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Biochemistry

Delineation of a region in the B2 bradykinin receptor that is essential for high-affinity agonist binding

(site-directed mutagenesis/binding site/transmembrane helix/neuropeptide receptor/G-protein-coupled receptor)

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ABSTRACT **We have made mutations in the predicted sixth transmembrane segment of a rat B2 bradykinin receptor and analyzed the variant proteins by expressing them in COS-1 cells. Two amino acid substitutions reduced the affinity of the receptor for bradykinin (Phe^{261} → Val by 1600-fold; Thr^{265} → Ala by 700-fold) with comparatively little effect on the affinity for the bradykinin antagonists NPC17731 and D-Arg-[Hyp^3,D-Phe^7]bradykinin (where Hyp is hydroxyproline). Three other substitutions (Gln^{262} → Ala, Asp^{268} → Ala, and Thr^{269} → Ala) modestly reduced the affinity for bradykinin and for the antagonist D-Arg-[Hyp^3,D-Phe^7]bradykinin. Even the most dramatically affected mutated receptors were still able to couple, after bradykinin binding, to phosphatidylinositol turnover. The data suggest that bradykinin directly contacts the face of the sixth transmembrane helix formed by the residues Phe^{261}, Gln^{262}, Thr^{265}, Asp^{268}, and Thr^{269} or that this face of the helix is the site of intraprotein contacts that serve to stabilize the agonist-binding conformation of the receptor.**

Bradykinin, a by-product of the blood clotting cascade or of plasma leakage into the extravascular space, is a potent mediator of pain and is also involved in inflammation and other pathological processes (1–3). There is currently intensive interest in the possible clinical uses of bradykinin antagonists (3–7). Bradykinin agonists may also be useful therapeutically, since there is evidence that kinins exert a cardioprotective effect in myocardial ischemia (8–10).

A first step in the rational design of bradykinin agonists and antagonists is the identification of residues in the bradykinin receptor that participate directly in ligand binding. This information can then be used to form a more refined picture of the geometry of the ligand binding site by examining the interaction with conformationally constrained ligands having different substituents, as illustrated by recent studies of ligand–receptor interactions in the neurokinin 1 receptor (11).

A B2 bradykinin receptor that may be responsible for many of the pharmacological actions of bradykinin has been cloned from human and rat tissues (12–15). It is a member of the family of G-protein-coupled receptors, and there is a high degree of conservation of the amino acid sequence between rat and human.

Here we present an analysis of the effect on ligand binding of amino acid substitutions in the predicted sixth transmembrane segment (16) of the B2 bradykinin receptor. We show that a narrowly delineated region in this transmembrane segment is essential for formation of a high-affinity binding site for bradykinin and that specific amino acids in this region interact strongly with bradykinin, but not with two bradykinin antagonists.

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METHODS

Molecular Biology. Isolation of a B2 bradykinin receptor cDNA from PC12 cells and its subcloning into the expression vector pLGP3 were described in the preceding paper (15). Single-stranded plasmid DNA containing uracil (17) was prepared by published techniques (18). Site-directed mutagenesis was performed as described by Kunkel *et al.* (17). In most cases the mutagenesis primer also introduced a translationally silent mutation that either created or destroyed a restriction enzyme recognition site, to facilitate initial identification of plasmids carrying the desired mutation. The presence of the desired mutation was confirmed by DNA sequencing by the dideoxy chain-termination method (19). The DNA sequence of the entire coding region was determined for those cDNAs encoding proteins that showed altered binding of bradykinin. Plasmid DNA was prepared by standard techniques (20) and was introduced into COS-1 cells by electroporation (21).

Functional Assays. Equilibrium binding of [^{3}H]bradykinin or [^{3}H]NPC17731 (DuPont/NEN) to COS-1 cells expressing mutated receptors was determined as described (15). K_d and B_{max} were estimated from these measurements by Scatchard analysis (22). The ability of unlabeled bradykinin or of unlabeled D-Phe^7-substituted kinins to compete for the binding sites was examined in equilibrium assays (15), and data were fitted to a single hyperbola by using a nonlinear least-squares program, yielding an estimate of IC_{50}. K_i was calculated by using the standard correction factor $(1 + [L]/K_L)^{-1}$, where [L] is the concentration of labeled ligand in the experiment, and K_L is the K_d for binding of the labeled ligand by the receptor measured in saturation binding experiments. Levels of inositol phosphates in control cells and in cells stimulated for 20 sec with bradykinin were measured according to Berridge *et al.* (23, 24) in cultures that had been incubated for 48 hr in medium containing [^{3}H]inositol.

