The N Terminus of Bradykinin B2 Receptor Is Adjacent to Extracellular Cys$^{20}$ and Cys$^{277}$ in the Receptor*

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Maryanne C. S. Herzig‡, Norman R. Nash§, Maureen Connolly¶, Donald J. Kyle‖, and
L. M. Fredrik Leeb-Lundberg**

From the ‡Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284; the
§Department of Neurology, Emory University, Atlanta, Georgia 30322; ¶Guilford Pharmaceuticals, Baltimore, Maryland
21224; and the ¶Department of Structural Biology and Medicinal Chemistry, Scios Inc., Sunnyvale, California 94086

Chemical cross-linking combined with site-directed mutagenesis was used to evaluate the role of extracellular
cysteines and their positions relative to the binding site for the agonist bradykinin (BK) in the human BK B2
receptor. All extracellular cysteines, Cys$^{20}$, Cys$^{103}$, Cys$^{184}$, and Cys$^{277}$, in the receptor were mutated to
serines, and single and double mutants were transfected into COS-7 cells. The Ser$^{20}$ and Ser$^{277}$ single mutants and
the Ser$^{103}$/Ser$^{277}$ double mutant bound [3H]BK and the antagonist [3H]N-PNP-17731 with pharmacological profiles
identical to the wild-type B2 receptor. In contrast, the Ser$^{103}$ and Ser$^{184}$ single mutants were unable to bind
either of the two radioligands. However, these mutants were still expressed as determined by immunoblotting
with anti-B2 receptor antibodies. Previous studies on the bovine B2 receptor showed that bifunctional re-
agents, which are reactive to amines at one end and to free sulfhydryls in the opposite end, cross-link the N
terminus of receptor-bound BK to a sulfhydryl in the receptor (Herzig, M. C. S., and Leeb-Lundberg, L. M. F.
(1995) J. Biol. Chem. 270, 20591–20598). Here, we show that m-maleimidobenzyol-N-hydroxysuccinimide ester
and 1,5-difluoro-2,4-dinitrobenzene cross-linked BK to the wild-type human B2 receptor and the Ser$^{20}$ and
Ser$^{277}$ single mutant receptors, whereas these reagents were unable to cross-link BK to the Ser$^{103}$/Ser$^{277}$ double
mutant. These results show that Cys$^{103}$ and Cys$^{184}$ are both required for expression of high affinity agonist
and antagonist binding sites in the human B2 receptor, while Cys$^{20}$ and Cys$^{277}$ are not required. Furthermore,
the results provide direct biochemical evidence that the N
terminus of BK, when bound to the B2 receptor, is
adjacent to Cys$^{277}$ in extracellular domain 4 and Cys$^{20}$ in
extracellular domain 1 of the receptor.

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*** To whom correspondence should be addressed: Dept. of Biochem-
istry, The University of Texas Health Science Center, 7703 Floyd Curl
Dr., San Antonio, TX 78284-7760. Tel.: 210-567-3766/3767; Fax: 210-
567-6585; E-mail: lundberg@bioc02.uthscsa.edu.

The abbreviations used are: BK, bradykinin; G-protein, guanine
nucleotide regulatory protein; GPCR, G-protein-coupled receptor; Gp-
p(NH)p, guanylyl-5’-yl imidodiphosphate; MBS, m-maleimidobenzyol-N-
hydroxysuccinimide ester; DFDNB, 1,5-difluoro-2,4-dinitrobenzene;
in human (3, 4), rat (5), mouse (6), and rabbit (7) has revealed
that this receptor belongs to the seven transmembrane-domain
GPCR superfamily. These receptors have an extracellular N
terminus named extracellular domain 1 (EC-I), an intracellular
C terminus named intracellular domain 4 (IC-IV), and seven
highly conserved transmembrane domains (TM-I–VII) inter-
rupted by three intracellular loops (IC-I–III) and three extra-
cellular loops (EC-II–IV) which alternate. The delineation of
the domains in GPCR that are involved in the binding of
ligands is a central question in the understanding of these
receptors and crucial in the development of specific receptor
antagonists. GPCRs for cationic amines bind agonists using
residues located exclusively within transmembrane domains
(8), while those for hydrophobic amines bind agonists using residues
located primarily in the extracellular domains (9). On the other
hand, the binding of peptide agonists to GPCRs appears to
involve residues located in both extracellular and transmem-
brane domains (8, 10, 11).

