The Sixth Transmembrane Domains of the Human B1 and B2 Bradykinin Receptors Are Structurally Compatible and Involved in Discriminating between Subtype-selective Agonists*

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In order to investigate the molecular basis for the ability of the human B1 and B2 bradykinin (BK) receptor subtypes to discriminate between subtype-selective ligands, we constructed chimeric proteins in which the sixth transmembrane domains (TM-VI) of these receptors were exchanged. The pharmacological profiles of the constructs were analyzed by radioligand binding in particulate preparations of transiently transfected HEK293 cells using the agonist [3H]des-Arg10-kallidin and the antagonist [3H]NPC17731. The ability of these constructs to transmit an intracellular signal was measured in transiently transfected A10 cells, a vascular smooth muscle cell line, by single cell Ca2+ imaging. Substitution of B1 TM-VI into the B2 receptor (B2(B1VI)) dramatically reduced the affinity of the B2-selective agonist BK, whereas the affinity of the B2-selective antagonist NPC17731 was unaltered. High affinity BK binding was fully regained when two residues, Tyr259 and Ala263, near the extracellular surface of TM-VI in B2(B1VI), were replaced with the corresponding residues in the wild-type B2 receptor, which are Phe259 and Thr263. The construct B1(B2VI), produced by substitution of B2 TM-VI into the B1 receptor, did not support high affinity binding of the B1-selective agonist des-Arg10-kallidin. In contrast to BK and des-Arg10-kallidin, the binding of the less subtype-selective agonist kallidin showed little sensitivity to TM-VI exchange. These results show that TM-VI in the human B1 and B2 BK receptor subtypes, although only 36% identical, are structurally compatible. Furthermore, this domain contributes significantly to the ability of these receptors to discriminate between the subtype-selective agonists BK and des-Arg10-kallidin.

Kinins are potent inflammatory mediators that stimulate a wide spectrum of activities such as vasodilatation, vascular permeability, and pain (1–3). Based on the ability of kinin peptides to contract and relax various vascular smooth muscle tissues, receptors for kinins have been classified into two subtypes, termed B1 and B2 (1, 4). The B2 receptor is the most prevalent subtype under nonpathological conditions, whereas the B1 receptor activity is normally low but increases following trauma (5). The two receptor subtypes can be distinguished by BK1, which binds primarily to the B2 receptor, and the carboxypeptidase fragments des-Arg9-BK and des-Arg10-Lys-BK, also known as des-Arg10-kallidin, which bind primarily to the B1 receptor. The cDNAs for these receptors have been cloned from several species including human (6–8), rat (9), mouse (10–12), and rabbit (13, 14). Analysis of their primary sequences has revealed that both receptors are members of the G-protein-coupled receptor superfamily which have seven hydrophobic domains that span the membrane. Although B1 and B2 receptors are coupled to similar signaling pathways (15, 16) and discriminate between ligands which differ only in their C-terminal amino acid (1, 4), the primary structures of these receptors are relatively different, with the human receptors exhibiting only 36% identity (6–8).

No information has yet been published on determinants involved in ligand binding to the B1 receptor, although some information is available about the determinants in the B2 receptor. In the rat B2 receptor, site-directed mutagenesis has shown that BK binding is dependent on two aspartate residues, Asp266 and Asp284, in EC-IV (17), and on Phe261 and Thr265 located below Asp266 in TM-VI (18, 19). In the human B2 receptor, cross-linking of BK with short bifunctional reagents has shown that the N terminus of BK when bound to the receptor is adjacent to Cys277 in EC-IV (20, 21). Furthermore, antibodies directed specifically against the C-terminal half of EC-IV in the human B2 receptor interfere with BK binding (22). Thus, it is possible that residues in EC-IV and the adjacent TM-VI form part of the binding site for BK in the B2 receptor. These results are supportive of a model of BK bound to the B2 receptor which proposes that the N terminus or the positively charged side chain of Arg1 interacts with BK (23, 24). Very little is known about the binding site(s) for antagonists in the B2 receptor, but all studies agree that this site(s) is at least in part different from that for agonists.