RESULTS

Initial Screening of Mutants in the Sixth Transmembrane Segment. Mutated bradykinin receptors were expressed in COS-1 cells, and equilibrium high-affinity binding of [^{3}H]bradykinin was examined with [^{3}H]bradykinin concentrations in the low nanomolar range. Two mutated receptors with the substitutions Thr^{265} → Ala (T265A) (Fig. 1) and F261V showed little binding in this range of [^{3}H]bradykinin concentrations. Three other mutated receptors—Q262A, D268A, and T269A—had an affinity for [^{3}H]bradykinin that was decreased by a factor of 5–20 relative to the affinity of the wild-type receptor (Table 1). The remaining mutated receptors—F259A, I263A, S264A (Fig. 1), F266A, and L267A—did not differ from the wild-type receptor in affinity

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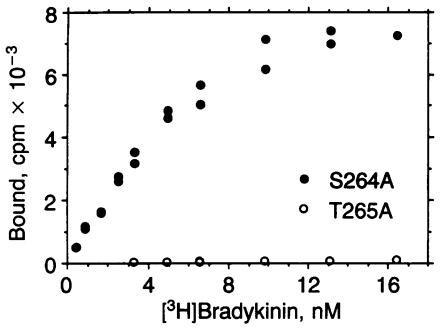

FIG. 1. Specific binding of [³H]bradykinin to COS-1 cells expressing mutated bradykinin receptors. ●, S264A; ○, T265A. K_d for binding to the S264A receptor was 9.1 nM in this experiment. There was negligible specific binding to the T265A receptor. Nonspecific binding in the presence of 20 μM unlabeled bradykinin, which was 110 cpm for S264A and 75 cpm for T265A at the highest concentration of [³H]bradykinin, has been subtracted.

for bradykinin (Table 1). In all cases where the mutated receptors exhibited high-affinity binding, the proteins were efficiently expressed. Levels of receptor expression, measured as the number of sites per cell transformed with DNA, were 2.9×10^5 to 2.3×10^6 sites per cell for wild-type receptor (15) and 7.8×10^4 to 1.3×10^6 sites per cell for mutated receptors.

To probe additional features of the ligand binding site, we examined the ability of the B2 antagonist D-Arg-[Hyp³,D-Phe⁷]bradykinin to compete with [³H]bradykinin for the binding site of the mutated receptors (Table 1). Two of the mutations, Q262A and D268A, that modestly reduced affinity for [³H]bradykinin also resulted in a 5- to 20-fold shift in affinity for the B2 antagonist. Another mutation, T269A, that modestly reduced the affinity for bradykinin had a lesser effect on the affinity for D-Arg-[Hyp³,D-Phe⁷]bradykinin, and the five mutations that did not affect affinity for [³H]bradykinin had no effect on affinity for the antagonist. Receptors with the substitutions T265A and F261V were not examined in this assay, since they failed to bind sufficient [³H]bradykinin.

T265A. While the substitution T265A nearly eliminated binding of [³H]bradykinin at nanomolar concentrations, it did not affect high-affinity binding of the antagonist [³H]NPC17731 (Fig. 2 and Table 1). The preservation of high affinity for [³H]NPC17731 constitutes direct evidence of correct folding and expression of the altered receptor. The number of [³H]NPC17731 binding sites present at the cell surface (average of 3.3×10^5 sites per cell) was comparable to the number of receptor sites in COS-1 cells expressing the wild-type receptor. Competition between unlabeled bradykinin and [³H]NPC17731 for the binding site demonstrated that bradykinin does bind to the mutated receptor, with a K_i, 1.3 μM, that represents a 700-fold shift in affinity relative to the wild-type receptor (Fig. 3 and Table 1). Similar competition experiments indicated a modest reduction in affinity for D-Arg-[Hyp³,D-Phe⁷]bradykinin, with an estimated K_i of 140 nM (Fig. 3 and Table 1). This decrease in affinity, which could be related to the fact that D-Phe⁷-substituted bradykinin analogues have partial agonist activity at B2 receptors, is small in comparison to the effect on [³H]bradykinin binding.

