Palmitoylation of the Human Bradykinin B_2 Receptor Influences Ligand Efficacy†

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Received August 1, 2001; Revised Manuscript Received October 17, 2001

ABSTRACT: To investigate the palmitoylation of the human bradykinin B_2 receptor, we have mutated individually or simultaneously into glycine two potential acylation sites (cysteines 324 and 329) located in the carboxyl terminus of the receptor and evaluated the effects of these mutations by transfection in COS-7, CHO-K1, and HEK 293T. The wild-type receptor and the single mutants, but not the double mutant, incorporated [^{3}H]palmitate, indicating that the receptor carboxyl tail can be palmitoylated at both sites. The mutants did not differ from the wild-type receptor for the kinetics of [^{3}H]bradykinin binding, the basal and bradykinin-stimulated coupling to phospholipases C and A_2, and agonist-induced phosphorylation. The nonpalmitoylated receptor had a 30% reduced capacity to internalize [^{3}H]bradykinin. This indicates that palmitoylation does not influence the basal activity of the receptor and its agonist-driven activation. However, the mutants triggered phospholipid metabolism and MAP kinase activation in response to B_2 receptor antagonists. Pseudopeptide and nonpeptide compounds that behaved as antagonists on the wild-type receptor became agonists on the nonpalmitoylated receptor and produced phospholipases C and A_2 responses of 25−50% as compared to that of bradykinin. These results suggest that palmitoylation is required for the stabilization of the receptor−ligand complex in an uncoupled conformation.

Bradykinin (BK)[1] is involved in a variety of physiological and pathological processes, including vasodilation, ion transfer in epithelia, and pain (*1*). Pharmacological and molecular studies have identified two G protein-coupled receptor (GPCR) subtypes for BK designated B_1 and B_2 (*2*−*4*). The B_2 subtype, constitutively synthesized in many organs, is responsible for most of the known physiological effects of BK. Sequence analysis of the human B_2 receptor suggests that this receptor may undergo post-translational modifications that can alter its configuration (*2*). Previous studies have documented the role of phosphorylation of the carboxyterminal region in the regulation of the B_2 receptor function (*5*−*7*). In addition, the receptor has potential palmitoylation sites. However, B_2 receptor palmitoylation has not been studied. Several GPCRs are covalently modified by fatty acids (for review, see refs *8*−*10*). The acylated amino acid is commonly a cysteine residue that is linked to palmitate by a thioester bond and located in the intracellular carboxyl tail of these receptors. Palmitoylation is thought to associate the receptor carboxyl tail with the plasma membrane, thus creating a fourth intracellular loop. Analysis of the amino acid sequence of the human B_2 receptor and alignment with the sequence of several other GPCRs reveal the presence of a highly conserved cysteine residue located at position 324 (Cys^{324}) in the carboxyterminal domain of the B_2 receptor. The B_2 receptor sequence contains an additional unconserved cysteine at position 329 (Cys^{329}). Covalent modification with palmitic acid may occur at these sites and determine structural conformation of the carboxyterminal region important for receptor function.

The present work is aimed at establishing whether the human bradykinin B_2 receptor is palmitoylated, identifying the palmitoylation site(s), and assessing the consequences of palmitoylation for receptor function. We show that the two cysteine residues located in the carboxyterminal domain of the receptor are palmitoylated and play a critical role in the response of the receptor to ligand binding.

EXPERIMENTAL PROCEDURES

Construction of the Mutant Receptor cDNAs. Mutant cDNAs were constructed (*11*) by using the previously cloned human renal BK B_2 receptor (B_2wt) cDNA placed under the control of a cytomegalovirus promoter into the eucaryotic expression vector pcDNA$_3$ (Invitrogen, Leek, The Nether-

† This work was supported by INSERM and by the Bristol−Myers−Squibb Institute for Medical Research (Princeton, NJ). A.P. was supported by fellowships from the Ministère de la Recherche, the Fondation de la Recherche Médicale, and the Société de Secours des Amis des Sciences. A.B. has received a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft, and I.D. is a research fellow of the Boehringer Ingelheim Fonds.

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[1] Abbreviations: B_2 receptor, type 2 bradykinin receptor; wt, wild-type; B_2wt, wild-type type 2 bradykinin receptor; BK, bradykinin; GPCR, G protein-coupled receptor; Cys or C, cysteine; Gly or G, glycine; Asn, asparagine; Trp, tryptophane; Asp, aspartic acid; PLC, phospholipase C; PLA_2, phospholipase A_2; BSA, bovine serum albumin; AA, arachidonic acid; IPs, inositol phosphates; PBS, phosphate-buffered saline.

10.1021/bi011600t CCC: $20.00
Published on Web 11/30/2001

lands) as a template in site-directed mutagenesis using the Transformer site-directed mutagenesis kit (2nd version, Clontech, Palo Alto, CA). We created three mutants corresponding to the substitution of one or two Cys of the cytoplasmic tail (Figure 1). These mutants, $C^{324}G$, $C^{329}G$, or $C^{324}G/C^{329}G$, were generated by a single nucleotide substitution (indicated by bold face type in the sequences given below) so as to create a glycine codon (underlined) at the appropriate site (numbering according to Hess et al. (*2*)). The nucleotide sequences of the oligonucleotide primers used were 5′-CCAGGGAGTG**G**GCCAGAAAGGG-3′ for $C^{324}G$ and 5′-GAAAGGGGGC**G**GCAGGTCAGAAC-3′ for $C^{329}G$. Both oligonucleotides were used to create $C^{324}G/C^{329}G$. All of the mutations were confirmed by sequencing using an Amplicycle sequencing kit (PerkinElmer, Langen, Germany).

Cell Culture and Receptor Expression. Cell culture and transfection were performed as previously described (*6*, *7*, *11*). Briefly, COS-7 and HEK 293T cells (ATCC, Rockville, MD) were grown in Dulbecco's modified eagle's medium (DMEM, Biological Industries, Israel) and CHO-K1 cells in Ham's F-12 medium (Seromed, Berlin, Germany). The mediums were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B. For immunoprecipitation and MAP kinase activation studies, transient receptor expression was achieved by transfecting COS-7, CHO-K1, or HEK 293T cells grown in 6- or 12-well plates using the Lipofectamine method (Life Technologies, Karlsruhe, Germany). Otherwise, COS-7 cells were grown in T75 flasks and transfected by using the DEAE-dextran method (*12*). They were then subcultured into 24-well plates, where binding, internalization, PLC, and PLA_2 assays were performed. To obtain CHO-K1 cell lines with a stable expression of receptors, transfection was performed using the Superfect transfection reagent (Qiagen, Courtaboeuf, France). Cell clones were selected with geneticin (0.75 mg/mL) for stable vector expression and with [^{3}H]BK binding experiments and coupling assays for B_2 receptor expression. All of the cell types were maintained at 37 °C in a humidified water-jacketed incubator with 5% CO_2. Functional studies of the transfected cells were done at confluence. Transfecting the $pcDNA_3$ vector without insertion created the control cells.

