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High-Affinity Binding of Peptide Agonists to the Human B1 Bradykinin Receptor Depends on Interaction between the Peptide N-Terminal L-Lysine and the Fourth Extracellular Domain of the Receptor

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ABSTRACT

The aim of this study was to identify the location of the N terminus of peptide agonist ligands when bound to the human B1 bradykinin (BK) receptor. To reach this aim, we exploited the fact that high-affinity binding of kinin peptides to the human B1 receptor subtype requires a peptide N-terminal L-Lys, whereas high-affinity binding to the B2 receptor subtype does not require this residue. This was done by comparing the affinities of BK, a B2 receptor-selective peptide, and kallidin or Lys-BK, a less receptor-selective peptide, for chimeric proteins in which each B1 receptor domain had been substituted in the human B2 receptor and expressed in HEK293 cells. Individual substitution of transmembrane domains 1–7 (TM-I–VII) and extracellular domains 1–4 (EC-I–IV) of the B1 receptor in the B2 receptor influenced the affinities of BK and Lys-BK approximately equally. In contrast, substitution of B1 EC-IV dramatically reduced the affinity and potency of BK, whereas these parameters for Lys-BK were essentially unaltered. Substitution of either the N- or C-terminal half of B1 EC-IV in the B2 receptor only had a limited effect on the peptide binding constants, indicating the involvement of multiple residues throughout this domain. Complementary mutations of the N-terminal residue in Lys-BK revealed that both the positive charge and the proper spatial orientation of this residue were required for interaction with B1 EC-IV. Thus, the N-terminal residue of peptide agonists when bound to the human B1 receptor is positioned extracellularly and interacts with EC-IV.

Kinins are proinflammatory peptide agonists 8 to10 amino acids in length that are released in response to tissue injury from kininogen precursors through the action of kallikreins (Proud and Kaplan, 1988; Bhoola et al., 1992). Receptors for kinins have been classified into two subtypes, termed B1 and B2 (Regoli and Barabe, 1980), which are both members of the G protein-coupled receptor superfamily (Hess et al., 1992; Menke et al., 1994). Bradykinin (BK) and kallidin, or Lys-BK, the first set of bioactive kinins formed, act on the B2 receptor, whereas the carboxypeptidase products desArg^{9}-BK and desArg^{10}-Lys-BK, the second set of bioactive kinins formed, act on the B1 receptor (Regoli and Barabe, 1980).

Kinins elicit pain, inflammation, and hyperalgesia (Proud and Kaplan, 1988; Dray and Perkins, 1993). Furthermore, animal models suggest that the B2 receptor participates in the acute phase of the inflammatory and pain response, whereas the B1 receptor participates in the chronic phase of the response (Proud and Kaplan, 1988; Farmer et al., 1991; Dray and Perkins, 1993). This schedule of receptor activities may be a consequence of receptor autoregulation by kinins, which favors B1 receptor expression (Phagoo et al., 1999). As a consequence of this interplay, the design of new and improved therapeutic agents that are intended to control kinin responses in inflammation would be greatly benefited by the parallel mapping of the ligand-binding sites in B1 and B2 receptors.

Previous mapping studies have focused almost exclusively on the B2 receptor subtype. We used a combination of crosslinking and mutagenesis to show that the N terminus of the agonist BK when bound to the human B2 receptor is adjacent to Cys^{277} in the fourth extracellular domain (EC-IV) (Herzig and Leeb-Lundberg, 1995; Herzig et al., 1996). Indeed, BK binding to the rat B2 receptor is directly dependent on two aspartate residues, Asp^{268} and Asp^{286}, in EC-IV that interact with either the N terminus or the guanidinium side chain of Arg^{1} in BK (Kyle et al., 1994; Novotny et al., 1994). Furthermore, the interaction of BK with the human B2 receptor is inhibited by antibodies raised against the C-termi-

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ABBREVIATIONS: BK, bradykinin; EC, extracellular domain; TM, transmembrane domain; DMEM, Dulbecco's modified Eagle's medium; WT, wild type; PCR, polymerase chain reaction; HEK, human embryonic kidney.

nal half of EC-IV (Abd Alla et al., 1996). Thus, the N-terminal residue of BK when bound to the B2 receptor is extracellular and adjacent to EC-IV. The C-terminal residue of kinin peptides is adjacent to a position facing the ligand-binding pocket approximately two turns into the helix of the third transmembrane domain (TM-III) (Fathy et al., 1998). This position was localized with a chimeric receptor strategy involving the identification of residues that enable human B2 and B1 receptors to discriminate between peptide ligands (Leeb et al., 1997; Fathy et al., 1998). The same strategy was used to identify this position in the B1 receptor as a counterion for the C terminus of B1 receptor-selective desArg peptide ligands (Fathy et al., 1998).

To complete the orientation of peptide agonists when bound to the human B1 receptor, we used the same chimeric strategy to locate the peptide N terminus. To do so, we took advantage of the fact that high-affinity binding to the human B1 receptor requires an N-terminal Lys in the peptide, whereas binding to the human B2 receptor does not require this residue (Regoli and Barabe, 1980). In other words, Lys-BK and desArg^{10}-Lys-BK bind with much higher affinity to the human B1 receptor than do their N-terminally truncated analogs BK and desArg^{9}-BK, whereas the human B2 receptor does not discriminate between the absence and presence of the N-terminal Lys. Our results show that the N-terminal residue of peptide agonists when bound to the B1 receptor is extracellular and adjacent to EC-IV. Thus, human B2 and B1 receptors, although only 36% homologous, orient their natural ligands in a very similar manner.

