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Discovery of a High Affinity Radioligand for the Human Orphan Receptor, Bombesin Receptor Subtype 3, Which Demonstrates That It Has a Unique Pharmacology Compared with Other Mammalian Bombesin Receptors*

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An orphan receptor discovered in 1993 was called bombesin receptor subtype 3 (BRS-3) because of 47–51% amino acid identity with bombesin (Bn) receptors. Its pharmacology is unknown, because no naturally occurring tissues have sufficient receptors to allow studies. We made two cell lines stably expressing the human BRS-3 (hBRS-3). hBRS-3 was overexpressed in the human non-small cell lung cancer cells, NCI-H1299, and the other was made in Balb 3T3 cells, which lack endogenous BRS-3. [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) (where Nle represents norleucine) was discovered to have high potency for stimulating inositol phosphate formation in both cell lines. [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13}, Nle^{14}]Bn-(6–14) bound to both cell lines with high affinity. Neither Bn nor 14 other naturally occurring Bn peptides bound to hBRS-3 with a K_d <1000 nM. Twenty-six synthetic peptides that are high affinity agonists or antagonists at other bombesin receptors had an affinity >1000 nM. Guanosine 5′-(β,γ-imido)triphosphate inhibited binding to both cells due to a change in receptor affinity. These results demonstrate hBRS-3 has a unique pharmacology. It does not interact with high affinity with any known natural agonist or high affinity antagonist of the Bn receptor family, suggesting the natural ligand is either an undiscovered member of the Bn peptide family or an unrelated peptide. The availability of these cell lines and the hBRS-3 ligand should facilitate identification of the natural ligand for BRS-3, its pharmacology, and cell biology.

Recently, an orphan receptor that is a member of the heptahelical superfamily of receptors was described in both human small cell lung cancer cells (1) and guinea pig uterus (2). Because this orphan receptor had a high degree of homology to mammalian bombesin receptors (*i.e.* 51–52% for the gastrin-releasing peptide receptor (GRP-R)[1] and 47% for the neuromedin B receptor (NMB-R) (1, 2)), it was named the BRS-3 for bombesin receptor subtype-3 in one study (1). Studies of the distribution of the receptor mRNA show that BRS-3 has a pattern of expression limited to rat secondary spermatocytes (1), guinea pig brain and pregnant uterus (2), and some tumor cell lines (various human small cell and non-small cell lung cancer cell lines (1), the human ductal breast cancer cell line T47D (3), and the human epidermal cancer cell line A431 (3)). However, the natural ligand that interacts with the BRS-3 is unknown, and its pharmacology is largely unknown because of the lack of a radioligand. In addition, little is known about the cellular basis of action of BRS-3 except that it is coupled to phospholipase C when expressed in *Xenopus* oocytes (1) or when transfected into Balb 3T3 cells (4). The ability to elucidate the pharmacology of the BRS-3 is not only limited by the lack of a radioligand but also by the lack of a cell containing native BRS-3 receptors in sufficient numbers to allow binding studies to identify a possible radioligand.

To deal with this latter issue, in the present study we have used two different strategies to produce cell lines stably expressing the human BRS-3 (hBRS-3) receptor whose pharmacology and coupling will probably closely resemble that of the native hBRS-3. Furthermore, we have discovered a unique ligand that is a synthetic analogue of bombesin-(6–14), which interacts with high affinity with the hBRS-3. With this radioligand, we demonstrate for the first time that the hBRS-3 possesses a unique pharmacology for mammalian bombesin receptors, that BRS-3 is G protein-coupled, and that none of the existing natural occurring bombesin-related peptides are the natural ligand for this receptor.

EXPERIMENTAL PROCEDURES

Materials—Balb 3T3 cells were obtained from ATCC, Rockville, MD; NCI-H1299 cells were a gift from Herb Oie of NCI-Navy Medical Oncology Branch, Naval Medical Center (Bethesda, MD); bacitracin and benzamidine were from Sigma; basal medium Eagle amino acid solution, Dulbecco's minimum essential medium, RPMI 1640, fetal bovine serum, G418 sulfate, and 0.1% trypsin in 1 mM EDTA were from Life Technologies, Inc.; Na^{125}I (2200 Ci/mmol) was from Amersham Life Science Inc.; [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from NEN Life Science Products; 1,2,4,6-tetra-

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[1] The abbreviations used are: GRP-R, gastrin-releasing peptide receptor; GRP, gastrin-releasing peptide; hGRP, human GRP; NMB, neuromedin B; hNMB, human NMB; NMB-R, NMB receptor; PG-L, pGlu-Gly-Gly-Gly-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2; BRS-3, bombesin receptor subtype 3; hBRS-3, human BRS-3; Bn, bombesin; NMC, neuromedin C; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; Nle, norleucine.

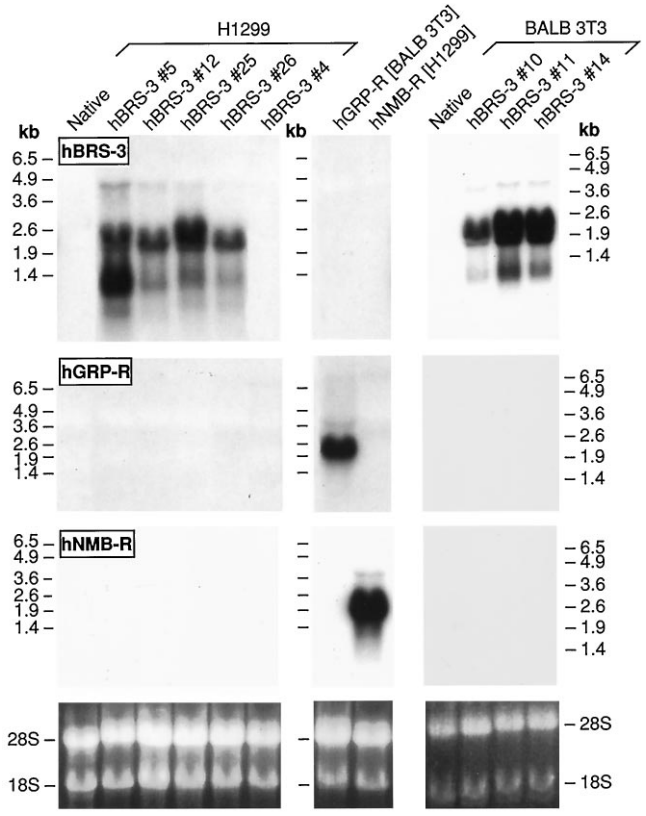

FIG. 1. **Northern blot analysis to detect hBRS-3, hGRP-R, and hNMB receptor mRNA in native H1299, Balb 3T3, and H1299 cells and Balb 3T3 cells stably transfected with hBRS-3 receptor.** 15 μg of total cellular RNA isolated from each of the indicated cell lines was analyzed by Northern blot and subsequent gene-specific hybridization as described under "Methods." The *top panel* shows the autoradiogram for hBRS-3 receptor mRNA; the *second panel* shows the hGRP-R; and the *third panel* shows the hNMB receptor. The *bottom panel* shows a photograph of the gel stained with ethidium bromide. H1299 native and Balb 3T3 native cells are cells not transfected with any of the three bombesin receptor subtypes. hBRS-3 clones 5 and 12 were transfected with hBRS-3 in the expression vector pcDNA3, whereas hBRS-3 clones 25, 26, and 4 were transfected with the hBRS-3 in the expression vector pCD2 as described under "Methods." This *figure* is representative of two others. *kb*, kilobase pairs.

