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Regulation of the Human Bradykinin B2 Receptor Expressed in sf21 Insect Cells: A Possible Role for Tyrosine Kinases

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Abstract The functional regulation of the human bradykinin B2 receptor expressed in sf21 cells was studied. Human bradykinin B2 receptors were immunodetected as a band of 75–80 kDa in membranes from recombinant baculovirus-infected cells and visualized at the plasma membrane, by confocal microscopy, using an antibody against an epitope from its second extracellular loop. B2 receptors, detected in membranes by [^{3}H-bradykinin] binding, showed a Kd of 0.66 nmol/L and an expression level of 2.57 pmol/mg of protein at 54 h postinfection. In these cells, bradykinin induced a transient increase of intracellular calcium ($[Ca^{2+}]_i$) in fura 2-AM loaded sf21 cells, and promoted [^{35}S]-GTP$_\gamma$S binding to membranes. The effects of bradykinin were dose dependent (with an EC_{50} of 50 nmol/L for calcium mobilization) and were inhibited by N-α-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8},D-phe^7]-Bk, a specific B2 receptor antagonist. When the B2 antagonist was applied at the top of the calcium transient, it accelerated the decline of the peak, suggesting that calcium mobilization at this point was still influenced by receptor occupation. No calcium mobilization was elicited by 1 μmol/L (Des-Arg^9)-Bk, a B1 receptor agonist that did not inhibit the subsequent action of 100 nmol/L bradykinin. No effect of bradykinin was detected in uninfected cells or cells infected with the wild-type baculovirus. Bradykinin-induced $[Ca^{2+}]_i$ mobilization was increased by genistein and tyrphostin A51. These tyrosine kinase inhibitors did not modify basal levels of $[Ca^{2+}]_i$. Homologous desensitization of the B2 receptor was observed after repeated applications of bradykinin, which resulted in attenuated changes in intracellular calcium. In addition, genistein promoted an increased response to a third exposure to the agonist when applied after washing the cells that had been previously challenged with two increasing doses of bradykinin. Genistein did not affect the calcium mobilization induced by activation of the endogenous octopamine G protein-coupled receptor or by thapsigargin. The B2 receptor, detected by confocal microscopy in unpermeabilized cells, remained constant at the surface of cells stimulated with bradykinin for 10 min, in the presence or absence of genistein. Agonist-promoted phosphorylation of the B2 receptor was markedly accentuated by genistein treatment. Phosphoaminoacid analysis revealed the presence of phosphoserine and traces of phosphothreonine, but not phosphotyrosine, suggesting that the putative tyrosine kinase(s), activated by bradykinin, could act in a step previous to receptor phosphorylation. Interestingly, genistein prevented agonist-induced G protein uncoupling from B2 receptors, determined by in vitro bradykinin-stimulated [^{35}S]-GTP$_\gamma$S binding, in membranes from bradykinin pretreated cells. Our results suggest that tyrosine kinase(s) regulate the activity of the human B2 receptor in sf21 cells by affecting its coupling to G proteins and its phosphorylation. J. Cell. Biochem. 76:658–673, 2000.

Key words: human bradykinin receptor; receptor desensitization; intracellular calcium; Sf21 cells

Bradykinin (Bk) is a vasoactive nonapeptide, generated at sites of tissue damage, that contributes to inflammation and pain. Long-term actions of Bk include activation of proliferation pathways. Cellular effects of this hormone are mediated through G-protein coupled receptors characterized by their seven transmembrane spanning helices. Currently, mammalian Bk receptors are classified in two types: B1 and B2; however, other types exist in nonmammalian

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species [Schroeder et al., 1997; Regoli et al., 1993, 1994]. In a wide variety of cells, Bk receptors are coupled to Pertussis toxin (PTX)-insensitive G proteins and phospholipase C. The release of calcium from intracellular stores, followed by influx of this cation from the extracellular space, is a typical response to the occupation of B2 receptor by agonists [Hall, 1992; Regoli et al., 1993, 1994]. In fibroblasts and endothelial cells, Bk activates diverse kinases, as a consequence of B2 receptor action, including isoforms of protein kinase C (PKC) and tyrosine kinases such as Src and Fak [Lee and Villereal, 1996; Rodriguez-Fernández and Rozengurt, 1996].

Homologous desensitization of the B2 receptor by GRK-2-catalyzed serine and threonine phosphorylation was recently reported [Blaukat et al., 1996]. Direct activation of PKC with active phorbol esters also induced some receptor phosphorylation [Blaukat et al., 1996]. Immunoblot analysis has evidenced the presence of phosphotyrosine in immunoprecipitated B2 receptors from unstimulated WI-38 human lung fibroblasts [Jong et al., 1993]. In addition, specific tyrosine residues in the intracellular domains of this receptor are required for the efficient coupling of B2 to the production of inositol phosphates [Prado et al., 1997]. A recent work determined the phosphorylation of these specific tyrosine residues by mass spectrometric analysis of rat B2 receptors isolated from transfected unstimulated CHO cells [Soskic et al., 1999]. The ability of Bk receptors to induce activation of tyrosine and serine-threonine kinases [Lee and Villereal, 1996], the fact that some PKC isoforms are regulated by tyrosine phosphorylation [Denning et al., 1996; Zang et al., 1997], and the ability of PKC to regulate the activity of GRK-2 [Chuang et al., 1995] opens the possibility that tyrosine kinases may regulate, in a direct or indirect way, the activity of B2 bradykinin receptor.

Baculovirus-mediated expression of serpentine receptors in insect cells is a useful system for the study of specificity of G-protein coupling [Barr et al., 1997; Leopold et al., 1997; Nishimura et al., 1998a; Richardson et al., 1992], calcium homeostasis [Hu et al., 1994] and homologous and heterologous regulation of several G-protein coupled receptors (e.g., serotonin, muscarinic, dopamine, endothelin, thrombine, histamine, neurokinin receptors) [Chen et al., 1996; Kukkonen et al., 1996, 1998; Satoh et al.,1997; Hu et al.,1994; Nishimura et al., 1998b; Fukushima et al., 1997; Beukers et al., 1997]. We explored the regulation of the human B2 receptor functionally expressed in sf21 insect cells. As expected, the recombinant B2 receptor was coupled to intracellular calcium mobilization. Bk induced homologous desensitization of the B2 receptor. Interestingly, inhibitors of tyrosine kinases increased ligand-promoted $[Ca^{2+}]_i$ mobilization, in vitro bradykinin-stimulated [^{35}S]GTPγS binding to membranes from agonist stimulated cells, and enhanced agonist-elicited phosphorylation of the B2 receptor. These data suggest a novel role for tyrosine kinases in the modulation of B2 receptor function.

MATERIALS AND METHODS

Reagents

All reagents used in the present study were analytical grade. Bradykinin, (Des-Arg^9)-Bk, (Des-Arg^9,Leu^8]-Bk, N-α-adamantaneacetyl-D-Arg-[Hyp^3, Thi^{5,8},D-phe^7]-Bk, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid (EGTA), and *N*-[2-hydroxyethyl]-piperazine-*N′*-[2-ethanesulfonic acid] (HEPES) were purchased from Sigma (St. Louis, MO). Genistein was purchased from RBI (Natick, MA), and tyrphostin A51 was obtained from Calbiochem (La Jolla, CA). Fura-2-AM was purchased from Molecular Probes (Eugene, OR). [^{3}H]Bk (90 Ci/mmol), [^{32}P]-orthophosphate (8,500–9,120 Ci/mmol) and [^{35}S]GTP-γ-S (1,250 Ci/mmol) were purchased from NEN Life Science Products, Boston, MA.

Cell Culture

Sf21 insect cells were obtained from Invitrogen (San Diego, CA) and cultured in Grace's medium (Sigma) supplemented with lactalbumin hydrolysate, yeastolate, 2 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Gibco, Grand Island, NY) as previously described [O'Relly et al., 1992]. Cells were grown on plastic tissue-culture dishes (Costar, Cambridge, MA).

Production of Recombinant Baculoviruses

The cDNA encoding the human type II bradykinin receptor (B2) was subcloned into the baculovirus vectors pBlueBac4 and pBlueBacHis2A (Invitrogen) using standard ligation techniques [O'Relly et al., 1992; Sambrook et al., 1989]. The pBlueBacHis2A vector adds an epitope (6

amino acids) to the N-terminal domain of the B2. We used this construct to immunoprecipitate the receptor using the commercial antibody Anti-Xpress (Invitrogen). The orientation of the cDNA was confirmed by restriction mapping. Recombinant baculoviruses were produced following the manufacturer instructions (Invitrogen). Baculoviruses were purified twice using the plaque assay and amplified. The titer of the amplified product was determined following the expression of β-galactosidase [O'Relly et al., 1992]. The final virus stock was maintained at 4°C until use. The virus multiplicity of infection used in this study was 1. All experiments were performed between 45 and 54 h postinfection.

[^{3}H]Bk Binding Experiments

Receptor expression and affinity was determined by [^{3}H]Bk saturation binding experiments as described elsewhere [Abd Alla et al., 1993; De Werd and Leeb-Lundberg, 1997] Membranes from B2 receptor baculovirus-infected sf21 cells, prepared as described for [^{35}S]GTPγS binding (see Membrane Preparation and [^{35}S]GTPγS Binding), were obtained 54 h postinfection. In brief, membranes (40 μg/tube in a final volume of 250 μl) were incubated in 25 mmol/L N-tris [Hydroxymethyl]methyl-2-amino ethane sulfonic acid (TES), pH 7.2, 0.5 mmol/L EDTA, 0.2 mmol/L $MgCl_2$, 1 mmol/L 1,10-phenantroline, and 140 μg/ml bacitracin supplemented with 0.1% bovine serum albumin (BSA) and increasing concentrations of [^{3}H]Bk for 45 min at 25°C. Specific binding was determined by subtraction of nonspecific binding that occurred in the presence of 100 nmol/L cold Bk from total binding, determined in the absence of unlabeled Bk. The bound radiolabeled ligand was separated from the free ligand by filtration over GF/C filters (Sigma), precoated with 0.3% polyethyleneimine, using a Brandell harvester. Filters were washed three times with ice-cold 50 mmol/L Tris, pH 7.4, 10 mmol/L $MgCl_2$ buffer, and bound radioactivity was determined by liquid scintillation counting.

