Bradykinin-induced Internalization of the Human B2 Receptor Requires Phosphorylation of Three Serine and Two Threonine Residues at Its Carboxyl Tail*

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Anne Pizard‡‡, Andree Blaukat¶¶, Werner Müller-Esterl¶¶, François Alhenc-Gelas‡, and Rabary M. Rajerison‡

From ‡INSERM Unité 367, 17 rue du Fer à Moulin 75005 Paris, France and the ¶Institute of Physiological Chemistry and Pathobiology, Johannes Gutenberg University at Mainz, Duesbergweg 6, D-55099 Mainz, Federal Republic of Germany

The binding of bradykinin (BK) to B2 receptor triggers the internalization of the agonist-receptor complex. To investigate the mechanisms and the receptor structures involved in this fundamental process of receptor regulation, the human B2 receptor was mutated within its cytoplasmic tail by complementary strategies of truncation, deletion, and amino acid substitution. Ligand binding, signal transduction, internalization as well as phosphorylation were studied for the mutated receptors expressed in COS, CHO, and HEK 293 cells. Truncation of 44 out of 55 amino acid residues of the receptor's cytoplasmic tail corresponding to positions 321–364 did not alter the kinetics of BK binding and the receptor coupling to phospholipase C and phospholipase A2. By contrast, truncations after positions 320 and 334, deletions within the segment covering positions 335–351, as well as alanine substitution of serine and threonine residues within segment 335–351 diminished the internalization capacity of the mutant receptors. Mutants with a markedly reduced internalization potential failed to produce BK-induced receptor phosphorylation suggesting that phosphorylation may be involved in receptor internalization. The mutagenesis approaches converged at the conclusion that three serines in positions 339, 346, and 348 and two threonines in positions 342 and 345, contained in a sequence segment that is highly conserved between species, have a critical role in the ligand-dependent internalization and phosphorylation of kinin receptors and can intervene in these processes in an alternative manner. However, mutants lacking these residues were still sensitive to dominant-negative forms of b-arrestin and dynamin, suggesting the existence of additional receptor structure(s) involved in the receptor sequestration through clathrin-coated vesicles.

to respond to agonists becomes altered after previous exposure to their ligands, a phenomenon referred to as desensitization. Desensitization can be a consequence of receptor endocytosis (1) and may also result from receptor phosphorylation that prevents further activation of G proteins (2, 3). Negative cooperativity in bradykinin (BK) binding due to receptor-receptor interaction in the cell membrane can also contribute to the desensitization of some receptors including the BK B2 receptor (4). For few GPCRs, internalization appears to be a prerequisite for resensitization prior to receptor recycling to the plasma membrane (5). Internalization is believed to involve clathrin-coated vesicles and/or caveolin-rich vesicles, and to result from an interaction of components of the endocytic machinery with specific motifs located in the cytoplasmic domains of the receptor (6). For many receptors, the carboxyl tail has been found to play a critical role in receptor internalization (2, 3, 7) but other critical motifs have been identified in the second or third intracellular loops (8, 9) as well as in the seventh transmembrane domain (10).

The molecular mechanisms that trigger internalization of the agonist-occupied receptor have been studied for only a few receptors. Phosphorylation has been implicated in the desensitization process uncoupling the receptor from G proteins, and in the initiation of internalization sequestering the receptor from the cell surface (2, 3). Indeed, mutations of potential phosphorylation sites of the GLP-1, muscarinic m3, and the cholecystokinin receptors have been shown to reduce their internalization (11–13). However, receptor internalization and desensitization are not always causally linked, e.g. mutations of certain GPCRs suppressed desensitization but not internalization (14, 15). Conversely mutations in some GPCRs altered internalization but not desensitization (10, 15). Clearly, the two processes can proceed independently although they may involve the same type of post-translational modifications such as phosphorylation.

Prototypical GPCRs that differ by their ligand-induced desensitization, phosphorylation, and internalization are the receptors for the vasoactive kinin peptides. Two subtypes of mammalian kinin receptors, B1 and B2, have been recognized so far (16–18). The B2 receptor is responsible for most of the physiological actions of BK including vasodilation (19) through activation of G proteins (20) that stimulate the activity of phospholipase C (PLC) and phospholipase A2 (PLA2) and increase the cytoplasmic calcium concentration, [Ca2+]; (21). More recently, other signaling pathways such as the mitogen-serum albumin; IP3, inositol phosphates; [Ca2+], intracellular Ca2+; CHO, Chinese hamster ovary; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.
activated protein (MAP) kinase pathway were found to be triggered via the B₂ receptor (22, 23). BK-induced B₂ receptor internalization has been reported in several cell systems including cultured human fibroblasts HF-15 (24, 25) and CHO-K1 cells transfected with the human B₂ receptor cDNA (4), and we have recently reported that in HF-15 cells BK induces the phosphorylation of serine and threonine residues located in the COOH terminus of the B₂ receptor (21). The time course of BK-stimulated phosphorylation paralleled the kinetics of desensitization/resensitization and of internalization/recycling of the receptor, suggesting that these phenomena may be interrelated (21).

The present work was aimed at studying the role of the COOH-terminal tail of the human renal B₂ bradykinin receptor (referred as wild-type, B₂wt) in BK binding, coupling to signal transduction pathways, internalization, and phosphorylation. For this purpose, we generated a series of human B₂ receptor mutants and transfected them into COS-7 cells, CHO-K1, and HEK 293 cells. Three complementary strategies were followed for mutagenesis, by generating mutants with truncated COOH-terminal tail, with mutants of deletion of internal regions of the COOH-terminal tail, and mutants with serine and/or threonine residue(s) replaced by alanine. This allowed us to demonstrate that a cluster of three serine and two threonine residues located in the center portion of the COOH-terminal tail region that becomes phosphorylated in response to BK is involved in the internalization of the B₂ receptor. Co-transfection of the B₂ wt and its mutants with β-arrestin and dynamin mutants documented a role of clathrin-coated pit pathway in receptor internalization.