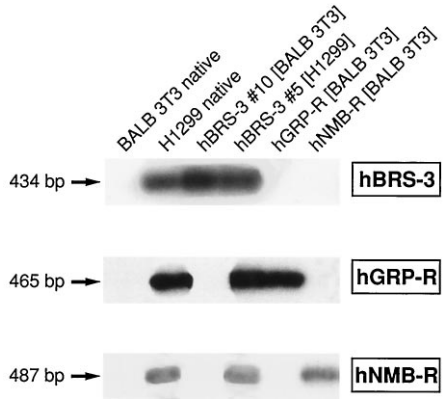

FIG. 2. **Autoradiograph of Southern blot after RT-PCR using gene-specific primers for hBRS-3, GRP-R, and NMB-R in native H1299, native Balb 3T3, and Balb or H1299 cells stably transfected with hBRS-3, hGRP receptor, or hNMB receptor.** Reverse transcription was performed using total cellular RNA. PCR was performed using gene-specific primers for human BRS-3, GRP receptors, or NMB receptor as described under "Methods." Hybridization was performed using ^{32}P-radiolabeled gene-specific probes as described under "Methods." The *top panel* shows the results with an hBRS-3-specific probe; the *middle panel* shows results using an hGRP receptor-specific probe; and the *bottom panel* shows results with an hNMB receptor probe.

chloro-3α-6α-diphenylglycouril (Iodo-Gen) was from Pierce; bombesin (Bn), gastrin-releasing peptide (GRP), neuromedin B (NMB), litorin, ranatensin, alytesin, neuromedin C (NMC), phyllolitorin, [Tyr^4]Bn, and rohdei-litorin were from Bachem (Torrence, CA); [D-Phe^6,β-Ala^{11},Phe^{13}, Nle^{14}]Bn-(6–14) and [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) were gifts from John Taylor of Biomeasure Inc. (Milford, MA).

Methods

Preparation of Peptides—The peptides were synthesized with solid-phase methods as described previously (5–7). Briefly, introduction of the reduced peptide bond was carried out by the standard methods described previously (5, 7) on methylbenzhydrylamine resin (Advanced Chem Tech, Louisville, KY). Alkylamide analogues were synthesized in a standard Leu-*O*-polystyrene resin by using tosyl group protection for the imidazole group of His. Peptide esters were prepared by standard, automated solid-phase techniques on Advanced Chem Tech ACT200 machines with Merrifield Leu-*O*-polystyrene resin and α-Boc protection for all amino acids and both the α and imidazole nitrogen of His in position 12 as described previously (6). Free peptides were then cleaved from the resin by transesterification with 10% triethylamine/methanol at 40 °C (2 days). Peptides were purified as described previously (5–7) to greater than 97% purity. Peptides were characterized by amino acid analysis and matrix-assisted laser desorption mass spectroscopy (Finnegan, Hemel Hemstead, UK).

Growth and Maintenance of Cells—Balb 3T3 and hBRS-3-transfected Balb 3T3 cells were grown in Dulbecco's minimum essential medium. NCI-H1299 and hBRS-3-transfected H1299 cells were grown in RPMI 1640. Both cell media were supplemented with 10% (v/v) fetal bovine serum (Life Technologies), penicillin (50 units/ml), and streptomycin (50 μg/ml) (Life Technologies) (plus 300 μg/ml G418 sulfate for stable transfectants). All cells were maintained at 37 °C in a 5% CO_2 atmosphere. Cells were passaged every 3–4 days at confluence after detaching the cells with 0.1% trypsin in 1 mM EDTA.

Isolation of Cellular RNA and Northern Blot—Cells were harvested in GIT buffer (4 M guanidine isothiocyanate, 30 mM sodium acetate, pH 7.0, and 1% (v/v) 2-mercaptoethanol), and total cellular RNA was isolated according to the method described by Davis *et al.* (8). Fifteen-μg samples were subjected to denaturing gel electrophoresis in formaldehyde agarose (0.22 M and 1% (w/v), respectively) and then transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) according to the method by Thomas (9). Total cellular RNA from some cell lines was isolated using the RNeasy Midi kit (QIAGEN Inc., Chatsworth, CA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Southern blot—For RT-PCR, first strand cDNA was synthesized using 1.0 μg of total cellular RNA with the first strand cDNA synthesis kit (Life Technologies). For amplification from first strand cDNAs, gene-specific primers for the hBRS-3 were used as described elsewhere (1). Nested PCR of the hBRS-3 was performed with the following primers: 5′-CAGAATCATCAAGCTCTGTG-3′ (sense) and 5′-AGTCTTCAGGATGGCATTGG-3′ (antisense). Gene-specific primers for the hGRP-R were as reported in Pansky *et al.* (10). The human NMB-R primers were as follows: 5′-CGGACTCTGCTGGAAAGGA-3′ (sense) and 5′-GACGTCTGCATGTCCATGG-3′ (antisense). PCR was carried out with the GeneAmp PCR System 9600 (Perkin-Elmer) using routine conditions and buffer provided by the manufacturer. PCR products were electrophoretically separated in 1.2% (w/v) SeaKem GTG agarose gels (FMC BioProducts, Rockland, ME) and transferred to nitrocellulose. Hybridization was carried out at 37 °C (Random Primers DNA labeling system probes (Life Technologies)) or at room temperature (end-labeled synthetic oligonucleotides) in a buffer containing 40% (v/v) formamide (Fluka Chemical, Switzerland), 4 × SSC (300 mM NaCl, 30 mM sodium citrate; Research Genetics, Huntsville, AL), 20 mM Tris, pH 7.5 (Quality Biological, Gaithersburg, MD), 10% (v/v) dextran sulfate (Oncor, Gaithersburg, MD), 1 × Denhardt solution (Digene Diagnostics, Beltsville, MD), and 20 μg/ml sonicated herring sperm DNA (Digene Diagnostics, Beltsville, MD). Nitrocellulose filters of Northern transfers were hybridized overnight with the full-length human GRP-R, hBRS-3, or human NMB-R (1, 11) cDNA, respectively, and radioactively labeled to a specific activity of about 1×10^9 cpm/μg DNA. Filters were washed with sequentially increasing stringency, ending with a final wash in 0.1 × SSC, 0.1% SDS (v/v) at 55 °C, air-dried, and exposed to x-ray films (XAR, Eastman Kodak Co.). Nitrocellulose filters from Southern transfers were hybridized at room temperature with ^{32}P-end-labeled, gene-specific synthetic oligonucleotides, washed at room temperature as described for Northern transfers, air-dried, and exposed to x-ray films for several hours.

TABLE I

Comparison of the amount of saturable binding of different bombesin receptor ligands to hBRS-3 transfected and nontransfected BALB 3T3 cells and H1299 cells

Native nontransfected Balb 3T3 cells, NCI-H1299 cells, or various hBRS-3-transfected cell lines identified by Northern blotting (1–3 × 10^6 cells/ml) were incubated with 75 pM[^{125}I-D-Tyr^0]NMB,[^{125}I-Tyr^4]Bn, ^{125}I-GRP,[^{125}I-D-Tyr^6]Bn-(6–13)ME, or [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) alone or with 1 μM unlabeled peptide. Results are expressed as the fmol/10^7 cells of each ligand saturably bound. Results are the means of six experiments, and in each experiment each value was calculated in duplicate.

Cell line	Ligand bound				
	[^{125}I-D-Tyr^0]NMB	[^{125}I-Tyr^4]Bn	^{125}I-GRP	[^{125}I-D-Tyr^6]Bn-(6–13) methyl ester	[^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14)
	fmol / 10^7 cells				
Balb 3T3 cells					
Nontransfected	0.2 ± 0.1	0.5 ± 0.2	0.1 ± 0.01	1.1 ± 0.5	1.7 ± 0.7
BRS-3-transfected					
Clone 10	0.2 ± 0.1	0.03 ± 0.01	0.03 ± 0.01	0.4 ± 0.1	85.2 ± 17[a,b]
Clone 14	0.3 ± 0.1	0.25 ± 0.10	0.8 ± 0.7	0.3 ± 0.1	42.1 ± 7.1[a,b]
NCI-H1299 cells					
Nontransfected	0.9 ± 0.4	1.3 ± 0.3[a]	1.7 ± 0.4[a]	1.8 ± 0.4[a]	2.5 ± 0.5[a]
BRS-3-transfected					
Clone 5	1.0 ± 0.3[a]	1.5 ± 0.3[a]	1.1 ± 0.3[a]	0.4 ± 0.1	8.2 ± 1.0[a,b]
Clone 26	0.4 ± 0.2	1.0 ± 0.3[a]	2.2 ± 0.8[a]	0.5 ± 0.2	7.4 ± 0.5[a,b]

[a] Significantly greater ($p < 0.05$) than binding with no cells added.
[b] Significantly greater ($p < 0.01$) than nontransfected cells.