The construction of chimeric proteins of receptors has been shown to be a fruitful approach to unraveling the structural elements involved in their abilities to discriminate between different ligands. In terms of G-protein-coupled receptors, this approach has worked best with receptor subtypes, as such receptors generally exhibit a relatively high degree of homology (25–31). The B1 and B2 BK receptor subtypes exhibit a surprisingly low overall degree of homology. However, the simi-
larity between the B1 and B2 receptors increases significantly when considering only those residues facing the ligand binding pocket proposed for G-protein-coupled receptors. In light of the potential role of TM-VI in agonist binding to the B2 receptor, we were interested in evaluating the involvement of this domain in the ability of human B1 and B2 receptors to discriminate between kinin ligands. Consequently, we constructed chimeric proteins in which TM-VI in these receptors were exchanged. The pharmacological profiles of these constructs and their abilities to signal were then determined in transfected cells by radioligand binding and single cell Ca\(^{2+}\) imaging.

**EXPERIMENTAL PROCEDURES**

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**Materials—**[3,4-prolyl-3,4-\(^{3}\)H]Des-Arg\(^{10}\)-kallidin (107 Ci/mmol), [2,3-prolyl-3,4-\(^{3}\)H]bradykinin (110 Ci/mmol), and [prolyl-3,4-\(^{3}\)H]NPC17731 (53.5 Ci/mmol) were obtained from DuPont NEN. NPC17731 and NPC18565 were generous gifts from Donald J. Kyle, Scios, Inc., Sunnyvale, CA. Kallidin was obtained from Peninsula (Belmont, CA), and des-Arg\(^{10}\)-kallidin and NPC349 were from Bachem (Torrance, CA). LipofectAMINE, DMEM, Leibovitz's L-15 medium, and Hank's balanced salt solution were from Life Technologies, Inc. Reagents for calcium phosphate transfections were purchased from 5 Prime (Boulder, CO). Fura-2/AM was from Molecular Probes (Eugene, OR). Enzymes were obtained from Life Technologies, Inc. and New England Biolabs (Beverly, MA). Sera and all other peptides and chemicals were from Sigma.

**Construction of Receptor cDNA—**The original human B1 and B2 receptor clones in vector pcDNA3 (Invitrogen) were kindly provided by J. Fred Hess, Merck Research Laboratories, West Point, PA. Fusions between the B1 and the B2 receptor cDNA clones were made using a modified PCR ligation-PCR protocol (32). The B2 clone was modified by introducing a XhoI site at the 3' end of the insert. Appropriate B1 and B2 clone fragments were amplified in 100-μl PCR reactions containing 10 ng of template DNA, 200 μM dNTPs, 100 pmol of each primer, 2.5 units of Phusion polymerase, and the reaction buffer supplied by the manufacturer (Stratagene, La Jolla, CA). T7 and SP6 were chosen as flanking primers, and internal primers were designed according to the desired fusion point in the chimeric construct. Appropriate gene fragments were amplified for 20 cycles and purified using 1% agarose gels and the QiaEx II Kit (Qiagen, Chatsworth, CA). The isolated PCR products were combined, phosphorylated, and ligated using T4 DNA ligase. In a second PCR, the fusion product was amplified for 25 cycles. Amplification products were purified as described above, cut with HindIII and XhoI, and ligated into the pcDNA3 vector. The identity of the chimeric insert was confirmed by cycle sequencing. Pure plasmid DNA for transfections into mammalian cells was isolated with the Qiagen Plasmid Maxi kit (Qiagen).