Only limited progress has been made in delineating the
binding site(s) for BK in the B2 receptor. Using site-directed
mutagenesis, residues located near the extracellular surface in
TM-VI were shown to be important for high affinity agonist
binding (12, 13). Furthermore, agonist binding was shown also
to be critically dependent on specific charged residues in EC-IV
(10). The involvement of extracellular residues in the binding of
BK to the B2 receptor is not surprising considering the hydro-
philic nature of this peptide. Interestingly, most mutations in
the B2 receptor that affect agonist affinity only minimally
affect antagonist affinity. Thus, the agonist and antagonist
binding sites in the B2 receptor do not appear to be identical
and may overlap only partially if at all.

Recently, we found that the N terminus of BK when bound
to the B2 receptor in bovine myometrial membranes can be cross-
linked by heterobifunctional cross-linkers to a sulfhydryl resi-
due(s) in the receptor (14). The sequence of the bovine B2
receptor is unknown. However, the sequences of the human
(3, 4), rat (5), mouse (6), and rabbit (7) B2 receptors contain
a conserved cysteine in each extracellular domain, as well as
different cysteines in the transmembrane and intracellular
domains. Considering that a sulfhydryl is located within the radii
of the linker arms of the cross-linkers attached to the N termi-

BES, N,N’-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid; CHAPS,
3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIPES,
1-(piperazin-1-ylmonomethyl)-2-aminoethane sulfonic acid; PBS, phosphate-buffered saline; WT, wild-
type; TM, transmembrane domain; EC, extracellular domain; IC, intra-
cellular domain; CHO, Chinese hamster ovary.
mutated to serines should enable us to identify directly the positioning of the N terminus of BK relative to specific cysteine residues in the receptor. In this report, we show the results of radioligand binding and cross-linking studies on WT human B2 receptors and receptors in which individual extracellular cysteines were replaced with serines.

### EXPERIMENTAL PROCEDURES

#### Materials—
[2,3-prolyl-3,4-\textsuperscript{H}]Bradykinin (110 Ci/mmol) and [prolyl-3,4-\textsuperscript{H}]HPNPC17731 (53.5 Ci/mmol) were purchased from DuPont NEN.

Fresh uteri from pregnant cows were from a local slaughterhouse.

Cell Culture—COS-7 cells (ATCC, Bethesda, MD) were seeded onto 10-cm dishes at a concentration of 8 \times 10^5 cells/dish and grown in complete medium (Ham's F-12 supplemented with 10% fetal bovine serum, glutamine, and 1% penicillin/streptomycin). COS-7 cells were of the highest grade available.

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Binding assays were initiated by addition of radioligand ([3H]BK and [3H]NPC17731, respectively). As shown in Table 1, the signal required 5–60 μg of protein/ml of the WT and the various mutant receptors. Binding assays were initiated by addition of radioligand (50 μl) with and without excess nonradiolabeled ligand to the receptor preparation (450 μl). After incubation for 60–90 min at 24°C, the assay was terminated by dilution with 4 ml of ice-cold PBS 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. Filters were counted for radioactivity in a Beckman LS5000TD scintillation counter.

Protein Cross-linking—Membranes were thawed and diluted to 5–60 μg/ml in cross-linking buffer (identical to binding buffer but with a pH = 7.2) and allowed to bind ligand for 60–90 min at 24°C as described above before addition of dimethyl sulfosuccinate (≤2% final concentration) with and without cross-linker; the incubation was continued for an additional 10 min. The cross-linking reaction was then quenched by a 1:1 dilution with 2% glycine in cross-linking buffer. In order to dissociate noncovalently bound radioligand, quenched samples were then diluted 4-fold with buffer containing 1 μM BK and 10 μM Gpp(NH)p and incubated for ≈180 min at 24°C, followed by filtration on GF/C filters as described above. Cross-linked ligand was the amount of radioligand remaining after dissociation. Specific cross-linked ligand was cross-linked radioligand minus nonspecific binding (as determined in the presence of 1 μM BK in the binding assay).