Western Blotting Analysis

Membranes from sf21 uninfected or B2-expressing cells were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 7 mol/L urea gels and transferred to nitrocellulose sheets using semi-dry blotting (Biorad, Hercules, CA). The sheets were treated as described by Abd Alla et al. [1996]. Briefly, sheets were blocked with 50 mmol/L Tris, 0.2 mol/L NaCl, pH 7.4 (buffer A), containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h. Antisera against the carboxyl-terminus of B2 receptor (intracellular domain; I D4 AS 346/2; Abd Alla et al., 1996] was diluted 1:1,000 in buffer A containing 2% bovine serum albumin. After 30 min of incubation at 37°C, the nitrocellulose sheets were washed five times for 15 min each with buffer A and incubated for 30 min with peroxidase-labeled $F(ab)_2$ fragments of mouse anti-rabbit antibody (1:5,000; Sigma). After extensive washing, bound antibody was visualized with Supersignal Ultra chemiluminescence detection kit (Pierce, Rockford, IL).

Fluorescence Microscopy

For detection of B2 receptors in sf21 cells, cells were grown on glass coverslips and infected with B2 baculovirus. The cells were washed with extracellular solution (10 mmol/L NaCl, 60 mmol/L KCl, 25 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 4 mmol/L D-glucose, 15 mmol/L HEPES, 100 mmol/L mannitol, 0.1% bovine serum albumin, pH 6.2) 48 h postinfection and stimulated as described in figure legends. Then cells were fixed with 3.0% paraformaldehyde in PBS for 30 min washed three times with phosphate-buffered saline (PBS), and incubated overnight at 4°C with a rabbit polyclonal antibody directed against amino acids 91–105 of the second extracellular loop of the receptor (AS 276) [Abd Alla et al., 1996] (1:100 dilution) in PBS supplement with 0.5% bovine serum albumin. The cells were then washed three times with PBS, and incubated for 4 h at room temperature with a secondary anti-rabbit antibody coupled to fluorescein isothiocyanate (Zymed, San Francisco, CA). The coverslips were embedded in immunofluorescence mounting medium (Dako, Carpinteria, CA) onto glass slides and fluorescence was observed using an MRC 1024 Biorad confocal microscope and analyzed with Laser Sharp ver. 2.1 T software. Uninfected Sf21 cells and B2-expressing cells where the anti-B2 antibody was omitted (negative controls) were simultaneously analyzed.

Intracellular Calcium Measurements

Confluent sf21 cells, expressing B2 receptor, were mechanically dispersed with a plastic pipette, and placed in the extracellular solution to a final concentration of 1.5–2 million cells/ml

and incubated with 5 μmol/L Fura-2-AM for 45 min at room temperature. After this, the cells were washed twice and incubated for 30 min in Fura-2-AM-free solution. Then the cells were washed three to four times and placed in the cuvette of an Aminco-Bowman series 2 luminescence spectrometer (SLM series II). The excitation wavelength alternated every second between 340 and 380 nm and the emission was collected at 510 nm. Each experiment was individually calibrated to obtain the maximum fluorescence after disrupting the cells with 0.1% Triton X-100; the minimum fluorescence was obtained after chelating the calcium in the solution with 20 mmol/L EGTA. The values obtained with this procedure were used to calculate the intracellular calcium concentration according to previously published equations [Grynkiewicz et al., 1985]. Some experiments were made in the absence of extracellular calcium; in those experiments, 5 mmol/L EGTA was added to the extracellular solution.

Phosphorylation of B2 Receptors

Sf21 cells, grown to a density of 2 million cells/ml in 10-cm culture dishes, were infected with the B2 baculovirus as described above. The day of the experiment, cells were maintained in extracellular solution for 1 h and then incubated in 3 ml of the same solution containing [^{32}P]-orthophosphate (0.2 mCi/ml) for 3 h at 22°C. Labeled cells were preincubated with genistein or vehicle and stimulated with Bk; then cells were washed twice with ice-cold PBS and solubilized with 1.0 ml of ice-cold solubilization buffer (10 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.05% SDS, 50 mmol/L NaF, 100 μmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, 10 mmol/L sodium pyrophosphate, 1 mmol/L p-serine, 1 mmol/L p-threonine, and the protease inhibitors: leupeptin 20 μg/ml, aprotinin 20 μg/ml, PMSF 100 μg/ml, bacitracin 500 μg/ml, and soybean trypsin inhibitor 50 μg/ml). The extracts were maintained for 1 h on ice, centrifuged at 12,700 *g* for 15 min at 4°C, and the supernatants were transferred to new tubes containing 5 μl of B2 antiserum against the carboxyl-terminus sequence of the receptor (intracellular domain; ID 4 AS 346/2; Abd Alla et al., 1996). On a different set of experiments, the supernatants of cells expressing the epitope-tagged B2 receptor were transferred to tubes containing 5 μl of the Xpress antibody (Invitrogen). Immunoprecipitation was performed as previously reported [Vázquez-Prado et al., 1997]. Briefly, tubes were incubated overnight at 4°C. Then 40 μl of protein A agarose beads (50% slurry) were added and incubated for 1 h at the same temperature. Beads were washed: (a) five times (1 ml/each) with a solution containing: 50 mmol/L HEPES, 50 mmol/L NaH_2PO_4, 100 mmol/L NaCl, pH 7.2, 1.0% Triton X-100, 0.05% SDS, and 100 mmol/L NaF; (b) once with 50 mmol/L Tris, 0.15 mol/L NaCl, pH 7.4; and (c) finally once with 10 mmol/L Tris, 0.15 mol/L NaCl, pH 7.4. Washed beads were incubated for 30 min at 65°C in 100 μl of Laemmli sample buffer containing 7 mol/L urea and 5% mercapto-ethanol. Samples were electrophoresed in 7 mol/L urea-containing 10% SDS-PAGE minigels, transferred to nitrocellulose and exposed to X-OMAT X-ray-film (Kodak, Rochester, NY) at −80°C with an intensifying screen. The amount of phosphorylated receptor was determined by densitometric analysis of autoradiographs or by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA. Storm 840).

Phosphoaminoacid Analysis

Identification of phosphoaminoacids was performed by two-dimensional thin-layer cellulose electrophoresis of receptors subjected to acid hydrolysis as described [Boyle et al., 1991; Vázquez-Prado et al., 1997]. Immunoprecipitated phosphorylated B2 receptors, from control or stimulated cells, were electrophoresed in 7 mol/L urea-containing 10% SDS-PAGE minigels, transferred to polyvinylidene difluoride (PVDF) membranes (Biorad), and subjected to hydrolysis in 6N HCl for 1 h at 110°C in a volume of 200 μl. Samples were evaporated to dryness in a vacuum concentrator (Speed Vac) and solubilized in a mixture of cold phosphoaminoacid standards (phosphoserine, phosphothreonine, and phosphotyrosine, 25 nmol each). Electrophoresis was performed using 750 V for each dimension in a cooled Hoefer Supersub chamber. For these experiments, cells were labeled with 0.5 mCi/ml [^{32}P]-orthophosphate for 5 h.

Membrane Preparation and [^{35}S]GTP-γ-S Binding

B2 expressing cultures, washed with extracellular solution, were scraped with a rubber policeman in buffer containing: 20 mmol/L HEPES, pH 7.5, 5 mmol/L EDTA, 100 μmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, 10 mmol/L sodium pyrophosphate, and the previ-

ously mentioned protease inhibitors. Scraped cells were disrupted with a Polytron for 30 s and membranes were prepared as previously described [Vázquez-Prado et al., 1997]. [^{35}S]GTP-γ-S binding was performed as described by Wieland and Jakobs [1994]. Briefly, the binding reaction was carried out for 5 min at 30°C in a volume of 250 µl buffer (50 mmol/L Tris, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 100 mmol/L NaCl, 0.1% BSA, pH 7.5, containing 1 µmol/L GDP) containing 0.2 nmol/L [^{35}S]GTP-γ-S. The reactions were initiated by the addition of membranes (25 µg protein/tube) and terminated by the addition of 2 ml of ice-cold buffer (50 mmol/L Tris, 10 mmol/L $MgCl_2$, pH 7.5), and filtration on Whatman GF/C filters using a Brandel harvester. Nonspecific binding was determined in the presence of 100 µmol/L cold GTPγS. Filters were washed three times and dried, and radioactivity was measured with a liquid scintillation counter (Beckman, Fullerton, CA, model LS6000SC). In some experiments, membranes were prepared from cells previously stimulated with Bk, in the presence or absence of genistein. Controls included unstimulated cells and cells preincubated with genistein, as described in the figure legends.

Data Analysis

The change in $[Ca^{2+}]_i$ was obtained from the difference between the peak reached after agonist application and the basal level. Statistical analysis was performed by the Student's *t* test using Sigma Plot software (Jandel Corp., Chicago, IL). To quantify fluorescence intensity of confocal images, the average intensity of each cell in the visual field was measured and the data were integrated using an image analysis program (VISILOG 5, NOESIS). Analysis of binding experiments and dose–response curves was performed with the GRAPH PAD PRISM 2.0 software (San Diego, CA).

