Correlations in Palmitoylation and Multiple Phosphorylation of Rat Bradykinin B2 Receptor in Chinese Hamster Ovary Cells*

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Vukic Soskic‡, Elke Nyakatura‡, Martin Roos‡, Werner Müller-Esterl§, and Jasminka Godovac-Zimmermann‡¶

From the ‡Institute of Molecular Biotechnology e.V., 07745 Jena, Germany, and the §Institute of Physiological Chemistry and Pathobiocchemistry, Johannes Gutenberg University at Mainz, 55099 Mainz, Germany

Rat bradykinin B2 receptor from unstimulated Chinese hamster ovary cells transfected with the corresponding cDNA has been isolated, and subsequent mass spectrometric analysis of multiple phosphorylated species and of the palmitoylation attachment site is described. Bradykinin B2 receptor was isolated on oligo(dT)-cellulose using N-(e-maleimidocaproyloxy)succinimide-Met-Lys-bradykinin coupled to a protected (dA)30-mer. This allowed a one-step isolation of the receptor on an oligo(dT)-cellulose column via variation solely of salt concentration. After enzymatic in-gel digestion, matrix-assisted laser desorption ionization and electrospray ion trap mass spectrometric analysis of the isolated rat bradykinin B2 receptor showed phosphorylation at Ser365, Ser371, Ser378, Ser380, and Thr374. Further phosphorylation at Tyr352 and Tyr461 was observed. Rat bradykinin receptor B2 receptor is also palmitoylated at Cys356. All of the phosphorylation sites except for Tyr461 cluster at the carboxyl-terminal domain of the receptor located on the cytoplasmic face of the cell membrane. Surprisingly, many of the post-translational modifications were shown by MS9 mass spectroscopic analysis to be correlated pairwise, e.g. phosphorylation at Ser365 and Ser371, at Ser378 and Ser380, and at Thr374 and Ser380 as well as mutually exclusive phosphorylation at Tyr352 and palmitoylation at Cys356. The last correlation may be involved in a receptor internalization motif. Pairwise correlations and mutual exclusion of phosphorylation and palmitoylation suggest critical roles of multiple post-translational modifications for the regulation of activity, coupling to intracellular signaling pathways, and/or sequestration of the bradykinin receptor.

Bradykinin, a member of the kinin family (1), is a nonapeptide with diverse biological activities ranging from a role in the inflammatory process to regulatory effects on vascular permeability, blood pressure, renal homeostasis, and pain generation (2, 3). Bradykinin mediates its physiological effects by binding to and activation of the bradykinin B2 receptor. Molecular cloning has revealed the primary structure of the B2 receptor (4) and classified it as a member of the G protein-coupled receptor superfamily. The consensus bradykinin receptor topology predicts four extracellular domains (ED1–4) and intracellular domains (ID1–4), each separated by seven transmembrane helical regions (TM1–7) spanning the lipid bilayer. B2 receptors are post-translationally modified by glycosylation (5), phosphorylation (6), and presumably by palmitoylation of the cytoplasmic surface.

Based on homology with other G protein-coupled receptors there have been indications regarding possible structural features probed by agonists, antagonists, and anti-idiotypic antibodies (5, 6), functional regions (7), and sites of post-translational modifications for the B2 receptor (8). Site-directed mutagenesis indicated the importance of Tyr residues and of ID4 for the signaling and the uptake of the B2 receptor in receptor in rat-1 cells transfected with wild and mutant receptor cDNAs (9). However, as yet there is still rather little direct evidence at the protein level for attributes such as the precise sites and the roles of glycosylation, palmitoylation, and phosphorylation of bradykinin B2 or other G protein-coupled receptors. Indeed phosphorylation sites for few G protein-coupled receptors have been mapped to date (10–12), and most of these sites reflect the in vitro phosphorylation of the isolated receptor.

