0026-895X/96/040716-12$3.00/0

MOLECULAR PHARMACOLOGY, 50:716–727 (1996).

Agonist Binding and Protein Kinase C Activation Stimulate Phosphorylation of the Gastrin-Releasing Peptide Receptor at Distinct Sites

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Received February 21, 1996; Accepted June 12, 1996

SUMMARY

Gastrin-releasing peptide and other bombesin-like peptides stimulate secretion, cell proliferation, and smooth muscle contraction via a family of G protein-coupled receptors that activate phospholipase C. Second messenger formation by one of these receptors, called BR1, is rapidly desensitized after treatment of cells with either agonists or the protein kinase C activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA). To determine whether receptor phosphorylation was involved in BR1 desensitization, we generated antibodies to a peptide corresponding to a unique sequence within the COOH terminus of this receptor. One antibody (BR1-517) immunoprecipitated 60% of the solubilized [^{125}I-Tyr^{4}]bombesin/receptor complex prepared from either Swiss 3T3 fibroblasts or CHO-K1 cells transfected to express high levels of mouse BR1 (CHO-mBR1). Furthermore, immunoprecipitation of photoaffinity-labeled receptors yielded the expected 87-kDa radiolabeled band on gel electrophoresis. Phosphorylation of this immunoprecipitated receptor protein was markedly stimulated when [^{32}P]orthophosphate-labeled Swiss 3T3 cells or CHO-mBR1 cells were treated with 100 nM bombesin for 5 min. $^{32}PO_4$ incorporation into immunoprecipitated receptor was detectable after 2 min and maximal after 15 min of bombesin treatment. Phosphoamino acid analysis showed ^{32}P labeling of serine and threonine but not tyrosine residues. Pretreatment of CHO-mBR1 cells with 100 nM TPA for 30 min also desensitized bombesin stimulation of inositol-1,4,5-trisphosphate formation. However, TPA did not increase $^{32}PO_4$ incorporation into the immunoprecipitated receptor, although protein kinase C inhibition potentiated bombesin-induced receptor phosphorylation. Subsequent studies showed that TPA did stimulate receptor phosphorylation, but the antibody did not recognize this phosphorylated state of the receptor. Thus, TPA decreased the efficiency of receptor immunoprecipitation, and subsequent incubation of receptor with alkaline phosphatase reversed this TPA inhibition. The differential specificity of the antibody for various phosphorylated forms of BR1 demonstrates that agonist-induced and TPA-induced phosphorylations of the receptor occur at distinct sites.

Desensitization of G protein-coupled receptors is recognized to be a complex process. In the case of the β-adrenergic receptor, which stimulates adenylyl cyclase via G_s, agonist-induced desensitization can occur as a result of receptor phosphorylation at unique sites by two types of kinases: the cAMP-dependent protein kinase and GRK (1). The extent to which this paradigm applies to G protein-coupled receptors that activate effectors other than adenylyl cyclase is not clear (2).

Three structurally related mammalian receptors mediate the multiple biological effects of the bombesin-like peptides (3). These receptors are homologous to the seven-transmembrane domain receptor family and can be distinguished pharmacologically as either GRP-preferring or neuromedin B-preferring subtypes (3, 4). Bombesin receptors are known to regulate a variety of biological processes, including body temperature, satiety, gastrointestinal peptide secretion, pancreatic enzyme secretion, pituitary and pancreatic hormone secretion, smooth muscle contraction, and cell proliferation (5). Agonist binding increases phospholipase C activity to generate the two second messengers Ins(1,4,5)P_3 and diacylglycerol, the latter producing PKC activation (6–8). Signal transduction from the receptor to phospholipase C is mediated by pertussis toxin-insensitive G proteins, most likely G_q/G_{11} (9–11).

This investigation was supported by the Texas Advanced Research Program (Grants 1823 and 11618–056) and a Pharmaceutical Manufacturers Association Foundation Advanced Predoctoral Fellowship (B.Y.W.).

ABBREVIATIONS: GRP, gastrin-releasing peptide; BR1, gastrin-releasing peptide-preferring bombesin receptor subtype 1; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate, DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; IP, inositol phosphate; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; TCA, trichloroacetic acid; TBS, Tris-buffered saline; ANB-NOS, *N*-5-azido-2-nitrobenzoyloxysuccinimide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GRK, G protein receptor kinase; mBR1, mouse BR1; CHO, Chinese hamster ovary; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

In the pancreatic β cell line HIT-T15, bombesin stimulation of both second messenger production and insulin secretion is desensitized within minutes (12, 13). Because agonist-induced receptor down-regulation and desensitization occur with similar kinetics, a decrease in receptor density may play a role in the desensitization process (12, 13). However, direct activation of PKC with TPA produces rapid bombesin receptor desensitization with no change in receptor levels (13). Thus, desensitization can occur independent of receptor down-regulation. Bombesin-stimulated second messenger formation is also desensitized by agonist and TPA pretreatment of Swiss 3T3 fibroblasts, which are mitogenically stimulated by bombesin activation of the BR1 receptor (11, 14, 15). Unlike in HIT-T15 cells, neither bombesin nor TPA leads to acute receptor down-regulation in Swiss 3T3 cells (14–16). Rather, PKC activation inhibits receptor/G protein coupling as shown by the observation that bombesin-induced IP production in permeabilized fibroblasts is blocked by TPA pretreatment, whereas guanosine-5′-*O*-(3-thio)triphosphate stimulation is unaffected (11). However, because bombesin pretreatment leads to receptor desensitization even in PKC-depleted cells, this enzyme cannot be essential for agonist-induced desensitization, and bombesin and TPA must elicit desensitization via distinct mechanisms (17). By analogy to other G protein-coupled receptors, the changes in bombesin receptor function induced by agonist binding and/or PKC activation may involve receptor phosphorylation. In the current study, we developed a specific, immunoprecipitating antibody to BR1 and used it to investigate the potential role of bombesin receptor phosphorylation in desensitization by bombesin and TPA.

Experimental Procedures

Materials. Synthetic bombesin and GRP(14–27) were obtained from Bachem California (Torrance, CA); [^{3}H]inositol (specific activity, 18.9 Ci/mmol) and carrier-free Na^{125}I were from Amersham (Arlington Heights, IL); carrier-free [^{32}P]orthophosphate was from ICN Biomedicals (Cleveland, OH); ANB-NOS, bicinchrominic acid protein assay reagent, and constant boiling 6 N HCl were from Pierce Chemical (Rockford, IL); pcDNA 3 was from InVitrogen (San Diego, CA); CNBr-activated Sepharose 4B was from Pharmacia (Piscataway, NJ); keyhole limpet hemocyanin and GF109203X were from Calbiochem-Novabiochem (San Diego, CA); gelatin, Tween-20 (enzyme immunoassay grade), goat anti-rabbit IgG horseradish peroxidase conjugate, and peroxidase substrate kit ABTS were from Bio-Rad (Richmond, CA); glutaraldehyde, protein A, deoxycholate, TPA, and bacterial alkaline phosphatase were from Sigma Chemical (St. Louis, MO); Centricon-30 was from Amicon (Beverly, MA); *N*-Glycanase was from Genzyme (Cambridge, MA); Immobilon-*p* polyvinylidene fluoride transfer membrane was from Millipore (Bedford, MA); plates and culture flasks were from Corning Glass Works (Corning, NY); DMEM, F12, glutamine, geneticin, and Freund's adjuvant were from GIBCO (Grand Island, NY); and fetal bovine serum and newborn calf serum were from JRH Biosciences (Lenexa, KS).

Generation of BR1 expressing CHO-K1 cells. A pGEM4 plasmid containing the entire coding region of mBR1 was generously provided by Dr. James F. Battey (National Institute of Neurological Disorders and Stroke, Bethesda, MD). The bombesin receptor was excised from pGEM4 with *Hin*dIII and *Apa*I and inserted into the same restriction sites in the mammalian expression vector pcDNA3. CHO-K1 cells, generously provided by Dr. Philip J. S. Stork (Vollum Institute for Advanced Biomedical Research, Oregon Health Science University, Portland, OR), were chosen for transfection because they lack endogenous bombesin receptors, as demonstrated by ligand binding. Transfection into CHO-K1 cells was accomplished by electroporation (420 V, 960 μF, 32 msec) using a Gene Pulser apparatus (BioRad) according to the method of Tykocinski *et al.* (18). After selection with 500 μg/ml geneticin (G418), clonal lines of G418-resistant cells were obtained by serial dilution and screened by [^{125}I-Tyr^4]bombesin binding as described previously (19). Clone CHO-mBR1 (CHO-K1 cells transfected to express high levels of mouse BR1) expresses a single class of high affinity (K_d = 5.1 nM at 4°) binding sites (1.1 × 10^6/cell) for bombesin.

Cell culture. Swiss 3T3 cells (generously provided by Dr. Kenneth Brown, Institute of Animal Physiology, Cambridge, UK) were grown in DMEM containing 10% (v/v) newborn calf serum at 37° in a humidified atmosphere of 5% CO_2/95% air. For membrane preparation and phosphorylation experiments, Swiss 3T3 cells were plated at a density of 2 × 10^5 cells/100-mm plate and used after 6–7 days.

CHO-K1 and CHO-mBR1 cells were grown in F12 medium supplemented with 10% (v/v) fetal bovine serum and 1 mM glutamine at 37° in a humidified atmosphere of 5% CO_2/95% air. For membrane preparation and phosphorylation experiments, these cells were plated at 3.5 × 10^5 cells/100-mm plate, the medium was changed after 3 days, and experiments were performed on day 4.

Measurement of [^{3}H]IP production. Swiss 3T3 cells were plated at a density of 1 × 10^5 cells/35-mm dish, and after 3–4 days, [^{3}H]inositol (2 μCi/2 μl) was added either directly to the growth medium or to fresh inositol-free DMEM containing 100 units/ml penicillin and streptomycin, 1 mM glutamine, and 10% (v/v) dialyzed fetal bovine serum. After 24–48 hr, the cells were incubated for 30 min in HBSS, pH 7.3 (118 mM NaCl, 4.6 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 5 mM HEPES, and 1 mg/ml BSA), to remove unincorporated [^{3}H]inositol and then treated with 100 nM bombesin in fresh HBSS. After the incubation times indicated, [^{3}H]IPs were extracted with 0.5 ml of ice-cold 20% (v/v) TCA and separated as described previously (19).

