Identification of the Critical Residues of Bradykinin Receptor B1 for Interaction with the Kinins Guided by Site-Directed Mutagenesis and Molecular Modeling

Sookhee N. Ha,*,‡ Pat J. Hey,§ Rick W. Ransom,§ Mark G. Bock,|| Dai-Shi Su,|| Kathryn L. Murphy,§ Ray Chang,§ Tsing-Bau Chen,§ Douglas Pettibone,§ and J. Fred Hess§

Basic Chemistry, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, Department of Medicinal Chemistry and Neuroscience, Merck Research Laboratories, West Point, Pennsylvania 19486, and Department of Medicinal Chemistry, Merck Research Laboratories, West Point, Pennsylvania 19486

Received April 5, 2006; Revised Manuscript Received September 29, 2006

ABSTRACT: We report the critical residues for the interaction of the kinins with human bradykinin receptor 1 (B1) using site-directed mutagenesis in conjunction with molecular modeling of the binding modes of the kinins in the homology model of the B1 receptor. Mutation of Lys^{118} in transmembrane (TM) helix 3, Ala^{270} in TM6, and Leu^{294} in TM7 causes a significant decrease in the affinity for the peptide agonists des-Arg^{10}kallidin (KD) and des-Arg^{9}BK but not the peptide antagonist des-$Arg^{10}Leu^{9}$KD. In contrast, mutations in TM2, TM3, TM6, and TM7 cause a significant decrease in the affinity for both the peptide agonists and the antagonist. These data indicate that the B1 bradykinin binding pocket for agonists and antagonists is similar, but the manners in which they interact with the receptor do not completely overlap. Therefore, there is a potential to influence the receptor's ligand selectivity.

Kinins play an important role in the pathophysiological processes of pain and inflammation (*1*, *2*). There are two types of kinin receptors, B1 and B2, and both of the human receptors have been cloned (*3*, *4*) and belong to G-protein-coupled receptor (GPCR) family A (*5*, *6*). Among the kinins, the pro-inflammatory peptide agonists, bradykinin and kallidin, selectively activate the B2 receptor, while their carboxypeptidase metabolites desArg^{9}BK and desArg^{10}KD act on the B1 receptor. The B1 receptor consists of 353 amino acids with seven helix transmembrane domains, a hallmark of the GPCR superfamily. Many studies indicate that the B1 receptor is involved in inflammatory pain and neutrophil infiltration through activation of a cytokine network (*7*−*9*). Therefore, it is of interest to develop an antagonist against B1 for treating pain and inflammation. Peptide antagonists selective for B1 versus B2 receptors have been reported (*10*−*19*) since the late 1970s. Recently, reports on non-peptide B1 receptor antagonists have begun to appear (*20*−*25*).

There have been relatively few efforts aimed at understanding the interaction between the bradykinin receptors and their ligands at the molecular level. The first homology model of the B2 receptor was constructed on the basis of the bacteriorhodopsin structure to probe the relationship between the length of the carbon chain of the NPC 18325-like peptide series and the affinity for the B2 receptor (*26*, *27*). Another prototypic study of the molecular mechanism of activation or inactivation of the B2 receptor was carried out using a homology model generated from the crystal structure of bovine rhodopsin (*28*−*30*) and mutation analysis (*31*). Several structures of unbound B1 and B2 receptor peptide antagonists have been determined by NMR spectroscopy and molecular modeling (*32*−*34*). There have been several mutagenesis studies in which amino acids in the bradykinin B1 and B2 receptors that participate in ligand binding have been examined. The results of this study are evaluated, relative to this previous work, in the Discussion.

The structures of the des-Arg kinins with their antagonists are shown in Figure 1. The antagonists were converted from the agonists in each case by changing the last residue from Phe to Leu (DALBK and DALK). The binding affinities of these peptide ligands range from subnanomolar to hundreds of nanomolar; des-Arg^{10}kallidin (DAK) exhibits a higher affinity for the human B1 receptor than des-Arg^{9}bradykinin (DABK). The kinin binding properties for 17 mutant bradykinin B1 receptor cDNAs transiently expressed in HEK293T cells have been assessed. To place the mutagenesis data in context, a molecular model of the human bradykinin B1 receptor has been generated on the basis of the crystal structure of bovine rhodopsin. The binding mode of the kinins was determined by the automated docking with an integration of the mutagenesis data. The binding mode was explored in an effort to gain insight into the molecular mechanism for receptor function. We describe here in conjunction with the homology model of the human bradykinin B1 receptor an investigation of the interaction of the kinins with the receptor.

EXPERIMENTAL PROCEDURES

Mutation. Point mutations were introduced into the human B1 bradykinin receptor in pcDNA3 using the Quick-Change reagents and protocol (Stratagene). Mutations were identified by DNA sequence analysis using BigDye terminator cycle

* To whom correspondence should be addressed. E-mail: sookhee_ha@merck.com. Phone: (732) 594-8474. Fax: (732) 594-4224. The coordinates of the homology model of the B1 receptor are available upon request from the corresponding author.

‡ Basic Chemistry.

§ Department of Medicinal Chemistry and Neuroscience.

|| Department of Medicinal Chemistry.

