We have studied the ligand-induced phosphorylation/dephosphorylation of the bradykinin B2 receptor endogenously expressed in human HF-15 fibroblasts. An antiserum (AS346) to a synthetic peptide (CRS36), derived from the extreme carboxyl terminus of the human B2 receptor, precipitated the receptor from solubilized membranes of HF-15 cells that had been labeled with [32P]orthophosphate. A low basal level of B2 receptor phosphorylation was found in the absence of a ligand. Stimulation of the cells with the B2 receptor agonists bradykinin, [Lys⁶,Hyp⁷]bradykinin, kallidin, and T-kinin resulted in a rapid and efficient phosphorylation of the receptor. The B2 receptor antagonist HOE140 and the B1 receptor agonist des-Arg⁵-bradykinin failed to induce significant phosphorylation of the B2 receptor. Phosphoamino acid analysis revealed that the B2 receptor is phosphorylated on serine and threonine, but not on tyrosine residues. The ligand-induced phosphorylation of the receptor was concentration-dependent, with an apparent EC₅₀ of 33 nM, and peaked at 1 min after challenge. The kinin-stimulated phosphorylation of the B2 receptor was rapid and transient and paralleled the kinetics of desensitization/resensitization of the receptor as followed by [Ca²⁺], release and radioligand binding assay, respectively. The ligand-induced phosphorylation of the B2 receptor was independent of the protein kinase C pathway. In vitro experiments suggest βARK1 (β-adrenergic receptor kinase) as a candidate kinase that could mediate the homologous B2 receptor phosphorylation. Inhibitors of protein phosphatases 1 and 2A effectively blocked the dephosphorylation, but did not affect the internalization of the B2 receptor, whereas inhibitors of receptor internalization delayed its dephosphorylation. These finding point to a role of ligand-induced phosphorylation in the desensitization and redistribution of the bradykinin receptor in human fibroblasts.

The nonapeptide bradykinin (NH₂-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-COOH), a prototypic member of the kinin family, mediates important biological processes such as hypotension, edema formation, pain sensations, smooth muscle contraction, and cell growth (1). Kinins are locally released on the surface of target cells due to limited proteolysis of their parental molecules, kininogens, by the kallikreins (2). The broad spectrum of their (patho)physiological activities is mediated by cognate kinin receptors that classify pharmacologically as B1 and B2 subtypes (3). B1 receptors respond preferentially to des-Arg¹⁰-kallidin, whereas B2 receptors are stimulated by bradykinin and kallidin (lysylbradykinin). The multiplicity of biological effects elicited by the kinins is reflected by the complexity of their signaling pathways. B2 receptors couple to various G proteins such as Gₓₐ, thereby triggering the inositol trisphosphate/Ca²⁺ pathway via phospholipase C-β and/or the arachidonic acid/prostaglandin pathway via phospholipase A₂ (4). Recent findings indicate that B2 receptors might also couple to the cAMP pathway via Gₛ (5). In human foreskin fibroblasts, bradykinin induces, via phospholipase C, a transient rise in [Ca²⁺], that is counteracted by Ca²⁺ extrusion (6). The increased [Ca²⁺], activates the nitric oxide/cGMP pathway and, together with diacylglycerol, drives the translocation and activation of protein kinase C, specifically of its isoforms α, ε, and η (7).

Given the remarkable pharmacological profile of these substances, it is evident that the activity of these peptides demands a careful control. Elaborate mechanisms exist that direct the kininogens to the surface of their target cells and allow kinin release at or next to its site of action (8). The liberated kinins are rapidly degraded in vivo by peptidases such as angiotensin-converting enzyme (kininase II), carboxypeptidase N (kininase I), and aminopeptidase P, which truncate and thereby inactivate the kinin peptides (9); the half-life of bradykinin in the plasma is <15 s (10). At the level of their receptors, the actions of kinins are restricted with respect to time and space by mechanisms involving receptor desensitization (11), internalization of the receptor-ligand complex (12, 13), loss of extracellular ligand-binding sites (14), and modulation of receptor affinity (11). Although tachyphylaxis and redistribution are well documented for the B2 receptor, the molecular mechanisms underlying these phenomena are not well understood. We hypothesized that reversible phosphorylation of kinin receptors might contribute to these phenomena.

Here we have set out to investigate the agonist-induced phosphorylation of the bradykinin B2 receptor in a nontransformed human cell line (HF-15 fibroblasts) that endogenously expresses a high copy number of the B2 receptor. Using an anti-peptide antibody cross-reactive with the native receptor, we demonstrate the ligand-induced phosphorylation on Ser and Thr residues located in the carboxyl-terminal domain of the B2 receptor. Our data suggest that ligand-induced reversible phosphorylation might play a crucial role in the regulation of responsiveness and availability of the endogenous B2 receptor from human fibroblasts.
EXPERIMENTAL PROCEDURES

Materials—Pro-mix™, S-[35S]-labeled amino acids (Pro-mix™), and [32P]orthophosphate were from Amersham Corp. Bisindolylmaleimide (GF109203), bovine serum albumin, and phospho-free Dulbecco's modified Eagle's medium (DMEM)1 were from AppliChem. Trypsin from bovine pancreas was from Biochrom. A23187, calphostin C, dibutyryl cGMP, epidermal growth factor, forskolin, fura-2/AM (1-[2-(5-carboxyoxazol)-2-yl]-6-amino benzofuran-5-oxyl-2'-(2'-aminomethyl-5'-ethylyphenoxy)ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester), H-8, HOE140, H-89, PKC-β, PKC-α, PKC-γ, PKC-δ, PKC-ε, PKC-ζ, PKC-η, PKC-θ, PKC-ι, PKC-µ, PKC-½, PKC-θ2, PKC-τ, PKC-ó, PKC-π, PKC-ρ, PKC-σ, PKC-τ2, Pluronic F-127, proline, proline-5 methyllysine, protease inhibitors, protein ladder markers (10–200 kDa) were from 1xray films were from Fuji. HOE140 ([D-Arg0, Hyp3, Thi5, D-Tic7, 1640 medium were from c.c. pro. Bacitracin was from Fluka. New RX rosporine, and thapsigargin were from Calbiochem. DMEM and RPMI medium; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid), MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis. 

