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Expression and Characterization of Cloned Human Bombesin Receptors

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SUMMARY

Little is known about the pharmacology or cell biology of human bombesin (Bn) receptors, because they are usually present at low levels and both subtypes are frequently present in the same tissues. Human gastrin-releasing peptide (GRP) receptors (huGRP-R) and human neuromedin B (NMB) receptors (huNMB-R) were stably transfected into BALB/3T3 fibroblasts. Both receptor types were glycosylated, with 35% of the huGRP-R and 38% of the huNMB-R representing carbohydrate residues. The extent of glycosylation of the transfected huGRP-R was the same as that seen in the human glioblastoma cell line U-118. Radiolabeled agonist ligands were rapidly internalized, whereas noninternalized ligand readily dissociated in a temperature-dependent fashion. The affinities of various agonists for binding to the huGRP-R were Bn (K_i = 1.4 ± 0.2 nM) = 4 × GRP = 300 × NMB. In contrast, affinities for the huNMB-R were NMB (K_i = 8.1 ± 5.2 nM) = 4 × Bn = 600 × GRP. [F_5-D-Phe^6,D-Ala^{11}]Bn(6–13)methyl ester was the most potent huGRP-R antagonist, whereas D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2 was the most potent huNMB-R antagonist. Agonist binding to either receptor type caused activation of phospholipase C and increased cellular [^{3}H]inositol phosphate levels. GRP was potent at increasing [^{3}H]inositol phosphate generation in cells expressing the huGRP-R (EC_{50} = 13.6 ± 1.3 nM), whereas NMB was similarly potent when acting upon cells expressing the huNMB-R (EC_{50} = 9.3 ± 1.4 nM). However, neither receptor type, when stimulated with agonist, caused an increase in cAMP levels. These data show that stably transfected huGRP-R exhibit similar pharmacology for agonists and antagonists, are appropriately glycosylated, and function similarly with respect to their ability to alter biological activity, compared with natively expressed receptors. Minimal native huNMB-R data are available for comparison, but in general the huNMB-R is similar to the rat NMB receptor in its pharmacology and cell biology.

Bn-related peptides act on diverse tissues, including the central nervous system [regulating satiety (1), thermoregulation (2), and circadian rhythm (3)] and the gastrointestinal tract [regulating pancreatic secretion (4), smooth muscle contractility (5), and the release of other gastrointestinal peptides (6)], and are involved in development [stimulating growth of chondrocytes *in utero* (7) and participating in normal lung development (8)]. Bn-related peptides also are involved in the regulation of thyrotropin release (9) and in immune function [stimulating chemotaxis and increasing natural killer cell activity and antibody-dependent cellular cytoxicity (10)] and function as potent growth factors for both normal and neoplastic tissues (11, 12).

In mammals the actions of the amphibian peptides Bn, litorin, and ranatensin are mediated by GRP and NMB. By pharmacological, functional, and cloning studies, these actions are now known to be mediated by two classes of receptors, the GRP-R and the NMB-R, which are widely expressed in the central nervous system and peripheral tissues (13). Each of these receptors is linked to activation of phospholipase C, whereas activation of the GRP-R in some cell types also acts to increase cAMP levels (12, 14). Activation of both receptor subtypes results in receptor internalization, down-regulation, and desensitization (12, 15). Recently, the huGRP-R and huNMB-R have been cloned and sequenced (16).

Although extensive information is available on the effects of this family of peptides in various species, including their possible physiological functions and pharmacological actions (11), relatively few such studies exist for humans. Recent studies show that Bn-related peptides in humans are potent satiety factors (17), can stimulate the release of different peptides (6), and can function as potent growth factors in various human tumors, including human small cell lung cancer cells, breast cancer cells, and prostatic adenocarcinoma cells (11, 18, 19).

ABBREVIATIONS: Bn, bombesin; GRP, gastrin-releasing peptide; NMB, neuromedin B; GRP-R, gastrin-releasing peptide receptor(s); NMB-R, neuromedin B receptor(s); huGRP-R, human gastrin-releasing peptide receptor(s); huNMB-R, human neuromedin B receptor(s); PNGase F, peptide-*N*^{4}-(acetyl-β-glucosaminyl)asparagine amidase; DSS, disuccinimidyl suberate; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; DMEM, Dulbecco's modified essential medium; cyclo-SS-octa, D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2; IP, inositol phosphates; [Ca^{2+}]$_i$, intracellular calcium concentration; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

However, little else is known about the action of GRP and NMB in humans. Understanding the pharmacological and cellular bases of action of these peptides and their receptors in humans, and not relying on data derived from nonhuman animals, is important for a number of reasons. Recent studies demonstrate that a number of gastrointestinal peptides may have different structure-function relationships in different species, with a given compound functioning as an agonist in one species but as an antagonist in another (20); therefore, extrapolation of animal-derived data to human tissues is not always correct. Furthermore, existing cell lines expressing human Bn receptors usually express both Bn receptor types, thus limiting their usefulness for exploring the specific characteristics of either the huGRP-R or the huNMB-R. The availability of cells possessing a homogeneous population of either huGRP-R or huNMB-R would allow their pharmacology, regulation, signal transduction properties, and cell biology to be explored. In addition, cells expressing such a homogeneous population of receptors would be useful for the screening of potential human Bn receptor antagonists.

In the current study we have stably expressed the huGRP-R and huNMB-R in murine BALB/3T3 cells, thereby facilitating the exploration of their pharmacology as well as the cellular basis of their action. We selected BALB/3T3 cells as hosts for the huGRP-R and huNMB-R because these cells do not express Bn receptors (14, 21) before transfection. Furthermore, recent detailed studies have demonstrated that, for both the murine GRP-R and the rat NMB-R, stable transfection of these receptors into this cell line yields receptors that are pharmacologically indistinguishable from their natively expressed counterparts (14, 21).

Experimental Procedures

Materials

Rat glioblastoma C_6 cells, human glioblastoma U-118 cells, and BALB/3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD), Swiss 3T3 fibroblasts were a gift from Dr. John Taylor (Biomeasure, Medford, MA), and DMEM, fetal bovine serum, and aminoglycoside G-418 were obtained from GIBCO (Waltham, MA).

Bovine serum albumin (fraction V) and HEPES were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); soybean trypsin inhibitor, EGTA, trypsin, and bacitracin were obtained from Sigma Chemical Co. (St. Louis, MO); Bn, GRP, and NMB were obtained from Peninsula Laboratories (Belmont, CA); $Na^{125}I$ was from Amersham (Arlington Heights, IL); *myo*-[2-^{3}H]inositol (16–20 Ci/mmol) and cAMP radioimmunoassay reagents were from New England Nuclear (Boston, MA); Dowex AG 1-X8 anion exchange resin (100–200 mesh, formate form), SDS, and 2-mercaptoethanol were from Bio-Rad (Richmond, CA); Hydro-Fluor scintillation fluid, methanol, acetic acid, and hydrochloric acid were from the J. T. Baker Chemical Co. (Phillipsburg, NJ); PNGase F was from Genzyme (Cambridge, MA); cholera toxin and forskolin were from Calbiochem (San Diego, CA); and MBS and DSS were from Pierce Chemical Co. (Rockville, IL).

Methods

Transfection of cell lines. BALB/3T3 fibroblasts devoid of GRP-R and NMB-R were selected by clonal expansion after assaying for GRP-R or NMB-R by RNase protection and binding studies, as described previously (21). These BALB/3T3 cells were stably transfected using a full length huGRP-R clone (huGRP-R-transfected cells) (16) or using a full length huNMB-R clone (huNMB-R-transfected cells) (16). In both cases the receptor was subcloned into a modified version of the pCD2 plasmid and transfected using calcium phosphate precipitation. Stable transfectants were isolated in the presence of 800 μg/ml aminoglycoside G-418, identified by binding studies, and then maintained in DMEM containing 10% fetal bovine serum and 270 μg/ml G-418. Cells were passaged every 3–5 days at confluence by splitting 1:4.

