THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 44, Issue of November 2, pp. 40431–40440, 2001
Printed in U.S.A.

Determination of Bradykinin B_2 Receptor *in Vivo* Phosphorylation Sites and Their Role in Receptor Function*

Received for publication, July 24, 2001
Published, JBC Papers in Press, August 21, 2001, DOI 10.1074/jbc.M107024200

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Reversible phosphorylation plays important roles in G protein-coupled receptor signaling, desensitization, and endocytosis, yet the precise location and role of *in vivo* phosphorylation sites is unknown for most receptors. Using metabolic ^{32}P labeling and phosphopeptide sequencing we provide a complete phosphorylation map of the human bradykinin B_2 receptor in its native cellular environment. We identified three serine residues, Ser^{339}, Ser^{346}, and Ser^{348}, at the C-terminal tail as principal phosphorylation sites. Constitutive phosphorylation occurs at Ser^{348}, while ligand-induced phosphorylation is found at Ser^{339} and Ser^{346}/Ser^{348} that could be executed by several G protein-coupled receptor kinases. In addition, we found a protein kinase C-dependent phosphorylation of Ser^{346} that was mutually exclusive with the basal phosphorylation at Ser^{348} and therefore may be implicated in differential regulation of B_2 receptor activation. Functional analysis of receptor mutants revealed that a low phosphorylation stoichiometry is sufficient to initiate receptor sequestration while a clustered phosphorylation around Ser^{346} is necessary for desensitization of the B_2 receptor-induced phospholipase C activation. This was further supported by the specifically reduced Ser^{346}/Ser^{348} phosphorylation observed upon stimulation with a nondesensitizing B_2 receptor agonist. The differential usage of clustered phosphoacceptor sites points to distinct roles of multiple kinases in controlling G protein-coupled receptor function.

G protein-coupled receptors (GPCRs)[1] constitute the largest family of proteins converting external stimuli into intracellular activity. They share a common deduced structure comprising seven α-helical transmembrane domains connected by extra- and intracellular loops. Through their intracellular domains they interact with heterotrimeric G proteins, which in turn modulate the activity of various effectors, such as adenylate cyclases, phospholipases, and ion channels. These effectors generate the intracellular second messengers, which ultimately evoke cellular responses (1). Signal transduction of GPCRs is carefully controlled: continuous or repeated agonist stimulation leads to an attenuation of the response, a phenomenon called desensitization. Although desensitization of receptor/G protein/effector systems generally involves perturbations of all three components, the impairment of the ability of receptors to activate G proteins appears to be the most important and seems to involve an agonist-induced receptor phosphorylation (2–4).

Much of the knowledge about the molecular mechanisms governing desensitization has come from studies of rhodopsin and β_2-adrenergic receptors (5–7). Rhodopsin, which is available in much greater quantities than any other GPCR, has been successfully subjected to mapping of *in vitro* (*i.e.* purified and reconstituted components) and in *vivo* (*i.e.* in cultured cells) phosphorylation sites in its C-terminal tail (8, 9). More recently, the identification of phosphorylation sites in the reconstituted β_2-adrenergic receptor by G protein-coupled receptor kinase (GRK) 2 and GRK5 was reported (10). The functional relevance of these *in vitro* phosphorylation sites has subsequently been challenged by mutagenesis studies that failed to correlate the presence of the mapped residues with cellular receptor-mediated functions (11). This discrepancy could be explained by the rather poor substrate specificity of GRKs in reconstituted systems resulting in the phosphorylation of sites not used *in vivo* (2). For instance, rhodopsin kinase (GRK1) phosphorylates the β_2-adrenergic receptor *in vitro*, and rhodopsin is an excellent substrate for GRK2 in reconstituted systems (4, 12). However, due to their differential tissue distribution both combinations are very unlikely to play a relevant role under physiological conditions. Thus, phosphorylation sites of GPCRs identified *in vitro* do not necessarily correlate with sites that tune receptor functions in a native cellular environment.

In many studies site-directed mutagenesis and total phosphorylation of GPCRs has been performed to screen for key amino acids involved in signal transduction. Using this approach, several mutant receptors have been generated that were useful to corroborate a role of GPCR phosphorylation in adaptation processes (5, 13, 14). For example, four consecutive serine residues in the third intracellular loop were identified as the major phosphorylation sites of the α_{2A}-adrenergic receptor in Chinese hamster ovary cells, and their phosphorylation correlated in an additive manner with the desensitization of the

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; B_2R, bradykinin B_2 receptor; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PLC, phospholipase C; PAGE, polyacrylamide gel electrophoresis; pS, phosphorylated serine.

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receptor-mediated reduction of cellular cAMP levels (13). A general limitation of the mutagenesis approach is that truncations, deletions, or exchanges of amino acids often affect receptor structure, trafficking, localization, and stability or intervene with GRK recognition. Therefore, mutagenesis studies may allow valuable deductions about *in vivo* phosphorylation sites but the unequivocal identification of these sites requires receptor purification and biochemical analysis. Because of the hydrophobic nature and inherently low expression of GPCRs this particularly challenging task has not yet been accomplished for a single non-rhodopsin GPCR.

In an effort to identify the *in vivo* phosphorylation sites of a prototypical GPCR, we chose to study the human bradykinin B_2 receptor (B_2R). Previous work has demonstrated that the B_2R desensitizes upon prolonged or repeated agonist stimulation (15–19), and that the agonist-induced B_2R phosphorylation and dephosphorylation correlate with its de- and resensitization (15). Furthermore, a cluster of serine and threonine residues located in the C-terminal tail of the B_2R has been suggested to hold potential phosphorylation sites (15, 17, 18). Using two-dimensional phosphopeptide mapping and Edman sequencing we report the complete *in vivo* phosphorylation pattern of the human B_2R and demonstrate that the differential usage of clustered phosphoacceptor sites contributes to the complex regulation of receptor sequestration and desensitization.

EXPERIMENTAL PROCEDURES

Reagents—[^{3}H]Bradykinin (1.48–4.07 TBq/mmol), [^{32}P]orthophosphate (360 MBq/ml), and *myo*-[^{3}H]inositol (2.96–4.44 TBq/mmol) were from Amersham; bradykinin was from Bachem; aprotinin (Trasylol™) was from Bayer; AG-X8 anion exchanger resin was from Bio-Rad; GF109203X and PMA were from Calbiochem; bacitracin and Pefabloc™ were from FLUKA; FR190997 was a kind gift from Fujisawa Pharmaceutical Co., LipofectAMINE™ and protein markers were from Life Technologies; cellulose thin layer chromatography (TLC) plates were from Merck; sequencing grade trypsin was from Promega; leupeptin was from Roche Molecular Bioscience; nitrocellulose membranes were from Schleicher & Schuell; phosphoamino acid standards were from Sigma; and protein A-agarose was from Zymed Laboratories Inc. All tissue culture reagents were from Sigma and Life Technologies.

Mutagenesis—Mutants of the human B_2R were generated using the Transformer site-directed mutagenesis kit from CLONTECH. The following constructs have been previously described: S339A, S348A, and ΔST (17). Point mutations were created by the same procedure to replace serine residues (by single nucleotide substitution) by alanine using the following oligonucleotide primers: 5′-GCACACTGCGGACCGCCATCTCCGTG-3′ for S346A, 5′-GAACGCCATGGGCACACTGCGGACCGCCATCTCCGTGG-3′ for S339A/S346A, 5′-GCGGACCGCCATCGCCGTGG-3′ for S346A/S348A, and 5′-GAACGCCATGGGCACACTGCGGACCGCCATCGCCGTGG-3′ for ΔS. All mutations were confirmed by sequencing using the Amplicycle™ kit (PerkinElmer Life Sciences).

