The N-terminal Amino Group of [Tyr\(^8\)]Bradykinin Is Bound Adjacent to Analogous Amino Acids of the Human and Rat B\(_2\) Receptor*

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To obtain data of the bradykinin B\(_2\) receptor’s agonist binding site, we used a combined approach of affinity labeling and “immuno-identification” of receptor fragments generated by cyanogen bromide cleavage. Domain-specific antibodies to the various extracellular receptor domains were applied to detect receptor fragments with covalently attached [\(^{125}\)I-Tyr\(^8\)]bradykinin. As a cross-linker we used the homobifunctional reagent disuccinimidyl tartarate (DST), which reacts preferentially with primary amines. With this technique a [\(^{125}\)I-Tyr\(^8\)]bradykinin-labeled receptor fragment derived from the third extracellular domain was identified. The e-amino group of lysine (Lys\(^{172}\)) of the human B\(_2\) receptor provides the only primary amino group within this receptor fragment. This strongly suggests that DST attached the N-terminal amino group of [Tyr\(^8\)]bradykinin to Lys\(^{172}\) of the human B\(_2\) receptor. Next we asked whether DST attaches [Tyr\(^8\)]bradykinin to the analoguous residue, Lys\(^{174}\) of the rat B\(_2\) receptor, which is 81% identical to the human B\(_2\) receptor, and we attempted to label the wild-type rat B\(_2\) receptor and a rat B\(_2\) receptor mutant where Lys\(^{174}\) had been exchanged for alanine. Affinity labeling of the wild-type rat B\(_2\) receptor worked efficiently, whereas DST did not attach detectable amounts of [\(^{125}\)I-Tyr\(^8\)]bradykinin to the K174A rat B\(_2\) receptor mutant. Taken together these observations indicate that the N-terminal amino group of [Tyr\(^8\)]bradykinin to the K174A rat B\(_2\) receptor mutant. The ligand binding sites of G protein-coupled receptors have been mapped using mutations and affinity labeling. These studies suggest that much of the ligand binding site of peptide hormone receptors such as the angiotensin AT\(_1\) receptor (1), the neurokinin NK-1 receptor (2, 3), the luteinizing hormone receptors such as the angiotensin AT\(_1\) receptor (1), and the interleukin-8 receptor (4) is within the extracellular loop regions. Cloning of the cDNA coding for a rat (6) and a human (7) B\(_2\) receptor revealed that the receptor for the peptide hormone bradykinin belongs to the family of G protein-coupled receptors. Mutation studies of the bradykinin B\(_2\) receptor identified residues important for agonist binding on putative transmembrane regions TM6 (8) and at the top of TM6 and TM7 (9). Few of the mutations were made within the extracellular domains, and it is not clear whether they form a contact region for B\(_2\) ligands. To assess the involvement of the extracellular domains in agonist binding without changing the receptor’s primary structure, we previously produced and characterized six antisera to the predicted extracellular domains (EDs)\(^3\) of the B\(_2\) receptor (10). Antibodies to the amino-half of the third extracellular domain (ED3\(_C\)) compete with bradykinin’s binding and are agonists (10). To further the analysis of bradykinin’s binding site(s), we combined affinity labeling and immunoidentification of [\(^{125}\)I-Tyr\(^8\)]bradykinin-labeled B\(_2\) receptor fragments generated by cyanogen bromide cleavage to identify a region(s) where the agonist bradykinin contacts the B\(_2\) receptor. We chose the amino group-specific homobifunctional cross-linker disuccinimidyl tartarate (DST) to localize the position of the N-terminal amino group of [\(^{125}\)I-Tyr\(^8\)]bradykinin when bound to the B\(_2\) receptor. A combined approach of affinity labeling and identification of ligand-labeled receptor fragments has been extensively used to determine ligand binding site(s) of membrane receptors, e.g. of the GABA\(_A\) receptor (11), of the renal V\(_2\) vasopressin receptor (12), or of the nicotinic acetylcholine receptor (13). In this study we used this technique to localize a B\(_2\) receptor region involved in the interaction with the agonist’s N terminus. This should contribute to a refinement of the present model of the B\(_2\) receptor (14), thereby facilitating the rational design of B\(_2\) agonists or antagonists.