F261V. Like the T265A mutation, the substitution F261V had only a small effect on the binding of [³H]NPC17731 (K_d 14 nM) (Table 1), and did not interfere with expression of high levels of receptor (average 3.9×10^5 sites per cell) at the cell surface. The K_i for unlabeled bradykinin binding, determined by competition with [³H]NPC17731, was dramatically shifted to 2.9 μM, a 1600-fold reduction in affinity compared to wild-type receptor (Fig. 3 and Table 1). The K_i for D-Arg-[Hyp³,D-Phe⁷]bradykinin was 24 nM (Fig. 3 and Table 1).

Coupling to Phosphatidylinositol Turnover. The mutated receptors with severely reduced affinity for bradykinin, F261V and T265A, were expressed in COS-1 cells and tested for their ability to couple bradykinin binding to phosphatidylinositol turnover. Because of the demonstrated low affinity of these mutated receptors for bradykinin, 100 μM ligand was used in assays of the F261V mutant, and 10 μM ligand in assays of the T265A mutant. Bradykinin increased inositol

Table 1. Effect of substitutions in the sixth transmembrane helix on affinity of the receptor for bradykinin and related peptides

	Bradykinin		NPC567*		NPC17731†	
Receptor	K_d, nM (range)	n	K_i, nM (range)	n	K_d, nM (range)	n
Wild type	4.4 (1.5–7.5)‡	16	7.5 (4.3–9.2)‡	6	3.6 (2.3–4.8)	2
Wild type	1.8 (1.4–2.2)§	2				
F259A	3.7 (3.2–4.0)	4	6.4 (6.1–6.7)	2		
F261V	2900 (2300–3500)§	2	24 (12–36)	3	14 (7.8–20)	2
Q262A	35 (28–42)	2	120 (54–210)	3		
I263A	8.3 (6.9–9.6)	2	6.4 (8.0–8.5)	2		
S264A	7.8 (6.5–9.1)	2	7.8 (7.5–8.0)	2		
T265A	1300 (770–1900)§	2	140 (140–140)	2	5.2 (3.2–7.2)	2
F266A	9.9 (7.8–12)	2	5.7 (5.1–6.2)	2		
L267A	8.9 (8.5–9.2)	2	4.2 (2.8–5.6)	2		
D268A	33 (20–53)	3	56 (38–80)	3		
T269A	33 (17–48)	2	21 (17–24)	2	9.6 (6.1–13)	2

The indicated receptors were expressed in COS-1 cells, and equilibrium binding measurements were performed as described in *Methods*. Values are given as mean (range) for n determinations. Values shown as K_d were determined by Scatchard analysis of binding data for [³H]bradykinin or [³H]NPC17731 as indicated. Values shown as K_i were determined from the IC_{50} for competition of the indicated unlabeled ligand with [³H]NPC17731 (F261V and T265A) or with [³H]bradykinin (all other receptors) as described in *Methods*. The shifts in affinity stated in the text are with reference to the corresponding value (K_d or K_i) for the wild-type receptor. The small discrepancy between K_d and K_i for bradykinin binding to the wild-type receptor probably reflects imprecision in the assumed value for the specific activity of the labeled bradykinin or for the concentrations of the labeled or unlabeled bradykinin stocks.

*D-Arg-[Hyp³,D-Phe⁷]bradykinin.

†D-Arg-[Hyp³,D-Hyp(*trans*-propyl ether)⁷,Oic⁸]bradykinin (where Oic is octrahydroindole-2-carboxylic acid).

‡Data from Nardone *et al.* (15).

§K_i values determined from competition assays between unlabeled bradykinin and [³H]NPC17731.