We report here on the isolation of rat B2 receptor from transfected Chinese hamster ovary (CHO) cells using oligo(dA) covalently linked to bradykinin via a specially developed bi-functional cross-linker. Affinity chromatography has been carried out under very mild conditions using oligo(dT) columns analogous to methods used for isolation of eukaryotic mRNA. In-gel digestion of electrophoretically separated receptor, subsequent peptide mass fingerprinting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) or electrospray ionization (ESI) and fragment analysis by tandem (MS/MS) mass spectrometry have been used to characterize post-translational modifications of this receptor and its multiply phosphorylated species. The patterns of post-translational modifications which have been observed for the receptor under in vivo conditions show correlations among the various modifications and provide new information on their possible role(s) in the functional regulation of the bradykinin B2 receptor.

EXPERIMENTAL PROCEDURES

Materials—Ham’s F-12 nutrient medium was from Life Technologies, Inc. Dithiothreitol Microselect, used for deprotecting the modified

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1 The abbreviations used are: ED, extracellular domain(s); ID, intracellular domain(s); TM, transmembrane domain(s); CHO, Chinese hamster ovary; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; EMCS, N-(e-maleimidocaproyloxy)succinimide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; SS-R, 1-O-dimethoxymethylxylidyldisulfide; (dA)9-9'-S-EMCS-BK, (dA)9-9'-S-N-(e-maleimidocaproyloxy)succinimide; MKBK, Met-Lys-bradykinin; EMC-MKBK, N-(e-maleimidocaproyloxy)succinimide Met-Lys-bradykinin; Pipes, 1,4-piperazinediethanesulfonic acid.
oligonucleotide, tetrabutylammoniumhydrogensulfate, and chymostatin were from Fluka. 1,10-Phenanthroline was from Merck. Heps, N'-[e-maleimidocaproyl]succinimide (EMCS), phenylmethanesulfonyl fluoride, CHAPS, Ellman's reagent, bacitracin, bovine serum albumin, and MES were from Sigma. The Aquaporin RP-300 A HPLC column and chemicals for peptide sequencing were from Applied Biosystems. DNase-free RNase A was from Boehringer Mannheim. Met-Lys-bradykinin was from Bachem; trypsin was from Promega. Other chemicals (from Merck and Roth) were of the best grade available.

**Synthesis of Polyadenylated Met-Lys-Bradykinin**—Met-Lys-bradykinin derivatized with EMCS at the ε-NH₂ group of Lys² was prepared by a synthesis similar to that used recently to prepare an analogous endothelin derivative (12). 1 mg (400 nmol) of Met-Lys-bradykinin was dissolved in 400 µl of 35% acetonitrile, 0.05% trifluoroacetic acid, further diluted with 50 mM sodium borate and 0.015% Triton X-305, pH 8.2, and a 10-fold excess (4 µmol) of the heterobifunctional cross-linker EMCS dissolved in 200 µl of acetonitrile was added. Subsequent steps in the reaction were carried out as described previously (12). The derivatized Met-Lys-bradykinin, N-[e-maleimidocaproyl]succinimide-Met-Lys-bradykinin (EMC-MKBK), was purified on an Aquaporin RP-300 column using a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The purity of the fraction containing EMC-MKBK was checked by protein mass spectrometry (found MH⁺ 1513.9, expected 1513.6). (dA)₃₀ was used for attachment of 30-mer (dA) to the EMCS-MKBK according to the procedure in Ref. 12. The (dA)₃₀-5''-EMC-MKBK was purified on a Sephasil C18 column by applying a linear gradient of 2–70% acetonitrile in 1.0 mM triethylammoniumacetate and 2 mM tetrabutylammoniumhydrogensulfate.

**Cell Culture and Membrane Preparation**—CHO cells transfected with rat B₂ receptor cDNA (4, 13) were grown in Ham's F-12 medium containing 10% fetal calf serum and 50% acetonitrile. The band corresponding to the B₂ receptor was cut out from the SDS-polyacrylamide gel electrophoresis to confirm derivatization at Lys² and by MALDI-TOF mass spectrometry (found MH⁺ 1513.9, expected 1513.6). (dA)₃₀-5''-SS-R was used for attachment of 30-mer (dA) to the EMCS-MKBK according to the procedure in Ref. 12. The (dA)₃₀-5''-EMC-MKBK was purified on a Sephasil C18 column by applying a linear gradient of 2–70% acetonitrile in 1.0 mM triethylammoniumacetate and 2 mM tetrabutylammoniumhydrogensulfate.