Cell Culture and Transfection—Human embryonic kidney cells HEK293T were grown to about 50–70% confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transfections were done in serum-free medium with the indicated cDNAs using 0.2–0.4 μg/well of a 24-well plate, 0.4–1 μg/well of a 12-well plate, 1–2 μg/well of a 6-well plate or 10 μg/10-cm dish by the LipofectAMINE™ method according to the suppliers manual. Cells were used for experiments 48–60 h after transfection. HF-15 human foreskin fibroblasts were grown to confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Ligand Binding and Receptor Internalization—Ligand binding and internalization were measured according to established procedures using [^{3}H]bradykinin (20). HEK293T cells grown on 12-well plates were used 40 h after transfection with B_2R constructs. Cells were washed twice with phosphate-buffered saline supplemented with 0.2% bovine serum albumin, 2 mM bacitracin, and incubated for 90 min at 4 °C in the same solution containing 5 nM [^{3}H]bradykinin in the absence ("total binding") or presence ("nonspecific binding") of 5 μM unlabeled bradykinin. Internalization of the receptor was initiated by incubating cells for different time periods at 37 °C. Cells were washed twice to remove free ligand, and the cell-bound [^{3}H]bradykinin was extracted with 0.2 M acetic acid, pH 2.8, 0.5 M NaCl, 0.2% bovine serum albumin, and radioactivity of the extract was measured ("surface-associated bradykinin"). The acid-stripped cells were dissolved in 1 M NaOH and the radioactivity of the lysate was determined ("intracellular bradykinin").

Phospholipase C Assays—Phospholipase C activity was measured by analyzing inositol phosphate accumulation (21). Cells grown on 24-well plates were labeled with 1 μCi/ml *myo*-[^{3}H]inositol for 24 h in inositol-free Ham's F-12 including 0.1% (w/v) bovine serum albumin, treated for 5 min with 10 mM LiCl and then challenged with 0.01–1 μM bradykinin for 10 min in the presence of 10 mM LiCl. Reactions were stopped by addition of 1 ml of ice-cold 10 mM formic acid. Water-soluble inositols were extracted for 2–12 h at 4 °C and separated by anion exchange chromatography using AG-X8 as a resin. Inositol phosphates were eluted with 2 M ammonium formate and quantified by liquid scintillation counting. Results were normalized for total labeling of lipid pools, which was calculated from the radioactivity in water-soluble extracts and cells. To follow desensitization, cells were pretreated with 100 nM bradykinin for 5 min. Following removal of excess of ligand by washing cells three times with medium, 10 mM LiCl was added 10 or 15 min after the initial stimulation and inositol phosphate levels were measured as described above.

^{32}P Labeling and Two-dimensional Mapping of Phosphorylation Sites—^{32}P labeling of cells (1–2 mCi/ml for 6–8 h), solubilization, and immunoprecipitation of B_2R using an antiserum against the C-terminal receptor domain were done as detailed previously (15). For HF-15 cells with endogenous B_2R, a 10-cm dish was used, whereas for transiently transfected HEK293T and COS-7 cells a well of a 6-well plate was sufficient for the following procedure. After 10% SDS-PAGE proteins were transferred onto nitrocellulose membranes using a semi-dry unit from Bio-Rad. Radiolabeled B_2R was detected by PhosphorImager (BAS2000, Fuji) analysis and tryptic digests were performed as described by Boyle *et al.* (22) with minor modifications. Briefly, membrane pieces containing the ^{32}P-labeled B_2R were cut out and blocked with 0.5% polyvinylpyrrolidon 40 in 0.6% acetic acid for 30 min at 37 °C. Following extensive washes with water, membrane bound B_2R was cleaved *in situ* with 1 μg of modified sequencing grade trypsin in 200 μl of 50 mM $(NH_4)HCO_3$ for 12 h at 37 °C. Released tryptic peptides were vacuum-dried and oxidized with 50 μl of performic acid for 1 h on ice. Reactions were stopped by dilution with 500 μl of 20% (v/v) ammonia solution. Thereafter samples were frozen, vacuum-dried, and a second digest was performed with 1 μg of trypsin in 50 μl of 50 mM $(NH_4)HCO_3$ for 12 h at 37 °C. Following vacuum drying, samples were dissolved in 5 μl of electrophoresis buffer (formic acid:acetic acid:water, 46:156:1790 (v/v/v)) and phosphopeptides were separated by electrophoresis on cellulose thin layer plates in a first dimension (2000 V, 40 min, electrophoresis buffer) and ascending chromatography in a second dimension (15 h, isobutyric acid, 1-butanol, pyridine, acetic acid, water, 1250:38:96:58:558 (v/v/v/v/v)). Phosphopeptides were detected by PhosphorImager analysis and eluted from the cellulose matrix with 2 × 250 μl of 20% (v/v) acetonitrile in a sonicated water bath for 15 min. Part of the extract (25–100 cpm) was hydrolyzed with 6 M HCl for 1 h at 110 °C and subjected to a phosphoamino acid analysis (15, 22). The second fraction (50–500 cpm) was sequenced by Edman degradation using a solid phase sequencer (ABI 477). Twenty sequencing cycles were collected, dried, and analyzed for their content of ^{32}P radioactivity using a PhosphorImager. Data obtained from Edman degradation (position of phosphoamino acids in tryptic peptides) and phosphoamino acid analysis (phosphorylated amino acid), the known sequence of tryptic peptides (Table I) and the anticipated transmembrane topography of the B_2R (23) were used to predict phosphorylation sites. The prediction was verified by *in vitro* mutagenesis of corresponding phosphoacceptor sites followed by two-dimensional phosphopeptide mapping.

RESULTS

Mapping of Serine 339, 346, and 348 as Major in Vivo Phosphorylation Sites of B_2 Receptor—To molecularly characterize the importance of receptor phosphorylation in signal transduction of the B_2R, we applied an analytical two-dimensional phosphopeptide mapping strategy to identify *in vivo* phosphorylation sites of the receptor (22). HF-15 human fibroblasts expressing 400–750 fmol of endogenous B_2R/mg of protein were labeled with [^{32}P]orthophosphate, stimulated with bradykinin, lysed, and B_2R was immunoprecipitated with a specific anti-peptide antibody (15). Following SDS-PAGE, proteins were transferred onto nitrocellulose membranes and the radio-