RESULTS

Functional Expression of Human B2 Receptor in sf21 Insect Cells

The human B2 bradykinin receptor was efficiently expressed in sf21 insect cells by the heterologous recombinant baculovirus system. [^{3}H]Bk binding experiments revealed a Kd of 0.66 ± 0.166 nmol/L (mean ± SEM, three determinations by triplicate) and expression level of 2.57 ± 0.55 pmol/mg (mean ± SEM) of protein in membranes obtained 54 h postinfection (Fig. 1A). This receptor was detected by Western-blot analysis as a band of 75–80 kDa in membranes from recombinant baculovirus-infected cells (Fig. 1B, lane B2R). No signal was detected in membranes from noninfected cells (Fig. 1B, lane NI). The presence of the B2 receptor on the plasma membrane of unpermeabilized cells was detected by confocal microscopy

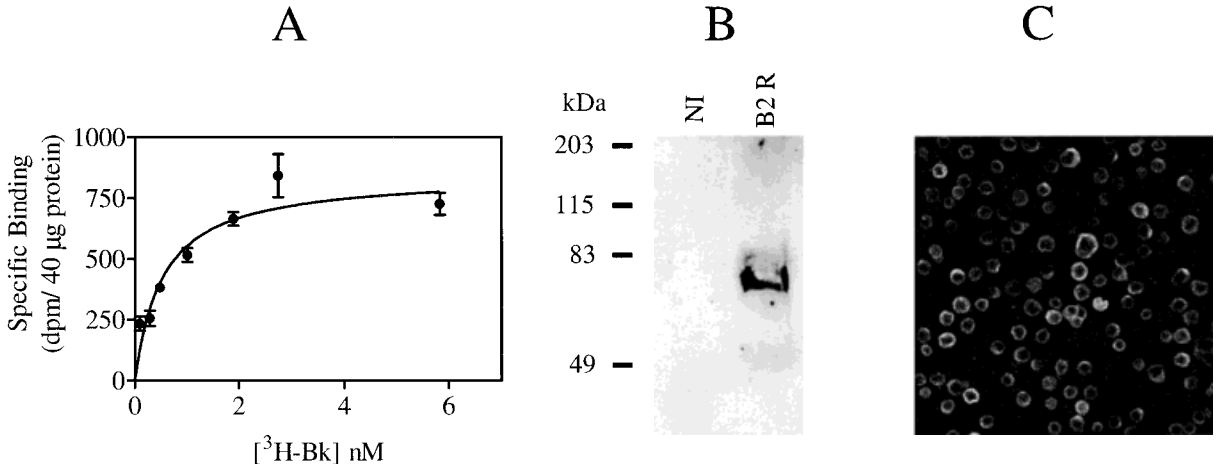

Fig. 1. A,B: [^{3}H Bk] saturation binding, Western blotting and surface expression of human B2 receptor in insect sf21 cells. A: [^{3}H]Bk saturation binding of human B2 receptor expressed in sf21 cells 54 h postinfection. Binding was done in membranes from infected cells as described in Materials and Methods. A representative curve from three experiments done in triplicate is shown. Scatchard analysis of the plotted data revealed a Kd of 0.66 nmol/L. B: Immunoblots of membranes of sf21 cells expressing the human B2 receptor (B2R) and membranes of noninfected cells (NI). Ten micrograms of membranes were loaded in each lane, and the immunoblots were probed by AS-346 antibody at a 1:1,000 dilution. NI were used as control. **C:** Expression of the receptor was examined 48 h postinfection by indirect immunoflorescence staining using an antibody against the second extracellular loop of the receptor (AS 276) and fluorescein-labeled goat anti-rabbit antibody.

using an antibody against an epitope (amino acids 91–105) from the second extracellular loop of the receptor [Abd Alla et al., 1996] followed by a fluorescein isothiocyanate–conjugated second antibody (Fig. 1C). Only background signal was detected in B2-expressing cells incubated exclusively with the second antibody and in noninfected cells incubated with the anti-B2 antibody followed by the fluorescent conjugate (data not shown). Fluorescence was detected at the plasma membrane of sf21 cells expressing B2 receptors, indicating that the mature protein reached the cell surface. In permeabilized cells, staining was also visible inside the cells, likely within the endoplasmic reticulum and Golgi compartments (data not shown).

Insect sf21 cells, infected with recombinant baculovirus containing the full-length human B2 receptor cDNA, responded to a stimulation with Bk with a transient rise in the $[Ca^{2+}]_i$. The magnitude of the response was concentration dependent, with an EC_{50} of 50 nmol/L (Fig. 2A). Concentrations <2.5 nmol/L did not produce a measurable response in $[Ca^{2+}]_i$. The maximum change in $[Ca^{2+}]_i$ was obtained with 100 nmol/L Bk. Expression of the B2 receptor in sf21 cells, tested between 45 and 48 h postinfection, did not modify the basal intracellular calcium compared to uninfected cells (190.17 ± 9.03 nmol/L and 175.98 ± 25.71 nmol/L, respectively; n = 15, $p < 0.05$). All subsequent experiments were performed within this time frame.

The specific B1 receptor agonist (Des-Arg^9)-Bk (1 µmol/L) did not modify basal $[Ca^{2+}]_i$ or the response to 100 nmol/L Bk (Fig. 2B, n = 4). The response to Bk (100 nmol/L) was completely blocked by the selective B2 receptor antagonist N-α-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8},D-phe^7]-Bk (N-α -Bk, 10 µmol/L; data not shown). Application of 10 µmol/L *N*-α -Bk at the peak of the Bk response (in the continuous presence of 100 nmol/L Bk) resulted in a rapid decline in $[Ca^{2+}]_i$ towards baseline (Fig. 2C). This result indicated that continuous receptor occupancy was required to maintain the $[Ca^{2+}]_i$ response.

Functional coupling between the B2 receptor and GTP-binding proteins was studied by analyzing Bk-promoted [^{35}S]GTP-γ-S binding. In membranes from B2 expressing sf21 cells, 1 µmol/L Bk increased [^{35}S]GTP-γ-S binding in vitro by 81% over basal levels (basal 1,250.22 ± 172.68 dpm/25 µg membrane protein, Bk 2274.28 ± 359.75 dpm/25 µg membrane protein; $P < 0.05$; n = 4, Fig. 2D).

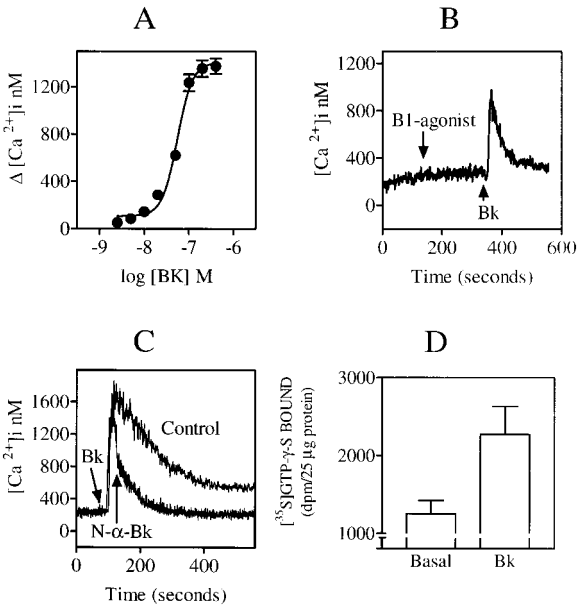

Fig. 2. A–C: Functional expression of human B2 receptor in insect sf21 cells. A: Dose–response curve to bradykinin (Bk) shows the $[Ca^{2+}]_i$ change induced by increasing concentrations of the agonist. Analysis of the dose–response curve revealed an EC_{50} of 50 nmol/L. B: Effect of 100 nmol/L Bk added after 1 µmol/L (Des-Arg^9)-Bk, a B1 receptor-specific agonist. C: Effect of 10 µmol/L N-α-adamantaneacetyl-D-Arg-[Hyp^3, Thi^{5,8},D-phe^7]-Bk (N-α-Bk), a B2-specific antagonist, on the calcium change induced by 100 nmol/L Bk. The antagonist was added at the peak of the Bk response (shown by the vertical arrow). **D:** Bk-elicited binding of [^{35}S]GTP-γ-S to membranes from cells expressing the B2 receptor. In vitro [^{35}S]GTP-γ-S binding was performed in the absence of any agent (basal) or in the presence of 1 µmol/L bradykinin (Bk). Nonspecific binding was subtracted from each value. Values represent the means ± standard errors from seven of independent determinations ($P < 0.05$).

Tyrosine Kinase Inhibitors Enhanced B2 Receptor Induced $[Ca^{2+}]_i$ Mobilization

Repeated applications of Bk resulted in attenuated changes in $[Ca^{2+}]_i$ (Fig. 3A), indicating that the B2 receptor desensitizes in the continuous presence of the agonist. This conclusion was further supported by the finding that a partial response to Bk was recovered after removing the agonist from the incubation medium and maintaining the cells in agonist-free solution for 15 min (Fig. 3B).