### RESULTS

**Expression of WT and Mutant B2 Receptors**—The expression of high affinity agonist and antagonist binding sites in the WT and serine mutant receptors was analyzed by radioligand binding using [3H]BK and [3H]NPC17731, respectively. As shown in Table I, both ligands bound with high affinities to the WT

| Construct | K\textsubscript{D(BR)} | K\textsubscript{D(NPC)} | K\textsubscript{D(BR)/K\textsubscript{D(NPC)}} | P\textsubscript{max(BR)/P\textsubscript{max(NPC)}} |
|-----------|-------------------------|------------------------|-----------------------------------------------|-----------------------------------------------|
| WT        | 0.110 ± 0.010           | 0.082 ± 0.011          | 1.36 ± 0.06                                   | 0.84 ± 0.13                                   |
| Ser\textsuperscript{20} | 0.173 ± 0.039          | 0.038 ± 0.004          | 4.44 ± 0.51                                   | 0.97 ± 0.09                                   |
| Ser\textsuperscript{777} | 0.185 ± 0.019          | 0.054 ± 0.012          | 3.66 ± 1.18                                   | 0.85 ± 0.25                                   |
| Ser\textsuperscript{20,777} | 0.147 ± 0.018          | 0.041 ± 0.005          | 3.60 ± 0.84                                   | 1.18 ± 0.14                                   |
| Ser\textsuperscript{103}  | ——                     | — — — — — —           | — — — — — —                                   | — — — — — —                                   |
| Ser\textsuperscript{184}  | ——                     | — — — — — —           | — — — — — —                                   | — — — — — —                                   |

\(a\) The K\textsubscript{D} values were determined in saturation binding experiments and are presented as the averages ± S.E. of at least two experiments.

\(b\) The values are averages ± S.E. of ratios from within individual experiments.

\(c\) No significant binding detected.
Procedures. Bands were visualized using ECL. Molecular mass standards are shown on the left side. k, kilodaltons.

In order to determine if the replacement of extracellular Cys20 and Cys277 with serines either individually or in combination in the human B2 receptor does not influence the pharmacological profile of the receptor.

Agnost Cross-linking to WT and Mutant B2 Receptors—We showed previously that the N terminus of BK when bound to the bovine B2 receptor can be cross-linked to a sulfhydryl residue in the receptor via the cross-linkers MBS and DFDNB (14). Here, we attempted to identify, using the human B2 receptor, the exact cysteine residue(s) to which BK can be linked. The effectiveness of MBS in cross-linking BK to receptor, the Ser^{20} and Ser^{277} single mutants, and the Ser^{20}/Ser^{277} double mutant. The ratio of the B_{max} value for ^{3}H/BK binding over that of ^{3}H/NPC17731 binding to each receptor construct was approximately 1, indicating that the two ligands identified the same number of binding sites in the constructs. In contrast, the ratio of the K_{D} value for ^{3}H/BK binding over that of ^{3}H/NPC17731 binding was different. Whereas the ratio for the WT receptor was approximately 1, the ratios for the Ser^{20} and Ser^{277} mutants were approximately 4 (Table I). With each mutant, the higher ratio was due to an increase in the K_{D} for agonist binding that was matched by a decrease in the K_{D} for antagonist binding.

In contrast to the Ser^{20} and Ser^{277} mutants, the Ser^{103} and Ser^{184} single mutants were unable to bind either radioligand. To ensure that the Ser^{103} and Ser^{184} single mutant receptors were expressed, these mutants were probed by Western blot analysis with anti-B2 receptor antibodies. Fig. 1 shows that the Ser^{103} (lane 3) and Ser^{184} (lane 4) mutant receptors as well as the WT receptor (lane 2) were expressed. As controls, non-transfected COS-7 cells (Fig. 1, lane 1) and B2 receptors from human foreskin fibroblasts (lane 5) were also immunoblotted. These results show that mutation of Cys^{20} and Cys^{277} to serines in the B2 receptor results in a receptor with retained high affinity binding sites for agonists and antagonists, while mutation of Cys^{103} and Cys^{184} to serines completely interferes with the folding of the receptor to form proper binding sites for these classes of ligands.