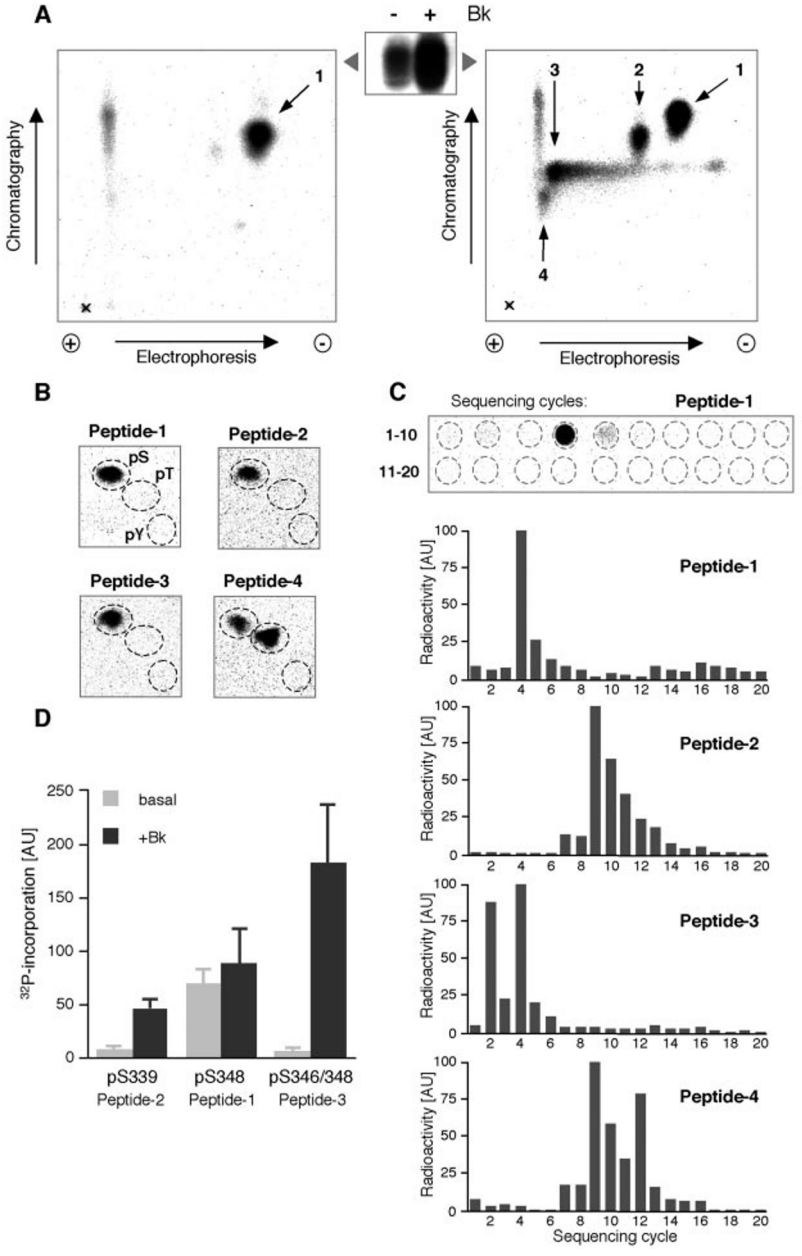

FIG. 1. **Mapping of serine 339, 346, and 348 as the major phosphorylation sites of B_2 receptor.** *A*, HF-15 cells grown on 10 cm-plates were labeled with [^{32}P]orthophosphate and left untreated or stimulated with 1 μM bradykinin (*Bk*) for 10 min. Following immunoprecipitation and 10% SDS-PAGE, isolated proteins were transferred onto nitrocellulose membranes and analyzed using a PhosphorImager (BAS2000, Fuji). The ^{32}P-labeled B_2R was *in situ* digested with trypsin and resulting peptides were separated on thin layer chromatography (TLC) plates by high voltage electrophoresis and ascending chromatography. A *cross* illustrates where samples were applied; + and − indicate the polarity during electrophoresis. Thereafter, phosphopeptides were localized by PhosphorImager analysis. *B*, peptides were eluted and a fraction was hydrolyzed, subjected to phosphoamino acid analysis followed by two-dimensional electrophoretic separation on TLC plates and PhosphorImager analysis. Phosphorylated amino acids were identified by commercial standards (locations indicated by *dashed circles*). *C*, major fractions of phosphopeptides were subjected to 20 cycles of Edman degradation and cleaved amino acids were collected and analyzed using a PhosphorImager to locate the position of the phosphorylation site(s) as exemplified for peptide 1. The content of ^{32}P radioactivity of each sequencing cycle was quantified and expressed in arbitrary units (*AU*). *D*, phosphopeptides on TLC plates were quantified using a PhosphorImager and the relative contributions of individual phosphopeptides to total basal (light) as well as bradykinin-mediated (dark) phosphorylation were determined. Mean ± S.D. from eight independent experiments are shown.

labeled B_2R was detected as a 55–68-kDa band by PhosphorImager analysis (Fig. 1*A*). Notably the broadness of the band and an occasionally observed doublet are likely due to heterogeneous glycosylation (24). In average a 4.0 ± 0.5-fold increase of the basal B_2R phosphorylation was observed in bradykinin-stimulated cells. *In situ* digestion with trypsin-generated phosphopeptides that were separated on thin layer plates by high voltage electrophoresis and ascending chromatography. PhosphorImager analysis revealed a major phosphopeptide in untreated cells that contained more than 80% of the total radioactivity (Fig. 1*A, peptide 1*). The same phosphopeptide was present in bradykinin-stimulated cells as well as three additional ^{32}P-labeled peptides (Fig. 1*A, peptides 2–4*). Total hydrolysis and phosphoamino acid analysis performed with a fraction of the material isolated from the spots revealed that peptides 1, 2, and 3 contained exclusively phosphoserine whereas peptide 4 carried the ^{32}P label both on serine and threonine (Fig. 1*B*). The major fraction of the peptides was used for solid phase Edman sequencing. Because peptide quantities were too low for chemical conversion and identification of released amino acids, cleavage products of 20 sequencing cycles were collected and analyzed for their ^{32}P content using a PhosphorImager. The majority of radioactivity from peptide 1 eluted in cycle 4 (Fig. 1*C*). Together with the finding that phosphate in peptide 1 is exclusively attached to serine (*cf.* Fig. 1*B*), we conclude that the fragment covering amino acid positions 345–351 of the human B_2R sequence (Ref. 23, Table I) is the only one present in the tryptic digest that can be serine phosphorylated at position 4, namely at Ser^{348} (pS348). Peptide 2 generated a peak of radioactivity in sequencing cycle 9 thus identifying Ser^{339} as the phosphorylation site (Fig. 1*C*, Table I). In the case of peptide 3, peaks of radioactivity were detected in cycles 2 and 4, whereas peptide 4 released ^{32}P in cycles 9 and 12 (Fig. 1*C*). Alignment with the sequence of tryptic peptides (Table I) and consideration of the data from the phosphoamino acid analysis (Fig. 1*B*) allowed the identification of Ser^{346} and Ser^{348} (peptide 3) and Ser^{339} and Thr^{342} (peptide-4) as B_2R phosphorylation sites.

For a more quantitative comparison, two-dimensional maps from several experiments were analyzed using a PhosphorIm-

TABLE I
A tryptic peptide pattern of the human B_2R was generated using the PEPTIDEMASS tool (45)

Positions (amino acids in the single letter code) are numbered according to the published human B_2R sequence (23), putative transmembrane segments are underlined, extracellular domains are italic, and identified phosphorylations sites are bold.

Position	Sequence of tryptic peptides
1–19	*MUNVTLQGPTLNGTFAQSR*
20–59	*CPQVEWLGWLNTIQ*PPFLWVLFVLATLENIFVLSVF CLHK
60–104	SSCTVAEIYLGNLAAADLILACGLPFWAITI*SNNFOW LFGETLCR*
105–128	VVNAIISMNLYSSICFLMLVSIDR
129–134	YLALVK
135–140	TMSMGR
141–142	MR
143–145	GVR
146–148	WAK
149–169	LYSLVIWGCTLLLSSPMLVF*R*
170–172	*TMK*
173–225	*EYSDEGHNVTAGVISYPSLIWE*VFTNMLLNVVGFLL PLSVITFCTMQIMQVLR
226–231	NNEMQK
232–233	FK
234–239	EIQTER
240	R
241–270	ATVLVLVVLLLFIICWLPFQISTFL*DTLHR*
271–281	*LGILSSCQOER*
282–310	*IIDVITQ*IASFMAYSNSCLNPLVYVIVGK
311	R
312–313	FR
314	K
315	K
316–326	SWEVYQGVCQK
327–330	GGCR
331–344	SEPIQMEN**S**MG**T**LR
345–351	T**S**I**S**VER
352–355	QIHK
356–363	LQDWAGSR
364	Q

ager. The contribution of the individual ^{32}P-labeled peptides to total phosphorylation was calculated for untreated and bradykinin-stimulated cells and corrected for the increase in total phosphorylation. The contribution of peptide 4 containing pS339/T342 to total receptor phosphorylation was always very low (<5%), and therefore this peptide was excluded from quantitative studies. As shown in Fig. 1*D*, phosphorylation of Ser^{348} in peptide 1 did not significantly change during agonist challenge on this relative scale. In contrast, phosphorylation of Ser^{339} and the double-labeled peptide 3 containing pS346/S348 increased about 10- and >40-fold, respectively, in the presence of bradykinin (Fig. 1, *A* and *D*).