To study the effect of tyrosine kinase inhibitors on homologous B2 receptor desensitization, we repeated the protocol of Bk applications of 100 nmol/L and 400 nmol/L, removed the agonist, and incubated the cells for 15 min in agonist-free solution containing 10 µmol/L genistein during the last 10 min. Interestingly, under these conditions, the third Bk application

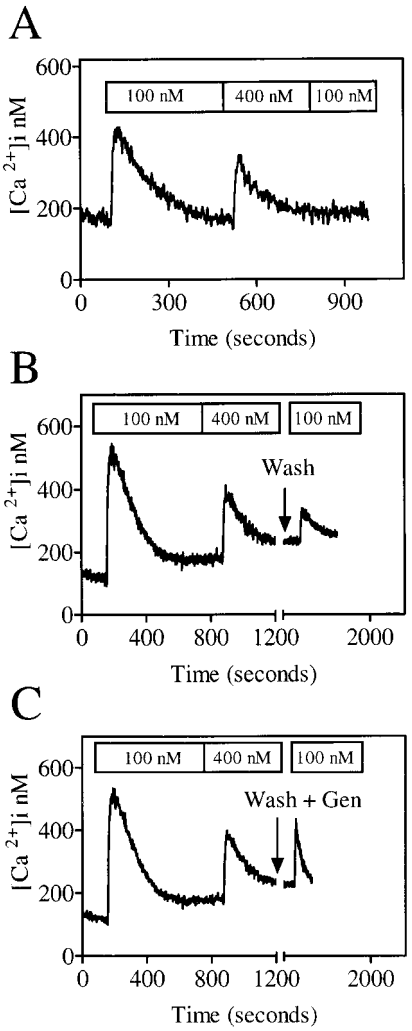

Fig. 3. A–C: Mobilization of $[Ca^{2+}]_i$ elicited by consecutive applications of Bk in B2 receptor-expressing sf21 cells. A: Typical response in calcium to three consecutive applications of Bk. Transient response induced by 100 nmol/L Bk (first application), 400 nmol/L Bk (second application), and 100 nmol/L Bk (third application). B: Similar protocol to that in A, but Bk was removed from the bath (Wash) before the third application. C: Similar protocol to the previous one, but in this case the cells were exposed to 10 µmol/L genistein (Wash + Gen) before the third stimulation with Bk. The time interval during wash was 15 min. The pause before the third application in B and C is indicated by a gap in the curve.

resulted in a greater response in $[Ca^{2+}]_i$ transients as compared to control cells (Fig. 3B and C).

To determine the availability of calcium from intracellular stores after repeated applications of Bk, we studied $[Ca^{2+}]_i$ transients induced by 500 µmol/L octopamine in cells in which two Bk stimulations had been elicited. Octopamine signals through an endogenous receptor linked to changes in $[Ca^{2+}]_i$ via a pertussis toxin–insensitive G protein and phospholipase C [Hu et al., 1994]. Figure 4A illustrates the changes in $[Ca^{2+}]_i$ induced by two subsequent applications of 100 nmol/L and 400 nmol/L of Bk. Under these conditions, octopamine produced a larger transient rise in $[Ca^{2+}]_i$ compared to the initial Bk application (Fig. 4A). These data demonstrated that the attenuated response to repeated applications of Bk were a result of homologous receptor desensitization and not of depletion of calcium stores.

To further explore the role of tyrosine kinases in agonist-promoted B2 receptor desensitization, sf21 cells were incubated with 10 µmol/L genistein for 2–10 min, then stimulated with Bk (100 → 400 nmol/L) followed by 500 µmol/L octopamine (Fig. 4B). Interestingly, genistein enhanced the response to the first (100 nmol/L) and second (400 nmol/L) Bk stimulations with no measurable effect on the octopamine response (Fig. 4B). No modification in the basal calcium concentration was induced by genistein (control = 192.37 ± 9 nmol/L; Gen = 195.33 ± 6.9 nmol/L, n = 8). In these experiments, genistein promoted a significant increase in the mobilization of calcium for both Bk applications compared to the control. The change in $[Ca^{2+}]_i$ elicited by 100 nmol/L Bk, in genistein-pretreated cells, was 483.17 ± 48.20 nmol/L compared to 287.76 ± 33.54 in control cells (Fig. 4C, n = 12; $P < 0.05$). The second Bk application, in genistein-pretreated cells, produced a change in $[Ca^{2+}]_i$ of 310.98 ± 31.5 nmol/L compared to 187.6 ± 16 in control cells (Fig. 4C, n = 11; $P < <0.05$). Interestingly, as observed in Figure 4A,B, a third stimulus, in this case with octopamine, gave a similar response in control and genistein-pretreated cells, suggesting regulation of B2 receptor activity by tyrosine kinase(s) at the level of receptor, or at a point of the signal transduction pathway not shared with octopamine receptors. Similar results were obtained after incubating the cells with 800 nmol/L tyrphostin A51 for 5 min, which increased the change in $[Ca^{2+}]_i$ for the first Bk application by 151 ± 8% (n = 5) and by 165 ± 11% (n = 7) for the second Bk application compared to control cells (data not shown).

Similar experiments were performed in the presence of 5 mmol/L EGTA. Under these conditions, a significant increase in the response to the second application of Bk (400 nmol/L) was observed in cells pretreated with genistein, whereas the first calcium transient remained uninfluenced by this tyrosine kinase inhibitor

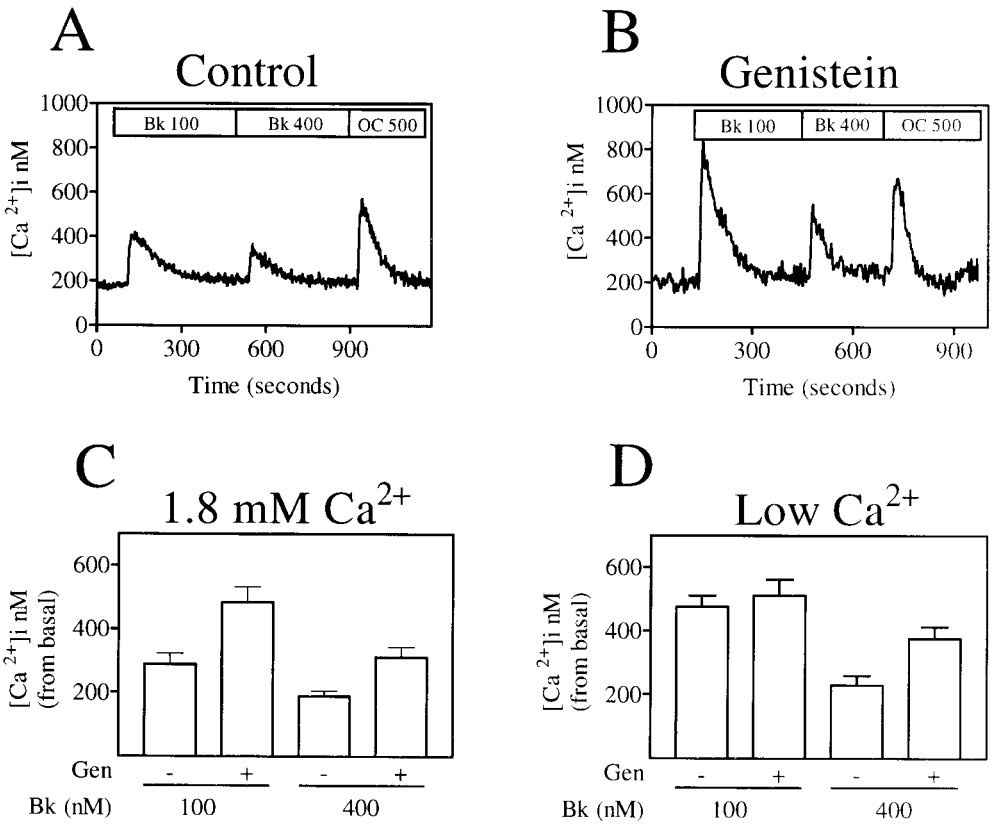

Fig. 4. A–C. Effect of genistein on the mobilization of $[Ca^{2+}]_i$ elicited by consecutive Bk applications in B2 receptor-expressing sf21 cells. A: Representative experiment illustrates the desensitization of the Bk receptor after agonist stimulation. The cells were exposed to 100 and 400 nmol/L Bk. After this, the cells were stimulated with 500 µmol/L octopamine (OC 500). B: Effect of 10 µmol/L genistein, preincubated for 10 min, in the response to 100 and 400 nmol/L Bk and 500 µmol/L octopamine (OC 500). The boxes indicate the time of application for each agonist. C: Change in $[Ca^{2+}]_i$ induced by the first (100 nmol/L) and second (400 nmol/L) Bk applications in genistein-treated and control cells in the presence of extracellular calcium (1.8 mmol/L Ca^{2+}), $P < 0.05$ for the effect of both Bk applications in the presence of genistein compared to control cells. **D:** Change in $[Ca^{2+}]_i$ induced by the first (100 nmol/L) and second (400 nmol/L) Bk applications in genistein-treated cells in the presence of 5 mmol/L EGTA (Low Ca^{2+}), $P < 0.05$ for the effect of 400 nmol/L Bk in genistein-treated cells compared to control. Values represent the means ± standard errors from six independent determinations.

(Fig. 4D). The peak of calcium elicited by a second application of BK increased from 228.91 ± 29.61 nmol/L in control cells to 375.97 ± 35.86 nmol/L in genistein-pretreated cells (n = 5; $P < 0.05$). The initial effect of Bk on $[Ca^{2+}]_i$ mobilization in the absence of extracellular calcium was unaffected by genistein (475.36 ± 34.62 and 509.89 ± 51.15 nmol/L in control and genistein-treated cells, respectively, n = 5; $P = 0.6$).

Tyrosine Kinase Inhibitors Did Not Affect Octopamine or Thapsigargin Elicited $[Ca^{2+}]_i$ Transients

The effect of tyrosine kinase inhibitors was tested on the endogenous octopamine receptor and on the thapsigargin (TG)-induced changes in $[Ca^{2+}]_i$. As illustrated in Figure 5A, addition of 500 µmol/L octopamine resulted in a typical transient change in $[Ca^{2+}]_i$. A second addition of 500 µmol/L octopamine produced an attenuated calcium response, suggesting that this receptor may also desensitize.