Peptide Synthesis and Production of Anti-peptide Antibodies—Peptide LHK10 derived from the first intracellular domain (ID1) of the rat B2 receptor sequence, peptide DR15 of ID2, peptide RNN16 of ID3, and peptide CRS36 of ID4 of the human B2 receptor sequence were synthesized by the solid-phase method using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemocycle (see Table 1). The peptides were coupled to the solid phase, keyhole limpet hemocyanin, by the carbodiimide method as described previously (17), except for peptide CRS36, which was used without prior conjugation. Female New Zealand White rabbits were immunized with the conjugates or the peptide alone (CRS36) according to established procedures. The antisera were tested for antigen specificity by the indirect solid-phase-linked enzyme-linked immunosorbent assay using microtiter plates (Maxisorb, Nunc) coated with a 2 μg/ml concentration of the peptide. The cross-reactivity with the native receptor was probed by immunoprecipitation (see below). For nomenclature of the B2 receptor domains, see Ref. 18.

Ligand Binding Studies—The binding activity of the receptor was tested on cell monolayers by the natural ligand, i.e. [H]bradykinin (19). Cells were grown on 24-well plates were washed twice with ice-cold HEPES-buffered RPMI 1640 medium and incubated for 90 min at 4°C with 200 μl of 5 nM [H]bradykinin, 2 μM bacitracin in the same medium in the absence (total binding) or in the presence (nonspecific binding) of 5 μM unlabeled bradykinin. The unbound ligand was removed by washing the adherent cells four times with 1 ml of ice-cold HEPES-buffered RPMI 1640 medium. The cells were dissolved in 1 M NaOH, and the radioactivity of the extract was measured. For internalization studies, [H]bradykinin binding was performed as described above, and internalization was initiated by incubating the cells for 30 min at 37°C. After removal of the free ligand by four washing steps with HEPES-buffered RPMI 1640 medium, surface-bound bradykinin was extracted with 500 μl of 2 M acetic acid, pH 2.8, 0.5 μM NaCl (13), and the radioactivity of the extract was measured. The radioactivity associated with the cells after pH 2.8 treatment was considered "intracellular" (13); it was determined after dissolving the cells in 1 M NaOH. To measure the recovery of binding, the cells were stimulated with 100 nM bradykinin for 5 min at 37°C. Bradykinin was removed by four washing steps with HEPES-buffered RPMI 1640 medium at 37°C. Fresh medium was added; the cells were incubated at 37°C for varying periods of time, and [H]bradykinin binding was determined as described above. As a control, unstimulated cells were assayed under otherwise identical conditions.

Membrane Preparations—The membrane fraction of HF-15 cells was prepared as described previously (19) with minor modifications. The cells were harvested in 20 mM Hepes, pH 6.8, 5 mM MgCl2 (buffer A) including protease inhibitors (0.1 mM Pefabloc SC®, 10 μg/ml each 1,10-phenanthroline, aprotinin, leupeptin, and pepstatin A) and homogenized by Ultraturrax treatment on ice. Crude membranes were collected by centrifugation at 30,000 × g for 30 min at 4°C and further homogenized by repeated passages through increasingly smaller cannulas. The membranes were layered on top of a stepwise gradient consisting of 3 ml each of 20, 45, 35, 25, and 0.5 (w/v) sucrose in buffer A and centrifuged at 100,000 × g for 90 min at 4°C in a swing-out rotor. The membrane fractions were collected, diluted (1:4) in buffer A, centrifuged (100,000 × g, 20 min, 4°C), and washed with buffer A including 250 mM NaCl and protease inhibitors. The membranes were washed with buffer A (without NaCl), centrifuged, resuspended in the same buffer, and stored at −80°C.

Measurement of Changes in Intracellular Free Ca2+—Intracellular free Ca2+ concentrations in HF-15 cells were determined by fura-2/AM as described previously (6) with minor modifications. Confluent HF-15 cells grown on 10-mm diameter coverslips were washed twice with minimum essential medium (MEM) 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 de-esterification of fura-2/AM. To monitor the changes in [Ca2+], the coverslips were mounted in a holder at an angle of 45° and placed into a thermostatted cuvette, and the fluorescence at 510 nm was measured. The excitation wavelength alternated between 340 and 380 nm in intervals of 600 ms. Changes in [Ca2+]i are given as the ratio of intensities at 340 and 380 nm, respectively.