10.1021/bi060673f CCC: $33.50
Published on Web 11/09/2006

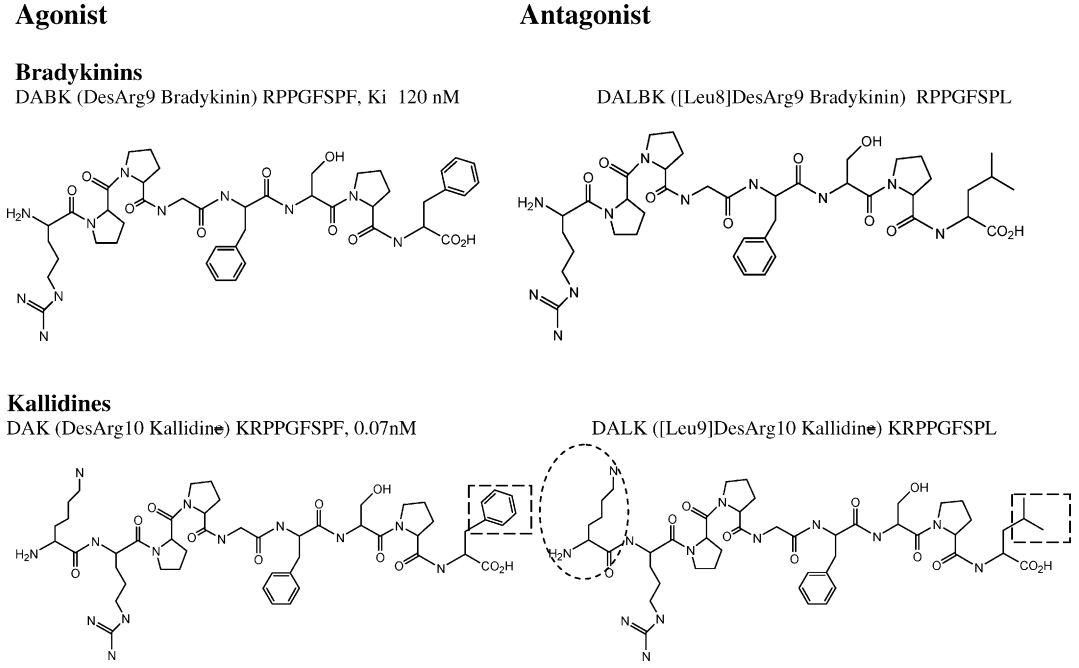

FIGURE 1: Structure of des-arginine kinins at the C-termini and their antagonists. Kallidins are one amino acid (lysine in the circle) longer at the N-terminus than bradykinins. The agonists become antagonists when the last residue, Phe, is changed to Leu as shown in the rectangles.

Table 1: Site-Directed Mutagenesis Data of the Kinins[a]

	K_i (nM)			
	DAKD	DALKD	DABK	K_D
wild type	**0.14 ± 0.05**	**0.26 ± 0.12**	**100 ± 25**	**27 ± 6.9**
I97F	**1.4 ± 0.55**	1.8 ± 0.81	**1700 ± 160**	45 ± 6.4
W98A	0.14 ± 0.1	0.28 ± 0.07	43 ± 42	28 ± 16
I113F	0.58 ± 0.04	0.8 ± 0.23	**1100 ± 190**	94 ± 32
I113S	0.38 ± 0.12	1.8 ± 0.97	830 ± 220	42 ± 18
N114A	0.16 ± 0.01	0.39 ± 0.02	170 ± 24	not determined
K118A	**16 ± 2.0**	0.84 ± 0.48	**2600 ± 1400**	**350 ± 60**
K118S	**14 ± 3.3**	0.67 ± 0.22	**2800 ± 1100**	**280 ± 54**
Y266A	0.16 ± 0.01	0.19 ± 0.15	43 ± 22	23 ± 17
Y266F	0.071 ± 0.024	0.22 ± 0.19	17 ± 11	19 ± 11
H267Q	0.07 ± 0.04	0.12 ± 0.08	***4.3 ± 0.30***	18 ± 11
A270T	**2.1 ± 0.52**	0.37 ± 0.04	**3000 ± 460**	**340 ± 190**
E273A	0.37 ± 0.09	**5.93 ± 0.91**	50 ± 7.0	**427 ± 38**
D291A	**120 ± 12**	**93 ± 19**	400 ± 200	**>5000**
L294A	0.51 ± 0.31	0.62 ± 0.29	**1450 ± 450**	37 ± 14
Q295A	0.07 ± 0.01	0.26 ± 0.02	310 ± 130	63 ± 23
N298S	0.07 ± 0.01	0.10 ± 0.03	**11 ± 2.9**	19 ± 11
F302A	**11 ± 5.3**	**3.9 ± 0.76**	**3000 ± 610**	204 ± 5.7

[a] Binding affinities in bold italic letters represent increases in potency of more than 10-fold relative to that of the wild-type B1 receptor, and those in bold represent decreases in potency of more than 10-fold relative to that of the wild-type B1 receptor.

sequencing chemistry followed by analysis on an ABI 377 DNA sequencer. To eliminate any errors from PCR, both strands of the entire coding sequence of the human B1 receptor were sequenced. DNA encoding the mutant B1 bradykinin receptors was transfected into either HEK293 cells by calcium phosphate or HEK293T cells by Lipofectamine2000. Membranes were prepared from transfected cells, and the pharmacological profile of the mutant receptor was determined by competition of specific binding of either the peptide antagonist [^{3}H]des-Arg^{10},Leu^{9}kallidin, or the agonist [^{3}H]des-Arg^{10}kallidin. Saturation binding with the appropriate radiolabeled peptide was performed so that a K_i value for each test compound (Table 1) could be determined using GraphPad Prism.

Binding Assays. Radioligand, [^{3}H]des-Arg^{10},Leu^{9}kallidin was purchased from New England Nuclear (NET 1096, 67 Ci/mmol). Whole cells from different human B1 mutation cells were prepared in PBS/EDTA buffer. After the medium was aspirated out, the attached cells were scraped off by adding 2 × 10 mL of a PBS/EDTA solution. The whole cells were spun down at 950 rpm for 15 min. The final cell pellets was brought up in an appropriate volume (45 mL per 175 cm^2 flask) of binding assay buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 100 mM MK-422, 140 μg/mL bacitracin, and 0.1% heat-denatured bovine serum albumin, adjusted to pH 7.4 with 6 N NaOH). To assess the specific binding of [^{3}H]des-Arg^{10},Leu^{9}kallidin, 0.25 mL of cell suspension was added to triplicate tubes containing 5 μL of either buffer (for total binding) or unlabeled des-Arg-kallidin (final concentration of 10 μM for nonspecific binding) or displacers (at desired final concentrations) and 10 μL of [^{3}H]-des-Arg^{10},Leu^{9}kallidin (final concentration of 0.25−0.5 nM). After incubation at 25 °C for 60 min, the incubation mixtures were filtered through glass fiber GF/B filters (presoaked in 0.2% polyethylenimine) on a Tomtec cell harvester and rapidly washed by two cycles of 1 mL of ice-cold HEPES buffer [20 mM HEPES, 125 mM NaCl, and 5 mM KCl (pH 7.4)]. The radioactivity trapped on the filters was counted by liquid scintillation counting (Packard 1900 beta counter).