Inclusion of 100 μM Gpp(NH)p, a non-hydrolyzable analog of GTP, in the radioligand binding assay decreased the specific binding of ^{3}H/BK to WT, Ser^{20}, Ser^{277}, and Ser^{20}/Ser^{277} by 28 ± 13%, 15 ± 4%, 24 ± 13%, and 41 ± 4%, respectively (mean ± S.E., n = 3). These results show that mutation of Cys^{20} and Cys^{277} to serines either individually or in combination does not interfere with the coupling of the B2 receptor to a G-protein.

Pharmacological Specificity of BK Binding to WT and Mutant B2 Receptors—In order to determine if the replacement of extracellular cysteines with serines in the B2 receptor alters the pharmacological profile of the receptor, the ability of various kinin agonists and a kinin antagonists to compete for ^{3}H/BK binding to the receptors was evaluated. As shown in Fig. 2A, ^{3}H/BK binding to the WT receptor was competed for by BK, a B2 receptor-specific agonist, and HOE140, a B2 receptor-specific antagonist, but not by the B1 receptor-specific agonist des-Arg^9-BK. Fig. 2 (B–D) shows that the Ser^{20}, Ser^{277}, and Ser^{20}/Ser^{277} mutant receptors displayed pharmacological profiles virtually identical to the WT receptor. Thus, replacement of extracellular Cys^{20} and Cys^{277} with serines either individually or in combination in the human B2 receptor does not influence the pharmacological profile of the receptor.

Agnost Cross-linking to WT and Mutant B2 Receptors—We showed previously that the N terminus of BK when bound to the bovine B2 receptor can be cross-linked to a sulfhydryl residue in the receptor via the cross-linkers MBS and DFDNB (14). Here, we attempted to identify, using the human B2 receptor, the exact cysteine residue(s) to which BK can be cross-linked. The effectiveness of MBS in cross-linking BK to the receptors was assessed in ^{3}H/BK binding experiments (Fig. 3) as described previously for the bovine B2 receptor (14). Membranes were allowed to bind ^{3}H/BK before addition of cross-linker. ^{3}H/BK cross-linked to the membranes was determined by dissociating noncovalently bound ^{3}H/BK. Each panel in Fig. 3 shows the total ^{3}H/BK binding, nonpecific ^{3}H/BK binding, and cross-linked ^{3}H/BK as a function of increasing concentration of MBS. In the absence of added MBS, cross-linked ^{3}H/BK equaled nonpecific ^{3}H/BK binding. Total and nonspecific ^{3}H/BK binding did not change significantly with increasing MBS concentration, indicating that ^{3}H/BK was cross-linked only to B2 receptors. The effectiveness of the cross-linker is seen in the difference between cross-linked ^{3}H/BK and nonpecific ^{3}H/BK binding. The maximum cross-linking efficiency of the WT human B2 receptor was 19.1 ± 5.4%. This efficiency was comparable with that for the B2 receptor expressed in bovine myometrial membranes (41.1 ± 8.1%). The

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**Fig. 1.** Expression of the human Ser^{103} and Ser^{184} B2 receptor mutants. Equal amounts of solubilized protein (20 μg) from non-transfected COS-7 cells (lane 1), CHO cells expressing the WT receptor (lane 2), COS-7 cells expressing the Ser^{103} (lane 3) and Ser^{184} mutant receptors (lane 4), and human foreskin fibroblasts were subjected to SDS-polyacrylamide gel electrophoresis and then immunoblotted with anti-B2 receptor antibodies. Fig. 1 shows that the Ser^{103} (lane 3) and Ser^{184} (lane 4) mutant receptors as well as the WT receptor (lane 2) were expressed. As controls, non-transfected COS-7 cells (Fig. 1, lane 1) and B2 receptors from human foreskin fibroblasts (lane 5) were also immunoblotted.