**Cell Culture and Transfection—**Transiently transfected HEK293 cells were used for analysis of radioligand binding. These cells were grown in DMEM supplemented with 10% heat-inactivated horse serum at 37°C in 10% CO\(_{2}\). At 24 h before transfection, the cells were seeded into 100-mm dishes at 60–80% confluency. Cells were transfected using the calcium phosphate precipitate method with overnight incubation in the presence of 15 μg of cDNA per dish. At 48–96 h after transfection, the cells were washed twice with ice-cold PBS and then pelleted by centrifugation at 2,000 g for 10 min. Transiently transfected A10 cells were used for analysis of intracellular Ca\(^{2+}\) mobilization. These cells were grown in DMEM supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 10% CO\(_{2}\). At 24 h before transfection, the cells were seeded at 80% confluency in 6-well plates containing 25-mm glass coverslips that had been pretreated by incubation in growth medium. Cells were transfected by incubation for 6 h in 1 ml of DMEM containing 6 μl of LipofectAMINE and 2 μg of cDNA per well. The cells were allowed to recover for about 48 h before loading with fura-2.

**Membrane Preparation—**Transfected HEK293 cells were resuspended in a buffer containing 25 mM Tris, pH 6.8, 0.5 mM EDTA, 0.2 mM MgCl\(_{2}\), and 1 mM 1,10-phenanthroline and homogenized using 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 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 1,000–4,000 dpm/assay of specific radioligand binding. Binding assays were performed in a total volume of 0.5–1 ml with either [\(^{3}\)H]Hides-Arg\(^{10}\)-KD, [\(^{3}\)H]HIBK, or [\(^{3}\)H]NPC17731 with or without varying concentrations of nonradioactive kinin peptides. After incubation for 60–90 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 then 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.

**Intracellular Ca\(^{2+}\) Mobilization—**Using a previously described protocol (16), A10 smooth muscle cells transfected with various cDNAs were incubated in modified Leibovitz's L-15 medium at room temperature with 4 μM fura-2/AM. The fura-2-loaded cells were equilibrated to 37°C in Hank's balanced salt solution with 1.3 mM Ca\(^{2+}\), pH 7.4, and stimulated by the addition of various ligands as noted in Fig. 4. The cytosolic free Ca\(^{2+}\) signal from individual cells was acquired and processed by an integrated digital imaging fluorescence microscopy system. Fura-2 was alternately excited at 340 and 380 nm, and the emissions were collected at 510 nm. The signals are presented in the figure as the ratio of bound/free Ca\(^{2+}\) (F\(_{340}/F_{380}\)).

**Measurement of Endogenous Receptor Activity—**Radioligand binding of [\(^{3}\)H]Hides-Arg\(^{10}\)-KD, [\(^{3}\)H]BK, and [\(^{3}\)H]NPC17731 to naive and mock-transfected cells demonstrated that HEK293 cells had no detectable endogenous B1 and B2 receptor activity. Furthermore, kinin agonists failed to produce a Ca\(^{2+}\) signal in naive and mock-transfected A10 cells.

**RESULTS AND DISCUSSION**

In order to determine if the sixth transmembrane domains (TM-VI) of the human B1 and B2 BK receptors are structurally compatible and contribute to the ability of these receptors to discriminate among kinin ligands, we created chimeric receptor constructs in which TM-VI in the two receptors were exchanged. The chimeric constructs are represented schematically in Fig. 1. The nomenclature used for these constructs was B1(B2VI) for a B1 receptor with a B2 TM-VI and B2(B1VI) for a B2 receptor with a B1 TM-VI. In addition, we created a receptor construct, B2(B1VI), which specifically in two residues in TM-VI of B2(B1VI) were replaced with the corresponding residues in the WT B2 receptor. A PCR-based mutagenesis protocol was chosen so as to place the boundaries between the different parts of the chimeric receptors exactly at the membrane boundaries proposed by Baldwin (33).