Treating the cells with 10 µmol/L genistein for 10 min did not alter the octopamine-induced change in $[Ca^{2+}]_i$, suggesting that the desensitization process of this G-protein-coupled receptor is not modulated by tyrosine kinases (Fig. 5A,B). In the presence of genistein, the first octopamine application produced a change in $[Ca^{2+}]_i$ of 172.47 ± 33.98 nmol/L compared to 175.97 ± 33.34 nmol/L in control cells ($P = 0.94$, n = 7). The second application in genistein-pretreated cells produced a calcium transient of 91.66 ± 20.58 nmol/L compared to 98.65 ± 33.22 nmol/L in control cells ($P = 0.82$, n = 11). This result is particularly interesting because

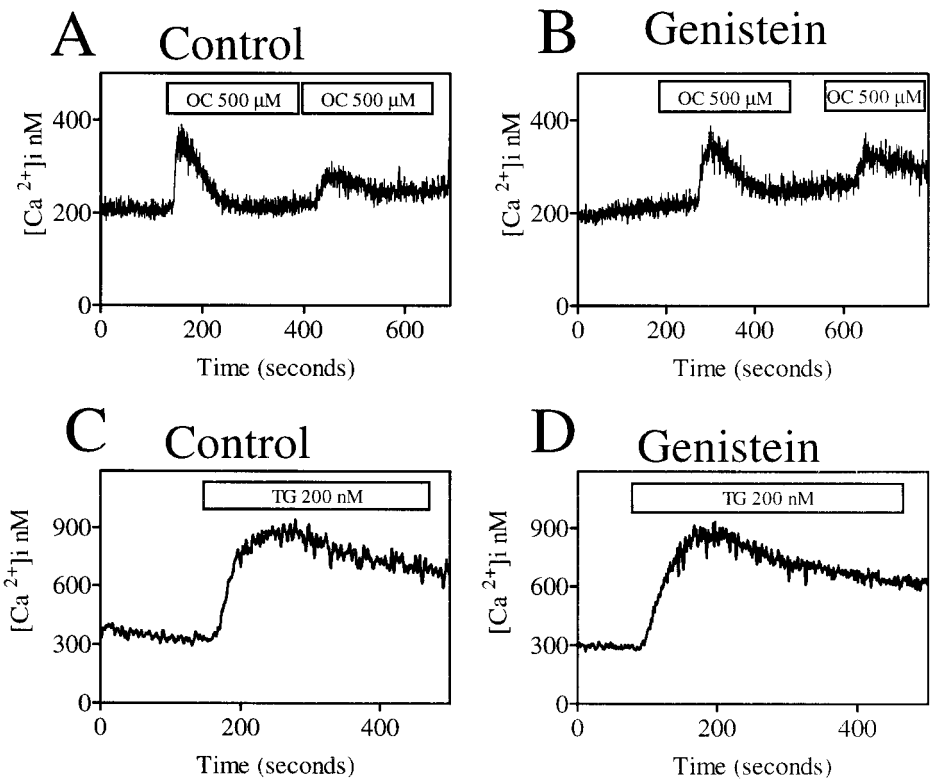

Fig. 5. A–D: Effect of genistein on octopamine- or thapsigargin-induced calcium responses in sf21 cells expressing B2 receptors. A: Representative trace of the change in $[Ca^{2+}]_i$ induced by two consecutive applications of 500 µmol/L octopamine (first and second peaks). B: Representative trace of the change in $[Ca^{2+}]_i$ induced by two consecutive applications of 500 µmol/L octopamine in cells incubated with 10 µmol/L genistein for 10 min. C: Representative trace of the change in $[Ca^{2+}]_i$ induced by 200 nmol/L thapsigargin (TG) in control cells and (D) shows change in $[Ca^{2+}]_i$ induced by 200 nmol/L thapsigargin (TG) in cells treated with 10 µmol/L genistein. Values represent the means ± standard errors from the number of independent observations. ($P = 0.36$ for TG and $P = 0.94$ and $P = 0.82$ for the first and second octopamine applications, comparing the respective peaks in control and genistein-treated cells.)

the octopamine and the B2 receptors could be using the same signaling cascade (pertussis-toxin-insensitive G-proteins and phospholipase C [PLC]) to induce the changes in $[Ca^{2+}]_i$ observed after agonist stimulation [Hu et al., 1994).

The TG-induced change in $[Ca^{2+}]_i$ was also insensitive to tyrosine kinase inhibitors, suggesting that release of calcium from intracellular stores by TG and the secondary calcium influx stimulated by the release (capacitative calcium entry) were not modulated by tyrosine kinases in sf21 cells (Fig. 5C,D). In genistein-treated cells, after two applications of Bk, TG induced a change in $[Ca^{2+}]_i$ of 653.42 ± 115.90 nmol/L compared to 742.29 ± 110.93 nmol/L in control cells (n = 10; $P = 0.36$).

Surface Expression of B2 Receptors in Bradykinin- and Genistein-Pretreated Cells

The effect of genistein on internalization of B2 receptors was determined by the intensity of fluorescence using confocal microscopy. Uniform staining at the cell surface for B2 receptors was observed in Bk-treated cells in the presence or absence of genistein, and in unstimulated cells (Fig. 6, upper panel). In control cells (basal), the mean of fluorescence intensity was 26.85 ± 3.97; for Bk-stimulated cells (Bk) it was 25.18 ± 4.26 and for genistein-pretreated cells (Gen → Bk) it was 23.72 ± 4.58 (Fig. 6, bottom panel). These results indicated that in the time frame in which the calcium experiments were performed, no significant internalization of B2 receptor occurred.

Genistein Increased Ligand-Promoted B2 Receptor Phosphorylation and Prevented Agonist-Induced G-Protein Uncoupling

Bk-promoted phosphorylation of the B2 receptor from sf21 insect cells loaded with [^{32}P]-orthophosphate is shown in Figure 7A. A radiolabeled band with a molecular weight of 75–80

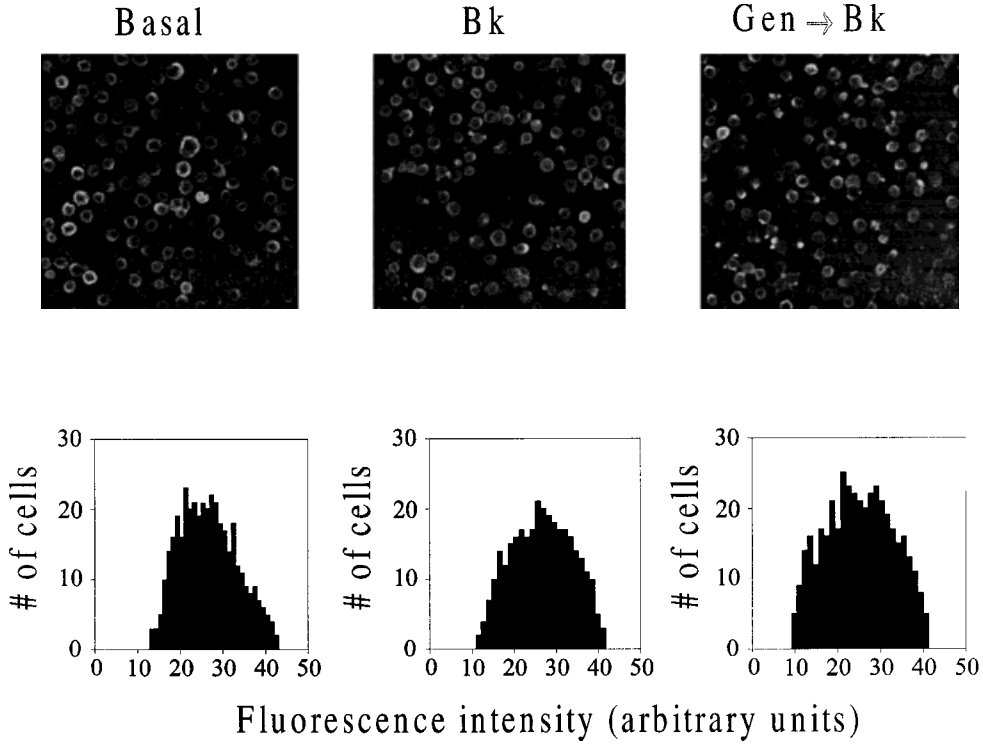

Fig. 6. Effect of Bk and genistein on the surface localization of B2 receptors visualized by confocal microscopy in sf21 cells. B2 expressing sf21 cells were grown on glass coverslips and left untreated (Basal) or treated with 100 nmol/L bradykinin (Bk) or 10 μmol/L genistein followed by BK stimulus, (Gen → Bk). Indirect immunoflorescence was performed with an antibody against the second extracellular loop of the receptor as described in Materials and Methods (upper panel). Bottom panel: The intensity of fluorescence for each treatment. The ordinate specifies the cell number, and the abscissa gives the relative fluorescence intensity.

kDa was immunoprecipitated using a B2 receptor-specific antibody (Fig. 7A). A band of a similar molecular weight was immunoprecipitated from sf21 cells expressing an N-terminally tagged B2 receptor by an antibody against the novel epitope (AntiXpress antibody, data not shown). Basal receptor phosphorylation, judged from semiquantitative receptor autoradiography, increased in cells treated for 10 min with 1 μmol/L Bk (100% vs. 133.3 ± 4.5% $P < 0.05$; Fig. 7B). The Bk-stimulated phosphorylation of the B2 receptor was further increased by preincubating the cells for 10 min with genistein (262 ± 18.63%, $P < 0.05$ compared to basal and Bk; Fig. 7B), whereas in the absence of Bk, no significant effect of genistein on phosphorylation of the B2 receptor was seen (94.4 ± 5.7% of basal). Phosphoaminoacid analysis revealed the presence of phosphoserine and traces of phosphothreonine in Bk-stimulated receptors (Bk), in receptors preincubated with genistein followed by agonist stimulation (Gen→Bk) (Fig. 7C), and in receptors isolated from control (basal) and genistein-preincubated cells (not shown).

