Many of the physiological functions of bradykinin are mediated via the B2 receptor. Little is known about binding sites for bradykinin on the receptor. Therefore, antisera against peptides derived from the putative extracellular domains of the B2 receptor were raised. The antibodies strongly reacted with their corresponding antigens and cross-reacted both with the denatured and the native B2 receptor. Affinity-purified antibodies to the various extracellular domains were used to probe the contact sites between the receptor and its agonist, bradykinin or its antagonist HOE140. Antibodies to extracellular domain 3 (second loop) efficiently interfered, in a concentration-dependent manner, with agonist and antagonist binding and vice versa. Antibodies to extracellular domain 4 (third loop) blocked binding of the agonist but not of the antagonist, whereas antibodies to extracellular domains 1 and 2 or to intracellular domains failed to block ligand binding. Antibodies to ectodomain 3 competed with agonistic anti-idiotypic antibodies for B2 receptor binding. Further, affinity-purified antibodies to the amino-terminal portion of extracellular domain 3 transiently increased intracellular free Ca²⁺ concentration and thus are agonists. The Ca²⁺ signal was specifically blocked by the B2 antagonist HOE140. By contrast, antibodies to the carboxyl-terminal segment of extracellular domain 4 failed to trigger Ca²⁺ release. The specific effects of antibodies to the amino-terminal portion of extracellular domain 3 suggest that this portion of the B2 receptor may be involved in ligand binding and in agonist function.

Physiological and pathophysiological processes are mediated by kinins and their receptors. Kinins are liberated by proteolytic cleavage of the precursor proteins kininogens (1); they decrease blood pressure, induce pain and inflammation, contract smooth muscles, and regulate ion fluxes (2). Receptors for kinins are classified pharmacologically into two major subtypes, B1 and B2 (3). The B1 receptors are triggered by carboxy-terminally truncated kinins such as [des-Arg¹⁰]kallikrein, whereas bradykinin is the agonist of B2 receptors. Molecular cloning has revealed the primary structures of the B1 (4) and the B2 receptors (5) and classified them as members of the G-protein-coupled receptor family that are thought to contain seven membrane spanning α-helices.

The signaling pathways of the B2 receptors have been explored in some detail. The bradykinin B2 receptor is preferentially coupled to G proteins of the Gα1 subtype (6), which activate the phospholipase C-mediated cascade. This results in the hydrolysis of inositol-containing lipids, the generation of inositol phosphates, and the transient rise of the intracellular free Ca²⁺ concentration (7). The initial increase of intracellular Ca²⁺ is followed by Ca²⁺ extrusion, which counteracts Ca²⁺ influx, thereby regulating total cell calcium (8). B2-mediated release of diacylglycerol, another hydrolysis product of phospholipase C, results in the translocation of specific protein kinase C isoforms (9). The B2 receptor is also coupled to the phospholipase A2 pathway, which releases the prostaglandin precursor, arachidonic acid (10).

Although the amino acid sequence of the B2 receptor has been deduced from its cDNA and its transmembrane topology has been predicted from the corresponding hydropathy plots, the specific role of the extracellular domains in ligand binding and in signal transduction is unknown. To address this question, we have raised antibodies against peptides derived from the ectodomains of the B2 receptor and used them to probe for the function(s) of the corresponding structures. Our data show that extracellular domain 3 is involved in ligand binding and may play an essential role in communicating the agonist signal through the receptor.

EXPERIMENTAL PROCEDURES

Materials—NaCl (17.4 Ci/mmol) and the chemiluminescence detection kit (ECL) were from Amersham Corp.; [2,3-proyl-3,4-²H]bradykinin (specific activity 98 Ci/mmol) was from DuPont NEN; iodogen (1,3,4,6-tetrachloro-3-6-diphenyl-glycoluril) and 1,5-difluoro-2,4-dinitrobenzene were from Pierce; Sephadex-G50 was from Pharmacia Biotech Inc.; Dowex 1 (1 x 8), wheat germ agglutinin (WGA) from ¹