Radioligand Binding and Internalization Studies with [^{3}H]-BK. Cells were incubated as previously described (*11*) in Hank's balanced salt solution (HBSS; 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 127 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 0.8 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 10 mM sodium acetate (pH 7.4)), containing protease and peptidase inhibitors (10^{-5} M captopril, 0.08 U/mL aprotinin, and 0.1 mg/mL bacitracin), 0.1% fatty-acid-free bovine serum albumin (BSA), and [^{3}H]-BK (110 Ci/mmol; NEN, Leblanc Mesnil, France).

For the equilibrium binding studies with [^{3}H]BK and the determination of receptor density, incubations were carried out at 4 °C for 6 h in the presence of 1.25×10^{-11} to 2.5×10^{-8} M [^{3}H]BK for COS cells or $10^{-11}-10^{-7}$ M [^{3}H]BK for CHO cells. Cells were extensively washed with HBSS, and bound [^{3}H]BK was determined by liquid scintillation counting (Optiphase HiSafe 3 and LKB 1211 Rackbeta, Wallac Oy, Turku, Finland). For the determination of specific binding, the assay included measurements of nonspecific binding in the presence of a 1000-fold excess of unlabeled BK; nonspecific binding was subtracted from the total binding determined in the absence of unlabeled BK. To express the data per milligram of cell protein, the protein content of three wells was measured using the Bio-Rad reagent for the Bradford assay (Bio-Rad, München, Germany) with BSA as a standard protein.

Internalization of [^{3}H]BK was studied by incubating transfected COS-7 cells with 2nM [^{3}H]BK at 37 °C for 2−70 min, in the absence (total binding) or presence (nonspecific binding) of 10 μM unlabeled BK. Internalization was followed immediately after the addition of BK. Each experiment included cells transfected with B_2wt as a control. After being extensively washed with HBSS, cell-surface bound radioligand was separated from internalized radioactivity by an acidic washing step (*13*) using 0.2 M acetic acid and 0.5 M NaCl (pH 2.5) before counting the radioactivity. To normalize the data from the different experiments, each value of internalized radioactivity (acid resistant) was first calculated as a fraction of the corresponding total specific binding (cell surface plus acid resistant). Then, the obtained fraction was converted into a percentage of the value at 70 min for the cells expressing B_2wt (*7*).

Measurement of Inositol Phosphate Production. Cells were loaded for 18 h at 37 °C with 3 μCi/mL myo[2-^{3}H]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 protease inhibitors as described previously, and stimulated for 15 min with BK or B_2 receptor antagonists (see the following discussion) at varying concentrations ($10^{-10}-10^{-5}$M). Reactions were terminated by the addition of 3% (w/v) ice-cold perchloric acid, and total [^{3}H]inositol phosphates were isolated using AG1-X8 anion-exchange column chromatography (formate form, 100−200 mesh; Bio-Rad, München, Germany) after the extraction of radioactive phospholipids with chloroform (*11*). The radioactivity measured in inositol phosphates is expressed in the percent of the total radioactivity incorporated into the cells.

Measurement of Phospholipase A_2 Activity. Cells were labeled with 1 μCi/mL [^{3}H]arachidonic acid (150−230 Ci/mmol; Amersham International) for 18 h. After repeated washing to eliminate the unbound radiolabel, PLA_2 activation experiments were performed at 37 °C for 10 min in HBSS containing 0.1% BSA, protease inhibitors, and the test compounds or vehicles. Radioactivity in the medium supernatant containing the released [^{3}H]arachidonic acid plus derived [^{3}H]-labeled metabolites was measured and expressed in the percent of the total radioactivity, comprising the medium plus cell-associated radioactivity (*11*).

Measurement of ERK2 Activity. Experiments were conducted in HEK 293T cells as previously described (*6*). Briefly, HEK 293T cells were transfected with cDNAs encoding the studied receptors and a hemaglutinin (HA)-tagged extracellular-regulated kinase 2 (ERK2). After a 5 min incubation period at 37 °C alone or with the addition of $10^{-8}-10^{-4}$ M HOE 140 and a B_2 receptor antagonist and two washes with PBS, cells were scraped and incubated with gentle rocking for 45 min at 4 °C in 0.5 mL of a lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM Pefabloc, 10 μg/mL leupeptin, and 1% trasylol). Then, equal amounts of lysates,

corresponding to about 200 μg proteins, were incubated with 5 μL of a polyclonal antiserum against the HA tag plus 35 μL of protein A agarose slurry for 3 h at 4 °C. Resultant precipitates were washed three times with a lysis buffer and two times with a kinase buffer (10 mM Tris (pH 7.5) and 10 mM $MgCl_2$) and incubated for 20 min at room temperature in 30 μL of a kinase buffer including 200 μM ATP, 1 μCi [γ-^{32}P]ATP (30 Ci/mmol; Amersham International), and 10 μg myelin basic protein (MBP, SIGMA, Schnelldorf, Germany). Reactions were stopped by adding 30 μL of an SDS sample buffer and heating for 2 min at 98 °C. Proteins were separated by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and gels were cut above the 30 KDa marker band. Upper parts were transferred onto nitrocellulose membranes (Sclheicher and Schuell, Dassel, Germany) using a semidry unit (Bio-Rad, München, Germany) and probed with 0.5 μg/mL anti-ERK2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to check for the equal immunoprecipitation of HA-ERK2 in all of the samples. Lower parts were stained with 0.2% Coomassie brilliant blue in 10% acetic acid and 12.5% isopropyl alcohol before monitoring MBP phosphorylation with phosphorimager (BAS2000, Fuji, Japan).

[^{35}S]-Labeling and Immunoprecipitation of Receptors. COS-7 and CHO-K1 cells were washed twice with sulfur-free HEPES-buffered DMEM, incubated for 30 min at 37 °C in the same medium, and labeled with 0.1 mCi/mL [^{35}S]-amino acids (Prox-mix, Amersham International) for 8 h (*5*). After three washes with 50 mM Tris (pH 7.5) and 150 mM NaCl, cells were scraped into an ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (w/v) NP-40, 0.5% deoxycholate, 0.1% SDS, 0.1 mM Pefabloc SC, and 10 μg/mL each of 1,10-phenantroline, aprotinin, leupeptin, and pepstatin A and then incubated for 45 min at 4 °C with gentle rocking. The resulting lysates were centrifuged for 10 min at 13 000 rpm, and the supernatants were precleared with 50 μL of a *Staphylococcus aureus* cell suspension (Pansorbin, Calbiochem, Sweden).