To explore the possible participation of tyrosine kinase(s) on B2 receptors–G-protein coupling, we determined the effect of genistein on agonist-modulated G-protein-receptor coupling. In vitro Bk-stimulated [^{35}S]GTPγS binding was performed in membranes from control (basal) cells, and from cells stimulated with Bk in the absence or presence of genistein. Coupling of B2 receptors to G proteins is demonstrated by the ability of Bk to elicit in vitro [^{35}S]GTPγS binding to membranes from control unstimulated cells (Fig. 2D and Fig. 7D, left pair of bars). In membranes from cells stimulated in vivo with Bk, by a protocol similar to the used for calcium determination (100 nmol/L Bk followed by 400 nmol/L Bk), no in vitro stimulation by Bk was detected by [^{35}S]GTPγS binding (Fig 7D, middle pair of bars). Bk was able to elicit in vitro [^{35}S]GTPγS binding to membranes from Bk-stimulated cells that were pre-

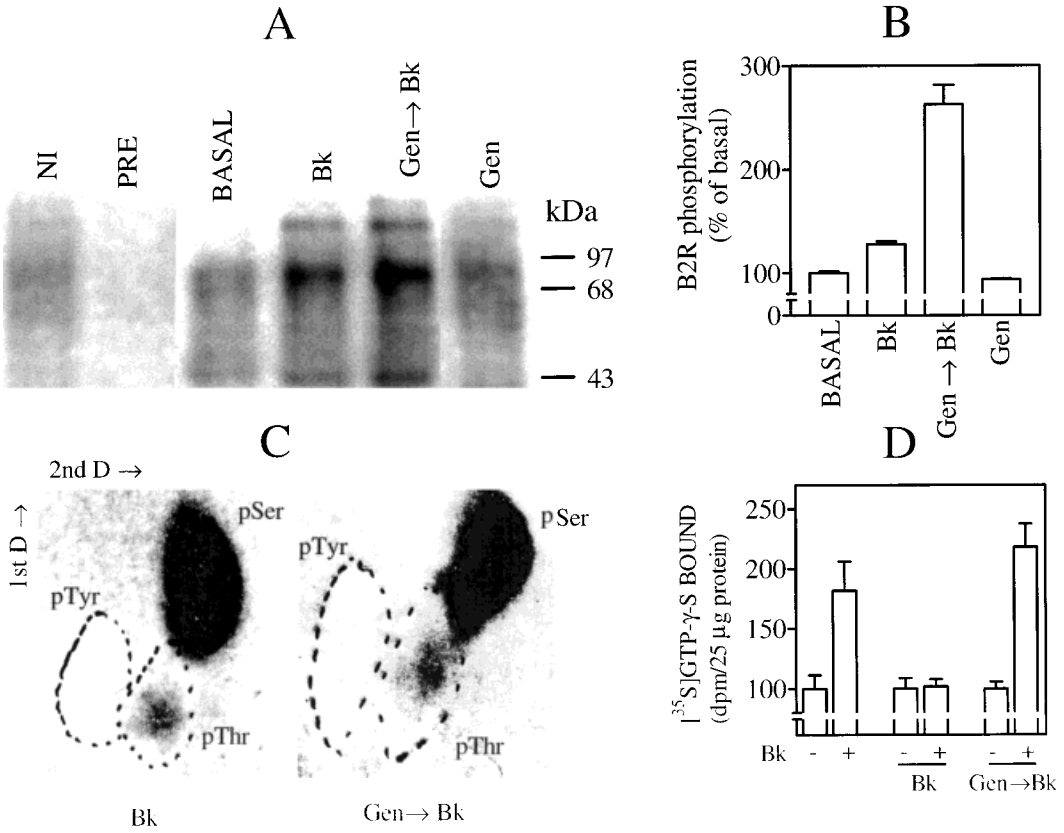

Fig. 7. **A,B:** Effect of Bk and genistein on the phosphorylation and G protein coupling of B2 receptor in sf21 cells. A: Sf21 cells expressing the human B2 receptor were radiolabeled with [^{32}P]-orthophosphate and incubated with vehicle (basal), 1 µmol/L bradykinin (Bk), 10 µmol/L genistein for 10 min, followed by 1 µmol/L Bk (Bk → Gen) or 10 µmol/L genistein (Gen). Immunoprecipitations were performed with an antibody against the carboxi-terminal region of the receptor as mentioned in Materials and Methods, or with preimmune serum as control (PRE). NI refers to noninfected cells in which the anti-B2 antibody was used. A representative autoradiograph of immunoprecipitated B2 is shown. Prestained molecular weight markers are indicated at the right. B: Means ± SE of percentage change in phosphorylation over the basal level of B2 receptor expressed in sf21 cells ($P < 0.05$, for comparison of Bk and Gen → Bk versus Basal and between them, n = 6). Data were obtained from densitometric analysis of the autoradiographies of immunoprecipitated phosphorylated receptors. **C:** Phosphoaminoacid analysis of hydrolysates from phosphorylated B2 receptors analyzed by two-dimensional thin-layer cellulose electrophoresis. A representative autoradiograph showing the results obtained for receptors from cells stimulated with Bk in the presence or absence of genistein, as described in (A), is shown. The electrophoretic migration of cold phosphoaminoacids used as standards is indicated. The experiment was repeated once with similar results. **D:** In vitro Bk-stimulated [^{35}S]GTPγS binding to membranes from cells preincubated with Bk in the presence or absence of genistein. Sf21 cells expressing B2 receptors were incubated without any agent or with Bk in the presence or absence of genistein as indicated below each pair of bars. Preincubation of cells with genistein (10 µmol/L) was for 10 min followed by agonist (100 nmol/L Bk for 10 min then 400 nmol/L Bk 10 min). In vitro [^{35}S]GTPγS binding to membranes was performed in the absence of any agent (−) or presence (+) of 1 µmol/L Bk as indicated in Materials and Methods. Plotted are the means ± SEM of 10 determinations using membranes obtained from two different cultures. Results obtained from cells preincubated with genistein in the absence of Bk were similar to those represented for the first pair of bars. ($P < 0.05$ for the in vitro effect of Bk in the first and third pair of bars in respect to their basal.)

incubated with genistein (Fig 7D, right pair of bars).

DISCUSSION

The data presented here show that the pharmacology of the recombinant B2 receptor expressed in sf21 cells is compatible with that of the B2 receptor from human cells [Hall, 1992] and that regulation of this receptor can be reproduced faithfully in insect cells. Expression of human B2 Bk receptor in sf9 insect cells was previously reported [Kunze et al., 1997; Dong et al., 1995; Hu et al., 1994]. In this work, we provide support to the hypothesis that regulation of the activity and phosphorylation of this receptor is influenced by tyrosine kinase(s).

Some discrepancy seems to exist between the affinity of recombinant B2 receptors measured by [^{3}H]Bk binding [Kd 0.66 nmol/L) and the efficiency of the receptors to stimulate intracellular calcium mobilization [EC_{50} 50 nmol/L). Although in the vast majority of cells expressing B2 receptors a close correlation exists between these parameters, differences in some cells that naturally express B2 receptors have also been detected. Examples include phosphoinositide turnover in guinea pig gallbladder cells [Falcone et al., 1993], murine fibrosarcoma HSDM1C1 cells [Sharif and Whiting, 1993], and colon muscle cells [Hasler et al., 1995] and B2 receptor phosphorylation in human HF-15 fibroblasts [Blaukat et al., 1996]. Indeed, heterogeneity in B2 receptor subpopulations defined by differences in their affinity for Bk have been reported [Baenziger et al., 1992; Dalemar et al., 1996]. Pizard et al. [1998] cited several studies that reported large variations, by more than four orders of magnitude in the affinity constants reported for Bk ranging from 0.003 to 50 nmol/L, and explored the reasons for this heterogeneity. They suggested a mechanism of negative cooperativity. Accordingly, B2 receptor molecules likely interact with each other, resulting in a decrease in the apparent affinity of the receptor for Bk because of an acceleration of bound ligand dissociation [Pizard et al., 1998]. In our transfected system, it is likely that the availability of G proteins is limiting. Because an agonist was used for the binding experiments, and assuming that the process of negative cooperativity is taking place, it remains possible that receptor species with low affinity for the agonist (in the uncoupled state) remained undetected. Binding experiments in which the antagonist [^{3}H]NPC17731, at concentration as high as 20 nmol/L, was used as radioligand did not reach saturation (results not shown). As discussed in detail by Kenakin [1997], this difference in the binding results for agonists and antagonists is frequently observed in the studies involving recombinant G-protein-coupled receptors in which the availability of G-proteins is limited.

B2 antagonist, when applied at the top of the calcium transient, accelerated the shutdown of the signal. These data suggest that calcium mobilization at this point still is influenced by receptor occupation and that the height and amplitude of the peak could be modified if receptor desensitization were prevented. In this situation, the increased response to Bk in the presence of genistein, even during the first challenge with the agonist, could be explained by a possible participation of tyrosine kinases regulating homologous receptor desensitization. The effect of genistein-sensitive tyrosine kinase(s) could also be attributed to a component of the signal transduction pathway downstream of the receptor that is not shared with octopamine or nonreceptor-activated calcium mobilization by TG. Our experiments, in which the effect of genistein on calcium mobilization was determined in the absence of extracellular calcium, suggest an effect of this inhibitor at two points of the process—one at the level of receptor desensitization, because its effect was only detected in the second challenge with the agonist; and the other at the level of calcium entry, because its effect over the initial stimulation with BK disappeared in the absence of extracellular calcium. In CHO cells expressing mouse B2 receptors, the effect of genistein on calcium mobilization revealed that the activity of tyrosine kinases is required for the sustained phase of the response but does not affect the initial peak of calcium elicited by Bk [Taketo et al., 1997]. Differences in the regulatory properties of human and mouse B2 receptors can be involved in the opposite effects observed for the action of genistein in Bk-elicited calcium mobilization in CHO and sf21 cells. Furthermore, it seems possible that different regulatory mechanisms, acting at the level of effector proteins, are required to maintain the sustained phase of the calcium response in each cell type. A diminished response of B2 and other serpentine receptors in the absence of extracellular calcium has been commonly observed [Harteneck et al., 1995]. Furthermore, inward calcium currents after agonist stimulation are detected by patch-clamp techniques a few milliseconds after agonist application, a lesser time than that required to reach the peak of calcium in the first challenge with the agonist [Vaca and Kunze, 1994]. Tyrosine kinase activity is required to sustain inward calcium currents in human foreskin fibroblasts [Lee et al., 1993]. No entry of calcium induced by mobilization from intracellular stores was detected in fibroblasts from c-Src knockout mice, and the process was recovered by transfection of the tyrosine kinase [Babnigg et al., 1997].