The pharmacological profiles of the WT receptors and chimeric receptor constructs were evaluated by determining the binding constants for various kinin agonist and antagonist ligands in equilibrium radioligand binding assays using membrane preparations of HEK293 cells transiently transfected with receptor cDNAs (Figs. 2 and 3). The amino acid sequences of the kinin ligands used in this study are shown in Table I, and a summary of the binding constants of the ligands is given in Table II. The different cDNAs were also transfected into a vascular smooth muscle cell line, A10, to assess their ability to mediate mobilization of intracellular Ca\(^{2+}\). This was done to ensure that the constructs were properly folded and functionally active in mammalian cells (Fig. 4). Such a cell line was chosen since the pharmacology of kinins was originally done on vascular smooth muscle tissue (1), and, more recently, native B1 and B2 receptors in cultured vascular smooth muscle cells from the rabbit superior mesenteric artery have been shown to mediate mobilization of intracellular Ca\(^{2+}\) (15, 16).

As shown in Fig. 2 and Table I, Scatchard analysis of saturation binding curves revealed that the WT B2 receptors and the B2 receptor-like chimeric constructs B2(B1VI) and B2(B1VI)C\(^{539}\) were identified using the antagonist [\(^{3}\)H]NPC17731. The WT B2 receptor and the B2(B1VI)C\(^{539}\) construct, but not the B2(B1VI)C\(^{539}\) construct, also supported high affinity binding of the agonist [\(^{3}\)H]HIBK (data not shown). The WT B1 receptor was identified by high affinity binding of the agonist [\(^{3}\)H]Hides-Arg\(^{10}\)-KD. The B1 receptor-like chimeric construct B1(B2VI) did not support high affinity binding of any of these radioligands.
shown in Table II and Fig. 4, BK was found to be a highly selective agonist ligand at the human WT B2 receptor subtype with an affinity for this receptor ($K_i = 1.82 \pm 0.10$ nM) which is over three orders of magnitude higher than for the human WT B1 receptor subtype. Removal of the C-terminal arginine in BK to form des-Arg9-BK produces a dramatic decrease in affinity for the B2 receptor and change in subtype selectivity (1,6,8). Addition of a lysine to the N-terminus of des-Arg9-BK produces the highly selective B1 agonist des-Arg10-KD. Des-Arg10-KD bound to the human WT B1 receptor with an affinity (0.098 ± 0.034 nM) which is over four orders of magnitude higher than that for the B2 receptor. Adding back the C-terminal arginine to des-Arg10-KD to form the agonist KD produced a decrease in affinity for the B1 receptor by about two orders of magnitude but an increase in affinity for the B2 receptor by over three orders of magnitude. In fact, KD bound almost equally well to the two receptor subtypes. The affinity of KD for the B2 receptor ($K_i = 3.61 \pm 0.08$ nM) was only 3.2-fold higher than that for the B1 receptor ($K_i = 11.7 \pm 1.3$ nM). The pharmacological profiles of the human WT B1 and B2 receptors, as determined with the radioligands used in this study, agrees with the pharmacology upon which these receptors were originally classified (1).

Several classes of BK peptide antagonists have been developed. The first generation of B2 antagonists, of which NPC349 is a representative example, were developed around the crucial replacement of L-Pro7 in BK with a D-aromatic amino acid resulting in moderately high affinities for the B2 receptor (34, 35). In our study, NPC349 bound to the human WT B2 receptor with relatively high affinity ($K_i = 20.5 \pm 1.2$ nM). However, we found that this antagonist exhibited very low selectivity between the receptor subtypes (Table II). In second generation BK peptide antagonists, a restricted $\beta$-turn was introduced in...
the C-terminal portion of the peptides, and this modification substantially increased affinities of these antagonists for the B2 receptor. NPC17731 is a representative example of this generation of antagonists (36–38). Indeed, in this study, NPC17731 bound with very high affinity to the B2 receptor ($K_I = 0.180 \pm 0.007$ nM) and exhibited a selectivity (700-fold) for this receptor subtype which was much higher than that of NPC349. Curiously, in the WT B1 receptor, high concentrations of NPC17731 enhanced by about 2-fold the binding of a $K_d$ concentration of the B1-selective agonist des-Arg$^{10}$-KD. This result shows that NPC17731 increased the affinity of des-Arg$^{10}$-KD for this receptor. NPC17731 must cause this effect by occupying a site in the B1 receptor, distinct from that of des-Arg$^{10}$-KD, which allosterically stabilizes an agonist-preferred state of this receptor.