**EXPERIMENTAL PROCEDURES**

**Construction of the Mutant Receptor cDNAs**—Mutant cDNAs were constructed by using the previously cloned B₂ wt cDNA placed under the control of cytomegalovirus promoter into the eukaryotic expression vector pcDNA3 (Invitrogen, Leek, Netherlands) (4) as a template in site-directed mutagenesis using the Transformer™ Site-directed Mutagenesis Kit (2nd version, CLONTECH, Palo Alto, CA). All mutations were used were: 5'-stop codons (underlined) terminating translation at positions 351, 334, 320, and 316, respectively, of the human B₂ receptor. Deletions substituting by Ala. The mutant S339A/T342A and 5'-GGAGGTGTACTAGGAGGTTG-3' for the mutant tR351, tI334, and tY320 lacked the terminal 13, 30, and 44 amino acid residues, respectively, of the human B₂ receptor. Deletions induced in which Ser339, Thr342, Thr345, Ser346, and Ser348 were simultaneously mutated for del[343–351]. The resulting mutants del[335–351], del[335–342], del[320–334], and del[316–320] were created by single nucletide substitutions (indicated by bold face in the sequences given below) at the appropriate sites so as to create stop codons (underlined) terminating translation at positions 351, 334, 320, and 316, respectively, of the protein sequence (numbering according to Hess et al. (17)). The nucletide sequences of the oligonucleotides primers used were: 5'-GGTCAGAACCGTAGATCCACAACCTCGC-3' for the mutant tR351, tI334, and tY320 were synthesized with 15 μg of each plasmid using Superfect Transfection Reagent (Qiagen, Courtaboeuf, France). For equilibrium studies and determination of receptor density or apparent affinity for [³H]BK, incubation was carried out at 4°C for 6 h in the presence of 1.25 × 10⁻¹⁰ to 2.5 × 10⁻⁸ M [³H]BK (CHO cells) or 10⁻¹⁰ to 10⁻⁷ M [³H]BK (CHO cells). The cells were extensively washed with HBSS and bound [³H]BK was determined in a liquid scintillation counter. Internalization of [³H]BK was studied by incubating the cells with 2 nM [³H]BK at 37°C for 2 to 70 min (4). Cells were washed with HBSS before cell surface-bound radioligand was separated from internalized radioactivity by an acidic washing step (27) with 0.2 M ascorbic acid, 0.5 M NaCl, pH 2.5. To study the role of protein kinase C (PKC) activity, cells were preincubated with 10⁻⁷ M phorbol 12-myristate 13-acetate (PMA) or 10⁻⁷ M staurosporine (Sigma, St-Qentin-Fallavier, France) for 30 min before and during the incubation with [³H]BK. To monitor the effect of phosphatase inhibitor, cells were incubated with 10⁻⁸ M okadaic acid (Sigma) 5 min prior to [³H]BK challenge, and internalization was followed for 10 min in the continuous presence of the phosphatase inhibitor. In some experiments, cells were transfected with hepatitis C virus non-structural protein 4A construct (kindly provided by F. Bessis, Institut Pasteur) and transiently co-transfected with 15 μg of each plasmid. Determination of specific binding, each assay included measurements of nonspecific binding in the presence of 1000-fold excess of unlabeled BK; nonspecific binding was determined according to the manufacturer’s instructions in three cells using BSA as the standard protein (Bio-Rad, Muenchen, Germany).

**Inositol Phosphate Production**—Cells were loaded for 18 h at 37°C with 3 μCi/ml myo-[³H]inositol (10–20 Ci/mmol, Amersham International) added to the culture medium. The cells were washed twice with HBSS, preincubated for 10 min at 37°C with 10 mM LiCl in HBSS containing 0.1% BSA and the protease inhibitors used for binding assay, and stimulated for 15 min with BK at varying concentrations (10⁻¹⁰ to 10⁻⁷ M). To study the role of PKC, cells were treated with PMA (10⁻⁷ M) or staurosporine (10⁻⁷ M) for 30 min before and during the incubation with BK. Reactions were terminated by addition of 3% (v/v) ice-cold perchloric acid and total [³H]inositol phosphate radioactivity was isolated using AG1-X8 anion exchange column chromatography (formate form, 100–200 mesh, Bio-Rad) after the radioactivity containing perchloric acid solution was neutralized with 5 M NaOH. For determination of radioactivity incorporated into the various compounds labeled with [³H]inositol.

**Measurement of Phospholipase A₂ Activity**—Cells were labeled with equilibrium with 1 μCi/ml [³H]Arachidonic acid (150–230 Ci/mmol, Amersham International) for 16 h. After washing steps to eliminate...
unbound radiolabel (4), phospholipase A2 activation experiments were performed at 37 °C for 10 min in HBSS containing 0.1% BSA, protease inhibitors, and the test compounds or vehicles. The medium radioactivity containing the released [3H]arachidonic acid plus derived [3H]-labeled metabolites was counted and expressed in percent of the total radioactive content plus cell-associated radioactivity.