To immunoprecipitate the B_2 receptor, 2.5 μL of the antiserum AS346 (*5*) diluted in 0.1 mL of 5% BSA in a lysis buffer was added. After the solution sat for 15 min at room temperature, 50 μL of Pansorbin was added, and the suspension was incubated for 10 min at room temperature. Precipitates were recovered by centrifugation for 2 min at 6000 rpm and washed three times with a lysis buffer and once with water. An SDS sample buffer (25 μL) was added to the immunoprecipitate followed by a 15 min incubation at 45 °C. Proteins were resolved by 10% SDS−PAGE in the presence of 5 M urea. After fixation with 20% (w/v) trichloroacetic acid for 20 min, the gels were washed several times with water and subjected to fluorography using 15% (w/v) sodium salicylate as a fluorophor.

Receptor Palmitoylation Studies. Experiments were conducted in COS-7 cells 40 h after transfection or in CHO-K1 cell lines at confluence. The cells were serum-starved for 20 h before metabolic labeling with 0.2 mCi/mL [9,10-^{3}H]-palmitic acid (60 Ci/mmol; Amersham International) in HEPES-buffered DMEM. Two washing steps with an ice-cold phosphate-buffered salt solution stopped the reaction. Cell lysis, solubilization, immunoprecipitation, and gel electrophoresis were done as detailed previously. In some experiments, the immunoprecipitate was treated at 37 °C with 1 M hydroxylamine at pH 7.5 for 30 min. After drying at 60 °C for 2 h, the gels were exposed to Kodak films (Eastman Kodak Company, Rochester, NY) for 1−3 weeks at −80 °C to reveal the [^{3}H]-labeled proteins.

Receptor Phosphorylation Studies. Phosphorylation studies were performed as previously described (*5*, *7*). COS-7 cells, 40 h after transfection, were washed twice with phosphate-free HEPES-buffered DMEM, incubated for 1 h at 37 °C, and labeled with 0.25 mCi/mL [^{32}P]orthophosphate (0.9−1.1 Ci/mmol; Amersham International) for 8 h in the same medium. After a 5-min exposure to 1 μM BK at 37 °C or to medium alone, cells were scraped into 1 mL of an ice-cold lysis buffer containing protease inhibitors and phosphatase inhibitors (50 mM NaF, 25 mM sodium pyrophosphate, and 1 mM sodium orthovanadate). Solubilization, immunoprecipitation, and gel electrophoresis were done as described previously. Proteins labeled by [^{32}P]orthophosphate were revealed by autoradiography using Kodak films.

Studies with B_2 Receptor Antagonists. The following compounds were studied alone or in combination with bradykinin for their effect on inositol phophate production and phospholipase A_2 activity in the CHO-K1 cell lines expressing the wild-type receptor or the mutants: HOE 140 or Icatibant (D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-bradykinin), a largely used potent pseudopeptide antagonist (*14*, *15*) (this compound was also tested for its effect on ERK2 activity in HEK 293T cells); LF 16.0335 (1-[[3-[(2,4-dimethylquinolin-8-yl)oxymethyl]-2,4-dichlorophenyl]sulfonyl]-2(*S*)-[[4-[4-(aminoiminomethyl)phenylcarbonyl]piperazin-1-yl]carbonyl]-pyrrolidine); and LF 16-0687 (1-[[[2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxy]methyl]phenyl]sulfonyl]-*N*-[3-[[4-(aminomethyl)phenyl]carbonylamino]propyl]-2(*S*)-pyrrolidinecarboxamide), two new potent nonpeptide antagonists (*16*, *17*).

RESULTS

Identification of Cys^{324} and Cys^{329} as the Palmitoylation Sites of the B_2 Receptor. To establish receptor palmitoylation and assess the role of carboxyl tail cysteine residues, [^{3}H]-palmitate-labeling experiments were performed in COS-7 cells transfected with an empty $pcDNA_3$ vector or with vectors containing the cDNAs encoding for the wild-type receptor (B_2wt) or the mutant receptors C^{324}G, C^{329}G, and C^{324}G/C^{329}G in which glycine was substituted for Cys^{324} or Cys^{329}. The amino acid sequence of the carboxyl tail of B_2-wt and these mutants is given in Figure 1. After cell labeling with [^{3}H]palmitic acid or [^{35}S]methionine, immunoprecipitation was performed using the antiserum AS346 which has been raised against a peptide derived from the distal part of the carboxyl tail of B_2wt (*5*). Figure 2 illustrates an example of fluorography after electrophoresis of the immunoreactive material obtained from COS-7 cells transfected with the same amount of cDNAs encoding for the various receptors. Immunoprecipitation resulted in a diffuse ^{3}H or ^{35}S radioactive band of 60−90 KDa, corresponding to the B_2 receptor (*5*), that was absent for cells transfected with empty $pcDNA_3$ (Figure 2, lane 2). The ^{35}S signal was of a similar magnitude for B_2wt and the various mutants, suggesting an identical recognition and immunoprecipitation of all of the receptors by AS346. By contrast, the ^{3}H signal varied from one mutant

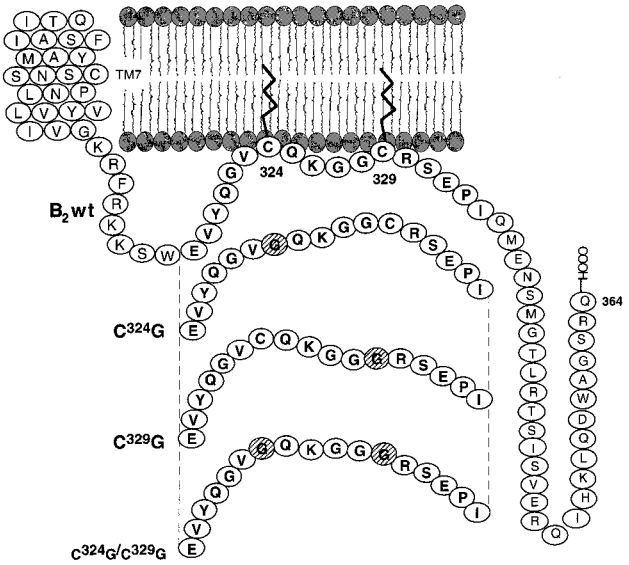

FIGURE 1: Schematic representation of the carboxyterminal domain of the human B_2 receptor and mutant receptors. (Top) Amino acid sequence of the receptor carboxyl tail with indication of the two palmitoylation sites corresponding to the cysteine residues in positions 324 and 329 (numbering according to Hess et al. (*2*)). (Bottom) Sequences of the mutated receptors generated by the replacement of Cys^{324}, Cys^{329}, or both cysteines by glycine.