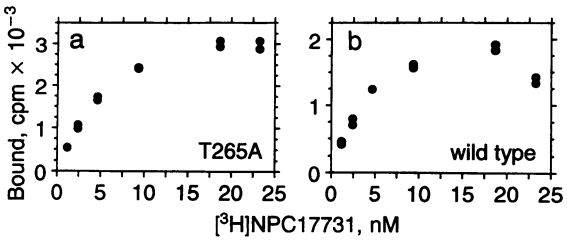

FIG. 2. Specific binding of [³H]NPC17731 to COS-1 cells expressing T265A receptor (*a*) or wild-type receptor (*b*). K_d was 7.2 nM for the T265A receptor and 4.8 nM for the wild-type receptor in the experiments shown.

bisphosphate and inositol trisphosphate in cells expressing either altered receptor. The increases in inositol trisphosphate (range of 1.7- to 4.5-fold for F261V and 1.7- to 2.0-fold for T265A in two experiments) were similar to the stimulation obtained in COS-1 cells expressing the wild-type B2 receptor. Incubation with 100 μM bradykinin did not affect the levels of inositol bisphosphate and trisphosphate in control COS-1 cells or in COS-1 cells transformed with cDNA encoding a nonfunctional bradykinin receptor.

DISCUSSION

This paper analyzes the contribution of amino acid residues in the predicted sixth transmembrane segment (16) of the B2 bradykinin receptor to binding of bradykinin. We have replaced 10 individual residues from position 259 to position 269 of the rat B2 receptor with alanine or valine, thus scanning the external portion of the sixth transmembrane segment except for the conserved proline at position 260. In this region we identified two mutations, T265A and F261V, that greatly reduced the affinity of the receptor for [³H]bradykinin and three other mutations, Q262A, D268A, and T269A, that modestly reduced the affinity.

The five substitutions F261V, Q262A, T265A, D268A, and T269A appear to affect binding of bradykinin through a nondisruptive alteration of receptor structure, most likely involving simply the loss of interactions made by the original side chain, rather than by causing abnormal folding of the receptor protein (discussed in refs. 25 and 26). Each mutation replaces an existing side chain with a smaller side chain, a

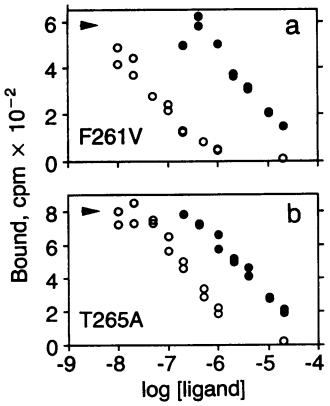

FIG. 3. Estimates of the affinities of mutated receptors for kinins by competition of the unlabeled peptides with [³H]NPC17731. (*a*) Binding of bradykinin (●; IC_{50} 4.5 μM, K_i 2.3 μM) and D-Arg-[Hyp³,D-Phe⁷]bradykinin (○; IC_{50} 47 nM, K_i 24 nM) to F261V. (*b*) Binding of bradykinin (●; IC_{50} 4.0 μM, K_i 1.9 μM) and D-Arg-[Hyp³,D-Phe⁷]bradykinin (○; IC_{50} 290 nM, K_i 140 nM) to T265A. Arrowheads in each panel represent binding of [³H]NPC17731 in the absence of competing unlabeled peptide. [³H]NPC17731 concentration was 14 nM in the measurements on F261V and 6 nM in the measurements on T265A.

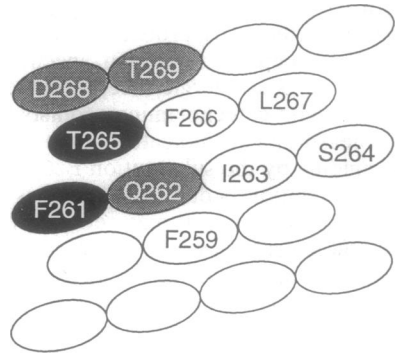

FIG. 4. Part of the predicted sixth transmembrane α-helix drawn in cylindrical projection. Residues analyzed in this work are labeled. Dark shading indicates the positions at which substitution of alanine or valine resulted in a large reduction in affinity for bradykinin, with less change or no change in affinity for the antagonists tested. Medium shading indicates the positions where substitution of alanine caused a modest reduction in affinity for bradykinin and for antagonist. The residues essential for high-affinity binding of bradykinin are located along one face of the α-helix.