1S Labeling and Immunoprecipitation—Confluent cells on six-well plates were washed twice with sulfur-free HEPES-buffered DMEM, incubated for 30 min at 37°C, and washed three times with 30 ml of 1% (w/v) bovine serum albumin, 0.1 mM 1,10-phenanthroline, aprotinin, leupeptin, and pepstatin A) with 100 mM NaOH. To measure the recovery of binding, the cells were treated with 5 μM [3H]bradykinin (19). After washing three times with 50 mM Tris, pH 7.5, 150 mM NaCl (Tris-buffered saline), cells were scraped into 1 ml of ice-cold lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and protease inhibitors (0.1 mM Pefabloc SC®, 10 μg/ml each 1,10-phenanthroline, aprotinin, leupeptin, and pepstatin A). Solubilization was carried out for 45 min at 4°C with gentle rocking. The lysates were centrifuged for 10 min at 13,000 rpm, and the supernatants were preclreated with 50 μl of a Staphylococcus aureus cell suspension (Pansorbin®). To immunoprecipitate the B2 receptor, 5 μl of antiserum AS346 diluted in 100 μl of 5% (w/v) bovine serum albumin solution in lysis buffer was added to the lysate and the resultant mixture was incubated for 15 min at room temperature. Fifty μl of Pansorbin® was added; the suspension was incubated for 10 min at room temperature; and the precipitate was recovered by centrifugation for 2 min at 6000 rpm. The resultant precipitates were washed three times with lysis buffer (see above), followed by a single wash with distilled water. To each precipitate was added 25 μl of SDS sample buffer (20), followed by incubation for 15 min at 45°C. The proteins were resolved by 10% polyacrylamide gel electrophoresis (PAGE) in the presence of 5 μl urea. After fixation with 20% w/v trichloroacetic acid for 20 min, gels were washed several times with water and subjected to fluorography using 15% (w/v) sodium salicylate as the fluorophor. For control, antisera were preabsorbed with their corresponding antigens coupled to a solid matrix (Affi-Gel 10, Bio-Rad) for 90 min at room temperature.

Receptor Phosphorylation—Confluent cells on 6-well plates were washed twice with phosphate-free HEPES-buffered DMEM, incubated for 1 h at 37°C in the same medium, and labeled with 0.25 μCi/ml [32P]orthophosphate for 12 h. The cells were exposed for varying time periods to varying concentrations of bradykinin at 37°C, washed three
times with Tris-buffered saline at 37 °C, and scapped into 1 ml of ice-cold lysis buffer containing inhibitors for proteases (see above) and for phosphatases (50 mM NaF, 25 mM sodium pyrophosphate, 1 mM sodium orthovanadate). Solubilization and immunoprecipitation were carried out as detailed above. The resultant precipitates were analyzed by 10% SDS-PAGE in the presence of 5 μurea and by autoradiography. For quantitative analyses, the bands corresponding to the receptor protein were excised from the gel, and their radioactivity was measured in a β-counter.

Limited Trypsin Digest—[^32P]-Labeled cells were stimulated with 1 μM bradykinin for 5 min. The medium was removed; the cells were washed twice with 6.5 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 150 mM NaCl, pH 7.4 (phosphate-buffered saline); and the surface proteins were partially digested with 0.025% (w/v) trypsin, 0.5 mM EDTA in phosphate-buffered saline for 20 min at 37 °C. As a control, the cells were incubated with phosphate-buffered saline alone under otherwise identical conditions. The reaction was stopped by applying 0.025% soybean trypsin inhibitor and 2.5 mM Pefabloc SC® in lysis buffer. Immunoprecipitations with the domain-specific antibodies anti-LHK10 (directed to ID1), anti-DRY16 (ID2), anti-RNN16 (ID3), and anti-CRS36 (ID4) were carried out as described above. The resultant B2 receptor fragments were analyzed by 15% SDS-PAGE and autoradiography.

**TABLE I**

| Designation  | Sequence                        | Positions  | Species | Antibserum* |
|--------------|---------------------------------|------------|---------|-------------|
| LHK10        | LHKTNCVTAE                      | 89–98      | Rat     | Anti-ID1 (AS280) |
| DRY16        | DRYLALKTSMGMRM                  | 159–174    | Rat/human | Anti-ID2 (AS277) |
| RNN16        | RNNMKKREVQTEKK                  | 257–272    | Rat     | Anti-ID3 (AS387) |
| CRS36        | CSHPQHMSMTGTLATISVERQHKLDWAGSIQ | 361–395    | Human   | Anti-ID4 (AS346) |

*Peptides are identified by their first three amino-terminal residues using the one-letter code, followed by the total number of residues constituting the peptide.

**RESULTS**

Production of Precipitating Antiserum to B2 Receptor—Immunization of rabbits was carried out with the synthetic peptide CRS36, derived from the carboxyl-terminal domain ID4 of the human B2 receptor (Table I). The titer of specific antipeptide antibodies was followed by the indirect enzyme-linked immunosorbent assay (data not shown). The cross-reactivity of antisemur AS346 with the human B2 receptor was demonstrated by Western blotting of the receptor recombinantly expressed in SF9 and COS-1 cells (data not shown). Immunoprecipitation of[^32P]-labeled COS-1 cells transfected with the human B2 receptor cDNA followed by reducing SDS-PAGE and autoradiography revealed a major band of 69 ± 5 kDa and an additional band of 52 ± 5 kDa (Fig. 1, lane 2). Minor bands of 29–31 kDa were also visible (lane 2), which may reflect proteolytic degradation products of the B2 receptor. The observed apparent molecular masses of 52 and 69 kDa match well with the previously reported masses of 47 and 69 kDa for the B2 receptor kinase (22) and of 1 μM bradykinin for 30°C. The reaction was stopped by addition of 1 μl of lysis buffer, and the B2 receptor was immunoprecipitated and analyzed as described above.