Phosphorylation of B_2 Receptor Mutants Lacking Identified Phosphorylation Sites—To validate the assignment of B_2R phosphorylation sites we used receptor mutants in which Ser^{339}, Ser^{346}, and Ser^{348} individually or in combinations were replaced by alanine residues. Comparable expression levels of all mutants were confirmed by binding assays and immunoprecipitation of ^{35}S-labeled proteins (not shown). Immunoprecipitation of the ^{32}P-labeled B_2R mutants revealed that the ligand-induced phosphorylation of S339A and S346A was slightly reduced whereas the basal phosphorylation in the absence of a ligand was essentially unchanged over the wild-type receptor (Fig. 2*A*). In contrast, we observed a complete lack of basal phosphorylation in S348A and S346A/S348A mutants, together with a significantly decreased bradykinin-induced phosphorylation. Basal phosphorylation of the double mutant S339A/S346A was slightly increased, and only a minor increment was observed upon bradykinin stimulation. Finally, the ΔS (S339A/S346A/S348A) and ΔST (S339A/S346A/S348A-T342A/T345A) mutants failed to produce any significant phosphorylation above background (Fig. 2*A*).

Mutant B_2R were further subjected to two-dimensional phosphopeptide mapping and the resultant phosphopeptides were characterized by phosphoamino acid analysis and Edman degradation. Surprisingly, a major phosphorylated peptide was seen in the S348A mutant in a similar location as peptide 1 in wild-type B_2R (Fig. 2*B, arrow*). Sequence and phosphoamino acid analysis revealed that the S348A mutant had a compensatory phosphorylation at Ser^{346} in position 2 of the corresponding peptide (Fig. 2*B*). In the single mutants S339A and S346A as well as in the double mutants S339A/S346A and S346A/S348A, phosphopeptides corresponding to peptides 2 and 3 of wild-type B_2R (*cf.* Fig. 1*A*) were absent from the two-dimensional maps (Fig. 2*C*) thus confirming our identification *of vivo* B_2R phosphorylation sites. Under these conditions, no phosphopeptide(s) of significant quantity was detected in the two-dimensional maps of the ΔS and ΔST variants. Taken together, we have identified (i) graded phosphorylation of four closely spaced residues, Ser^{348} > Ser^{346} > Ser^{339} ≫ Thr^{342}, (ii) basal phosphorylation at a single site, Ser^{348}, (iii) bradykinin-induced phosphorylation at two major sites, Ser^{346} and Ser^{339}, and (iv) combined phosphorylation at Ser^{346} and Ser^{348}.

Identification of Kinases That Can Phosphorylate the B_2 Receptor—Co-expression of receptors with GRKs is commonly used to obtain information about the nature of kinases involved in GPCR phosphorylation (25–29). To identify potential kinase(s) executing B_2R phosphorylation and to locate their corresponding substrate sites we analyzed the phosphopeptide patterns of B_2R co-transfected with human GRK2–6 in HEK293T cells. Immunoprecipitates from ^{32}P-labeled cells indicated that total basal and bradykinin-mediated B_2R phosphorylation did not significantly change upon co-expression with GRK2, GRK3, GRK5, or GRK6 (Fig. 3*A*). In contrast, expression of GRK4α drastically increased the basal level of ^{32}P incorporation into B_2R. However, phosphopeptide maps revealed quantitative changes in the distribution of phosphopeptides for the various GRKs. For example, ^{32}P labeling of peptide 3 containing pS346/pS348 was enhanced 1.5–3-fold as compared with mock-transfected cells in the order GRK6 < GRK5 < GRK2 < GRK4α < GRK3. Most prominently, GRK4α elevated the basal phosphorylation of Ser^{339} and Ser^{346}/Ser^{348} 15- and 24-fold, respectively. These results suggest that several endogenous GRKs may phosphorylate the B_2R and that the various GRKs, even without apparent effect on total GPCR phosphorylation levels, may induce distinct phosphorylation patterns with possible functional consequences for receptor desensitization and sequestration.

We have previously observed that activators of protein kinase C may induce a ligand-independent phosphorylation of the B_2R (15). Under identical conditions PMA pretreatment significantly reduced the bradykinin-induced PLC stimulation (not shown), indicating that an agonist-independent ("heterologous") receptor phosphorylation may negatively affect signal transduction of the B_2R.

To identify residue(s) in the B_2R sequence targeted by PKC we analyzed two-dimensional phosphopeptide maps from ^{32}P-labeled HF-15 cells treated with PMA. PhosphorImager analysis revealed a new spot (peptide 5) that partially overlapped with the major phosphopeptide 1 containing pS348 (Fig. 3*B*). Peptides 1 and 5 were isolated avoiding cross-contamination, and sequence and phosphoamino acid analysis confirmed the identity of pS348 in peptide 1 (Fig. 3*C*). Peptide 5 showed ^{32}P-labeled serine in position 2 suggesting residue Ser^{346} in peptide 345–351 as potential PKC phosphorylation site (Table