The abbreviations used are: WGA, wheat germ agglutinin; CHO, Chinese hamster ovary; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; E64, N-[L-(3-carboxyoxazol-2-carbonyl)-leucyl]-agmatin; ED, extracellular domain; fur-a(2)A(2)M; G(2)-[2(S-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N′,N′-tetraacetic acid, pentacetoxymethyl ether]; HMEM, minimum essential medium buffered with 20 mM Na⁺-HEPES, pH 7.4, 1.8 mM Ca²⁺; HPP-HOE140, 3-(4-hydroxy-phenyl-propionyl)-HOE140; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PIPES, pi-
The pVL1392 vector (kindly provided by Dr. H. Reiländer, Frankfurt) was used for the transfection of CHO cells with the rat B2 receptor cDNA using the Lipofectin transfection method as described (8). CHO cells were transfected with the rat B2 receptor cDNA and gelatin was from Merck. All other chemicals were of analytical grade. Hydroxyphenyl-propyl)-HOE140 (HPP-HOE140) was from Hoechst; Triticum vulgaris (2) was obtained from Desitrix; polyvinylidene difluoride (PVDF) sheets were from Millipore; Affi-Gel 10 and nonfat dry milk was from Bio-Rad; MaxiSorb titer plates were from Nunc; HOE140 and 3-(4-

cyanin and fura-2/AM were from Calbiochem; polyvinylidene difluoride (PVDF) sheets were from Millipore; Affi-Gel 10 and nonfat dry milk was from Bio-Rad; MaxiSorb titer plates were from Nunc; HOE140 and 3-(4-

D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-Tic-Oic-Arg; Hyp, 4-hydroxyproline; Thi, Cysteine residue given in parenthesis is not present in the native sequence. All antisera were raised in rabbit.

Positions in the sequences of the rat B2 receptor (5) and of the human B2 receptor (11). The single-letter code for amino acid residues is used. 

The purity and specificity of the antibodies were analyzed by Edman degradation and electrospray mass spectrometry. Peptides were covalently coupled to the carrier protein, keyhole limpet hemocyanin, by maleimidocaproyl N-hydroxysuccinimide (17). Rabbits were immunized with the conjugates (18). Peptide MLN33 was used for immunization without prior coupling to a carrier protein. Antisera were tested for antigen specificity and cross-reactivity with homologous human or rat peptides by the indirect enzyme-linked immunosorbent assay (ELISA) (19) using microtiter plates (MaxiSorb, Nunc) coated with 2 μg/ml of the peptide or 0.5 μg/ml of the conjugate.

Western Blotting and Immunoprinting—Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride sheets using semidyry blotting (20). The sheets were treated with 50 mM Tris, 0.2 mM NaCl, pH 7.4 (buffer A), containing 5% (v/v) of nonfat dry milk and 0.1% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, pH 8.5. Proteins were desalted and concentrated using a Centricon filtration unit, exclusion limit 30,000 Da. The purity and specificity of the antibodies were analyzed by SDS-PAGE and enzyme-linked immunosorbent assay, respectively.

Lectin Affinity Chromatography of B2 Receptor—WGA was covalently coupled to Affi-Gel 10 (5 mg/ml of gel) according to the manufacturer’s instructions (Bio-Rad). The antigen (5 mg/ml of gel) was applied and incubated under gentle agitation for 12 h at 4°C. The affinity matrix was washed three times with PBS, and the bound antibodies were eluted with 0.2 M glycine, pH 2.5, and immediately neutralized with 1 M KOH. Antibodies were desalted and concentrated using a Centricon filtration unit, exclusion limit 30,000 Da. The purity and specificity of the antibodies were analyzed by SDS-PAGE and enzyme-linked immunosorbent assay, respectively.

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SDS sample buffer (15) and applied to polyacrylamide gel electrophoresis (PAGE).

Receptor Radioiodination of Peptides and Antibodies—Peptides or antibodies (1 μg each) dissolved in 100 μl of PBS were incubated with 2 μCi of carrier-free Na-125I on a solid phase of iodogen (100 μg/tube) for 10 min (16). Unreacted iodine was separated by gel filtration over Sephadex G50 columns or by anion exchange chromatography over Dowex-1.

Synthesis of Peptides and Production of Anti-peptide Antibodies—Peptides derived from the rat or human B2 receptor sequence (Fig. 1) were synthesized by solid phase peptide synthesis using the Fmoc (N-(9-fluorenylethoxycarbonyl) or the t-Boc (t-butoxycarbonyl) chemistry (Table I). 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-hydroxysuccinimide (17). Rabbits were immunized with the conjugates (18). Peptide MLN33 was used for immunization without prior coupling to a carrier protein. Antisera were tested for antigen specificity and cross-reactivity with homologous human or rat peptides by the indirect enzyme-linked immunosorbent assay (ELISA) (19) using microtiter plates (MaxiSorb, Nunc) coated with 2 μg/ml of the peptide or 0.5 μg/ml of the conjugate.