**Phosphoamino Acid Analysis**—The immunoprecipitated[^32P]-labeled B2 receptor was subjected to SDS-PAGE, and the corresponding band of 69 kDa was cut from the unixed dried gel and extracted for 12 h at room temperature with 50 mM ammonium bicitrate, pH 7.5, containing 0.1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. The extracted protein was precipitated with 20% (v/v) trichloroacetic acid and centrifuged. The pelleted protein was dried and hydrolyzed in 5.6 M HCl for 0.1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. The extracted protein was partially digested with 0.025% (w/v) trypsin, 0.5 mM EDTA in phosphate-buffered saline alone under otherwise identical conditions. The reaction was stopped by applying 0.025% soybean trypsin inhibitor and 2.5 mM Pefabloc SC® in lysis buffer. Immunoprecipitations with the domain-specific antibodies anti-LHK10 (directed to ID1), anti-DRY16 (ID2), anti-RNN16 (ID3), and anti-CRS36 (ID4) were carried out as described above. The resultant B2 receptor fragments were analyzed by 15% SDS-PAGE and autoradiography.

**FIG. 1. Specificity of the antiserum to the bradykinin B2 receptor.** COS-1 cells were transfected with a plasmid containing the human B2 cDNA (lanes 2–4) or with the same vector lacking the B2 insert (lane 1). Following metabolic[^32P]methionine labeling, the cells were lysed, and the B2 receptor was immunoprecipitated with antisemur AS346 (anti-CRS36 antisemur (α-CRS); lanes 1 and 2), the corresponding preimmune serum (preim; lane 3), or antisemur AS346 preadsorbed on an Affi-Gel 10 column of the cognate immunogen, peptide CRS36 (pread; lane 4). Immunoprecipitates were analyzed by reducing 10% SDS-PAGE and fluorography. Relative molecular masses (in kilodaltons (K)) of standard proteins are indicated on the left, and relative sizes of the immunoprecipitated bands are given on the right. The receptor protein enriched from human fibroblasts and of ~70 kDa for the same receptor previously cross-linked to a radiolabeled ligand (19). This conclusion is corroborated by the finding that immunoprecipitation of mock-transfected cells that lack an endogenous B2 receptor produced only background staining (lane 1). The specificity of antisemur AS346 was further documented by the application of the corresponding preimmune serum, which failed to give a specific response (lane 3). Furthermore, preadsorption of AS346 on a CRS36-Affi-Gel 10 column completely prevented the precipitation of the radiolabeled proteins, thus underlining the specificity of the antisemur (lane 4). Similar band patterns were obtained when lysates of[^35S]-labeled HF-15 cells were immunoprecipitated with AS346 (data not shown).

**Ligand-induced Phosphorylation of Human B2 Receptor in HF-15 Cells**—We applied antisemur AS346 to follow the ligand-induced phosphorylation of the B2 receptor endogenously expressed by the human foreskin fibroblast cell line. HF-15 cells loaded with[^32P]orthophosphate were challenged with 1 μM bradykinin or buffer alone, harvested, lysed, immunoprecipitated with AS346, and subjected to SDS-PAGE. The application of buffer alone failed to produce significant signals indicative of phosphorylated proteins (Fig. 2A, lane 1). Stimulation with bradykinin revealed two major phosphoproteins of 69 and 52 kDa (lane 2). Quantitative analysis of the incorporated radiolabel indicated that bradykinin increased the incorporation of[^32P]phosphate into the 52/69-kDa bands by a
Bradykinin B2 Receptor Phosphorylation

32369

Fig. 2. Bradykinin-induced phosphorylation of the human B2 receptor. A, HF-15 cells were radiolabeled with [32P]orthophosphate and incubated for 15 min with vehicle (lane 1), with 1 μM bradykinin (Bk; lanes 2, 4, and 5), or with a mixture of 1 μM bradykinin and a 1 μM concentration of the B2 antagonist HOE140 (HOE; lane 3). The cells were lysed, and immunoprecipitations were performed with antiserum AS346 (lanes 1–3), preimmune serum (preim; lane 4), or antiserum AS346 preabsorbed on peptide CRS36-Affigel-Gel (pread; lane 5). Immunoprecipitates were analyzed by reducing 10% SDS-PAGE and autoradiography. B, shown is [32P]phosphate incorporation after a 15-min incubation with buffer alone (lane 1) or with 1 μM bradykinin (lane 2), kallidin (lane 3), [Lys6,Hyp3]bradykinin (lane 4), des-Arg4-bradykinin (lane 5), des-Arg9-bradykinin (lane 6), T-kinin (lane 7), ornithokinin (lane 8), NPC567 (lane 9), or HOE140 (lane 10). Relative molecular masses (in kilodaltons [K]) of standard proteins (on the left) and of receptor bands (on the right) are given.

factor of up to 6 over the basal level (cf. lanes 1 and 2). Ligand-induced phosphorylation was completely prevented by co-administration of the B2 receptor antagonist HOE140 (1 μM), demonstrating that the observed protein phosphorylation is B2 receptor-mediated (lane 3). Bands of varying intensity were seen at the top of the gel (lanes 1–5). They likely represent nonspecific aggregation product(s) unable to penetrate the matrix. A band at the front of the gel that was found in the absence or presence of specific antibodies likely represents 32P-labeled nucleotides, inorganic phosphate, and/or phospholipids (not shown in Fig. 2).