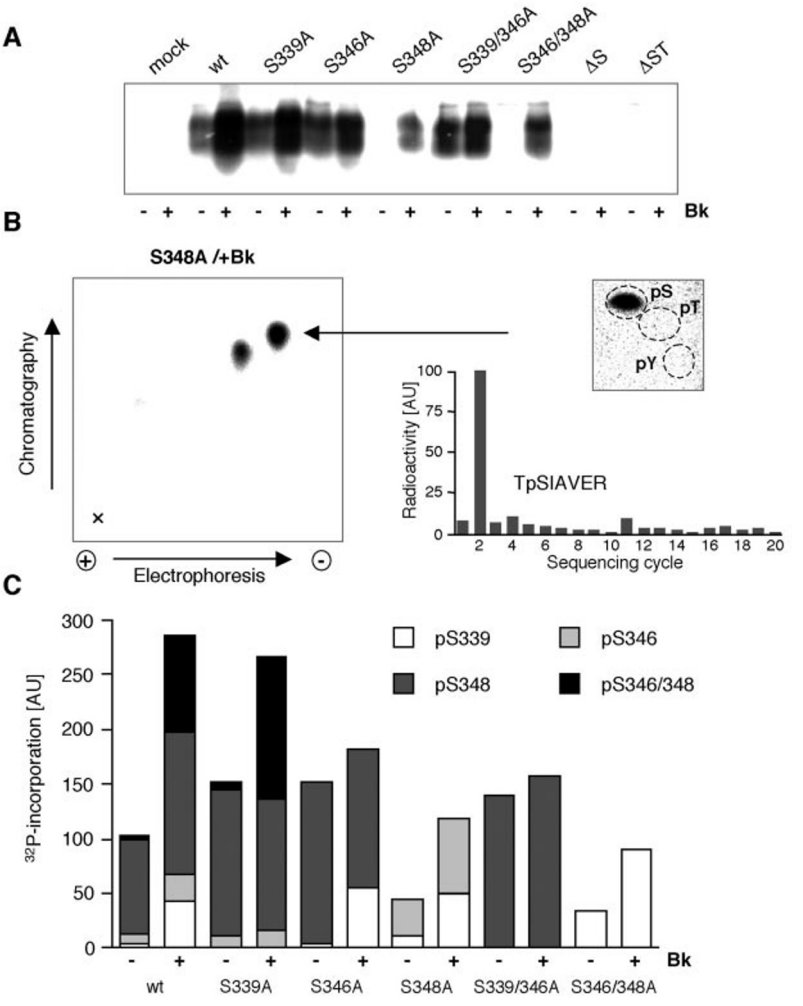

FIG. 2. **Phosphorylation of B_2 receptor mutants lacking the mapped phosphorylation sites.** HEK293T cells were transfected with the wild-type human B_2R or the following mutants: S339A, S346A, S348A, S339A/S348A, S346A/S348A, S339A/S346A/S348A (ΔS) or S339A/S346A/S348A-T342A/T345A (ΔST). *A*, cells were labeled with [^{32}P]orthophosphate, stimulated with 1 μM bradykinin (*Bk*) for 5 min or not (−), lysed, and B_2R was analyzed by immunoprecipitation and autoradiography. *B*, tryptic phosphopeptides were separated and visualized using a PhosphorImager. A representative two-dimensional map including phosphoamino acid analysis and quantification of Edman sequencing cycles of the major spot (*arrow*) from the S348A mutant is shown. *C*, the relative contributions of individual phosphopeptides to total receptor phosphorylation were determined and expressed in arbitrary units (*AU*). *Bars* in the stack diagram indicate phosphopeptides pS339 (*white*), pS346 (*light gray*), pS348 (*gray*), and pS346/S348 (*black*). Means calculated from at least three independent experiments are shown.

I). When HEK293T cells expressing the S346A mutant B_2R were treated with PMA, total phosphorylation did not change and spot 5 failed to appear whereas spot 1 was present (Fig. 3*B*). Together these findings point to Ser^{346} as the major PKC target site in the B_2R. This notion was confirmed by the repression of PMA-induced Ser^{346} phosphorylation in the presence of PKC inhibitors (not shown). Quantitative evaluation of two-dimensional maps revealed a 3-fold increase in Ser^{346} phosphorylation upon PMA treatment, and a 40% decrease in ^{32}P incorporation in the major B_2R phosphorylation site Ser^{348} (Fig. 3*D*). PKC stimulation did not produce any double phosphorylated peptide 4 bearing pS346/S348 (Fig. 3, *B* and *D*), suggesting that the basal phosphorylation at Ser^{348} and the PKC-mediated phosphorylation at Ser^{346} are mutually exclusive. A thorough review of two-dimensional phosphopeptide maps revealed the presence of low amounts of peptide 5 with a single phosphorylation at Ser^{346}, partially overlapping with highly abundant peptide 1 comprising pS348 (*cf.* Figs. 1*A* and 3*B*). This indicates that a PKC-mediated phosphorylation of Ser^{346} also occurs under physiological conditions in the absence of exogenous PKC activators.

Time Course and Dose Dependence of Bradykinin-stimulated B_2 Receptor Phosphorylation—Having identified the principal phosphoacceptor sites in intact cells we tested whether the phosphopeptide pattern of the B_2R changes during the time course of stimulation. We followed the kinetics of phosphorylation of the endogenous B_2R in HF-15 fibroblasts over a period of 60 min and found that bradykinin-induced B_2R phosphorylation is a fast process reaching a maximum after 5 min and decreasing almost to basal levels after 60 min (Fig. 4*A*, *bottom panel*). The appearance of pS339 and pS346/S348 strictly followed this time course, whereas pS346 or pS348 remained constant over the entire period of the experiment (Fig. 4*A, top panel*).

Analyzing the dose-dependence of B_2R phosphorylation, a half-maximum effect was observed with ~10 nM bradykinin, and ≥100 nM of the ligand was sufficient to trigger full receptor phosphorylation (Fig. 4*B, bottom panel*). Whereas pS348 was constant and independent of the applied bradykinin concentration, single phosphorylation at Ser^{346} increased up to 2.5-fold during stimulation with low ligand concentrations (≤1 nM Bk), remained constant at intermediate bradykinin concentrations (1–100 nM), and decreased at the highest agonist concentrations (≥100 nM). Phosphorylation of Ser^{339} and Ser^{346}/Ser^{348} increased markedly in correlation with the dose of the ligand (Fig. 4*B*, *top panel*). While pS339 reached saturation levels at 0.1–1 μM bradykinin, pS346/S348 strongly increased up to 10 μM bradykinin, *i.e.* the maximum ligand concentration used in our experiments. Thus, a PKC-dependent B_2R phosphorylation at Ser^{346} preferentially prevailed at low agonist concentrations, whereas pS339 and pS346/S348 were induced by moderate agonist concentrations, and phosphorylation at Ser^{346}/Ser^{348} was dominant at high bradykinin concentrations.

Role of Specific Phosphorylation Sites in Receptor Sequestration and Desensitization—Next we analyzed the role of the various B_2R phosphorylation sites in modulating receptor functions. First we studied B_2R internalization in HEK293T cells transfected with different B_2R constructs using [^{3}H]bradykinin