**Affinity Purification of Bradykinin B₂ Receptor**—CHO membranes (600 µl) were suspended in 2 volumes of 20 mM potassium phosphate, pH 7.4, containing 4 mM CHAPS, 500 mM NaCl, 20 mM EDTA (buffer A). The mixture was stirred gently at 4 °C for 1 h and then centrifuged at 100,000 × g for 1 h at 4 °C. The supernatants were incubated with 0.2 mol of (dA)₃₀-5''-EMC-MKBK for 1 h at 4 °C, 30 mg of oligo(dT)-cellulose was added, and the suspension was agitated gently at 4 °C for 2 h. The-cellulose was pelleted by centrifugation (4 °C, 1,000 × g, 5 min) and packed into a microcolumn. The column was washed with 10 ml of buffer A at 4 °C and afterward eluted with 10 ml Tris/HCl, pH 7.4, 4 mM CHAPS, 1 mM EDTA. Fractions (50 µl each) containing B₂ receptor were identified by SDS-polyacrylamide gel electrophoresis and immunoblot analysis.

**Identification of Bradykinin B₂ Receptor**—Rat bradykinin B₂ receptor was isolated from CHO cells using a “fishhook” strategy similar to that recently applied successfully for isolation of another peptide receptor, endothelin B receptor (12). The fishhook consists of the receptor ligand (here MLBK) and a (dA)₉₀ polynucleotide joined via the heterobifunctional crosslinking reagent EMCS which, in addition to a N'-hydroxyssuccinimide-activated ester, also possesses a thiol-selective maleimido group. This linker was attached to MKBK via the ε-amino group of Lys², and the thiol-selective maleimido group was used to attach (dA)₉₀. The final product, (dA)₉₀-5''-S-EMC-MKBK, was subsequently added to a suspension of CHO cell membranes solubilized in the non-ionic detergent CHAPS. B₂ receptors bound to (dA)₉₀-5''-S-EMC-MKBK were subsequently added to a suspension of CHO cell membranes solubilized in the non-ionic detergent CHAPS. B₂ receptors bound to (dA)₉₀-5''-S-EMC-MKBK were subsequently added to a suspension of CHO cell membranes solubilized in the non-ionic detergent CHAPS. B₂ receptors bound to (dA)₉₀-5''-S-EMC-MKBK were purified in a manner similar to that used for the isolation of eukaryotic mRNA: absorption on oligo(dT)-cellulose, washing in the presence of high salt concentrations, which favor the formation of a poly(dA-dT) double helix, and elution with very low salt concentrations that destabilize a poly(dA-dT) double helix. Recombinant B₂ receptor is highly expressed in the transfected CHO cells used for the present experiments (1.3 pmol/mg of protein (13)) and shows a strong band at about 42 kDa in SDS-polyacrylamide gel electrophoresis of total membrane proteins (lane 1, Fig. 1). This band is strongly enriched relative to other membrane proteins by the fishhook purification strategy (lane 2, Fig. 1). Subsequent analysis of this band (Fig. 1, lane 2) revealed that it contains highly pure B₂ receptor suitable for detailed analysis by mass spectrometry. By comparison with intensities from the unpurified extract of membrane proteins, we estimate that about 70% of cellular B₂ receptor was recovered after fishhook purification.