Mutagenesis and Pharmacological Characterization. A series of site-directed mutations of the human B1 receptor were created for the study of the interaction of the receptor with peptide agonists and antagonists. The selection of amino acid residues to be mutated was based upon previous mutagenesis studies, with either human bradykinin B1 or B2 (11 mutants), as well as the molecular model of the B1 receptor (six mutants). The mutants were transiently expressed in HEK-293 cells and their pharmacological properties characterized by testing the ability of kinins to compete with specific binding of a radiotracer. For most mutants that were tested, the affinity of B1 for the peptide antagonist [^{3}H]-

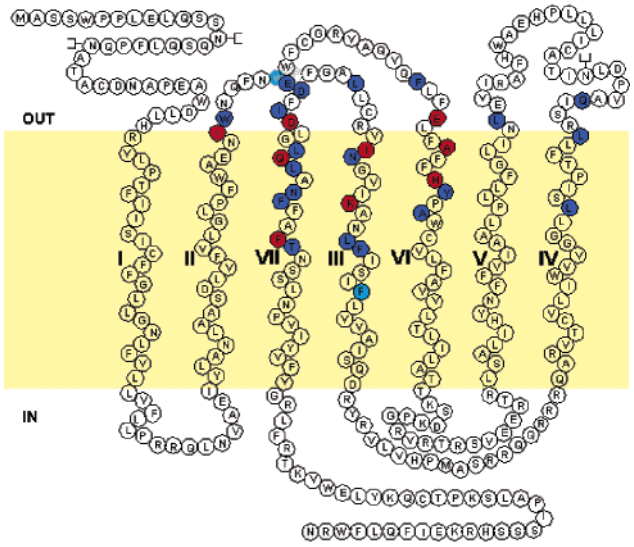

FIGURE 2: Snake diagram of the B1 receptor. Residues that alter the binidng afiinity upon mutation are colored red, minor changes light blue, and residues that have not been changed blue.

des-Arg^{10},Leu^{9}kallidin was sufficient to enable the use of this radioligand in competition binding studies. In contrast, mutation of either of the acidic residues flanking the fourth extracellular domain, Glu^{273} and Asp^{291}, to alanine resulted in a significant decrease in affinity for [^{3}H]des-Arg^{10},Leu^{9}-kallidin; therefore, a nonpeptide B1 receptor antagonist (*24*) was utilized in evaluating these mutants. The pharmacological profile determined by competition binding analysis of the mutant B1 receptors is summarized in Table 1, and residues that were mutated are shown in the snake diagram of Figure 2.

Homology Modeling and Docking of Kinins. It is known that the GPCRs maintain their characteristic folds through structural mimicry, although a low degree of homology exists between GPCRs (*35*). Likewise, although the low level of homology between the B1 receptor and bovine rhodopsin (36.6% similar and 19.8% identical in sequence), they share a series of highly conserved key residues in the transmembrane helices unique to GPCR family A. An alignment of the amino acid sequences of the human B1, rat B1, and human B2 receptors with the bovine rhodopsin sequence was created with Fasta and adjusted to align those key conserved residues as shown in bold letters in Figure 3.

A homology model was generated with the homology module in MOE using the crystal structure of bovine rhodopsin [PDB entry 1F88 (*28*)] as a template. Diverse conformations of the loops were generated with the Mcloop module in MIX, a modeling suite at Merck. To select what we view as the most appropriate model, we took into account the size of the crevice between the transmembrane helices and the extracellular loops that is available for ligand binding. The kinins were docked into the active site with the docking module Flog in MIX. The binding poses with high ranking scores (*36*) were visually inspected, and one pose that agrees most with the mutation data was selected. Finally, the complex of the selected pose of the kinins and the B1 receptor was refined with molecular dynamics at room temperature and energy minimization.

As shown in Figure 4, the N-terminus of DALK is facing TM6 and TM7 and the C-terminus TM3 in the crevice surrounded by residues Ile^{97}, Trp^{98} (TM1), Ile^{113}, Asn^{114}, Lys^{118} (TM3), Ala^{264}, Tyr^{266}, Glu^{273} (TM6), Asp^{291}, Gln^{295}, Asn^{298}, Phe^{302} (TM7), and Glu^{273} (EC3). For the agonist DAK, the carboxylic group of the last residue Phe is interacting with the side chain of Lys^{118} while the phenyl ring makes hydrophobic contact with Ala^{270} and Leu^{294} (Figure 5). For the antagonist DALK, we suggest that Leu of DALK makes weaker contact with Lys^{118}, Ala^{270}, and Leu^{294} (Figure 5) than Phe of the agonist, DAK.

RESULTS AND DISCUSSION

Mutations in the Second Extracellular Domain. The acidic amino acid residues flanking the second extracellular domain, Glu^{273} and Asp^{291} in the human B1 receptor, were found to be involved in binding of peptide to the rat B2 receptor (*37*). We made analogous mutations in the human B1 receptor, Glu^{273}Ala and Asp^{291}Ala. A dramatic decrease in affinity for DAK and DALK is observed, indicating that these residues play a critical role in binding both peptide agonists and antagonists. This loss of affinity for the peptide ligands required the use of a radiolabeled nonpeptide ligand, *N*-{2-[4-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]ethyl}-2-[(2*R*)-1-(2-naphthylsulfonyl)-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl]acetamide, for the pharmacological analysis of these mutants (*24*). As shown in Figure 4, the first and second basic residues of the kinins, Lys^{1} and Arg^{2}, respectively, are located near Asp^{291} and Glu^{273}.