strategy that minimizes the probability of global changes in protein structure (25, 26). Further, several experimental criteria support the conclusion that the amino acid replacements have not caused global alterations of protein structure. First, comparable levels of wild-type and mutated receptors are expressed at the cell surface in COS-1 cells transformed with the corresponding cDNAs. Second, the receptor proteins are folded correctly to produce high-affinity binding sites for the B2 antagonists D-Arg-[Hyp³,D-Phe⁷]bradykinin and NPC17731, even when high-affinity binding of bradykinin is compromised. Finally, the F261V and T265A receptors are able to assume functional resting and active conformations, since they can activate phosphatidylinositol turnover in response to bradykinin binding.

In the following discussion, we consider three simple ways in which a nondisruptive removal of amino acid side chains could result in a substantially lower affinity for bradykinin and lead to a strong discrimination between bradykinin and bradykinin antagonists, traits seen most vividly in the mutants T265A and F261V. (*i*) The residues we have mutated may be located in the binding site and directly contact bradykinin. (*ii*) The residues we have mutated may come into direct contact with bradykinin after a bradykinin-induced conformational change. (*iii*) The residues we have mutated may be located at a site remote from the ligand binding site, and their mutation may have an allosteric effect on bradykinin binding. For some mutations a combination of these mechanisms might act to reduce the affinity for bradykinin.

Direct Contact with Agonist. One explanation for our results is that residues F261 and T265 are part of a contact site for agonist. These residues, together with the adjacent residues Q262, D268, and T269, are aligned on one face of a predicted α-helix and present a relatively hydrophilic surface (Fig. 4). Under this interpretation, Q262, D268, and T269 in the B2 bradykinin receptor might form a common contact site for both agonist and antagonist kinin peptides, while F261 and T265 interact more strongly with agonists.

The equilibrium binding measurements indicate that the complex of bradykinin with the T265A mutant is less stable than its complex with wild-type receptor by 3–4 kcal/mol, suggesting, according to this contact-site model, that the low affinity of the T265A mutant for bradykinin reflects loss of a hydrogen bond between T265 and a charged group of the ligand (26, 27). The destabilization of bradykinin binding to the T269A mutant relative to wild-type receptor, which is only ≈1.5 kcal/mol, could be accounted for by loss of an

uncharged hydrogen bond (26, 27). Hydrogen bonding interactions have been extensively probed by mutation of other G-protein-coupled receptors, and two specific serine hydroxyl groups of the β-adrenergic receptor have been identified that interact with catecholamine agonists (28).

Our binding measurements indicate that the destabilization of bradykinin binding by the substitution F261V is ≈4 kcal/mol. If the destabilization is due to simple loss of an interaction with bound bradykinin, it may reflect formation of a cavity in the mutated protein, where the close van der Waals contact between the ligand binding site and the complementary surface of the ligand is interrupted. Comparable energetic contributions of buried nonpolar side chains to protein folding have been measured experimentally in a *Bacillus amyloliquefaciens* ribonuclease (barnase), where the buried side chains were truncated by Ile → Ala or Phe → Leu substitutions (29). The participation of nonpolar as well as polar interactions in high-affinity binding of a small peptide ligand has been illustrated in the three-dimensional structure of a complex of angiotensin with antibody (30).

A part of the sixth transmembrane helix of other G-protein-coupled receptors corresponding to the region we have examined may participate in ligand binding. Thus, retinal can be crosslinked to residues in the corresponding part of the sixth transmembrane helix of rhodopsin (31), and specific residues in the sixth transmembrane segment of opsin and related proteins have a role in determining the spectral characteristics of rhodopsin and of cone photopigments (32–35). Residues F289 and F290 in the sixth transmembrane segment of the β-adrenergic receptor (which correspond to F261 and Q262 in the B2 bradykinin receptor) and residue Y506 in the M3 muscarinic acetylcholine receptor (which corresponds to F261) have been thought to interact selectively with agonists (36–38). General structural models that have been proposed for the G-protein-coupled receptors also are consistent with contact between the ligand and side chains projecting from the sixth transmembrane helix at the positions occupied by F261 and T265 in the bradykinin receptor (16, 39).