CHO-mBR1 cells were seeded at a density of 2 × 10^5/35-mm plate and fed 2 days later. Experiments were initiated on the fourth day by incubating the cells with 2 μCi of [^{3}H]inositol in the inositol-free medium described above. After 24 hr, the cells were washed and incubated in fresh HBSS containing 100 nM bombesin for the times indicated. When specified, 10 mM LiCl was added at the same time as peptide. [^{3}H]IPs were collected and separated as described above.

In desensitization experiments, cells were incubated in HBSS with or without (control) 100 nM bombesin. After 5 min, the medium was aspirated, and the cells were rapidly rinsed four times with 1 ml of warm (37°) HBSS. The cells were then incubated with fresh HBSS without or with 100 nM bombesin for 15 sec. The reaction was stopped, and [^{3}H]Ins(1,4,5)P_3 was measured as described above.

Antibody production. A peptide (Table 1) corresponding to residues 347–358 of the Swiss 3T3 mouse bombesin receptor (20, 21) with an added carboxyl-terminal tyrosine was synthesized by Dr. William T. Moore (University of Texas Medical School Analytical Chemistry Center, Houston, TX) on an ABI model 430A peptide synthesizer (Applied Biosystems, Norwalk, CT) using *N-tert*-butoxycarbonyl/benzyl solid-phase methodology (22). The peptide was cleaved and deblocked using hydrogen fluoride and purified by high pressure liquid chromatography. Peptide structure was verified by fast atom bombardment/mass spectrometry (23).

The peptide was coupled to keyhole limpet hemocyanin using glutaraldehyde (24) and injected intradermally into male New Zealand White rabbits (Ray Nichols, Lumberton, TX). The initial immunization was in Freund's complete adjuvant, whereas subsequent monthly booster injections were in Freund's incomplete adjuvant. Sera were tested by enzyme-linked immunosorbent assay as described previously (25).

Affinity purification of the peptide antibody. Peptide (1 mg) was coupled to CNBr-activated Sepharose 4B (100 mg/300 μl) according to the manufacturer's directions and then packed into a 2-ml disposable column. The column was pre-equilibrated at 4° with TBS (20 mM Tris, 100 mM NaCl, pH 7.4); then, antiserum BR1-517 (4 ml)

TABLE 1

Alignment of the peptide antigen with the carboxy-terminal region of bombesin receptor subtypes

The carboxy-terminal amino acid sequences of bombesin receptor subtypes are shown, starting from the putative palmitoylated cysteine. Underlining indicates the amino acid residues that match those in the peptide antigen used for antibody production. Receptor sequences were from the following sources: mBR1 (accession no. M35328, M57922), human BR1 (no. M73481), human NMB-R (no. M73482), and human BRS-3 (no. L08893).

Receptor subtype	Amino acid sequence	Position
Peptide antigen	N R S H S T G R S T T C Y	
mBR1	C C Q P G L M N R S H S T G R S T T C M	340–359
Human BR1	C C Q P G L I I R S H S T G R S T T C M	340–359
Human NMB-R	C G R K S Y Q E R G T S Y L L S S S A V	341–360
Human BRS-3	C C K A E R P E P P V A D T S L T T L A	347–366

diluted with 4 ml of TBS was passed over the packing five times at 4°. The column was sequentially washed with 50 ml of TBS; 20 ml of 0.2 M glycine, pH 3.0; and 10 ml of TBS to remove nonspecific antibodies. The peptide-adsorbed antibodies were then eluted with 30 ml of 100 mM triethylamine, pH 11.5, and 5 M LiCl. Fractions (1 ml) were collected into 255 μl of 1 M sodium acetate, pH 6.8. Pooled fractions containing specific antibody, as identified by enzyme-linked immunosorbent assay, were immediately concentrated using a Centricon-50, and the buffer was exchanged for TBS containing 0.02% sodium azide and 0.1% BSA. The purified antibody was stored at 4°.

Membrane preparation and receptor binding. Cell monolayers were scraped into ice-cold phosphate-buffered saline (10 mM Na_2PO_4, 150 mM NaCl, pH 7.4) and pelleted. The cells were then resuspended in imidazole homogenization buffer (4 mM imidazole, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4) at 4° and lysed with 10 strokes of a Polytron homogenizer at 900 rpm. After centrifugation at 500 × *g* for 10 min, the supernatant was centrifuged at 10,000 × *g* for 30 min at 4°. For storage, the membrane pellet was resuspended in glycyl-glycine buffer (20 mM glycyl-glycine, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.2) and frozen at −70°.

For binding, [Tyr^4]bombesin and GRP(14–27) were radioiodinated using chloramine-T, reduced using dithiothreitol, and purified by reverse-phase high performance liquid chromatography to a specific activity of 2200 Ci/mmol as described previously (19). The binding reaction usually contained 200 μg of membranes in 1 ml of HEPES binding buffer (50 mM HEPES, 7 mM $MgCl_2$, 2 mM EDTA, 2 units/ml of bacitracin, 0.5% BSA, pH 7.4) containing [^{125}I-Tyr^4]bombesin or [^{125}I]GRP(14–27) at a concentration of 0.3 nM with or without 100 nM bombesin. After 20 min at 30°, the binding reaction was stopped by the addition of 4 ml of ice-cold HEPES binding buffer. Samples were centrifuged at 10,000 × *g* for 10 min, and the radioactivity associated with the pellet was measured with a Pharmacia LKB gamma spectrometer at an efficiency of 75%. Specific binding was calculated as the difference between the amount of radioligand bound in the absence (total binding) and the presence (nonspecific binding) of 100 nM bombesin.

Photoaffinity labeling of BR1 and immunoprecipitation. After binding, [^{125}I]GRP(14–27) bound membrane pellets were resuspended in HEPES binding buffer without BSA to a final concentration of 0.2 mg/ml. A freshly prepared solution of ANB-NOS (10 mM in dimethylsulfoxide) was added to a final concentration of 0.1 mM. The membranes were incubated in the dark on ice for 10 min and then irradiated at 254 nm (Mineralight model R-52, Ultraviolet Products, San Gabriel, CA) for 5 min. The reaction was terminated by the addition of 1 M Tris·HCl, pH 8, and the membranes were pelleted.

For gel electrophoresis, the membranes were dissolved in SDS sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 20% glycerol, and 50 mM dithiothreitol) by boiling for 5 min and resolved by SDS-PAGE on 8% acrylamide gels according to the method of Laemmli (26). The gels were dried on filter paper and exposed to Amersham Hyperfilm with DuPont intensifying screens at −70°.

For immunoabsorption, affinity labeled membranes were solubilized for 1 hr at 4° at a protein/detergent ratio of 1:3 in HEPES binding buffer containing 1.5 mg/ml deoxycholate, 100 μg/ml benzamidine, and 10 μg/ml concentration each of chymostatin, pepstatin, antipain, and leupeptin. After centrifugation at 100,000 × *g* for 1 hr at 4°, purified antibody BR1-517 was added to the supernatant and incubated at 4° for 2–20 hr. Protein A/Sepharose 4B (20 μl of a 50% slurry) was added, and the incubation was continued for another hour. After centrifugation at 10,000 × *g* for 1 min, the immunoprecipitate was washed twice with ice-cold HEPES binding buffer containing 1.5 mg/ml deoxycholate and then dissolved in SDS sample buffer. The samples were analyzed by SDS-PAGE and autoradiography as described above.

***In vivo* ^{32}P labeling and immunoprecipitation.** Cells grown in 100-mm dishes were washed twice with Tris buffer, pH 7.4 (1 mM Tris, 136 mM NaCl, 2.6 mM KCl) and incubated with 3 ml of phosphate-free DMEM containing 10% (v/v) dialyzed newborn calf serum and 1–3 mCi of [^{32}P]orthophosphate for 3 hr at 37° in a humidified atmosphere of 5% CO_2/95% air. Bombesin or TPA was then added, and the incubation was continued for the stated times. The cells were then rinsed with cold Tris buffer, and membranes were immediately prepared in imidazole homogenization buffer containing 50 mM sodium fluoride and 1 mM sodium vanadate. The membrane pellets were washed once with immunoprecipitation buffer (10 mM Na_2HPO_4, pH 7, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate) and then solubilized in immunoprecipitation buffer containing 1.5 mg/ml deoxycholate, 100 μg/ml benzamidine, and 10 μg/ml concentration each of chymostatin, pepstatin, antipain, and leupeptin. The membrane protein concentration was assessed using the bicinchrominic acid protein assay according to the manufacturer's directions. Aliquots of the solubilized membranes containing equal amounts of protein (~50 μg) were precleared by incubation with a 1:500 dilution of preimmune sera followed by precipitation with protein A/Sepharose 4B. After the addition of purified antibody BR1-517, the samples were incubated overnight at 4° and then precipitated with protein A/Sepharose 4B. The immunoprecipitates were washed extensively, solubilized in 2× SDS sample buffer, and subjected to SDS-PAGE and autoradiography. For quantification of ^{32}P incorporation, the radioactive bands were localized by autoradiography and excised, and Cherenkov counts were measured in an LKB Wallac 1209 Rackbeta liquid scintillation counter (Gaithersburg, MD). Background radioactivity, measured in an adjacent section of the gel in the same lane, was subtracted.

Deglycosylation and dephosphorylation of mBR1. After immunoprecipitation, the bombesin receptor was deglycosylated according to the method of Kris *et al.* (27). Briefly, the washed immunoprecipitates from 200 μg of membrane protein were solubilized in 75 μl of 0.5% SDS at 56° for 1 hr and then boiled for 5 min. Aliquots (30 μl) were incubated overnight at 37° with 32.4 μl of 50 mM sodium phosphate, pH 8.6, 15 μl of 7.5% Nonidet P-40, 1.8 μl of *N*-glycanase (250 units/ml), and 0.4 μl of a protease inhibitor cocktail containing 50 μg/ml bacitracin and 10 μg/ml concentration each of soybean trypsin inhibitor and leupeptin. The reaction was terminated by the addition of 14 μl of 6× SDS sample buffer and boiling for 5 min. The samples were analyzed by SDS-PAGE and autoradiography as described above.