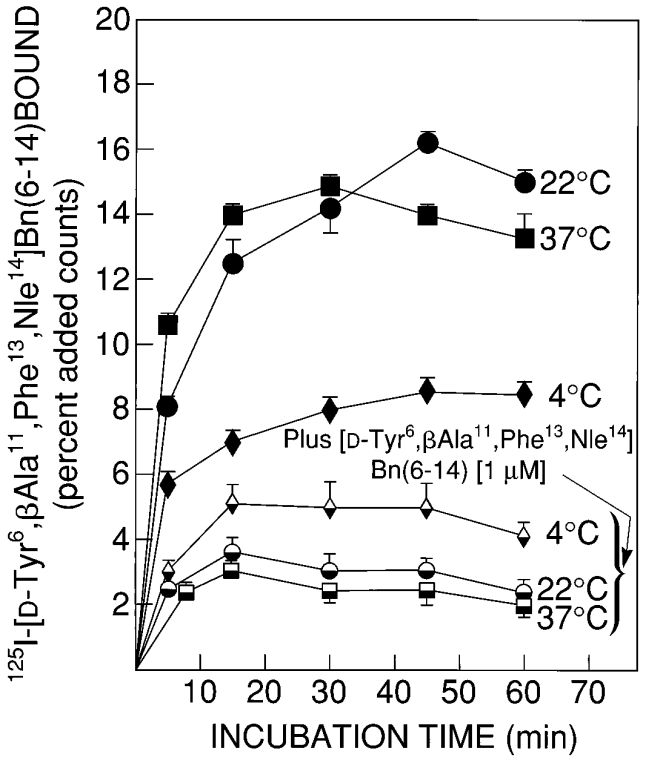

FIG. 3. **Time and temperature dependence of binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to hBRS-3-transfected H1299 cells.** hBRS-3-transfected H1299 cells (1 × 10^6 cells/ml) (clone 5) were incubated with 50 pM [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) at the indicated temperature. At the indicated time, 100-μl aliquots were removed, and total and nonsaturable binding (plus 1 μM [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14)) were determined at each temperature using centrifugation as described under "Methods." Results are the mean ± S.E. from three experiments, and each point was determined in duplicate.

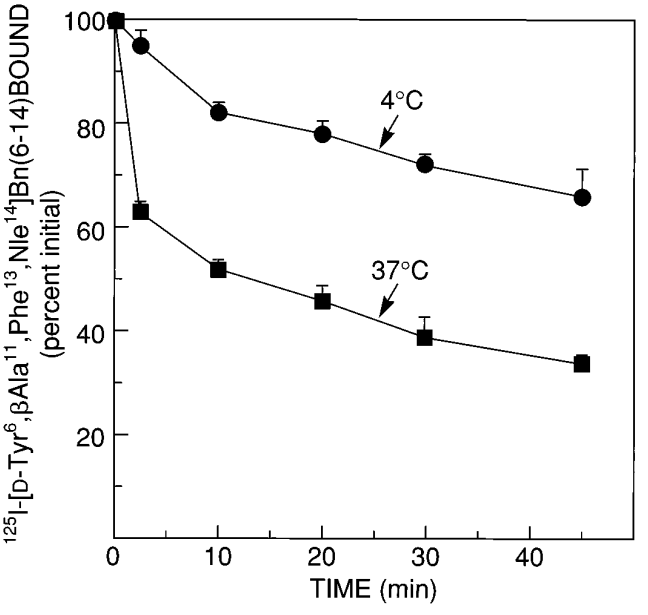

FIG. 4. **Time and temperature dependence of dissociation of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) from BRS-3-transfected 1299 cells.** After incubation of hBRS-3-transfected H1299 cells (1.5 × 10^6 cells/ml) (clone 5) for 45 min at 25 °C with 50 pM [[^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14), the cells were diluted 100-fold in incubation buffer at the indicated temperature and incubated for the indicated time prior to filtering the cells on GF/B filters. Results are expressed as the percentage of the ligand saturably bound at time 0 (*percent initial*). The results are the mean ± S.E. from three experiments, and each point was determined in duplicate.

Plasmids—The hBRS-3 cDNA was amplified by PCR from the original cDNA clone (1) generating a 1.3-kilobase pair fragment that included the coding region and an additional 62 base pairs of the immediate 3′-untranslated region. A sequence encoding the flag epitope tag (5′-GACTACAAGGACGACGATGACAAG-3′) was inserted between the first (Met) and second (Ala) amino acid residue of the coding region during PCR extension from the original clone. The epitope-tagged BRS-3 cDNA was cloned into the *Eco*RI site of the mammalian expression vectors pcDNA3 (Invitrogen; San Diego, CA) and modified pCD2, respectively. The correct DNA sequence of the inserts in the expression plasmids was verified by automatic sequencing on both strands (model A373, Applied Biosystems, Perkin-Elmer).

Stable Transfection—Fifteen μg of plasmid DNA (human epitope-tagged BRS-3 cDNA in the mammalian expression vectors pCD2 and pcDNA3 (Invitrogen; San Diego, CA)) was used for transfection of NCI-H1299 cells with 25 μl of lipofectAMINE (Life Technologies). Balb 3T3 cells were transfected using the $CaPO_4$ precipitation method as described by Davis *et al.* (8). Three days after transfection, cells were split in a ratio of 1:3, and the selection antibiotic G418 (Life Technologies) was added to the regular growth medium at a concentration of 800 μg/ml. Single colonies were isolated 2 weeks later and expanded in growth medium containing G418 (300 μg/ml).

Preparation of Membranes from Native Balb 3T3 Fibroblasts and NCI-H1299 Cells or hBRS-3-transfected Balb 3T3 and NCI-H1299 Cells—The homogenizing buffer contained 50 mM Tris (pH 7.4), 0.2 mg/ml soybean trypsin inhibitor, 0.2 mg/ml benzamidine, and 0.1%