In analogy with B2 agonists, the removal of the C-terminal

![Graph of competition binding isotherms](image_url)
arginine from the B2 antagonist NPC17731 to form the des-Arg10 analog NPC18565 resulted in a dramatic decrease in affinity for the B2 receptor and an increase in affinity for the B1 receptor. The affinity of NPC18565 for the B1 receptor (Kd = 0.071 ± 0.009 nM) was found to be 368-fold higher than that for the B2 receptor. Furthermore, this ligand behaved as an antagonist at the B1 receptor2 (data not shown). Removal of the C-terminal arginine from HOE140, another B2-selective antagonist, also produces a B1 antagonist (39).

Ligand Binding to the Chimeric B2(B1VI) and B1(B2VI) Constructs—As described above, the affinity and selectivity of peptide agonists for the B1 and B2 receptors is greatly influenced by removing and adding basic residues at the N and C termini of the peptide. Thus, it is reasonable to suggest that at least some of the determinants in the B1 and B2 receptors which are involved in discriminating between peptide agonists are those that interact with the N- and C-terminal regions of these peptides. Site-directed mutagenesis suggests that BK when bound to the B2 receptor contacts residues in TM-VI and the adjacent EC-IV (17–19). In addition, chemical cross-linking indicates that this contact involves the N-terminal region of the peptide (20, 21). Consequently, we investigated the role of TM-VI in the ability of the human B1 and B2 receptors to discriminate between kinin peptides. This was done by exchanging these domains between the two receptor subtypes.

Substitution of B1 TM-VI into the B2 receptor to make the construct B2(B1VI) reduced by 75-fold the affinity of the B2-selective agonist BK (Table II). This result indicates that B2 TM-VI contains specific determinants which support high affinity BK binding that are not present in B1 TM-VI. Interestingly, this substitution reduced the affinity of KD only 6.8-fold. That the binding of KD was only slightly affected by the exchange of TM-VI is not surprising since KD bound to both WT B1 and B2 receptors almost equally well. B2(B1 VI) did not support binding of the B1-selective agonist des-Arg10-KD indicating that TM-VI is not sufficient to support binding of this ligand. The ability of these agonists to bind to B2(B1VI) correlated well with their ability to mobilize intracellular Ca2+ (Fig. 4).

Substitution of B2 TM-VI into the B1 receptor to make the construct B1(B2VI) completely prevented high affinity binding of the B1-selective agonist des-Arg10-KD. In addition, and not surprisingly, this construct did not support high affinity binding of the B2-selective ligands BK and NPC17731. Without high affinity binding sites in the B1(B2VI) construct for any radioligands used in this study, we were unable to determine a detailed pharmacological profile of this construct. However, this construct was apparently not misfolded, as it did respond to high concentrations of des-Arg10-KD and to the less subtype-selective agonist KD in the functional assay (Fig. 4). The lack of high affinity binding of des-Arg10-KD to B1(B2VI) suggests that B1 TM-VI does carry important determinants for the binding of this agonist. Without a high affinity radioligand for assay of B1(B2VI), it was not possible to determine the sensitivity of KD binding to this exchange.