**Fig. 2.** Pharmacological specificity of ^{3}H/BK binding to human WT and serine mutant B2 receptors. Membranes (5–250 μg of protein) of CHO cells stably expressing the human WT receptor (Human WT), and COS-7 cells transiently expressing the human Ser^{20} mutant receptor (Ser^{20}), the human Ser^{184} mutant receptor (Ser^{184}), and the human Ser^{20}/Ser^{277} double mutant receptor (Ser^{20}+Ser^{277}) were incubated with a constant concentration of ^{3}H/BK (1 nM) in the absence and presence of increasing concentrations of BK (C), HOE140 (□), and desArg^9-BK (▲). The results are presented as percent of control, where control refers to specific ^{3}H/BK binding to membranes as determined in the presence of 1 μM BK. 100% control represents 7890 fmol/mg of protein (Human WT), 1130 fmol/mg of protein (Ser^{20}), and 790 fmol/mg of protein (Ser^{277}), and 150 fmol/mg of protein (Ser^{20}/Ser^{277}) of ^{3}H/BK binding. The results are the averages ± S.E. of two to four experiments with each point assayed in duplicate.
maximum cross-linking efficiencies of the Ser<sup>20</sup> and Ser<sup>277</sup> mutants were 20.6 ± 5.3% and 16.3 ± 3.0%, respectively. These results indicate that mutation of either Cys<sup>20</sup> or Cys<sup>277</sup> to a serine did not interfere with the ability of MBS to cross-link BK to the receptor. In contrast, no cross-linking of BK to the Ser<sup>20</sup>/Ser<sup>277</sup> mutant was observed. Fig. 4 shows that mutation of either of these cysteines to a serine also did not interfere with the ability of 1 mM DFDNB to cross-link BK, whereas, again, no significant cross-linking of BK to the Ser<sup>20</sup>/Ser<sup>277</sup> mutant was observed. These results show that either Cys<sup>20</sup> or Cys<sup>277</sup> can provide the sulphydryl necessary for MBS and DFDNB to cross-link BK to the human B2 receptor and excludes any other sulphydryl. In agreement with previous studies of the B2 receptor in bovine myometrial membranes (14), MBS was unable to cross-link the antagonist [H]NPC17731 when bound to either the wild-type or mutant human receptors (data not shown).

**DISCUSSION**

In this study, we analyzed the role of the four cysteines located on the extracellular surface of the BK B2 receptor and their positioning relative to the N terminus of BK when bound to the receptor. Our results show that Cys<sup>103</sup> in EC-II and Cys<sup>184</sup> in EC-III are essential for formation of high affinity agonist and antagonist binding sites in the receptor, while Cys<sup>20</sup> in the EC-I and Cys<sup>277</sup> in the EC-IV are not essential. We further demonstrate that either Cys<sup>20</sup> or Cys<sup>277</sup> in the B2 receptor provides the sulphydryl necessary for the bifunctional reagents MBS and DFDNB to cross-link receptor-bound BK to the receptor. Consequently, these residues must be located within the radius of the linker arms of MBS (9.9 Å) and DFDNB (3 Å) when attached to the N terminus of the receptor-bound BK.