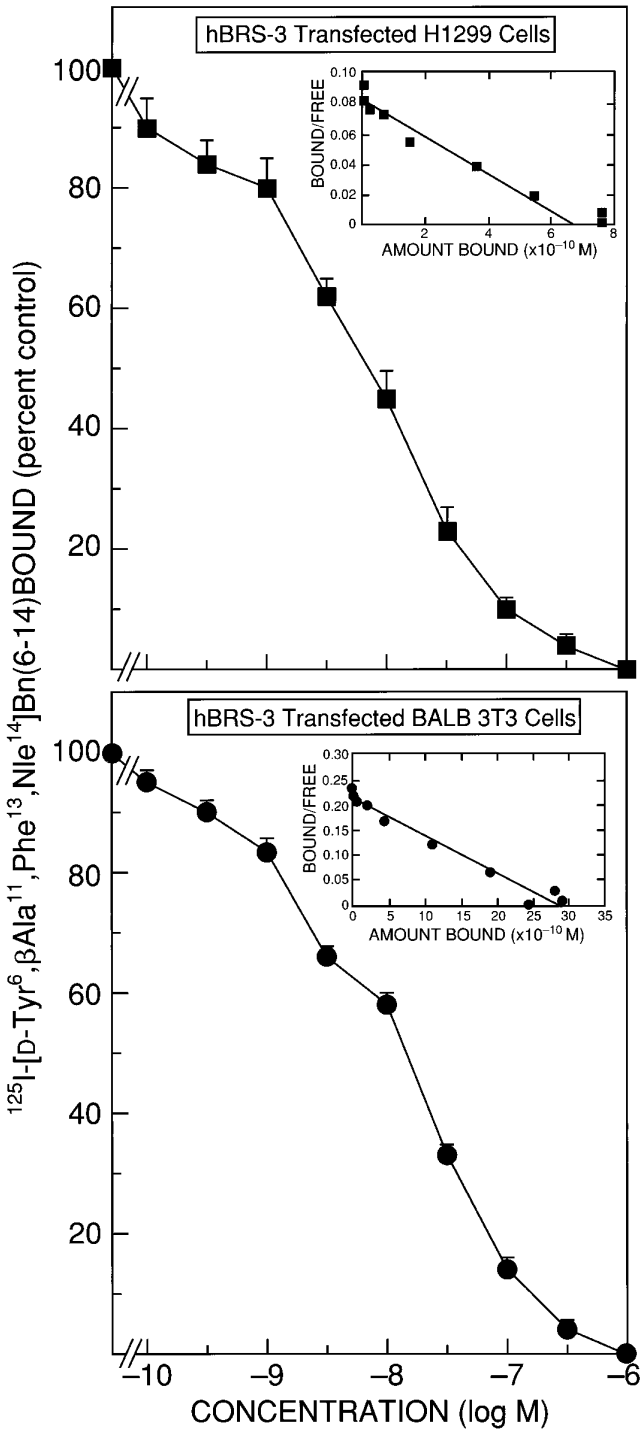

FIG. 5. **Receptor number and affinity for [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) of BRS-3 receptors on hBRS-3-transfected H1299 cells (*top*) and hBRS-3-transfected Balb 3T3 cells (*bottom*).** hBRS-3-transfected H1299 cells (1.5×10^6 cells/ml) or hBRS-3-transfected Balb 3T3 cells (1×10^6 cells/ml) were incubated for 45 min at 25 °C with 50 pM [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) with or without the indicated concentration of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14). Results are expressed as the percentage of saturable binding seen with no [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) added. The *inset* shows the results of the dose-inhibition plotted in the form of Scatchard. Results are the means ± S.E. of four experiments, and in each experiment the point was determined in duplicate.

bacitracin. 1×10^7 cells/ml were homogenized at 4 °C with a polytron (Brinkman Instruments) at the speed of 6 for 30 s. The homogenized suspension was centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant was removed and recentrifuged at 20,000 rpm for 20 min. The pellet was resuspended in homogenizing buffer and stored at −20 °C.

TABLE II

Ability of various agents to alter binding to the hBRS-3 receptor

A suspension (1×10^6 cells/ml) of hBRS-3-transfected Balb 3T3 cells (clone #10) was incubated with 50 pM[^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) either alone or with 1 μM [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14), or with the indicated concentration of the unlabeled peptides listed. Results are expressed as the percentage of the saturable binding with no unlabeled peptide. Results are means ± S.E. from three separate experiments, and in each experiment each point was determined in duplicate.

Agent added[a]	[^{125}I-D-Tyr^6,β-Ala^{11}, Phe^{13},Nle^{14}]Bn-(6–14) binding (percentage of control)
[D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14)(0.1 μM)	9 ± 4[b]
Secretin (1 μM)	88 ± 2
CCK-8 (1 μM)	91 ± 4
Substance P (1 μM)	95 ± 2
VIP (1 μM)	97 ± 3
Gastrin-17-I (1 μM)	96 ± 2
Gastrin-17-II (1 μM)	97 ± 5
Endothelin-1 (1 μM)	90 ± 8
Endothelin-3 (1 μM)	93 ± 6
PACAP-27 (1 μM)	98 ± 4
CGRP (1 μM)	92 ± 2
Carbachol (100 μM)	94 ± 5

[a] VIP, vasoactive intestinal peptide; CCK-8, COOH-terminal octapeptide of cholecystokinin; PACAP-27, pituitary adenylate cyclase-activating peptide; CGRP, calcitonin gene-related peptide.

[b] $p < 0.01$ compared with saturable binding with no agent added.

Preparation of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14), [^{125}I-Tyr^4]Bn, [^{125}I-DTyr^6]Bn-(6–13)methyl ester, [^{125}I-Tyr^0]NMB, and ^{125}I-GRP—The radioligands were prepared by a modification of the methods described previously (12, 13). To 0.8 μg of IODO-GEN in a reaction vial, 20 μl of KH_2PO_4 (pH 7.4), 8 μg of peptide in 4 μl of water and 2 mCi (20 μl) of $Na^{125}I$ were added and incubated at room temperature for 6 min. The incubation was stopped by the addition of 100 μl of distilled water, and 300 μl of 1.5 M dithiothreitol was added. The iodination mixture was incubated 80 °C for 60 min. [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) and [^{125}I-D-Tyr^6]Bn-(6–13)methyl ester (13) did not undergo the incubation with dithiothreitol. Radiolabeled peptides were separated using a Sep-Pak and high pressure liquid chromatography as described previously (12, 13). Radioligands were stored with 0.5% bovine serum albumin at −20 °C.

Binding of ^{125}I-Labeled Peptides to Transfected and Untransfected Cells—The standard binding buffer contained 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1.0 μM $MgCl_2$, 0.01% soybean trypsin inhibitor, 0.2% (v/v) amino acid mixture, 0.2% bovine serum albumin, and 0.1% bacitracin. Incubations contained 50 pM ^{125}I-labeled ligand and $1–2 \times 10^6$ cells/ml (unless otherwise stated) and were for the indicated durations at the indicated temperatures. Nonsaturable binding was the amount of radioactivity associated with the cells in incubations containing 50 pM radiolabeled ligand and 1 μM unlabeled ligand. Nonsaturable binding was <10% of total binding in all experiments.

Binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to cell membranes—The standard membrane binding buffer contained 10 mM HEPES (pH 7.4) 118 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.2 mg/ml benzamidine, 0.2 mg/ml soybean trypsin inhibitor, 0.1% bacitracin, and 0.2% (w/v) bovine serum albumin. The binding assay contained 50 pM of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) and 200 μg of cell membrane protein in a 300-μl incubation volume with or without unlabeled ligands. The nonsaturable binding of radiolabeled ligand was the amount of radioactivity associated with the cell membranes in incubations containing 50 pM ligand plus 1 μM of unlabeled ligand and was <15% of the total binding. Incubations were performed at 22 °C for 45 min.

RESULTS

Preparation of hBRS-3-transfected Cells—Two different cell lines stably expressing transfected hBRS-3 receptors were made. BRS-3 receptors are reported to exist on some small cell and non-small cell lung cancer cell lines such as NCI-H1299 (1) but not on Balb 3T3 cells. To determine whether any of the bombesin receptors existed in the Balb 3T3 cells or the human