Experimental Procedures

Materials. [2,3-*prolyl*-3,4-^{3}H]Bradykinin (110 Ci/mmol), [*prolyl*-3,4-^{3}H]NPC17731 (53.5 Ci/mmol), des-Arg^{10}-[3,4-*prolyl*-3,4-^{3}H]kallidin (107 Ci/mmol), des-Arg^{10}-[Leu^{9}][3,4-*prolyl*-3,4-^{3}H]kallidin (107 Ci/mmol), and [^{3}H]*myo*-inositol (10–20 Ci/mmol) were obtained from DuPont/NEN (Boston, MA). Kallidin or L-Lys-BK was obtained from Peninsula Laboratories, Inc. (Belmont, CA), and desArg^{10}-Lys-BK was from Bachem (Torrence, CA). NPC17731, NPC18565, D-Lys-BK, Ala-BK, and Arg-BK were synthesized at Purdue Pharma, L.P. (Ardsley, NY) following previously reported procedures (Kyle et al., 1991). Dulbecco's modified Eagle's medium (DMEM), Leibovitz's L-15 medium, PBS, and Hanks' balanced salt solution were from Life Technologies (Gaithersburg, MD). Reagents for calcium phosphate transfections were purchased from 5 Prime → 3 Prime (Boulder, CO). Enzymes were obtained from Life Technologies, New England Biolabs (Beverly, MA), and Stratagene (LaJolla, CA). Sera and all other peptides and chemicals were from Sigma Chemical Co. (St. Louis, MO).

Construction of Receptor cDNA. The original human wild type (WT) B1 and B2 receptor clones in vector pcDNA3 (Invitrogen, San Diego, CA) were kindly provided by J. Fred Hess, Merck Research Laboratories, West Point, PA, and fusions between the B1 and the B2 receptor cDNA clones were made with a modified polymerase chain reaction (PCR)-ligation-PCR protocol as previously described (Leeb et al., 1997; Fathy et al., 1998).

Cell Culture and Transfection. Human embryonic kidney (HEK293) cells were grown in DMEM supplemented with 10% heat-inactivated horse serum at 37°C in 10% CO_2. At 24 h before transient transfections, the cells were seeded into 100-mm dishes or 6-well plates at 60 to 80% confluency. The cells were then transfected with the calcium phosphate precipitate method with overnight incubation in the presence of 15 μg of cDNA per 100-mm dish and 2 μg per well in 6-well plates. The cells were then further incubated for an additional 72 to 96 h after transfection.

Membrane Preparation. Transfected HEK293 cells were washed twice with ice-cold PBS and then pelleted by centrifugation at 2000*g* for 10 min. The cells were then resuspended in a buffer containing 25 mM 2-{[2(hydroxymethyl)ethyl]amino}ethanesulfonic acid, pH 6.8, 0.5 mM EDTA, 0.2 mM $MgCl_2$, and 1 mM 1,10-phenanthroline and homogenized on ice with an ultra-turrax at 20,500 rpm for 10 s. Membranes were isolated by centrifugation at 45,000*g* for 30 min at 4°C. The pellets were then resuspended in the above-mentioned buffer supplemented with 0.1% BSA and 0.014% bacitracin (binding buffer).

Radioligand Binding. Membranes were diluted in binding buffer to give a signal of 1,000 to 4,000 dpm/assay of specific radioligand binding. Binding assays were performed in a total volume of 0.5 ml with either [^{3}H]BK, [^{3}H]NPC17731, [^{3}H]desArg^{10}-Lys-BK, or [^{3}H]desArg^{10}[Leu^{9}]-Lys-BK with or without varying concentrations of nonradioactive kinin peptides. After incubation for 60 to 90 min at room temperature, assays were terminated by dilution with 4 ml of ice-cold PBS/0.3% BSA and rapid vacuum filtration on Whatman GF/C filters previously soaked in 1% polyethyleneimine. The trapped membranes were washed with an additional 2×4 ml of ice-cold PBS/0.3% BSA. The filters were then counted for radioactivity in a Beckman LS5000TD scintillation counter. Binding constants were calculated with Radlig (Biosoft, Ferguson, MO).

Phosphoinositide Hydrolysis. Cells were assayed essentially as described in Tropea et al. (1992), with a few modifications. Briefly, transfected HEK293 cells grown in 6-well dishes were incubated with 1 μCi/ml [^{3}H]*myo*-inositol in DMEM, 5% heat-inactivated horse serum at 37°C for 24 h in 10% CO_2. Before experimentation, the cells were washed four times with 1 ml of Leibovitz's L-15 medium, pH 7.4, at room temperature and incubated in Leibovitz's L-15 medium, 50 mM LiCl for 30 min. Following replacement with 2 ml of the same medium, the cells were incubated with or without agonists at 37°C

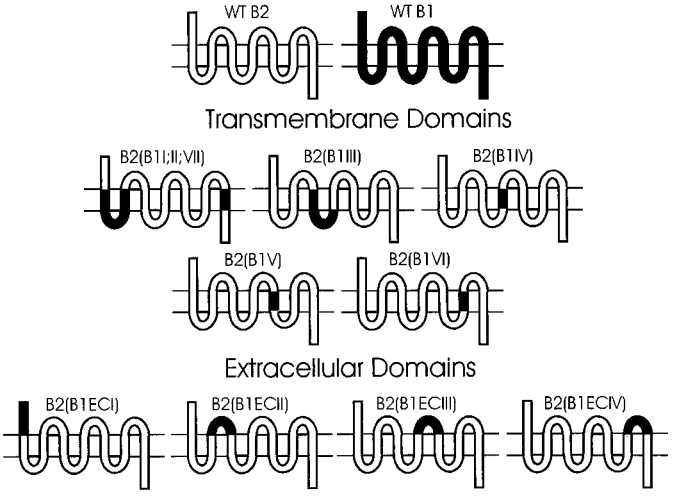

Fig. 1. Schematic representations of the human B1 and B2 BK receptors. Serpentine diagram of the B2 (open) and B1 (filled) receptors, and the B1 and B2 receptor chimeras used in this study. Based on numbering from the first and third methionine in the B1 and B2 receptors, respectively, as previously described (Hess et al., 1992; Menke et al., 1994), the boundaries of the exchanged domains in the chimeras were as follows: B2(B1I; II; VII), Pro^{41} → Trp^{98} and Leu^{292} → Gly^{316} of B1 for Pro^{34} → Ser^{91} and Val^{285} → Gly^{309} of B2; B2(B1III), Val^{112} → Arg^{155} of B1 for Val^{105} → Lys^{148} of B2; B2(B1IV), Val^{156} → Ile^{178} of B1 for Leu^{149} → Met^{171} of B2; B2(B1V), Phe^{200} → Tyr^{223} of B1 for Val^{195} → Met^{218} of B2; B2(B1VI), Thr^{248} → Leu^{272} of B1 for Ala^{241} → Leu^{265} of B2; B2(B1ECI), Met^{1} → Leu^{40} of B1 for Met^{1} → Gln^{33} of B2; B2(B1ECII), Asn^{99} → Arg^{111} of B1 for Asn^{92} → Arg^{104} of B2; B2(B1ECIII), Gln^{179} → His^{199} of B1 for Lys^{172} → Glu^{194} of B2; and B2(B1ECIV), Glu^{273} → Asp^{291} of B1 for Asp^{266} → Asp^{284} of B2.

