Investigation of the Extracellular Accessibility of the Connecting Loop between Membrane Domains I and II of the Bradykinin B2 Receptor*

(Received for publication, September 8, 1998, and in revised form, December 22, 1998)

Ursula Quitterer‡, Essam Zaki§, and Said AbdAlla§

From the ‡Institut für Pharmakologie und Toxikologie der Universität, Versbacher Straße 9, 97078 Würzburg, Germany and the §Genetics Engineering and Biotechnology Research Institute (GEBRI), Alexandria, Egypt

In analogy to the structure of rhodopsin, the seven hydrophobic segments of G-protein-coupled receptors are supposed to form seven membrane-spanning α-helices. To analyze the topology of the bradykinin B2 receptor, we raised site-directed antibodies to peptides corresponding to the loop regions and the amino and carboxyl terminus of this receptor. We found that a segment with predicted intracellular orientation according to the rhodopsin model, the connecting loop between membrane domains I and II of the bradykinin B2 receptor, was accessible to site-directed antibodies on intact fibroblasts, A431 cells, or COS cells expressing human B2 receptors. Extracellular orientation of this loop was further confirmed by the substituted cysteine accessibility method which showed that exchange of cysteine 94 for serine on this loop by point mutagenesis suppressed the effect of thiol modification by a membrane impermeant maleimide. In addition, this segment seemed to be involved in B2 receptor activation, since (i) thiol modification of cysteine 94 partially suppressed B2 receptor activation, and (ii) site-directed antibodies to the connecting loop between membrane domains I and II were agonists. The agonistic activity of the antibodies was suppressed by the B2 antagonist HOE140 confirming the B2 specificity of the antibody-generated signal. The extracellular orientation of the connecting loop between membrane domains I and II was confirmed by site-directed antibodies to the connecting loop region between membrane domains I and II and to determine the agonist-binding site (13). We present here that the connecting loop between membrane domains I and II faces the extracellular side suggesting a membrane topology of the B2 receptor with five membrane spanning and two re-entrant membrane segments which is different from rhodopsin.

**EXPERIMENTAL PROCEDURES**

Materials—Na[125]I (17.4 Ci/mg), the chemiluminescence detection kit (ECL), and [2-3-prolyl-3,4-3H]bradykinin (specific activity 78 Ci/mmol) were from Amersham; IODO-GEN (1,3,4,6-tetrachloro-3a-diphenyl-glycoluril) and disuccinimidyl tartarate were from Pierce; Dowex AG 1-X8, wheat germ agglutinin, N-acylglucosamine, and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin were from Sigma; myo-[2-3H]inositol (specific activity 17 Ci/mmol) was from NEN Life Science Products Inc.; stillbensulfonate maleimide1 (4-acetamido-4-[maleimidylstilbene-2,2′-disulfonic acid) was from Molecular Probes; bradykinin, kallidin, and HOE140 were from Bachem.

Cell Culture and Cell Transfection—Human foreskin fibroblasts, HF-15 (14), A431, and COS cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and kept in a humidified 5% CO₂, 95% air atmosphere at 37 °C. COS cells of transfected COS cells were determined on adherent cells (15) with minor modifications. Adherent COS cells on gelatin-coated (0.1% in phosphate-buffered saline) 12-well plates were labeled with myo-[2-3H]inositol (2 μCi/ml, specific activity 17 Ci/mmol) for 12 h in inositol-free RPMI medium supplemented with 1% (v/v) fetal calf serum. Prior to the start of the experiment, the cells were switched to serum-free RPMI medium and kept for 30 min. This medium was replaced by 1 mL of methionine-free RPMI medium supplemented with 10% (v/v) fetal calf serum and kept in a humidified 5% CO₂, 95% air atmosphere at 37 °C.

Construction of Expression Vectors—The cDNAs coding for rat B2 receptor mutants (B2–88Ser; B2–94Ser; B2–88/94Ser) were constructed by overlap extension using the polymerase chain reaction as described (15). Identity of the constructs was confirmed by DNA sequencing.

Determination of Inositol Phosphate Levels—Inositol phosphate levels of transfected COS cells were determined on adherent cells (15) with minor modifications. Adherent COS cells on gelatin-coated (0.1% in phosphate-buffered saline) 12-well plates were labeled with myo-[2-3H]inositol (2 μCi/ml, specific activity 17 Ci/mmol) for 12 h in inositol-free RPMI medium supplemented with 1% (v/v) fetal calf serum.