Desensitization is a regulatory mechanism that terminates agonist-induced second mes-

senger cascades for a wide variety of receptors. There is ample experimental evidence indicating that phosphorylation of G-protein-coupled receptors is involved in the desensitization mechanism [Alblas et al., 1996; Cyr et al., 1993; Lefkowitz et al., 1993]. Serine/threonine kinases such as protein kinase C- and G-protein-coupled receptor kinases (GRKs) such as the β-adrenergic receptor kinases play important roles in the desensitization and internalization of adrenergic receptors [Lefkowitz et al., 1993]. Regulation of B2 receptor activity by ligand-promoted serine and threonine phosphorylation has been demonstrated [Blaukat et al., 1996], and the potential role of protein kinase C in the desensitization of the B2 receptor has been highlighted [Clerk et al., 1996; Luo et al., 1992]. Absence of tyrosine phosphorylation of radiolabeled B2 receptor has been recently reported [Blaukat et al., 1996]; according to those data, it could be hypothesized that the tyrosine kinase inhibited by genistein produced an indirect effect on the receptor function. This hypothesis is further supported by the fact that phosphoserine and traces of phosphothreonine were the phosphoaminoacids detected by two-dimensional thin-layer cellulose electrophoresis from acid hydrolysates of the B2 recombinant receptors isolated from sf21 cells. In this regard, it has been demonstrated that isoforms of PKC (particularly PKC δ) are negatively regulated by phosphorylation [Denning et al., 1996; Zang et al., 1997] and that PKC activity regulates positively the activity of GRK-2 [Chuang et al., 1996, 1995]. The effect of phorbol esters on the phosphorylation of neurokinin receptor in Sf9 cells has been reported, suggesting the presence of endogenous PKC isoforms [Albas et al., 1995]. Furthermore, members of the heterotrimeric GTP-binding proteins in these cells have been detected [Nishimura et al., 1998; Leopoldt et al., 1997]. However, because tyrosine phosphorylation, detected by anti-phosphotyrosine antibodies, has been reported to occur in B2 receptors endogenously expressed in WI-38 human lung fibroblasts, a direct effect of genistein cannot be ruled out [Jong et al., 1993]. Also, the presence of phosphotyrosine that remained under the limits of detection of our system cannot be ruled out. In this respect, the detection of phosphotyrosine, by mass spectrometric analysis, has recently been reported in intracellular tyrosines from B2 receptors isolated from unstimulated transfected CHO cells [Soskik et al., 1999]. Interestingly, these residues correspond to intracellular tyrosines that have been reported as essential for receptor-elicited phosphoinositide turnover [Prado et al., 1997].

Mutation of rat B2 receptor at Tyr131 significantly reduced IP_3 formation and arachidonic acid release in response to Bk, whereas mutation of Tyr321 and Tyr131 had a profound effect on receptor internalization [Prado et al., 1997]. Thus, accumulating evidence suggests that stimulation of B2 receptors may induce tyrosine phosphorylation of downstream effectors, and in turn that tyrosine phosphorylation may affect B2 receptor signaling by a direct or indirect mechanism. Our data regarding the ability of genistein to prevent Bk-elicited uncoupling of B2 receptors from G proteins detected by in vitro [^{35}S]GTPγS binding to membranes from cells that were stimulated with Bk in the presence of genistein, together with the absence of phosphotyrosine in the phosphoaminoacid analysis, support the alternative of an indirect role for tyrosine phosphorylation on the increased ability of B2 receptors to elicit intracellular calcium mobilization, by an effect at the level of receptor–G-protein coupling interface, and on the increased phosphorylation of receptors (mainly at serine residues).

The fact that the genistein effect on intracellular calcium mobilization was observed a few minutes after its application suggests that genistein-reduced receptor desensitization does not involve receptor internalization, which is an event that usually requires several minutes to occur. This is further supported by the results illustrated in Figure 3, in which genistein was applied several minutes after exposing the receptor to two consecutive Bk applications. Even under these conditions, genistein produced a typical increment in the Bk-induced change in $[Ca^{2+}]_i$, as compared to control cells, indicating that the receptors were still available in the plasma membrane, but they were not fully responsive to Bk (in the absence of tyrosine kinase inhibitors). During the time in which the studies on calcium mobilization were performed, no change in the expression of the receptor at the cell surface were detected by indirect immunofluorescence in unpermeabilized cells, suggesting that no major internalization of receptors occurred during this period. To the best of our knowledge, no studies on the ability of sf21 insect cells to support the internalization of B2 receptors have been reported.

Because we did not follow the internalization of radioligand bound to receptors, our results regarding the permanence of receptors, detected by immunofluorescence, can also be interpreted on the basis of a rapid receptor recycling that maintains a constant number of receptors on the cell surface. Other G-protein-coupled receptors whose internalization has been demonstrated in insect cells are the human β_2 adrenergic receptor and the human dopamine D1 receptor. In both examples, 30% of receptor internalization was detected [Kleymann et al., 1993; Trogadis et al., 1995].

An enigma of the present study is that increased B2 receptor phosphorylation of the B2 receptor comes along with attenuated receptor desensitization, whereas previous studies have shown that ligand-promoted B2 receptor phosphorylation is causally linked to its desensitization [Blaukat et al., 1996]. The distinct possibility, however, remains that in the presence of genistein, the B2 receptor is phosphorylated on multiple residues that are not phosphorylated by homologous mechanisms, or that the activity of other proteins, which are able to influence receptor–G-protein coupling, can be altered by phosphorylation. In this sense, it has been reported that tyrosine phosphorylation of Gαq/11 diminishes its coupling to muscarinic M1 receptors [Humemori et al., 1997]. At the present time, we cannot exclude the possibility that the observed effects are cell type specific and not a general phenomenon also observed in mammalian cells. Our preliminary results with human fibroblasts expressing endogenous B2 receptors show that in these cells, they are also subject to genistein-responsive regulation (data not shown). We anticipate that sf21 insect cells provide a useful system to further explore the mechanisms that modulate receptor activity by tyrosine kinases.

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REFERENCES

Abd Alla S, Buschko J, Quitterer U, Maidhof A, Haasemann M, Breipohl G, Knolle J, Müller-Esterl W. 1993. Structural features of the human bradykinin B2 receptor probed by agonists, antagonists, and anti-idiotypic antibodies. J Biol Chem 268:17277–17285.

Abd Alla S, Quitterer U, Grigoriev S, Maidhof A, Haasemann M, Jarnagin K, Müller- Esterl W. 1996. Extracellular domains of the bradykinin B2 receptor involved in ligand binding and agonist sensing defined by antipeptide antibodies. J Biol Chem 271:1748–1755.

Alblas J, Etten I van, Khanum A, Moolenaar WH. 1995. C-terminal truncation of the neurokinin-2 receptor causes enhanced and sustained agonist-induced signaling. Role of receptor phosphorylation in signal attenuation. J Biol Chem 270:8944–8951.

Alblas J, Etten IV, Moolenaar WH. 1996. Truncated desensitization-defective neurokinin receptors mediate sustained MAP kinase activation, cell growth and transformation by a Ras-independent mechanism. EMBO J 15: 3351–3360.

Babnigg G, Bowersox SR, Villereal ML. 1997. The role of pp60 c-src in the regulation of calcium entry via store-operated calcium channels. J Biol Chem 272:29434–29437.

Baenziger NL, Jong YJI, Yocum SA, Dalemar RL, Wilhelm B, Vavrek R, Stewart JM. 1992. Diversity of B2 bradykinin receptors with nanomolar affinity expressed in passaged IMR90 human lung fibroblasts. Eur J Cell Biol 58:71–80.

Barr AJ, Brass LF, Manning DR. 1997. Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. A direct evaluation of selectivity in receptor G protein coupling. J Biol Chem 272:2223–2294.

Beukers MW, Klaassen CH, De Grip WJ, Verzijl D, Timmerman H, Leurs R. 1997. Heterologous expression of rat epitope-tagged histamine H2 receptors in insect Sf9 cells. Br J Pharmacol 122:867–874.

Blaukat A, Alla SA, Lohse MJ, Müller-Esterl W. 1996. Ligand-induced phosphorylation/dephosphorylation of the endogenous bradykinin B2 receptor from human fibroblasts. J Biol Chem 271:32366–32374.

Boyle WJ, van der Geer P, Hunter T. 1991. Phosphopeptide mapping and phosphoaminoacid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol 201:110–149.

Cauzabon S, Parker PJ, Strosberg AD, Couraud PO. 1993. Endothelins stimulate tyrosine phosphorylation and activity of p42/mitogen-activated protein kinase in astrocytes. Biochem J 293:381–386.

Chen X, Earley K, Luo W, Lin SH, Schilling WP. 1996. Functional expression of a human thrombin receptor in Sf9 insect cells, evidence for an active tethered ligand. Biochem J 314 (Pt 2):603–611.

Chuang TT, Iacovelli L, Sallese M, De Blasi A. 1996. G protein-coupled receptors: heterologous regulation of homologous desensitization and its implications. Trends Pharmacol Sci 17:416–421.

Chuang TT, LeVine H III, De Blasi A. 1995. Phosphorylation and activation of beta-adrenergic receptor kinase by protein kinase C. J Biol Chem 270:18660–18665.

Clerk A, Gillespie-Brown J, Fuller SJ, Sugden PH. 1996. Stimulation of phosphatidylinositol hydrolysis, protein kinase C translocation, and mitogen-activated protein kinase activity by bradykinin in rat ventricular myocytes: dissociation from the hypertrophic response. Biochem J 317:109–118.