For phosphatase treatment, membranes were prepared in the

presence of phosphatase inhibitors as described for the ^{32}P-labeling studies and incubated with [^{125}I]GRP(14–27) for 20 min at 37°; then, photoaffinity cross-linking was carried out as described above. The membrane pellets were washed once with phosphatase buffer (20 mM HEPES, pH 8, 25 mM KCl, 15 mM $MgCl_2$) and then solubilized in this buffer containing 1.5 mg/ml deoxycholate, 0.1% SDS, 100 μg/ml benzamidine, and 10 μg/ml concentration each of chymotrypsin, pepstatin, antipain, and leupeptin as described above. After the addition of bacterial alkaline phosphatase (5 units) to the solubilized membranes (~45 μg/sample), samples were incubated for 1 hr at 37°, conditions that have previously been shown to dephosphorylate other G protein-coupled receptors (28). Receptors were then immunoprecipitated as described above and analyzed by SDS-PAGE and autoradiography. The radioactivity incorporated into the photoaffinity labeled receptor band was quantified using a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

For quantification of the ^{125}I-labeled receptor protein by the PhosphorImager, each receptor band on a gel was circumscribed by identical size rectangles, and the radioactive signal within each rectangle was measured. The same size rectangle was used to circumscribe a region below each receptor band, and the radioactivity in the latter region was taken as a measure of the intrasample background. The background in each lane was subtracted from the receptor signal to generate the results shown.

Phosphoamino acid analysis. Phosphorylation and immunoprecipitation of the receptor were carried out as described above. After separation by SDS-PAGE, phosphorylated proteins were electrophoretically transferred to polyvinylidene fluoride membranes at 0.1 A for 30 min and then at 0.5 A for 45 min in Dunn's carbonate buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 0.1% SDS, and 20% methanol). The phosphorylated bombesin receptor band was localized by autoradiography, excised, and hydrolyzed with 6 N HCl at 110° for 0.5 or 2 hr. Nonradioactive phosphorylated amino acid standards (1 μl each of 10 mM phosphoserine, 10 mM phosphothreonine, and 10 mM phosphotyrosine in water) were added, and the hydrolysate was dried by vacuum centrifugation. After resuspension in 3 μl of 7.8% acetic acid/2.5% formic acid, the samples were spotted onto a cellulose plate (catalogue no. 13255, Eastman Kodak, Rochester, NY) and analyzed by two-dimensional thin layer chromatography on a Hunter Thin Layer Peptide Mapping System (model HTLE-7000, C.B.S. Scientific, Del Mar, CA). Separation was achieved in the first dimension at 1100 V for 60 min with 7.8% acetic acid/2.5% formic acid and in the second dimension at 1300 V for 45 min with 5% acetic acid/0.5% pyridine. The phosphorylated amino acid standards were visualized by staining with 0.25% ninhydrin in acetone, and the ^{32}P-labeled amino acids were detected by autoradiography.

Results

Bombesin receptor desensitization in CHO cells. We found that Swiss 3T3 cells contain ~100,000 bombesin receptors/cell, which is consistent with the range of 50,000–240,000 receptors/cell reported by other investigators (14, 29). To increase the sensitivity with which receptor phosphorylation could be detected, we transfected CHO-K1 cells with the mBR1 cDNA to generate a cell line, CHO-mBR1, that expresses $4.5 \pm 0.8 \times 10^5$ receptors/cell (K_d for [^{125}I-Tyr^4]bombesin = 0.26 ± 0.08 nM at 4°, n = 5). We then compared the ability of bombesin to stimulate phosphatidylinositol hydrolysis in Swiss 3T3 cells with that in CHO-mBR1 cells. The dose response for bombesin stimulation of [^{3}H]IP formation was determined during a 30-min incubation of CHO-mBR1 cells in the presence of 10 mM LiCl to inhibit IP hydrolysis. The ED_{50} value for bombesin was 0.18 ± 0.03 nM (data not shown), which is similar to its reported potency in Swiss 3T3 cells (30). We next examined the time course for bombesin stimulation of Ins(1,4,5)P_3 formation (Fig. 1). This experiment was carried out in the absence of LiCl to ensure that phosphatidylinositol depletion did not occur during the incubation (Fig. 1, *top*). In Swiss 3T3 fibroblasts, [^{3}H]Ins(1,4,5)P_3 levels increased to a peak value at 5–15 sec of bombesin stimulation and then slowly decreased, although they were still elevated 5 min after the addition of bombesin (Fig. 1, *top*). In CHO-mBR1 cells, bombesin also produced a maximal increase in [^{3}H]Ins(1,4,5)P_3 levels at 15 sec (Fig. 1, *top*). [^{3}H]Ins(1,4,5)P_3 formation subsequently declined gradually and returned to the basal level after 30 min of bombesin treatment (Fig. 1 and data not shown). Because the dose response and time course for bombesin-stimulated IP formation were comparable in the two cell lines, signaling by the transfected mBR1 receptor in CHO-K1 cells was similar to that of the endogenous receptor in Swiss 3T3 cells.

By rechallenging the cells with fresh peptide, we next determined whether the decrease in [^{3}H]Ins(1,4,5)P_3 levels observed after 5 min of bombesin stimulation resulted from homologous desensitization. Cells were incubated for 5 min without or with 100 nM bombesin, quickly washed, and then challenged in a subsequent 15-sec incubation (Fig. 1, *bottom*). In Swiss 3T3 cells, the initial 15-sec stimulation with 100 nM bombesin produced a 9-fold increase in [^{3}H]Ins(1,4,5)P_3 production (Fig. 1, *bottom left*). After 5 min of bombesin pretreatment, the level of [^{3}H]Ins(1,4,5)P_3 formed in the following 15-sec incubation without peptide was 2.5-fold higher than that in the untreated control cells, showing that the bombesin pretreatment had a residual effect. This residual effect is likely to be due to continued occupancy and stimulation of the receptor by prebound bombesin, which dissociates slowly compared with the short (15-sec) challenge incubation. Consistent with this interpretation, [^{3}H]Ins(1,4,5)P_3 production was not stimulated above the residual level by the addition of fresh bombesin in the second incubation. Thus, Swiss 3T3 cells were partially desensitized by the 5-min bombesin pretreatment. Similar results were observed in CHO-mBR1 cells. The initial incubation with 100 nM bombesin for 15 sec increased [^{3}H]Ins(1,4,5)P_3 levels 8.5-fold over basal levels (Fig. 1, *bottom middle*). After a 5-min preincubation with bombesin, the [^{3}H]Ins(1,4,5)P_3 levels were higher than in nonpretreated cells, and a second challenge with bombesin again failed to increase [^{3}H]Ins(1,4,5)P_3 beyond that produced by the pretreatment alone. The residual stimulation observed after bombesin pretreatment is consistent with the time course shown in Fig. 1 (*top*), and the inability of freshly added agonist to further stimulate second messenger formation clearly demonstrates that the decreased response was due not to peptide degradation but rather to partial desensitization.

Previous studies have shown that acute treatment of Swiss 3T3 cells with TPA leads to activation of PKC and to desensitization of bombesin-stimulated second messenger formation (13, 15). We observed the same behavior in the transfected CHO cells (Fig. 1, *bottom right*). In two independent experiments, preincubation of CHO-mBR1 cells with 100 nM TPA for 30 min did not significantly affect basal [^{3}H]Ins(1,4,5)P_3 levels but inhibited bombesin stimulation by 65%.

These results show that the transfected mBR1 receptor is functional in CHO cells and that both homologous and het-

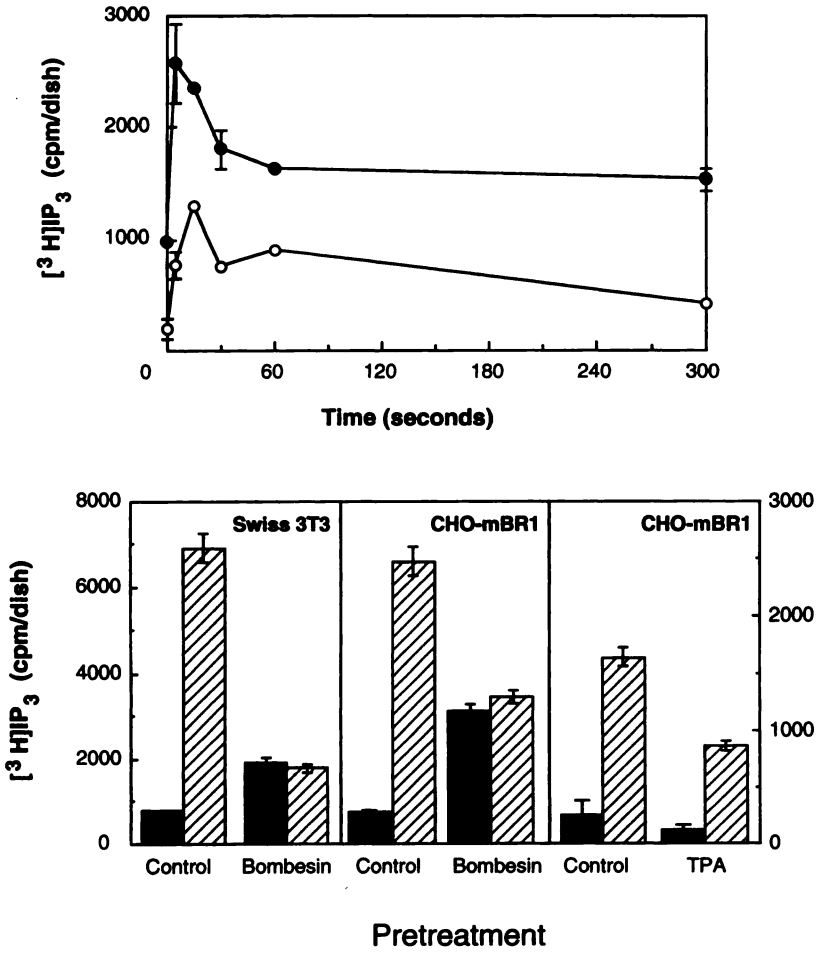

Fig. 1. Desensitization of bombesin-stimulated IP production. *Top*, Swiss 3T3 fibroblasts (6×10^5/dish, ●) and CHO-mBR1 cells (1.3×10^6/dish, ○) were prelabeled with [^{3}H]inositol (1 μCi/plate) at 37° for 24–48 hr. Bombesin (100 nM) was added at t = 0, and the incubation was continued for the indicated times. The reaction was stopped by the addition of TCA, and accumulated [^{3}H]Ins(1,4,5)P_3 was determined as described in Experimental Procedures. *Points*, mean of duplicate dishes; *bars*, range. *Bottom*, after prelabeling with [^{3}H]-inositol, Swiss 3T3 fibroblasts or CHO-mBR1 cells were incubated at 37° in HBSS either alone (*Control*) or in the presence of 100 nM bombesin for 5 min (*left* and *middle*) or with 100 nM TPA for 30 min (*right*). The pretreatment media were then removed, and the cells were incubated at 37° in buffer without (*shaded bars*) or with (*striped bars*) 100 nM bombesin. The challenge incubation was stopped after 15 sec by the addition of TCA, and [^{3}H]Ins(1,4,5)P_3 production was measured. *Bars*, mean ± standard error of triplicate dishes.

erologous receptor desensitizations are comparable to those observed in Swiss 3T3 fibroblasts.