**Generation of Mutant B2 Receptors**—To study the functional role of the COOH-terminal tail region corresponding to intracellular domain ID-4 of the B2wt we produced three truncation variants with progressive deletions of the COOH-terminal portion of the receptor. These truncation mutants are designated by “T” followed by the name (single letter code) and position of their carboxyl-terminal amino acid, i.e. T351, T334, and T320; they lack the COOH-terminal 14, 30, or 44 residues of the native receptor (Fig. 1). Three deletion mutants are designated by “del” followed by the positions delimiting the deleted region, i.e. del[335–351], del[335–342], and del[343–351] lacking 17, 8, and 9 residues, respectively, of the COOH-terminal tail region (Fig. 1). We also created 7 receptor mutants in which alanine (Ala) was substituted for serine (Ser) or threonine (Thr) residue(s), singly or combined (Table I). In the mutant B2AST we exchanged 5 Ser/Thr residues located in the center portion of the COOH-terminal domain, i.e. at positions 339, 342, 345, 346, and 348 (Fig. 1). We have also constructed 3 mutants where Ser/Thr residues have been replaced by Ala in other regions of the receptor, i.e. T237A, S316A, and S331A (not shown). The rationale for the design of the various mutants is given below.

**Expression and Characterization of Mutant B2 Receptors**—The mutant receptors were characterized for their ligand binding and signal transduction properties. Ligand binding was assessed by incubating transfected cells with a wide range of [3H]BK concentrations at 1.25 × 10^{-11} M to 2.5 × 10^{-8} M for COS-7 cells (Table I) and at 10^{-11} M to 10^{-7} M for CHO-K1 cells (Table II). The incubation was carried out for 6 h under equilibrium conditions at 4 °C to suppress internalization. In COS cells (Table I) the kinetics of [3H]BK binding displayed negative cooperativity as previously observed for the human B2wt in CHO-K1 cells (4). The receptor density determined by Scatchard analysis (31) was similar for the B2wt and mutant receptors except for tY320 and B2AST which were 10- and 5-fold less expressed, respectively. The kinetics of [3H]BK binding estimated from Hill analysis of the binding data (32) were similar for the various receptors since half-maximum binding (K_{D,50}) occurred within a narrow range (2.3 to 6.3 nM) of [3H]BK concentrations. Regarding the signal transduction properties, the basal IPs production was similar for B2wt and the mutant receptors demonstrating that none of the mutations resulted in a constitutive activation of PLC. All mutant receptors were able to trigger PLC activation in response to 10^{-11} to 10^{-7} M BK and responded to BK with maximum stimulation (4.1–8.5-fold) and EC_{50} values (0.2–0.8 nm) in the same order of magnitude as those observed with B2wt with the exception of tY320 which had a higher EC_{50} value (2.2 ± 0.9 nm).

Then, we choose to stably express in CHO-K1 cells the mutant tY320 bearing the most pronounced structural change and also the largest deleted mutant, del[335–351] lacking the 5 Ser and Thr residues. The B2wt CHO cell line was obtained as described previously (4). In these cells (Table II), tY320 also exhibited a higher EC_{50} value for PLC activation, a greater EC_{50} value for PLAc activation and a lower affinity for [3H]BK. By contrast, the mutant del[335–351] exhibited a slightly higher affinity for [3H]BK and exhibited a 50- and 50-fold lower EC_{50} value for PLC and PLAc activation, respectively, compared with B2wt (Table II). These data indicate that the COOH-terminal tail is not necessary for signal transduction but that its conformation influences the efficiency of BK binding and coupling to PLC and PLAc.

**Internalization of [3H]BK by Mutant B2 Receptors**—In COS-9 cells (Table I) the COSB2wt which were transfected with 15 μg of cDNA and challenged with 2 nM [3H]BK (Fig. 2), the internalization occurred with a t_{1/2} of 12 min at 37 °C and...
FIG. 1. Schematic representation of the COOH-terminal tail of B2wt and mutant receptors. Top, mutated amino acid residues are indicated by their position within the B2wt sequence; the numbering is according to Hess et al. (39). Ser and Thr residues which were replaced by Ala are marked by asterisks. Center, cytoplasmic tail truncations were performed at 3 sites to generate tR351, tI334, and tY320. Bottom, deletion of segments holding 17, 8, and 9 residues generated mutants del[335–351], del[335–342], and del[343–351], respectively; the deleted segments are identified by their positions (given in brackets) and marked by bold lines.

TABLE I

Bradykinin B2 Receptor Internalization and Phosphorylation

In each binding experiment, up to eight mutants were studied together with the B2wt as control. Cells were used 72 h after transfection, and incubated at 4 °C for 6 h with seven increasing concentrations of [3H]BK (from $1.25 \times 10^{-11}$ to $2.5 \times 10^{-8}$ M), before determination of specific binding (see “Experimental Procedures”). Data were first plotted using Scatchard coordinates. Given the curvilinear character of the plots obtained, binding values at the three highest [3H]BK concentrations were used to estimate the maximal binding capacity ($B_{\text{max}}$). All binding values were then plotted using Hill coordinates to estimate the [3H]BK concentration ($K_{\text{app}}$) corresponding to half-saturation of binding sites. In each PLC activation experiment out of the 14 performed, up to six mutants were studied together with the B2wt as control. Cells at 72 h after transfection were incubated at 37 °C for 15 min with $10^{-11}$ to $10^{-7}$ M BK and 10 mM LiCl, before determination of IPs production (see “Experimental Procedures”). For each mutant receptor, results are the mean ± S.E. of at least three independent experiments, each performed in triplicate.