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Lectin Affinity Chromatography of B2 Receptor—WGA was covalently coupled to Affi-Gel 10 (10 mg/ml of gel). B2 receptors from HF-15 cells were solubilized with 4 mM CHAPS in 20 mM PIPES, pH 6.8. The solution was diluted with an equal volume of 20 mM PIPES, pH 6.8, adjusted to 1 mM NaCl, 10 mM MnCl2, 100 mM CaCl2, and incubated for 4 h at 4°C with the WGA affinity matrix. After extensive washing, bound proteins were eluted by a 30-min incubation with an equal volume of 20 mM PIPES, 1 mM NaCl, 100 mM MnCl2, 100 mM CaCl2, 0.5 mM N-acetyl glucosamine, pH 6.8. Proteins were desalted and precipitated by 80% (v/v) acetone (21) and recovered by centrifugation. The protein pellet was dissolved in 2% (w/v) SDS, 5 mM EDTA, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.01% (w/v) bromophenol perazine-N, N'-bis-(2-ethanesulfonic acid); TM, transmembrane segment.
blue, 67.5 mM Tris-HCl, pH 6.7 by boiling for 5 min, and proteins were resolved on 10% (w/v) polyacrylamide gels containing 0.1% (w/v) of SDS (15).

Immunofinity Chromatography of the B2 Receptor—Affinity-purified domain-specific antibodies were covalently bound to Affi-Gel 10 (15 mg/ml gel). Membranes of SF9 cells infected with baculovirus encoding the human B2 cDNA (100 pmols of B2 receptor/20 mg of total membrane protein) were solubilized with 2% (w/v) sodium deoxycholate in PBS including 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml E64, and 2 μM leupeptin. The deoxycholate was diluted to 0.1% (w/v) by the addition of 20 mM HEPES, pH 7.4, containing 150 mM NaCl, 1 mM EDTA (buffer B). Then 10% (w/v) glycerol and 0.1% (w/v) Triton X-100 were added, and the solution was applied to the immunoaffinity matrix for an overnight incubation. The affinity matrix was extensively washed with buffer B, and bound proteins were eluted with 0.2 M glycine, pH 2.5 supplemented with 10% (w/v) 1,4-dioxane. The eluted protein fraction was neutralized with 1 M Tris, pH 8.0, and concentrated by Centricon filtration (exclusion limit 30,000 Da). The purity of the enriched B2 receptor was assessed by SDS-PAGE and silver staining. For NH2-terminal sequencing, proteins from three experiments were pooled, applied to a ProSpin sample preparation cartridge, and sequenced on a 477 A protein sequencer equipped with an on-line 120A PTH Analyzer (Applied Biosystems).

Affinity Cross-linking of the B2 Receptor—B2 agonist or antagonist was cross-linked to the B2 receptor as described previously (22) with minor modifications. B2 receptors of HF-15 cells were enriched by WGA affinity chromatography, and the eluted proteins were desalted by dialysis prior to ligand binding and cross-linking with 1 mM dithiothreitol and 1 mM EDTA (buffer C) with or without 2 μM leupeptin. Cross-linking to recombinant B2 receptors of CHO (1.5 μg/ml gel) or SF9 cells (4–5 μg/ml of protein) and SF9 cells (4–5 μg/ml of protein) was performed on intact cells or without prior enrichment of receptor protein.

Competition Studies with Radiolabeled Ligands—Membranes or confluent HF-15 cells on 24-well plates in 0.5 ml of RPMI 1640 containing protease inhibitors and buffer with 20 mM Na+-HEPES, pH 7.4 (binding buffer) were incubated with 100 nM [3H]HPP-HOE140 (0.5 μM, specific activity 1367 Ci/mmol) or with [3H]bradykinin (2 μM, specific activity 98 Ci/mmol) in the presence of increasing concentrations of affinity-purified antibodies (5 × 10−8 M to 1 × 10−5 M). After 2 h of incubation at 4°C, the cells were washed three times with ice-cold medium. The cells were dissolved in 1% (w/v) NaOH, and radioactivity was determined.

Competition Studies with Iodinated Antibodies—Confluent HF-15 cells on 24-well plates were washed twice with binding buffer (see above). Then 0.5 ml of binding buffer was added to each well. For competition studies cells were incubated at 4°C with 125I-labeled immunoselected antibodies (1 × 10−8 M; specific activity 0.02 Ci/mg) in the presence or absence of 1 × 10−9 M bradykinin or HOE 140. After 2 h of incubation at 4°C, the cells were washed three times with ice-cold medium and dissolved in 1% (w/v) NaOH, and radioactivity was determined.