Specific immunoprecipitation of BR1. A polyclonal rabbit antibody was generated against a 13-amino acid peptide corresponding to a unique sequence in the carboxyl-terminal region of BR1 (Table 1). Fig. 2 shows that this antiserum efficiently and specifically immunoprecipitated the bombesin receptor. After incubation of Swiss 3T3 membranes with [^{125}I-Tyr^4]bombesin, the receptor/ligand complex was solubilized with deoxycholate and then immunoprecipitated with varying concentrations of serum. The efficiency of immunoprecipitation was concentration dependent with a 1:1000 dilution of antiserum precipitating 60% of the added [^{125}I]bombesin/receptor complex (Fig. 2, *top*). However, the ineffectiveness of higher serum concentrations to further increase immunoprecipitation efficiency suggested that multiple receptor forms were present (see below). In five independent experiments, 60 ± 7% of the [^{125}I-Tyr^4]-bombesin/BR1 complex prepared from Swiss 3T3 fibroblasts was precipitated by the peptide antibody. Similar results were observed for receptors from CHO-mBR1 cells (data not shown). Immunoprecipitation was blocked when 7 μM antigen peptide was added to the antibody incubation, and preimmune serum precipitated <5% of the ligand/BR1 complex (Fig. 2, *bottom*).

The biochemical nature of the protein recognized by antibody BR1-517 is further characterized in Fig. 3. Receptors were first identified in membranes by covalently coupling specifically bound [^{125}I]GRP(14–27) with the photoaffinity cross-linker ANB-NOS (Fig. 3). The major protein radiolabeled in both Swiss 3T3 membranes (Fig. 3A) and CHO-mBR1 membranes (Fig. 3B) migrated as a broad band with a molecular mass of ~87 kDa. [^{125}I]GRP(14–27) incorporation into these bands was blocked when binding was performed in the presence of 100 nM bombesin, demonstrating that the labeling was saturable. No labeling was observed in untransfected CHO-K1 cells, which do not express bombesin receptors.

To determine whether the antiserum would immunoprecipitate the receptor protein, Swiss 3T3 and CHO-mBR1 membranes were photoaffinity labeled as described above and solubilized with deoxycholate. After immunoprecipitation and SDS-PAGE, a broad 87-kDa band was evident in samples incubated with BR1-517 antiserum but not with preimmune serum (Fig. 3). Therefore, the antipeptide antibody specifically recognizes the 87-kDa BR1 protein.

Bombesin-stimulated receptor phosphorylation. To determine the effect of bombesin on BR1 phosphorylation, Swiss 3T3 fibroblasts and CHO-mBR1 cells were metabolically labeled with [^{32}P]orthophosphate and then incubated for 5 min without or with 100 nM bombesin. Membranes were then prepared, solubilized, and immunoprecipitated with affinity purified antibody. In both Swiss 3T3 fibroblasts (Fig. 4) and CHO-mBR1 cells (Fig. 5A), bombesin markedly stimulated ^{32}P incorporation into a broad 87-kDa band that comigrated with photoaffinity labeled receptor. $^{32}PO_4$ incorpora-

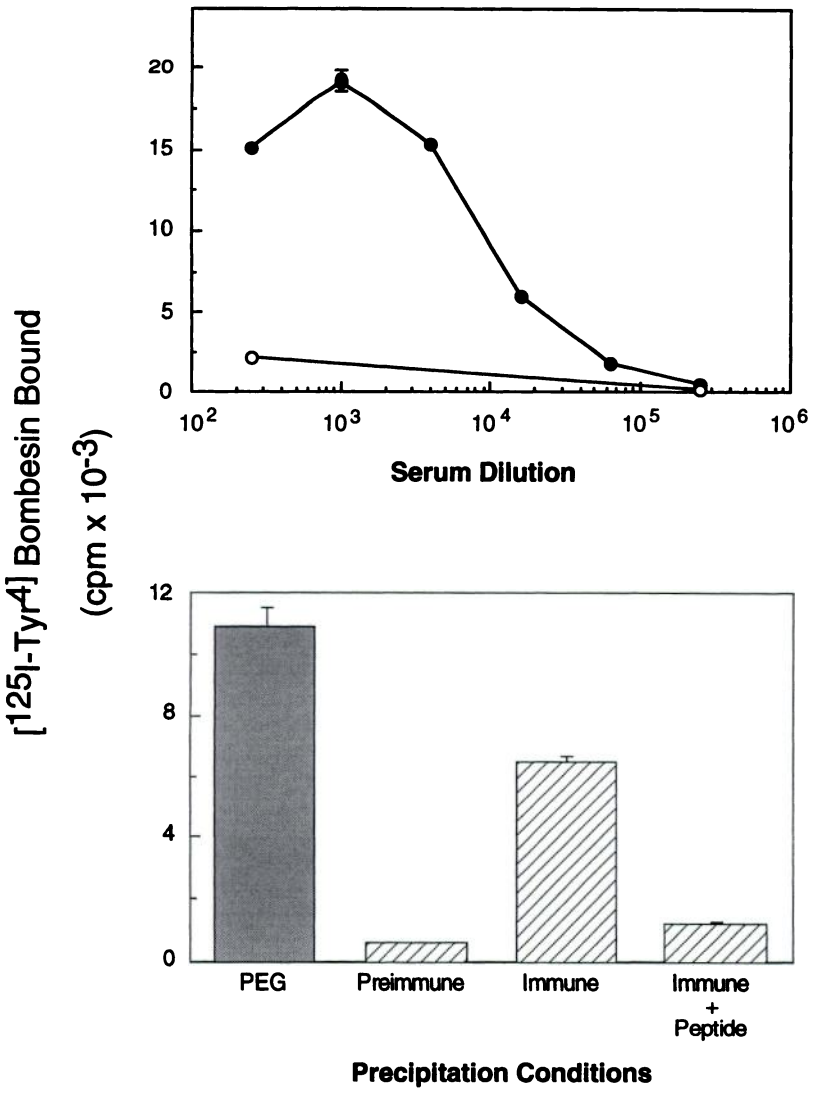

Fig. 2. Immunoprecipitation of the [^{125}I-Tyr^4]bombesin/receptor complex. Swiss 3T3 membranes (100 μg) were incubated with [^{125}I-Tyr^4]bombesin (5 × 10^5 cpm/ml; 0.14 nM) at 37° in the absence or presence of 100 nM bombesin. After centrifugation, membranes were solubilized with detergent, and the amount of [^{125}I-Tyr^4]bombesin/receptor complex in the soluble fraction was measured by precipitation with PEG. Aliquots of solubilized receptor were then incubated with sera for 2 hr at 4°, and the radioactivity precipitated with Protein A-Sepharose 4B was determined as described in Experimental Procedures. *Top*, solubilized proteins were incubated with either preimmune serum (○) or antiserum BR1-517 (●) at the concentrations indicated. The soluble fraction contained 33,314 ± 346 cpm of [^{125}I-Tyr^4]bombesin/receptor complex as determined by PEG precipitation before the addition of antiserum. *Bottom*, solubilized proteins were incubated with preimmune serum (1:10 dilution), antiserum (1:10 dilution), or antiserum plus 7 μM antigen peptide.

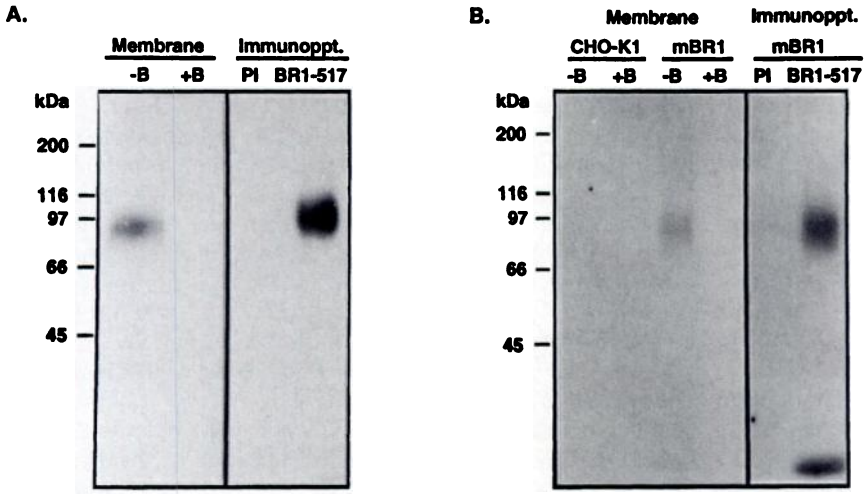

Fig. 3. Immunoprecipitation of affinity-labeled receptors. Membranes (200 μg/ml) from Swiss 3T3 fibroblasts (A) and CHO-K1 or CHO-mBR1 cells (B) were incubated at 30° for 20 min with [^{125}I]GRP(14–27) (10^6 cpm/ml; 0.28 nM) in the absence (−*B*) or presence (+*B*) of 100 nM bombesin. After the binding incubation, 0.1 mM ANB-NOS was added, and cross-linking was performed as described in Experimental Procedures. One aliquot of each reaction was directly analyzed by SDS-PAGE and autoradiography (*Membrane*). The membranes in replicate aliquots were solubilized and immunoprecipitated with either preimmune serum (*PI*) or BR1-517 antiserum before electrophoresis of the precipitated proteins (*Immunoppt.*).

tion was stimulated 2.6-fold in Swiss 3T3 cells and 3-fold in CHO-mBR1 cells. The addition of 7 μM peptide antigen completely blocked immunoprecipitation of the 87-kDa $^{32}PO_4$-labeled protein in both CHO-mBR1 cells (Fig. 5A) and Swiss 3T3 fibroblasts (data not shown).