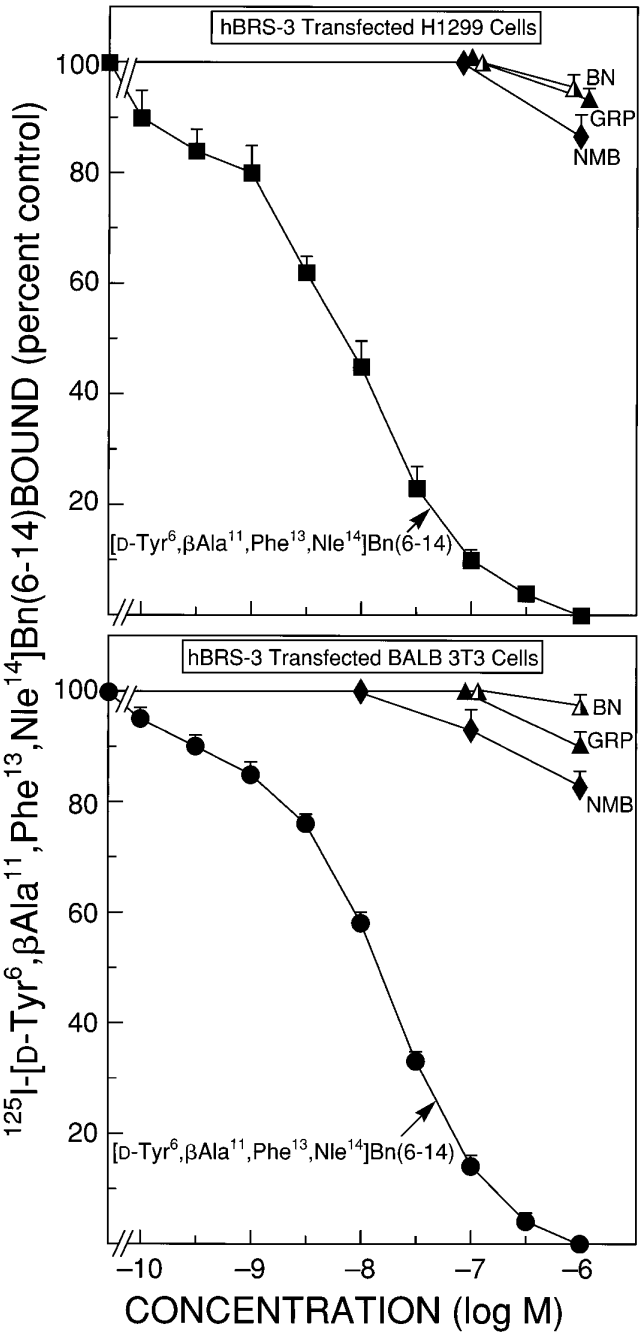

FIG. 6. **Comparison of the ability of [D-Tyr^6,β-Ala^{11},Phe^{13}, Nle^{14}]Bn-(6–14) and the natural occurring Bn peptides (Bn, GRP, NMB) to inhibit binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13}, Nle^{14}]Bn-(6–14) to hBRS-3-transfected H1299 cells (*top*) or hBRS-3-transfected Balb 3T3 cells (*bottom*).** The experimental conditions were the same as outlined in the Fig. 5 legend except that the indicated concentration of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14), Bn, GRP, or NMB was added. Results are expressed as the percentage of the saturable binding with no unlabeled peptide added (*percent control*). Results are the mean ± S.E. of four experiments, and in each experiment results were determined in duplicate.

non-small cell lung cancer cell line, NCI-H1299, which we planned to use to make stable cell lines containing BRS-3, we initially used Northern blot analysis (Fig. 1) and did not detect hBRS-3 mRNA in either cell line; nor was the hGRP receptor or hNMB receptor mRNA detected (Fig. 1). In contrast, employing RT-PCR and Southern blot analysis, the BRS-3 receptor was present in native H1299 cells but not in native Balb 3T3 cells (Fig. 2, *top panel*, *lanes 1* and *2*). Similarly, the hGRP receptor (Fig. 2, *middle panel*, *lanes 1* and *2*) and hNMB receptor (Fig. 2, *bottom panel*, *lanes 1* and *2*) were present in native H1299 cells but not in native Balb 3T3 cells. Binding studies on native H1299 cells with an NMB-R ligand ([^{125}I-D-Tyr^0]NMB) (14, 15)), a GRP-R receptor agonist ligand ([^{125}I-Tyr^4]Bn (16), ^{125}I-GRP (15)), or antagonist ligand ([^{125}I-D-Phe^6]Bn-(6–13)methyl ester (13)) showed low levels of GRP receptors (Table I). In contrast, native Balb 3T3 cells did not demonstrate significant saturable binding with any of the four NMB-R or GRP-R ligands (Table I).

H1299 and Balb 3T3 cells stably expressing the hBRS-3 were identified by Northern blot analysis (Fig. 1). The results from five clones stably expressing hBRS-3 in NCI-H1299 cells (H1299 clones 4, 5, 12, 25, and 26) and three clones in Balb 3T3 cells (Balb hBRS-3 clones 10, 11, and 14) are shown in Fig. 1. Radiolabeled NMB-R or GRP-R receptor ligands (*i.e.* [^{125}I-D-Tyr^0]NMB, [^{125}I-Tyr^4]Bn, ^{125}I-GRP, [^{125}I-D-Tyr^6]Bn-(6–13)-methyl ester) did not demonstrate increased saturable binding to these clones compared with the results in the native nontransfected cells (Table I). Furthermore, 15 natural occurring bombesin-related peptides were examined for the ability to increase [^{3}H]inositol phosphates in a number of these hBRS-3-transfected cell lines, but none caused significant increases (2-fold increase) at concentrations <30–100 nM. In screening studies of various synthetic bombesin-related peptides in one of the authors' laboratories (E. R. S.) using hBRS-3 expressed in *Xenopus* oocytes, it was found that the synthetic Bn-related peptide, [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) elicited a calcium response at concentrations <100 nM. Preliminary studies in a number of hBRS-3-transfected H1299 and Balb 3T3 cell lines demonstrate that this peptide stimulated >5-fold increase in [^{3}H]inositol phosphates with detectable effects at <100 nM (data not shown).

Characterization of hBRS-3 Ligand—In an effort to create a radioligand that would bind to BRS-3 with high affinity, we synthesized [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) and iodinated the peptide. [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) demonstrated no saturable binding to nontransfected Balb 3T3 cells (Table I), a low but significant level ($p < 0.05$) of binding to nontransfected NCI-H1299 cells that have low levels of hBRS-3 receptors (Figs. 1 and 2), and significant binding to each of 10 different H1299 clones and four Balb 3T3 clones that had been stably transfected with hBRS-3 receptors. The results from four clones (H1299 clones 5 and 26 and Balb 3T3 clones 10 and 14) are shown in Table I. One NCI-H1299 clone (number 5) and one hBRS-3-transfected Balb 3T3 clone (number 10) demonstrating high binding were used to characterize hBRS-3 pharmacology in the following studies.

Binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to either hBRS-3-transfected H1299 cells (clone 5) (Fig. 3) or hBRS-3-transfected Balb 3T3 cells (data not shown) was time- and temperature-dependent. Binding was rapid at 22 °C or 37 °C, reaching maximal by 20 min and then was constant for 40 min (Fig. 3). Binding at either temperature was reduced >85% by the addition of unlabeled [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) (Fig. 3). Decreasing the temperature to 4 °C slowed the rate of binding such that by 60 min only 50% of the maximal binding seen at 22 or 37 °C was seen (Fig. 3).

Binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to both cell lines was reversible, and the dissociation rate was temperature-dependent. In hBRS-3-transfected H1299 cells (clone 5) (Fig. 4) or hBRS-3-transfected Balb 3T3 cells (clone 10) (data not shown), 37% of the ligand rapidly dissociated within the first 2 min, and an additional 30% slowly dissociated over an additional 40 min at 37 °C. The dissociation rate was markedly slowed at 4 °C such that after a 45-min incubation

TABLE III

Comparison of the affinity of naturally occurring bombesin-related peptides for the hBRS-3, the GRP receptor, and the NMB receptor

Cells (1–2 × 10^6/ml) were incubated with 50 pM[^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) (hBRS-3-transfected cells), [^{125}I-Tyr^4]Bn (rat pancreatic acini), or [^{125}I-D-Tyr^0]NMB (rNMB-R-transfected cells) for 45 min at 22 °C (transfected cells) or 30 min at 37 °C (pancreatic acini). Increasing concentrations of unlabeled peptide were added, and dose-response curves were analyzed using a least-squares, curve-fitting program (LIGAND). K_i values were calculated using the method of Cheng and Prusoff (15). Values are mean ± S.E. from at least four experiments. >10,000 means the affinity was greater than 10,000 nM.