| Receptors | [3H]BK binding | PLC activation |
|-----------|----------------|----------------|
|           | $K_{\text{app}}$ | $B_{\max}$ | % of total radioactivity | Basal activity | Maximal effect | EC$_{50}$ |
| B2wt      | 4.4 ± 0.5 | 5.5 ± 1.0 | 0.7 ± 0.1 | 4.2 ± 0.4 | 0.4 ± 0.1 |
| Truncation mutants | | | | | | |
| tR351     | 4.4 ± 0.1 | 9.5 ± 3.1 | 0.6 ± 0.1 | 5.3 ± 0.5 | 0.8 ± 0.2 |
| tI334     | 2.5 ± 0.3 | 4.0 ± 0.5 | 1.0 ± 0.1 | 5.1 ± 0.7 | 0.2 ± 0.1 |
| tY320     | 2.9 ± 1.0 | 0.8 ± 0.2 | 0.5 ± 0.1 | 4.1 ± 0.3 | 2.2 ± 0.9 |
| Deletion mutants | | | | | | |
| del[335–351] | 2.4 ± 0.2 | 6.1 ± 1.5 | 0.7 ± 0.1 | 5.5 ± 1.1 | 0.2 ± 0.1 |
| del[335–342] | 2.3 ± 0.3 | 4.9 ± 1.1 | 0.7 ± 0.1 | 5.5 ± 0.6 | 0.3 ± 0.1 |
| del[343–351] | 2.7 ± 0.2 | 6.3 ± 1.5 | 0.8 ± 0.1 | 5.6 ± 0.5 | 0.2 ± 0.1 |
| Ser/Thr point mutants | | | | | | |
| S339A     | 5.4 ± 0.9 | 7.6 ± 2.2 | 0.5 ± 0.1 | 8.5 ± 0.8 | 0.5 ± 0.3 |
| T342A     | 5.6 ± 0.7 | 5.5 ± 0.3 | 0.5 ± 0.1 | 6.2 ± 0.7 | 0.2 ± 0.1 |
| S346A     | 5.1 ± 0.2 | 5.8 ± 0.4 | 0.7 ± 0.1 | 5.1 ± 1.2 | 0.5 ± 0.1 |
| S339A/T342A | 3.7 ± 0.4 | 4.6 ± 4.6 | 0.7 ± 0.1 | 6.8 ± 2.1 | 0.2 ± 0.1 |
| T345A/S346A | 6.3 ± 1.5 | 9.1 ± 2.6 | 0.7 ± 0.1 | 6.9 ± 0.7 | 0.3 ± 0.1 |
| S339A/T345A/S346A | 4.3 ± 0.7 | 5.8 ± 1.5 | 0.8 ± 0.1 | 6.5 ± 2.1 | 0.4 ± 0.2 |
| B2ΔST     | 2.8 ± 0.2 | 1.8 ± 0.2 | 0.7 ± 0.2 | 5.2 ± 0.1 | 0.2 ± 0.1 |

* Ratio between maximal ($10^{-7}$ M BK) and basal IPs productions.
For each type of experiment mutants were studied together with the B2wt as control. CHO-K1 stable transfected cells were incubated overnight at 4 °C with 30 increasing concentrations of [3H]BK (from 10⁻¹¹ to 10⁻⁷ M) before determination of specific binding (see “Experimental Procedures”). Data were obtained as explained in the legend of Table I. In coupling experiments, cells were incubated at 37 °C with 10⁻¹¹ to 10⁻⁷ M BK for 10 min before measurement of PLA₂ activation and 15 min in presence of 10 mM LiCl before determination of IPs production (see “Experimental Procedures”). For each mutant receptor, results are the mean ± S.E. from at least three independent experiments.

| Receptors          | [3H]BK binding | PLC activation | PLA₂ activation |
|--------------------|----------------|----------------|-----------------|
|                    | Kᵦᵢₜ,app pmol [3H]BK bound/mg protein | Maximal effect° | EC₅₀  | Maximal effect° | EC₅₀  |
| B₂wt               | 22.7 ± 6.3     | 2.2 ± 0.3      | 39.3 ± 0.93     | 6.27 ± 0.09 | 0.92 ± 0.07 |
| Truncation mutant  |                |                |                 |            |           |
| tY320              | 294 ± 153      | 1.3 ± 0.3      | 25.5 ± 1.87     | 1.14 ± 0.43 | 1.47 ± 0.25 |
| Deletion mutant    |                |                |                 |            |           |
| del[335–351]       | 4.4 ± 1.1      | 6.5 ± 0.2      | 20.3 ± 0.80     | 0.024 ± 0.002 | 11.57 ± 1.95 | 0.018 ± 0.002 |

* Ratio between maximal (10⁻⁷ M BK) and basal IPs productions.

Fig. 2. [3H]BK internalization in COS-7 cells transfected with B2wt and mutant receptors. Cells (used 72 h after transfection) were incubated with 2 nM [3H]BK at 37 °C for the times indicated. Unbound radioactivity was removed at 4 °C before the cell surface-associated and the internalized radioactivities were separated and quantitated as described under “Experimental Procedures.” Specific binding was calculated as the difference of total binding and nonspecific binding in the presence of unlabeled BK (10⁻⁷ M). Internalized radioactivity represents the acid-resistant fraction (%) of the total specific binding. Results are given as the percentage of the control, i.e. B2wt internalization at 70 min representing 60.3 ± 2.5% of the [3H]BK bound (100% corresponding to 0.18 ± 0.01 pmol/mg of protein). For point mutations, the data for five representative mutants (out of 10) are presented. Values are means ± S.E. from at least three independent experiments each performed in duplicate. T₅₃³⁴–₃₅⁵A, T₃₄₅A/S₃₄₆A.