Immunofluorescence Studies of A431 Cells—The human epithelial cell line, A431 was grown on glass coverslips for 48 h. Three h before the experiment, the medium was replaced by RPMI 1640 supplemented with 0.5% (v/v) fetal calf serum. Prior to immunofluorescence, cells were washed three times with 60 mM PIPES, 25 mM HEPES, 10 mM EDTA, 2 mM Mg(CH3COO)2, pH 6.9, and fixed for 30 min with 3% (w/v) paraformaldehyde in the same buffer adjusted to pH 7.5. Excess paraformaldehyde was quenched by the addition of 50 mM NH4Cl in PBS, pH 7.4; this was followed by 30 min of incubation with PBS, pH 7.4, containing 0.3% (w/v) gelatin. The cells were treated for 1 h at room temperature with anti-peptide antisera, 1:100 in 0.3% gelatin/PBS. The first antibody was detected using a rhodamine-coupled donkey anti-rabbit immunoglobulin, 1:100 in 0.3% gelatin/PBS. Controls included antisera preincubated with 2 μM of their respective antigens. The coverslips were embedded in Moviol® and viewed with an Ortho-plan microscope (Leitz).

Flow Cytometric Analysis—Confluent HF-15 cells (0.5–1 pmol of B2 receptor/mg of protein) were harvested using PBS, 0.5 mM EDTA, 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 × 106) were incubated with 2 μM fura-2/AM (Molecular Probes) in HMEM containing 0.04% (w/v) pluronic F-127. After a 45-min incubation at 30°C, the cells were washed twice and incubated for 1 h at 4°C. After washing three times, fluorescence isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Sigma), diluted 1:80 (v/v), was added to the cells. The cells were incubated for 1 h at 4°C, washed, fixed with 2% (w/v) of formaldehyde, and analyzed on a FACScan (Becton Dickinson) using the LYSIS program.

Measurement of Changes in Intracellular Free Ca2+ Concentration—Intracellular free Ca2+ concentration, [Ca2+]i, of HF-15 cells was determined by fura-2/AM as described previously (8) with minor modifications. Confluent HF-15 cells grown on 10-mm diameter glass coverslips were washed twice with minimum essential medium buffered with 20 mM Na+-HEPES, pH 7.4 (HMEM), and incubated with 2 μM fura-2/AM in HMEM containing 0.04% (w/v) pluronic F-127. After a 45-min incubation at 30°C, the cells were washed twice and incubated in HMEM for another 30 min to allow for complete deesterification of fura-2/AM. For determination of a change in [Ca2+]i, the coverslips were mounted in a holder at an angle of 45° and put into a thermostatted quartz cuvette, and fluorescence at 510 nm was determined. The excitation wavelength alternated between 340 and 380 nm in intervals of 600 ms. Changes in [Ca2+]i, are given as the ratio of 340 and 380 nm.

RESULTS

Selection of Peptides for Immunizations—To raise antisera that cross-react with four predicted extracellular domains (EDs) of the B2 receptor, we selected six segments from ED1 through ED4 of the B2 receptor so that the peptides included a cysteine residue if available (Table I). The peptides covered the entire sequence of the putative ectodomains of the B2 receptor; there were two overlaps of 1 and 3 residues between the peptides selected from ED3 and ED4, respectively (Fig. 1). Two
additional peptides chosen from the putative intracellular domains (IDs), ID2 and ID4, served as controls. The peptides were covalently coupled to a carrier protein, keyhole limpet hemocyanin, and used for immunization except for MLN33, which was directly used without prior conjugation. The resultant antisera recognized their cognate antigens as verified by the indirect enzyme-linked immunosorbent assay, and cross-reacted with the sequences from the human or the rat B2 receptor, respectively (not shown). A single peptide, designated CWN12, which is derived from ED4 and covers the center portion of peptide SGC18, failed to produce a significant titer of specific antibodies (not shown).

Cross-reactivity with the Denatured B2 Receptor—To analyze the interaction of the anti-peptide antisera with B2 receptors we used HF-15 fibroblast-derived B2 receptor, partially purified by WGA affinity chromatography for immunoprint analysis (Fig. 2A). Antiserum directed against EDs 1, 2, 3, and 4 (lanes 2–7) and to ID2 or ID4 (lanes 8 and 9) efficiently recognized a protein of 69 ± 3 kDa. A protein of similar molecular mass, 69 ± 5 kDa, was stained when HOE140-labeled B2 receptor was identified by antiserum to HOE140 (Fig. 2A, lane 1) (22). Likewise, when [125I-Tyr0]bradykinin was cross-linked to the B2 receptor in HF-15 membranes and the sample was resolved by SDS-PAGE followed by autoradiography, a single radiolabeled band of 69 ± 3 kDa was seen (Fig. 2A, lane 10). In some cases bands of higher molecular mass were seen, which might represent aggregated B2 receptors (Fig. 2A, lanes 7–9).