**Identification of Tryptic Fragments of Bradykinin B₂ Receptor**—Bands corresponding to Coomasie Blue-stained bradykinin B₂ receptor, run off the SDS-polyacrylamide gels and subjected to in-gel tryptic digestion. MALDI mass spectrometry was used for the initial analysis of the entire mixture of tryptic peptides. From the MALDI spectra it was possible to identify peptides from the extracellular domains ED3 as well as peptides from cytoplasmic regions ID2, ID3, and ID4. Peptides containing TM helical regions TM3–TM5 joined to the corre-
sponding loop region(s) could also be observed (Fig. 2 and Table I). In the present studies we did not observe peptides from domains ED1, TM1, ID1, TM2, and ED2 (Fig. 2) presumably because this region of the B2 receptor has only two internal sites for tryptic hydrolysis at positions 10 and 91 and therefore would be expected to yield large, hydrophobic peptides except for the extreme amino-terminal peptide of 10 residues.

Identification of Post-translationally Modified Peptides—The MALDI mass measurements gave clear indications of which peptides were post-translationally modified (Table I). We observed phosphorylation for peptides 6–7, 26–31, 28–31, and 32–33. Peptides 6–7, 26–31, and 28–31 showed an increased mass of 80 Da, which is characteristic for peptides with single phosphorylation sites. For peptide 32–33, a mass consistent with a diphosphorylated peptide was observed. For peptide 30–31, palmitoylation was suggested by an increase in mass of 238 Da. The identity of the peptides was confirmed by electrospray ion trap mass spectrometry of the unseparated peptide mixture with subsequent MS/MS analysis of selected fragments of interest. It is known that in electrospray ion trap mass spectrometry the phosphorylated peptides partially lose the $\text{H}_3\text{PO}_4$ moiety in the mass spectrometer, thus producing a pair of peaks separated by a mass difference of 98 Da (18). In accordance with this, we observed that phosphopeptides 6–7, 26–31, and 28–31 could be identified by a pair of masses 80 Da higher (the mass of $\text{H}_3\text{PO}_4$ minus H$_2$O) and 18 Da lower than expected based on the amino acid sequence. For the peptide 32–33, the loss of two $\text{H}_3\text{PO}_4$ moieties confirmed the presence of two phosphorylation sites. We conclude that intracellular domains ID2 and ID4 of the rat B2 receptor are modified by phosphorylation (ID2, ID4) and palmitoylation (ID4).

Identification of Phosphorylation Sites—For the peptide 26–31, a m/z of 1992.2 Da was observed in the MALDI-TOF spectra. There were two pairs of peaks separated by 98 Da (m/z 1992.2 and 1895.8 Da). In addition the MS/MS ESI spectrum of peptide 26–31 (Fig. 3A) showed a y ion series, y15, y14, y13, y12, y9, y8, y7, and a b ion series, b15, b14, b13, b12, b11, b9, b8, and b7, which was sufficient to identify Tyr$^{162}$ and not Ser$^{344}$ in the amino acid sequence $^{344}\text{FRKKSREYV}\cdot\text{P}\cdot\text{QAICRK}^{358}$ as the site of phosphorylation. The presence of the w11 ion confirmed that Ser$^{344}$ is not phosphorylated. For peptide 6–7 an increase of 80 Da was observed, suggesting that the peptide 16$^{\text{VY}}$LALVKTMSMG$^{171}$ is phosphorylated at Tyr$^{161}$ or Ser$^{165}$. MS/MS analysis generated y12 and y11 ions as well as b12, b11, b10, and b7 ions that were sufficient to determine that Tyr$^{161}$ is the phosphorylation attachment site.