TABLE 1
Amino acid sequences of B1 and B2 BK receptor ligands

Ligand	Sequence[a]
Agonists	
BK	Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg
desArg^{9}BK	Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe
desArg^{10}Lys-BK	Lys - Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe
Lys-BK	Lys - Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg
Antagonists	
NPC17731	D -Arg - Arg - Pro - Hyp - Gly - Phe - Ser - X - Oic - Arg
NPC18565	D -Arg - Arg - Pro - Hyp - Gly - Phe - Ser - X - Oic
desArg^{10}[Leu^{9}]Lys-BK	Lys - Arg - Pro - Pro - Gly - Phe - Ser - Pro - Leu

[a] Oic is L-[(3aS,7aS)-octahydroindol-2-yl-carbonyl]; X, D-Hype(*trans*-propyl).

TABLE 2
K_D values for agonists and antagonists on the WT B1 and B2 BK receptors and chimeric B1/B2 receptor constructs expressed in HEK293 cells

Construct	Agonists		Antagonists	
	BK	DesArg^{10}-Lys-BK	NPC17731	NPC18565
		nM[a]		
WTB2	0.192 ± 0.030[b]	>5000	0.180 ± 0.007[b]	24.6 ± 3.6[b]
WTB1	2880 ± 710	0.098 ± 0.034[c]	224 ± 18[d]	1.32 ± 0.15
B2(B1I;II;VII)	0.580 ± 0.271	>1000	0.393 ± 0.192	20.6 ± 9.1
B2(B1III)	U.B.[b]	U.B.[b]	U.B.[b]	
B2(B1IV)	1.68 ± 1.16	>1000	0.377 ± 0.197	16.0 ± 3.3
B2(B1V)	0.213 ± 0.064	>1000	0.373 ± 0.386	36.4 ± 18.3
B2(B1VI)	U.B.[c]	U.B.[c]	0.181 ± 0.016[c]	9.71 ± 0.50[c]
B2(B1ECI)	4.10 ± 1.20	>1000	1.60 ± 0.33	380 ± 76.0
B2(B1ECII)	0.547 ± 0.022	>1000	0.239 ± 0.039	10.0 ± 0.9
B2(B1ECIII)	0.377 ± 0.030	>1000	0.094 ± 0.033	1.95 ± 0.71
B2(B1ECIV)	2.63 ± 0.16	173 ± 16.9	0.083 ± 0.019	14.4 ± 1.4

U.B., undetectable binding.
[a] The constants are presented as K_D values. The values were calculated by the Radlig program with data obtained from hot and cold saturation binding experiments with the agonists [^{3}H]BK and [^{3}H]desArg^{10}-Lys-BK and antagonists [^{3}H]NPC17731 and [^{3}H]desArg^{10}[Leu^{9}]-Lys-BK, and the values are presented as means ± S.E. of three experiments.
[b] From Fathy et al. (1998).
[c] From Leeb et al. (1997).
[d] EC_{50} value; NPC17731 increased the binding of [^{3}H]desArg^{10}[Leu^{9}]-Lys-BK.

TABLE 3
K_D and K_I values for BK and Lys-BK on the WT B1 and B2 BK receptors and chimeric B1/B2 receptor constructs expressed in HEK293 cells

Construct	[^{3}H] Agonist			[^{3}H] Antagonist		
	BK	Lys-BK	$K_{D(BK)}/K_{D(Lys-BK)}$	BK	Lys-BK	$K_{I(BK)}/K_{I(Lys-BK)}$
			nM[a]			
WTB2	0.192 ± 0.030[b]	0.157 ± 0.007[b]	1.22	1.92 ± 0.24[b]	4.30 ± 0.27[b]	0.447
WTB1	2880 ± 710	11.7 ± 1.3[c]	60.3	21700 ± 8700	441 ± 40	49.3
B2(B1I;II;VII)	0.580 ± 0.271	0.670 ± 0.010	0.866	0.547 ± 0.228	3.45 ± 1.77	0.166
B2(B1IIIS^{111})	0.566 ± 0.149[b]	0.779 ± 0.157[b]	0.727	137 ± 19[b]	117 ± 32[b]	1.17
B2(B1IV)	1.68 ± 1.16	3.01 ± 0.16	0.558	3.32 ± 0.74	8.34 ± 2.34	0.398
B2(B1V)	0.213 ± 0.064	0.417 ± 0.177	0.551	0.478 ± 0.475	4.41 ± 0.91	0.108
B2(B1VIF^{259},T^{263})	0.294 ± 0.111	0.148 ± 0.013	1.99	1.75 ± 0.04[c]	0.333 ± 0.054[c]	5.26
B2(B1ECI)	4.10 ± 1.20	5.58 ± 2.60	0.735	38.6 ± 10.8	48.3 ± 19.2	0.799
B2(B1ECII)	0.547 ± 0.022	0.709 ± 0.071	0.772	0.807 ± 0.126	2.35 ± 0.148	0.343
B2(B1ECIII)	0.377 ± 0.030	0.315 ± 0.060	1.20	0.302 ± 0.221	3.16 ± 0.749	0.096
B2(B1ECIV)	2.63 ± 0.16	0.579 ± 0.177	4.54	793 ± 341	0.458 ± 0.006	1730

[a] The constants are presented as K_D ([^{3}H] Agonist) and K_I values ([^{3}H] Antagonist). The values were calculated by the Radlig program with data obtained from hot and cold saturation-binding experiments and competition-binding experiments with the agonists [^{3}H]BK and [^{3}H]desArg^{10}-Lys-BK and the antagonists [^{3}H]NPC17731 and [^{3}H]desArg^{10}[Leu^{9}]-Lys-BK, and the values are presented as means ± S.E. of three experiments.
[b] From Fathy et al. (1998).
[c] From Leeb et al. (1997).

for 20 min. Inositol phosphates were then extracted and isolated with anion exchange chromatography.