Specificity of the Anti-B2 Antisera—To assess the specificity of the antisera, we used membranes from CHO cells and SF9 cells that overexpress the rat or the human B2 receptor for Western blotting and immunoprinting. The results are exemplified for the antiserum to the first ectodomain, ED1. In Triton X-114-extracted membranes of recombinant CHO cells expressing the rat B2 cDNA, anti-ED1 detected a single 69 ± 3-kDa protein (Fig. 2B, lane 1). No specific staining was found in nontransfected cells, Fig. 2B, lane 2. As control B2 receptor from CHO cells that was labeled by HOE140 was detected using anti-HOE140 antiserum (Fig. 2B, lane 3). Binding of the antibodies to the B2 receptor was suppressed when cross-linking was performed in the presence of a 1,000-fold molar excess of bradykinin (Fig. 2B, lane 4). We further tested the specificity of the anti-ED1 antiserum using CHAPS-extracted membranes of SF9 cells infected with recombinant baculovirus encoding the human B2 cDNA, and detected three protein bands of 38, 41, and 45 ± 5 kDa (Fig. 2C, lane 1). Proteins with a molecular mass of approximately 75–80 kDa are likely to be dimerized B2 receptors that aggregated probably during sample preparation of SF9 cell membranes expressing high amounts of B2 receptor (22). Membranes from mock-infected SF9 cells did not show any specific bands (Fig. 2C, lane 2). As a control HOE140 was cross-linked to the B2 receptor followed by detection with anti-HOE140 antiserum. Proteins of similar molecular weight were stained, Fig. 2C, lane 3. This staining was suppressed by a 1,000-fold molar excess of bradykinin (Fig. 2C, lane 4). The differences in the apparent molecular masses of recombinant B2 receptors from SF9 cells and of B2 receptors of HF-15 fibroblasts and the occurrence of multiple immunoreactive bands in SF9 cells are likely caused by incomplete glycosylation, characteristic for glycoproteins expressed in SF9 cells (23).

Affinity Purification and Amino-terminal Sequence Analysis of the B2 Receptor—Is the protein identified by immunostaining with anti-peptide antisera the authentic B2 receptor? We enriched the receptor protein from SF9 membranes by immunofinity chromatography using a mixture of immunoselected anti-peptide antibodies to the extracellular domains. Edman degradation of the protein revealed an amino-terminal sequence of Met-Leu-Asn-Val-Thr-Xaa-Gln-Gly-Xaa-Thr-Leu-Asn-Gly-Thr-Phe-Ala-Xaa-Ser, where Xaa stands for an unidentified residue. This sequence is identical with the human B2 receptor sequence starting at the third in-frame initiator codon (11); note that the construct in baculovirus had been engineered such that only the most 3′-located initiator codon was available. These data demonstrate that the affinity-purified anti-peptide antibodies selectively enrich B2 receptor from solubilized SF9 membranes.

Fluorescence-activated Cell Sorting (FACS) Analysis of Native B2 Receptors on HF-15 Fibroblasts—To test the reactivity of the antibodies with native B2 receptor we performed FACS analysis of HF-15 cells that were stained by antisera to the various extracellular domains ED1 to ED4 (Fig. 3A). Antiserum to extracellular domains ED1 to ED4 bound to B2 receptors of intact HF-15 cells (Fig. 3A, I–VI) as demonstrated by increased fluorescence intensity in comparison with preimmune serum (Fig. 3A, VII), suggesting that the various anti-peptide antibodies cross-react to similar extents with the B2 receptor. No specific staining was observed with antisera to intracellular domain ID2 or ID4, exemplified for anti-iD2 (Fig. 3A, VII). This finding is in agreement with the hypothetical model of the B2 receptor (cf. Fig. 1).

Redistribution of B2 Receptor Detected by Antibodies to Extracellular Domains—The successful binding of anti-peptide antisera to cellular B2 receptors allowed us to examine the fate of the B2 receptor after its activation by an agonist. In these
Studies we applied a mixture of the various anti-ED antibodies. HF-15 cells were preincubated at 37°C for 60 min in the absence (Fig. 3B, I) or presence of 1 μM bradykinin (Fig. 3B, III). Pretreatment of the cells by the B2 agonist drastically reduced the antibody binding to B2 receptors (Fig. 3B, IIII). Thus, after agonist treatment the antigenic epitopes are no longer available for antibody binding. Preincubation with 1 μM of the agonist, HOE140, did not change the overall fluorescence intensity (Fig. 3B, IV). Together these data suggest that our anti-peptide antibodies readily recognize the extracellular domain(s) of the B2 receptor and that, like other G-protein-coupled receptors, sequestration and/or internalization modifies the accessibility of extracellular domains for antibody detection.