1 The abbreviations used are: stillbensulfonate maleimide, 4-acetamido-4-[maleimidylstilbene-2,2′-disulfonic acid, HOE140, n-Ang-Ang-Pro-Hyp-Gly-Thr-Ser-Tic-Oε-Arg; kallidin, [Lys⁸]bradykinin; bradykinin, Ang-Pro-Pro-Gly-Phε-Ser-Pro-Phe-Arg; fura-2/AM, 1-[2-(carboxyoxazol-2-yl)-4-aminobenzofuran-5-oxyl]-2-[2′-amino-5′-methylphenoxy]-ethane-N,N,N′,N′-tetraacetic acid, pentacacetoxymethylster; [Ca²⁺], intracellular [Ca²⁺].

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
to the experiment, cells were washed twice with incubation buffer (138 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.6 mM CaCl₂, 20 mM Na-HEPES, pH 7.2) and stored for 5 min in incubation buffer with 10 mM LiCl. Then the cells were placed to 37 °C and the experiment was started by the addition of ligand or buffer as indicated. After 20 min total inositol phosphates were extracted (15). After thiold modification, cells were preincubated with 100 μM of the membrane impermeant thiol-specific reagent stibenedisulfonate maleimide (Molecular Probes) for 5 min at room temperature.

**Determination of Changes in [Ca²⁺]ᵢ—Bradykinin- or antibody-induced changes in the intracellular free Ca²⁺ concentration, [Ca²⁺]ᵢ, of adherent HF-15 or COS cells seeded on glass coverslips was determined on cells loaded with 2 μM fura-2/AM as described previously (16). Changes in [Ca²⁺]ᵢ, are given as the the ratio between 340/380 nm.**

**Synthesis of Peptides and Production of Antibodies—** Production of domain-specific antisera to the putative intracellular domains of the bradykinin B₂ receptor was performed as described previously (12). Briefly, peptides derived from the rat B₂ receptor sequence (see Fig. 1A) were synthesized by solid phase peptide synthesis using the Fmoc (N-(9-fluorenylmethyloxycarbonyl) or the t-butyloxycarbonyl chemistry. Peptides purified by high performance liquid chromatography were routinely analyzed by Edman degradation and electrospray mass spectrometry. Peptides were covalently coupled to the carrier protein keyhole limpet hemocyanin by maleimidocaproyl N-hydrazinocinimide (peptides I-II and III-IV) or 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (peptides V-VI and C₁₄₀) (Fig. 1A). Antisera to a peptide derived from the connecting loop between membrane domains III and IV and to the carboxyl terminus (C₁₄₀) have been described previously (12). Rabbits were immunized with the conjugates, and the antisera were tested for cross-reactivity with the respective human or rat peptides by the indirect enzyme-linked immunosorbent assay. Immunoselection of the antibodies was routinely performed as described (12).

**Western Blotting—** Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride sheets using semidyry blotting (17). The blotting membrane was treated with 50 mM Tris, 0.2 mM NaCl, pH 7.4 (buffer A), containing 5% (w/v) of nonfat dry milk and 0.1% (w/v) of Tween 20 for 1 h. Antisera were diluted 1:1000 in buffer A containing 2% (w/v) of bovine serum albumin. After 30 min of washing, bound antibody was visualized with a chemiluminescence reagent (Molecular Probes) for 5 min at room temperature.

**Flow Cytometric Analyses—** HF-15, A431, or COS cells (90% confluency) were detached by 0.5 mM EDTA in phosphate-buffered saline and washed twice with ice-cold RPMI 1640 containing 0.1% (w/v) bovine serum albumin. Cells (1 x 10⁶) were suspended in the incubation medium containing 10 mM LiCl and incubated for 1 h at 4 °C. After washing three times, fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma) was added. After extensive washing, bound antibody was visualized with a chemiluminescence detection kit (ECL, Amersham).

**Lectin Affinity Chromatography of the Human B₂ Receptor—** Enrichment of the B₂ receptor from HF-15 cells was performed as described previously (12).