Preparation of peptides. [D-Tyr^0]NMB, [F_5-D-Phe^6,D-Ala^{11}]-Bn(6–13)methyl ester, [Leu^{14},Ψ13–14]Bn, and cyclo-SS-octa were synthesized using solid-phase methods, as described previously. Peptides were purified on a 2.5- × 90-cm Sephadex G-25 column, followed by elution with a linear gradient consisting of acetonitrile and 0.1% trifluoroacetic acid, using an Eldex Chromatrol gradient controller and 1.5- × 50-cm Vydac C_{18} silica (10–15-mm) columns. Peptides were further purified by rechromatography on the same column when necessary, to >97% purity.

Binding studies. [^{125}I-D-Tyr^0]NMB (2200 Ci/mmol), ^{125}I-GRP (2200 Ci/mmol), and [^{125}I-Tyr^4]Bn (2000 Ci/mmol) were prepared using Iodo-Gen and purified using high pressure liquid chromatography, according to previously published methods (22). Binding studies were performed by suspending disaggregated cells in binding buffer containing 75 pM levels of either [^{125}I-D-Tyr^0]NMB or [^{125}I-Tyr^4]Bn and 3 × 10^6 cells/ml, for 30 min at 22°. Nonsaturable binding of either radiolabeled peptide was the amount of radioactivity associated with transfected cells when the incubation mixture contained either 1 μM NMB or 1 μM Bn. Nonsaturable binding was <10% of total binding in all experiments, and all values in this paper are reported as saturable binding.

Internalization of [^{125}I-D-Tyr^0]NMB or [^{125}I-Tyr^4]Bn. Cells were disaggregated, washed, and resuspended in binding buffer as described above, and then 3 × 10^6 cells/ml were incubated with 75 pM radiolabeled peptide for various times at 4°, 22°, or 37°. After incubation, 100-μl samples were added to 1.0 ml of 0.2 M acetic acid in 0.5 M NaCl, pH 2.5, for 5 min at 4°, to remove surface-bound radioligand, as described previously (14, 21, 22). In all cases, parallel incubations were conducted in the presence of 1 μM NMB or 1 μM Bn to determine changes in nonsaturable binding. Results are expressed as the percentage of saturably bound radiolabeled peptide that is internalized.

Cross-linking of huGRP-R and huNMB-R. Cell membranes were prepared by growing huGRP-R-transfected cells or huNMB-R-transfected cells to confluence, washing them once in binding buffer, and then resuspending them in homogenization buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.2 mg/ml soybean trypsin inhibitor, 100 mM phenylmethylsulfonyl fluoride). Cells were homogenized on ice using a Polytron homogenizer (Beckman Instruments, Fullerton, CA) at speed 6 for 30 sec. The homogenate was then centrifuged at 1500 rpm for 10 min in a Sorval RC-5B Superspeed centrifuge (DuPont, Wilmington, DE); the supernatant was removed and recentrifuged at 20,000 rpm for 20 min. The pellet was resuspended in homogenization buffer and stored at −70°.

Cell homogenates were diluted to the concentration of 0.5 mg of protein/ml with homogenization buffer supplemented with 0.2% bovine serum albumin and 0.1% bacitracin. Aliquots (500 μl) were preincubated with 0.5 nM [^{125}I-D-Tyr^0]NMB or 0.5 nM ^{125}I-GRP at 22°, in 1.6-ml polypropylene tubes. After a 15-min incubation, the reaction mixture was centrifuged at 10,000 × *g* for 3 min. The pellet was washed twice in 1 ml of cross-linking buffer (50 mM HEPES, pH 7.5, 5 mM $MgCl_2$) (at 4°) and resuspended in 200 μl of cross-linking buffer containing 1 mM MBS as the cross-linking agent for huNMB-R or 1 mM DSS as the cross-linking agent for huGRP-R. After cross-linking at 22° for 30 min, the reaction was stopped by addition of 25 μl of 1 M glycine. After 10 min on ice, the sample was centrifuged at 10,000 × *g* for 3 min. The supernatant was aspirated and the pellet was resuspended in 100 μl of 120 mM Tris·HCl, pH 6.8. A 6-μl aliquot of the mixture was reserved to determine protein concentration. Cross-linked membranes were solubilized by addition of 25 μl of gel loading buffer (0.4 M Tris·HCl, pH 6.8, 20%, w/v, SDS, 50%, v/v, glycerol, 0.05%, w/v, bromphenol blue, 0.5 M dithiothreitol) at 22° for 60 min. After adjustment of the protein concentration, cell membranes (10 μg of

protein/lane) of either type were applied to the gel and were subjected to SDS-polyacrylamide gel electrophoresis. For the GRP-R-transfected cell membranes, the Laemmli buffer system (375 mM Tris·HCl, pH 8.8, 0.1% SDS, 10% acrylamide, for the gel; 25 mM Tris, 192 mM glycine, 0.1% SDS, for electrodes) was used, whereas for the NMB-R-transfected cell membranes the Weber and Osborn buffer system was used [100 mM sodium phosphate, 0.1% SDS, for the gel (6% acrylamide) and the electrodes] as described previously (23). In both cases electrophoresis was carried out at a constant current of 40 mA/gel. Gels were stained with 0.1% (w/v) Coomassie blue R-250 in 40% (v/v) ethanol/10% (v/v) acetic acid and destained with 10% (v/v) ethanol/7.5% (v/v) acetic acid. After overnight destaining, gels were equilibrated in 45% (v/v) ethanol/5% (v/v) glycerol for 30 min and dried in a slab gel drier (model SE 540; Hoefer Scientific Instruments, San Francisco, CA). Dried gels were exposed to storage phosphor screens for 3 days at 22° and processed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

PNGase F treatment of membrane receptor proteins. Cross-linked membrane proteins were denatured by a 3-min incubation at 95° in 80 μl of 50 mM Tris·HCl, pH 7.7, containing 50 mM EDTA, 50 mM 2-mercaptoethanol, and 0.5% (w/v) SDS. To a 20-μl aliquot containing 40 μg of membrane protein, 10 μl of 7.5% Nonidet P-40 were added. The mixture was incubated with 10 units/ml PNGase F, in a volume of 60 μl, for 18 hr at 37°. The reaction was halted using 4% SDS, and the samples were subsequently analyzed by SDS-polyacrylamide gel electrophoresis.

Measurement of phosphoinositides. Total phosphoinositides in cells transfected with either the huNMB-R or huGRP-R were determined as described previously, with minor modifications (14, 21, 22). Cells were grown to confluence in 24-well plates in regular medium and then were loaded for 24 hr at 37° with 100 Ci/ml *myo*-[2-^{3}H]inositol in DMEM containing 2% fetal bovine serum. Cells were washed and incubated in phosphoinositide buffer (binding buffer additionally containing 10 mM $LiCl_2$) for 15 min and then for 60 min at 37° with agonists at various concentrations. Reactions were halted by addition of 1% HCl in methanol, and total [^{3}H]IP were isolated by anion exchange chromatography as described previously (14, 21, 22).

Measurement of cAMP levels. Cells were mechanically disaggregated and washed twice in binding buffer. Cells (5×10^6/ml) were incubated with various peptides for 60 min at 37°, after which cAMP was solubilized by the addition of 2 volumes of ice-cold ethanol. Peptide effects were measured in the presence of 30 μM forskolin or 100 ng/ml cholera toxin. When forskolin was used cells were preincubated for 30 min with forskolin, and when cholera toxin was used cells were preincubated for 60 min with cholera toxin before exposure to peptide. cAMP was measured by radioimmunoassay as described previously (14), with all samples diluted so that the values remained in the linear portion of the standard curve.