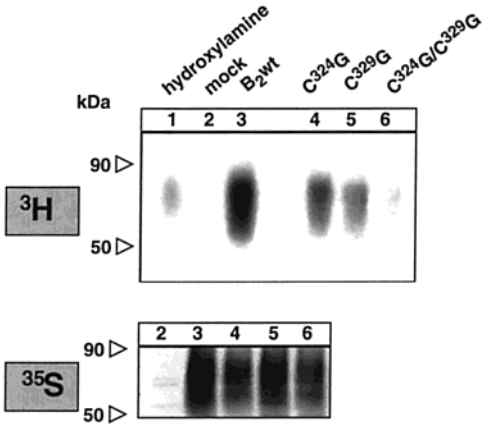

FIGURE 2: Incorporation of [^{3}H]palmitic acid and [^{35}S]methionine into B_2wt and mutant receptors. COS-7 cells were metabolically labeled with [^{3}H]palmitic acid (upper panel) or [^{35}S]-labeled amino acids (lower panel). B_2wt or mutant receptors were immunoprecipitated from COS-7 cell lysates and characterized by SDS−PAGE electrophoresis followed by autoradiography. Lane 1 corresponds to immunoprecipitate from B_2wt-transfected cells treated with 1 M hydroxylamine at a neutral pH. Relative molecular masses determined with standard proteins are indicated on the left. Data are representative of three independent experiments with similar results.

to another. It was of a reduced magnitude for the single mutants $C^{324}G$ (lane 4) and $C^{329}G$ (lane 5) and was almost undetectable for the double-substitution mutant $C^{324}G/C^{329}G$ (lane 6) as compared to B_2wt (lane 3). These results strongly suggest that palmitate is linked to Cys^{324} and Cys^{329} in B_2-wt. This conclusion is also supported by the finding (lane 1) that the neutral 1 M hydroxylamine treatment of immunoprecipitate from B_2wt-transfected cells resulted in an ^{3}H signal as low as the signal obtained with the double-substitution mutant $C^{324}G/C^{329}G$. Indeed, acylation of a cysteine residue is sensitive to neutral hydroxylamine treatment, whereas the hydrolysis of ester bonds to serine and threonine residues occurs only at an alkaline pH (*18*).

Functional Characterization of the Palmitoylation-Deficient Mutant Receptors. Table 1 summarizes the data of experiments designed to characterize the kinetics of [^{3}H]BK binding and BK-induced PLC activation in transfected COS-7 cells. [^{3}H]BK binding was monitored at 4 °C to avoid temperature-dependent modifications of the receptor triggered by the agonist (*11*). Under these conditions, all of the mutants exhibited the phenomenon of negative cooperativity in BK binding that we previously observed for B_2wt in CHO-K1 and COS-7 cells (*7*, *11*). The kinetics of [^{3}H]BK binding estimated from a Hill plot analysis of data obtained with increasing amounts of labeled BK (1.25×10^{-11} to 2.5×10^{-8} M) were indistinguishable for the various receptors. Half-maximal binding occurred within a narrow range of [^{3}H]-BK concentration (2.4−4.1 nM; Table 1). All of the mutants were able to positively couple to PLC in response to 10^{-11} to 10^{-7} M BK. They responded to BK with maximal stimulation (3.7- to 5.6-fold of the basal value) and EC_{50} values (0.4−1.0 nM) in the same order of magnitude as those observed with B_2wt. Also, basal IPs production was similar in cells expressing the B_2wt or the mutant receptors (0.6−0.8% of total radioactivity; Table 1). These results indicate that the mutations of Cys^{324} and Cys^{329} have no effect on the level of constitutive activity of the receptor and do not alter the kinetics of BK binding and BK-induced stimulation of PLC.

Table 1: [^{3}H]BK Binding and BK-Induced PLC Activation Characteristics of B_2wt and Mutant Receptors in COS-7 Cells[a]

receptor	B_2wt	$C^{324}G$	$C^{329}G$	$C^{324}G/C^{329}G$
	[^{3}H]BK Binding			
Bmax (pmol of [^{3}H]BK bound/mg of protein)	5.49 ± 1.34	5.55 ± 1.44	8.08 ± 1.71	8.59 ± 2.14
KDapp (nM)	3.99 ± 0.63	2.45 ± 0.35	3.33 ± 0.75	4.05 ± 1.07
	PLC Activation			
basal value (% of total radioactivity)	0.65 ± 0.08	0.80 ± 0.08	0.60 ± 0.09	0.72 ± 0.06
BK activation[b]	4.42 ± 0.41	3.72 ± 0.41	5.63 ± 1.17	5.50 ± 0.86
EC_{50} (nM)	0.41 ± 0.06	0.81 ± 0.17	0.81 ± 0.25	1.03 ± 0.50

[a] In each experiment, mutants were studied together with B_2wt as a control. Cells were used 72 h after transfection and incubated at 4 °C for 6 h with seven increasing concentrations of [^{3}H]BK (from 1.25×10^{-11} to 2.5×10^{-8} M) before determination of specific binding (see the Experimental Procedures). Data were first plotted using Scatchard coordinates. Given the nonlinear character of the plots obtained (see also Pizard et al. (*11*)), binding values at the three highest [^{3}H]BK concentrations were used to estimate the maximal binding capacity (Bmax). All of the binding values were then plotted using Hill coordinates to estimate the [^{3}H]BK concentration (KDapp), corresponding to half-saturation of binding sites (see Pizard et al. (*11*)). For determination of IPs production, cells at 72 h after transfection were incubated at 37 °C for 15 min with 10^{-11}−10^{-7} M BK and 10 mM LiCl. Results are the mean ± SEM of three to five independent experiments, each performed in triplicate. [b] The values represent a ratio between maximal (10^{-7} M BK) and basal IPs productions.