Peptides added[a]	K_i			
	hBRS-3-transfected cells		Rat pancreatic acini	rNMB-R-transfected Balb 3T3 cells
	NSCLC (1299)	Balb 3T3		
	nM		nM	nM
[D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14)	4.2 ± 1	8.9 ± 0.7	0.99 ± 0.16	0.36 ± 0.06
Bombesin	>10,000	>10,000	4 ± 1	34 ± 2
GRP	>10,000	>10,000	18 ± 5	440 ± 70
NMB	2800 ± 900	4800 ± 400	248 ± 5	4 ± 1
Ranatensin	7400 ± 1400	6900 ± 2000	2 ± 1	13 ± 2
Litorin	3900 ± 500	4100 ± 400	6 ± 1	7 ± 1
Alytesin	5100 ± 1400	3600 ± 1200	62 ± 7	460 ± 70
[Leu^8]Phyllolitorin	6200 ± 2000	5400 ± 1900	420 ± 45	>10,000
Phyllolitorin	3600 ± 1300	2100 ± 200	240 ± 50	47 ± 3
PG-L	3600 ± 1400	5300 ± 1400	3 ± 1	210 ± 20
Rohdei-litorin	2700 ± 300	3100 ± 700	31 ± 4	460 ± 20
Neuromedin C	>10,000	>10,000	20 ± 12	140 ± 10
Xenopus NMB	>10,000	7100 ± 2500	180 ± 30	340 ± 60
SAP bombesin	>10,000	7100 ± 950	3200 ± 400	>10,000
[Phe^{13}]Bombesin	7100 ± 1150	6600 ± 1750	0.77 ± 0.15	350 ± 50
Frog GRP-10	8800 ± 300	>10,000	130 ± 7	>10,000

[a] *Xenopus* NMB, [Gln^3,Ile^6]NMB (20); SAP bombesin, [Ser^3,Arg^9,Phe^{13}]bombesin (4); frog GRP-10, [Ser^{19}]GRP-(18–27) (21).

only 25% dissociated at 4 °C (Fig. 4).

To assess ligand stability, hBRS-3-transfected H1299 cells (1 × 10^6/ml) were incubated with 200 pM [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) for 15 min at 37 °C, which resulted in maximal binding (Fig. 3), and the supernatants were analyzed using high pressure liquid chromatography. 93± 1% of the radiolabeled peptide eluted in the same peak as [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) not exposed to cells (data not shown).

hBRS-3 Receptor Pharmacology—[D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) inhibited binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) in a dose-dependent manner in both hBRS-3-transfected H1299 cells (Fig. 5, *top*) or hBRS-3-transfected Balb 3T3 cells (Fig. 5, *bottom*). Detectable inhibition of binding in each cell line occurred with 0.1 nM [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14); half-maximal inhibition occurred at 4.9 nM with hBRS-3-transfected H1299 cells and 8.9 nM with hBRS-3-transfected Balb 3T3 cells; and complete inhibition occurred at 1 μM. Analysis of the [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) dose-inhibition curve using a least-squares, curve-fitting program (LIGAND) (17) demonstrated the binding was best fitted by a single binding site model (Fig. 5, *inset*). hBRS-3-transfected H1299 cells had an affinity of 4.2 ± 1 nM for [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14), and with hBRS-3-transfected Balb 3T3 cells the affinity was 8.9 ± 0.7. hBRS-3-transfected H1299 cells (clone 5) had a binding capacity of 1.52 ± 0.13 fmol/mg protein (458 ± 40 fmol/10^6 cells), which was 4-fold lower than the binding capacity of hBRS-3-transfected Balb 3T3 cells (clone 10) of 6.7 ± 0.5 fmol/mg protein (2690 ± 180 fmol/10^6 cells).

To assess the specificity of the binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to hBRS-3-transfected Balb 3T3 cells (Table II) or H1299 cells (data not shown), the ability of various peptides or neurotransmitters that interact with receptors different from the bombesin receptor family was tested (Table II). At concentrations that cause a maximal effect at their receptors, none of these agents inhibited binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to hBRS-3-transfected Balb 3T3 cells (Table II) or hBRS-3 H1299-transfected cells (data not shown).

To assess the affinity of the hBRS-3 receptor for known, naturally occurring Bn-related peptides, the affinities for bombesin, GRP, NMB (Fig. 6), and 12 other natural occurring members of the bombesin family of peptides (Table III) were determined in hBRS-3-transfected H1299 cells (Fig. 6, *top*; Table III) and Balb 3T3 cells (Fig. 6, *bottom*; Table II). Both cell lines had almost no affinity for bombesin or GRP (>10,000 nM) (Fig. 6, Table III). NMB had a 300–400-fold lower affinity in both hBRS-3-transfected cell lines than [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) (Fig. 6, Table III). Each of the 12 other natural occurring bombesin-related peptides (4, 18–20) had low affinity (>2 μM) for the hBRS-3 receptors in each of the hBRS-3-transfected cell lines (Table III). These results differed markedly from the ability of these natural occurring bombesin-related peptides to interact with the GRP receptor in rat pancreatic acini or the NMB receptor in rNMB-transfected Balb 3T3 cells (Table III). Specifically, six of these peptides ([Phe^{13}]Bn (4), NMC, PG-L (18), litorin, ranatensin, bombesin) had high affinity (<5 nM) for the GRP receptor (Table II), and two had high affinity (<10 nM) for the NMB receptor (NMB, litorin).

Five different classes of GRP receptor antagonists have been described, and two classes of NMB receptor antagonists (21–24) have been described. Twenty-two members of each of these classes of GRP receptor or NMB receptor antagonists (Fig. 7) or closely related synthetic peptides (Table IV) were tested for the ability to interact with the hBRS-3 receptors in each of the transfected cell lines, which was compared with their ability to interact with the native GRP receptor in rat pancreatic acini and the NMB receptor in rNMB-R-transfected Balb 3T3 cells. Each of the 22 bombesin receptor antagonists had low affinity (>1 μM) for the hBRS-3 receptors on each of the two different transfected cell types (Table III) including three members of the [D-Phe^{12}]Bn (clones 1–3, Table IV) class of GRP receptor and NMB receptor antagonists (23, 25, 26), three members of the bombesin pseudopeptide class of GRP receptor antagonists (clones 4–6, Table IV) (7, 27, 28), two members of the D-Pro^{13} pseudopeptide class of potent GRP receptor antagonists (clones 7 and 8, Table IV), 11 members of the des-Met^{13} classes of amides, esters, alkylamides, and hydrazides (clones 9–18, Table IV) (which function as selective GRP receptor antagonists