pmol/mg protein (not shown) with the remark that the internalization was more efficient (72.6 ± 1.5% (n = 7) of the bound radioactivity) in CHÓ than in COS cells. Regarding the effects of the receptor mutations, most of the data presented below were from COS-7 cells transfected with 15 μg of cDNA. In these cells, it was apparent that the serial truncations of the COOH-terminal tail region gave distinct phenotypes: tI335 which lacks the extreme 14 COOH-terminal residues had an unchanged internalization capacity whereas tI334 displayed a markedly reduced capacity to internalize [3H]BK, i.e. 35% of that of the B2wt (100%). Further truncations of the receptor exemplified by mutant tY320 did not result in a greater reduction of the internalization capacity (Fig. 2). These findings suggest that the COOH-terminal portion distal to Arg³⁵¹ is dispensable for receptor internalization while the segment spanning positions 335 to 351 comprising 17 residues appears to be of critical importance to receptor sequestration. To further test this hypothesis, we constructed a deletion mutant, del[335–351] which selectively lacks segment Gln³³⁵ to Arg³⁵¹ and retains the portion distal to Arg³⁵¹ (Fig. 1). The internalization capacity of del[335–351] was similar to that of tI3³⁴ demonstrating that the 13 most COOH-terminal residues of the B2 receptor (positions 352 to 364) cannot rescue this phenotype. In addition, the same internalization rate was observed over a wide range of density of this mutant receptor del[335–351] that was established by transfecting cells with 0.75 to 15 μg of cDNA (not shown). To pinpoint the region(s) important for internalization, we generated mutants where the proximal 8 residues of the critical Gln³³⁵ to Arg³⁵¹ segment are removed, del[335–342] or where the distal 9 residues are deleted, del[335–342]. The effect of the largest deletion mutant (del[335–351]) was almost quantitatively reproduced by deletion mutant del[335–342] (24.7 ± 5.2 and 30.7 ± 5.2%, respectively, of B2wt). However, del[343–351] also resulted in a reduced internalization capacity (56.5 ± 4.5% of B2wt), suggesting that the adjacent segments spanning positions 335 to 342 and 343 to 351 do not function in a simple additive manner for receptor internalization.

Because the segment of the COOH-terminal domain of the B2wt that appears to interfere with receptor internalization contains 5 residues of serine/threonine that may be critical for receptor sequestration, we constructed mutant receptors where a single residue was changed to alanine, i.e. S₃₃₉A, T₃₄₂A, and S₃₄₈A, or two adjacent residues were substituted at the same position, i.e. T₃₄₂A/S₃₄₅A/S₃₄₆A. The internalization capacity was reduced in the order Thr³₄₂ > Thr₃₄₅/Ser³₄₆ > Ser³₃₉ > Ser³₄₈ although the level (41.1 ± 4.5% to 88.3 ± 3.1% of B2wt) was not as dramatic as for del[335–351] and tI3³³⁴ (24.7 and 35.1% of B2wt, respectively) (Fig. 2). By contrast, the simultaneous replacement of distant Ser/Thr residues, i.e. S₃₃₉A/T₃₄₂A and S₃₃₉A/T₃₄₅A/S₃₄₆A, produced a markedly reduced internalization (27.6 ± 2.6 and 32.4 ± 1.8% of B2wt, respectively) similar to that observed for del[335–351] and tI3³³⁴ (Fig. 2). This increment was not further increased by the substitution of...
Cultured during 48 h after transfection for transient expression or stable CHO cell lines at confluence were incubated with 2 nM \(^{3}H\)BK at 37 °C for 70 min. Data were obtained as described in the legend of Fig. 2. Results (mean ± S.E.) are given as the percent of the control, i.e. \(B_2\)wt internalization which represents 57.4 ± 3.1% of the corresponding total specific binding in HEK 293 cells and 72.6 ± 1.5% and 89.6 ± 1.9% in transiently and stably expressing CHO cells, respectively; ***, p < 0.005, test ANOVA.

All 5 Ser/Thr residues of the Gln\(^{335}\) → Arg\(^{351}\) segment, i.e. S339A/T342A/T345A/S346A/S348A (Fig. 2, note that this mutant \(B_2\)wt represents a full-length receptor protein). Together these findings suggest that Ser and Thr residues within the 17-residue segment spanning positions 335 to 351 contribute differentially to receptor endocytosis and can very likely intervene in this process in an alternative manner. Mutations of Ser/Thr residues external to this segment, e.g. T237A, S316A, and S331A neither altered the binding capacity nor the internalization rate of the resultant receptor mutants (data not shown).

**Internalization of \(^{3}H\)BK by Mutant \(B_2\) Receptors Expressed in CHO-K1 and HEK 293 Cells**—Additionally to their characterization in the COS-7 cells, the wild type and some mutant receptors were tested for their ability to internalize \(^{3}H\)BK when expressed in CHO-K1 cells (stably and transiently) and in HEK 293 (transiently). The results summarized in Fig. 3 demonstrated that in those cells the truncation mutant \(t\)Y320 and the deletion mutant del[335–351] as well as the point mutation \(B_2\)ΔεST receptor all exhibited a markedly decreased ability to internalize \(^{3}H\)BK compared with the \(B_2\)wt, like in COS-7 cells. However, the fraction of the \(^{3}H\)BK binding which was internalized differed slightly from one cell type to another. For the control \(B_2\)wt, this fraction was of 72.6 ± 1.5% in CHO-K1, 57. ± 3.1% in HEK 293, and 60.3 ± 2.5% in COS-7. These observations can be linked to the differential amount of GRKs and arrestins expressed in these cell types reported by Menard et al. (33). It should be pointed out that the deletion mutant del[335–351] exhibited in every cell type the same reduced internalization rate than \(B_2\)ΔεST supporting our hypothesis that phosphorylation of Ser and Thr within the segment 335–351 is crucial for an optimal internalization of the BK \(B_2\) receptor.