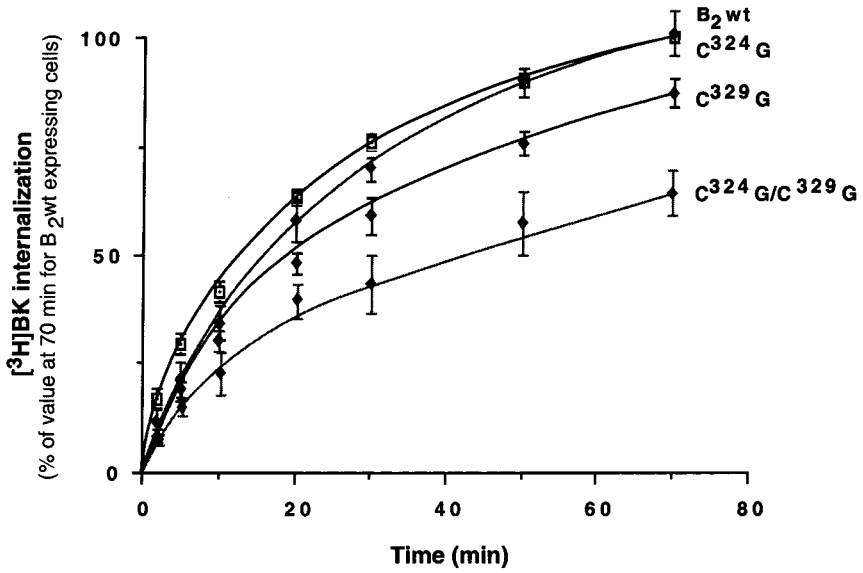

FIGURE 3: [^{3}H]BK internalization in COS-7 cells transfected with B_2wt or mutant receptors. Cells, used 72 h after transfection, were incubated with 2 nM [^{3}H]BK at 37 °C for the time indicated in the presence or absence of 10 μM unlabeled BK. Unbound radioactivity was removed at 4 °C before cell surface-associated and internalized radioactivities were separated and quantitated, as described in the Experimental Procedures. After subtraction of nonspecific binding, the fraction of ^{3}H radioactivity that was internalized (acid resistant) was calculated for each incubation time and each type of transfected cells. Then, each fraction was converted into a percentage of the value at 70 min for the cells expressing the B_2wt receptor; this latter represented 60.3 ± 2.5% of the [^{3}H]BK bound (corresponding to 0.11 ± 0.01 pmol/mg of protein, $n = 22$). Values are means ± SEM from three to five independent experiments, each performed in duplicate. Internalization by $C^{324}G/C^{329}G$ and $C^{329}G$ mutants is statistically different from that by B_2wt after 10 and 30 min, respectively; those mutants differed from each other after 50 min ($p < 0.05-0.001$, Bonferroni one-way ANOVA test).

To examine whether these mutations influence receptor trafficking, [^{3}H]BK internalization was measured at 37 °C in COS-7 cells using 2 nM of [^{3}H]BK (Figure 3). In cells expressing B_2wt, the internalization represented 60.3 ± 2.5% ($n = 22$) of bound [^{3}H]BK at 70 min. The single mutations $C^{324}G$ and $C^{329}G$ had only a minor effect on the internalization, but when these mutations were combined, the internalization was reduced by approximately 30% as compared to B_2wt and represented only 45.2 ± 4.5% ($n = 4$) of the bound [^{3}H]BK at 70 min. This suggests that the substitution of the two cysteines of the receptor carboxyl tail makes the receptor less susceptible to the BK-induced sequestration processes.

We have previously demonstrated that B_2 receptor phosphorylation is required for subsequent internalization after agonist activation (*7*). Therefore, we studied the BK-induced phosphorylation of $C^{324}G$, $C^{329}G$, and the double mutant. Immunoprecipitation was carried out after [^{32}P] labeling of cells transfected with the various receptors and stimulated with 1 μM BK. The data (Figure 4) show that, for each receptor, BK treatment resulted in an increase in the phosphorylation level, even for the double mutant receptor $C^{324}G/C^{329}G$, which exhibited a reduced internalization. We could not find major differences in the ligand-induced overall phosphorylation of B_2wt and mutated receptors.

In summary, the data obtained in transfected COS-7 cells indicate that the kinetics of [^{3}H]BK binding, the basal and agonist-stimulated coupling to PLC, and the phosphorylation capacity of the receptor were unchanged after palmitoylation-site mutation, whereas the internalization capacity of the nonpalmitoylated mutant was moderately reduced by 30% as compared to B_2wt.

Stable Expression of the Mutants in CHO-K1 Cells: Studies of Antagonists. To further characterize the functional consequences of receptor palmitoylation defects and examine their effect on the pharmacological profile of the B_2 receptor, we established CHO-K1 cell lines which stably express the mutant receptors. CHO-K1 cell lines have the advantage that, at a comparable level of receptor expression, BK-induced stimulation of PLC activity is much more pronounced than in COS-7 cells (*7*; compare also the data of Tables 1 and 2 in this study), suggesting a more efficient G protein coupling of the receptor. Furthermore, PLA_2 activation can be followed in CHO-K1 but not in COS-7 cells (*7*, *11*). We used the stable CHO-K1 cell lines to examine the recognition of pharmacological ligands by the receptors. A cell line expressing B_2wt was used as a control (*11*). Two independent $C^{324}G/C^{329}G$ mutant expressing clones displaying a large difference in their receptor expression levels were studied to assess the effect of variations in receptor density on the pharmacological profile. We first established that the palmi-

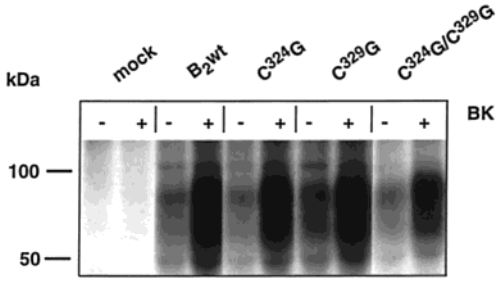

FIGURE 4: BK-induced phosphorylation of B_2wt and mutant receptors. COS-7 cells used 40 h after transfection were radiolabeled with [^{32}P]orthophosphate and incubated for 5 min without (−) or with 1 μM BK (+). Cells were lysed and B_2 receptors immunoprecipitated. Radiolabeled proteins were analyzed by reducing 10% SDS−PAGE and autoradiography. Relative molecular masses determined with standard proteins are indicated on the left. Data are representative of three independent experiments with similar results.