Results

Initial studies were performed to confirm the presence of huGRP-R on huGPR-R-transfected cells and huNMB-R on huNMB-R-transfected cells and to structurally compare the receptors in the two cell types (Figs. 1 and 2). In huGRP-R-transfected cells a single broad protein band of 60 ± 1 kDa was cross-linked using ^{125}I-GRP (Fig. 1, *middle*), whereas for huNMB-R-transfected cells a single band of 72 ± 1 kDa was cross-linked using [^{125}I-D-Tyr^0]NMB (Fig. 2, *left*). Recently, some human small cell lung cancer cells and glioma and glioblastoma cell lines have been shown to possess GRP-R (24). To structurally compare the transfected huGRP-R with the native receptor, similar cross-linking studies were performed using the human glioblastoma cell line U-118. Similarly to the cells stably transfected with the huGRP-R, a single band of 60 ± 1 kDa was seen (Fig. 1, *left*). In contrast to the huGRP-R,

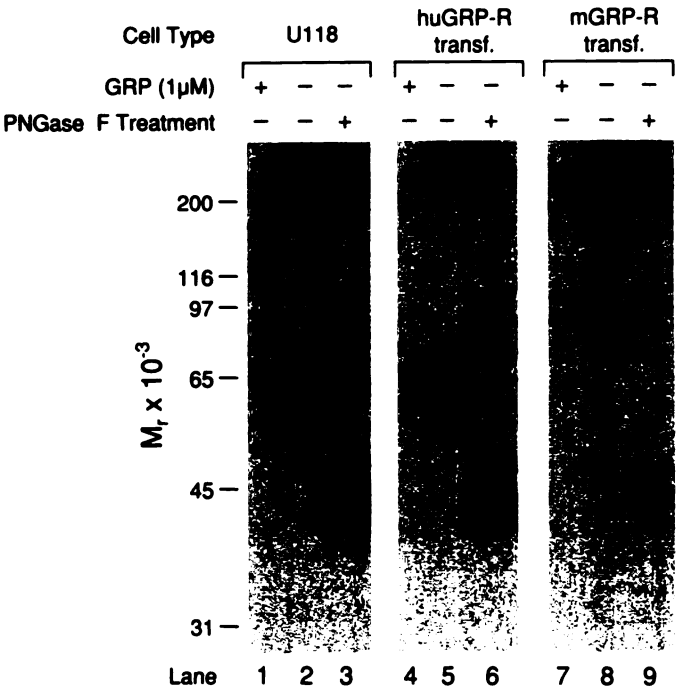

Fig. 1. Affinity labeling and deglycosylation of the huGRP-R on huGRP-R-transfected cells (*middle*) and human glioblastoma U-118 cell membranes (*left*) and of the mouse GRP-R on mouse GRP-R-transfected cell membranes (*right*). Membranes were prepared as outlined in Experimental Procedures. GRP-R cross-linking was performed using ^{125}I-GRP and radiolabeled peptide was cross-linked using 1 mM DSS, as described in Experimental Procedures, after binding in the presence or absence of 1 μM GRP. This experiment is representative of two others.

the mouse GRP-R transfected into the same BALB/3T3 cells gave a broad protein band of 822 kDa (Fig. 1, *right*), which is identical to the molecular mass of the GRP-R found in native murine Swiss 3T3 cells (14, 24). Although some human small cell lung cancer cell lines, including NCI-H209, NCI-510, and NCI-H1373, are reported to possess huNMB-R (16, 25), because of low receptor numbers an insufficient amount of binding of [^{125}I-D-Tyr^0]NMB to these cell lines was observed (data not shown) to allow cross-linking to be carried out, and therefore comparative data for a nontransformed, natively expressed, huNMB-R could not be obtained. With the rat NMB-R transfected into the same BALB/3T3 cells, a single broad protein band of 631 kDa was seen (Fig. 2, *right*), which was similar to the value reported for the native rat NMB-R on rat C_6 glioma cells. Treatment with PNGase F resulted in a deglycosylated receptor, with a molecular mass of 43 ± 1 kDa for the huGRP-R and mouse GRP-R for both the transfected cells and the glioblastoma cell line U-118 (Fig. 1) and with a molecular mass of 43 ± 1 kDa for the huNMB-R and rat NMB-R on transfected cells (Fig. 2). These values agree closely with the molecular masses of these receptors predicted from the amino acid sequence (16).

The time and temperature dependence of [^{125}I-Tyr^4]Bn binding to huGRP-R-transfected cells (Fig. 3, *left*) and that of [^{125}I-D-Tyr^0]NMB binding to huNMB-R-transfected cells (Fig. 3, *right*) were similar. For both cell types ligand binding was rapid at both 37° and 22°. Binding decreased with time for both cell types at 37°, whereas it remained relatively constant between 30 and 60 min of incubation at 22°. Half-maximal binding of radiolabeled ligand at 37° or 22° was observed

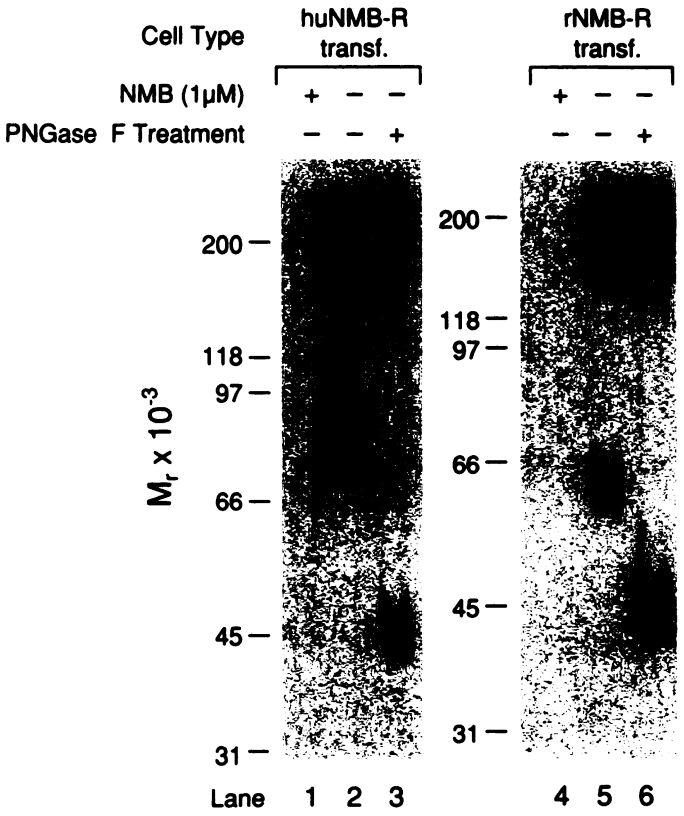

Fig. 2. Affinity labeling and deglycosylation of the hNMB-R on huNMB-R-transfected cells (*left*) and the rat NMB-R on rat NMB-R-transfected cells (*right*). NMB-R cross-linking was performed using [^{125}I-D-Tyr^0]NMB with 1 mM MBS, in the presence or absence of 1 μM NMB. After cross-linking, membranes were deglycosylated by incubation with 10 units/ml PGNase F, as described in Experimental Procedures. The experiment is representative of two others.

between 4 and 5 min for both cell types (Fig. 3). Reducing the incubation temperature to 4° resulted in a decreased rate and amount of binding to both cell types (Fig. 3). Addition of 1 μM Bn to huGRP-R-transfected cells incubated with [^{125}I-Tyr^4]Bn or addition of 1 μM NMB to huNMB-R-transfected cells incubated with [^{125}I-D-Tyr^0]NMB at 4°, 22°, or 37° reduced binding by >90% (Fig. 3).

To compare the ability of huNMB-R-transfected cells and huGRP-R-transfected cells to interact with the two naturally occurring mammalian Bn-related peptides, GRP and NMB, complete dose-inhibition studies for these agonists (Fig. 4) were performed. For huGRP-R-transfected cells, GRP was the most potent at inhibiting binding of [^{125}I-Tyr^4]Bn, causing detectable inhibition at 100 pM, half-maximal inhibition at approximately 3 nM, and complete inhibition at 1 μM (Fig. 4, *left*). To determine receptor affinity and number of binding sites, a saturation analysis was performed by adding increasing amounts of unlabeled peptide to a fixed concentration of radioligand. Analysis of the ability of Bn to inhibit [^{125}I-Tyr^4]Bn binding using the least-squares curve-fitting program LIGAND demonstrated that the data were best fit by a single binding site model (K_i = 1.4 ± 0.2 nM). Bn was 4-fold more potent than GRP (K_i = 6.2 ± 1.3 nM) (Table 1) and was >300-fold more potent than NMB (K_i = 437 ± 30 nM) (Table 1). In contrast, for huNMB-R-transfected cells NMB was the most potent at inhibiting the binding of [^{125}I-D-Tyr^0]NMB, with detectable inhibition being observed at 0.1 nM, half-maximal inhibition at approximately 8 nM, and complete inhibition at 1 μM (Fig. 4, *left*). Analysis of the binding data using the least-squares curve-fitting program LIGAND demonstrated that the data were best fit by a single-binding site model. NMB (K_i = 8.1 ± 5.2 nM) was 4-fold more potent than Bn (K_i = 32 ± 3 nM) (Table 1) and was 650-fold more potent than GRP (K_i = 5080 ± 770 nM) (Table 1).