To further address the antigen specificity of the immunoprecipitation, we employed the preimmune serum from the same rabbit (Fig. 2A, lane 4) and the corresponding immune serum that had been preincubated with peptide CRS36 from the carboxyl-terminal domain of the B2 receptor that is not shared by any other known protein sequence (lane 5). Under these conditions, no specifically labeled bands were observed. Hence, the 69-kDa band likely represents the authentic human bradykinin B2 receptor, whereas the 52-kDa band might be due to a degradation product of the receptor or could reflect alternative initiation or splicing of the B2 pre-mRNA (24). We have not further addressed these latter possibilities.

Because antiserum AS346 was raised against the carboxyl-terminal portion of the B2 receptor that contains multiple potential phosphorylation sites, we tested whether the antiserum precipitates the phosphorylated and non-phosphorylated forms of the B2 receptor to equivalent degrees. The amount of immunoprecipitated 35S-labeled B2 receptor from fibroblasts did not significantly differ in the absence or presence of 1 μM bradykinin (data not shown), suggesting that the ligand-induced phosphorylation of the B2 receptor does not interfere with its immunoprecipitation.

Specificity of Receptor Agonists Inducing B2 Receptor Phosphorylation—We tested the specificity of phosphorylation with respect to ligands of the B2 receptor other than bradykinin (Fig. 2B, lane 2). Stimulation with B2 receptor agonists such as kallidin (Lysylbradykinin) (lane 3), [Lys6,Hyp3]bradykinin (lane 4), and rat T-kinin (isoleucylserylbradykinin) (lane 7) also affected phosphorylation of the receptor protein, although to varying degrees. Unlike rat T-kinin, the avian kinin (ornithokinin) ([Thr4,Leu7]bradykinin) failed to produce phosphorylation of the B2 receptor (lane 2). Likewise, HOE140 (lane 3) also failed to trigger B2 receptor phosphorylation. Two representative B2 receptor antagonists, NPC567 (lane 9) and HOE140 (lane 10) at 1 μM concentrations did not induce phosphorylation of the receptor. Due to the low level of basal [32P]phosphate incorporation into the B2 receptor (lane 1), we were unable to assess the potential effect of HOE140 as an inverse agonist. The agonist-induced phosphorylation was also seen for the human B2 receptor recombinantly expressed in COS-1, Chinese hamster ovary, and SF9 cells, but not in the corresponding mock-transfected cells, and for the endogenous B2 receptor present in primary cultures of human umbilical vein endothelial cells (data not shown).

Is the ligand-induced modification of the bradykinin receptor due to homologous phosphorylation? To this end, we applied ligands that address overlapping signaling pathways as bradykinin (i.e. angiotensin II, endothelin-1, arginine vasopressin, ATP, and norepinephrine) or ligands known to be engaged in cross-talk with kinin-stimulated pathways (i.e. epidermal growth factor and insulin). None of these ligands applied at concentrations of 0.1–10 μM produced significant B2 receptor phosphorylation (data not shown). Likewise, incubation of HF-15 cells with these agents prior to bradykinin challenge (0.01–1 μM) was without effect on the bradykinin-triggered B2 receptor phosphorylation (data not shown). Taken together, these findings suggest that the B2 receptor is phosphorylated in response to a cognate agonist by homologous phosphorylation in varying cellular contexts.

Phosphoamino Acid Analysis and Identification of Phosphorylated Domain—The cDNA sequence of the human B2 receptor predicts, for its intracellular domains, several potential phosphorylation sites that conform to the consensus sequences of various protein kinases. To identify the type of phosphorylated amino acid(s), we stimulated HF-15 cells with 1 μM bradykinin, immunoprecipitated the phosphorylated receptor from the solubilized membrane fractions, and purified the 32P-labeled receptor by preparative SDS-PAGE. The relevant band of 69 kDa was cut out, eluted, hydrolyzed in 5.6 N HCl, and subjected to two-dimensional phosphoamino acid analysis (Fig. 3A). The corresponding autoradiogram indicates that the B2 receptor is phosphorylated on serine residues (major) and also on threonine residues (minor), but not on tyrosine residues.

To localize phosphorylation sites within the receptor structure, we stimulated radiolabeled HF-15 cells with bradykinin and treated the intact cells with trypsin. The human B2 receptor sequence contains six potential trypsin cleavage sites of (Arg/Lys)-Xaa in its predicted extracellular domains (E-Ds), i.e. one site each in ED1 and ED2 and two sites each in ED3 and ED4. Limited proteolysis at these sites should generate a set of fragments that expose the intact intracellular domains connected to the predicted transmembrane regions and varying portions of the extracellular domains. Following trypsinization,
Bradykinin B2 Receptor Phosphorylation

A. *32P*-labeled immunoprecipitates from HF-15 cells were resolved by SDS-PAGE. The band corresponding to the B2 receptor (69 kDa) was excised from the unfixed dried gels, extracted, and hydrolyzed in 5.6 n HCl. Phosphorylated amino acids were resolved by two-dimensional thin-layer electrophoresis at the pH values indicated and identified by a set of standard phosphoamino acids. B, *32P*-labeled HF-15 cells were stimulated with 1 μM bradykinin for 10 min, and the intact cells were subjected to limited proteolysis by 0.025% (w/v) trypsin for 20 min. Following inactivation of the proteinase by a molar excess of soybean trypsin inhibitor, the cells were lysed, and the resultant receptor fragments were immunoprecipitated by domain-specific anti-peptide antibodies (α-ID1 to α-ID4) directed to various intracellular domains (lanes 1–4). The immunoprecipitates were analyzed by reducing 15% SDS-PAGE and autoradiography. As a control, intact receptor was applied (lane 5). Relative molecular masses (in kilodaltons (K)) of standard proteins (on the left) and of receptor bands (on the right) are given.