**Flow Cytometric Analyses—** HF-15, A431, or COS cells (90% confluency) were detached by 0.5 mM EDTA in phosphate-buffered saline and washed twice with ice-cold RPMI 1640 containing 0.1% (w/v) bovine serum albumin, 20 mM Na-HEPES, pH 7.4 (incubation medium). Cells (1 x 10⁶) were suspended in the incubation medium containing 10 mM LiCl and incubated for 1 h at 4 °C. After washing three times, fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma) was added. After extensive washing, bound antibody was visualized with a chemiluminescence detection kit (ECL, Amersham).
Fig. 1. Cross-reactivity of site-directed antibodies with the B₂ receptor. A, topology model of the human bradykinin B₂ receptor (9) with the extended amino terminus as determined by AbdAlla et al. (30). Filled black circles indicate the position of the peptides used to generate domain-specific antisera of this study and dotted circles indicate positions of peptides used to generate antibodies by which the orientation of the extracellular segments was determined previously (12). Arrows indicate the analogous positions of cysteines 88 and 94 of the rat B₂ receptor sequence. B, immunoblot of B₂ receptors enriched by wheat germ agglutinin affinity chromatography from HF-15 cells. Twenty μg of protein containing 0.1 pmol of B₂ receptor were applied per lane. The blots were probed by antisera (dilution 1:1000) to the connecting loop I-II (lane 1 and 5), to the connecting loop III-IV (lane 2), to the connecting loop V-VI (lane 3) and to the carboxyl terminus (lane 4) in the absence (lanes 1–4) or presence of 20 μM immunizing peptide (lane 5). Bound antibody was visualized by the chemiluminescence detection method. As a control, HOE140 was cross-linked by diisuccinimidyl tartarate (1 mM) to the B₂ receptor and visualized by anti-HOE140 antibodies (lane 6). Immunoprint of B₂ receptors transiently expressed in COS cells (lane 7) probed by anti-I-II antibodies. As a control membranes of mock-transfected COS cells were applied (lane 8). The membrane preparation of transfected COS cells contained about 10–15 fmol of B₂ receptor/μg of protein.

Redistribution of B₂ Receptors Detected by Antibodies to the Connecting Loop I-II—We further analyzed the orientation of the connecting loop between membrane domains I-II on two different B₂ receptor expressing cells: A431 cells and B₂ receptor-transfected COS cells (Fig. 2B). In addition to HF-15 cells, intact A431 cells were stained by antibodies to the connecting loop between membrane domains I-II (Fig. 2B, panel 2) suggesting that the B₂ receptor topology was similar on fibroblasts and on epithelial cells. Relative fluorescence intensity of A431 cells was increased only 10-fold above control compared with a 100-fold increase on HF-15 cells (Fig. 2B, panels 1 and 2). This finding may indicate that the amount of B₂ receptors/cell of A431 cells is much lower than that of HF-15 cells, a conclusion which is in agreement with the 75-fold increased EC₅₀ value for the bradykinin-induced rise in [Ca²⁺] as determined by site-directed antibodies (Fig. 3A). Therefore we asked whether the B₂ receptor contains two cysteines at position 88 and 94 of the B₂ receptor sequence. The connecting loop between membrane domains I-II of the B₂ receptor contains two cysteines at position 88 and 94 (Fig. 1A). Cysteine modification often alters the function of membrane proteins (20) without modifying the receptor’s primary sequence. The connecting loop between membrane domains I-II of the B₂ receptor contains cysteine(s) for B₂ receptor activation. Most cellularly accessible cysteine(s) for B₂ receptor activation. Most

Transfected cells, and that the epitope(s) recognized by these antibodies is (are) similarly accessible from the extracellular side of cells expressing recombinant or native B₂ receptors.