Immunofluorescence of A431 Cells—To immunovisualize the B2 receptor on cells other than fibroblasts, the epidermoid carcinoma cell line A431 was chosen. A strong immunostaining of the plasma membrane of fixed, nonpermeabilized cells was observed with a mixture of antibodies against ED1 to ED4 (Fig. 4A). A specific staining of the outer rims of the cells was also observed when the individual antisera against extracellular domains, ED1, ED2, ED3N, and ED4C, were used (Fig. 4e to 4h). The cells exhibit a punctuated labeling, which may be due to receptor clustering and/or high receptor density in pseudopodia and microvilli of A431 cells; this latter notion was confirmed by electron microscopy (not shown). The presence of the cognate antigen for each antibody abrogated the specific immunostaining (b). No staining was seen with preimmune serum (c), with antiserum to an unrelated peptide (d), or with an antiserum to intracellular domain, ID2 (l). Hence the anti-peptide antibodies are useful to probe for the B2 receptor on the surface of various cell types (Figs. 3 and 4).

Blockade of Bradykinin Binding by Anti-ED Antibodies—We asked whether our antibodies interfered with the binding of bradykinin to the B2 receptor. HF-15 cell membranes were preincubated for 2 h at 4°C with 250 nM affinity-purified antibodies to the various extracellular domains. This was followed by the addition of 2 nM [3H]bradykinin and further incubation for 60 min at 4°C. The unbound radioligand was separated by filtration through GF/C glass filters, and the filter-bound radioactivity was determined. Controls were done in the absence (total binding) or presence of 2 μM of the unlabeled ligand, bradykinin (Fig. 5A, columns 1 and 2). Out of six antisera tested only antibodies against the amino-terminal portion of extracellular domain 3, ED3N, and to the carboxy-terminal segment of extracellular domain 4, ED4c, interfered with bradykinin binding to the B2 receptor (Fig. 5A, columns 5 and 8). Antibodies to other segments of the extracellular domains (Fig. 5A, columns 3, 4, 6, and 7) and to the intracellular domains (not shown) had no effect on [3H]bradykinin binding. These data suggest that domains ED3 and ED4 of the B2 receptor might be critically involved in ligand binding.

Concentration-dependent Displacement of Radioligand by Anti-ED Antibodies—To further analyze the involvement of extracellular domains 3 and 4 in agonist and antagonist binding, we tested whether the effect of the antibodies is dose-de-
Intact HF-15 cells were incubated for 2 h at 4 °C with 250 nM affinity-purified antibodies to ED1, ED2, ED3N, ED3C, ED4N, or ED4C, followed by the incubation with 2 nM [3H]bradykinin. Nonspecific binding and total binding were determined in the presence or absence of 2 μM unlabeled bradykinin. Following filtration and washing, filter-bound radioactivity was determined. B and C, competition of increasing concentrations of antibodies with radioligands. Intact HF-15 cells containing 15–20 fmol of B2 receptor/well were incubated at 4°C with 2 nM of [3H]bradykinin (B) or with 0.5 nM of [125I]HPP-HOE140 (C) in the presence of increasing concentrations of anti-ED3N (●), anti-ED3C (○), or anti-ED4C (□). The results (mean ± S.E.) from three independent experiments are given as percentage of maximum specific binding in the absence of competitor (100%).

**Fig. 5.** Displacement of radioligands by domain-directed antibodies. A, membranes of HF-15 cells (100 μl containing 30–50 fmol of B2 receptor) were incubated for 2 h at 4 °C with 250 nM affinity-purified antibodies to ED1, ED2, ED3N, ED3C, ED4N, or ED4C, followed by the incubation with 2 nM [3H]bradykinin. Nonspecific binding and total binding were determined in the presence or absence of 2 μM unlabeled bradykinin. Following filtration and washing, filter-bound radioactivity was determined. B and C, competition of increasing concentrations of antibodies with radioligands. Intact HF-15 cells containing 15–20 fmol of B2 receptor/well were incubated at 4°C with 2 nM of [3H]bradykinin (B) or with 0.5 nM of [125I]HPP-HOE140 (C) in the presence of increasing concentrations of anti-ED3N (●), anti-ED3C (○), or anti-ED4C (□). The results (mean ± S.E.) from three independent experiments are given as percentage of maximum specific binding in the absence of competitor (100%).