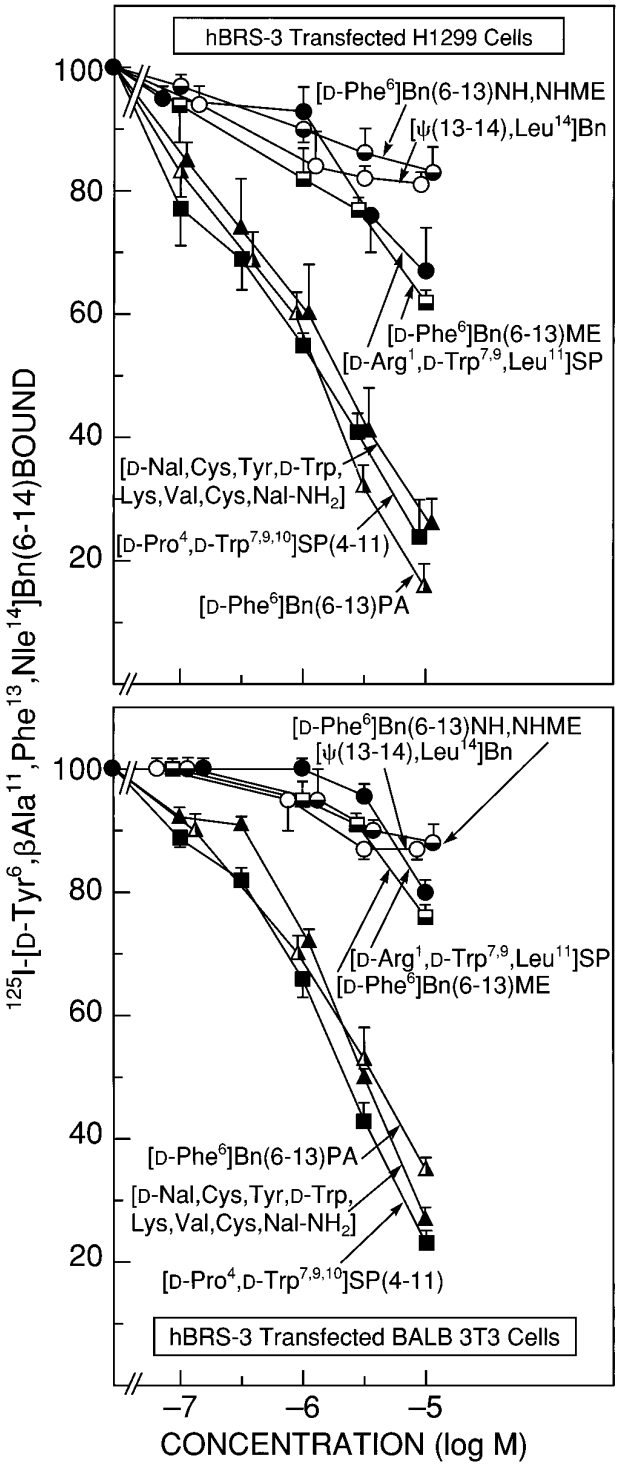

FIG. 7. **Ability of various classes of Bn receptor antagonists to inhibit binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to hBRS-3 receptors in hBRS-3-transfected H1299 (*top*) or Balb 3T3 cells (*bottom*).** The experimental conditions were the same as in Fig. 5 except that the indicated concentration of antagonist was added. Results are expressed as the percentage of saturable binding seen with no unlabeled peptide present. Results are the mean ± S.E. from at least three experiments, and in each experiment each point was determined in duplicate. *SP*, substance P. The other abbreviations are defined in Table IV.

(6, 29–31)), and one member (clone 20, Table IV) of the D-amino acid-substituted octapeptide analogues of somatostatin (which function as NMB receptor antagonists (22)). The D-amino acid-substituted analogues of substance P, or the substance P-(4–11) class of antagonists (which function as receptor antagonists for GRP receptor, NMB receptor, and other receptors (21, 32–34)), had similar low affinities for the hBRS-3 receptor as those reported for the GRP receptor or the NMB receptor (Fig. 7, Table IV). Four synthetic bombesin-related agonists with substitutions in similar positions to [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) (clones 23–26, Table IV) also had low affinity for the hBRS-3 on each transfected cell line (Table IV).

To determine whether hBRS-3 was coupled to G proteins, the ability of the nonhydrolyzable guanine nucleotide analogue Gpp(NH)p to alter binding of [^{125}I-D-Tyr^6,β-Leu^{11},Phe^{13},Nle^{14}]Bn-(6–14) to each of the hBRS-3-transfected cell lines was examined (Fig. 8). Gpp(NH)p caused a dose-dependent 70% decrease in binding in hBRS-3-transfected H1299 cell membranes (Fig. 8, *top*) and a 45% decrease in hBRS-3-transfected Balb 3T3 cell membranes (Fig. 8, *bottom*). Gpp(NH)p caused a detectable decrease with 30 nM Gpp(NH)p, a half-maximal effect at 0.25–0.3 μM, and a maximal effect at 100 μM (Fig. 8). To determine whether the decrease in binding of [^{125}I-D-Tyr^6,β-Leu^{11},Phe^{13},Nle^{14}]Bn-(6–14) was due to a change in receptor number or affinity, the effect of 100 μM Gpp(NH)p on the dose-inhibition curve of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) was determined (Fig. 9). With both hBRS-3-transfected cell types, Gpp(NH)p caused a decrease in hBRS-3 receptor affinity with no change in hBRS-3 receptor number (B_{max}). Specifically, in hBRS-3-transfected H1299 cell membranes the receptor affinity for [D-Phe^6,β-Leu^{11},Phe^{13},Nle^{14}]Bn-(6–14) decreased significantly ($p < 0.001$) from 4.94 ± 0.13 nM to 11.37 ± 0.10 nM with 100 μM Gpp(NH)p, whereas there was no change in receptor capacity (0.33 ± 0.1 to 0.35 ± 0.05 pmol/mg protein). Similarly in hBRS-3-transfected Balb 3T3 cell membranes with the addition of 100 μM Gpp(NH)p, hBRS-3 receptor affinity for [D-Phe^6,β-Leu^{11},Phe^{13},Nle^{14}]Bn-(6–14) decreased 2-fold (from 13.5 ± 0.17 nM to 25.0 ± 2.2 nM ($p < 0.01$), whereas there was no significant change in the receptor number (8.4 ± 0.10 pmol/mg protein) without 100 μM Gpp(NH)p and 8.8 ± 1.9 pmol mg/protein with 100 μM Gpp(NH)p.

DISCUSSION

In the present study, we describe for the first time a comprehensive analysis of the pharmacology of the orphan receptor, BRS-3, using a newly discovered novel ligand with high affinity for this receptor. To achieve this it was first necessary to prepare stable cell lines expressing the hBRS-3 that would be similar in their receptor pharmacology to the native hBRS-3 receptor. This was necessary because, although previous studies have shown the hBRS-3 to exist on certain tumor cell lines (1) such as various human small cell and non-small cell line cancer cells (1), the human ductal breast cancer cell line T47D (3), and the human epidermal cancer cell line A431 (3), their receptor number was too low to allow ligand studies of direct interaction with the hBRS-3. Therefore, two strategies were used to produce stable cell lines that would resemble native cells possessing this receptor. A stable hBRS-3 cell line in Balb 3T3 cells was made, because in previous studies (12, 14, 35, 36) we had demonstrated Balb 3T3 fibroblasts do not possess receptors for the bombesin receptor family (GRP-R, NMB-R, BRS-3) (12, 14, 35, 36). However, these cells closely resemble Swiss 3T3 cells, which possess murine GRP-R and have been extensively used to study the GRP and NMB receptors (36, 37). Specifically, when the mouse GRP receptor (36), human NMB receptor (35), human GRP receptor (35), or rat NMB receptor (14) was expressed in Balb 3T3 cells, all four mammalian bombesin receptors behaved in a fashion indistinguishable from native cells expressing these receptors in interacting with ligands, in G protein coupling, and in their affinity for agonists and antagonists. The second strategy we used was to overex-

TABLE IV

Affinity of various synthetic GRP and NMB receptor agonists and antagonists for hBRS-3 receptors and other bombesin receptors

Cells (1–2 × 10^6/ml) were incubated with 50 pM[^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) (hBRS-3-transfected cells), [^{125}I-Tyr^4]Bn (rat pancreatic acini), or [^{125}I-D-Tyr^0]NMB (rNMB-R-transfected cells) for 45 min at 22 °C (transfected cells) of 30 min at 37 °C (pancreatic acini). Increasing concentrations of unlabeled peptide were added, and dose-response curves were analyzed using a least-squares, curve-fitting program (LIGAND). K_i values were calculated using the method of Cheng and Prusoff (15). Values are mean ± S.E. from at least four experiments. >10,000 means the affinity was greater than 10,000 nM.