Numerous classes of Bn receptor antagonists have been described (20). The abilities of three peptides, representing different classes of antagonists, to interact with the GRP-R on huGRP-R-transfected cells and with the NMB-R on huNMB-R-transfected cells were determined (Fig. 5). For huGRP-R-transfected cells, [F$_5$-D-Phe^6,D-Ala^{11}]Bn(6–13)methyl ester was the most potent at inhibiting the binding of [^{125}I-Tyr^4]Bn, with half-maximal inhibition being observed at 0.9 ± 0.1 nM. This antagonist was >8-fold more potent than [Leu^{14},Ψ13–14]Bn (K_i = 7.7 ± 0.3 nM) and was >5000-fold more potent than cyclo-SS-octa (K_i = 4570 ± 230 nM). In contrast, only cyclo-SS-octa was potent at inhibiting the binding of [^{125}I-D-Tyr^0]-NMB to huNMB-R-transfected cells (K_i = 605 ± 23 nM). Both [F$_5$-D-Phe^6,D-Ala^{11}]Bn(6–13)methyl ester and [Leu^{14},Ψ13–14]-Bn, at the maximal concentration used (10 μM), displaced <30% of [^{125}I-D-Tyr^0]NMB binding to huNMB-R-transfected cells (K_i > 10,000 nM).

The kinetics of binding were further examined by investigating the reversibility of binding of [^{125}I-Tyr^4]Bn to huGRP-R-transfected cells (Fig. 6, *left*) and of [^{125}I-D-Tyr^0]NMB to huNMB-R-transfected cells (Fig. 6, *right*). At 37° the dissociation of [^{125}I-Tyr^4]Bn bound to huGRP-R-transfected cells was slower than the dissociation of [^{125}I-D-Tyr^0]NMB bound to huNMB-R-transfected cells (Fig. 6). Specifically, by 15 min approximately 20% of bound ligand dissociated from huGRP-R-transfected cells, increasing to 53% by 60 min. In contrast, 58% of bound ligand dissociated from huNMB-R-transfected cells by 15 min, and this increased to 76% by 60 min. Lowering the temperature to 4° slowed the dissociation of bound ligand from either cell type, with <10% being dissociated by 60 min (Fig. 6). The incompleteness of dissociation at 37° suggested the possibility of peptide internalization in each of the cell types.

To determine whether internalization of ligand was occurring, acid-stripping experiments were performed to remove surface-bound ligand at various times and temperatures (Fig. 7). After incubation for 5 min at 37°, the time of half-maximal binding for both cell types (Fig. 3), 46 ± 3% of bound radiolabel was internalized by huGRP-R-transfected cells (Fig. 7, *left*), whereas 27 ± 3% of radiolabel was internalized by huNMB-R-transfected cells (Fig. 7, *right*). Maximal internalization at 37° was achieved by approximately 60–90 min in both cell types (Fig. 7). Internalization at 22° similarly was more rapid and complete with the huGRP-R-transfected cells (62 ± 1% internalized by 15 min and 75 ± 4% internalized by 90 min) than with the huNMB-R-transfected cells (29 ± 5% internalized by 15 min and 63 ± 3% internalized by 90 min). In both cell systems receptor internalization rates were markedly inhibited at 4°, with >90% of bound ligand existing in an acid-strippable form after a 90-min incubation (Fig. 7).

To determine whether transfected human Bn receptors activated phospholipase C, we determined the ability of the two mammalian Bn-related peptides to increase [^{3}H]IP in both cell types (Fig. 8). In huGRP-R-transfected cells a maximally effective dose of GRP (i.e., 1 μM) caused a 3.2-fold increase in

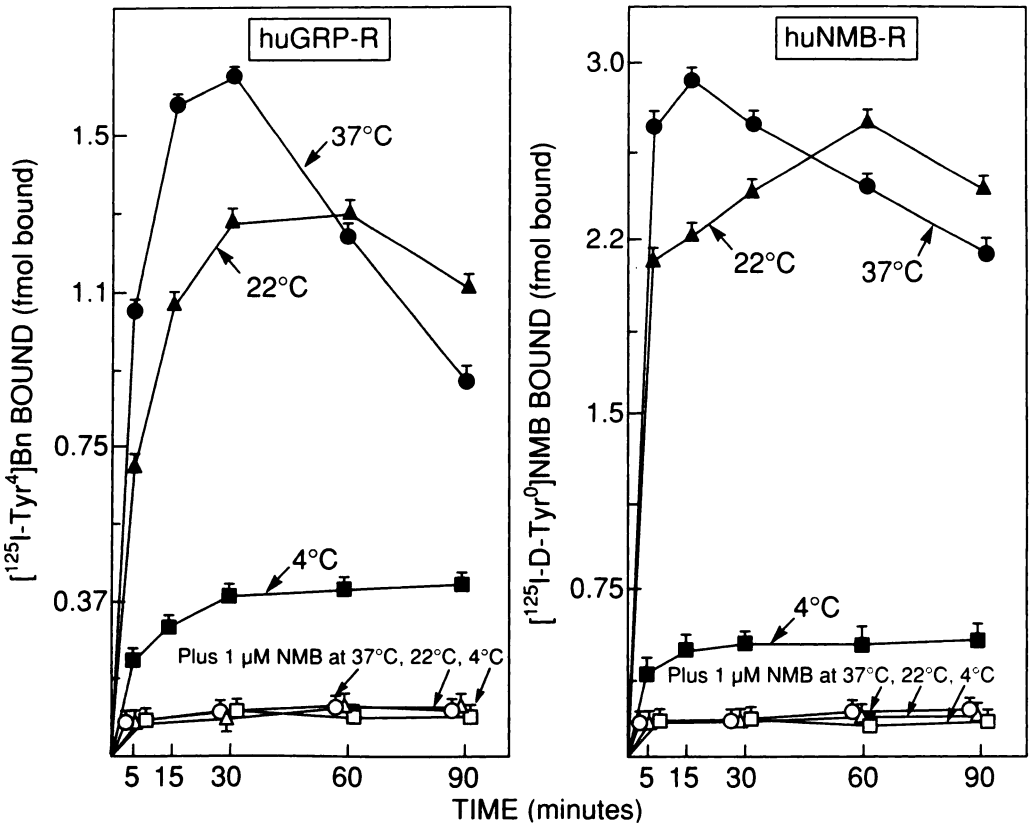

Fig. 3. Time- and temperature-dependent binding of [^{125}I-Tyr^4]Bn to huGRP-R-transfected cells (*left*) and [^{125}I-D-Tyr^0]NMB to huNMB-R-transfected cells (*right*). For both cell types, 3 × 10^6 cells/ml were incubated with 75 pM radiolabeled peptide alone (*closed symbols*) or with 1 μM unlabeled peptide (*open symbols*). At the indicated times and temperatures, 100-μl samples were taken and processed as described in Experimental Procedures. Results are expressed as fmol bound at each time point. In each experiment each value was determined in triplicate, and the results are given as the means ± standard errors of at least three separate experiments.