EXPERIMENTAL PROCEDURES

Materials—\(\text{Na[\(^{125}\)I]}\) (17.5 Ci/mg) and the chemiluminescence detection kit (ECL) were from Amersham Corp.; [\(2,3\)-prolyl-3,4-\(^3\)H]bradykinin (specific activity, 98 Ci/mmol) was from NEN DuPont; iodine (1,3,4,6-tetrachloro-3a-6a-diphenyl-glycoluril) and DST were from Pierce; Sephadeh G-50 and protein A-Sepharose were from Pharmacia Biotech Inc.; cyanogen bromide, Dowex AG1 \(\times 8\), and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin were from Sigma; Centricron-30 filters were from Amicon; nitrocellulose sheets were from Schleicher & Schüll; Affi-Gel 10 was from Bio-Rad; Lipo- fectAMINE was from Life Technologies, Inc. All other chemicals were of analytical grade.

Cell Culture and Cell Transfection—HF-15 cells (15) expressing 0.5–1 pmol B\(_2\) receptor/mg protein at passages 6–11 were maintained in Dulbecco’s modified Eagle’s medium, and COS-1 cells were grown in RPMI 1640 medium. Both media were supplemented with 10% (v/v) fetal bovine serum, and cells were kept in a 5% \(\text{CO}_2/95\%\) air atmosphere. COS-1 cells were transfected with wild-type and K174A-mutated rat B\(_2\) receptor cDNA by Lipo- fectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. The rat B\(_2\) receptor mutant was made by a modification of the polymerase chain reaction mutagenesis method (16) in a cassette encompassing the unique BglII

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and PouII sites of the protein coding region. DNA was transiently expressed in COS-1 using the eukaryotic expression vector pCDLSRz59, which is a derivative of the SRα promoter vector pCDLSRz296 (17). The mutagenesis cassette was completely sequenced to confirm the identity of the mutant.

**Competition and Saturation Binding of Radiolabeled Ligands—**
Binding of [3H]bradykinin and of [125I]-labeled antibodies to intact HF-15 or COS-1 cells were performed as described previously (10). Enriched B2 receptor fractions were recovered by boiling of the Sepharose in SDS sample buffer. Proteins were separated by Tricine-SDS-PAGE (24) under reducing conditions, and [125I]-Tyr8-bradykinin-labeled receptor fragments were identified by autoradiography of the gels for 3–6 days.

**RESULTS AND DISCUSSION**

**Affinity Labeling of the B2 Receptor by [125I-Tyr8]Bradykinin—**
We previously characterized domain-specific antibodies to the extracellular domains of the B2 receptor. Antibodies to the amino half of ED3N (Fig. 1, A and B) compete with bradykinin for binding to the B2 receptor and are agonists (10). To further analyze the agonist binding site(s), we used an affinity labeling-based approach. For affinity labeling, we chose [125I-Tyr8]bradykinin as B2 agonist (Fig. 1B). This ligand binds to the B2 receptor of HF-15 cells with a K0 of 3 ± 0.5 × 10−9 M. This value is close to the affinity of bradykinin, which binds to B2 receptors of HF-15 cells with a K0 value of 1.5 ± 0.4 × 10−9 M. In contrast to [125I-Tyr8]bradykinin, which was used for previous studies (25), the N terminus of [Tyr8]bradykinin is not modified. [125I-Tyr8]bradykinin is an agonist, it stimulates similarly to bradykinin the phospholipase C pathway with an EC50 of 0.8 ± 0.2 × 10−8 M (not shown). This value was determined for the phospholipase C-mediated rise in the intracellular free Ca2+ concentration monitored by fura-2 (26). To detect a contact region between the agonist’s N-terminal amino group and the B2 receptor, we chose the homobifunctional amino group-specific cross-linker DST. Affinity labeling of the human B2 receptor of HF-15 cells by [125I-Tyr8]bradykinin is shown in Fig. 2 (lane 1). The labeled B2 receptor has a molecular mass of 69 ± 4 kDa. This value is in good agreement with the molecular mass of the B2 receptor determined previously (10). A 1000-fold molar excess of unlabeled [Tyr8]bradykinin suppressed the labeling of the B2 receptor (Fig. 2, lane 2), indicating that we specifically labeled B2 receptors.