For the peptide 32–33, $^{359}\text{GGCMGESVQMENSMGTLRT}$-

**Table I**

| Peptide* | Residues | Expected mass MH | Measured mass |
|----------|----------|------------------|--------------|
| 5        | 137–160  | 2826.4           | 2827.0       |
| 6–7      | 161–172  | 1369.7           | 1369.3       |
| 6–7      | 161–172  | 1369.7           | 1488.0*      |
| 7–9      | 167–177  | 1281.6           | 1281.8       |
| 11–12    | 181–204  | 2803.5           | 2805.3       |
| 13–14    | 205–223  | 2208.4           | 2209.5       |
| 14–20    | 208–271  | 7459.9           | 7463.6       |
| 16–17    | 252–262  | 1375.7           | 1379.4       |
| 18–21    | 263–272  | 1264.5           | 1265.1       |
| 20–21    | 266–272  | 861.0            | 862.0        |
| 25–31    | 343–358  | 2068.4           | 2067.5       |
| 26–31    | 344–358  | 1912.2           | 1992.2*      |
| 26–32    | 344–376  | 4540.2           | 4539.0       |
| 28–31    | 347–358  | 1480.7           | 1558.6*      |
| 28–32    | 347–376  | 3349.9           | 3350.7       |
| 30–31    | 350–358  | 1109.3           | 1349.1**     |
| 32–36    | 359–396  | 4193.7           | 4194.9       |
| 32–33    | 359–383  | 2646.0           | 2806.4*      |
| 33–34    | 377–387  | 1283.4           | 1283.4       |

*Peptides corresponding to putative tryptic hydrolysis at Lys or Arg residues were numbered consecutively from the NH$_2$ terminus of the bradykinin B$_2$ receptor (4, 30). Di- and tripeptides with mass <400 Da are not shown. Longer peptides arising from incomplete tryptic hydrolysis are shown as the component peptides.

$^a$K ion. $^*$, one or more phosphate groups. $^{**}$, palmitoylation.
SISVDR\textsuperscript{383}, the MALDI-TOF mass spectra gave a parent ion with an m/z of 2806.4 corresponding to this peptide with two phosphate groups attached. No ions corresponding to this peptide with more or fewer than two phosphate groups could be detected. Given that this peptide has six potential phosphorylation sites, Ser at positions 365, 371, 378, and 380 and Thr at 374 and 377, this was a first indication that phosphorylation of these sites. Further evidence for correlation among the sites that are phosphorylated in this peptide has been obtained from ion trap ESI mass spectra after fragmentation of this peptide (Fig. 3B). Initial inspection of the MS/MS spectra clearly indicated a very complicated phosphorylation pattern for this peptide. From the $y^{2+}$ and $b^{2+}$ series of fragments, clear evidence of concurrent phosphorylation at Ser\textsuperscript{365} and Ser\textsuperscript{371} was obtained from the fragments $y^{2+}12$ and $b^{2+}14$-pp (Fig. 4A, note that one or two attached phosphate groups are indicated by -p or -pp in the following text and in Fig. 4). Other fragments that would be consistent with this phosphorylation pattern include $y^{2+}18$-p, $y^{2+}16$-p, and $y^{2+}5$ as well as $b^{2+}19$-pp, $b^{2+}17$-pp, and $b^{2+}12$-p. Similarly, the fragment $y^{2+}6$-pp-98, which corresponds to the peptide $y^{2+}6$-pp with the loss of two phosphate groups, gives clear evidence for concurrent phosphorylation at Ser\textsuperscript{377} and Ser\textsuperscript{380}. Other fragments that would be consistent with concurrent phosphorylation of these two serines include $y^{2+}18$-pp, $y^{2+}14$-pp, $y^{2+}12$-pp, and $y^{2+}4$-pp as well as $b^{2+}21$-p. (Fig. 4) There are three fragments, $y^{2+}7$p, $y^{2+}6$p, and $b^{2+}18$p which cannot be consistent with the above two types of phosphorylation and which require phosphorylation at one of Ser\textsuperscript{365}, Ser\textsuperscript{371}, or Thr\textsuperscript{374} as well as at one of Thr\textsuperscript{377}, Ser\textsuperscript{378}, or Ser\textsuperscript{380}. Evidence for concurrent phosphorylation at Thr\textsuperscript{374} and Ser\textsuperscript{380} was obtained by MS\textsuperscript{3} analysis of the $y$ series fragment $y^{2+}12$-pp (Figs. 3B, inset, and 4B).