**Fig. 6.** Competition of radiolabeled anti-ED antibodies and of anti-idiotypic antibodies with bradykinin or HOE140. Confluent HF-15 cells were incubated for 2 h at 4 °C with 10 nM [125I]-labeled anti-ED3N (panel A) or [125I]-anti-ED4C (panel B) in the absence or presence of 10 μM bradykinin, HOE140, (des-Arg9)bradykinin, or cognate peptides KDY13 of anti-ED3N and SGC18 of anti-ED4C. In panel C, HF-15 cells suspended in medium were incubated with 10 nM [125I]-labeled anti-idiotypic antibodies in the absence or presence of 10 μM bradykinin or HOE140 or of a 100-fold molar excess (1 μM) of anti-ED3N or anti-ED4C. Following incubation, the cells were washed and lysed, and the cell-associated radioactivity was determined. The results (mean ± S.E.) from three independent experiments are presented.

The binding of [125I]HPP-HOE140 (Fig. 5C). Other antibodies such as anti-ED3C did not interfere with the binding of the ligands (Fig. 5, B and C). We conclude that at least part of the contact site for bradykinin and/or for HOE140 may be near or within the amino-terminal portion of ED3. The carboxyl-terminal portion of ED4 may contribute to the receptor binding of the agonist but not of the antagonist.

**Displacement of [125I]-Labeled Anti-ED Antibodies by B2 Ligands**—Anti-ED antibodies interfere with radioligand binding to the B2 receptor. This may either indicate that they are competitive inhibitors for the binding site or that the antibodies act allosterically by inducing and/or stabilizing a receptor conformation unable to bind the ligand. To discriminate between these possibilities competition binding between the unlabeled ligands, bradykinin and HOE140, and the [125I]-labeled antibodies anti-ED3N and anti-ED4C was done with HF-15 fibroblasts. The binding of 10 nM [125I]-labeled anti-ED3N to the receptor was reduced by 80% in the presence of 10 μM bradykinin, and it was abolished by 10 μM HOE140 (Fig. 6A). At the same concentration the cognate peptide, KDY13, completely displaced radiolabeled anti-ED3N; the B1 receptor agonist, (des-Arg9)bradykinin, had no effect (Fig. 6A). In the case of [125I]-
labeled anti-ED4C antibodies, no inhibition of binding was seen in the presence of 10 μM bradykinin or HOE140, whereas displacement was observed by the same concentration of the cognate peptide, SGC18, Fig. 6B. We conclude that antibodies to ED3N, but not antibodies to ED4C, are competitive with B2 receptor ligands. Anti-ED4C may cause an allosteric alteration and stabilize a conformation of the receptor unable to bind agonists.

Displacement of Anti-idiotypic Antibodies by Antibodies to ED3N—To further address the interaction between anti-ED3N and the kinin receptor we applied anti-idiotypic antibodies that had been raised against the idiotype, monoclonal antibody MBK3 to bradykinin (24). These antibodies have previously been shown to bind to and stimulate in an agonist-like manner the human or mouse B2 receptor (24). Competition experiments demonstrate that bradykinin and HOE140 interfere with 125I-labeled anti-idiotypic antibodies for receptor binding (Fig. 6C). Antibodies to ED3N displaced the radiolabeled anti-idiotypes, although not completely, whereas antibodies to ED1, ED2, and ED4C had no effect (anti-ED4C is shown in Fig. 6C). These findings indicate that the majority of the anti-idiotypic antibodies are likely to bind to ED3N, and that interaction sites for bradykinin, HOE140, anti-idiotypic antibodies, and anti-ED3N antibodies with the external portion of the receptor are mutually overlapping.

Agonist-like Effects of Antibodies to ED3N—Our finding that antibodies to ED3N interfere with the receptor binding of bradykinin and anti-idiotypic antibodies prompted us to ask whether anti-ED3N itself is an agonist. Therefore, we measured intracellular free Ca2+ in HF-15 fibroblasts treated with anti-ED3N. At a concentration of 250 nM anti-ED3N, transiently increased [Ca2+]i in an agonist-like manner, Fig. 7A. This effect is mediated by the B2 receptor because a 10-fold molar excess of the B2 antagonist, HOE140, prevented the Ca2+ transient (Fig. 7B). Antibodies to the distal portion of the same domain, ED3C, or to other ectodomains such as ED4, were without effect (Fig. 7, C and D). Hence polyclonal antibodies to the amino-terminal portion of ectodomain 3 are agonists.