Mutations in the Third Transmembrane Helix. An extensive survey of amino acid residues in the third transmembrane helix (TM3) of the rat bradykinin B2 receptor did not reveal any residues involved in peptide binding (*38*). However, the analysis of B2−B1 chimeric receptors (*39*) demonstrated the importance of the third transmembrane helix in discriminating between subtype selective ligands. These investigations demonstrated that substitution of the third transmembrane helix of the B2 receptor with the third transmembrane helix of the B1 receptor resulted in a chimeric receptor with decreased affinity for the B2 receptor agonist BK. The chimeric receptor's decreased affinity for BK was proposed to be due to the Lys^{118} residue in the B1 receptor, since substitution of this residue with the residue in the B2 receptor, Ser, restored the affinity for BK. Several independent lines of evidence (*40*, *41*) also indicated that the C-terminus of the peptide agonists interacts with Lys^{118} and that this interaction is important for activation of the B1 receptor. Our competition binding studies indicate that the Lys^{118} mutant B1 receptor undergoes a significant decrease in affinity for the peptide agonists DAK and DABK, whereas the binding of the peptide antagonist DALK to the mutant receptor is unaffected, as shown in Table 1. The binding modes for the agonist and antagonists are slightly different at the C-terminal end because of a change in structure. In DALK, Leu^{9} replaces Phe^{9} of DAK, and this dictates local conformational change. In DAK, the phenyl ring is located near Ala^{270} and Leu^{294} while the carboxylic group is in the proximity of Lys^{118} and forms a stable salt bridge. In DALK, the Leu side chain causes the peptide to adopt a different conformation in which the Leu side chain is located farther from these two amino acids and the C-terminal carboxylic group is some distance from Lys^{118}. This binding mode is consistent with the data showing that mutations of Leu^{294},

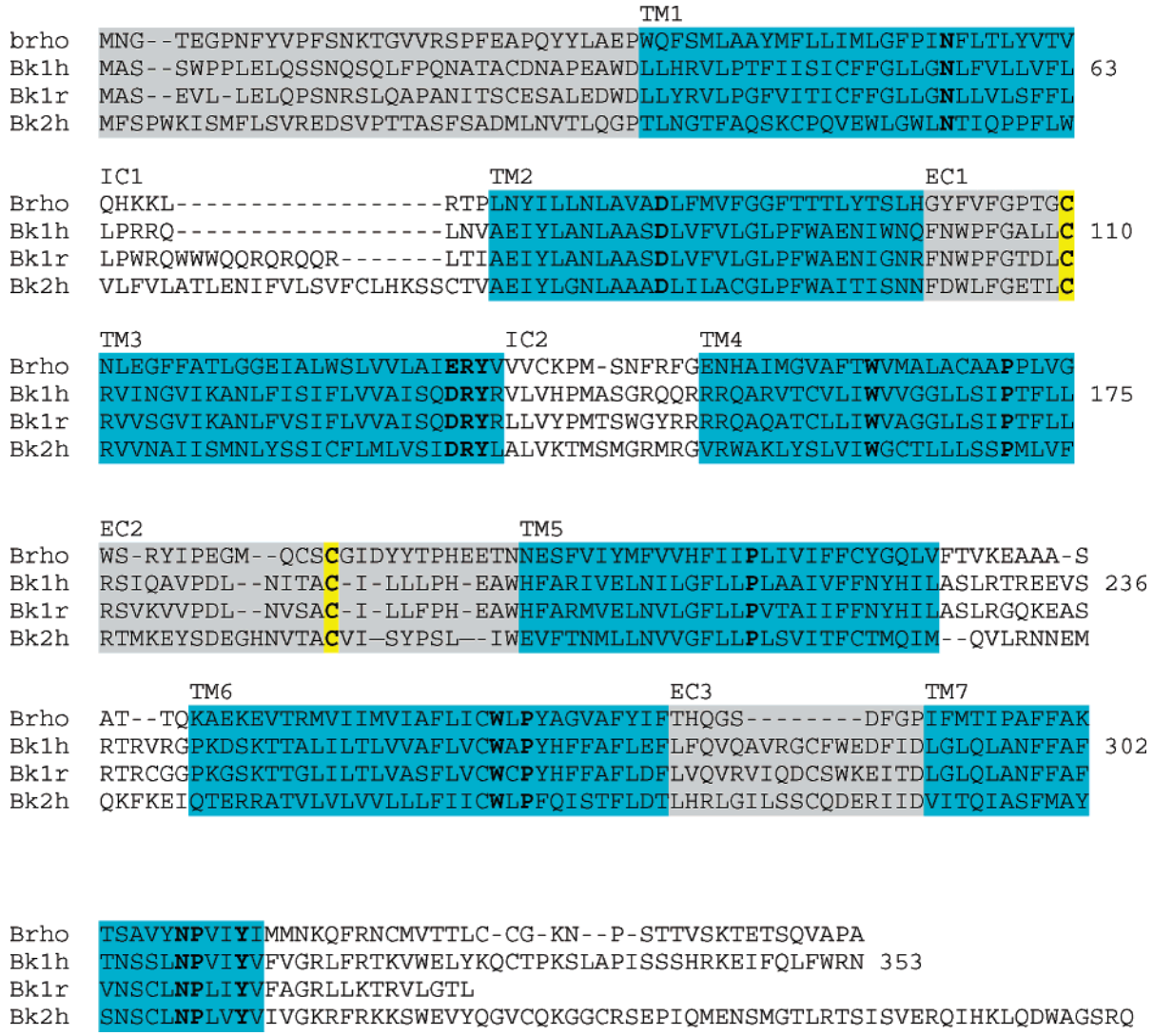

FIGURE 3: Alignment of amino acid sequences of bovine rhodopsin (Brho), the human B1 receptor (Bk1h), the rat B2 receptor (Bk1r), and the human B2 receptor (Bk2h). Transmembrane helices are colored with a blue background and extracellular loops gray. TM denotes transmembrane helices, EC extracellular loops, and IC intracellular loops. Highly conserved residues are marked with bold letters, and the cysteines of the disulfide bond are colored with a yellow background. A dash denotes a gap.

Ala^{270}, and Lys^{118} decrease the affinity of the mutant receptor for peptide agonists, but less for the antagonist DALK.

Asn^{114} is located on the same helical face as Lys^{118} of TM3 in the B1 receptor model, and mutations of Asn^{114} to Ala and Ser, the residue in the rat bradykinin B2 receptor, did not alter the binding affinity for DABK, DAK, or DALK. As shown in Figure 4, Asn^{114} is located at the corner of the hydrophobic pocket; for Phe^{6} of DALK, mutation to Ser or Ala with a small side chain would have a minor influence. In contrast, Asn^{114} is important for binding of nonpeptidic antagonists in the dihydroquinoxalinone series of compounds to the B1 receptor (*25*, *42*). The agonists and antagonists occupy a similar region of the B1 receptor, and the details of interaction vary depending on the chemical structures of the compounds.