Results

Construction of B1 and B2 Receptor Chimeras. To identify the location of the N terminus of peptide agonists when bound to the human B1 receptor, we created a series of basic chimeric receptor constructs in which TM-I–VII and EC-I–IV in the B1 receptor were individually substituted in the corresponding positions in the B2 receptor (Fig. 1). The nomenclature used for these constructs was, e.g., B2(B1IV) for a B2 receptor with a B1 TM-IV and B2(B1ECIV) for a B2 receptor with a B1 EC-IV. The pharmacological and functional profiles of the WT and chimeric receptor constructs were determined by radioligand binding and by agonist-stimulated phosphoinositide hydrolysis in transfected HEK293 cells with a variety of kinin peptide agonists and antagonists (Table 1).

Pharmacological Characterization of WT B1 and B2 Receptors and B1 and B2 Receptor Chimeras. The pharmacological profiles of WT B2 and B1 receptors and chimeric B1/B2 receptor constructs were analyzed by radioligand binding with the high-affinity B2-selective peptide agonist [^{3}H]BK and antagonist [^{3}H]NPC17731 and the high-affinity B1-selective peptide agonist [^{3}H]desArg^{10}-Lys-BK and antagonist [^{3}H]desArg^{10}[Leu^9]-Lys-BK. As shown in Table 2,

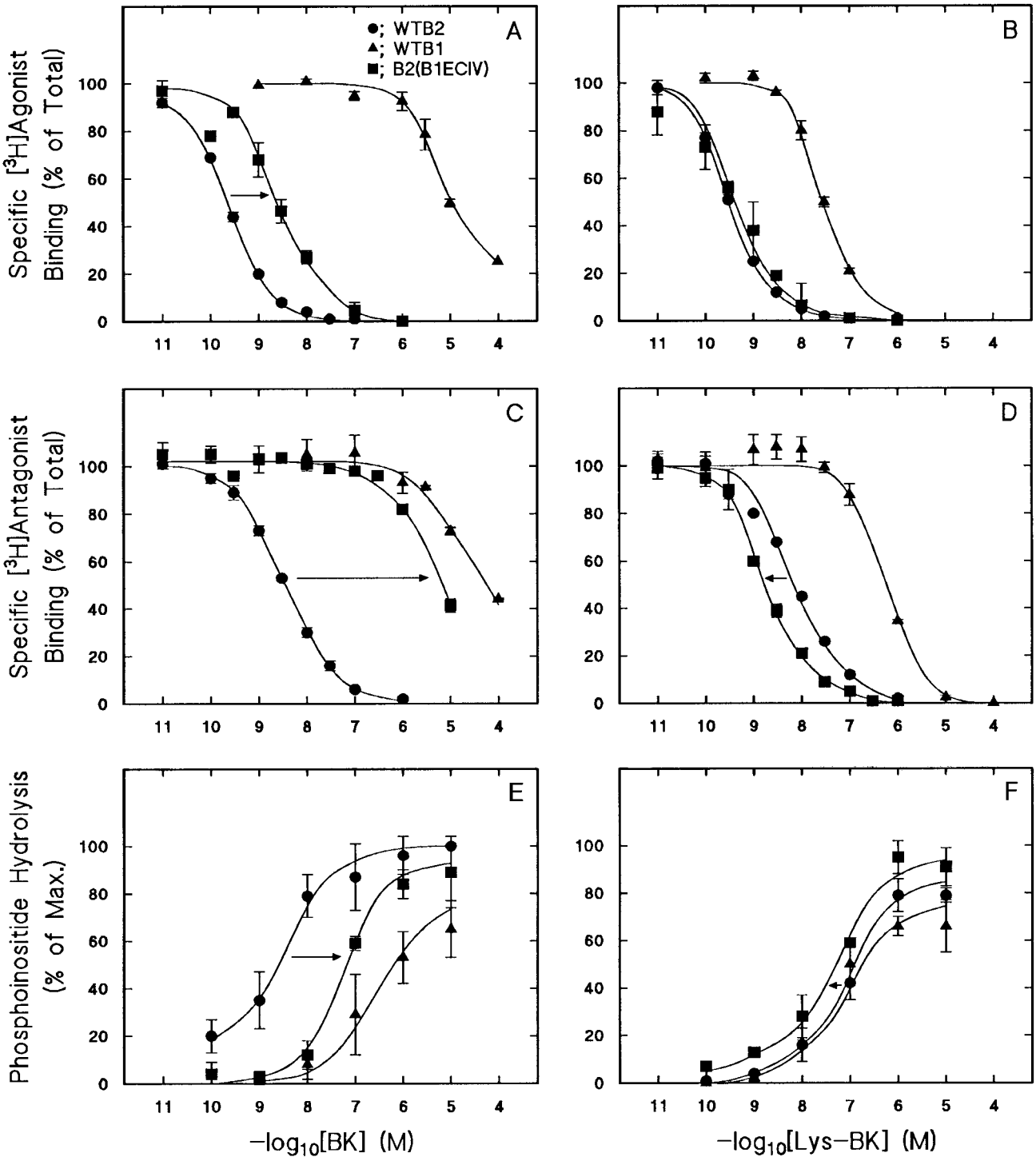

Fig. 2. BK and Lys-BK binding and function isotherms on human WT B1 and B2 receptors and B2(B1ECIV). HEK293 cells were transfected with WT B2 (●), WT B1 (▲), and B2(B1ECIV) (■). A–D, particulate preparations of the cells were incubated with K_D concentrations of [^{3}H]agonist (A and B), including [^{3}H]BK or [^{3}H]desArg^{10}Lys-BK, or [^{3}H]antagonist (C and D), including [^{3}H]NPC17731 or [^{3}H]desArg^{10}[Leu^9]-Lys-BK in the absence and presence of increasing concentrations of BK (A and C) or Lys-BK (B and D) as indicated and assayed as described in *Experimental Procedures*. E and F, cells were incubated in the absence and presence of increasing concentrations of BK (E) or Lys-BK (F) as indicated and then assayed for [^{3}H]inositol phosphates as in *Experimental Procedures*. The results are presented as percentage of maximum stimulation, where 100% maximum stimulation is the response of WTB2 to BK and WTB1 to desArg^{10}-Lys-BK and were 104,079 ± 13,189 and 66,026 ± 4,584 dpm (n = 2). The results are presented as means ± S.E. of three experiments with each point assayed in duplicate. Some points have error bars that are smaller than the symbols. K_D and K_I values are given in Table 3. Arrows indicate the effect of the substitution.