Cyr CR, Rudy B, Kris RM. 1993. Prolonged desensitization of the human endothelin A receptor in Xenopus oocytes. J Biol Chem 268:26071–26074.

Dalemar LR, Jong YJI, Wilhelm B, Baenziger NL. 1996. Protein kinases A and C rapidly modulate expression of human fibroblast B2 bradykinin receptor affinity forms. Eur J Cell Biol 69:236–244.

Denning MF, Dlugosz AA, Threadgill DW, Magnuson T, Yuspa SH. 1996. Activation of the epidermal growth factor receptor signal transduction pathway stimulates tyrosine phosphorylation of protein kinase C delta. J Biol Chem 271:5325–5331.

De Weerd WFC, Leeb-Lundberg LMF. 1997. Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Gα subunits Gαq and Gαi in caveolae in DDT_1 MF-2 smooth muscle cells. J Biol Chem 272:17858–17866.

Dong Y, Kunze DL, Vaca L, Schilling WP. 1995. Ins(1,4,5)P3 activates Drosophila cation channel Trpl in recombinant baculovirus-infected Sf9 insect cells. Am J Physiol 269: C1332–C1339.

Falcone RC, Hubbs SJ, Vanderloo JD, Prosser JC, Little J, Gomes G, Aharony D, Krell RD. 1993. Characterization of bradykinin receptors in guinea pig gall bladder. J Pharmacol Exp Ther 266:1291–1299.

Fukushima Y, Asano T, Saitoh T, Anai M, Funaki M, Ogihara T, Katagiri H, Matsuhashi N, Yazaki Y, Sugano K. 1997. Oligomer formation of histamine H2 receptors expressed in Sf9 and COS-7 cells. FEBS Lett 409:283–286.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hall JM. 1992. Bradykinin receptors: pharmacological properties and biological roles. J Pharmacol Ther 56:131–190.

Harteneck C, Obukhov AG, Zobel A, Kalkbrenner F, Schultz G. 1995. The Drosophila cation channel trpl expressed in insect Sf9 cells is stimulated by agonists of G-protein-coupled receptors. FEBS Lett 358:297–300.

Hasler WL, Kurosawa S, Takahashi T, Feng H, Gaginella TS, Owyang C. 1995. Bradykinin acting on B2 receptors contracts colon circular muscle cells by IP3 generation and adenylate cyclase inhibition. J Pharmacol Exp Ther 273:344–350.

Hu Y, Rajan L, Schilling WP. 1994. Ca2+ signaling in Sf9 insect cells and the functional expression of a rat brain M5 muscarinic receptor. Am J Physiol 266:C1736–C1643.

Humemori H, Inoue T, Kume S, Sekiyama N, Nagao M, Itoh H, Nakanishi S, Mikoshiba K, Yamamoto T. 1997. Activation of the G protein Gq/11 through tyrosine phosphorylation of the α subunit. Science 276:1878–1881.

Jong YJI, Dalemar LR, Wilhelm B, Baezinger NL. 1993. Human bradykinin B2 receptors isolated by receptor-specific monoclonal antibodies are tyrosine phosphorylated. Proc Natl Acad Sci USA 90:10994–10998.

Kenakin T. 1997. Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. Trends Pharmacol Sci 12:456–464.

Kleymann G, Boege F, Hahn M, Hampe W, Vasudevan S, Reilander H. 1993. Human beta 2-adrenergic receptor produced in stably transformed insect cells is functionally coupled via endogenous GTP-binding protein to adenylyl cyclase. Eur J Biochem 213:797–804.

Kukkonen JP, Nasman J, Ojala P, Oker-Blom C, Akerman KE. 1996. Functional properties of muscarinic receptor subtypes Hm1, Hm3 and Hm5 expressed in Sf9 cells using the baculovirus expression system. J Pharmacol Exp Ther 279:593–601.

Kukkonen JP, Nasman J, Rinken A, Dementjev A, Akerman KE. 1998. Pseudo-noncompetitive antagonism of M1, M3, and M5 muscarinic receptor-mediated Ca^{2+} mobilization by muscarinic antagonists. Biochem Biophys Res Commun 243:41–46.

Kunze DL, Sinkins WG, Vaca L, Schilling WP. 1997. Properties of single Drosophila Trpl channels expressed in Sf9 insect cells. Am J Physiol 272:C27–C34.

Lee KM, Villereal ML. 1996. Tyrosine phosphorylation and activation of $pp60^{c\text{-}src}$ and $pp60^{FAK}$ in bradykinin-stimulated fibroblasts. Am J Physiol 270 (Cell Physiol 39): C1430–C1437.

Lee KM, Toscas K, Villereal ML. 1993. Inhibition of bradykinin- and thapsigargin-induced Ca2+ entry by tyrosine kinase inhibitors. J Biol Chem 268:9945–9948.

Lefkowitz RJ, Cotecchia S, Kjelsberg MA, Pitcher J, Koch WJ, Inglese J, Caron MG. 1993. Adrenergic receptors: recent insights into their mechanism of activation and desensitization. Adv Second Messenger Phosphoprotein Res 28:1–9.

Leopoldt D, Harteneck C, Nurnberg B. 1997. G proteins endogenously expressed in Sf9 cells: interactions with mammalian histamine receptors. Naunyn Schmiedebergs Arch Pharmacol 356:216–224.

Luo H, Lindeman RP, Chase HS Jr. 1992. Participation of protein kinase C in desensitization to bradykinin and to carbachol in MDCK cells. Am J Physiol 262:F499–F506.

Nishimura K, Frederick J, Kwatra MM. 1998a. Human substance P receptor expressed in Sf9 cells couples with multiple endogenous G proteins. J Recept Signal Transduct Res 18:51–65.

Nishimura K, Warabi K, Roush ED, Frederick J, Schwinn DA, Kwatra MM. 1998b. Characterization of GRK2-catalyzed phosphorylation of the human substance P receptor in Sf9 membranes. Biochemistry 37:1192–1198.

O'Relly DR, Miller LK, Luckow UA. 1992. Baculovirus expression vectors: a laboratory manual. New York: Freeman.

Pizard A, Marchetti J, Allegrini J, Alhenc-Gelas F, Rajerison RM. 1998. Negative cooperativity in the human bradykinin B2 receptor. J Biol Chem 273:1309–1315.

Prado GN, Taylor L, Polgar P. 1997. Effects of intracellular tyrosine residue mutation and carboxyl terminus truncation on signal transduction and internalization of the rat bradykinin B2 receptor. J Biol Chem 272:14638–14642.

Regoli D, Jukic D, Gobeil F, Rhaleb NE. 1993. Receptor for bradykinin and related kinins: a critical analysis. Can J Physiol Pharmacol 71:556–567.

Regoli D, Gobeil F, Nguyen QT, Jukic D, Seoane PR, Salvino JM, Sawutz DG. 1994. Bradykinin receptor types and B_2 subtypes. Life Sci 55:735–749.

Richardson RM, Hosey MM. 1992. Agonist-induced phosphorylation and desensitization of human m2 muscarinic cholinergic receptors in Sf9 insect cells. J Biol Chem 267:22249–22455.

Rodriguez-Fernandez JL, Rozengurt E. 1996. Bombesin, bradykinin, vasopressin and phorbol esters rapidly and transiently activate Src family tyrosine kinases in Swiss 3T3 cells. J Biol Chem 271:27895–27901.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Satoh M, Miyamoto C, Terashima H, Tachibana Y, Wada K, Watanabe T, Hayes AE, Gentz R, Furuichi Y. 1997. Human endothelin receptors ET(A) and ET(B) expressed in baculovirus-infected insect cells-direct application for signal transduction analysis. Eur J Biochem 249:803–811.

Schroeder C, Beug H, Müller-Esterl W. 1997. Cloning and functional characterization of the ornithokinin receptor. Recognition of the major kinin receptor antagonist, HOE140, as a full agonist. J Biol Chem 272:12475–12481.

Sharif NA, Whiting RL. 1993. The neuropeptide bradykinin stimulates phosphoinositide turnover in HSDM1C1 cells: B2-antagonist-sensitive responses and receptor binding studies. Neurochem Res 18:1313–1320.

Soskic V, Nyakatura E, Roos M, Müller-Esterl W, Godovac-Zimmermann J. 1999. Correlations in palmitoylation and multiple phosphorylation of rat bradykinin B2 receptor in chinese hamster ovary cells. J Biol Chem 274:8539–8545.

Taketo M, Yokoyama S, Kimura Y, Higashida H. 1997. Ca2+ release and Ca2+ influx in Chinese hamster ovary cells expressing the cloned mouse B2 bradykinin receptor: tyrosine kinase inhibitor-sensitive and -insensitive processes. Biochim Biophys Acta 1355:89–98.

Trogadis JE, Ng GY, O'Dowd BF, George SR, Stevens JK. 1995. Dopamine D1 receptor distribution in Sf9 cells imaged by confocal microscopy: a quantitative evaluation. J Histochem Cytochem 43:497–506.

Vaca L, Kunze DL. 1994. Depletion of intracellular Ca2+ stores activates a Ca(2+)-selective channel in vascular endothelium. Am J Physiol 267:C920–C925.

Vázquez-Prado J, Medina LC, García-Sáinz JA. 1997. Activation of endothelin ET_A receptors induces phosphorylation of α_{1b}-adrenoreceptors in rat-1 fibroblasts. J Biol Chem 272:27330–27333.

Wieland T, Jakobs KH. 1994. Measurement of receptor-stimulated guanosine 5′-O-(γ-thio)triphosphate binding by G proteins. Methods Enzymol 237:3–12.

Zang Q, Lu Z, Curto M, Barile N, Shalloway D, Foster DA. 1997. Association between v-Src and protein kinase C delta in v-Src-transformed fibroblasts. J Biol Chem 272: 13275–13280.