**Immunoprecipitation of Mutant Receptors**—The above observations made it of interest to study the receptor phosphorylation, especially to demonstrate whether the Ser and Thr residues discussed above are implicated. To this end, transfected cells with the various truncated, deleted and point mutated receptor mutants were labeled with \(^{35}S\)methionine or \(^{32}P\)orthophosphate, and incubated with or without 1 \(\mu\)M BK for 5 min prior to cell lysis. For immunoprecipitation of the receptor we applied antisera AS346 which has been raised against a peptide derived from the COOH terminus of the \(B_2\)wt (positions 329 to 364) (21). In \(^{35}S\) labeling experiments (Fig. 4d), immunoprecipitation of \(B_2\)wt resulted in a diffuse band of 60–100 kDa that was superimposable with the immunoreactive band found in \(^{32}P\) labeling experiments (Fig. 4, a-c). All mutant receptors including \(B_2\)ΔεST were readily immunoprecipitated by the antisera except for deletion mutants del[343–351] and del[335–351] which were precipitated at a 3–4-fold lower efficiency, and truncation mutants \(t\)R351, \(t\)I334, and \(t\)Y320 which failed to react. These findings localize major immunogenic epitope(s) recognized by antisera AS346 to the extreme COOH-terminal receptor portion distal of residue Arg\(^{351}\).

**Phosphorylation of Mutant Receptors**—Under basal conditions, i.e. in the absence of BK, a significant phosphorylation was observed for \(B_2\)wt and mutant receptors except for del[335–351], del[343–351], and S348A suggesting that Ser at position 348 may represent a major target site for ligand-independent phosphorylation. BK dramatically increased the phosphorylation level of \(B_2\)wt as well as of T237A, S316A, and S331A where Ser and Thr residues external to the Gln\(^{335}\) → Arg\(^{351}\) segment had been mutated (data not shown). In marked contrast, BK failed to increase the phosphorylation level of del[335–351], \(B_2\)ΔεST, and S339A/T342A/S345A/S346A/S348A (Fig. 4); these mutants are characterized by a markedly reduced internalization capacity (Figs. 2 and 3). In all mutants exhibiting an “intermediate” internalization phenotype such as del[335–342], del[343–351], S339A, T342A, S348A, S339A/T342A, and T345A/S346A BK was still able to increase the phosphorylation (Fig. 4). Together these results demonstrate that the Ser and Thr residues phosphorylated upon ligand stimulation are located within the Gln\(^{335}\) → Arg\(^{351}\) segment. The data also point to a close relationship between internalization and phosphorylation implying that BK promotes receptor internalization by homologous phosphorylation. To further test this hypothesis, we investigated with the \(B_2\)wt whether PKC is involved in the internalization and phosphorylation processes. It was apparent that \(^{3}H\)BK binding was not sensitive to application, before and during the ligand incubation, of 0.1 \(\mu\)M PMA or staurosporine, an activator and inhibitor of PKC, respectively (not shown). Under the same conditions, there was also no change in \(^{3}H\)BK internalization and BK stimulation of PLC. Concurrently, an immunoprecipitation of the \(B_2\)wt showed that, unlike BK, PMA did not increase the receptor phosphorylation (not shown). Thus, PKC regulation of the \(B_2\)wt showed that, unlike BK, PMA did not increase the receptor phosphorylation (not shown).
Fig. 4. BK-induced phosphorylation of B2wt and mutant receptors. COS cells used 40 h after transfection were radiolabeled with 35S-labeled amino acids (panel d) or with 32Porthophosphate and incubated for 5 min with (+) or without (−) 1 μM BK. The cells were lysed, and the receptors immunoprecipitated with antiserum AS346. The radiolabeled proteins were analyzed by reducing 10% SDS-PAGE and autoradiography. Relative molecular masses of standard proteins (not shown) are indicated on the left. Data are representative of three independent experiments with similar results. TS345–346A, T345A/S346A; S339A/T342A; S339A/T342A; S339A/T345A/S346A; S339A/T345A/S346A.

FIG. 5. Effects of phosphatase inhibitor treatment on B2wt and B2ΔST receptor internalization and phosphorylation. COS cells were incubated with 0.1 μM okadaic acid for 5 min prior to and during a 10-min incubation period with 2 nM [3H]BK at 37 °C. Results are expressed in percent of internalized radioactivity in untreated B2wt cells which represents 34.4 ± 2.2% of total bound radioactivity. a, 32P radiolabeled transfected cells were exposed to 0.1 μM okadaic acid in the presence or absence of 1 μM BK for 5 min before analysis of antiserum AS346-immunoprecipitated proteins as described in the legend to Fig. 4; *, p < 0.05, test ANOVA.