Table 2: [^{3}H]BK Binding and BK-Induced PLC and PLA_2 Activation Characteristics of B_2wt and Mutant Receptors in CHO-K1 Cells[a]

receptor	B_2wt	$C^{324}G$	$C^{329}G$	$C^{324}G/C^{329}G$ A	$C^{324}G/C^{329}G$ B
			[^{3}H]BK Binding		
Bmax (pmol of [^{3}H]BK bound/mg of protein)	2.2 ± 0.3	7.2 ± 1.2	5.2 ± 1.6	5.4 ± 0.9	1.6 ± 0.6
KDapp (nM)	22.7 ± 6.3	17.0 ± 9.2	8.6 ± 2.9	13.0 ± 4.9	17.7 ± 2.2
			PLC Activation		
basal value (% of total radioactivity)	1.84 ± 0.39	1.99 ± 0.36	2.09 ± 0.14	1.83 ± 0.12	1.60 ± 0.15
BK activation[b]	39.31 ± 0.93	64.97 ± 1.23	45.09 ± 2.39	69.75 ± 0.96	36.18 ± 6.34
EC_{50} (nM)	0.61 ± 0.08	0.30 ± 0.06	0.23 ± 0.06*	0.41 ± 0.03	0.36 ± 0.05
			PLA_2 Activation		
basal value (% of total radioactivity)	1.50 ± 0.13	1.31 ± 0.12	1.46 ± 0.13	1.46 ± 0.13	nd[c]
BK activation[b]	6.27 ± 0.09	9.92 ± 1.62	7.43 ± 0.08	10.26 ± 0.38	nd
EC_{50} (nM)	0.92 ± 0.07	0.66 ± 0.17	0.20 ± 0.01**,§	0.81 ± 0.16	nd

[a] For each experiment, mutants were studied together with B_2wt as a control. Two independent clones (A and B) with different receptor expression levels (see Bmax) were studied for the $C^{324}G/C^{329}G$ mutant. CHO-K1 cells were incubated overnight at 4 °C with thirty increasing concentrations of [^{3}H]BK (from 10^{-11}−10^{-7} M). Data were obtained as explained in the legend of Table 1. In the phospholipases activation experiments, cells were incubated at 37 °C with 10^{-11} to 10^{-7} M BK for 10 min before measurement of PLA_2 activation and 15 min in the presence of 10 mM LiCl before determination of IPs production. Results are the mean ± SEM of three to five independent experiments. Symbols: (*) for $p < 0.05$ and (**) for $p < 0.01$ as compared to B_2wt and (§) for $p < 0.05$ as compared to $C^{324}G/C^{329}G$ (Bonferroni one-way ANOVA test). [b] The values represent maximal (10^{-7} M BK) IPs or AA production minus basal production, expressed in a percent of the total radioactivity. [c] nd: not determined.

Table 3: Effects of BK and B_2 Receptor Antagonists on PLC and PLA_2 Activity in CHO-K1 Cells Expressing B_2wt or the $C^{324}G/C^{329}G$ Mutant[a]

receptor	B_2wt	$C^{324}G/C^{329}G$ A	$C^{324}G/C^{329}G$ B
Bmax (pmol of [^{3}H]BK bound/mg of protein)	2.2 ± 0.3	5.4 ± 0.9	1.6 ± 0.6
	PLC Activation		
basal value (% of total radioactivity)	1.84 ± 0.39	1.83 ± 0.12	1.60 ± 0.15
BK[b]	39.31 ± 0.93	69.75 ± 0.96	36.18 ± 6.34
HOE 140[b]	0.94 ± 0.56	35.07 ± 1.66	19.29 ± 5.23
LF 16.0335[b]	−0.08 ± 0.3	16.21 ± 0.24	4.96 ± 1.36
LF 16-0687[b]	0.34 ± 0.28	25.94 ± 0.60	8.11 ± 1.40
BK + HOE 140[b]	6.08 ± 1.01	52.58 ± 1.55	28.04 ± 4.43
BK + LF 16.0335[b]	5.62 ± 0.25	34.61 ± 0.82	21.74 ± 4.92
BK + LF 16-0687[b]	0.40 ± 0.28	27.41 ± 0.29	13.42 ± 1.94
	PLA_2 Activation		
basal value (% of total radioactivity)	1.50 ± 0.13	1.46 ± 0.13	nd[c]
BK[b]	6.27 ± 0.09	10.26 ± 0.38	nd
HOE 140[b]	0.42 ± 0.11	4.30 ± 0.12	nd
LF 16.0335[b]	−0.29 ± 0.09	2.31 ± 0.24	nd
LF 16-0687[b]	0.45 ± 0.17	1.84 ± 0.17	nd
BK + HOE 140[b]	1.02 ± 0.42	5.08 ± 0.24	nd
BK + LF 16.0335[b]	0.80 ± 0.13	4.16 ± 0.06	nd
BK + LF 16-0687[b]	0.98 ± 0.12	2.99 ± 0.67	nd

[a] Data were obtained as explained in the legend of Table 1. Cells were incubated at 37 °C with various amounts of ligand for 10 min before the measurement of PLA_2 activation and 15 min in the presence of 10 mM LiCl before the determination of IPs production. Results are the mean ± SEM of three to five independent experiments. [b] The values represent IPs or AA production at maximal concentration of BK (10^{-7} M) or antagonists (10^{-5} M) minus the corresponding basal production, all expressed in a percent of the total radioactivity. [c] nd: not determined.

toylation of the various receptors occurred in CHO-K1 cells with a pattern similar to that observed in COS cells (data not shown). The results given in Table 2 show that all of the mutants were able to bind [^{3}H]BK and to trigger PLC as well as PLA_2 activation in response to BK stimulation with kinetics similar to those of B_2wt. BK-stimulated IPs production and arachidonic acid release were related to the receptor expression level, but there was no influence of the mutations (Table 2). These results confirm the observations made in COS cells that the various mutations of the receptor have no major effect on the basal activity of the receptor, the characteristics of BK binding, and BK activation of PLC. In addition, they show that BK stimulation of PLA_2 is not influenced by the mutations.

We observed that the inhibitory effect of B_2 receptor antagonists on BK-induced activation of PLC and PLA_2 was reduced in the nonpalmitoylated $C^{324}G/C^{329}G$ mutant (25−70% inhibition instead of 85−99% in B_2wt). This is illustrated in Table 3, presenting data of competition experiments using BK and the pseudopeptide antagonist HOE 140 or two nonpeptide antagonists, LF 16.0335 and LF 16-0687. The loss of the antagonist property of these compounds on BK-induced PLC and PLA_2 activation was, in fact, related to their agonist effect on the mutant (Table 3 and Figure 5). HOE 140 elicited a stronger activation than LF 16.0335 and LF 16-0687 in both PLC and PLA_2 assays. The maximum effect of HOE 140 corresponded to roughly 50% of the effect produced by BK. The same effect was observed for the two clones of the $C^{324}G/C^{329}G$ mutant that differed by a factor of 3 in their Bmax and had either a lower or higher expression level than the B_2wt expressing clone (Table 3). The EC_{50} values for PLC activation for these two clones