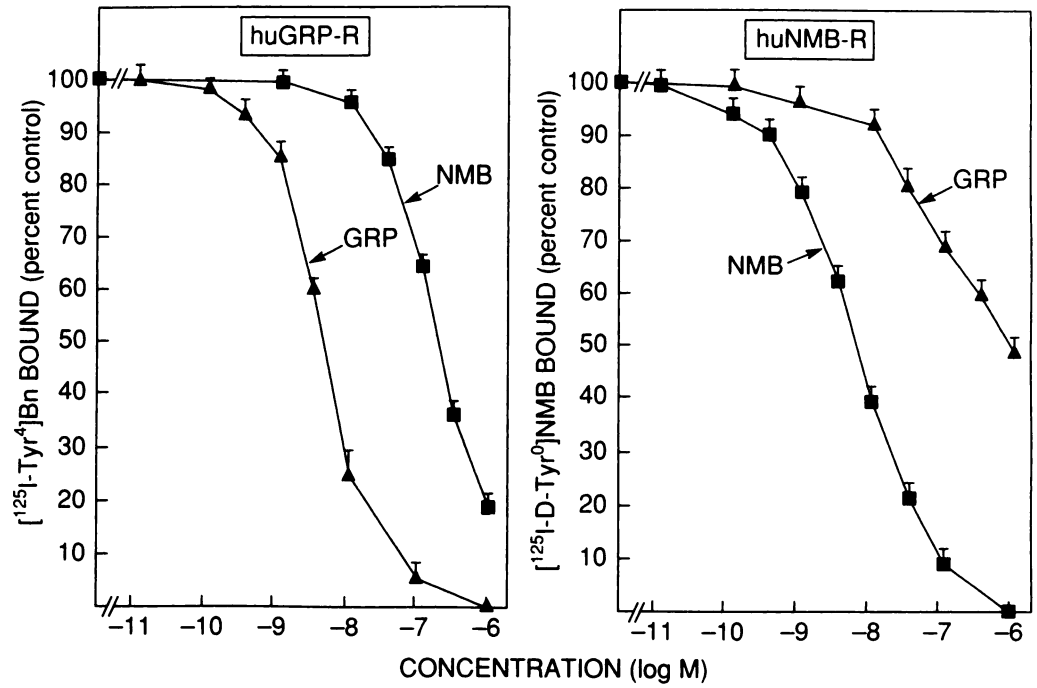

Fig. 4. Comparison of the ability of various Bn-related agonists to inhibit the binding of [^{125}I-Tyr^4]Bn to huGRP-R-transfected cells (*left*) and [^{125}I-D-Tyr^0]NMB to huNMB-R-transfected cells (*right*). For both cell types, 3 × 10^6 cells/ml were incubated with 75 pM radiolabeled peptide alone or with the indicated concentrations of the unlabeled Bn-related peptide. Data are expressed as the percentage of saturably bound radioactivity in the absence of nonradioactive peptide. For each experiment each value was determined in duplicate, and the results are the means ± standard errors of at least three separate experiments.

[^{3}H]IP. GRP was the most potent (EC_{50} = 13.6 ± 1.3 nM) at increasing [^{3}H]IP and was 100-fold more potent than NMB (EC_{50} = 1410 ± 171 nM) (Fig. 8, *left*). In huNMB-R-transfected cells a maximally effective concentration of NMB (i.e., 1 μM) caused a 19-fold increase in [^{3}H]IP. NMB (EC_{50} = 9.3 ± 1.4 nM) was 96-fold more potent than GRP (EC_{50} = 891 ± 67 nM) at increasing [^{3}H]IP in the huNMB-R-transfected cells (Fig. 8, *right*).

In addition to activating phospholipase C, Bn stimulation of the murine GRP-R expressed on Swiss 3T3 cells results in increased cellular cAMP levels (12, 14). However, the same receptor transfected into murine BALB/3T3 cells fails to increase cAMP levels (14). Therefore, we determined whether either transfected human Bn receptor type increased cellular cAMP levels when activated. When huNMB-R- or huGRP-R-transfected cells were preincubated with 30 mM forskolin or 100 ng/ml cholera toxin, basal cAMP levels were increased approximately 2-fold (Table 2). Whereas further incubation

TABLE 1

Binding affinities of various Bn-related agonists and receptor antagonists for different mammalian Bn receptors

Binding was performed as detailed in Experimental Procedures. Data for rat NMB-R-transfected BALB/3T3 cells (14, 21), C_6 glioblastoma cells (29), rat pancreatic acini (20, 35), mouse GRP-R-transfected BALB/3T3 cells (14), mouse pancreatic acini (20, 28), Swiss 3T3 cells (12, 14, 29), and guinea pig pancretic acini (4, 20) have been published previously or were obtained from binding studies with these cells. All values represent the mean ± standard error of at least three separate experiments, with each experiment performed in duplicate.

	NMB-R			GRP-R					
	Human transfected cells	Rat		Human transfected cells	Mouse			Rat pancreas	Guinea pig pancreas
		Transfected cells	C_6 Cells		Transfected cells	Pancreas	Swiss 3T3 cells		
				K_i (nM)					
Agonists									
NMB	8.1 ± 5.2	4.2 ± 0.6	3.3 ± 0.8	437 ± 30	174 ± 4	230 ± 80	42 ± 5	248 ± 5	1,500 ± 150
Bn	32 ± 3	34 ± 2	21 ± 7	1.4 ± 0.2	0.9 ± 0.3	6.8 ± 1.3	1.3 ± 0.2	4.1 ± 1.0	3.6 ± 0.5
GRP	5,080 ± 770	439 ± 66	403 ± 58	6.2 ± 1.3	3.1 ± 1.4	6.0 ± 2.0	1.6 ± 0.2	18 ± 5	11 ± 2
Antagonists									
[F_5-D-Phe^6-D-Ala^{11}]Bn(6-13)-methyl ester	>10,000	>10,000	620 ± 120	0.9 ± 0.1	ND[a]	ND	0.2 ± 0.1	5.5 ± 1.4	6 ± 1
[Leu^{14},γ13-14]Bn	>10,000	>10,000	>10,000	7.7 ± 0.3	87 ± 11	ND	65 ± 6	434 ± 65	60± 6
Cyclo-SS-octa	605 ± 23	220 ± 36	60 ± 9	4,570 ± 230	ND	ND	>10,000	>10,000	ND

[a] ND, no data.

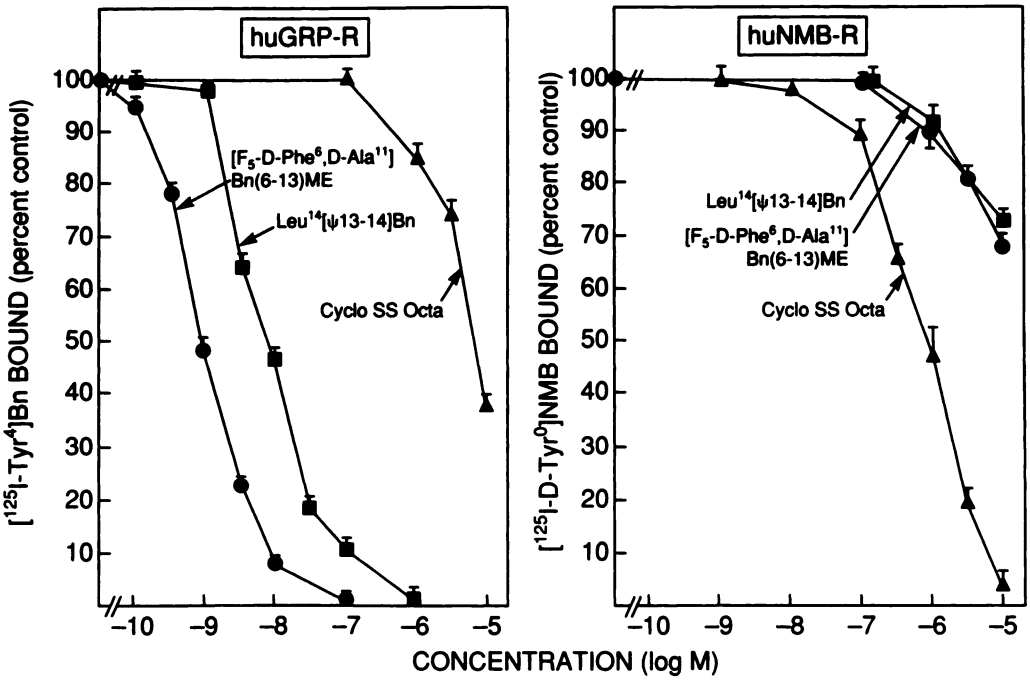

Fig. 5. Ability of various Bn receptor antagonists to inhibit the binding of [^{125}I-Tyr^4]Bn to huGRP-R-transfected cells (*left*) and [^{125}I-D-Tyr^0] NMB to huNMB-R-transfected cells (*right*). Experimental conditions were similar to those described in the legend to Fig. 3. For each experiment, each value was determined in duplicate, and the results are the means ± standard errors of at least three separate experiments.

with Bn increased cAMP levels 10.8-fold in Swiss 3T3 cells, Bn, GRP, and NMB failed to significantly increase cAMP levels further in the huNMB-R- and huGRP-R-transfected cell lines (Table 2).

