinin binding site. In these models of the HOE-140 hydrophobic pocket composed of Phe261, Trp286 from TM-6 and Tyr277 from TM-7 may contribute to interactions with the large $\beta$-turn-forcing residues, Tic and Oic. Models built with this arrangement for the HOE-140 binding pocket also suggest that Asp286 does not contact Arg286 of HOE-140 as it does in the BK models. One possible arrangement has the Arg$^\gamma$ of HOE-140 making a salt bridge with Asp286. However, the recent observation that antibodies that are directed to the carboxyl half of extracellular domain 4 and the top of TM-7 are unable to inhibit HOE-140 binding suggests that HOE-140 interacts weakly with the top of TM-7 (56).

The lack of parallelism of receptor mutations and ligand changes on the BK and Phe5-HOE-140 affinity was unexpected. Furthermore, a number of alanine amino acid replacements were found that affected HOE-140 binding but not BK binding; these included P254A (6.8-fold), Q262A (4.1-fold), and Y297A (6.6-fold). The size of these effects is small compared with the size of mutations that affect BK binding. These differences in magnitude suggest that BK makes a few specific and strong contacts to the receptor, while HOE-140 makes many weaker contacts on the receptor. These data imply that although these similar compounds are classified as competitive it can not be automatically assumed that they make the same atomic interactions within the receptor; furthermore, the data suggest that each may have distinct binding interactions. These interpretations suggest that in spite of the structural similarity of bradykinin and HOE-140 and their competitive behavior they may occupy sites that are one helical turn removed from each other. This observation may fit with the widely noted observation by medicinal chemists that agonists and antagonists frequently have divergent structure activity relationships (58, 59).

The results and the derived models for bradykinin's interaction with its receptor present a picture that is somewhat different from the picture presented for substance P, angiotensin, and interleukin-8 when these GPC-7TM receptors contact their ligands (60–64). These ligands all have several contacts with extracellular regions of the receptor; albeit for most of these cases the number of transmembrane mutations that have been made is small. It is possible that the bradykinin receptor ligand binding site represents an exceptional peptide receptor whose site is more akin to the small amine and rhodopsin ligand pockets; however, a more unifying view might be that peptides bind to their receptors using the extracellular sequences to gain most of their binding energy (affinity) and specificity while a small part of the ligand interacts with helical regions near TM-5, TM-6, and TM-7. This kind of a model has been presented for the carboxyl end of the 74-residue C5a (65) and is implicated for the amino end of chemokines, such as interleukin-8 and monocyte chemotactic peptide-1 (66–68). These ligands all appear to have many high affinity contacts with extracellular regions and a small region of the ligand, which contributes minimal binding energy but confers the ability to be an agonist. These results and the size of bradykinin, 9 amino acids, suggest that the bradykinin binding site may be similar.
to other peptide binding sites using both extracellular sequences and helical sequences for affinity; however, BK may have more of the affinity-determining sequences in helical regions because of its small size and compact conformation.

Acknowledgments—We thank Dr. Howard Parmes, Emma Shelton, and Tanya Veronion for the synthesis and purification of [3H]Phe5-

REFERENCES

1. Proud, D., and Kaplan, A. P. (1988) Annu. Rev. Immunol. 6, 49–83
2. Hall, J. M. (1993) Pharmacol. & Ther. 56, 131–190
3. Dray, A., and Perkins, M. (1993) Trends Neurosci. 16, 99–104
4. McEarchern, A. E., Shelton, E. R., Bhakta, S., Obernolte, R., Bach, C., Zupp, P., Fuijishi, J., and Jarnagin, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7724–7728
5. Park, J., Freedman, R., Bach, C., Yee, C., Bohlwid, M., Kanninihi, H., Muller-Esterl, W., and Jarnagin, K. (1994) Braz. J. Med. Biol. Res. 27, 1707–1724
6. Hess, J. F., Borkowski, J. A., Young, G. S., Strader, C. D., and Ransom, R. W. (1992) Biochem. Biophys. Research Commun. 184, 260–268
7. Menke, J. G., Borkowski, J. A., Bierlo, K. K., MacNeil, T., Derrick, A. W., Schnee, K. A., Ransow, R. W., Strader, C. D., Linemeyer, B. L., and Hess, J. F. (1994) J. Biol. Chem. 269, 21585–21586
8. Costello, A. H., and Hargraves, R. E. (1989) Eur. J. Pharmacol. 171, 259–263
9. Steranka, L. R., Dehaas, C. J., Vavrek, R. J., Stewart, J. M., Enna, S. J., and Synder, S. H. (1987) Eur. J. Pharmacol. 136, 261–262
10. Steranka, L. R., Manning, D. C., Dehaas, C. J., Perkany, J. W., Borosky, S. A., Connor, J. R., Vavrek, R. J., and Synder, S. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3245–3249
11. Heapy, C. G., Shaw, J. S., and Farmer, S. C. (1993) Br. J. Pharmacol. 108, 209–213
12. Griesbacher, T., Sutliff, R. L., and Lembeck, F. (1994) Br. J. Pharmacol. 112, 1094–1096
13. Weipert, J., Hoffmann, H., Siebeck, M., and Whalley, E. T. (1988) Br. J. Pharmacol. 94, 282–284
14. Whalley, E. T., Solomon, J. A., Modaffer, D. M., Bonham, K. A., and Cheronis, J. C. (1992) Agents Actions Suppl. 38, 413–420
15. Wilson, D. D., De Garavilla, L., Kuhn, W., Togo, J., Burch, R. M., and Steranka, L. R. (1989) Circ. Shock 29, 95–101
16. Proud, D. (1994) J. Biol. Chem. 269, 2021–2031
17. Austin, C. E., Foreman, J. C., and Scadding, G. K. (1994) Br. J. Pharmacol. 111, 969–971
18. Allen, N. C., Brundish, D. E., Martin, J. R., and Wade, R. (1981) J. Chem. Soc. 2640–2648
19. Evans, E. A. (1974) Tritium and Its Compounds, pp. 326–330, Butterworth & Co., Ltd., London
20. Sambriski, J., Price, E. F., and Manatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Fossanby, J. R. (1983) Culture of Animal Cells: A Manual of Basic Technique, Alan R. Liss, Inc., New York
22. Villarreal, X. C., and Long, G. L. (1991) Anal. Biochem. 2040–2048
23. Sved, B. (1993) Trends in Cell Biology, 3, 460–468
24. Ariens, E. J. (1971) in Drug Design (Ariens, E. J., ed) p. 176, Academic Press, Inc., New York
25. Martina, Y. C., Bush, E. N., and Kyncl, J. J. (1990) in Drug Design (Ariens, E. J., ed) p. 176, Academic Press, Inc., New York
26. Martina, Y. C., Bush, E. N., and Kyncl, J. J. (1990) in Drug Design (Ariens, E. J., ed) p. 176, Academic Press, Inc., New York
27. Brown, J. M., and Grubman, S. L. (1994) J. Biol. Chem. 269, 7457–7470
28. Wu, C.-F., Chuang, C.-T., Chuang, C.-Y., and Wang, M.-T. (1994) Pharmacol. & Ther. 63, 455–470
29. Oxford, J. B., and Oxford, J. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1094–1101
30. Schwartz, B. (1994) Immunology Today 15, 374–379