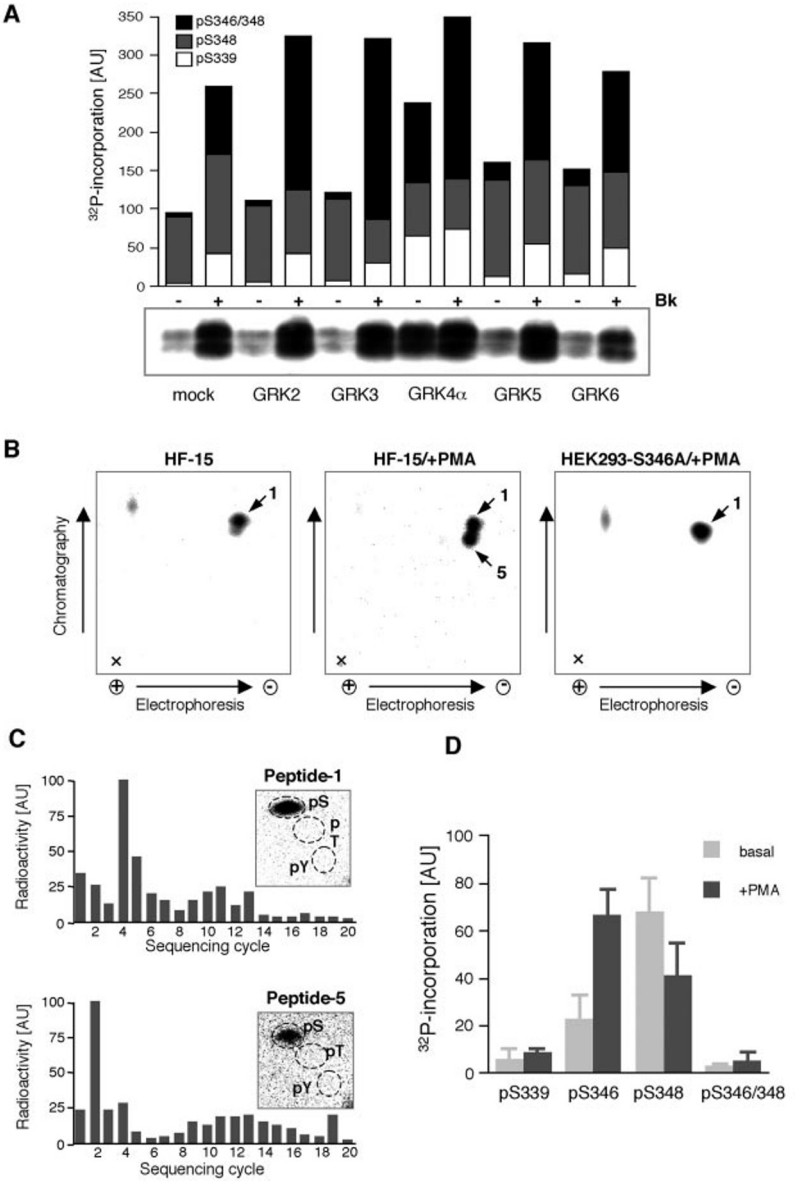

FIG. 3. **Identification of kinases that can phosphorylate the B_2 receptor.** *A*, HEK293T cells grown on 6-well plates were co-transfected with the human B_2R and human GRK2, GRK3, GRK4α, GRK5, and GRK6. Cells were labeled with [^{32}P]orthophosphate, left untreated (−) or stimulated with 1 μM bradykinin (Bk) for 5 min, lysed, and B_2R was isolated by immunoprecipitation. Following 10% SDS-PAGE and transfer onto nitrocellulose, B_2R was cleaved with trypsin and the resulting phosphopeptides were separated. Spots were quantified using a PhosphorImager and relative contributions of individual phosphopeptides (*white,* pS339; *gray*, pS348; and *black*, pS346/S348) to total receptor phosphorylation were calculated and expressed in arbitrary units (*AU*). Mean from three independent experiments are shown. *B*, HF-15 cells or HEK293T cells transfected with the S346A mutant were labeled with [^{32}P]orthophosphate and left untreated (−) or stimulated with 1 μM PMA (+*PMA*) for 10 min. Following immunoprecipitation, SDS-PAGE, transfer onto nitrocellulose membranes, and tryptic digest resulting peptides were separated and localized by PhosphorImager analysis. *C*, Edman sequencing and phosphoamino acid analysis of peptides 1 and 5. *D*, phosphopeptides were quantified and relative contributions of the individual phosphopeptides to total receptor phosphorylation were calculated and expressed in arbitrary units (*AU*). Mean ± S.D. from three independent experiments are shown.

as a probe. The internalization process of wild-type B_2R was fast with a half-maximal effect after 5–10 min and a maximum of almost 70% internalized receptors after 60 min (Fig. 5*A*). Single or double mutations of serine residues in B_2R caused only minor effects on the internalization capacity of the corresponding constructs. The time course and the extent of ΔS sequestration were initially similar to wild-type B_2R, but diverged beyond 20 min of incubation such that only 40% of receptors were internalized after 60 min. In contrast, internalization of the ΔST mutant was clearly reduced at all time points tested, and the majority of mutant receptors (>80%) remained surface exposed during the whole experiment. These results suggest that the initiation of B_2R sequestration requires only a low stoichiometry of phosphorylation without any obvious prevalence for specific residue(s) and that additional, probably phosphorylation-independent processes may be involved in the relocation of the receptor (17).

We also determined desensitization of the bradykinin-mediated PLC activation of wild-type B_2R and various phosphorylation-deficient mutants. To circumvent the problem that bradykinin, which has a high affinity to the B_2R cannot be properly washed out after receptor stimulation, we adapted an alternative protocol that monitors signal duration as a measure of receptor desensitization (21). In transfected HEK293T cells inositol phosphate levels triggered by wild-type B_2R were reduced by ~20% after 10 or 15 min of delayed accumulation as compared with control (Fig. 5*B*). Minor variations were seen with S339A and S348A mutants although difference to wild-type B_2R did not reach statistical significance. Under the same conditions the S346A mutant showed a slight increase of inositol phosphates, while an augmented second messenger accumulation was particularly evident and significant for the S339A/S346A, S346A/S348A, ΔS, and ΔST mutants. We conclude that Ser^{346} is a critical residue for desensitization of the B_2R, and that a clustered phosphorylation of Ser^{346} and at least one additional serine residues seems to be necessary for full desensitization of B_2R-mediated PLC activation.

To further confirm the role of receptor phosphorylation in desensitization we analyzed the pattern of B_2R phosphorylation upon stimulation of cells with FR190997. This synthetic non-peptidic agonist that has been reported to mediate a sustained activation of B_2R indicative of reduced desensitization (30, 31). Stimulation of B_2R-expressing HEK293T cells with increasing concentrations of FR190997 led to a dose-dependent rise in receptor phosphorylation (Fig. 5*C*) comparable to that observed with bradykinin (*cf*. Fig. 4*B*). However, analysis of the

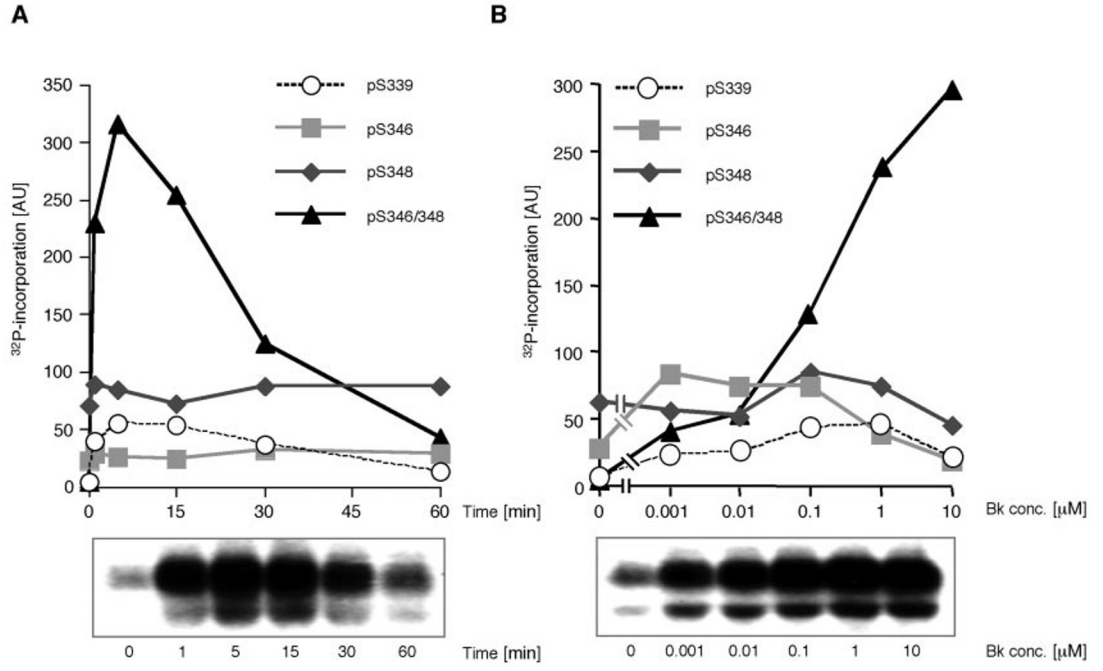

FIG. 4. **Dose dependence and time course of agonist stimulated B_2 receptor phosphorylation.** HF-15 cells grown on 6-well plates were labeled with [^{32}P]orthophosphate and stimulated for different time periods (0, 2.5, 5, 15, 30, and 60 min) with 1 μM bradykinin (*A*) or challenged with increasing concentrations (0, 0.001, 0.01, 0.1, 1, and 10 μM) of bradykinin for 5 min (*B*). Following immunoprecipitation, tryptic digest and two-dimensional separation, phosphopeptides were quantified using a PhosphorImager. The relative contributions of individual phosphopeptides (pS339, ○; pS346 ▨; pS348, *shaded diamond*; and pS346/S348, ▲) to total receptor phosphorylation of typical experiments expressed in arbitrary units (*AU*) are shown.

phosphopeptide pattern revealed a significantly reduced ^{32}P incorporation in peptide 3 containing pS346/S348 as well as in peptide 2 representing pS339 (Fig. 5*D*). These findings are in accord with the observation of a sustained B_2R signaling upon FR190997 stimulation (30, 31), and they lend further support to our hypothesis that phosphorylation of Ser^{346} in tandem with Ser^{348} is an important event during desensitization of B_2R-mediated signal transduction.