**Affinity Chromatography of [125I-Tyr8]Bradykinin-labeled B2 Receptors—**
To enrich [125I-Tyr8]bradykinin-labeled B2 receptor, we used affinity chromatography with anti-bradykinin antibodies. The antisemur cross-reacted with [Tyr8]bradykinin in enzyme-linked immunosorbent assay (not shown), its cross-reactivity with [Tyr8]bradykinin covalently attached to the B2 receptor was verified in immunoblots of [Tyr8]bradykinin-labeled B2 receptors (Fig. 3A, lane 1). The anti-bradykinin antibodies stained a protein of 69 kDa. In some experiments a degradation product of 46 kDa was seen (not shown). Aggregation products of higher molecular mass are probably due to the high amount of B2 receptor applied to each lane (10). For control, the affinity labeling was performed in the presence of 1, 10, 100, or 500 nM of the B2 antagonist HOE140 (Fig. 3A, lanes 2–5), which dose-dependently suppressed the subsequent staining with anti-bradykinin antibodies. This observation demonstrates that the anti-bradykinin antibodies cross-react with [Tyr8]bradykinin-labeled B2 receptors. The antibodies were used to enrich [125I-Tyr8]bradykinin-labeled B2 receptors. The enriched B2 receptor fraction was visualized by autoradiography after Tricine-SDS-PAGE (Fig. 3B, lane 1).

**Chemical Cleavage of the B2 Receptor by Cyanogen Bromide—**
To identify the region within the B2 receptor to which [125I-Tyr8]bradykinin had been attached by DST, the affinity-enriched B2 receptor was chemically cleaved by cyanogen bro-
mide. Cyanogen bromide specifically cleaves after methionine residues. The human B2 receptor sequence contains 17 methionines, nine expected cyanogen bromide cleavage fragments vary between 2,000 and 12,000 Da, and four of these larger fragments cover the entire sequence of the putative extracellular domains (Fig. 1A). The cleaved B2 receptor was separated by Tricine-SDS-PAGE, and autoradiography of the gel visualized [125I]-labeled receptor fragments of about 6,000 Da and some aggregation products of 12,000 Da (Fig. 3, lane 2). This experiment indicates that we efficiently cleaved the B2 receptor, though we cannot completely rule out the possibility that some partial cleavage may have occurred.

Immunoprecipitation of [125I-Tyr8]Bradykinin-labeled B2 Receptor Fragments by Domain-specific Antibodies—After chemical cleavage we identified the receptor fragments with covalently attached [125I-Tyr8]bradykinin. To this end we immunoprecipitated the receptor fragments by six different domain-specific antibodies to the extracellular domains of the B2 receptor (Ref. 10 and Fig. 1A). Antibodies to a peptide ED1A (Fig. 1A) derived from the extended N-terminal region of the human B2 receptor, which has been identified previously as the real start site of the human B2 receptor protein (27), have not been included in this study. The six different domain-specific antibodies immunoprecipitate the intact B2 receptor of HF-15.
The B2 Receptor’s Agonist Binding Site