A variant of the contact-site hypothesis is that the side chain of F261 or T265 in the wild-type receptor is involved in local intraprotein interactions that position other essential residues in the binding site for interaction with ligand—for example, through hydrogen bonding, amino–aromatic interactions, or aromatic–aromatic interactions.

Direct Contact with Agonist Only After an Agonist-Induced Conformational Change. Given that G-protein-coupled receptors undergo conformational changes following ligand binding, a second possibility is that bradykinin does not interact with F261 or T265 until after it has induced a conformational change in the receptor. Based on the precedent of rhodopsin (40, 41), the bradykinin receptor probably undergoes a series of conformational changes within microseconds to milliseconds after binding bradykinin. In the course of these conformational changes, some interactions of bradykinin with the initial conformation of the wild-type receptor may be lost, and there may be other energetic costs of the conformational change, with these energetic costs being compensated by new bradykinin–receptor interactions. A residue whose contribution to binding depends on the conformational change would not interact with antagonists, because antagonists do not trigger the necessary conformational change. A corollary, important in analyzing the effect of amino acid substitutions in the bradykinin receptor and other G-protein-coupled receptors, is that equilibrium binding assays are likely to probe different conformations of the receptor depending on whether an agonist or an antagonist ligand is used.

Allosteric Action Preferentially Destabilizing the Conformation That Binds Agonist. A third possibility is that the amino acid substitutions studied are at a position remote from the ligand binding site and reduce affinity for agonist indirectly by favoring a naturally occurring conformation of the receptor that binds agonist with low affinity. This model implies that the substitutions F261V or T265A in the bradykinin receptor stabilize a conformation of the wild-type B2 receptor that binds antagonist with high affinity and that binds bradykinin with relatively low affinity. It is plausible that the initial unliganded conformation of the receptor is such a conformation, since the conformational change induced by bradykinin—a unimolecular reaction occurring after the binding step—would then be driven by the additional binding energy gained through the conformational change. However, since the initial receptor conformation is probably converted rapidly to other conformations after ligand binding, its affinity has not been determined experimentally.

There are clear precedents for allosteric effects on ligand binding to G-protein-coupled receptors. One is provided by replacements of the conserved aspartate residue in the predicted second transmembrane segment of several receptors (42–48). The possibility that mutation of this residue affects ligand binding allosterically was raised for the β-adrenergic receptor (43) and is supported by evidence that this negatively charged side chain confers sensitivity to Na^+ on the α_2-adrenergic receptor and on the dopamine D2 receptor (46, 47), a conclusion indicating that it is exposed to the cytoplasm rather than available to interact with ligand (49). In line with this interpretation, substitution for this conserved aspartate frequently results in uncoupling of a receptor from intracellular signaling pathways (44, 48, 50), indicating a restriction on the ability of the mutated receptors to assume the active conformation. Another precedent for allosteric effects on agonist binding is provided by point mutations at A293 in the third intracellular loop of the α_{1B}-adrenergic receptor. Substitutions at A293 result in altered agonist binding and in a basal activation of inositol phosphate production in the absence of agonist (51). The basal activation of inositol phosphate production is suppressed by antagonists, indicating that substitutions at this position stabilize an activated conformation of the receptor (51).

Conclusion. In conclusion, our mutational studies of the predicted sixth transmembrane segment of the B2 bradykinin receptor have yielded valuable information about the interaction of the receptor with bradykinin. We have identified the specific residues F261 and T265 of the B2 bradykinin receptor as involved either in contact with bradykinin or in the agonist-induced conformational change. These experiments provide a focus for detailed probing of specific ligand–binding site interactions—for example, by analyzing binding of a series of chemically different ligands at receptors with single substitutions for F261 or T265. In the absence of high-resolution structural analyses of G-protein-coupled receptors, our study of the sixth transmembrane helix and further mutational studies of other transmembrane helices will be an essential foundation for the rational design of agonists and antagonists active at the B2 bradykinin receptor.

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