The substitution of B1 TM-VI into the B2 receptor (B2B1VI) increased the affinity of the B2 antagonist NPC349 by 3.5-fold (Table II). This preference for B1 TM-VI may in part explain the relatively high affinity of NPC349 for the B1 receptor and shows that this ligand can sense changes in this domain. Indeed, in the rat B2 receptor, mutation of Gln262 to alanine near the extracellular surface of TM-VI interferes moderately with the binding of the first generation antagonist D-Arg-[Hyp3,D-Phe7]BK (18). This residue corresponds to Gln260 in the human B2 receptor and His267 in the human B1 receptor. In contrast, the binding of NPC17731 was not sensitive to substitution of B1 TM-VI into the B2 receptor. Together with the lack of effects of any of the point mutations in TM-VI made to date in the rat B2 receptor on the binding of NPC17731 (18), these results show that if this antagonist interacts with B2 TM-VI, this interaction is different from that of BK. Thus, unlike subtype-selective agonists, the selectivity of NPC17731 for the B2 receptor is not determined by TM-VI. The des-Arg10 analog NPC18565, which binds with moderately high affinity to the B2 receptor, also showed minimal sensitivity to this exchange.

Ligand Binding to the B2(B1VI)[F259,F263]T265—Previous mutagenesis studies of the rat B2 receptor showed that residues located near the extracellular surface in TM-VI are important for high affinity BK binding to this receptor (18, 19). Specifically, replacement of Phe261 with alanine decreases BK affinity by 1,600-fold, while replacement of Thr265 with either alanine or valine decreases BK affinity by 700-fold. Replacement of Gln262 with alanine causes a more modest 10-fold decrease in the affinity of BK for the rat B2 receptor. These residues correspond to Phe259 Thr263, and Gln260, respectively, in the human B2 receptor, and Tyr266, Ala270, and His267, respectively, in the human B1 receptor. While Gln→His and possibly also Phe→Tyr, are relatively conserved changes, the Thr→Ala, is a drastic change. In an attempt to assess the significance of Phe→Tyr and Thr→Ala on the ability of the human

### Table II

| Ligands       | B1          | Constructs       | B2          | B2(B1VI)     | B2(B1VI)[F259,F263]T265 |
|---------------|-------------|------------------|-------------|--------------|-------------------------|
|               | Kd a       | Ki a             | Kd b       | Ki b         | Kd c                   |
| Des-Arg10-KD  | 0.098 ± 0.034 | >5000            | 1.62 ± 0.10 | >5000        | 1.39 ± 0.15             |
| KD            | 11.7 ± 1.3  | 3.61 ± 0.08      | 24.7 ± 1.3  | >5000        | 0.333 ± 0.054           |
| NPC349        | 39.4 ± 0.2  | 20.5 ± 1.2       | 5.80 ± 0.34 | 0.025 ± 0.013 | 6.04 ± 0.56             |
| NPC17731      | 126 ± 2d   | 0.180 ± 0.007    | 0.181 ± 0.016 | 0.025 ± 0.013 | 6.04 ± 0.56             |
| NPC18565      | 0.071 ± 0.009 | 26.1 ± 3.2      | 9.71 ± 0.50 | 6.04 ± 0.56  | 6.04 ± 0.56             |

a The Kd values were determined in saturation binding experiments (Fig. 2) and are presented as the average ± S.E. of at least three experiments.

b The Ki values were determined in competition binding experiments (Fig. 3) using Ki concentrations of either [3H]des-Arg10-KD (WT B1) or [3H]NPC17731 (WT B2, B2(B1VI), and B2(B1VI,F259,T265)) and are presented as the average ± S.E. of at least three experiments. Ki was calculated using the equation Kd = IC50/[1 + (L/Ki)].

c The binding constant of a ligand was determined either as a Kd or a Ki value.

d EC50 value; NPC17731 increased the binding of [3H]des-Arg10-KD (Fig. 3).