Two of the four extracellular cysteines in the B2 receptor, Cys<sup>103</sup> in EC-II and Cys<sup>184</sup> in EC-III (Fig. 5), are conserved in most members of the GPCR superfamily of which the sequence is known (8, 18). Studies with two members, rhodopsin and the β-adrenergic receptor, have revealed that these two cysteines are required for proper expression of the receptor (19–22). Most likely, these residues are linked in a disulfide bond that stabilizes the correctly folded conformation of the receptors. The Ser<sup>103</sup> and Ser<sup>184</sup> mutants of the B2 receptor were expressed in the membrane of COS-7 cells. However, these mutants did not express any high affinity binding sites for either the agonist BK or the antagonist NPC17731. Thus, in consensus with other members of the GPCR family, the B2 receptor appears to be absolutely dependent on these cysteines for expression of high affinity ligand binding sites. Conversely, Cys<sup>20</sup> in EC-I and Cys<sup>277</sup> in EC-IV are semi-conserved in the GPCR family and are found in the BK B2 receptor (3–6), the BK B1 receptor (23, 24), the neuropeptide Y1 receptor (25), the angiotensin II type 1 receptor (26), the endothelin receptors (27, 28), and the interleukin 8 receptor (29). In the current secondary structure model of GPCR based originally on the crystal structure of
bacteriorhodopsin (30, 31) and since modified by the recent crystal structure of rhodopsin (32) and hydrophathy analysis of over 200 members of the receptor superfamily (33), the seven transmembrane helices are believed not to be in a linear array but rather to be folded back upon themselves juxtapositioning TM-I and TM-VII and bringing the EC-I in relatively close proximity to EC-IV (Fig. 5). Thus, it is reasonable to suspect that a disulfide bond may exist between the cysteines in EC-I and in EC-IV and may be required to stabilize the folding of these GPCRs. The Ser20 and Ser277 mutants of the B2 receptor were expressed and displayed pharmacological profiles identical to that of the WT receptor. Thus, a disulfide bond between Cys20 and Cys277 in the B2 receptor, if present, is not critical for expression of high affinity agonist and antagonist binding sites. Furthermore, these residues do not appear to be involved directly as determinants in either agonist or antagonist binding to this receptor. Dithiothreitol had little effect on the binding of BK to the B2 receptor in both bovine myometrial membranes (14) and intact rat myometrial cells.2 Thus, even though disulfide bonds between some extracellular cysteines in the B2 receptor may be crucial during insertion of the receptor into the membrane for formation of proper binding sites, reducing such bonds in receptors already expressed in the membrane does not denature the agonist binding site. In contrast, expression of angiotensin II type 1 receptors in which the cysteines in EC-I and EC-IV are replaced with glycines resulted in a 10-fold decrease in angiotensin II affinity (34). Furthermore, the binding of angiotensin II to the WT receptor is sensitive to dithiothreitol. Thus, high affinity agonist binding to the angiotensin II type 1 receptor may be dependent on a disulfide between the cysteines in EC-I and EC-IV. The binding of losartan, a high affinity non-peptide angiotensin II type 1 receptor antagonist, was not sensitive to mutation of the above cysteines (34). Interestingly, the affinity of agonist binding decreased and the affinity of antagonist binding increased upon mutation of Cys20 and Cys277 to serines either singly or in combination. According to a three-state model of agonist and antagonist binding to the B2 receptor that we proposed (35, 36), these results suggest that these mutations favor the antagonist-stabilized inactive state of the receptor. This conclusion was supported by the increased Gpp(NH)p sensitivity of BK binding to at least one of the mutants, Ser20/Ser277. As the shift in agonist and antagonist affinity was observed with both the single and double mutants, it is possible that the normal equilibrium between conformational states of the receptor requires a disulfide bond between Cys20 and Cys277.

The cross-linking studies presented here using the human B2 receptor are a direct extension of our previous studies using the B2 receptor in bovine myometrial membranes (14). In the previous studies, we showed that heterobifunctional reagents such as MBS, which are reactive to amines at one end and to free sulfhydryls in the opposite end, and DFDNB, which is usually considered to be homobifunctional in activity and structure, cross-link the N terminus of receptor-bound BK to a sulfhydryl in the receptor. Consequently, we concluded that the N terminus of BK when bound to the receptor is adjacent to a cysteine in the receptor. The agonist binding site on the B2 receptor was distinguished from the antagonist binding site by the fact that these cross-linking reagents were completely unable to cross-link either NPC17731 or HOE140, two high affinity receptor antagonists, to the receptor. Molecular modeling studies (37), which are supported by experimental site-directed mutagenesis studies (10), suggest that the N terminus of BK when bound to the receptor is extracellular. Consequently, four cysteine residues, Cys20, Cys103, Cys184, and Cys277, in the receptor are candidates for providing the sulfhydryls necessary for MBS and DFDNB to cross-link BK to the receptor. As described above, two of these cysteines, Cys103 and Cys184, are presumed to be involved in a disulfide bond and, consequently, are not believed to be accessible for reaction with the cross-linkers. By identifying which, if any, of the above cysteines are critical for cross-linking, we would be able to identify which extracellular domains are adjacent to the BK N terminus. In this study, we show that the BK N terminus can be cross-linked by MBS and DFDNB to the receptor through both Cys20 in the EC-I and Cys277 in EC-IV. Indeed, only with the substitution of serine residues for both of these cysteine residues could the cross-linking of BK to the B2 receptor by MBS and DFDNB be prevented. These results exclude all other cysteines present in the receptor or any non-receptor cysteines in the membrane as anchors for the cross-linking. Therefore, we conclude that both EC-I and EC-IV of the receptor are positioned near the N terminus of BK when located in the agonist binding site. These biochemical results directly support the most recent structural model of GPCR described above in which the seven transmembrane helices are folded back upon themselves so that TM-I is adjacent to TM-VII. Indeed, without such an oval array of the seven transmembrane helices, it is difficult to envision how both EC-I and EC-IV could be within reach of the linker arm of the cross-linkers.