To further test whether the 87-kDa phosphorylated protein was BR1, immunoprecipitated proteins were subjected to deglycosylation. After *N*-glycanase treatment of samples from CHO-mBR1 cells (Fig. 5B) and Swiss 3T3 fibroblasts (data not shown), the apparent molecular sizes of both the $^{32}PO_4$-labeled protein and photoaffinity-labeled receptor were decreased to ~45 kDa. The observed decrease in recep-

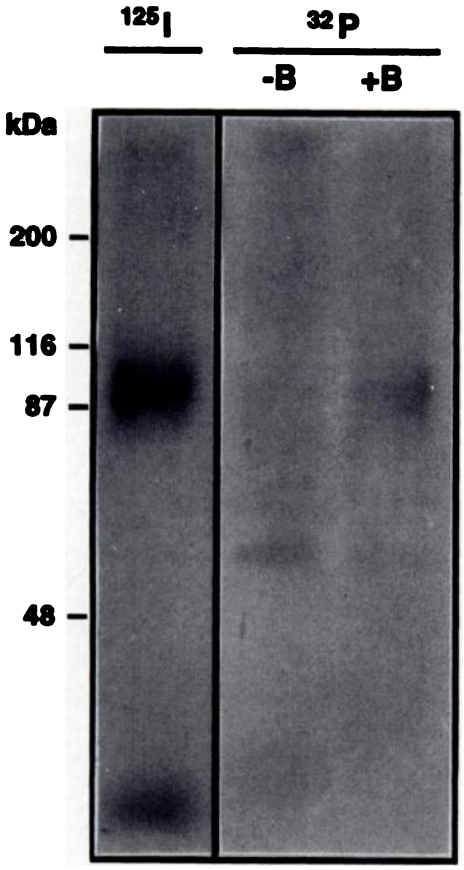

Fig. 4. Effect of bombesin on receptor phosphorylation in Swiss 3T3 cells. *^{125}I*, Swiss 3T3 membranes (200 μg/ml) were incubated with [^{125}I]GRP(14–27) (10^6 cpm/ml; 0.28 nM) at 30° for 20 min and then cross-linked with 0.1 mM ANB-NOS. *^{32}P*, Swiss 3T3 cells (2×10^6/dish) were metabolically labeled with [^{32}P]orthophosphate for 3 hr at 37°, incubated for 5 min either without (*−B*) or with (*+B*) 100 nM bombesin, and used for membrane preparation. Both ^{125}I- and ^{32}P-labeled membranes were subsequently solubilized and immunoprecipitated with antiserum BR1-517 as described in Experimental Procedures. Immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

tor mass after *N*-glycanase treatment is consistent with previous studies showing that BR1 is glycosylated on asparagine (31). Therefore, these results confirm that the 87-kDa $^{32}PO_4$-labeled protein represents phosphorylated BR1 and support the conclusion that bombesin stimulates $^{32}PO_4$ incorporation into BR1 in both Swiss 3T3 fibroblasts and CHO-mBR1 cells. To facilitate receptor detection and analysis, subsequent experiments were performed in CHO-mBR1 cells because receptor density in this cell line was 5 times higher than in Swiss 3T3 cells.

The time course of bombesin-stimulated receptor phosphorylation is shown in Fig. 6. Increased phosphorylation was detected 2 min after the addition of 100 nM bombesin, the earliest time tested. Maximum BR1 phosphorylation occurred after 15 min of bombesin stimulation and was sustained for at least an additional 15 min. At peak phosphorylation, $^{32}PO_4$ incorporation into BR1 was increased 9.5-fold over basal.

Phosphoamino acid analysis was performed on immunoprecipitated receptor from $^{32}PO_4$-labeled CHO-mBR1 cells treated with 100 nM bombesin for 15 min. After acid hydrolysis for 2 hr at 110°, two-dimensional thin layer chromatography showed $^{32}PO_4$ incorporation into both phosphoserine and phosphothreonine (Fig. 7). There was no labeling of phosphotyrosine. Even when acid hydrolysis conditions were optimized for phosphotyrosine detection (30 min at 110°), this amino acid was not labeled (data not shown). Therefore, agonist binding increases phosphorylation of mBR1 on serine and threonine residues.

Involvement of PKC in bombesin receptor phosphorylation. Because activation of PKC by TPA produces bombesin receptor desensitization (Fig. 1), we determined whether the receptor was phosphorylated after PKC stimulation (Fig. 8). CHO-mBR1 cells were prelabeled with [^{32}P]orthophosphate and incubated with 100 nM TPA for 30 min. The receptor was subsequently purified by immunoprecipitation as described above. Surprisingly, there was no detectable increase in $^{32}PO_4$-labeled BR1 in the immunoprecipitate from TPA-treated cells, although in the same experiments, 100 nM bombesin stimulated receptor phosphorylation by ~5-fold (Fig. 8). Furthermore, the PKC inhibitor GF109203X (32) enhanced bombesin-stimulated receptor phosphorylation by 41% in Fig. 8 (*top*) and an average of 40 ± 8% in four independent experiments. Similarly, chronic TPA pretreatment, which down-regulates PKC (33), enhanced bombesin-induced phosphorylation by 2-fold (Fig. 8, *bottom*).

The unexpected observation that TPA did not increase $^{32}PO_4$ labeling of BR1 could be explained if the antibody was unable to recognize and immunoprecipitate the receptor after it was phosphorylated by PKC. To test this hypothesis, we compared the efficiency of immunoprecipitation for receptors prepared from untreated cells with that from cells preincubated with TPA. After incubation with 100 nM TPA for 30 min, membranes were prepared and bound with [^{125}I-Tyr^4]bombesin as usual. The receptor/hormone complex was then solubilized, quantified by polyethylene glycol precipitation, and immunoprecipitated as described above. Consistent with previous observations (13, 15), TPA pretreatment did not affect ligand binding to the membrane receptor (data not shown). However, the efficiency for immunoprecipitation of the solubilized [^{125}I]bombesin/receptor complex was decreased 45 ± 2% by the TPA pretreatment (n = 2). To test whether this decrease in immunoprecipitation efficiency was due to BR1 phosphorylation, we next determined whether alkaline phosphatase could restore the ability of the antibody to recognize the TPA-treated receptor. In previous studies, dephosphorylation was more effective when receptors were denatured before enzyme treatment (28). Therefore, we used photoaffinity-labeled receptors for this experiment (Fig. 9). Membranes from control and TPA-treated cells were covalently cross-linked to [^{125}I]GRP(14–27), solubilized under denaturing conditions, and treated with alkaline phosphatase before immunoprecipitation. After gel electrophoresis, the radioactivity in the receptor band was quantified. Fig. 9 shows that TPA markedly decreased immunoprecipitation of BR1. However, treatment with alkaline phosphatase increased the immunoprecipitation of the TPA-pretreated receptor by 8-fold (Fig. 9). Interestingly, phosphatase treatment also increased the amount of BR1 immunoprecipitated from control cells (Fig. 9). Therefore, after phosphatase exposure, receptors from control and TPA-pretreated cells were bound equally to the antibody.

Taken together, these results indicate that receptor phosphorylation by PKC prevents immunoprecipitation by the BR1-517 antibody. Therefore, TPA did not affect $^{32}PO_4$ in-

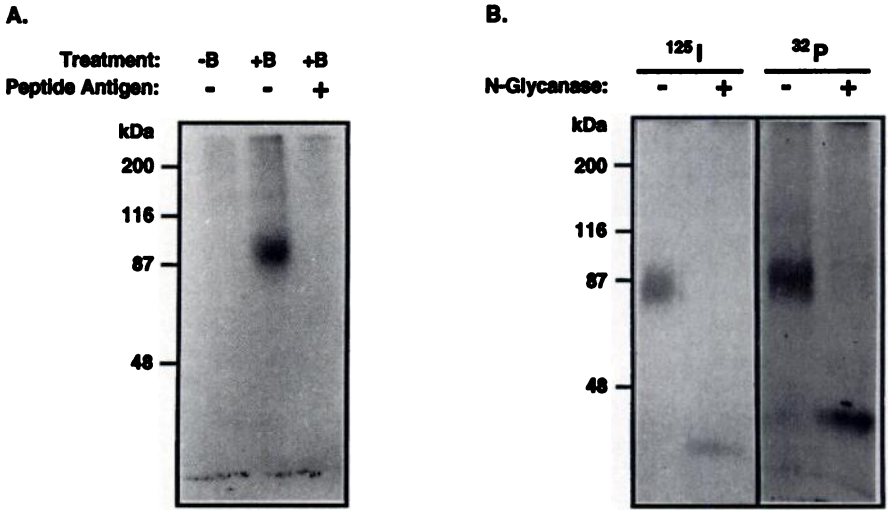

Fig. 5. Effect of bombesin on receptor phosphorylation in CHO-mBR1 cells. A, CHO-mBR1 cells (6×10^6/dish) were metabolically labeled with [^{32}P]orthophosphate for 3 hr. After the addition of 100 nM bombesin to the samples shown (+*B*), the incubation was continued for 5 min. Membranes were prepared and solubilized as described in Experimental Procedures. Immunoprecipitation with antibody BR1-517 was performed in the absence or presence of 1 μM peptide antigen as indicated, and precipitated proteins were then analyzed by SDS-PAGE and autoradiography. B, ^{32}P-labeled membranes were prepared from cells treated with 100 nM bombesin for 5 min as described above. Photoaffinity-labeled membranes (^{125}I) were prepared as described in the legend to Fig. 4. Both ^{125}I- and ^{32}P-labeled membranes were solubilized, immunoprecipitated with antibody BR1-517, and then incubated without or with *N*-glycanase to remove asparagine-linked carbohydrates. Reaction products were analyzed by SDS-PAGE and autoradiography.