Whether the critical residues in the Gln335 → Arg351 and Arg331 → Arg351 segment are essential for receptor endocytosis through clathrin-coated vesicles. For this purpose, we exposed cells expressing the B2wt or the mutant receptor del[335–351] to a standard solution or to hypertonic solution containing 0.4 M sucrose for 30 min prior to and for 10 min after the addition of [3H]BK, and measured the internalization of the radioligand. Both the B2wt as well as the mutated receptor showed reduction of [3H]BK internalization by roughly 70% (not shown). The same results were obtained whether receptors were expressed in COS-7 cells or CHO-K1 cells. This suggests that the clathrin-mediated, sucrose-sensitive endocytosis of the bradykinin B2 receptor might not require the Ser/Thr phosphorylation residues in the Gln335 → Arg351 segment. To further document this hypothesis, we tested the ability of wild type and dominant-negative mutant β-arrestin and dynamin to influence the sequestration of B2wt and the residual sequestration of B2ΔST receptors in COS-7 cells. Fig. 6 shows that unlike for the β-adrenergic receptor (33), the co-transfection with β-arrestin did not increase the internalization of [3H]BK by either the B2wt or B2ΔST receptors, but co-expression of β-arrestin (319–418) or dynamin K44A mutants inhibited internalization of [3H]BK endocytosis for both receptors. One possibility to explain the inability of the overexpression of wild type β-arrestin to increase significantly the internalization rate of both receptors, is that the endogenous proteins may be sufficient to mediate maximal internalization of these receptors. In any case these observations indicate that although clathrin-mediated endocytosis may be important for B2 receptor internalization this mechanism does not exclusively involve the phosphorylation of Ser/Thr residues in the Gln335 → Arg351 segment.

DISCUSSION

Post-stimulatory desensitization and cellular redistribution of GPCRs are important mechanisms that regulate the availability and signaling capacity of hormonal effector systems. For instance, ligand-induced internalization of the β-adrenergic receptor allows the assembly of a scaffold of signaling factors of the mitogen-activated protein kinase system on intracellular
vesicles and thus connects this receptor to the mitogenic pathways of the cell (37). Accumulating evidence suggests that specific sequence motifs of the intracellular loops and/or reversible modifications such as acylation and phosphorylation of the cytoplasmic domains of the receptors play critical roles in these processes (38). However, the precise molecular mechanisms driving desensitization and internalization of GPCRs have often remained unknown.

The present work demonstrates that a short sequence segment covering positions 335 to 351 of the bradykinin B2 receptor COOH-terminal tail plays a major role in the agonist-induced internalization and phosphorylation of the receptor, but is not necessary for PLC and PLA2 activation. Five amino acid residues, namely Ser in positions 339, 346, and 348 and Thr in positions 342 and 345, were shown to be involved in these functions and their respective contribution was analyzed. Complementary approaches for receptor modifications, i.e. truncation, deletion, and mutation of single or multiple residues were used to get insights into the structure-function relationships within the COOH-terminal tail. Most of the mutations resulted in alteration in the receptor capacity to undergo internalization and these alterations were consistent in different cell types. A reduction was always observed, indicating that the COOH-terminal tail possesses positive rather than negative motifs for internalization; at maximum we observed almost 80% reduction in the receptor sequestration in COS-7 cells. This contrasts with the findings that the human β2-adrenergic receptor truncated at position 365 was internalized to a greater extent than the wild type receptor in these cells (14). Our data show that the COOH-terminal tail contributes significantly to the internalization of the B2wt, although other structures such as the intracellular loops may also be involved, albeit with a reduced efficiency (39).

The maximum reduction in receptor internalization was obtained with three distinct types of mutations and the effect of these mutations was consistent in the three different cell types tested COS-7, CHO-K1, and HEK 293. Substitution of the 5 Ser/Thr residues in the Gln335 → Arg351 segment (B2ΔST) produced a similar impairment in internalization as the deletion of the entire segment (del[335–351]) or the truncation in position 334 (tI334). Furthermore, substitution of only two (Ser339 and Thr342) or three (Ser339, Thr345, and Ser346) of these residues resulted in the same effect. This signifies that substitution of the critical Ser/Thr residues rather than a global structural change was responsible for the observed effects. Deletions which remove the proximal residues Ser339 and Thr342 (del[335–342]) or the distal residues Thr345, Ser346, and Ser348 (del[343–351]) each gave a phenotype intermediate to that of B2wt and del[335–351]. Intermediate phenotypes were also observed for individual (S339A, T342A, and S348A) or combined replacements (T345A/S346A) or S339A, T342A, and S348A) or combined replacements (T345A/S346A) or combined replacements (T345A/S346A) of these residues resulted in the same effect. This signifies that substitution of the critical Ser/Thr residues rather than a global structural change was responsible for the observed effects. Deletions which remove the proximal residues Ser339 and Thr342 (del[335–342]) or the distal residues Thr345, Ser346, and Ser348 (del[343–351]) each gave a phenotype intermediate to that of B2wt and del[335–351]. Likewise, the combined effects of individual substitutions exceeded the effect of a simultaneous mutation of all 5 residues in B2ΔST. This indicates that the contribution of the non-mutated residues is smaller in the mutated receptors than in the intact receptor and suggests that the Ser/Thr residues of the Gln335 → Arg351 segment do not operate in a simple additive manner but operate in coordination to optimize receptor sequestration.

The present study shows that B2 receptor internalization is independent of the affinity for BK or of the efficiency of PLC and PLA2 coupling as reported for the truncated human platelet activating factor receptor (40). The COOH-terminal tail segment downstream of Tyr320 as well as the proximal residues Thr345, Ser346, and Ser348 do not function in the coupling of the receptors to G proteins stimulating the PLC pathway. These observations are in agreement with those of Faussner et al. (7) who reported that truncations either at Gly327 or Lys348 diminished the capacity to internalize BK but left the capacity to activate PLC unchanged. These and our results are, however, inconsistent with a recent report (39) claiming compromised ligand internalization associated with reduced PLC activity for a rat B2 receptor mutant devoid of the terminal 34 residues (corresponding to tR331 for the human receptor). Differences in species (rat versus man) and expression systems (Rat-1 versus COS-7, CHO-K1, and HEK 293) may help explain some of the observed discrepancies.