the cells were lysed; membrane proteins were solubilized in the presence of trypsin inhibitors; and receptor fragments were immunoprecipitated by antibodies to the various intracellular domains of the B2 receptor, ID1–ID4 (Table I). A single antisera, anti-CR536 to ID4 (AS346), brought down two phosphorylated fragments of 6 and 8 kDa, which correspond to ID4 attached to the seventh transmembrane region and short fragment(s) of ED4 (Fig. 3B, lane 4). For comparison, the corresponding precipitation with the phosphorylated receptor from non-trypsinized cells is shown (lane 5). A strong band of 5 kDa that is found in the presence or absence of antibodies likely represents a nonspecific band at the front of the gel (see above). Antisera to peptides derived from the other intracellular domains failed to precipitate radiolabeled B2 receptor fragments (lanes 1–3). Unlike AS346, these anti-peptide antibodies are not suitable for immunoprecipitation of the intact B2 receptor; however, they efficiently precipitate *35S*-labeled fragments of ≤35 kDa from the partial tryptic digest of the receptor (data not shown). None of these antisera precipitated phosphorylated fragment(s) in the range of 7–15 kDa (lanes 1–3), as would be expected for the corresponding phosphorylated fragments derived from ID1–ID3. The possibility remains that phosphorylation of their cognate epitopes abrogates antibody binding. In all, we conclude that the agonist-dependent phosphorylation of the bradykinin B2 receptor occurs at multiple sites, and at least a fraction thereof is located in its carboxyl-terminal domain, ID4.

Concentration Dependence and Time Course of Phosphorylation/Dephosphorylation—To study the ligand-induced phosphorylation in more detail, we applied varying concentrations of bradykinin (0.1 nM to 10 μM) under otherwise identical conditions for 5 min at 37 °C (Fig. 4A). Quantitative analysis of the *32P* incorporation into the 69-kDa band revealed a concentration dependence with a threshold of ~1 nM, which is close to the apparent Kd of 2 nM for bradykinin. Phosphorylation plateaus at ≥1 μM bradykinin (Fig. 4B). The apparent EC50 was 33 ± 17 nM bradykinin (eight independent determinations). At maximum, the total increase over basal phosphorylation was >4-fold; we chose the corresponding bradykinin concentration (1 μM) for most of the subsequent experiments.

To follow the time course of B2 receptor phosphorylation, we added bradykinin at a final concentration of 1 μM to the medium of HF-15 cells and analyzed aliquots of them at 0.5, 1, 2.5, 5, and 10 min after the challenge (Fig. 5A). Thirty s after bradykinin stimulation, a clear phosphorylation signal was seen, indicating that the phosphorylation of the B2 receptor is an immediate response to bradykinin exposure. The intensity of receptor phosphorylation peaked at 5 min (Fig. 5A) and rapidly declined after prolonged incubation (data not shown). The ratio of the phosphorylated bands at 69 and 52 kDa was almost constant over the entire observation period, suggesting that phosphorylation of the two proteins occurs simultaneously. Under the conditions of our experiments, we have not observed a conversion of the 69-kDa band to the 52-kDa band or vice versa. In a second experiment, we investigated the time course of B2 receptor dephosphorylation following a single pulse of bradykinin. To this end, HF-15 cells were stimulated
with 0.1 μM bradykinin for 5 min, and then the cells were rapidly washed four times with prewarmed culture medium. The resultant phosphorylation pattern was analyzed at varying time points elapsed after the end of challenge (Fig. 5B). Phosphorylation was strongest immediately after removal of the ligand (t = 0) and rapidly declined thereafter (t = 5 min) almost to control levels (t = 15–60 min). Taken together, our findings demonstrate that the ligand-induced phosphorylation of the B2 receptor is concentration-dependent, rapid, and reversible.

Resensitization of B2 Receptor following Bradykinin Challenge—We wondered whether the kinetics of the ligand-induced phosphorylation/dephosphorylation paralleled that of receptor desensitization/resensitization. To this end, we stimulated HF-15 cells with bradykinin, washed the cells extensively to remove the ligand, incubated the cells in a bradykinin-free medium, and followed the time course of receptor resensitization by measuring the ligand-induced change in [Ca^{2+}]. At 2.5 min after the initial challenge, the cells were unresponsive to a second stimulation with bradykinin (Fig. 6A). After 5 min, a moderate response in [Ca^{2+}] was seen; after 10 min, the response was largely although not completely restored. Full recovery of the response was seen 15–20 min after the primary challenge (data not shown).

In a parallel set of experiments, we followed the apparent number of binding sites for the radioligand, [3H]bradykinin. The number of binding sites was set at 100% before challenge (Fig. 6B). After 2.5 min, the apparent number of binding sites dropped to 34 ± 5%. The recovery was slow, with 52 ± 16% after 30 min and 88 ± 9% after 60 min, indicating that a fraction of the receptors were not available on the surface of the fibroblasts. The lack of full restoration of receptor number on the fibroblast surface might reflect partial down-regulation of the receptor after ligand challenge. These findings are compatible with the notion that ligand-induced phosphorylation might spur desensitization and subsequently down-regulation of the B2 receptor.