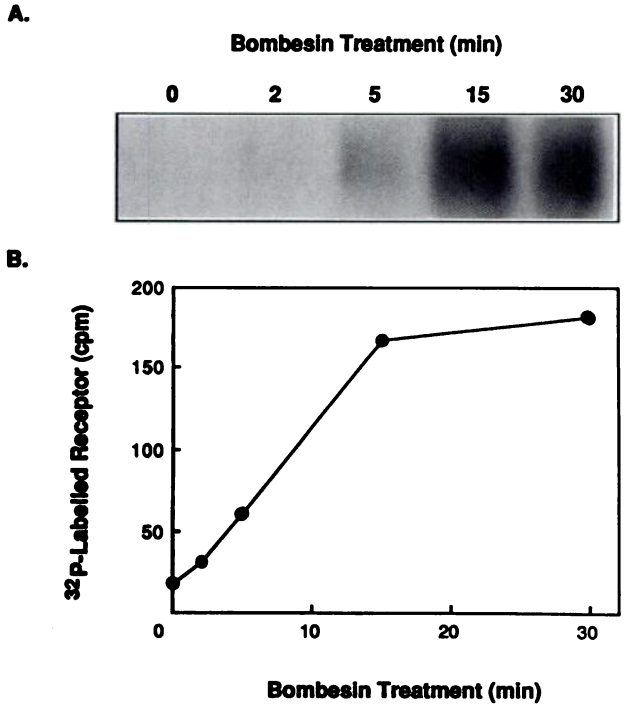

Fig. 6. Time course of bombesin-stimulated receptor phosphorylation. CHO-mBR1 cells (6×10^6/dish), metabolically labeled with [^{32}P]-orthophosphate, were incubated with 100 nM bombesin for the times shown. Membranes were prepared, solubilized, and immunoprecipitated with antibody BR1-517 as described in Experimental Procedures. *Top*, autoradiogram of the 87-kDa receptor band after SDS-PAGE. To quantify bombesin receptor phosphorylation, the 87-kDa bands were cut from the gel, and the associated Cherenkov radiation was measured in a liquid scintillation counter (*bottom*).

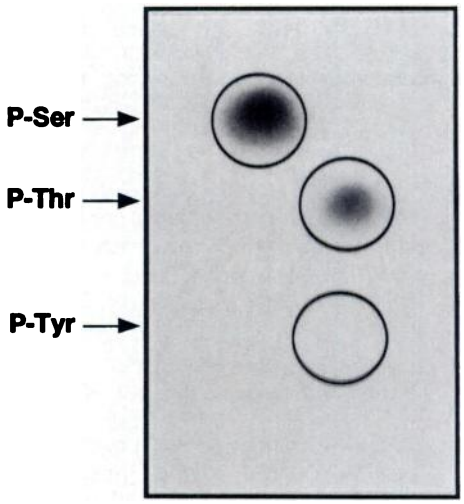

Fig. 7. Analysis of phosphorylated amino acids in the bombesin receptor. CHO-mBR1 cells (6×10^6/dish), metabolically labeled with [^{32}P]orthophosphate, were incubated with 100 nM bombesin for 15 min. Membranes were prepared, solubilized, immunoprecipitated with antibody BR1-517, and subjected to SDS-PAGE. Separated proteins were electrophoretically transferred from the gel to polyvinylidene difluoride membranes. The band at 87 kDa was excised and hydrolyzed with 6 N HCl for 2 hr. The products were subjected to two-dimensional thin layer chromatography and analyzed by autoradiography as described in Experimental Procedures. *Circles*, positions of the nonradioactive phosphoamino acids added as internal standards.

corporation into the immunoprecipitated receptor because the antibody could not recognize the receptor after PKC phosphorylation. In contrast, bombesin markedly stimulated $^{32}PO_4$ labeling of the receptor, showing that the phosphorylation triggered by hormone binding did not block receptor immunoprecipitation. Therefore, PKC and bombesin must lead to receptor phosphorylation at distinct sites.

Discussion

In the current study, we investigated the potential role of receptor phosphorylation in the acute desensitization of the BR1 bombesin receptor by agonist and by the PKC activator

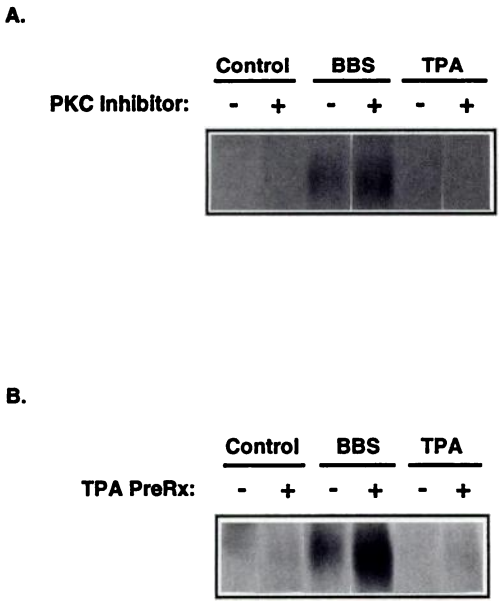

Fig. 8. Effect of PKC activation and inhibition on receptor phosphorylation. CHO-mBR1 cells (6×10^6/dish) were incubated with $^{32}PO_4$ for 3 hr. Either bombesin (*BBS*) (100 nM) or TPA (100 nM) was then added to the samples indicated, and the incubation was continued for 15 or 30 min, respectively. Membranes were prepared, solubilized, and immunoprecipitated with antibody BR1-517. The immunoprecipitated receptor was subjected to SDS-PAGE and autoradiography: the 87-kDa receptor band is shown. *Top*, PKC inhibitor GF109203X (3.5 μM) was added for the last 2 hr of the labeling reaction. *Bottom*, cells were incubated with or without 100 nM TPA for 26 hr before metabolic labeling.

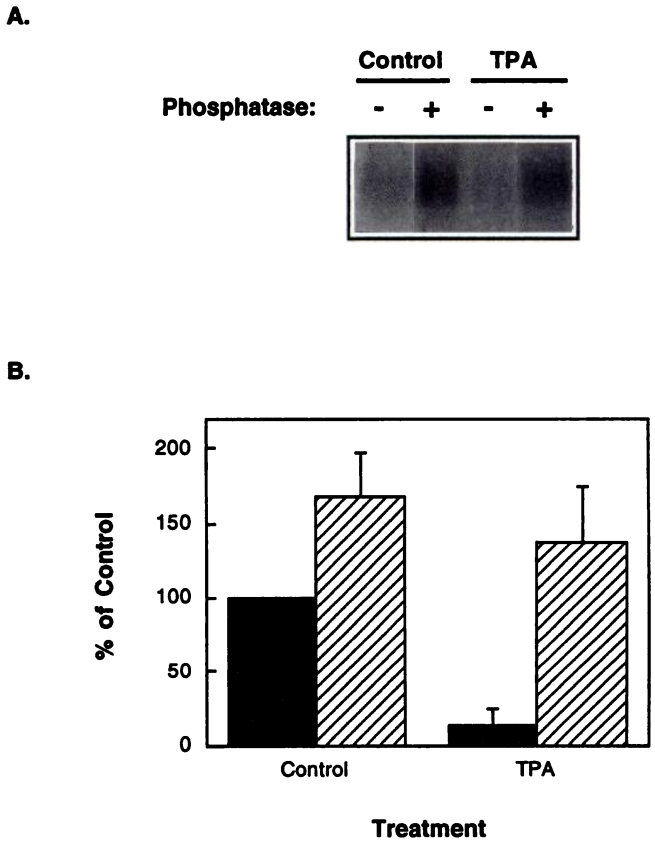

Fig. 9. Effect of dephosphorylation on receptor immunoprecipitation. CHO-mBR1 cells were incubated without or with 100 nM TPA for 30 min at 37°. Membranes were prepared in the presence of phosphatase inhibitors, incubated with [^{125}I]GRP(14–27) (0.28 nM), and cross-linked with 0.1 mM ANB-NOS. Photoaffinity-labeled receptors were solubilized in 1.5 mg/ml deoxycholate and 0.1% SDS and incubated either without or with bacterial alkaline phosphatase. Samples were then immunoprecipitated and analyzed by SDS-PAGE and autoradiography (*top*). Radioactivity in the 87-kDa bands was quantified with a PhosphorImager and expressed as a percentage of the untreated control sample (*bottom*). Values represent the mean ± standard deviation of two experiments.

TPA. We show that BR1 is partially phosphorylated under basal conditions and that both bombesin and TPA stimulate receptor phosphorylation concomitant with receptor desensitization. However, bombesin-stimulated phosphorylation is not mediated by PKC, and distinct receptor sites are targeted for phosphorylation by bombesin- and TPA-stimulated kinases.

We observed that bombesin treatment of Swiss 3T3 cells led to a biphasic increase in intracellular Ins(1,4,5)P_3, as previously reported (34). Maximal Ins(1,4,5)P_3 accumulation was achieved at 5–15 sec of bombesin stimulation and then declined over the next 15–30 min. Such a biphasic response in the absence of Li^+ indicates a decrease in the rate of phosphatidylinositol bisphosphate hydrolysis in the continuous presence of agonist and is characteristic of receptor desensitization (2). Indeed, after a 5-min exposure of Swiss 3T3 cells to bombesin, a second acute (15 sec) challenge with the peptide produced no stimulation beyond that remaining from the first treatment. The residual Ins(1,4,5)P_3 stimulation presumably results from continued receptor occupancy by bombesin. However, attempts to verify this conclusion by washing the cells with acid to dissociate prebound bombesin before the challenge incubation gave inconclusive results: the acid wash by itself affected Ins(1,4,5)P_3 formation. Nevertheless, the data clearly show that the bombesin receptor is desensitized.