DISCUSSION

The implication of receptor phosphorylation in regulation of GPCR functions has been studied for more than a decade (2–4, 32). Most of the knowledge has come from *in vitro* studies using purified components for reconstitution and/or from mutagenesis approaches targeting anticipated phosphoacceptor sites (2). To date the biochemical identification of *in vivo* phosphorylation sites of a GPCR and characterization of their biological role(s) has not been successful for any GPCR but rhodopsin (2, 8, 33, 36). In this report we present the precise mapping of phosphorylation sites of the human bradykinin B_2 receptor in its native cellular environment. This approach has allowed us to discriminate between (i) constitutive phosphorylation of human B_2R on Ser^{348}, (ii) homologous phosphorylation at Ser^{339} and/or Ser^{346} in tandem with Ser^{348}, (iii) and heterologous phosphorylation of Ser^{346} (Fig. 6). We were also able to follow discrete, but important changes in the phosphopeptide pattern of the B_2R upon GRK co-expression, during the kinetics of agonist stimulation and over a broad range of ligand concentrations. At last, the phosphorylation of specific residues was correlated with the initiation of receptor internalization and the regulation of its desensitization. This is thus the first report about two-dimensional mapping of *in vivo* phosphorylation sites of a non-rhodopsin GPCR with a detailed analysis of the importance of specific phosphoacceptor sites in controlling GPCR functions.

A highly sensitive strategy combining two-dimensional phosphopeptide mapping together with Edman degradation was employed to directly identify basal and agonist-induced phosphorylation sites of the human B_2R. A recent mass spectrometry study described the constitutive phosphorylation of six out of seven possible serine/threonine residues in the C terminus of rat B_2R that had been purified from transfected CHO cells (35). However, this report did not provide any quantitative information about the relative frequency of the identified phosphopeptides and did not distinguish between basal, heterologous, and agonist-induced phosphorylation. The present study was performed in an analytical scale, which enabled us to follow B_2R phosphorylation with temporal resolution in different physiological situations. We could clearly discriminate a basal phosphorylation at Ser^{348} and an agonist-mediated phosphorylation of Ser^{339} and/or Ser^{346} in tandem with Ser^{348} in native and recombinant cells. These markedly distinct phosphorylation patterns were not expected, since the two-state model of receptor activation would suggest rather quantitative differences that should reflect the equilibrium between inactive and active, subsequently phosphorylated receptors. The finding that various GRKs affect the relative abundance of phosphorylation of specific phosphoacceptor sites in the B_2R underlines the power of our approach in identifying subtle positional changes that would escape total phosphorylation studies but may affect receptor fine-tuning. An unanticipated result of our experiments is the functionally compensatory phosphorylation of Ser^{346} that was found when the major phosphorylation site Ser^{348} had been mutated to alanine. This alternative phosphorylation points to a rather relaxed substrate specificity of the B_2R kinase(s) that could scan from the receptor C terminus toward the membrane-inserted region for appropriate phosphoacceptor sites. Such a "sliding kinase" mechanism is also supported by the observed quantitative differences in the contribution of identified serine residues to total B_2R phosphorylation, *i.e.* Ser^{348} > Ser^{346} > Ser^{339} (Fig. 6). Based on mass spectrometry

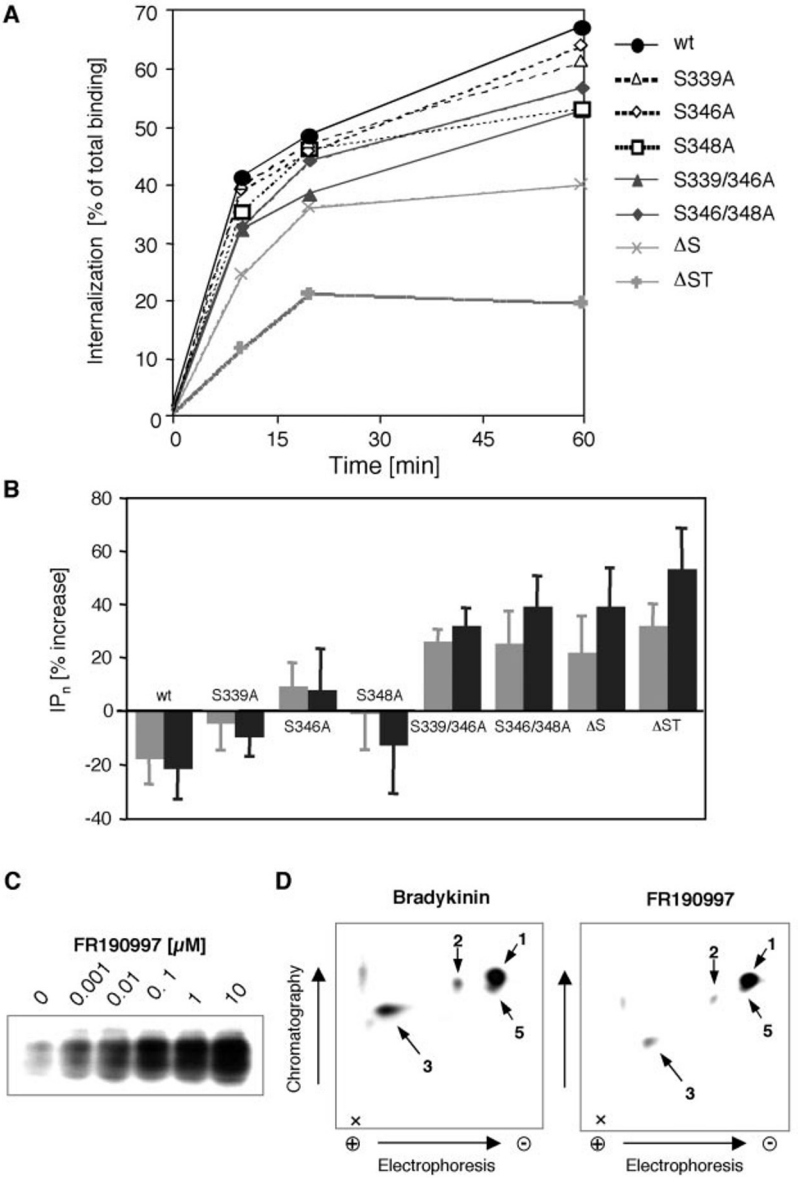

FIG. 5. **Role of specific phosphorylation sites in the regulation of receptor sequestration and desensitization of the bradykinin-induced phospholipase C activation.** *A*, HEK293T cells were transfected with the wild-type human B_2R (●) or the following mutants: S339A (△), S346A (◇), S348A (□), S339A/S346A (◬), S346A/S348A (*shaded diamond*), ΔS (×), and ΔST (*shaded cross*). Internalization was studied after binding of 5 nM [^{3}H]bradykinin at 4 °C by shifting the temperature to 37 °C for the indicated time periods. Extracellular and internalized ligand was separated and quantified by liquid scintillation counting. Means from a typical experiment performed in triplicates are shown. *B*, desensitization of the B_2R-induced PLC activation was studied by following intracellular inositol phosphate accumulation in HEK293T cells transfected with the indicated B_2R mutants and pretreated with 100 nM bradykinin for 5 min. After removal of excess of ligand and a lag phase of 10 (*gray bars*) or 15 min (*black bars*) 10 mM LiCl was added and inositol phosphate (IP_n) accumulation was measured for 10 min. Mean ± S.D. from a typical experiment performed in triplicates are shown. *C*, ^{32}P-labeled HEK293T cells were stimulated with increasing doses of the non-peptidic B_2R agonist FR190997, B_2R was immunoprecipitated, resolved by 10% SDS-PAGE, and visualized using a PhosphorImager. *D*, typical two-dimensional maps of tryptic phosphopeptides of B_2R from cells treated either with 10 nM bradykinin (*left panel*) or 10 nM FR190997 (*right panel*) are shown.

studies, a similar GRK-driven sequential modification has been suggested for the C-terminal portion of rhodopsin (36). However, in other GPCRs such as the α_{2A}-adrenergic receptor the phosphorylation of multiple serines in the third intracellular loop seems to occur independently of any directional order (13).