substitution of B1 TM-I/IC-I/TM-II/TM-VII in the B2 receptor resulted in a chimera, B2(B1I;II;VII), with a pharmacological profile very similar to that of the WT B2 receptor. The same profile was observed when each of these domains had been substituted individually (data not shown). A typical B2 receptor profile also was observed following substitution of B1 TM-V in the B2 receptor [B2(B1V)] (Table 2). However, substitution of B1 TM-IV in the B2 receptor to make B2(B1IV) resulted in a decrease (9-fold) in the affinity of the agonist BK without a significant effect on the affinity of the antagonist NPC17731. Thus, B1 TM-IV may contain residues that discriminate slightly against the binding of B2 receptor-selective agonists. However, among the TM domains in the B1 receptor, B2 receptor-selective peptide ligands are discriminated against primarily by TM-III and TM-VI as previously described (Leeb et al., 1997; Fathy et al., 1998).

Substitution of B1 EC-II and B1 EC–III individually in the B2 receptor to make B2(B1ECII) and B2(B1ECIII), respectively, yielded chimeras with pharmacological profiles very similar to that of the WT B2 receptor (Table 2). However, substitution of B1 EC-I in the B2 receptor to make B2(B1ECI) decreased the affinities of all B2-selective peptide ligands, including both the agonist BK (21-fold) and the antagonist NPC17731 (9-fold) as well as of the B1-selective peptide antagonist NPC18565 (15-fold). Substitution of B1 EC-IV to make B2(B1ECIV) also influenced the affinities of several ligands. However, contrary to B1 EC-I the effect of B1 EC-IV was specific for peptide agonists with a 14-fold drop in the affinity of BK. Interestingly, this substitution increased the affinity of the B1-selective agonist desArg^{10}-Lys-BK by >30-fold. Together with previously published observations (Leeb et al., 1997; Fathy et al., 1998), these results show that the binding B2-selective peptide agonists in the human B1 receptor is discriminated against primarily by TM-III, TM-VI, EC-I, and EC-IV, whereas peptide antagonist binding is discriminated against primarily by EC-I and TM-III.

N-Terminal Residue of Peptide Agonists Interacts with Fourth EC Domain of B1 Receptor. To directly investigate the positioning of the peptide agonist N terminus in the human B1 receptor, we took advantage of the fact that this receptor discriminates between peptide agonists with and without an N-terminal Lys, whereas the human B2 receptor does not. As shown in Table 3, this difference in receptor discrimination is clearly depicted in the binding constants of BK and Lys-BK for these receptors. To more directly portray this difference, we calculated the ratios of the BK and Lys-BK binding constants, $K_{D(BK)}/K_{D(Lys\text{-}BK)}$ and $K_{I(BK)}/K_{I(Lys\text{-}BK)}$, for these receptors, which were 60 and 49, respectively, for the WT B1 receptor and 1.2 and 0.45, respectively, for the WT B2 receptor. The discriminatory property of the WT B1 receptor also was observed in the binding of the B1-selective agonists desArg^9-BK and desArg^{10}-Lys-BK. The K_D values of these peptides for this receptor were 640 and 0.098 nM, respectively, yielding an affinity ratio of 6531. The affinities of desArg^9-BK and desArg^{10}-Lys-BK for the WT B2 receptor and many of the primarily B2 receptor-based chimeras used in this study were too low (K_D and K_i >5000 nM) to accurately determine their affinity ratios on these constructs. Thus, we determined the ratios for BK and Lys-BK in each of the receptor chimeras to identify the B1 receptor domain that is responsible for the discrimination. For B1 TM-III and TM-VI, we used the chimeras B2(B1IIIS^{111}) and B2(B1IVF^{259},T^{263}), where we have previously shown that high-affinity [^{3}H]BK and [^{3}H]NPC17731 binding is restored to that of the WT B2 receptor by internal domain substitutions (Leeb et al., 1997; Fathy et al., 1998). As shown in Table 3, in most of the chimeras the ratios varied only minimally (<2-fold for $K_{D(BK)}/K_{D(Lys\text{-}BK)}$ and <10-fold for $K_{I(BK)}/K_{I(Lys\text{-}BK)}$) from those for the WT B2 receptor. The major exception was B2(B1ECIV) in which $K_{D(BK)}/K_{D(Lys\text{-}BK)}$ and $K_{I(BK)}/K_{I(Lys\text{-}BK)}$ were ~4- and 3900-fold higher, respectively, than those for the WT B2 receptor (Table 3). The primary effect of the B1 EC-IV substitution was to decrease the potency of BK as determined by competition with both [^{3}H]agonist (Fig. 2A) and [^{3}H]antagonist binding (Fig. 2C), and by the ability of BK to stimulate phosphoinositide hydrolysis (Fig. 2E), whereas the same parameters for Lys-BK were essentially unaltered (Fig. 2, B, D, and F). These results show that it is EC-IV in the human B1 receptor that enables it to discriminate between peptide agonists with and without an N-terminal Lys.

Multiple B1 Receptor Residues Contribute to Interaction with N-Terminal Residue of Peptide Agonists. To investigate in finer detail the epitope in B1 EC-IV that is interacting with the peptide Lys, the N- and the C-terminal halves of B1 EC-IV were substituted individually in the B2 receptor. In B2(B1ECIV(N)), in which the N-terminal half of B1 EC-IV was substituted, residues 266 to 276 in the WT B2 receptor were replaced with the corresponding residues in the WT B1 receptor, which are residues 273 to 283. In B2(B1ECIV(C)), in which the C-terminal half was substituted, residues 277 to 284 in the WT B2 receptor were replaced with the corresponding residues in the WT B1 receptor, which are residues 283 to 291. As shown in Table 4, the K_I values for BK and Lys-BK binding to B2(B1ECIV(N)) were 2-fold higher and 4-fold lower, respectively, than those for the WT B2 receptor, whereas in B2(B1ECIV(C)) the values were 12-fold higher and 5-fold lower, respectively. Clearly, neither of the partial B1 EC-IV substitutions accounted for the 413-fold increase and 9-fold decrease in the K_I values for BK and