Mutation of the Sixth Transmembrane Helix. Previous mutagenesis studies of the rat and human B2 receptor identified several residues in TM6 that are critical for high-affinity binding of BK (*31*, *38*, *43*). These studies indicated that the most critical residue is Phe^{261}; mutating this residue to Ala resulted in an approximately 2000-fold decrease in affinity for BK. A molecular model of the rat B2 receptor indicated that Phe^{261}, along with residues Leu^{104}, Val^{108}, and Ile^{112} in TM3, forms a hydrophobic cavity that interacts with Pro^{7} of BK (*26*). To begin to delineate the amino acid residues in TM6 that may be important in the B1 receptor for binding the kinin peptides, we examined the effect of mutating the corresponding residue in the B1 receptor (see Figure 3). In sharp contrast to the results obtained with the Phe^{261}Ala B2 receptor, the analogous B1 receptor mutant, Tyr^{266}Ala, had no effect on peptide binding. Furthermore, substitution of Tyr^{266} with Phe, the amino acid present in the B2 receptor, only slightly altered the affinity of the B1 receptor for the agonists or the antagonists. In Figure 4, Tyr^{266} is located close to Pro^{8} of the peptide, and removal of an OH group from Tyr^{266}, by mutation to Phe, would strengthen the hydrophobic interaction between Pro^{8} and residue Phe^{266}, resulting in an increase in the level of receptor binding as shown in Table 1.

The role of B1 receptor residue Ala^{270} was also examined. Mutation of the corresponding residue in the rat B2 receptor, Thr^{265}, to Ala resulted in a 300−500-fold loss of affinity for BK (*38*, *43*). Furthermore, in the B2−B1 chimeric receptor, both Phe^{261} and Thr^{265} were substituted to restore BK binding

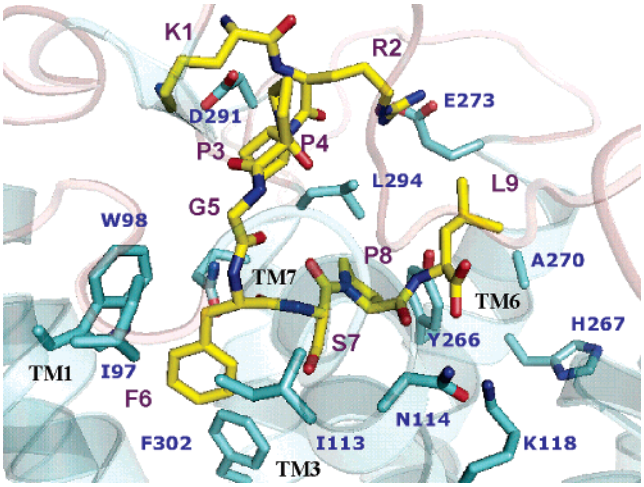

FIGURE 4: View of DALK at the binding site of the B1 receptor model. The kinin is interacting mainly with TM1, TM3, TM6, and TM7. DALK is colored yellow; TM1, TM3, and their residues are colored cyan and TM6, TM7, and their residues gold. Lys^1 and Arg^2 of DALK are located near D291 and E273. Phe^6 occupies the hydrophobic pocket surrounded by F302, I97, I113, and W98. C-Terminal Leu^9 of DALK is located near A270, L294, and K118.

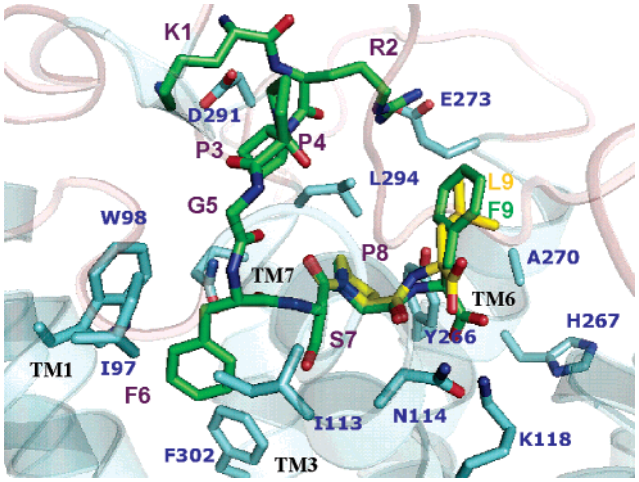

FIGURE 5: Possible docked DAK and DALK at the binding site of the B1 receptor model. DAK is colored green and DALK yellow. Lys118 is interacting with the carboxlyic group of Phe^9 in DAK, while the acidic group of Leu in DALK is farther from Lys^{118}.

(*44*). To explore the role of Ala^{270} in the B1 receptor, we replaced it with the residue present in the B2 receptor. The B1 receptor mutant $Ala^{270}Thr$ underwent a slight loss in binding affinity for the antagonist DALK but a more than 20-fold decrease in binding affinity for the agonists DABK and DAK. A similar result was observed when Leu^{294} was mutated to Ala. This result was discussed in the third membrane section in conjunction with the interaction of C-terminal carboxylic group with Lys^{118}. The binding modes of the agonists and the antagonists slightly differ in the C-terminus, as shown in Figure 5, in that the side chains of residues Ala^{270} and Leu^{294} are located near Phe^8 of the agonists while Leu^8 of the antagonist is farther from these two amino acids. The B2 receptor mutant $Gln^{262}Ala$ underwent a 4−8-fold decrease in affinity for BK (*38*, *43*). We mutated the analogous residue in the B1 receptor, His^{267}, to the B2 receptor residue, Gln. Although located in the proximity of the compounds, this mutation had no effect on the binding affinity for the kinins. This may due to the fact that the Gln residue has characteristics similar to those of His, and both of the residues are hydrophilic.

Amino Acid Residues in the Seventh Transmembrane Helix. A study using an approach of B1−B2 chimeric receptors coupled with site-directed mutagenesis identified two residues, Leu^{294} and Phe^{302}, in the seventh transmembrane helix as playing a role in ligand binding (*45*). These residues were initially identified by their ability to restore binding of $desArg^{10}$kallidin to a chimeric receptor in which the seventh transmembrane domain of the B1 receptor was replaced with the corresponding domain of the B2 receptor. Likewise, similar investigations were conducted to characterize mutant B1 receptors in which the endogenous residue was replaced with the corresponding B2 residue. We observed a marked decrease in binding affinity of both the agonist and antagonist to the mutant $Phe^{302}Ala$ B1 receptor. In our model, as shown in Figure 4, the benzene ring of Phe^6 occupies the hydrophobic crevice created by Phe^{302}, Ile^{113}, Trp^{98}, and Ile^{97}. Replacing the aromatic ring of Phe^{302} with a smaller aliphatic residue, Ala, would weaken the α stacking interactions with the benzene ring of Phe^6 of the kinins.