Data from GRK co-expression experiments and the finding that ^{32}P labeling of Ser^{348} remained largely invariable during agonist challenge suggests that either a non-GRK activity constantly provides new pS348 for subsequent GRK-mediated Ser^{346} phosphorylation or that synchronous *de novo* phosphorylation of Ser^{346}/Ser^{348} occurs (Fig. 6*B*). The former hypothesis would support a priming function of pS348 as it has been proposed for other GPCRs such as rhodopsin, the μ-opioid and the A_3 adenosine receptor (36–38). Such a priming phosphorylation of B_2R at Ser^{348} would also convert Thr^{345} and Ser^{346} to consensus sites for further GRK-mediated phosphorylation (29).

In contrast to the bradykinin-induced dual phosphorylation of Ser^{346} and Ser^{348}, PKC selectively triggered phosphorylation of Ser^{346} that appeared to block rather than promote subsequent Ser^{348} phosphorylation. The PKC-induced phosphorylation of Ser^{346} in the B_2R could be involved in agonist-independent, heterologous desensitization as it has been suggested for other GPCRs (2, 28, 33, 34, 37, 39). This hypothesis is supported by our finding that carbachol stimulation of co-expressed $G\alpha_q$-coupled m_1 and m_3 but not of $G\alpha_i$-coupled m_2 muscarinic receptors resulted in a moderate but significant increase of B_2R single Ser^{346} phosphorylation (data not shown). However, results from our analysis of the dose dependence of B_2R phosphorylation also suggest that PKC-mediated phosphorylation contributes to homologous B_2R desensitization upon stimulation with low doses of bradykinin. Based on inhibitor studies, such a scenario was earlier proposed for protein kinase A in regulating β_2-adrenergic receptor signaling (40).

Receptor internalization has been implicated in desensitization of GPCRs, although it is often too slow ($t_{1/2} \approx 5$–20 min) for a significant contribution to acute desensitization that usually occurs within the first few minutes of agonist challenge (2, 4). The sequestration of β_2-adrenergic receptors and the acidification of the corresponding intracellular compartments have been suggested to constitute the initial steps of resensitization, because both processes were found to be essential for GPCR dephosphorylation (41, 42). Indeed B_2R internalization seems to be necessary for its full dephosphorylation and subsequent resensitization (15, 43) but in addition, receptor phosphorylation was shown to initiate internalization of the B_2R (17). The

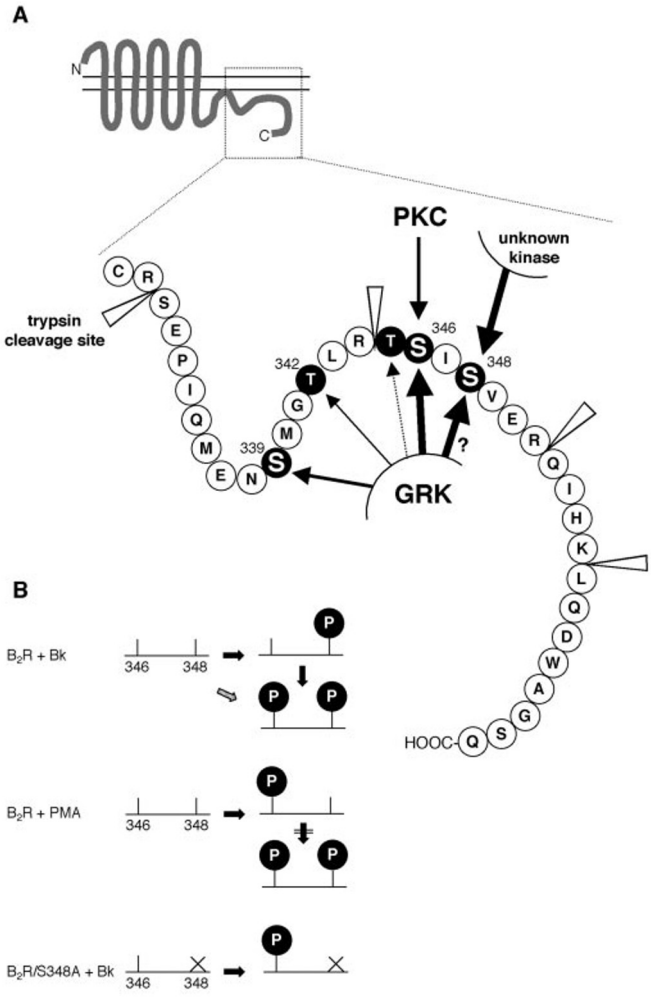

FIG. 6. ***In vivo* phosphorylation sites present in the B_2R tail domain.** *A*, schematic representation of the amino acid sequence (*single letter code*) of the B_2R C terminus starting from the potential palmitoylation sites (indicated by *serpentine lines*). Identified phosphoacceptor sites are *numbered* and *highlighted*, and candidate kinases are indicated. The thickness of the *arrows* point to the relative quantity of phosphate incorporation. *B*, phosphorylation reactions occurring upon stimulation of B_2R with bradykinin and PMA and S348A mutant with bradykinin.

fact that receptor mutants with deletions of two principal phosphorylation sites (S339A/S346A or S346A/S348A) do not display significant changes in their sequestration kinetics demonstrates that a low stoichiometry of phosphorylation is sufficient to trigger B_2R internalization. A relaxed phosphorylation requirement with respect to the location of phosphoacceptor sites and stoichiometry has been proposed for internalization of m_2 muscarinic and *N*-formylpeptide receptors (14, 44). Even a B_2R mutant with all three major phosphorylation sites replaced (ΔS) allowed internalization of a sizable receptor fraction (≈40%). The finding that this fraction was further reduced in the ΔST variant, where five potential phosphorylation sites (3 serines and 2 threonines) have been replaced, could be explained by the minute levels of Thr^{342} and Thr^{345} phosphorylation in the ΔS mutant that became obvious after pretreatment of cells with serine/threonine phosphatase inhibitors (data not shown).

Unlike the low stoichiometry phosphorylation requirement for receptor internalization, we found that tandem phosphorylation of Ser^{346} with Ser^{339} or Ser^{348} is necessary and sufficient to desensitize the B_2R-mediated PLC activation. These data correlate well with the findings of Leeb-Lundberg and co-workers (18) who described an increased spontaneous activity of a B_2R mutant replacing, among other residues, Ser^{346} and Ser^{348}. However, single phosphorylation of the major acceptor site Ser^{348} that was also found in the absence of ligand does not affect receptor signaling by itself, but may prime the B_2R for desensitization. Other examples for the critical role of clustered phosphoserine and phosphothreonine residues in desensitization have been reported for the m_2 muscarinic and *N*-formylpeptide receptors (14, 44). Furthermore, the observation that FR190997, an agonist capable of sustained signaling, is a weak inducer of Ser^{346}/Ser^{348} phosphorylation points out the importance of this tandem phosphorylation for B_2R desensitization and provides an intuitive explanation for the delayed B_2R desensitization upon FR190997 stimulation (30, 31).

The results from this comprehensive two-dimensional mapping study of *in vivo* phosphorylation sites demonstrate the power of this analytical method to reveal subtle temporal and positional changes in the phosphorylation pattern that translate into substantial alterations in the functional capacity of a prototypic GPCR. Future studies will unravel whether the molecular insights into differential phosphorylation requirements for internalization and desensitization of B_2R hold for GPCRs in general.

Acknowledgments—We thank Lars Rönnstrand for advice on the two-dimensional phosphopeptide mapping technique and Carl-Henrik Heldin, Stefan Offermans, and Lars Rönnstrand for critical reading of the manuscript.

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