**FIG. 2.** Cross-linking of [¹²⁵I-Tyr⁸]bradykinin to the human B₂ receptor of HF-15 cells by DST. Solubilized B₂ receptors (0.5–1 pmol B₂ receptor/mg protein) were incubated at 4°C with [¹²⁵I-Tyr⁸]bradykinin (5 nM) in the absence (lane 1) or the presence (lane 2) of a 1000-fold molar excess of bradykinin. Proteins were precipitated by antibodies. Bound antibodies were visualized with the chemiluminescence method, and immunoblots were probed by affinity-purified anti-bradykinin antibodies. [Tyr ⁸]bradykinin (5 nM) was cross-linked with anti-bradykinin antibodies. [¹²⁵I-Tyr⁸]bradykinin (5 nM) was attached to an amino acid that provides a free primary amino group (Fig. 1). Hence immunoprecipitation is expected to be equally effective with these domain-specific antibodies. A cyanogen bromide cleavage site lies within ED₃C of the rat B₂ receptor, Lys₁₇₄. We made a rat B₂ receptor mutant where Lys₁₇₄ was exchanged for alanine. To control the specificity of immunoprecipitation, we performed the reaction in the presence of 10 μM of the cognate peptides. The presence of the ED₃N or of the ED₃C peptide suppressed the immunoprecipitation with anti-ED₃N antibodies (Fig. 1A and Ref. 10). Hence immunoprecipitation is expected to be equally effective with these domain-specific antibodies. A cyanogen bromide cleavage site lies within ED₃C. Chemical cleavage may remove part of the epitope of the anti-ED₃C antibodies. This fact may reduce efficiency of immunoprecipitation with anti-ED₃C antibodies. After immunoprecipitation the immunoprecipitated proteins were separated by Tricine-SDS-PAGE under reducing conditions. Autoradiography of the gels visualized receptor fragments with covalently attached [¹²⁵I-Tyr⁸]bradykinin (Fig. 4A, lanes 3 and 4). For comparison, free [¹²⁵I-Tyr⁸]bradykinin was included (Fig. 4A, lane 7). Antibodies to the third extracellular domain (anti-ED₃N and anti-ED₃C) precipitated a 125I-labeled receptor fragment with a molecular mass of 6–8 kDa (Fig. 4A, lanes 3 and 4), whereas antibodies to extracellular domains ED₃B (ED1), ED2, ED₄N, and ED₄C did not enrich detectable amounts of [¹²⁵I-Tyr⁸]bradykinin-labeled receptor fragments. The molecular mass of 6–8 kDa of the labeled receptor fragment is in good agreement with the calculated molecular mass of the cyanogen bromide cleavage fragment derived from ED₃ including the molecular mass of [¹²⁵I-Tyr⁸]bradykinin. The amount of ¹²⁵I-labeled receptor fragments precipitated by ED₃C antibodies was less than the amount obtained with anti-ED₃N. Both antibodies bind to intact B₂ receptors with a similar affinity of 40 nM as determined with affinity-purified ¹²⁵I-labeled antibodies (not shown). A cyanogen bromide cleavage site lies within the C-terminal portion of the peptide used to raise anti-ED₃C antibodies (Fig. 1A). Chemical cleavage may have removed part of the epitope of these antibodies, thereby explaining the lower efficiency in immunoprecipitation (see above). In addition to the receptor fragment of 6–8 kDa, a ¹²⁵I-labeled fragment of about 2.5 kDa was seen in some experiments (Fig. 4A, lanes 3 and 4). This smaller fragment may represent a degradation product generated during the overnight incubation.

**FIG. 3.** Autoradiography of immunoprecipitated and cyanogen bromide-cleaved B₂ receptors. [¹²⁵I-Tyr⁸]bradykinin was cross-linked to solubilized B₂ receptors of HF-15 cells. [¹²⁵I-Tyr⁸]bradykinin-labeled B₂ receptors were enriched by affinity chromatography with anti-bradykinin antibodies and cleaved by cyanogen bromide. Receptor fragments were immunoprecipitated with six different domain-specific antibodies, proteins were separated by Tricine-SDS-PAGE under reducing conditions, and gels were autoradiographed (A, lanes 1–6). For comparison, free [¹²⁵I-Tyr⁸]bradykinin was applied (A, lane 7). In B, immunoprecipitation of B₂ receptor fragments was performed by anti-ED₃N (lanes 1 and 2) or anti-ED₃C (lanes 3 and 4) antibodies in the absence (lane 1) and 3) or the presence (lanes 2 and 4) of 10 μM of the cognate peptide. A typical experiment is shown that is representative of three independent experiments each with similar results.