The three concurrent phosphorylation patterns shown in Fig. 4C suffice to explain all observed fragments. Evidence that other patterns of phosphorylation probably do not exist was obtained by MS\textsuperscript{3} analysis of fragments $y^{2+}16$-p, which indicated that when Ser\textsuperscript{365} is phosphorylated, the only concurrent phosphorylation site is Ser\textsuperscript{371}, and of $y7$-pm which indicated that Thr\textsuperscript{377} is probably never phosphorylated, at least under the conditions to which the CHO cells were subjected in the present experiments. Given that phosphorylation of the five sites Ser\textsuperscript{365}, Ser\textsuperscript{371}, Ser\textsuperscript{378}, Ser\textsuperscript{380}, and Thr\textsuperscript{374} could occur in 10 different combinations, the present evidence that only three of these combinations occur is strong evidence for coordinated phosphorylation. We confirmed these results with many more NH\textsubscript{2}-terminal ($b^{2+}H\textsubscript{2}O$, $b^{2+}NH\textsubscript{3}$, $b^{2+}H\textsubscript{2}PO\textsubscript{4}$), COOH-terminal ($y^{2+}H\textsubscript{2}O$, $y^{2+}NH\textsubscript{3}$, $y^{2+}H\textsubscript{2}PO\textsubscript{4}$), and internal fragment ions, which for clarity have been omitted from further discussion here, but were of considerable help during the course of spectra analysis.

**Palmitoylation of B$_2$ Receptor**—The increase of 238 Da in the mass of peptide 30–31 indicated the presence of a palmitate residue (Table I). The MS/MS analysis generated $y^{2+}$, $y^{5+}$, $y^{7+}$, $y^{62}$, $y^{52}$, $y^{42}$, $y^{32}$, and $b^{42}$, $b^{52}$, $b^{62}$, $b^{72}$, $b^{82}$ ions showing that Cys\textsuperscript{356} in peptide $34^\text{PPKRSREVYPCQACR}K$\textsuperscript{358} is palmitoylated (Fig. 3C). MS/MS analysis of this peptide showed no phosphorylation at Tyr\textsuperscript{452} (Fig. 3C). Conversely, MS/MS analysis of peptide $34^\text{PPKRSRE}VYPCQACR$K\textsuperscript{358} demonstrated phosphorylation at residue Tyr\textsuperscript{452} but not palmitoylation.
tion at Cys356 (Fig. 3A). This result was confirmed by MS/MS analysis of peptide 347KSREVY(Pi)QAICRK358 (not shown). In the MALDI mass spectra we were able to find masses corresponding to peptides 25–31, 26–33, and 28–32 (Table I) with no phosphorylation at Tyr352 or palmitoylation at Cys356, but no masses were detected which correspond to peptides with simultaneous phosphorylation and palmitoylation of Tyr352 and Cys356, respectively. Together these results indicate a mutually exclusive palmitoylation at Cys356 or phosphorylation at Tyr352 of intracellular domain ID4 of the rat B2 receptor.

**DISCUSSION**

Post-translational modifications such as phosphorylation are a widespread mechanism thought to regulate the desensitization, internalization, and resensitization of G protein-coupled receptors. For two prototypic receptors, i.e. rhodopsin and β2-adrenergic receptor, the phosphorylation sites have been studied in some detail (9–11, 19–21). Typically serine and threonine residues located in the carboxyl-terminal domains of these receptors are phosphorylated in response to light (rhodopsin) or ligand (adrenergic receptors). Because of their extremely low abundance in the cell, ligand-dependent G protein-coupled receptors have been notoriously refractory to chemical analysis of phosphorylation sites in vivo, and major discrepancies between the mapping of phosphorylation sites in vitro and the effect of corresponding mutations in vivo have been reported (22). In the present work we took advantage of a highly sensitive technique, i.e. mass fingerprinting (23, 24), to map post-translational modification sites from a typical G protein-coupled hormone receptor isolated from transfected CHO cells by a novel fishhook technique. Tryptic peptides could be recovered from a large part of the sequence of the B2 receptor including, except for Thr92 and Thr95 of ID1, all those sequence