Our work provides strong evidence that phosphorylation is the triggering signal that enters the B2wt in the internalization process. Several lines of evidence support this notion: (i) BK stimulation induces internalization and homologous receptor

**FIG. 6.** Effects of β-arrestins or/and dynamin co-transfection on B2wt and B2ΔST receptor internalization. COS cells were transfected with 15 µg of expression vector for B2wt and B2ΔST, together with 15 µg of empty vector or wt β-arrestin, rat, or bovine β-arrestin (319–418) mutants, or human dynamin-K44A, as indicated. Then, 72 h after the transfection, the cells were incubated with 2 nM [3H]BK at 37 °C for 10 min. Results are given as the percent of the control, i.e. B2wt or B2ΔST-mediated internalization which represents 26.8 ± 4.1 and 4.1 ± 0.8% of the total bound radioactivity at 10 min, respectively. Data are the mean ± S.E. of eight determinations. They were analyzed using unpaired Student’s t test (*, p < 0.05, when compared with the corresponding B2wt and B2ΔST control cells).
phosphorylation; (ii) the kinetics of receptor internalization and recycling parallel those of receptor phosphorylation and dephosphorylation in HF-15 cells (24); (iii) inhibition of phosphatase activity which delays B2wt receptor dephosphorylation increases [3H]BK internalization but neither alters the internalization of [3H]BK by B2AST nor the phosphorylation state of this mutant receptor; (iv) PMA and staurosporine treatment which fail to change [3H]BK internalization do not affect receptor phosphorylation; (v) mutations ty320, del[335–351], B2AST, and S339AT345AS346A that suppress homologous receptor phosphorylation impair receptor internalization in all cell types tested; (vi) mutation of three phosphorylation sites, i.e. Ser339, Thr342, and Ser346, suffices to abolish homologous receptor phosphorylation and to markedly reduce internalization capacity of the human B2 receptor. Remarkably Ala substitution of 3 out of 14 potential phosphorylation sites (Ser, Thr, and Tyr) present in the 4 intracellular domains are sufficient to abolish ligand-induced phosphorylation and to markedly reduce the internalization capacity of the human B2 receptor studied in COS-7 cells. This observation points to the fact that the remaining non-mutated Ser/Thr residues cannot rescue the altered phosphorylation and internalization phenotype. On the contrary, the contributions of the non-mutated residues to internalization appear to be curtailed in these mutants.

The question whether BK-sensitive phosphorylation sites can serve as recognition motifs for receptor interaction with clathrin-coated vesicles was not elucidated in the present work. Indeed, the mutant del[335–351] with reduced internalization capacity and lacking these sites still exhibited a sensitivity to the desensitization that we have previously characterized in COS-7 expressing either the B2wt or mutant receptors, probably because of a differential expression of the human B2 receptor and its residual internalization still involves clathrin-coated vesicles. The internalization that was not sensitive to β-arrestin and dynamin mutants could reflect sequestration of the receptor in caveolae, like that described for the B2 receptor in DDT1 MF-2 smooth muscle cells by de Weerd et al. (25), and in A431 cells by Haasemann et al. (41). Interestingly, the desensitization that we have previously characterized in the CHO-K1 cells expressing the B2wt (4) did not occur in COS-7 expressing either the B2wt or mutant receptors, probably because of a differential expression of the human B2 receptor desensitization machinery between the two cell types. The lack of desensitization in COS-7 cells, together with the decreased internalization observed in some mutants, is, however, of interest because it indicates that receptor internalization is not a prerequisite for the desensitization, as reported by others (10).

In conclusion, this work has allowed us to establish that optimal internalization and phosphorylation of the B2wt require the integrity of Ser339 Ser346 Ser348 Thr342, and Thr345 residues, located in a short segment of 17 residues (Glu335 → Arg351) in the central portion of the COOH-terminal tail. However, the mutation of these residues is not sufficient to completely suppress the sequestration through clathrin-coated vesicles, suggesting that the internalization of the [3H]BK-B2 receptor complex proceeds from different mechanisms involving distinct receptor structures. The critical Ser/Thr residues are flanked by 2 acidic residues, Glu in positions 337 and 350 delimiting a core sequence of 14 residues, Glu337 → Glu350. Previous in vitro studies with the β-adrenergic receptor have pointed to the potential importance of acidic residues juxtaposed to Ser/Thr residues (42). Comparison of the human Glu337 → Glu350 segment with the sequences of other vertebrate kinin receptors (17, 18, 43) demonstrates that this cassette is well conserved among the kinin receptors: 9 of the 14 residues (65%) are invariant including all serine and threonine residues (Ser339 Ser346, Ser348 Thr342, and Thr345), a centrally located positive residue of arginine (Arg344), and the proximal acidic residue (Glu337) whereas the distal acidic residue is conserved (Glu350 or Asp350). By contrast the overall sequence identity of intracellular domain ID4 is poor (15/64 corresponding to 23%). Our notion that the Glu337 → Glu350 cassette plays a central role for receptor phosphorylation and internalization is further strengthened by the finding that the replacement of COOH-terminal tail region of the wild-type human B2 receptor which fails to undergo ligand-induced phosphorylation and internalization, by the homologous region of the B2 receptor holding the Glu337 → Glu350 cassette confers the capacity to the B2/B2 receptor chimera for ligand-induced internalization (7) and phosphorylation.2

One limitation of the present approach is highlighted by the finding of alternative phosphorylation of closely spaced Ser/Thr residues within the putative phosphorylation cassette. Hence we cannot draw firm conclusions as to relative importance, functional hierarchy, and/or sequential modification of the critical Ser/Thr residues. Studies aimed at the precise mapping of the phosphorylation sites in vivo and at the elucidation of the sequence of phosphorylation events in the human B2 bradykinin receptor are underway.

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