Thiol Modification of Cysteines 88 and 94 of the B₂ Receptor—Besides site-directed antibodies, cysteine modification by membrane impermeable thiol-modifying reagents is another means to determine the topology of polytopic membrane proteins (19–21) without modifying the receptor’s primary sequence. The connecting loop between membrane domains I-II of the B₂ receptor contains two cysteines at position 88 and 94 (Fig. 1A). Cysteine modification often alters the function of membrane proteins (20). Therefore we asked whether the B₂ receptor contains a free thiol group on its surface which is important for receptor function. We treated intact COS cells expressing the wild-type rat B₂ receptor with the membrane-impermeant thiol-specific probe, stilbenedisulfonate maleimide (19) and measured B₂ receptor activation. In the presence of 100 μM stilbenedisulfonate maleimide, the bradykinin-induced increase of inositol phosphate levels was reduced by 30 ± 6% (Fig. 3A). The concentration of bradykinin necessary to produce half-maximal activation decreased from 1.4 ± 0.2 × 10⁻¹⁰ μM to 9 ± 3 × 10⁻¹⁰ μM in the presence of stilbenedisulfonate maleimide (Fig. 3A). These data suggest the importance of an extracellularly accessible cysteine(s) for B₂ receptor activation. Most cysteines within the extracellular side of the B₂ receptor are supposed to be linked in disulfide bridges (8). Therefore we next asked whether a cysteine(s) in the connecting loop between membrane domains I-II had been modified by stilbenedisulfonate maleimide. To this end three different B₂ receptor mutants...
were created by point mutation of cysteine(s) to serine(s) at positions 88, 94, and 88/94. All three B2 receptor mutants were not different from the wild-type B2 receptor in their affinity for \([\text{H}]\text{bradykinin} (K_D = 0.6 \pm 0.2 \times 10^{-9} \text{ M})\) and their EC\(_{50}\) values for the bradykinin-induced rise in inositol phosphate levels (1.5 \pm 0.3 \times 10^{-9} \text{ M}) determined after transient expression in COS cells. Next, the bradykinin-induced rise in inositol phosphate levels was determined in the absence or presence of stilbenedisulfonate maleimide. Similarly as on the wild-type B2 receptor expressing cells, stilbenedisulfonate maleimide decreased the bradykinin-induced rise in inositol phosphate levels on cells expressing the B2 receptor mutant where cysteine 88 was replaced by serine (Fig. 3B). By contrast, on cells expressing mutants B2–88Ser and B2–88/94Ser, stilbenedisulfonate maleimide did not significantly decrease inositol phosphate levels after bradykinin stimulation (Fig. 3B). This finding suggests that cysteine 94 within the connecting loop between membrane domains I-II is accessible from the extracellular side and (ii) the extracellular orientation of this receptor loop seems to be involved in B2 receptor activation.

**Agonist-like Activity of Antibodies to the Connecting Loop**

**between Membrane Domains I and II on B2 Receptors**—To further analyze the potential involvement of the connecting loop I-II in B2 receptor activation, the effect of the anti-I-II antibodies on B2 receptor activation was determined. Immunoselected anti-I-II antibodies (100 nm) activated B2 receptors of transfected COS cells and of HF-15 cells (Fig. 4, panels 1 and 3) as determined by the transient rise in \([\text{Ca}^{2+}]_i\) of fura-2 labeled cells. No significant signal was obtained with mock-transfected COS cells (Fig. 4, panel 2) under the conditions applied, or after application of unrelated antibodies (Fig. 4, panel 5). The signal was suppressed when the cells had been pretreated for 5 min with a 100-fold molar excess of the B2 antagonist HOE140 thereby confirming the B2 specificity of the signal (Fig. 4, panels 4 and 6). Thus, anti-I-II antibodies are capable to (partially) activate the B2 receptor and therefore are agonists.

**Displacement of Antibodies to the Connecting Loop**

**between Membrane Domains I and II by B2 Ligands**—We previously demonstrated that antibodies to the connecting loop between membrane domains I-II are accessible to thiol modifying agent stilbenedisulfonate maleimide on intact cells. Similar results were obtained with 10 \(\mu\text{M}\) biotin maleimide (3-(N-maleimidopropionyl)biocytin), another membrane impermeant thiol modifying agent (not shown). These findings extend the data obtained with the site-directed antibodies: (i) the connecting loop between membrane domains I and II is accessible from the extracellular side and (ii) the extracellular orientation of this loop was demonstrated in a variety of experiments performed in triplicate.
Fig. 4. Changes in the intracellular free calcium concentration \([\text{Ca}^{2+}]\) of cells stimulated by antibodies to the connecting loop between membrane domains I and II (anti-I-II). Adherent COS cells (panels 1, 2, 4, and 5) or HF-15 cells (panels 3 and 6) on glass coverslips were labeled by fura-2/AM, and changes in \([\text{Ca}^{2+}]\) were monitored. COS cells were transfected by the human B2 receptor cDNA (panels 1, 4, and 5) 48 h before the experiment. As a control mock-transfected COS cells were used (panel 2). At the time indicated by an arrow, 100 nM immuno-selected anti-I-II antibodies (panels 1–4 and 6) or the same concentration of unrelated antibodies (panel 5) were added to the cells. Where indicated, COS or HF-15 cells had been pretreated by the B2 antagonist HOE140 (10 \(\mu\)M) for 5 min to control the B2 specificity of the signal (panels 4 and 6). Changes in \([\text{Ca}^{2+}]\) are given as the ratio between 340 and 380 nm. A representative experiment is shown which has been reproduced at least four times with similar results.