**DISCUSSION**

In these studies antibodies directed to putative extracellular domains of the bradykinin B2 receptor were prepared. These antibodies were used to map extracellular domains involved in ligand binding. This approach of ligand binding site mapping is complementary to the site-directed mutagenesis studies (25–27). The antibody approach reduces the commonly voiced concern about site-directed mutagenesis, that the mutation changes the receptor structure and binding ability without being located at the ligand binding site.

Our experiments show that the amino-terminal portion of ectodomain 3, ED3N, is involved in agonist binding and sensing because (i) antibodies to this segment competed with bradykinin for binding to the B2 receptor, (ii) bradykinin almost completely abolished the binding of radiolabeled anti-ED3N to B2 receptors, and (iii) anti-ED3N antibodies were agonists. A direct contact between the ED3N segment and bradykinin is uncertain; however, the mutual competition of ED3N antibodies and bradykinin suggests such a possibility. The ED3N region is also involved in binding of the antagonist, HOE140, because (i) anti-ED3N blocked HOE140 binding to B2 receptors, (ii) HOE140 completely abolished the binding of radiolabeled anti-ED3N to B2 receptors, and (iii) HOE140 nearly completely blocked anti-ED3N-induced cytosolic Ca2+ increase, i.e. anti-ED3N agonism.

The agonistic effect of anti-ED3N antibodies demonstrates that this receptor region can assume or can be induced to assume conformation(s) that transmit the signal to the G-protein. If one considers a two-domain model of G-protein-coupled receptors as suggested by the observation that transmembrane regions (TMs) 1–5 and TMs 6–7 need not be covalently connected for G-protein-coupled receptors to bind and signal (28, 29), then anti-ED3N might push apart the two domains, allowing access of the G-protein to the intracellular loops. Alternatively, anti-ED3N might stabilize the R* activated form of the receptor which is at equilibrium with the R inactive form under basal conditions (30). Autoantibodies to extracellular domains of the adrenergic and muscarinic receptors have been detected in the serum of patients with myocardial diseases or malignant hypertension (31–33). These antibodies are directed to the same extracellular loop as is anti-ED3N, interfere with ligand binding, and are agonists. These similarities between antibodies to extracellular domains of cationic amine receptors and of a peptide receptor emphasize the common molecular mechanisms governing the action of G-protein-coupled receptors.

A few attempts have been made to elucidate the binding site of the B2 receptor using site-directed mutagenesis (26, 27). Alanine substitutions of negatively charged residues were...
made at the TM7/TM4 boundary, D268A contained in the ED4 peptide, and at the TM6/TM4 boundary, D268A contained in the ED4 epitope. The D268A change reduced slightly the affinity of bradykinin and did not change the affinity with the related antagonists HOE140 or NPC17761 (26). However, anti-ED4A antibodies had no effect on bradykinin or HOE140 binding. The D268A mutation had larger effects on the bradykinin affinity and small effects on antagonist binding affinity. In accordance with that observation, anti-ED4A antibodies reduced bradykinin binding but had no effect on antagonist binding. Finally an alanine substitution, E179A, contained in the ED33 epitope, also caused a small reduction in bradykinin affinity (26). Thus the mutagenesis and the antibody methods for probing ligand binding sites concur in their indication that the charged residues Asp268 and Glu179 and associated peptide regions may be involved in agonist binding.

Our anti-ED4A antibody does not confirm the involvement of Asp268 in bradykinin binding; however, we note that D268 is the amino-terminal residue of the ED4 peptide, DTL12; and thus, the anti-ED4A antibodies may not bind the Asp268 residue as part of an extended protein chain in the same way as when it is the first residue of a peptide. The finding that anti-ED4 inhibits bradykinin binding but is unable to inhibit HOE140 inhibiting suggests that the binding sites for peptidic agonists and antagonists on B2 receptors do not perfectly overlap. This conclusion agrees with the suggestions, derived from site-directed mutagenesis studies, that agonists and antagonists do not bind to identical sites on the receptor (26, 34).

These studies, which used anti-extracellular domain antibodies covering all the extracellular domains of the bradykinin receptor, demonstrate that the binding of agonists by the receptor involves extracellular regions at the top of TM4, ED3a, and to a lesser extent at the top of TM7, ED4c. In contrast, the binding of antagonists is only affected by antibodies directed to the top of TM4, ED3a. Furthermore, the anti-ED3a antibodies are agonists, suggesting that the TM4 to TM5 loop, ED3, is important for signal transduction. These studies also point to the importance of extracellular domains for binding and signal transduction in this member of the G protein-coupled receptor family and demonstrate the utility of epitope-specific antibodies in defining functionally important regions of receptors.

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