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2 D. J. Kyle, personal communication.
B1 and B2 receptors to discriminate between kinin agonists, we replaced Tyr\textsuperscript{259} and Ala\textsuperscript{263} in B2(B1VI) with the corresponding residues in the WT B2 receptor (Fig. 1). This construct, B2(B1VI;F\textsuperscript{259},T\textsuperscript{263}), which differs only in two amino acids from B2(B1VI), bound BK with an affinity which is identical to that for the WT B2 receptor (Table II). Furthermore, B2(B1VI;F\textsuperscript{259},T\textsuperscript{263}) was responsive to BK stimulation in the functional assay (Fig. 4). Since, Phe\textsuperscript{259} and Thr\textsuperscript{263} now exist in a domain, B1 TM-VI, which is only 36% identical to the natural domain for these residues, the complete restoration of BK affinity upon replacement of these residues is most probably due to direct interaction of BK with these residues rather than to a conformational change in the receptor which favors BK binding. Furthermore, the presence of a tyrosine and alanine at these respective positions in the B1 receptor must deny BK important contact points in this receptor. The affinity of des-Arg\textsuperscript{10}-KD for B2(B1VI;F\textsuperscript{259},T\textsuperscript{263}) remained very low. Whether or not high affinity des-Arg\textsuperscript{10}-KD binding is sensitive to mutation of Tyr\textsuperscript{266} and Ala\textsuperscript{270} in TM-VI of the WT B1 receptor remains to be determined.

Replacement of Tyr\textsuperscript{259} and Ala\textsuperscript{263} in B2(B1VI) with phenylalanine and threonine, respectively, to make B2(B1VI;F\textsuperscript{259},T\textsuperscript{263}) not only restored the WT B2 receptor affinity of KD but increased it by 10-fold (Table II). One possible explanation for this result is that KD can use both B1 and B2 agonist-specific contact points in TM-VI of this construct. This replacement also moderately increased by 6- and 7-fold the affinities of NPC349 and NPC17731, respectively. Thus, even though the binding of these antagonists does not depend on residues in TM-VI crucial for BK binding, these ligands apparently can sense changes in this domain. In contrast, the binding of the B1 antagonist NPC18565 was insensitive to these replacements.

Conclusions—The significance of the results obtained in this study are severalfold. First, our results directly support the general model that G-protein-coupled receptors fold their hydrophobic, transmembrane domains in a similar fashion regardless of their sequence homology. Human B1 and B2 TM-VI, which exhibit only 36% identity, when exchanged with their respective receptor counterparts, support not only a proper binding pocket but also coupling to intracellular effectors. Thus, all the interhelical contacts necessary for the tertiary structure of the B1 and B2 receptor are maintained in these chimeric proteins. Furthermore, one would assume that the positions of Phe\textsuperscript{259} and Thr\textsuperscript{263} near the extracellular surface of this domain are crucial for high affinity binding of the B2-selective agonist BK. Residues important for the binding of the B1-selective agonist des-Arg\textsuperscript{10}-KD are unknown, but some of them may also be located in TM-VI as the binding of this agonist was also sensitive to TM-VI exchange. On the other hand, the binding of the less...
subtype-selective agonist KD showed little sensitivity to TM-VI exchange, which may mean that this agonist can use either B1 or B2 agonist-specific determinants in this domain. NPC17731 binding in the B2 receptor was insensitive to TM-VI exchange indicating that the selectivity of NPC17731 for this receptor is not determined by TM-VI. Also, these results provide further evidence that the agonist and antagonist binding sites in the B2 receptor are not identical. A differential role for TM-VI in agonist and antagonist binding to these receptors may be expected as this domain is adjacent to IC-III which is directly implicated in G-protein coupling in this family of receptors (40).

The specific involvement of residues in TM-VI in ligand binding to other G-protein-coupled receptors appears to vary with the receptor system and depends on whether agonist or antagonist binding is considered. The binding of some small monoamine agonists are moderately sensitive to mutation of residues near the extracellular surface in TM-VI (40, 41). The role of this domain in the binding of peptide agonists other than kinins may be limited. The neurokinin-1 receptor does not expect as this domain is adjacent to IC-III which is directly implicated in G-protein coupling in this family of receptors (40).

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