Discussion

This study provides the first systematic characterization of the pharmacology and cell biology of human Bn receptors using huGRP-R and huNMB-R stably transfected into BALB/3T3 cells and demonstrates that they are a good model system for such studies of human Bn receptors. Previous cross-linking studies have demonstrated that the huGRP-R expressed by small cell lung cancer cells and by a human duodenal tumor cell line (24, 26) has a molecular weight of 62,000. After PNGase F treatment, a single band of M_r 40,000 (24) was seen, demonstrating that the huGRP-R was approximately 35% glycosylated. Similarly, in the present study we found that in the human glioblastoma cell line U-118 the natively expressed huGRP-R had a molecular weight of 60,000 and that 34% of this weight was due to glycosylation. An identical result was found with the transfected huGRP-R, demonstrating that in BALB/3T3 cells this receptor is glycosylated to a similar extent as the natively expressed receptor. Species-appropriate glycosylation also was observed with the mouse GRP-R transfected into the same cell type (27), demonstrating that GRP-R glycosylation in BALB/3T3 cells appears to be similar to that observed in native tissues, regardless of the species origin of the receptor. The mouse GRP-R has four potential glycosylation sites, all of which are utilized (13, 27), and is 47% glycosylated in pancreatic acinar cells (28) and Swiss 3T3 cells (12, 26, 27) and when expressed in stably transfected BALB/3T3 cells (27). In contrast, the huGRP-R has only two potential *N*-linked glycosylation sites (16), which likely explains the difference in percentage glycosylation between the mouse GRP-R and the huGRP-R. No comparable data exist for natively

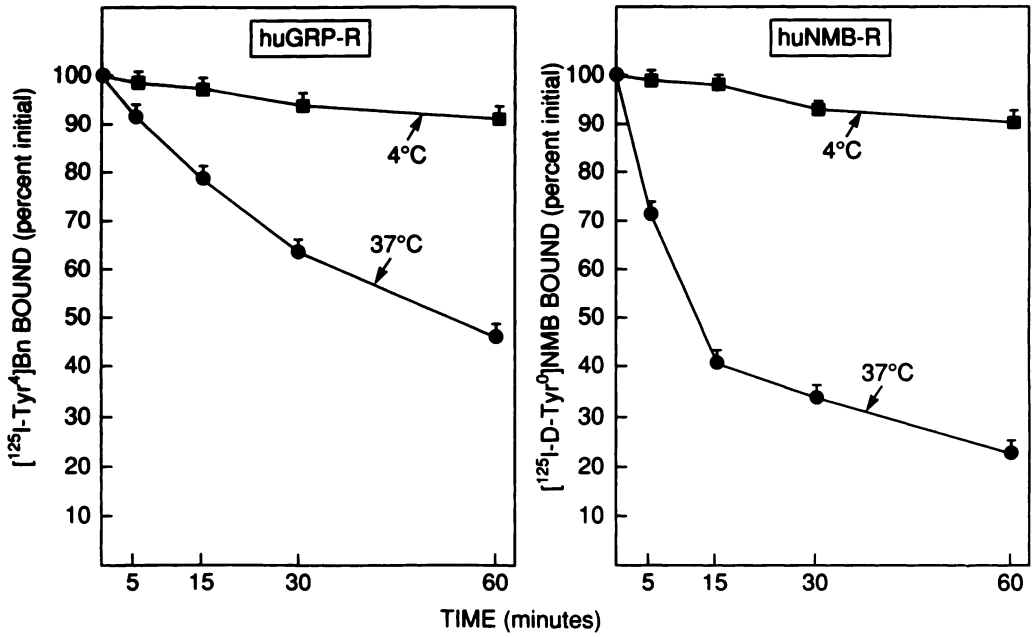

Fig. 6. Dissociation of [^{125}I-Tyr^4]Bn bound to huGRP-R-transfected cells (*left*) and [^{125}I-D-Tyr^0]NMB bound to huNMB-R-transfected cells (*right*). For both transfected cell types, 3 × 10^6 cells/ml were incubated in binding buffer with 75 pM radiolabeled peptide at 22° for 60 min. At that time, aliquots were diluted 50-fold with binding buffer at 4° or 37° and incubated at the indicated temperature for the indicated times. Results are expressed as the percentage of saturable binding at the beginning of the second incubation. In each experiment each value was determined in triplicate, and each data point is the mean ± standard error of at least three separate experiments.

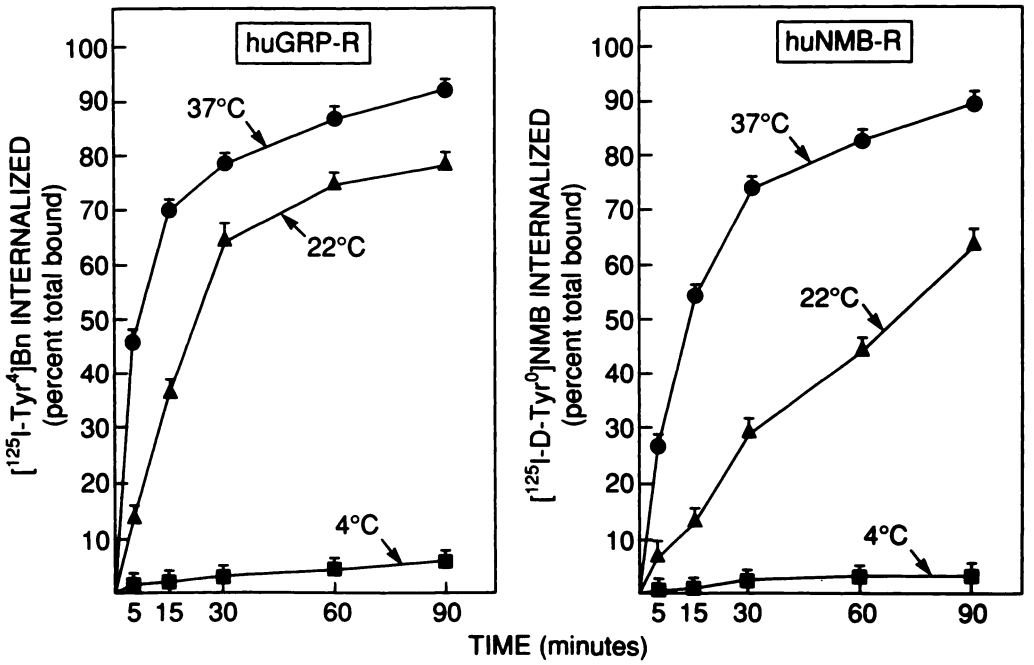

Fig. 7. Time and temperature dependence of internalization of the huGRP-R in huGRP-R-transfected cells (*left*) and the huNMB-R in huNMB-R-transfected cells (*right*). Surface-bound ligand was the proportion of saturably bound counts removed by acid stripping, as described in Experimental Procedures, whereas the internalized ligand was the proportion not removed. Results are expressed as the proportion of total saturable ligand at any time point and temperature that was not acid strippable. For each experiment each value was determined in triplicate, with each data point representing the mean ± standard error of at least three separate experiments.

expressed huNMB-R. In the present study with the transfected huNMB-R, a single cross-linked protein band of M_r 72,000 was seen. Deglycosylation studies demonstrated that 38% of the molecular weight of the huNMB-R was due to glycosylation. Similarly, in recent cross-linking studies the rat NMB-R was shown to have a molecular weight of 63,000, with 32% of the weight being due to glycosylation of the natively expressed NMB-R either by rat C_6 glioblastoma cells or by the stably transfected BALB/3T3 cells. The difference in glycosylation between species is again likely due to the fact that the huNMB-R has three potential *N*-linked glycosylation sites (16), whereas the rat NMB-R has only two such sites (13). In a recent study it was demonstrated that each potential glycosylation site on the rat NMB-R was glycosylated (23); therefore, the additional potential glycosylation site in the huNMB-R also is likely glycosylated, thus accounting for the greater degree of glycosylation of the huNMB-R versus the rat NMB-R.