To facilitate biochemical analysis of receptor phosphorylation, we stably expressed mBR1 in CHO-K1 cells, a line that lacks endogenous bombesin receptors but provides an environment in which other phospholipase C-coupled receptors are appropriately desensitized (2). In the resulting clonal isolate, named CHO-mBR1, bombesin also elicited a biphasic stimulation of Ins(1,4,5)P_3 formation, with [^{3}H]Ins(1,4,5)P_3 levels reaching a peak at 15 sec and then slowly returning toward basal values. As in Swiss 3T3 cells, pretreatment of CHO-mBR1 cells with bombesin for 5 min caused partial desensitization such that the Ins(1,4,5)P_3 levels produced in the second challenge incubation were lower than produced by the initial stimulation. Furthermore, pretreatment of CHO-mBR1 cells with TPA also desensitized the Ins(1,4,5)P_3 response to subsequent bombesin stimulation, as previously reported in other cell types (13, 15, 17, 35). Because bombesin responsiveness is regulated similarly in CHO-mBR1 and Swiss 3T3 fibroblasts, the transfected cell line not only serves as an abundant source of BR1 protein but also provides a valid model with which to study the mechanisms of bombesin receptor desensitization.

To rapidly and specifically purify the bombesin receptor, we raised antibodies to a peptide corresponding to a unique carboxyl-terminal sequence within BR1. The resulting BR1-517 antiserum efficiently immunoprecipitated the solubilized [^{125}I-Tyr^4]bombesin/receptor complex as well as the photoaffinity-labeled 87-kDa receptor protein. Furthermore, recep-

tor immunoprecipitation was blocked by low concentrations of the carboxyl-terminal antigen peptide, demonstrating that the immunoprecipitating antibody recognized this epitope of the receptor. We concluded that the antibody must be able to bind unphosphorylated receptor protein because the photoaffinity-labeled membrane receptor was immunoprecipitated, yet the receptor from control cells contained no detectable $^{32}PO_4$. However, immunoprecipitation of the receptor from control cells never exceeded 60% efficiency, even at supramaximal antiserum titers, suggesting that a portion of the receptor population was not recognized by the antibody. Nevertheless, the observation that bombesin markedly increased the amount of $^{32}PO_4$-labeled receptor in immunoprecipitates indicated that the antibody also recognized a phosphorylated form of BR1.

Incubation of either Swiss 3T3 cells or CHO-mBR1 cells with bombesin elevated receptor phosphorylation within 5 min. In CHO-mBR1 cells, receptor phosphorylation was detectable by 2 min, the earliest time point examined; was maximal by 15 min; and remained elevated after 30 min of continuous exposure to agonist. While these studies were in progress, Kroog *et al.* (36) reported that bombesin stimulated the phosphorylation of an epitope-tagged mBR1 transfected into Balb 3T3 fibroblasts, although they were unable to detect phosphorylation of the endogenous receptor in Swiss 3T3 cells. Interestingly, in their study, the receptor was maximally phosphorylated within the first minute of bombesin treatment. This difference in the time course of receptor phosphorylation could result from differences in the nature or concentration of the kinases present in Balb 3T3 and CHO-K1 cells. However, an alternate explanation derives from the results of experiments with TPA, which indicate that different phosphorylated states of the receptor are recognized by the antibodies used in the two studies.

Acute TPA exposure both desensitized bombesin-stimulated Ins(1,4,5)P_3 formation and enhanced receptor phosphorylation. Increased receptor phosphorylation was demonstrated by the effect of TPA to decrease the efficiency of receptor immunoprecipitation and by the ability of alkaline phosphatase treatment of the receptor to reverse this inhibition. Because the TPA-induced phosphorylation interfered with antibody binding to the receptor, we did not detect TPA-stimulated $^{32}P0_4$ incorporation into BR1 with our carboxyl-terminal antiserum. In agreement with these conclusions, Kroog *et al.* (36), using an antibody directed to the third intracellular loop of BR1, found that TPA did increase $^{32}P0_4$ labeling of the bombesin receptor. Because their third loop antibody immunoprecipitated the receptor after both TPA- and bombesin-induced phosphorylation, Kroog *et al.* detected $^{32}P0_4$ labeling of BR1 by both PKC-dependent and -independent pathways. In contrast, we detected only PKC-independent $^{32}P0_4$ incorporation into the bombesin receptor. It is perhaps not surprising, then, that the time course of bombesin-stimulated phosphorylation seemed to be different in the two studies. Certainly the temporal sequence in which different receptor residues are phosphorylated is likely to have significant physiological consequences and will be an important area for investigation. Interestingly, the rate of bombesin-induced BR1 phosphorylation in our study correlates with both the rate of receptor internalization reported by Kroog *et al.* (36) and the time course for the decrease in Ins(1,4,5)P_3 levels that we observed in the continued presence of bombesin. These results therefore indicate that phosphorylation may be responsible for receptor desensitization and/or receptor internalization.

Because our antibody does not recognize the mBR1 receptor phosphorylated after TPA stimulation but does recognize the receptor phosphorylated by agonist stimulation, it follows that BR1 phosphorylation must occur at different sites under these two conditions and that bombesin-induced receptor phosphorylation is not mediated by PKC. This conclusion is further supported by our observation that PKC blockade did not reduce (in fact it enhanced) bombesin-stimulated receptor phosphorylation. Although the kinase or kinases that are responsible for the PKC-independent phosphorylation of mBR1 have not been defined, likely candidates are members of the GRK family (37, 38). GRKs are serine and threonine kinases that preferentially phosphorylate the agonist-occupied form of seven-transmembrane-domain receptors and thereby cause receptor/G protein uncoupling (37, 38). The involvement and specificity of the known GRKs in bombesin receptor desensitization remain to be determined.

The receptor hyperphosphorylation produced by bombesin in cells depleted of PKC was unexpected. This apparent hyperphosphorylation may result from the selectivity of our antibody for different phosphorylation states of the receptor. Because alkaline phosphatase treatment increased immunoprecipitation of receptors from control as well as TPA-treated cells, we concluded that even in the basal state, BR1 was partially phosphorylated at a "PKC" site and that this phosphorylation could reduce the efficiency of receptor immunoprecipitation. When PKC is inhibited, basal receptor phosphorylation at this site must be reduced, enabling more of the receptor to be immunoprecipitated. Therefore, even if bombesin-stimulated receptor kinases could phosphorylate "PKC site"-phosphorylated and -unphosphorylated receptors equally well, increased immunoprecipitation of the latter would augment $^{32}PO_4$ labeling of the immunoprecipitated receptor in PKC-depleted cells. However, such a technical explanation is less likely because Kroog *et al.* also observed increased bombesin-induced $^{32}PO_4$ incorporation into the receptor in PKC-depleted cells and their antibodies did not exhibit the same selectivity for different phosphorylation states of the receptor as did ours (236). An alternate explanation for the receptor hyperphosphorylation observed in cells depleted of PKC is that phosphorylation at the "PKC" site makes the receptor a poorer substrate for a receptor kinase. This mechanism has recently been shown to explain the *in vitro* phosphorylation of the m1 muscarinic receptor, which also couples to phospholipase C via the G_q family of G proteins (39). Haga *et al.* (39) showed that purified m1 receptors were phosphorylated by GRK2 and PKC at distinct, mutually exclusive sites: GRK2 phosphorylated the receptor in the third intracellular loop, whereas PKC targeted sites in the carboxyl-terminal tail. Furthermore, the order of phosphorylation by the two kinases affected the stoichiometry of receptor phosphorylation. Prior phosphorylation of m1 receptors by PKC attenuated subsequent phosphorylation by GRK2 (39). A similar interaction would account for the potentiation of agonist-stimulated bombesin receptor phosphorylation in PKC-depleted cells. Therefore, such cross-talk between G protein receptor kinases and second messenger-regulated protein kinases may generally be used by cells to precisely control receptor function. Clearly, identification of

the receptor residues phosphorylated will be a necessary first step toward determining the functional significance of BR1 phosphorylation and the nature of the interaction between the multiple kinases apparently involved. The results from such an analysis will undoubtedly be complex; our observation that bombesin increases receptor phosphorylation on both serine and threonine residues shows that the PKC-independent pathway by itself leads to receptor phosphorylation on multiple sites.

Although our results do not identify the receptor sites phosphorylated, they suggest a likely possibility: that amino acids within the antibody epitope are modified by PKC activation. Phosphorylation of individual amino acids can dramatically alter the binding of site-specific antipeptide antibodies (40). In fact, such phosphorylation state-dependent antibodies have been successfully exploited to study the functional significance of receptor phosphorylation (40–42). The antigen peptide that we used to generate our bombesin receptor antibodies contains serine/threonine within a potential PKC consensus site (RxxSTxR) (43). Furthermore, a carboxyl-terminal region of the receptor containing this site has been implicated in receptor endocytosis (44). Although additional studies will be required to determine whether our antibody binds directly at a PKC phosphorylation site, the availability of a phosphorylation state-dependent antibody will undoubtedly be useful in examining the functional importance of both PKC-dependent and -independent pathways of BR1 phosphorylation.

In conclusion, we demonstrated that bombesin leads to the rapid phosphorylation of its receptor on both serine and threonine residues. Although PKC activation also increases receptor phosphorylation, the phosphorylation induced by bombesin occurs independent of PKC, and distinct receptor sites are targeted in the two pathways. Because PKC-mediated phosphorylation prevents our carboxyl-terminally directed antibody from recognizing the receptor, we suggest that a likely target for TPA-stimulated receptor phosphorylation will be within the antibody epitope. The role of this and other phosphorylation sites in bombesin receptor regulation remains to be determined.

Acknowledgments

We thank Dr. William T. Moore and the Analytical Chemical Center for peptide synthesis, Dr. James F. Battey for the bombesin receptor cDNA, Drs. William Hipkin and Dale Hereld for helpful suggestions, and Dr. Mari Haddox and Barbara Cochran for expert advice on phosphoamino acid analysis.