For the second site specific mutation characterized, Leu^{294}-Ala in the B1 receptor, we observed a trend of a decreasing affinity only for the agonists DAK and DABK. Therefore, Leu^{294} plays a role in the ability of the B1 receptor to bind the agonists as explained in the third transmembrane section. From the docking model, Leu^{294} and Ala^{270} are located near the benzene ring of Phe^8 of the agonists.

Mutations in TM7 of the rat bradykinin B2 receptor revealed that Gln^{290} participates in ligand binding (*38*). Substitution of these residues with Ala resulted in a modest decrease in the affinity for bradykinin. We prepared and analyzed the analogous mutation in the human B1 receptor, $Gln^{295}Ala$. There was no discernible impact of the mutation on the affinity of the mutant receptor for kinins. Although located in the proximity, in our model, the side chain of Gln^{295} is pointing away from the binding site, with no direct interaction with any kinin residues.

Other Mutations Based on the Human B1 Receptor Model. The initial phase of the mutagenesis analysis, described above, built upon information derived from previous mutagenesis studies with the B2 receptor and B1−B1 receptor chimers. The data from this portion of study were incorporated into the model of the human B1 receptor that was constructed using the bovine rhodopsin crystal structure as a template. The construction of the model led to the design of an additional set of mutants that were prepared and analyzed. Three mutations impacted the affinity of the B1 receptor for the kinins, $Ile^{97}Phe$ in TM1 and $Ile^{113}Phe$ and $Ile^{113}Ser$ in TM3. The impact of the mutation on binding affinity is large for both agonists and antagonists. Ile^{113}, Ile^{97}, Phe^{302}, and Trp^{98} form a hydrophobic pocket to accommodate Phe^6 of the kinins. The $Ile^{97}Phe$ and $Ile^{113}Phe$ mutations seemed to create a steric hindrance for Phe^6 of the kinins, which is optimally situated.

CONCLUSION

The aim of our investigation was to understand the interaction of the human B1 bradykinin receptor with the kinins, both the agonists and the antagonists, using molecular

Table 2: Key Residues of the B1 Receptor that Interacts with DALK[a]

residue in DALK	residues in the B1 receptor model
Lys^{1}	Asp^{291} (TM7), Asp^{288} (EC3)
Arg^{2}	Glu^{273} (TM6)
Pro^{3}	Ile^{192} (EC2)
Pro^{4}	Pro^{182} (EC2)
Phe^{6}	Ile^{97} (TM2), Trp^{98} (TM2), Ile^{113} (TM3), Gln^{295} (TM7), Phe^{302} (TM7)
Ser^{7}	Asn^{114} (TM3)
Pro^{8}	Tyr^{266} (TM6), His^{267} (TM6)
Leu^{9}	Lys^{118} (TM3), Ala^{270} (TM6), Leu^{294} (TM7)

[a] Transmembrane helices are given in parentheses.

modeling guided by site-directed mutagenesis. The properties of binding of more than 30 mutant receptors to the kinins were assessed. To place the mutagenesis data in context, a homology model of the human B1 receptor was constructed from the crystal structure of bovine rhodopsin and binding models for DAK, DABK, and DALK have been developed. Mutagenesis data for the kinins were put into the context of the receptor model by comparing the binding modes and the binding affinity of the kinins. They were also compared with the results of the mutation data of kinins of the B1 and B2 receptors from the literature.

Table 2 summarizes the interaction of DALK with the surrounding residues of the B1 receptor at the binding site based on this study. The kinins are interacting primarily with the residues in TM1, TM3, TM5, and TM6. Mutations of Glu^{273} in EC3 and Asp^{291} and Phe^{302} in TM7 cause a significant decrease in the affinity for both antagonists and agonists. Mutations of Ile^{113} in TM3 and Ile^{97} in TM1 cause a moderate decrease in affinity for both agonists and antagonists. Mutation of Lys^{118} in TM3, Leu^{294} in TM7, and Ala^{270} in TM6 resulted in a larger decrease in the binding affinity of the agonists than of the antagonists. A possible explanation was given in terms of a binding pose in the Results and Discussion. Interestingly, mutations of Tyr^{266} and His^{267} in TM6 and Asn^{298} cause the increase in the binding affinity of the kinins, particularly for DABK. Although we do not have a plausible explanation for this, it is assumed from either the enhancement of the hydrogen bond or the release of the steric hindrance.

Taken together, these data indicate that the peptide agonists and antagonists occupy the same binding pocket but there are slight variations in C-terminus of the peptide and the amino acids of the receptor that are important for their interaction within this binding pocket, which presumably reflects conformational differences between these two highly similar compounds. Lys^{118} in TM3 is known as a critical amino acid for agonist binding in the B1 receptor but is not necessary for antagonist binding. We postulate that the antagonists studied herein bind to an inactive form of the receptor; furthermore, the current homology model of the B1 receptor is based on inactive forms of bovine rhodopsin. Further work is required to understand the structural changes that occur in the receptor to generate an active form that is stabilized by binding of agonist to the receptor, where Lys^{118} is known to play an important role. The binding poses of the antagonists are in good agreement with the binding affinities of the kinins. The model was refined iteratively by integration of the mutagenesis data into the docking modes of the kinins at the binding site of the receptor. Together with mutation data, the binding model was used to understand the kinins and to gain insight into the molecular mechanism for receptor function.