**Fig. 4. Schematic of the analysis of phosphorylation patterns for the diphosphorylated peptide 359GGCMGESVQMEMSTMGTLRTSISVDR.** Panel A, ESI ion trap MS/MS fragmentation of the parent ion m/z 2806.4. The standard numbering of the b/1 series ions (above) and y/1 series ions (below) is indicated on the sequence of this peptide. The six potential phosphorylation sites are indicated by the encircled serine and threonine residues and the amino acid residue numbers at the top of the figure. Starting either from the parent ion b/1 25-pp or the parent ion y/1 25-pp, b/1 series and y/1 series fragment ions that were observed are shown above or below the peptide sequence, respectively. The number of attached phosphate groups is indicated by the label of the ion, e.g. y/1 18-p indicates the peptide fragment VQMENSMGTLRTSISVDR with one attached phosphate group. Fragment ions that can arise from diphosphorylation at Ser365 and Ser371, at Ser378 and Ser380, and at Thr374 and Ser380 are indicated by solid, dash, and dot-dash lines, respectively. Fragment ions that may arise from more than one of the diphosphorylation patterns are indicated by dotted lines. Open, gray, and solid circles denote phosphorylation sites that, respectively: are not phosphorylated (○), belong to a group of potential phosphorylation sites where the actual site of phosphorylation cannot be ascertained from the fragment ion (□), or are phosphorylated (●). For example, the ions y12-pp and y18-p indicate phosphorylation at Ser371 with a second phosphorylation site at any one of Ser378, Ser380, Thr374, or Thr377. Panel B, ESI ion trap MS3 analysis of peptide y12-12-pp. Panel C, the three diphosphorylation patterns that are necessary and sufficient to explain all MS results.
regions that might be expected to show post-translational modifications by phosphorylation or fatty acid attachment. MALDI, ESI mass spectrometry and MS² analysis revealed that the B₂ receptor is phosphorylated at Ser³⁶⁵, Ser³⁷¹, Ser³⁷⁸, Ser³⁸⁰, and Thr³⁷⁴. Further, we observed phosphorylation at Tyr¹⁶¹ and at Tyr³⁵² and palmitoylation at Cys³⁵⁶.

A number of previous studies on bradykinin B₂ receptor cloned into CHO cells (13, 25–28) have indicated that binding affinities for bradykinin and physiological responses to cell stimulation with bradykinin are similar to those observed in primary cell types. Because the receptor was isolated from unstimulated cells, the observed phosphorylation sites most probably represent the “basal” pattern of phosphorylation caused by intrinsic receptor activity (22), although there might be some contribution from accidentally released ligands, e.g., from culture medium supplemented with fetal calf serum, which is a rich source of kinogenins (29). However, basal phosphorylation in the absence of a ligand, which was not inhibited by antagonists, has previously been shown for human bradykinin B₂ receptor in human foreskin fibroblast cells (30), suggesting that such basal phosphorylation may not be unusual.

Phosphorylation and palmitoylation are major mechanisms that modulate signal transduction and internalization of many G protein-coupled receptors (31–35). Except for Ser³⁶⁵, all post-translational modification sites observed in the present work are strongly conserved in all known B₂ receptors, and the present work provides some new information on the possible roles of post-translational modification of these sites. For example, residue Tyr¹⁶¹ is highly conserved in all known sequences of B₂ receptor with Tyr¹⁶¹ being a part of the DRY motif that is conserved in the family of G protein-coupled receptors. Because Tyr¹⁶¹ seems to play a significant role in G protein coupling of the B₂ receptor (9), our present results suggest that phosphorylation of this critical residue may modulate the signaling capacity of this receptor. Similar phosphorylation of the DRY motif might be important for other G protein-coupled receptors.