There is no immediate consensus for the exact location of the agonist and antagonist binding sites in the B2 receptor. However, several studies using different techniques including chemical cross-linking (14) and site-directed mutagenesis (10, 12, 13) show that these two sites are not identical and may be only partially overlapping. Mutagenesis studies using the rat B2 receptor have revealed some information regarding the possible location of the agonist binding site. Individual mutations of two conserved aspartate residues, Asp266 and Asp286 in EC-IV, which correspond to Asp265 and Asp284 in the human receptor (Fig. 5), to alanines resulted in a 17- and 25-fold decrease in BK affinity, respectively, and the double mutation resulted in a 500-fold decrease (10). These results are in agreement with a model of BK bound to the B2 receptor proposed by Kyle based on structural homology modeling, molecular modeling, and systematic conformational searching methods of BK and the receptor (37). In this model, Asp266 and Asp286 in the receptor interact electrostatically with the N-terminal amino group, the guanidinyl side chain, or both of Arg3 in BK, a residue absolutely crucial for receptor binding. Immediately below EC-IV in TM-VI of the rat receptor residues crucial for high affinity agonist binding have also been identified. Replacement of Phe201 with alanine decreased agonist affinity by 1,600-fold (12, 13), while replacement of Thr265 with either alanine or valine decreased agonist affinity by 700-fold (12).
more modest decrease of 10-fold in the agonist affinity was observed when Gln\textsuperscript{262} and Thr\textsuperscript{267} were replaced with alanines (12). These studies suggest that residues located near the extracellular side in TM-VI and in EC-IV are parts of an agonist binding site in the B2 receptor. Anti-peptide antibodies developed against the C-terminal half of EC-IV of the human B2 receptor inhibited agonist binding providing further support for the involvement of this domain in this interaction. Interestingly, antibodies against the N-terminal half of EC-III inhibited both agonist and antagonist binding. Thus, determinants in EC-III and possibly in TM-IV and TM-V may be involved in binding both classes of ligands (16).

In conclusion, based on molecular modeling and site-directed mutagenesis, the consensus is evolving that regions in EC-IV and TM-VI are critical in the binding of agonists to the B2 receptor. However, these approaches clearly have limitation. The former approach is purely theoretical, while the latter approach may be either an effect of altering directly a ligand binding determinant in the receptor or an effect of a conformational change in the receptor, which is induced at a site distant to the binding site. Our approach using chemical cross-linking combined with site-directed mutagenesis probes residues that are located at a distance no greater than the linker arm of the cross-linking reagent from the bound ligand. The results that we have obtained indicate that the cysteines in EC-IV and EC-I of the B2 receptor can exist at a distance as short as 3 Å from the N terminus of the bound BK. Considering the current secondary structure model of GPCR, our results directly support the positioning of the N terminus of BK bound to the B2 receptor at the extracellular surface below EC-IV. Cys\textsuperscript{277} is positioned 11 residues beyond Asp\textsuperscript{266} and 7 residues before Asp\textsuperscript{284}, residues believed to be located at the interface between TM-VI and TM-VII, respectively. This pair of acidic residues is also thought to participate in an electrostatic interaction(s) with the N-terminal Arg\textsuperscript{1} in BK. The straight chain distance between Asp\textsuperscript{266} and Cys\textsuperscript{277}, which is about 40 Å, and between Asp\textsuperscript{284} and Cys\textsuperscript{277}, which is about 30 Å, indicates that EC-IV must be folded to align this cysteine and these aspartates with the BK N terminus. However, given the fact that both MBS and DFDNB can cross-link to BK at these cysteines, there must be some flexibility either in the MBS linker arm or in EC-IV and EC-I. Interestingly, DFDNB cross-linking of BK to these cysteines, there must be some flexibility either in the MBS linker arm or in EC-IV and EC-I. Indeed, this result is not unexpected with this short cross-linker, since according to our model Cys\textsuperscript{20} may be located slightly further away from Cys\textsuperscript{277} from the BK N terminus. Finally, NPC17731 could not be cross-linked either to the WT or mutant receptors under any conditions. When taken in consideration with the lack of effect of mutation of the aspartates in EC-IV on NPC17731 binding (13), these results suggest that antagonist binding to the B2 receptor involves at least some determinants that are different from those involved in agonist binding.

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