TABLE 4
K_I values for kinin analogs on WT B1 and B2 BK receptors and chimeric B1/B2 receptor constructs expressed in HEK293 cells

Ligand	WTB1	WTB2	B2(B1ECIV)	B2(B1ECIV(N))	B2(B1ECIV(C))
			nM[a]		
BK	21700 ± 8700	1.92 ± 0.24[b]	793 ± 341	3.15 ± 1.84	22.4 ± 12.1
L-Lys-BK	441 ± 40	4.30 ± 0.27[b]	0.458 ± 0.006	1.06 ± 0.658	0.854 ± 0.363
D-Lys-BK	2820 ± 150	4.56 ± 0.55	121 ± 35		
Ala-BK	12000 ± 4300	3.84 ± 0.17	414 ± 108		
Arg-BK	1830 ± 180	7.14 ± 1.74	44.0 ± 5.0		

[a] The constants are presented as K_I values. The values were calculated by the Radlig program with data obtained from competition-binding experiments with the agonists antagonists [^{3}H]NPC17731 and [^{3}H]desArg^{10}[Leu^9]-Lys-BK, and the values are presented as means ± S.E. of three experiments.
[b] From Fathy et al. (1998).

Lys-BK, respectively, caused by the entire B1 EC-IV substitution. Thus, the ability of the human B1 receptor to interact with the peptide N-terminal Lys reside in multiple residues distributed throughout EC-IV.

Interaction of N-Terminal Residue of Peptide Agonists Depends on Charge and Proper Spatial Orientation of Residue. To determine the nature of the interaction of the peptide N-terminal L-Lys with B1 EC-IV, complementary mutations were made in the peptide ligand. Figure 3A and Table 4 show that addition of L-Ala to BK did not significantly restore the affinity of the peptide for B2(B1ECIV). However, an increase in the peptide affinity was observed following addition of L-Arg. Thus, the side chain charge of the residue is important for binding. Interestingly, addition of D-Lys only partially restored the affinity of the peptide. We conclude from these results that both the charge and the spatial orientation of the N-terminal residue are critical for high-affinity interaction with B1 EC-IV.

As shown in Fig. 3B and Table 4, the WT B2 receptor was completely unable to discriminate between the three peptide analogs. However, the WT B1 receptor bound these peptides with an order of potency identical to that of B2(B1ECIV). These results show that EC-IV is directly responsible for the interaction with the peptide N-terminal L-Lys in the WT B1 receptor.

Discussion

In this study, we show that high-affinity binding of peptide agonist ligands to the human B1 receptor depends on an ionic and stereospecific interaction of an N-terminal L-Lys in the peptide with EC-IV in the receptor. The specific nature of this interaction was revealed by using chimeras of human B2 and B1 BK receptors and complementary mutations in the kinin peptide agonists for these receptors. Substitution of B1 EC-IV in the B2 receptor decreased the affinity of BK, a B2 receptor-selective peptide, whereas this substitution had essentially no effect on the affinity of Lys-BK, a less receptor-selective peptide, and slightly enhanced the affinity des-Arg^{10}-Lys-BK, a B1 receptor-selective peptide. N-Terminal extension of BK unveiled that the inhibitory effect of B1 EC-IV was neutralized by the proper spatial orientation of a positive charge at the N terminus, which was optimal with L-Lys. The same peptide requirements were necessary for binding to the WT B1 receptor, indicating that this is a biologically relevant mechanism.

The mechanism underlying B1 EC-IV inhibition of BK binding and the neutralization of this inhibitory action by addition of an N-terminal L-Lys is not clear but seems to involve the coordinated effect of multiple residues in both the N- and C-terminal halves of this domain that prevents BK from reaching critical binding epitopes located in TM-VI below EC-IV (Fig. 4). However, Lys^1 in Lys-BK may neutralize this effect and open the binding pocket. This mechanism may be related to that which is thought to occur with two conserved aspartates located at the N- and C-terminal ends of EC-IV in the B2 receptor and which are Asp^{268} and Asp^{286} in the human receptor (Fig. 4). Individual alanine mutation of these residues in the rat B2 receptor resulted in only limited decreases (19- and 28-fold, respectively) in the affinity of BK, whereas simultaneous mutation of these residues resulted in a dramatic decrease (500-fold) in the BK affinity (Novotny et al., 1994). These aspartates are thought to jointly coordinate the interaction of the receptor with the N terminus and/or with the guanidinium group in the side chain of Arg^1 in BK (Kyle et al., 1994), a residue that is critical for B2 receptor