Out of 7 Ser/Thr residues present in the carboxyl-terminal domain ID4 of the B₂ receptor, five phosphorylation sites at Ser³⁶⁵, Ser³⁷¹, Ser³⁷⁸, Ser³⁸⁰, and Thr³⁷⁴ have been identified. Previous work has demonstrated that truncation of 34 residues at the carboxyl-terminal tail of the rat B₂ receptor has a major impact on receptor internalization (36) thus further supporting the notion that phosphorylation and sequestration of the receptor may be causally linked (9). An unexpected finding of this study is that phosphorylation of region ID4 of the B₂ receptor involves three different molecular species with diphosphorylation at Ser³⁶⁵ and Ser³⁷¹ at Ser³⁷⁸ and Ser³⁸⁰ and at Thr³⁷⁴ and Ser³⁸⁰. The observations that only diphosphorylation apparently occurs and that the sites are correlated has important implications for attempts to analyze functional aspects of the bradykinin B₂ receptor, e.g., it seems essential to know the patterns of correlated phosphorylation prior to mutation of phosphorylation sites and the measurement of resultant physiological responses. Indeed, the present results suggest that in mutation experiments it is potentially possible not only to block phosphorylation at some sites, but perhaps also to create novel phosphorylation patterns that do not exist in the natural receptor, with potentially aberrant physiological responses (21).

Present results also allow a new suggestion for the role in internalization of the Tyr³⁵² and Cys³⁵⁶, both of which are strongly conserved in bradykinin B₂ receptors. Site-directed mutagenesis of rat-1 cells suggested that these residues may define an important site in B₂ receptor internalization but may contribute to the specificity of the site by phenol ring itself or the amino acid bulk rather than by phosphorylation (9). Our results showed that rat B₂ receptor is phosphorylated at Tyr³⁵² in CHO cells. The phosphorylated residue Tyr³⁵² is sequentially close to Cys³⁵⁶, which our results showed to be palmitoylated.

Another unanticipated finding of this study is the apparently mutually exclusive phosphorylation at Tyr³⁵² and palmitoylation at Cys³⁵⁶. Because these residues are sequentially juxtaposed one may speculate that in the nonacylated form of the B₂ receptor residue Tyr³⁵² is available to tyrosine kinase(s), whereas anchoring of the corresponding region in the membrane in the palmitoylated form prevents their access. This scenario is reminiscent of observations made for the β₂-adrenergic receptor (37) where a complete desensitization of the nonpalmitoylated receptor was observed because of hyperphosphorylation of two residues (Ser³⁴⁵/Ser³⁴⁶) located next to the critical Cys³⁴¹ residue used for fatty acid derivatization (38). Because of the many divergent roles that have been postulated for receptor palmitoylation (39–42), the direct demonstration in this work of fatty acid derivatization of the bradykinin B₂ receptor at Cys³⁵⁶ suggests that further investigation of the role of palmitoylation in the kinin receptor is needed.

It is clear from the present results that it is not sensible to speak of “the basal state” of bradykinin receptor in terms of a single molecular species. It is highly likely that the same applies to “the stimulated state” and that, as is beginning to be evident with other receptors (9–11, 19–21), a very complex set of temporal and spatial changes involving a substantial number of correlated post-translational modifications of the receptor will have to be elucidated in order to relate such modifications to physiological pathways. The present results demonstrate that such modifications can be detected and directly characterized for receptors modified under in vivo conditions and provide indications of the kinds of directed mutagenesis experiments that are likely to be helpful in resolving the probably very complex relationships between post-translational modifications and physiological pathways for bradykinin and probably other G protein-coupled receptors.

At the present state of knowledge about G protein-coupled receptors, the large number of post-translational modifications observed for the B₂ receptor, and particularly the correlation of modifications at different sites, was unanticipated and suggests that the number and diversity of post-translational modifications of such receptors, together with concomitant roles in signal transduction pathways, may be much more complex than presently realized. Taken together with recent analogous results on endothelin B receptor (12), it appears that the types of isolation procedures and mass spectrometry analyses established in the present work for direct analysis of modifications in in vivo systems at the protein level will be suitable, perhaps essential, for relating specific post-translational modifications to functional states of the bradykinin receptor. In this context, it may be noted that we have recently established “functional proteomics” methods for observing complex, time-dependent phosphorylation/dephosphorylation responses for large numbers of downstream proteins following receptor stimulation (44, 45). Together with the present methods, this potentially allows correlation under in vivo conditions of specific post-translational modifications of bradykinin receptor with downstream responses.

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