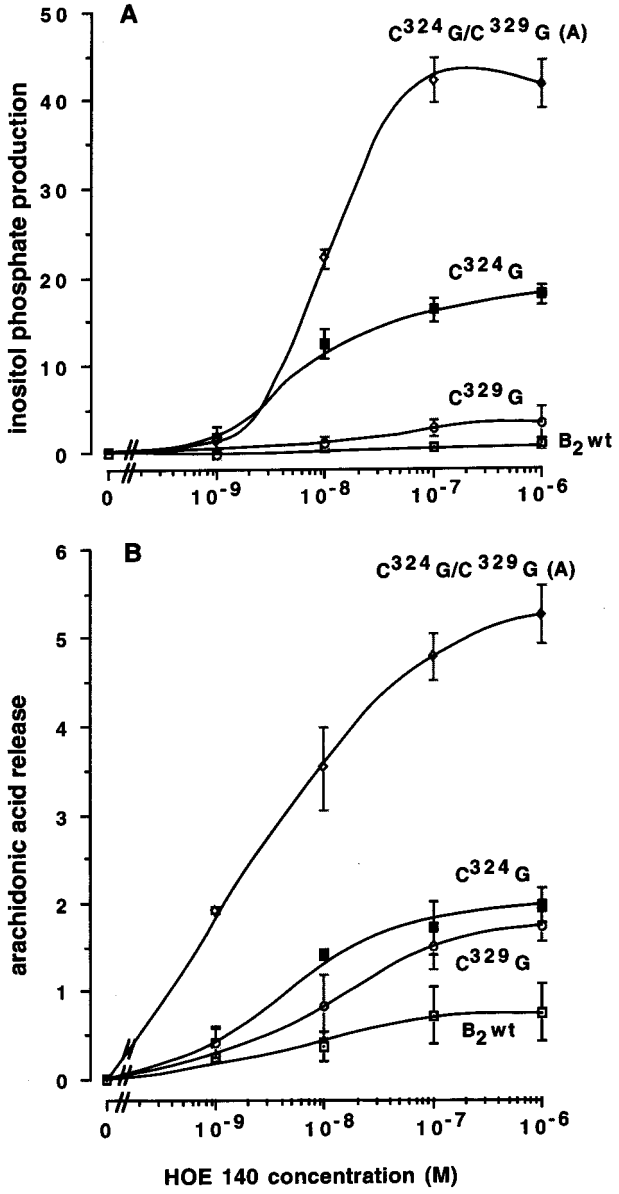

FIGURE 5: Effect of HOE 140 on PLC and PLA_2 activity in CHO-K1 cells expressing B_2wt or mutant receptors. Experiments were conducted as described in the legend of Table 1 except that CHO-K1 cells were incubated with $10^{-9}-10^{-6}$ M HOE 140 instead of BK. Inositol phosphate production (panel A) and arachidonic acid release (panel B) were expressed in a percentage of the total radioactivity incorporated in cells, after subtraction of the corresponding basal value. Results are means ± SEM of three to five independent experiments, each performed in duplicate.

were 2.9 ± 0.6 and 3.0 ± 0.5 nM, respectively. The EC_{50} value in the PLA_2 assay was 2.5 ± 0.9 nM, as determined for one of the clones (clone A, Table 3). LF 16.0335 and LF 16-0687 also displayed EC_{50} values in the nanomolar range (1.8 ± 1.2 (PLC) and 8.8 ± 5.8 nM (PLA_2) for LF 16.0335 and 0.3 ± 0.3 (PLC) and 5.3 ± 3.3 nM (PLA_2) for LF 16-0687). An agonist effect of a smaller magnitude was also observed on the single cysteine mutants (Figure 5). On B_2wt, LF 16.0335 was an antagonist, whereas HOE 140 and LF 16-0687 may be very weak partial agonists (Table 3). Thus, the mutations produced both an enhancement of agonism and a creation of agonism from an antagonism.

It is also known that the B_2 receptor is coupled in several cells to MAP kinase activation, potentially involved in the proliferative response observed upon BK treatment (*19*−*21*). We, therefore, tested whether the B_2 receptor deficient for palmitoylation responded to HOE 140 by activation of the MAP kinase pathway in transfected HEK 293T cells. The application of HOE 140 increased the activity of ERK2 in a dose-dependent manner when these cells were transfected with this kinase and the nonpalmitoylated mutant receptor $C^{324}G/C^{329}G$ (Figure 6). The maximal effect corresponded to a 7.5-fold increase and was achieved with 1 μM of HOE 140. Cells expressing B_2wt also responded to HOE 140 but with only a 2-fold increase in ERK2 activity, a response which is very small as compared to the 30- to 40-fold increase observed with BK (*6*).

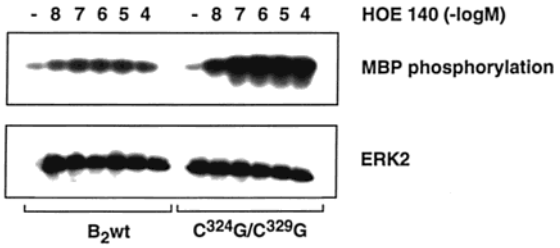

FIGURE 6: HOE 140-induced ERK2 activation by B_2wt and the nonpalmitoylated $C^{324}G/C^{329}G$ mutant. HEK 293T cells were cotransfected with HA-tagged ERK2 and B_2wt or $C^{324}G/C^{329}G$. After a 5 min application of HOE 140, ERK2 activity (upper panel) was determined after immunoprecipitation of the kinase with a polyclonal anti-HA antibody and using myelin basic protein (MBP) as a substrate. Reactions were carried out for 20 min at room temperature. Phosphorylated MBP was detected by autoradiography. A western blot (lower panel) with a polyclonal anti-ERK2 antibody was performed to confirm that equal amounts of ERK2 were present in the precipitates. The autoradiogram and western blot shown are representative of three independent experiments with similar results.

These observations indicate that mutation of the cysteine residues of the cytoplasmic tail results in B_2 receptors that positively couple to signaling pathways upon interaction with compounds that normally stabilize an uncoupled form of the receptor.