Fig. 5. Competition binding studies of antibodies to the connecting loop between membrane domains I and II (anti-I-II) with B2 ligands. Confluent HF-15, A431, or B2 receptor-transfected COS cells seeded on 24-well plates were incubated at 4 \(^\circ\)C with 10 nM immuno-selected anti-I-II antibodies in the absence (control) or presence of \(1 \mu\)M bradykinin, kallidin, HOE140, or the cognate peptide. After washing, bound antibodies were detected by iodine-labeled secondary antibodies. Values are given as % of control (= 100%) and are the means of three different experiments (± S.E.). Control values were 46,600 ± 5,800 cpm/well for HF-15 cells, 39,570 ± 2,120 cpm/well for A431 cells, and 320,300 ± 12,030 cpm/well for B2 receptor-transfected COS cells.

Topological modeling of G-protein-coupled receptors relies on the assumption that the seven hydrophobic segments form seven membrane-spanning \(\alpha\)-helices according to the structures of rhodopsin (2) or bacteriorhodopsin (1). Since this assumption was in conflict with the positive inside rule (7) for the connecting loop between membrane domains I-II of the bradykinin B2 receptor (8, 9), we attempted to determine the orientation of the connecting loop. Two different approaches were applied: (i) accessibility to site-directed antibodies and (ii) the substituted cysteine accessibility method which combines thiol modification by a membrane impermeant thiol-specific reagent with point mutagenesis. With both methods we found that the connecting loop between membrane domains I-II is not involved in the binding of agonists to the B2 receptor. By contrast, the presence of a 1000-fold molar excess of B2 antagonists as demonstrated for HOE140, suppressed the binding of anti-I-II antibodies to HF-15, A431, or B2 receptor-transfected COS cells (Fig. 5). Similar results were obtained with NPC 17773, another B2-specific antagonist (not shown).

**DISCUSSION**

Topological modeling of G-protein-coupled receptors relies on the assumption that the seven hydrophobic segments form seven membrane-spanning \(\alpha\)-helices according to the structures of rhodopsin (2) or bacteriorhodopsin (1). Since this assumption was in conflict with the positive inside rule (7) for the connecting loop between membrane domains I-II of the bradykinin B2 receptor (8, 9), we attempted to determine the orientation of the connecting loop. Two different approaches were applied: (i) accessibility to site-directed antibodies and (ii) the substituted cysteine accessibility method which combines thiol modification by a membrane impermeant thiol-specific reagent with point mutagenesis. With both methods we found that the connecting loop between membrane domains I-II is not involved in the binding of agonists to the B2 receptor. By contrast, the presence of a 1000-fold molar excess of B2 antagonists as demonstrated for HOE140, suppressed the binding of anti-I-II antibodies to HF-15, A431, or B2 receptor-transfected COS cells (Fig. 5). Similar results were obtained with NPC 17773, another B2-specific antagonist (not shown).
bradykinin or kallidin to the B₂ receptor. Chemical cross-linking studies (27) and site-directed mutagenesis (28, 29) suggest that the agonist- and antagonist-binding sites to the B₂ receptor are not identical and may be only partially overlapping. When we tested the effect of a 1000-fold molar excess of the B₂ antagonist HOE140 on the binding of anti-I-II antibodies to the B₂ receptor, we found that in contrast to B₂ agonists, HOE140 almost completely suppressed antibody binding. However, the binding of iodine-labeled HOE140 was not affected by the presence of the antibodies. There are two possible explanations for these findings: either (i) the connecting loop I-II forms a contact site of HOE140 to the B₂ receptor which is different from the agonist-binding site, or (ii) the B₂ antagonist induces or stabilizes a receptor conformation which is not accessible for the anti-I-II antibodies. Future studies applying site-directed mutagenesis and/or ligand cross-linking will have to determine which of these two possibilities is true thereby shedding more light on the question of how the atypical topology of the B₂ receptor may affect receptor functioning.

Acknowledgments—We thank Dr. W. Müller-Esterl (University of Mainz, Germany) for support in the field of bradykinin receptors, Dr. A. A. Roscher (Munich, Germany) for HF-15 cells and Dr. M. AlAwady, (University of Cairo, Egypt) for initial help in raising anti-peptide antisera.

REFERENCES