Previous studies have provided evidence that GRP, NMB, and other Bn-related peptides can activate either GRP-R or NMB-R but differ in their relative affinities in different species (20, 22, 29). In the present study, the transfected huGRP-R had the highest affinity for the amphibian peptide Bn, whereas GRP was 4-fold less potent and NMB was >300-fold less potent. A similar result was seen in rat pancreas, guinea pig pancreas, and rat pancreatic AR-42J cells, where Bn was 3–10 times more potent than GRP and >60 times more potent than NMB (see Table 1). In contrast, with the mouse GRP-R (expressed by either mouse pancreas, mouse Swiss 3T3 cells, or stably transfected BALB/3T3 cells), Bn and GRP have almost equal high affinity and have only a 30–40-fold higher affinity

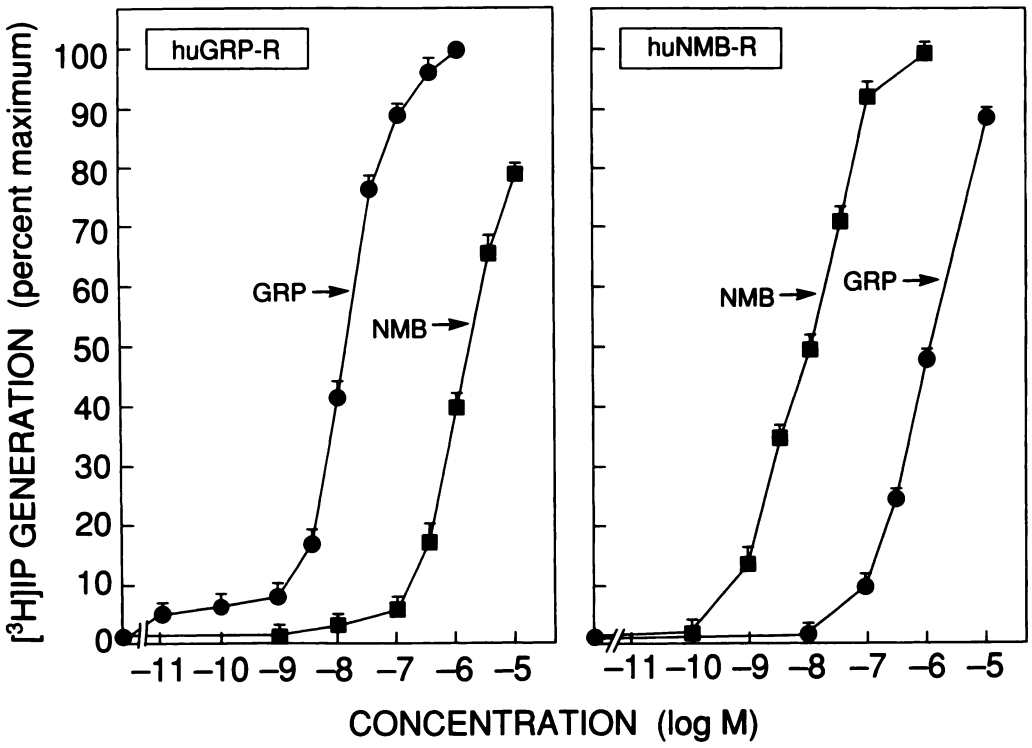

Fig. 8. Ability of Bn-related peptides to stimulate [^{3}H]IP generation in huGRP-R-transfected cells (*left*) or in huNMB-R-transfected cells (*right*). Confluent cells were incubated with 100 Ci/ml *myo*-[2-^{3}H]inositol for 24 hr, after which they were exposed to the indicated Bn-related peptides at the indicated concentrations for 60 min. Data are expressed as the percentage of maximal increase obtained using 1 μM Bn in huGRP-R-transfected cells or 1 μM NMB in huNMB-R-transfected cells. For huGRP-R-transfected cells, control and 1 μM Bn-stimulated values for [^{3}H]IP were 7,100 ± 1,100 dpm and 23,000 ± 2,200 dpm, respectively. For huNMB-R-transfected cells, control and 1 μM NMB-stimulated values for [^{3}H]IP were 9,800 ± 900 dpm and 185,000 ± 20,000 dpm, respectively. For each experiment, each value was determined in duplicate, with each data point representing the mean ± standard error of at least three separate experiments.

TABLE 2

Ability of Bn-related agonists to increase cAMP levels in huNMB-R- and huGRP-R-transfected cells and in Swiss murine 3T3 cells

For all cell types, 3 × 10^6 cells/ml were preincubated with 100 ng/ml cholera toxin for 60 min at 37° or with 30 μM forskolin for 30 min at 37°. After forskolin preincubation, the cells were further stimulated with the indicated peptides for 30 min. Basal cAMP levels were 18 pmol/10^6 cells for Swiss 3T3 cells, 27 pmol/10^6 cells for huNMB-R-transfected cells, and 139 pmol/10^6 cells for huGRP-R-transfected cells. Each value represents the fold increase (mean ± standard error) from three separate experiments, with each experiment performed in triplicate.

	Relative cAMP level		
	huNMB-R-transfected cells	huGRP-R-transfected cells	Murine Swiss 3T3 cells
No additions	1	1	1
Cholera toxin (100 ng/ml)	1.6 ± 0.4	1.4 ± 0.3	2.2 ± 0.4
Forskolin (30 μM)	2.1 ± 0.6	2.2 ± 0.4	2.9 ± 0.3
Forskolin (30 μM) + NMB (1 μM)	2.8 ± 0.6	3.3 ± 0.6	3.6 ± 0.9
Forskolin (30 μM) + Bn (1 μM)	2.5 ± 0.7	3.3 ± 0.5	10.8 ± 1.1[a]
Forskolin (30 μM) + GRP (1 μM)	2.9 ± 0.7	3.1 ± 0.6	6.3 ± 0.5[a]

[a] Value is significantly different from the value obtained in the presence of forskolin alone; $p < 0.05$.

than NMB (12, 14, 28). At present, the basis for this difference in the relative affinities and selectivity of these three naturally occurring agonists for the GRP-R in mice, compared with humans and other species, is unclear. The affinities of these three agonists for the transfected huGRP-R are in reasonable agreement with results from studies on human cells containing native huGRP-R. In human pancreatic acinar membranes Bn was the most potent at inhibiting binding (IC_{50} = 1 nM) and was 8-fold more potent than GRP-R (30). In the human colon cancer cell line NCI-H716 Bn was 3-fold more potent than GRP and 350-fold more potent than NMB (31), in the small cell lung cancer cell line NIH-H345 Bn was 5-fold more potent than GRP, and with the huGRP-R in the human gastric cancer cell line St42 (32) Bn was 2-fold more potent than GRP and 100-fold more potent than NMB. In biological studies assessing the ability of Bn-related peptides to cause contraction of isolated human circular jejunal smooth muscle cells, Bn was 20 times more potent than GRP (33). However, in the human duodenal tumor cell line HuTu-80 Bn and GRP were equipotent and >100-fold more potent than NMB (26), whereas in the human breast cancer cell line T-47D (19) GRP was reported to be 10-fold more potent than Bn. At present, it is unclear whether the differences in the relative affinities of these three Bn-related peptides for the huGRP-R reported in these various studies are due to different experimental conditions, intrinsic differences of these multiple tumor cell lines due to alterations in G protein coupling or glycosylation, or some other factors. The degree of glycosylation may represent a hitherto underappreciated reason for alterations in receptor affinity for ligands. Indeed, in a recent study (27) the extent of glycosylation of the mouse GRP-R was shown to affect receptor affinity and G protein coupling. Furthermore, in a study of the huGRP-R in T-47D breast cancer cells (19), which has a higher affinity for GRP than Bn, the cross-linked receptor had a molecular weight of 75,000. This molecular weight is similar to that reported for human glioma cells (24) and thus supports the contention that differences in glycosylation may represent an important factor contributing to the differing affinities of the huGRP-R for GRP and Bn in different human cell lines.