Cellular Kinases Involved in B2 Receptor Phosphorylation—In an attempt to identify the kinase(s) involved in B2 receptor phosphorylation, we investigated the effects of various kinase activators and inhibitors on the agonist-induced B2 receptor phosphorylation. Activation of protein kinase C by 2 μM PMA in the absence of a ligand resulted in a minor although significant incorporation of [32P]phosphate into the B2 protein (Fig. 7A, lane 3). The PMA-induced phosphorylation was ~30% of the bradykinin-induced phosphorylation (cf. lanes 2 and 3). Following long-term pretreatment of the cells with 0.2 μM PMA and thus protein kinase C down-regulation, the PMA-induced phosphorylation of the B2 receptor was drastically reduced to control levels (Fig. 7B, lanes 1 and 3), whereas the bradykinin-mediated phosphorylation was unaffected (lane 2). Treatment of the cells with a 10 μM concentration of the ionophore A23187, which increases [Ca^{2+}], and thereby activates Ca^{2+}-dependent kinases (Fig. 7, A and B, lane 4), failed to produce significant B2 receptor phosphorylation. Similar results were obtained with 50 μM forskolin (Fig. 7, A and B, lane 5), which elevates intracellular cAMP levels and activates cAMP-dependent protein kinase(s). Under the same experimental conditions, we were unable to find a significant effect of PMA, protein kinase C down-regulation, or forskolin on bradykinin-provoked Ca^{2+} transients (data not shown). Other activators of intracellular protein kinases of the A, C, or G type such as ionomycin, thapsigargin, dibutyryl cGMP, or (S,R)-cAMP-S applied at concentrations of 1–10 μM failed to produce significant ligand-independent phosphorylation of the B2 receptor (data not shown). Likewise, preincubation of the fibroblasts with inhibitors of protein kinases such as calphostin C, bisindolylmaleimide (GF109203), staurosporine, H-8, (R)-cAMP-S, KN-62, or KT5823 at concentrations of 1–10 μM failed to affect significantly the bradykinin-induced phosphorylation of the B2 receptor (data not shown). These findings indicate that the bradykinin-induced receptor phosphorylation in human fibroblasts is likely to be independent of protein kinases of the A, C, G, or Ca^{2+}/calmodulin-dependent type.

In Vitro Phosphorylation of Human B2 Receptor by βARK1—To address the possibility that the ligand-induced phosphorylation of the bradykinin B2 receptor is mediated by a G protein-coupled receptor kinase, we performed in vitro phosphorylation experiments using the native B2 receptor from human fibroblasts and a G protein-coupled receptor kinase (βARK1) that had been purified from baculovirus-infected Sf9 cells. By sucrose density gradient centrifugation, we prepared HF-15 cell membranes that had a [3H]bradykinin binding activity of 1.6–3.0 pmol/mg of protein. In vitro phosphorylation of the B2 receptor present in the membrane fraction was carried...
Bradykinin B2 Receptor Phosphorylation

**Fig. 7. Effect of second messenger-dependent kinases on B2 receptor phosphorylation.** HF-15 cells were metabolically labeled with [γ-32P]ATP and exposed for 15 min at 37 °C to 1 μM bradykinin (Bk; lane 2), 2 μM PMA (lane 3), 10 μM A23187 (lane 4), or 50 μM forskolin (For; lane 5). As a control, buffer was applied (lane 1). A, cells were without pretreatment. B, cells were incubated with 0.2 μM PMA for 20 h to down-regulate the protein kinase C pathway. Following cell lysis, the immunoprecipitated proteins were analyzed by reducing 10% SDS-PAGE and autoradiography. Relative molecular masses (in kilodaltons (K)) of receptor bands are given on the left. C, to quantify the incorporated radioactivity, the receptor bands were excised and measured in a β-counter. Means ± S.D. from eight independent experiments are presented. The control was done with buffer alone.

out as described (21). The reaction buffer contained 1 μCi of [γ-32P]ATP in the absence or presence of 0.5 pmol of B2 receptor, 50 nM βARK1, or 1 μM bradykinin. Following immunoprecipitation and SDS-PAGE, a phosphorylated band of 69 ± 5 kDa was visible in the corresponding autoradiogram (Fig. 8, lane 5). Little or no staining was seen in the controls in which the B2 receptor (lane 1), βARK1 (lanes 2 and 3), or bradykinin (lanes 1, 2, and 4) had been omitted. We were unable to see a significant effect on the βARK1-catalyzed B2 receptor phosphorylation when Gp subunits were added to the reaction mixture (data not shown). Collectively, these results suggest that βARK1 is a candidate G protein-coupled receptor kinase mediating the ligand-induced phosphorylation of the B2 receptor.

**Identification of Phosphatases Involved in B2 Receptor De-phosphorylation**—We wondered about the nature of the enzyme(s) and mechanisms involved in the rapid dephosphorylation of the bradykinin receptor after termination of agonist exposure. To this end, we performed pulse-chase experiments in which 100 nM bradykinin was applied to human HF-15 cells for 5 min in the presence of [γ-32P]ATP, followed by removal of the medium, extensive washes to remove trace amounts of the ligand, and incubation of the cells with fresh medium at t = 0 min. This protocol resulted in a rapid dephosphorylation of the receptor (cf. Fig. 5B). The continuous presence of 0.1 μM calyculin A, a potent inhibitor of phosphatases 1 and 2A, during the chase period drastically reduced the rate of receptor dephosphorylation (Fig. 9A). Under these conditions, ~80% of the B2 receptor phosphorylation persisted for at least 240 min (Fig. 9C). Note that B2 receptor phosphorylation was markedly increased by calyculin A even under control conditions, i.e., in the absence of exogenous bradykinin (Fig. 9A, first lane). This finding might indicate a ligand-independent phosphorylation due to spontaneous receptor activation, although we have not ruled out the possibility of endogenous production of small amounts of bradykinin by HF-15 cells.