References

1. Kobilka, B. Adrenergic receptors as models for G protein-coupled receptors. *Annu. Rev. Neurosci.* **15**:87–114 (1992).
2. Wojcikiewicz, R. J. H., A. B. Tobin, and S. R. Nahorski. Desensitization of cell signalling mediated by phosphoinositidase C. *Trends Pharmacol. Sci.* **14**:279–285 (1993).
3. Kroog, G. S., R. T. Jensen, and J. F. Battey. Mammalian bombesin receptors. *Med. Res. Rev.* **15**:389–417 (1995).
4. Jensen, R. T., and D. H. Coy. Progress in the development of potent bombesin receptor antagonists. *Trends Pharmacol. Sci.* **12**:13–19 (1991).
5. Sunday, M. E., L. M. Kaplan, E. Motoyama, W. W. Chin, and E. R. Spindel. Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. *Lab. Invest.* **59**:5–24 (1988).
6. Takuwa, N., Y. Takuwa, W. E. Bollag, and H. Rasmussen. The effects of bombesin on polyphosphoinositide and calcium metabolism in Swiss 3T3 cells. *J. Biol. Chem.* **262**:182–188 (1987).
7. Swope, S. L., and A. Schonbrunn. The biphasic stimulation of insulin secretion by bombesin involves both cytosolic free calcium and protein kinase C. *Biochem. J.* **253**:193–202 (1988).
8. Woll, P. J., and E. Rozengurt. Bombesin, and bombesin antagonists: studies in Swiss 3T3 cells and human small cell lung cancer. *Br. J. Cancer* **57**:579–586 (1988).
9. Fischer, J. B., and A. Schonbrunn. The bombesin receptor is coupled to a guanine nucleotide-binding protein which is insensitive to pertussis and cholera toxins. *J. Biol. Chem.* **263**:2808–2816 (1988).
10. Offermanns, S., E. Heiler, K. Spicher, and G. Schultz. Gq and G11 are concurrently activated by bombesin and vasopressin in Swiss 3T3 cells. *FEBS Lett.* **349**:201–204 (1994).
11. Plevin, R., S. Palmer, S. D. Gardner, and M. J. O. Wakelam. Regulation of bombesin-stimulated inositol 1,4,5-trisphosphate generation in Swiss 3T3 fibroblasts by a guanine-nucleotide-binding protein. *Biochem. J.* **268**:605–610 (1990).
12. Swope, S. L., and A. Schonbrunn. Characterization of ligand binding and processing by bombesin receptors in an insulin-secreting cell line. *Biochem. J.* **247**:731–738 (1987).
13. Swope, S. L., and A. Schonbrunn. Desensitization of islet cells to bombesin involves both receptor down-modulation and inhibition of receptor function. *Mol. Pharmacol.* **37**:758–766 (1990).
14. Brown, K. D., M. S. Laurie, C. J. Littlewood, D. M. Blakeley, and A. N. Corps. Characterization of the high-affinity receptors on Swiss 3T3 cells which mediate the binding, internalization, and degradation of the mitogenic peptide bombesin. *Biochem. J.* **252**:227–235 (1988).
15. Brown, K. D., D. M. Blakeley, M. H. Hamon, S. Laurie, and A. N. Corps. Protein kinase C-mediated negative-feedback inhibition of unstimulated and bombesin-stimulated polyphosphoinositide hydrolysis in Swiss-mouse 3T3 cells. *Biochem. J.* **245**:631–639 (1987).
16. Millar, J. B. A., and E. Rozengurt. Chronic desensitization to bombesin by progressive down-regulation of bombesin receptors in Swiss 3T3 cells. *J. Biol. Chem.* **265**:12052–12058 (1990).
17. Walsh, J. H., M. Bouzyk, and E. Rozengurt. Homologous desensitization of bombesin-induced increases in intracellular Ca2+ in quiescent Swiss 3T3 cells involves a protein kinase C-independent mechanism. *J. Cell. Physiol.* **156**:333–340 (1993).
18. Tykocinski, M. L., H. K. Shu, D. J. Ayers, E. I. Walter, R. R. Getty, R. K. Groger, C. A. Hauer, and M. E. Medif. Glycolipid reanchoring of T-lymphocyte surface antigen CD8 using the 3′ end sequence of decay-accelerating factor's mRNA. *Proc. Natl. Acad. Sci. USA* **85**:3555–3559 (1988).
19. Williams, B. Y., and A. Schonbrunn. Bombesin receptors in a human duodenal tumor cell line: binding properties and function. *Cancer Res.* **54**:818–824 (1994).
20. Spindel, E. R., E. Giladi, P. Brehm, R. H. Goodman, and T. P. Segerson. Cloning and functional characterization of a complementary DNA encoding the murine fibroblast bombesin/gastrin-releasing peptide receptor. *Mol. Endocrinol.* **4**:1956–1963 (1990).
21. Battey, J. F., J. M. Way, M. H. Corjay, H. Shapira, K. Kusano, R. Harkins, J. M. Wu, T. Slattery, E. Mann, and R. I. Feldman. Molecular cloning of the bombesin/gastrin-releasing peptide receptor from Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* **88**:395–399 (1991).
22. Merrifield, R. B., L. D. Vizioli, and H. G. Boman. Synthesis of the antibacterial peptide cecropin A (1–33). *Biochemistry* **21**:5020–5031 (1982).
23. Moore, W. T., and R. M. Caprioli. Monitoring peptide synthesis stepwise by mass spectrometry, in *Techniques in Protein Chemistry II* (J. Villafranca, ed.). Academia Press, San Diego, 511 (1991).
24. Current protocols, in *Molecular Biology*. Greene Publishing Associates/Wiley-Interscience, 11.15.2 (1992).
25. Deb, S., K. Hashizume, K. Boone, J. N. Southard, F. Talamantes, A. Rawitch, and M. J. Soares. Antipeptide antibodies reveal structural and functional characteristics of rat placental lactogen-II. *Mol. Cell. Endocrinol.* **63**:45–56 (1989).
26. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
27. Kris, R. M., R. Hazan, J. Villines, T. W. Moody, and J. Schlessinger. Identification of the bombesin receptor on murine and human cells by cross-linking experiments. *J. Biol. Chem.* **262**:11215–11220 (1991).
28. Hereld, D., R. Vaughan, J. Y. Kim, J. Borleis, and P. Devreotes. Localization of ligand-induced phosphorylation sites to serine clusters in the C-terminal domain of the Dictyostelium cAMP receptor, cAR1. *J. Biol. Chem.* **269**:7036–7044 (1994).
29. Zachary, I., and E. Rozengurt. High-affinity receptors for peptides of the bombesin family in Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* **82**:7616–7620 (1990).
30. Heslop, J. P., D. M. Blakeley, K. D. Brown, R. F. Irvine, and M. J. Berridge. Effects of bombesin and insulin on inositol(1,4,5)trisphosphate and inositol(1,3,4)trisphosphate formation in Swiss 3T3 cells. *Cell* **47**:703–709 (1986).
31. Kusui, T., R. V. Benya, J. F. Battey, and R. T. Jensen. Glycosylation of bombesin receptors: characterization, effect on binding, and G-protein coupling. *Biochemistry* **33**:12968–12980 (1994).
32. Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Bourseir, F. Loriolle, L. Duhamel, D. Charon, and J. Kirilovski. The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266**:15771–15781 (1991).

33. Kikkawa, U., and Y. Nishizuka. The role of protein kinase C in transmembrane signalling. *Annu. Rev. Cell. Biol.* **2**:149–178 (1986).
34. Oberhuber, H., K. Maly, F. Uberall, J. Hoflacher, A. Kiani, and H. H. Grunicke. Mechanisms of desensitization of the Ca^{2+}-mobilizing system to bombesin by Ha-ras: independence from down-modulation of agonist-stimulated inositol phosphate production. *J. Biol. Chem.* **266**:1437–1442 (1991).
35. Blakeley, D. M., A. N. Corps, and K. D. Brown. Bombesin, and platelet-derived growth factor stimulate formation of inositol phosphates and Ca2+ mobilization in Swiss 3T3 cells by different mechanisms. *Biochem. J.* **258**:177–185 (1989).
36. Kroog, G. S., E. Sainz, P. J. Worland, M. A. Akeson, R. V. Benya, R. T. Jensen, and J. F. Battey. The gastrin-releasing peptide receptor is rapidly phosphorylated by a kinase other than protein kinase C after exposure to agonist. *J. Biol. Chem.* **270**:8217–8224 (1995).
37. Haga, T., K. Haga, and K. Kameyama. G protein-coupled receptor kinases. *J. Neurochem.* **63**:400–412 (1994).
38. Premont, R. T., J. Inglese, and R. J. Lefkowitz. Protein kinases 3. Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J.* **9**:175–182 (1995).
39. Haga, K., K. Kameyama, T. Haga, U. Kikkawa, K. Shiozaki, and H. Uchiyama. Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C. *J. Biol. Chem.* **271**:2276–2782 (1996).
40. Czernik, A. J., J.-A. Girault, A. C. Nairn, J. Chen, G. Snyder, J. Kebabian, and P. Greengard. Production of phosphorylation state-specific antibodies. *Methods Enzymol.* **201**:264–283 (1991).
41. Coghlan, M. P., T. S. Pillay, J. M. Tavare, and K. Siddle. Site-specific anti-phosphopeptide antibodies: use in assessing insulin receptor serine/threonine phosphorylation state and identification of serine-1327 as a novel site of phorbol ester-induced phosphorylation. *Biochem. J.* **303**:893–899 (1994).
42. Nakazawa, K., S. Mikawa, T. Hashikawa, and M. Ito. Transient and persistent phosphorylation of AMP-type glutamate receptor subunits in cerebellar Purkinje cells. *Neuron* **15**:697–709 (1995).
43. Kennelly, P. J., and E. G. Krebs. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266**:15555–15558 (1991).
44. Benya, R. V., S. Fathi, J. F. Battey, and R. T. Jensen. Serines and threonines in the gastrin-releasing peptide receptor carboxyl terminus mediate internalization. *J. Biol. Chem.* **268**:20285–20290 (1993).

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