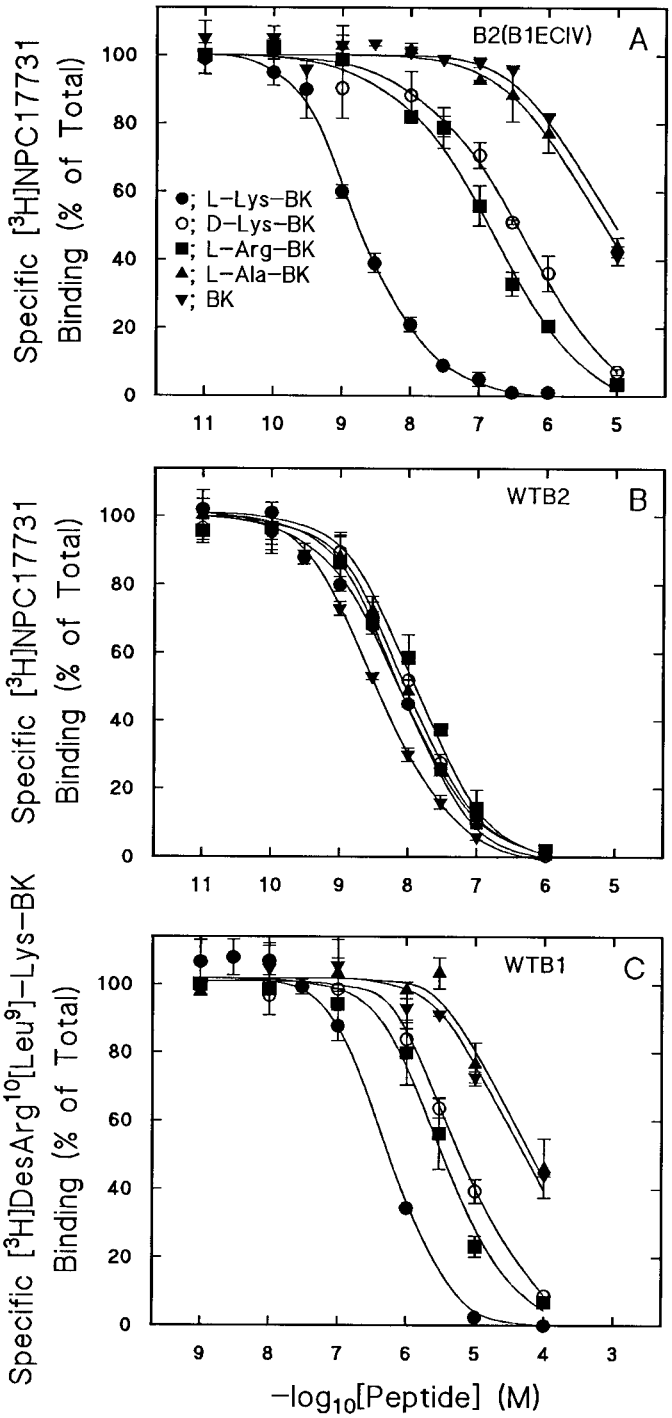

Fig. 3. Kinin peptide binding isotherms on WT B2 and B1 BK receptors and B2(B1ECIV). Particulate preparations of HEK293 cells expressing B2(B1ECIV) (A), WTB2 (B), or WTB1 (C) were incubated with K_D concentrations of [^{3}H]antagonist, including [^{3}H]NPC17731 (A and B) or [^{3}H]desArg^{10}[Leu^9]-Lys-BK (C) in the absence and presence of increasing concentrations of L-Lys-BK (●), D-Lys-BK (○), L-Arg-BK (■), L-Ala-BK (▲), and BK (▼) as indicated and assayed as described in *Experimental Procedures*. The results are presented as means ± S.E. of three experiments with each point assayed in duplicate. Some points have error bars that are smaller than the symbols. K_I values are given in Table 4.

binding (Regoli and Barabe, 1980). Given that the addition of amino acids at the N terminus of BK has little effect on B2 receptor binding, the aspartates most likely interact with the side chain guanidinium group. These aspartates are conserved in the B1 receptor being Glu^{273} and Asp^{291}, respectively, in the human receptor, and they presumably interact with Arg^{2} in Lys-BK and $desArg^{10}$-Lys-BK, a residue that is critical for B1 receptor binding (Regoli and Barabe, 1980). This conservation argues that the decrease in BK affinity observed following substitution of B1 EC-IV in the B2 receptor cannot be attributed to the absence of these two negatively charged residues. Interestingly, Asp^{291} is conserved in the AT1 angiotensin II receptor being Asp^{281} in rat AT1 receptor, and this residue is thought to interact with the side chain guanidinium group of Arg^{2} in angiotensin II (Hjorth et al., 1994; Feng et al., 1995). Furthermore, Asp^{1} in this peptide, which is equivalent to Lys^{1} in Lys-BK, is thought to interact with extracellular residues. However, the removal of Asp^{1} in angiotensin II is not as detrimental to binding to the AT1 receptor as the removal of Lys^{1} in Lys-BK and $desArg^{10}$-Lys-BK is to binding to the human B1 receptor (Timmermans et al., 1993).

In addition to the B1 and B2 BK receptors and the AT1 receptor, other G protein-coupled receptors also rely on EC-IV for ligand binding, discrimination, and/or function. In the follicle-stimulating hormone receptor, EC-IV is apparently involved in both hormone binding and stimulation of cAMP production (Ryu et al., 1998), and in the CC chemokine receptor 5 this domain is important for coreceptor activity with HIV (Alkhatib et al., 1997). Furthermore, EC-IV is critical for ligand discrimination in the human δ-opioid receptor (Wang et al., 1995; Varga et al., 1996).

Unlike the human, rabbit, and porcine B1 receptors, which bind $desArg^{10}$-Lys-BK with considerably higher affinity than $desArg^{9}$-BK, the mouse and rat B1 receptors bind these two peptide agonists with approximately equally high affinity (Hess et al., 1996; Ni et al., 1998). Interestingly, rodent kininogens differ from the human ones in that the amino acid preceding the BK sequence is Arg rather than Lys (Furuto-Kato et al., 1985; Sueyoshi et al., 1990; Hess et al., 1996). In other words, in rodent tissue kallikrein produces Arg-BK rather than Lys-BK. Thus, the rodent B1 receptors may have undergone coevolution with their kininogens to compensate for the absence of an N-terminal Lys in their ligands to remain functionally relevant.

Previous studies have concluded that BK, when bound to the human B2 receptor subtype, reaches from the extracellular surface of the receptor adjacent to EC-IV, where the BK N terminus is positioned (Novotny et al., 1994; Herzig and Leeb-Lundberg, 1995; Abd Alla et al., 1996; Herzig et al., 1996), down two helical turns along the interior face of TM-VI and residues Thr^{263} and Phe^{259} (Nardone and Hogan, 1994; Leeb et al., 1997), and across into a pocket bordered by the interior face of TM-III, and presumably TM-IV, -V, and -VI, adjacent to a position in TM-III that is occupied by Ser^{111} and where the BK C terminus is located (Fathy et al., 1998). This position is occupied by Lys^{118} in the human B1 receptor, which serves as a counterion for the C terminus of B1-selective desArg peptide ligands when bound in this receptor (Fathy et al., 1998). It appears that TM-VI provides some contact points for peptide agonists also in the B1 receptor even though their identity is currently unknown (Leeb et al., 1997). However, it is clear from the results presented in this report that in the B1 receptor, the N terminus of peptide agonists is extracellular and adjacent to EC-IV. Consequently, human B2 and B1 receptors, although only 36% homologous, orient their natural ligands BK and $desArg^{10}$-Lys-BK, respectively, in very similar manners. The binding energy for $desArg^{10}$-Lys-BK in the human B1 receptor appears to be provided primarily by ionic interactions contributed by the amino acid side chains at positions 1 and 2 in the peptide as discussed herein and by the C terminus at position 9 (Fathy et al., 1998), whereas positions 3 through 6 serve primarily a structural role (Regoli and Barabe, 1980; Tancredi et al., 1997). However, the binding energy for BK in the B2 receptor seems to be provided by nonionic interactions