REFERENCES

1. Dray, A., and Perkins, M. N. (1993) Bradykinin and inflammatory pain, *Trends Neurosci. 16*, 99−104.
2. Dray, A. (1997) Kinins and their receptors in hyperalgesia, *Can. J. Phys. Pharmacol. 75*, 704.
3. Hess, J. F., Borkowski, J. A., Young, G. S., Strader, C. D., and Ransom, R. W. (1992) Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor, *Biochem. Biophys. Res. Commun. 184*, 260−268.
4. Menk, J. G., Borkowski, J. A., Bierilo, K. K., MacNeil, T., Derrik, A. W., Schenck, K. A., Ransom, R. W., Strader, C., Linemyer, D., and Hess, J. F. (1994) Expression cloning of a human B1 bradykinin receptor, *J. Biol. Chem. 269*, 21583−21586.
5. Gether, U. (2000) Uncovering Molecular Mechanisms Involved in Activation of G Protein-Coupled Receptors, *Endocr. Rev. 21*, 90−113.
6. Marceau, F. (1995) Kinin B_1 receptors: A review, *Immunopharmacology 30*, 1−26.
7. Marceau, F., Hess, J. F., and Bachvarov, D. R. (1998) The B_1 Receptors for Kinins, *Pharmacol. Rev. 50*, 357−386.
8. Schremmer-Danninger, E., Offner, A., Siebeck, M., and Roscher, A. A. (1998) B1 bradykinin receptors and carboxypeptidase M are both upregulated in the aorta of pigs after LPS infusion, *Biochem. Biophys. Res. Commun. 243*, 246−252.
9. Decarie, A., Raymond, P., Gervais, N., Couture, R., and Adam, A. (1996) Serum interspecies differences in metabolic pathways of bradykinin and [des-Arg9]BK: Influence of enalaprilat, *Am. J. Physiol. 270*, 1340−1347.
10. Regoli, D., Barabe, J., and Park, W. K. (1977) Receptor for Bradykinin in Rabbit Aortae, *Can. J. Physiol. Pharmacol. 55*, 855−867.
11. Rigoli, D., and Barabe, J. (1988) Pharmacology of Bradykinin and Related Kinins, *Pharmacol. Rev. 32*, 1−46.
12. Galoppini, C., Meini, S., Tancredi, M., Di Fenza, A., Triolo, A., Quartara, L., Maggi, C. A., Formaggio, F., Toniolo, C., Mazzucco, S., Papini, A., and Rovero, P. (1999) A New Class of Pseudopeptide Antagonists of the Kinin B_1 Receptor Containing Alkyl Spacers, *J. Med. Chem. 42*, 409−414.
13. Gobeil, F., Charland, S., Filteau, C., Stephen, I., Neugebauer, W., and Regoli, D. (1999) Kinin B1 Receptor Antagonis Containing α-Methyl-L-Phenylalanine: In Vitro and in Vivo Antagonistic Activities, *Hypertension 33*, 823−829.
14. Gobeil, F., Neugebauer, W., Filteau, C., Jukic, D., Nsa Allogho, S., Pheng, L. H., Nguyen-Le, X. K., Bloiun, D., and Regoli, D. (1996) Structure-Activity Studies of B_1 Receptor-Related Peptides Antagonists, *Hypertension 28*, 833−839.
15. Stewart, J. M., Gera, L., Hanson, W., Zuzack, J. S., Burkard, M., McCullough, R., and Whalley, E. T. (1996) A New Generation of Bradykinin Antagonists, *Immunopharmacology 33*, 51−60.
16. Hanson, W. L., McCullough, R. G., Selig, W. M., Wieczorek, M., Ross, S., Whalley, E. T., Stewart, J. M., and Gera, L. (1996) In Vivo Pharmacological Profile of Novel, Potent, Stable BK Antagonists at B_1 and B_2 Receptors, *Immunopharmacology 33*, 191−193.
17. Tancredi, M., Galoppini, C., Meini, S., Quartara, L., Maggi, C. A., and Rovero, P. (1997) Synthesis and Biological Activity of New Bradykinin Pseudopeptide B_1 Receptor Agonists Containing Alkylic Spacers, *Bioorg. Med. Chem. Lett. 7*, 2661−2664.
18. Rovero, P., Vigano, S., Pegoraro, S., and Quartara, L. (1995) Synthesis of the Bradykinin B_1 Antagonist [desArg^{10}]HOE 140 on 2-Chlorotrityl Resin, *Lett. Pept. Sci. 2*, 319−323.
19. Groblewski, T., Maigret, B., and Bonnafous, J. C. (2001) Control of Conformational Equilibria in the Human B_2 Bradykinin Receptor Modeling of Nonpeptidic Ligand Action And Comparison to the Rhodopsin Structure, *J. Biol. Chem. 276*, 41100−41111.
20. Gougat, J., Muneaux, C., Muneaux, Y., Perreaut, P., and Planchenault, C. (1997) Nouveaux derives de *N*-(arylsulfonyl)-aminoacides ayant une affinite pour les recepteurs de la bradykinine [New derivatives of *N*-(arylsulfonyl) amino acids with an affinity for bradykinin receptors], WO 97/25315.
21. Horlick, R. A., Ohlmeyer, M. H., Stroke, I. L., Strohl, B., Pan, G., Schilling, A. E., Pardkar, V., Quintero, J. G., You, M., Riviello,