Calyculin A, an inhibitor of receptor internalization (25), considerably slowed down receptor dephosphorylation, although to a lesser degree than calyculin A (Fig. 9B). In the presence of 0.25 mg/ml calyculin A, which inhibits internalization of bradykinin-binding sites by ~80% (data not shown), half-maximal dephosphorylation was seen after 30–60 min and complete dephosphorylation after 180 min following removal of the ligand. Our combined data indicate that internalization might be a prerequisite for dephosphorylation of the B2 receptor by phosphatases 1 and/or 2A.

**DISCUSSION**

Receptor-mediated signaling in eukaryotic cells requires a finely tuned balance between activated and inactivated receptor states (26). Multiple mechanisms regulate G protein-coupled receptor activity and availability, e.g., ligand-induced phosphorylation and palmitoylation, sequestration and internalization, and down-regulation and recycling of the receptors (27, 28). Deletion and mutagenesis studies have stressed the importance of phosphorylation in the desensitization of adrenergic and muscarinicergic receptors (29–31). The development of efficient purification procedures has allowed the in vitro study of the ligand-induced phosphorylation of these receptors by reconstitution assays using the purified proteins (22, 32). In the case of peptide hormone receptors, their low copy number on native cells has hampered the analysis of ligand-induced receptor phosphorylation, although a few examples of such work exist (33, 34). Most of these studies used recombinantly expressed receptors; however, quantitative as well as qualitative discrepancies in the phosphorylation of endogenous versus recombinant receptors may exist (35). In this situation, we sought to study the ligand-induced phosphorylation of a prototypic peptide hormone receptor in its genuine cellular context by a direct immunoprecipitation technique.

Our data indicate that the endogenously expressed bradyki-
The ligand-induced phosphorylation of the B2 receptor is not strictly homologous under the conditions of our experiments. This finding does not rule out the possibility that heterologous desensitization of kinin receptors may exist, e.g., further downstream on the level of the inositol 1,4,5-trisphosphate receptor (44).

Our phosphoamino acid analyses of the B2 receptor prove that Ser residues are the prime targets for ligand-induced phosphorylation, whereas Thr is less frequently modified. We were unable to detect Tyr phosphorylation both by phosphoamino acid analysis and by Western blotting using a monoclonal antibody to phosphotyrosine, PY20 (data not shown). This latter finding contrasts with a previous report in which phosphorylation of the B2 receptor from WI-38 human lung fibroblasts was shown to occur on Tyr residue(s) by the PY20 antibody (45). Because the cells were not stimulated by bradykinin, the distinct possibility remains that Tyr phosphorylation was heterologously induced by an unknown ligand. Our preliminary mapping study indicates that the carboxyl-terminal domain ID4 of the B2 receptor is phosphorylated by receptor kinase(s). Considering the lack of Ser residues in ID3 and the absence of G protein-coupled receptor kinase consensus sites in ID1 and ID2, one might speculate that the cytoplasmic tail of the receptor is the major attack site for kinase(s). This hypothesis is in line with novel findings for the β2-adrenergic receptor, where in vitro phosphorylation sites of βARK1 and GRK5 (G protein-coupled receptor kinase) were mapped to ID4 (46). We have not made further attempts to precisely map the phosphorylation sites; however, site-directed mutagenesis in combination with Edman degradation of isolated receptor fragments should eventually allow the identification of relevant phosphorylation site(s) in the B2 receptor. The lack of sufficient amounts of highly purified receptor has prevented the quantitative analysis of the stoichiometry of B2 receptor phosphorylation, even under in vitro conditions where we used a crude membrane fraction of the receptor.

The ligand-induced phosphorylation of the B2 receptor is transient, even in the continuous presence of bradykinin. Hence, the action of a putative B2 receptor kinase(s) is rapidly counteracted by protein phosphatase(s). Our inhibition studies point to a role of phosphatases 1 and/or 2A, although the low discriminating power of phosphatase inhibitors and the well discriminate among the various possibilities.
documented problems with whole cell dephosphorylation studies (47) do not allow further conclusions as to the nature of the responsible enzyme(s). Another obvious candidate for such an activity is the recently described G protein-coupled receptor phosphatase 2A (48). Our findings that calyculin A does not interfere with the internalization of receptor-ligand complexes (data not shown), whereas concanavalin A, an inhibitor of receptor internalization (25), considerably delays the dephosphorylation of the B2 receptor (49), point to a role of protein kinase C-dependent phosphorylation in vitro and in human leukocytes.

An unanticipated finding of this study is the PMA-induced ligand-independent phosphorylation of the B2 receptor. Because PMA is a major activator of protein kinases C, our results suggest a role of these enzymes in the regulation of the "basal" phosphorylation of the B2 receptor. On the other hand, the ligand-induced phosphorylation of the B2 receptor is totally independent of protein kinase C, thus ruling out the possibility that this enzyme class is a mediator of homologous B2 receptor phosphorylation. The possibility remains that protein kinase C acts at the level of the competent receptor kinase. Chuang et al. (50) have shown that protein kinase C has the capacity to phosphorylate and thus to activate βARK1 in vitro and in human leukocytes. More recent studies by Winstel et al. (51) point to a role of protein kinase C-dependent phosphorylation in the activation and translocation of βARK1 to the cell membrane. The precise molecular machinery that regulates the reversible phosphorylation of kinin receptors by homologous and heterologous signals remains to be explored. Such studies into the molecular mechanisms modulating kinin receptor activity might also have important therapeutic implications with regard to the anti-hypertensive effects of angiotensin-convertin- converting enzyme/kinase II inhibitors.

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