DISCUSSION

Most GPCRs have one or more cysteine residues in the proximal part of the carboxyterminal cytoplasmic tail near the seventh transmembrane domain (*22*). Receptor palmitoylation may occur at these cysteines. The two cysteine residues of the human B_2 BK receptor carboxyl tail, Cys^{324} and Cys^{329}, are located 15 and 20 amino acids after the predicted seventh transmembrane domain. The present study shows that these two cysteines can be palmitoylated and probably play an important role in the conformation of the B_2 BK receptor tail. This is based on the findings that this receptor incorporated [^{3}H]palmitic acid in metabolically labeled cells, that the incorporated [^{3}H]palmitate was released by neutral hydroxylamine treatment, and that [^{3}H]palmitate incorporation was prevented by the mutation of Cys^{324} and Cys^{329}. The mutagenesis approach cannot, however, allow for the conclusion that the two cysteines are always simultaneously palmitoylated in the natural receptor. Suppression of one cysteine can indeed result in an alternative palmitoylation of the other cysteine, even if the palmitoylation enzyme has a lower affinity for the residue than for the mutated cysteine in the wild-type receptor. However, the observation that the single mutants lacking either Cys^{324} or

Cys^{329} exhibited a reduced incorporation of [^{3}H]palmitate as compared to B_2wt suggests that both cysteines are indeed palmitoylated in the human B_2 receptor. For the rat receptor, tandem mass spectrometry analysis suggested that the cysteine residue equivalent to human receptor Cys^{324} is palmitoylated (*23*). This study did not reveal whether the cysteine residue equivalent to human Cys^{329} is palmitoylated or not.

There appears to be no common rule applicable to all GPCRs concerning the effects of palmitoylation-site mutation on receptor function. In the B_2 receptor, substitution of either Cys^{324}, Cys^{329}, or of both cysteines by glycine suppressing palmitoylation caused no changes in the kinetics of BK binding and basal or BK-stimulated coupling to PLC and PLA_2. The fact that the nonpalmitoylated receptor mutant resembles the wild-type receptor in coupling capacity to signaling pathways has been observed for several other members of the GPCR family, while for a few receptors, the coupling efficiency was found to be reduced (*8*−*10*).

Similar to what was observed for m_2 muscarinic and α_2-adrenergic receptors (*24*, *25*), the basal and agonist-induced phosphorylation of the nonpalmitoylated human B_2 receptor were unaltered as compared to B_2wt. This contrasts with the β_2-adrenergic receptor, where the palmitoylation-defective mutant exhibited a constitutive hyperphosphorylation and failed to undergo additional phosphorylation after agonist treatment (*26*, *27*). The β_2-adrenergic receptor mutant behaved as a constitutively desensitized receptor (*27*, *28*). Because in the wild-type receptor the palmitate turnover was increased following agonist treatment (*29*−*32*), it has been proposed that the alterations in palmitoylation induced by the mutation or by the agonist lead to an increased susceptibility of the receptor carboxyl tail phosphorylation sites to kinase action and subsequent desensitization. This is not the case for the B_2 receptor, and the nonpalmitoylated receptor coupled to signaling pathways in response to BK to a similar extent as B_2wt, indicating that this mutant is not in a desensitized state, unlike the β_2-adrenergic receptor.

The only detectable alteration in the receptor response to BK after palmitoylation-site mutation was a change in the internalization of [^{3}H]BK. It was moderately reduced by 30% in the nonpalmitoylated $C^{324}G/C^{329}G$ mutant as compared to that of B_2wt. Single mutation at position 324 had no effect, in agreement with the observation of Faussner et al. (*33*), and mutation at position 329 caused only a slight reduction. This suggests that palmitoylation is necessary for an optimal efficiency of the internalization process but that a single palmitoylation may be sufficient for this function. The mutation effect may be also due, at least in part, to additional structural modifications such as kinks and helix disruption in the C-terminal domain induced by the replacement of cysteine by glycine. Among the other GPCRs studied, carboxyl tail cysteine mutation had no consistent effect on internalization, because either no change, a decrease, or an increase in the internalization capacity was observed (*8*).

Our observation that palmitoylation-defective human B_2 receptors positively couple to intracellular signaling pathways in response to antagonists has not been reported for any other GPCRs. A stimulatory effect was observed with a pseudopeptide B_2 receptor antagonist as well as with two nonpeptide antagonists. These compounds were able to trigger PLC and PLA_2 activation with EC_{50} values in the nanomolar concentration range, similar to the natural agonist BK. The response was more pronounced with the nonpalmitoylated $C^{324}G/C^{329}G$ mutant than after a single-cysteine mutation, suggesting a correlation with the receptor palmitoylation level. Furthermore, an activation of the MAP kinase signaling pathway by HOE 140 was observed with the nonpalmitoylated receptor. Because their phospholipases and ERK2 stimulatory effect was 25−50% of that of BK, these antagonists should be considered as partial agonists on the modified receptor. Recognition of antagonists as agonists by a mutated B_2 receptor has already been reported after a mutation of Asn^{113} and Trp^{256} in the human receptor (*34*). Those amino acids are located in transmembrane domains III and VI that are thought important for transduction of the ligand binding signal into receptor activation and coupling (*35*). The vasopressin V_2 receptor is another example of a GPCR where mutation of an amino acid, Asp^{130} close to transmembrane domain III, leads to the recognition of antagonists as agonists (*36*). However, these B_2-receptor and V_2-receptor mutants had acquired a constitutive coupling activity and a marked increase in the sensitivity to the agonist. In the case of the carboxyl tail cysteine mutations of the human B_2 receptor, measurements of basal PLC, PLA_2, and ERK2 activities indicated that no constitutive activation has occurred. Thus, the capacity to couple positively to cellular effectors in response to antagonists can be dissociated from the constitutive level of coupling of the receptor. The conformational alteration of the receptor induced by the carboxyl tail cysteine mutations is probably more subtle than that induced by the transmembrane domain mutations and results in a facilitated receptor-G protein interaction only after an additional alteration induced by ligand binding.

Our observation can explain the partial agonist effect observed with HOE 140 in some cellular systems (*37*, *38*), whereas in other systems, this compound was considered as a neutral antagonist or even as an inverse agonist (*39*). Palmitoylation-defective B_2 receptor mutants may indeed represent a model of naturally occurring partially palmitoylated or nonpalmitoylated forms of the receptor that positively couple to signaling upon antagonist binding.

The present study shows that palmitoylated cysteines are an important determinant of antagonist−B_2 receptor complex conformation, and it suggests that these cysteines influence the receptor coupling−uncoupling capacity to G proteins. Further studies can determine whether this is a general function of palmitoylation in the GPCR family.

ACKNOWLEDGMENT

We thank Dr. Didier Pruneau from Laboratoire Fournier (Daix, France) and Dr. Klaus Wirth from Aventis (Frankfurt am Main, Germany) for the generous gift of B_2 receptor antagonists and Dr. Jeannine Marchetti and Dr. David A. Mc Conner for constructive discussions.

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BI011600T