In contrast, few cell lines express sufficient huNMB-R to permit a systematic comparison of the data obtained herein using the stably transfected huNMB-R cells with data on cells natively expressing this receptor. In general, however, the pharmacology of the huNMB-R was similar to data obtained from rats, where this receptor has been extensively characterized (21, 22, 29). Indeed, the huNMB-R had relatively high affinity for both NMB and Bn, with GRP having a >800-fold lower affinity for this receptor than the huGRP-R. The only comparable data for natively expressed huNMB-R is from the NCI-

H345 small cell lung cancer cell line (25). For this cell line, one pharmacological analysis revealed that NMB had a 100-fold higher affinity than Bn and a 50-fold higher affinity than GRP (25), whereas in a study evaluating changes in $[Ca^{2+}]_i$ in the same cell line NMB was only 3-fold more potent than Bn (16). The reasons accounting for the differences in relative affinities of GRP and NMB for the huNMB-R, as well as their differing abilities to alter $[Ca^{2+}]_i$, between NCI-H345 cells and the stably huNMB-R-transfected cells studied herein are unclear but are likely multifactorial. Potential factors include noncomparable experimental conditions, alterations in peptide degradation, and different G protein populations in the different cell types. However, it also is important to realize that NCI-H345 cells express both huNMB-R and huGRP-R and that both classes of receptors can interact with both peptides. Earlier studies of the huNMB-R on NCI-H345 cells were not performed on cells possessing a pure population of receptors and are therefore difficult to interpret. However, in terms of selectivity for Bn receptors, the pharmacology of the human receptors is similar to that reported in various animal studies (22, 29), with NMB having >50-fold higher selectivity for the NMB-R than the GRP-R. In contrast, Bn interacts with high affinity with both transfected human Bn receptor subtypes, whereas GRP is >400-fold more selective for the huGRP-R than the huNMB-R. Because GRP and Bn share a similar carboxyl-terminal heptapeptide, with nine of the 10 carboxyl-terminal amino acids being identical in the biologically active portion of the molecule (34), this increased selectivity of GRP over Bn for huGRP-R suggests that the amino-terminal amino acids of GRP are important for determining this selectivity for the various human Bn receptors.

Previous studies have demonstrated, with Bn receptors from rats, mice, and guinea pigs (12, 14, 21, 29), that the radiolabeled agonist ligands are rapidly internalized and degraded. Results in the present study suggest that similar phenomena occur with huGRP-R and huNMB-R. With both human receptors, rapid temperature-dependent internalization was seen. Furthermore, with both human receptors binding decreased with time at 37°, which in animal studies was shown to be due to degradation of the radiolabeled agonists. In those studies the broad-spectrum protease inhibitor bacitracin, which was included in the incubation medium in the present study, was shown to markedly inhibit this degradation, whereas neutral endopeptidase or amino peptidase inhibitors were largely without effect (29).

In previous studies, six chemically different classes of Bn receptor antagonists have been described (20, 35). Almost no data are available on the ability of these antagonists to interact with human Bn receptors. In the present study, representative compounds from three of these classes of antagonists were examined for their abilities to interact with and distinguish the two human Bn receptor subtypes. Similarly to studies in other species (20), the [des-Met^{13}]Bn analogue [F_5-D-Phe^6,D-Ala^{11}]-Bn(6–13)methyl ester had high affinity for huGRP-R and had >10,000-fold selectivity for the huGRP-R over the huNMB-R. A recent study has demonstrated that this analogue functions as a GRP-R antagonist in humans *in vivo* and inhibits Bn-stimulated enzyme secretion and gastric emptying (36). Closely related [des-Met^{13}]Bn antagonists have been shown to inhibit Bn-stimulated increases in $[Ca^{2+}]_i$ in NCI-H345 small cell lung cancer cells (25) and to inhibit GRP-stimulated chloride currents in *Xenopus* oocytes after injection of huGRP-R mRNA but do not inhibit NMB-stimulated changes in chloride currents after injection of huNMB-R mRNA (16). These results, coupled with their marked selectivity for the huGRP-R, suggest that this class of antagonists will be useful for exploring the role of the GRP-R in mediating various physiological functions. The pseudopeptide analogue [Leu^{14},Ψ13–14]Bn also had high affinity for the huGRP-R and a 1000-fold selectivity for this receptor over the huNMB-R. This analogue has been shown to inhibit Bn-stimulated increases in [^{3}H]IP levels, $[Ca^{2+}]_i$, and proliferation of NCI-H345 cells (37). In contrast to these two antagonists, but similarly to results reported recently for rats, the somatostatin octapeptide analogue cyclo-SS-octa was more selective for the NMB-R than the GRP-R. However, it was pointed out that the use of this particular analogue may be limited because it has both somatostatin agonist activity and some μ-opioid antagonist activity, although structure-function studies suggest that these can be separated from the NMB-R inhibitory activity. The present study suggests that an additional problem also exists for this class of antagonists in humans, in that cyclo-SS-octa has a much lower affinity for the huNMB-R (K_i = 605 nM) than the rat NMB-R (K_i = 40–220 nM), as well as being less selective for this receptor than for the huGRP-R.

In all species examined, GRP-R and NMB-R activation results in activation of phospholipase C, which results in an increase in IP and mobilization of cellular calcium (12, 22, 38). In the present study we found that both huGRP-R and huNMB-R activate phospholipase C and increase [^{3}H]-IP levels. These results are similar to studies using human small cell lung cancer cells possessing either GRP-R or NMB-R (25, 37, 39), in which the addition of Bn-related peptides increases phosphatidylinositol turnover and $[Ca^{2+}]_i$. Furthermore, the GRP-R antagonist [D-Phe^6]Bn(6–13)methyl ester inhibited GRP-stimulated increases in $[Ca^{2+}]_i$ without altering the increase caused by NMB in the human small cell lung cancer cell line NCI-H345 (16). This demonstrates that activation of both huGRP-R and huNMB-R activates phospholipase C. Similarly, activation of the GRP-R on the human pancreatic tumor cell line Capan (40) and the human duodenal cancer cell line HuTu 80 (26) also has been shown to activate phospholipase C, whereas stimulation of GRP-R on human endometrial stromal cells (41) has been shown to increase the activity of phospholipase C and to increase $[Ca^{2+}]_i$ and IP levels. Thus, activation of both huGRP-R and huNMB-R, either stably transfected in BALB/3T3 cells or in human tissues, results in phospholipase C activation. The ability to readily detect changes in [^{3}H]IP consequent to receptor activation, coupled with the ability to easily measure binding to huGRP-R or huNMB-R in the present study, suggests that these two transfected cell lines should be excellent systems for exploring additional ligand or receptor structure-function relationships for the human Bn receptors.

In some cell systems, such as Swiss 3T3 cells (12, 14), activation of the GRP-R also increases cAMP levels. In contrast, activation of the rat NMB-R natively expressed by C_6 glioblastoma cells (22) or by rat pancreatic acini, or of the rat NMB-R transfected into BALB/3T3 cells (21), does not result in an increase in cAMP levels. In the present study activation of neither the huGRP-R nor the huNMB-R resulted in increases in cAMP levels. The failure to observe an increase in cAMP levels with activation of either human Bn receptor could be either due to altered G protein-

receptor coupling or due to the fact that neither human Bn receptor type natively acts to increase cAMP levels. In a recent study (14), BALB/3T3 cells stably transfected with the mouse GRP-R derived from Swiss 3T3 cells, which increases cAMP levels in Swiss 3T3 cells (12, 14, 42), did not show increased cAMP levels. That study demonstrated that BALB/3T3 cells possessed abundant G_s; thus, the failure to increase cAMP levels could not be explained on the basis of inadequate G protein availability. Also, forskolin and cholera toxin increased cAMP levels in the transfected BALB/3T3 cells, further demonstrating that adenylate cyclase could be directly activated in these cells (14). This suggests that mouse GRP-R activation of cAMP production may be peculiar to Swiss 3T3 cells and that the failure of this same receptor to increase cAMP levels in BALB/3T3 cells cannot be blamed on the lack of appropriate machinery necessary to generate this cyclic nucleotide. At present, however, the possibility still cannot be completely excluded that activation of either human Bn receptor may be coupled to adenylate cyclase, in addition to activating phospholipase C, and that this dual activation pathway was not detected in the present study because of differences in G protein coupling between the transfected cells and cells natively expressing these receptors.

In conclusion, in the present study we demonstrate that huGRP-R and huNMB-R can be stably transfected into BALB/3T3 cells. We show that the transfected receptors exhibit similar pharmacology for agonists and antagonists, that they undergo identical glycosylation, and that they function similarly with respect to their ability to alter biological activity, compared with natively expressed receptors. The availability of these cells should greatly facilitate further studies of human Bn receptor pharmacology and receptor modulation.

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