ED₃N, ED₄N, and ED₄C, which were used to raise antibodies (Fig. 1A, lanes 3 and 4). For comparison, free [¹²⁵I-Tyr⁸]bradykinin was included (Fig. 4A, lane 7). Antibodies to the third extracellular domain (anti-ED₃N and anti-ED₃C) precipitated a 125I-labeled receptor fragment with a molecular mass of 6–8 kDa (Fig. 4A, lanes 3 and 4), whereas antibodies to extracellular domains

**FIG. 4.** Autoradiography of immunoprecipitated and cyanogen bromide-cleaved B₂ receptors. [¹²⁵I-Tyr⁸]bradykinin was cross-linked to solubilized B₂ receptors of HF-15 cells. [¹²⁵I-Tyr⁸]bradykinin-labeled B₂ receptors were enriched by affinity chromatography with anti-bradykinin antibodies and cleaved by cyanogen bromide. Receptor fragments were immunoprecipitated with six different domain-specific antibodies, proteins were separated by Tricine-SDS-PAGE under reducing conditions, and gels were autoradiographed (A, lanes 1–6). For comparison, free [¹²⁵I-Tyr⁸]bradykinin was applied (A, lane 7). In B, immunoprecipitation of B₂ receptor fragments was performed by anti-ED₃N (lanes 1 and 2) or anti-ED₃C (lanes 3 and 4) antibodies in the absence (lane 1) and 3) or the presence (lanes 2 and 4) of 10 μM of the cognate peptide. A typical experiment is shown that is representative of three independent experiments each with similar results.
With a combined approach of affinity labeling and immunodetection of receptor fragments generated by cyanogen bromide cleavage, we identified Lys172 within the amino half of ED3 (ED3N) of the human B2 receptor to be located near the agonist’s N-terminal amino group. This is in agreement with our previous finding that antibodies to ED3N mutually compete with B2 agonists for binding to the B2 receptor (10). The lack to attach significant amounts of [125I-Tyr8]bradykinin to a rat B2 receptor mutant where the analogous lysine Lys174 had been replaced by alanine supports the observation made with the human B2 receptor and indicates that [Tyr8]bradykinin’s N terminus is bound to the analogous position of the rat and of the human B2 receptor. Previous work with heterobifunctional cross-linkers gave evidence that the bradykinin’s N terminus was bound adjacent to a sulfhydryl group of the bovine B2 receptor (28). Our data do not exclude such a possibility because (i) the cysteine providing the free sulphydryl group is not identified, and due to the lack of a three-dimensional structure its potential distance to Lys174 (or Lys172) of the rat or human B2 receptor is not determined, and (ii) we performed the cross-linking at 4°C and used the homobifunctional amino group-specific cross-linker DST with a spacer of four carbon atoms (6.4 Å), whereas cross-linking of the bradykinin’s N terminus to a sulfhydryl group of the B2 receptor worked efficiently at room temperature with heterobifunctional linkers, e.g. m-maleimidobenzoyl-N-hydroxysuccinimide ester with a spacer arm between the two reactive groups of >9.9 Å (28).

A model of the rat B2 receptor’s agonist binding site suggests that the N-terminal amino and guanidino group of Arg1 of bradykinin interact directly with negatively charged amino acids in extracellular domains ED3 and/or ED4 (14). This hypothesis was supported by rat B2 receptor mutants. Replacing either Asp268 or Asp269 of ED4 with alanine reduces the affinity of bradykinin to the mutant receptors 19- or 28-fold, respectively (8), and a cluster mutation where the negatively charged amino acids of ED3, Asp175 and Glu178,179, were exchanged for alanine resulted in a 12-fold loss in bradykinin affinity (8). But a direct interaction of bradykinin’s N terminus with a distinct B2 receptor domain had not been identified. Our data based on affinity labeling of the human and of the rat B2 receptor give strong evidence that the N-terminal region of [Tyr8]bradykinin is bound adjacent to Lys172 or Lys174, respectively, of ED3. This finding is complementary to the observations made with anti-peptide antibodies, because domain-specific antibodies to ED3N and bradykinin are mutually competitive in binding to the B2 receptor (10). Furthermore our experiments with the human and rat B2 receptor indicate that the location of [Tyr8]bradykinin’s N terminus is analogous within the human and the rat B2 receptor.

In addition to being a potential contact site, the interaction of the agonist bradykinin with ED3N may be essential for the induction or stabilization of the active receptor conformation because anti-ED3N antibodies are B2 agonists (10). A refined model of the B2 receptor’s agonist binding site based on the finding that the N-terminal amino group of [Tyr8]bradykinin is proximal to Lys174 of the rat or Lys172 of the human B2 receptor may advance the rational design of B2 agonists and antagonists (29) in the future.

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