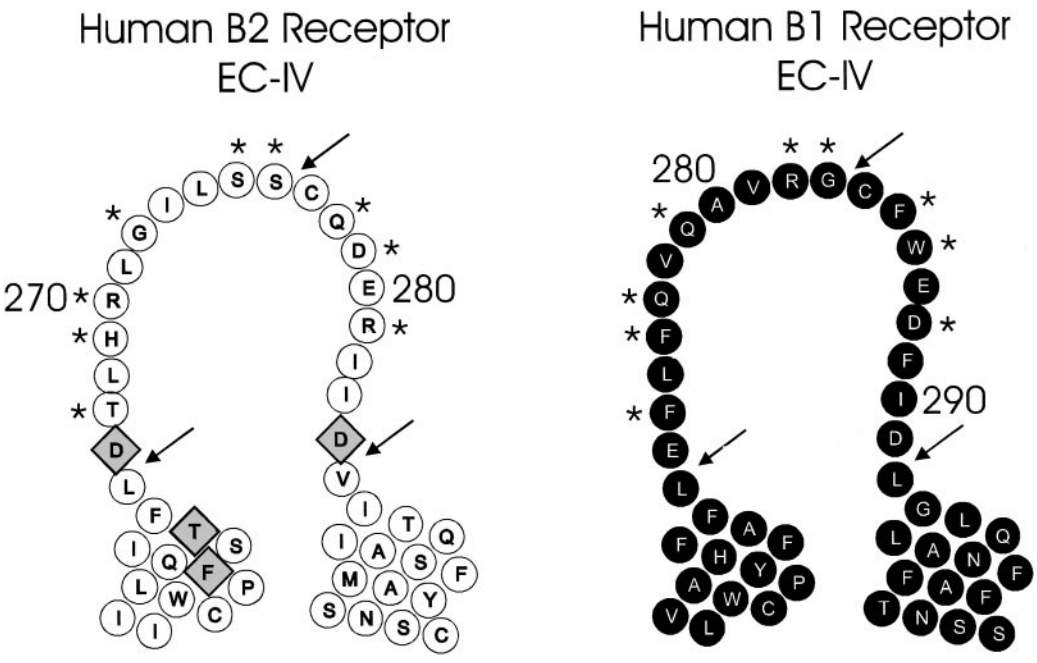

Fig. 4. Sequence comparison of amino acids in EC-IV of human B1 and B2 BK receptors. Amino acid sequences of the EC-IV in the B2 (open) and B1 (closed) receptors. Indicated are residues critical for BK binding in the B2 receptors (boxed), nonconserved residues (*), and boundaries of the B2(B1ECIV), B2(B1ECIV(N)), and B2(B1ECIV(C)) chimeras (arrows). The numbering is as described in Fig. 1.

contributed by residues located throughout the peptide and only partially by ionic interactions by the side chain at position 1 (Regoli and Barabe, 1980; Novotny et al., 1994; Tancredi et al., 1997). Interestingly, the AT1 receptor, which is ~30% homologous to the BK receptors and binds the structurally related ligand angiotensin II, seems to orient its ligand in a similar fashion. In the rat AT1 receptor, the side chain of Arg^{2} in angiotensin II is thought to interact with Asp^{281} in EC-IV as discussed above (Hjorth et al., 1994; Feng et al., 1995), whereas Lys^{199} in TM-V that is juxtaposed to the position in TM-III occupied by Ser^{111} and Lys^{118} in the B2 and B1 receptor, respectively, provides a counterion for the C terminus of the ligand (Noda et al., 1995; Yamano et al., 1995).

The C-terminal residues of peptide antagonists when bound in the B2 and B1 receptors also are located adjacent to Ser^{111} and Lys^{118}, respectively, in TM-III (Fathy et al., 1998). Interestingly, in the B2 receptor TM-III also was recently shown to be important in receptor function (Marie et al., 1999). However, the location of the antagonist N terminus is unknown in both receptor subtypes but appears to be different from that of peptide agonists, at least for second-generation B2 receptor antagonists such as NPC17731 and HOE140 (Abd Alla et al., 1996; Herzig et al., 1996) and the B1 receptor-selective NPC17731 analog NPC18565 as shown herein.

The decrease in the apparent affinity of BK following substitution of B1 EC-IV in the B2 receptor as measured with [^{3}H]NPC17731 as the radioligand was considerably greater than that observed with [^{3}H]BK. Furthermore, the B_{max} measured with [^{3}H]BK was approximately one-fiftieth of that measured with [^{3}H]NPC17731 (data not shown). It has been previously postulated based on studies in the NK1 neurokinin receptor that this effect is due to conformational changes that prevent the receptor from adopting an active, agonist-preferred conformation (Schwartz et al., 1997). Thus, the ability of an agonist such as BK to compete for binding with an antagonist such as NPC17731 is impaired by the inability of the receptor to isomerize to an active, agonist-preferred state. However, given that Lys-BK, which is also an agonist, is in fact slightly enhanced in its ability to compete with [^{3}H]NPC17731 binding by B1 EC-IV substitution, the notion that the receptor is unable to adopt an agonist-preferred state does not seem to be the case. Instead, it is a loss in the ability specifically of BK to promote the conformational effect that allows BK to compete with antagonist binding. An N-terminal L-Lys in the peptide ligand restores to the ligand the ability to cause this effect. This conclusion is in accordance with results in the CCKA cholecystokinin receptor where a methionine in EC-III was found to be necessary to coordinate the binding of sulfated agonist ligands and drive the receptor into the required conformation (Gigoux et al., 1998). In the absence of the methionine, both the ligand binding density and ability to compete with antagonists were dramatically impaired.

In summary, we have shown in this study that the N-terminal residue of kinin peptide agonists when bound in the human B1 receptor is extracellular and adjacent to EC-IV. Furthermore, high-affinity binding to this receptor depends on the direct interaction of an L-Lys at the peptide N terminus with this receptor domain. This study completes the description of the orientation of peptide agonists when bound to the B2 and B1 receptors and show that they are oriented in a similar fashion in these receptors regardless of the relatively low homology of the receptors.

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