C., Thorn, M. B., Damaj, B., Fitzpatrick, D., Dolle, M. M., Webb, M. L., Baldwin, J. J., and Sigal, N. H. (1999) Small molecule antagonists of the bradykinin B_1receptorm, *Immunopharmacology 43*, 169−177.
22. Bock, M. G., and Longmore, J. (2000) Bradykinin antagonists: New opportunities, *Curr. Opin. Chem. Biol. 4*, 401−406.
23. Su, D.-S., Markowitz, M. K., Murphy, K. L., Wan, B. L., Zrada, M. M., Harrell, C. M., O' Malley, S. S., Hess, F. J., Ransom, R. W., Chang, R. S., Wallace, M. A., Raab, C. E., Dean, D. C., Pettibone, D. J., Freidinger, R. M., and Bock, M. G. (2004) Development of an Efficient and Selective Radioligand for Bradykinin B_1 Receptor Occupancy Studies, *Bioorg. Med. Chem. Lett. 14*, 6045−6048.
24. Ransom, R. W., Harrell, C. M., Reiss, D. R., Murphy, K. L., Chang, R. S., Hess, F. J., Miller, P. J., Hey, P. J., Kunapuli, P., Su, D. S., Markowitz, M. K., Wallace, M. A., Raab, C. E., Jones, A. N., Dean, D. C., Pettibone, D. J., Freidinger, R. M., and Bock, M. G. (2004) Pharmacological characterization and radioligand binding properties of a high-affinity, nonpeptide, bradykinin B1 receptor antagonist, *Eur. J. Pharm. 499*, 77−84.
25. Ha, S. N., Hey, P. J., Ransom, R. W., Harrell, C. M., Jr., Murphy, K. L., Chang, R., Chen, T.-B., Su, D.-S., Markowitz, M., Bock, M. G., Freidinger, R. M., and Hess, F. J. (2005) Binding modes of dihydroquinoxalinones in a homology model of bradykinin receptor 1, *Biochem. Biophys. Res. Commun. 331*, 159−166.
26. Chakraborty, S., Connolly, M., and Kyle, D (1995) Novel pseudopeptides with high affinities for the human bradykinin B2 receptor, *Pept. Res. 8*, 16−19.
27. Kyle, D. (1994) Structural Features of the Bradykinin Receptor As Determined by Computer Simulations, Mutagenesis Experiments, and Conformationally Constrained Ligands: Establishing the Framework for the Design of New Antagonists, *Braz. J. Med. Biol. Res. 27*, 1757−1779.
28. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. B., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor, *Science 239*, 739−745.
29. Okada, T., Fusiyosi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) Fundamental Role of Internal Water Molecules in Rhodopsin Revealed by X-ray Crystallography, *Proc. Natl. Acad. Sci. U.S.A. 99*, 5982−6002.
30. Teller, D. C., Okada, T., Beshenke, C. A., Palczewski, K., and Stenkemp, R. E. (2001) Three Dimensional Structure of Rhodopsin, a Model of G protein-Coupled Receptors, *Biochemistry 40*, 7761−7772.
31. Marie, J., Richard, E., Pruneau, D., Paquet, L. C., Siatka, C., Larguier, R., Ponce, C., Vassault, P., Groblewski, T., Maigret, B., and Bonnafous, J. C. (2001) Control of conformation equilibria in the human B2 bradykinin receptor. Modeling of nonpeptidic ligand action and comparison to the rhodopsin structure, *J. Biol. Chem. 276*, 41100−41111.
32. Pellegrini, M., Gobbo, M., Rocchi, R., Peggion, E., Mammi, S., and Mierke, D. F. (1996) Threonine bradykinin: Conformational study of a flexible peptide in dimethyl sulfoxide by NMR and ensemble calculations, *Biopolymers 40*, 561−569.
33. Giragossian, C., Nardi, E., Savery, C., Pellegrini, M., Meini, S., Maggi, C. A., Papini, A. M., and Mierke, D. F. (2001) Structural characterization of lipopeptide agonists for the bradykinin B2 receptor, *Biopolymers 58*, 511−520.
34. Thurieau, C., Feletou, M., Hennig, P., Raimbaud, E., Canet, E., and Fauchere, J. L. (1996) Design and synthesis of new linear and cyclic bradykinin antagonists, *J. Med. Chem. 39*, 2095−2101.
35. Ballesters, J. A., Sji, L., and Javitch, J. A. (2001) Structural Mimicry in G protein-Coupled Receptors: Implication of the high resolution structure of rhodopsin for structure-function analysis of rhodopsin-like receptors, *Mol. Pharmacol. 4*, 561−574.
36. Miller, M. D., Kearsley, S. K., Underwood, D. J., and Sheridan, R. P. (1994) FLOG: A system to select 'quasi-flexible' ligands complementary to a receptor of known three-dimensional structure, *J. Comput.-Aided Mol. Des. 8*, 153−174.
37. Novotny, E., Bednar, A. D., Connolly, M. A., Connor, J. R., and Stormann, T. M. (1994) Mutation of aspartate residues in the third extracellular loop of the rat B2 receptor decreases affinity for bradykinin, *Biochem. Biophys. Res. Commun. 201*, 523−530.
38. Jarnagin, K., Bhakta, S., Zuppan, P., Yee, C., Ho, T., Phan, T., Tahilramani, R., Pease, J. H., Miller, A., and Freedman, R. (1996) Mutation in the B2 bradykinin receptor reveal a different pattern of contacts for peptidic agonists and peptidic antagonists, *J. Biol. Chem. 271*, 28277−28286.
39. Fathy, D. B., and Mathis, S. A. (1998) A single position in the third transmembrane domains of human B1 and B2 bradykinin receptor is adjacent to and discriminates between the C-terminal residues of subtype-selective ligands, *J. Biol. Chem. 273*, 12210−12218.
40. Drouin, J. N., Gauderau, P., St. Pierre, S. A., and Regoli, D. (1979) Strucure-acitivity studies of [des-Arg9]-bradykinin on the B1 receptor of the rabbit aorta, *Can. J. Physiol. Pharmacol. 57*, 562−566.
41. Drouin, J. N., Gauderau, P., St. Pierre, S. A., and Regoli, D. (1979) Biological activities of kinins modified at the N- or at the C-terminal end, *Can. J. Physiol. Pharmacol. 57*, 1018−1023.
42. Su, D.-S., Markowitz, M. K., Diprado, R. M., Murphy, K., Harrell, C. M., O' Malley, S. S., Ransom, R., Chang, R. L., Ha, S., Hess, F. J., Pettibone, D. J., Mason, G. S., Boyce, S., Fredinger, R. M., and Bock, M. G. (2003) Discovery of a Potent, Non-peptide Bradykinin B1 Receptor Antagonist, *J. Am. Chem. Soc. 125*, 7516−7517.
43. Nardon, J., and Hogan, P. G. (1994) Delineation of a region in the B2 bradykinin receptor that is essential for high affinity agonist binding, *Proc. Natl. Acad. Sci. U.S.A. 91*, 4417−4421.
44. Leeb, T., Mathis, S. A., and Leeb-Lundberg, L. M. F. (1997) The sixth transmembrane domains of the human B1 and 2 bradykinin receptors are structurally compatible and involved in discriminating between subtype-selective agonists, *J. Biol. Chem. 272*, 311−317.
45. Bastian, S., Prunau, S., Loillier, B., Robert, C., Bonnafous, J. C., and Paquet, J. L. (2000) Identification of a key region of kinin B1 receptor for high affinity binding of peptide antagonists, *J. Biol. Chem. 275*, 6107−6113.

BI060673F