Analogue number	Peptides added[a]	K_i			
		hBRS-3-transfected cells		Rat pancreatic acini	rNMB-R-transfected Balb 3T3 cells
		NSCLC (1299)	Balb 3T3		
		nM		nM	nM
1	[D-Phe^{12}]Bn	1700 ± 400	>10,000	>10,000	>10,000
2	[Tyr^4,D-Phe^{12}]Bn	4200 ± 600	>10,000	>10,000	1900 ± 100
3	[D-Phe^6,D-Phe^{12},Leu^{14}]Bn	3900 ± 700	>10,000	430 ± 60	2300 ± 300
4	[Leu^{13},ψ(13–14),Leu^{14}]Bn	7600 ± 2100	>10,000	430 ± 60	>10,000
5	[D-Phe^6,Leu^{13},ψ(13–14),Cpa^{14}]Bn-(6–14)	1100 ± 200	>10,000	42 ± 5	2700 ± 200
6	[D-Phe^6,Leu^{13},ψ(13–14),D-Phe^{14}]Bn-(6–14)	1800 ± 300	>10,000	97 ± 13	4000 ± 600
7	[(3-Ph-Pr^6)-His^7,D-Ala^{11},D-Pro^{13},ψ(13–14),Pro^{14}]Bn-(6–14)NH_2	2300 ± 500	6800 ± 900	0.74 ± 0.04	>10,000
8	[(3-Ph-Pr^6)-Pro^7,β-Ala^{11},D-Pro^{13},ψPhe^{14}]Bn-(6–14)	3000 ± 400	7800 ± 700	580 ± 80	2500 ± 1800
9	[D-Phe^6]Bn-(1–13)NH_2	2400 ± 500	>10,000	450 ± 90	>10,000
10	[D-Phe^6]Bn-(6–13)NH_2	5700 ± 2400	>10,000	27 ± 6	10,800 ± 1100
11	[D-Phe^6]Bn-(6–13)methyl ester	2800 ± 900	5300 ± 2000	10 ± 1	7700 ± 1100
12	[D-Phe^6,D-Ala^{11}]Bn-(6–13)methyl ester	2800 ± 700	>10,000	3 ± 1	11,000 ± 5000
13	[D-Phe^6,*N*-Me-D-Ala^{11}]Bn-(6–13)methyl ester	4500 ± 800	>10,000	71 ± 11	>10,000
14	[D-Phe^6]Bn-(6–13)propylamide	2200 ± 500	1900 ± 300	6 ± 1	4600 ± 600
15	[D-Phe^6]Bn-(6–13)hexylamide	1700 ± 600	3200 ± 100	100 ± 10	>10,000
16	[D-Tyr^6,D-Ala^{11}]Bn-(6–13)butylamide	2400 ± 800	5300 ± 1400	6 ± 1	2300 ± 560
17	[D-Phe^6]Bn-(6–13)NHN(ME_2)	>10,000	>10,000	3200 ± 400	>10,000
18	[D-Phe^6]Bn-(6–13)$NHNH_2$	>10,000	>10,000	1200 ± 400	>10,000
19	[D-Phe^1,β-Leu^8,des-Met^9]Litorin	>10,000	>10,000	76 ± 13	>10,000
20	[D-Na1,Cys,Tyr,D-Typ,Lys,Val,Cys,Na1-NH_2	2200 ± 500	2800 ± 200	>10,000	220 ± 40
21	[D-Arg^1,D-Trp^{7,9},Leu^{11}]Substance P	>10,000	>10,000	11,300 ± 1800	4100 ± 800
22	[D-Pro^4,D-Trp^{7,9,10}]Substance P-(4–11)	1100 ± 5500	2300 ± 400	>10,000	2500 ± 600
23	[D-Phe^6]Bn-(6–14)	3100 ± 1004	>10,000	2 ± 0.1	14 ± 2
24	[D-Phe^6,D-Ala^{11},Leu^{14}]Bn-(6–14)	1400 ± 200	>10,000	13 ± 3	7600 ± 1100
25	[D-Cpa^1,D-Ala^6,Leu^8,Nle^9]Litorin	2100 ± 500	>10,000	65 ± 8	>10,000
26	[D-Phe^1,Nle^9]Litorin	2300 ± 300	3200 ± 300	7 ± 1	50 ± 1

[a] ψ; pseudopeptide bond, (*i.e.*, CONH changed to Ch_2NH);Ph-Pr, phenylpropanolamine; Cpa, chlorophenylalamine; Nal, β-naphthylalanine; ME, methyl.

press the hBRS-3 in the human non-small cell lung cancer cell line, NCI-H1299, which has been reported to express a low level of GRP-R, hNMB receptor, and hBRS-3 mRNA (1, 11), a conclusion we confirmed in the present study.

From ligand studies of these two different hBRS-3-containing cell lines produced by these two different strategies, a number of results support the conclusion that we are assessing interaction with the hBRS-3 and that it probably represents the true pharmacology of this receptor. First, the novel hBRS-3 ligand, [^{125}I-D-Tyr^6,β-Leu^{11},Phe^{13},Nle^{14}]Bn-(6–14), did not interact with native Balb 3T3 cells not transfected with the hBRS-3, which do not possess native BRS-3 receptors. However, after hBRS-3 was stably transfected, a number of Balb 3T3 cells were identified and characterized that all demonstrated marked increased binding of this ligand. However, no increased binding of the NMB receptor ligand, [^{125}I-D-Tyr^0]NMB, or of the GRP receptor ligands, [^{125}I-Tyr^4]Bn (13, 16, 38), ^{125}I-GRP (39, 40), or [^{125}I-D-Tyr^6]Bn-(6–13)methyl ester (13), was seen demonstrating the specific acquisition of only the hBRS-3 receptor. Second, nontransfected NCI-H1299 non-small cell lung cancer cells bound very low levels of this ligand as well as two GRP receptor ligands, which was consistent with the low level of expression of native hBRS-3 and hGRP receptors and mRNA found in this cell line in the present study and reported in a previous study (11). Similar to the results with the Balb 3T3 cells, after transfection of NCI-H1299 cells with hBRS-3 there was only increased binding of the novel hBRS-3 ligand and no increased binding of ligands that specifically interacted with NMB receptors or GRP receptors. Third, the novel hBRS-3 ligand interaction with the hBRS-3 receptors on each transfected cell line was characteristic of receptor interaction in that it was high affinity, saturable, time- and temperature-dependent, and reversible (41). Fourth, the novel hBRS-3 ligand binding was specific for the hBRS-3 receptor on each of these stable cell lines because unrelated peptides had no effect on the binding of this ligand. Fifth, both hBRS-3-transfected cell lines acquired high affinity (4–8 nM) for the novel hBRS-3 ligand but not for any of the 15 natural occurring bombesin-related agonists or 19 synthetic peptides that are known to function as GRP receptor or NMB receptor agonists or antagonists. This finding excludes the possibility the ligand was interacting with a GRP or NMB receptor. That the pharmacology observed probably represents the true pharmacology of the hBRS-3 receptor is supported by the observation that both cell lines demonstrated similar pharmacology.

Our results demonstrate that the hBRS-3 has a unique pharmacologic profile for a member of the bombesin receptor family. Its pharmacology differs in a number of ways from that for either the GRP receptor or NMB receptor. First, some peptides such as litorin, ranatensin, and bombesin have a high affinity for all other Bn receptors. However, each of these three peptides has a low affinity (>1 μM) for the hBRS-3. Second, a number of Bn-related peptides have selective high affinity for either the GRP receptor (GRP, NMC, PG-L, [Phe^{13}]bombesin) or the NMB receptor (NMB), and none of these peptides had high affinity for the hBRS-3. Third, a number of Bn peptides have relatively low affinities for both the NMB receptor and GRP receptor ([Leu^8]phyllolitorin, phylolitorin, rohdei-litorin), raising the possibility they could interact with a significantly different subtype of bombesin receptor. However, each of these peptides was also found to have low affinities in the micromolar range for the hBRS-3. Fourth, six different classes of GRP receptor (21, 24) or NMB receptor (22) antagonists have been described, some of which are highly selective. Representative

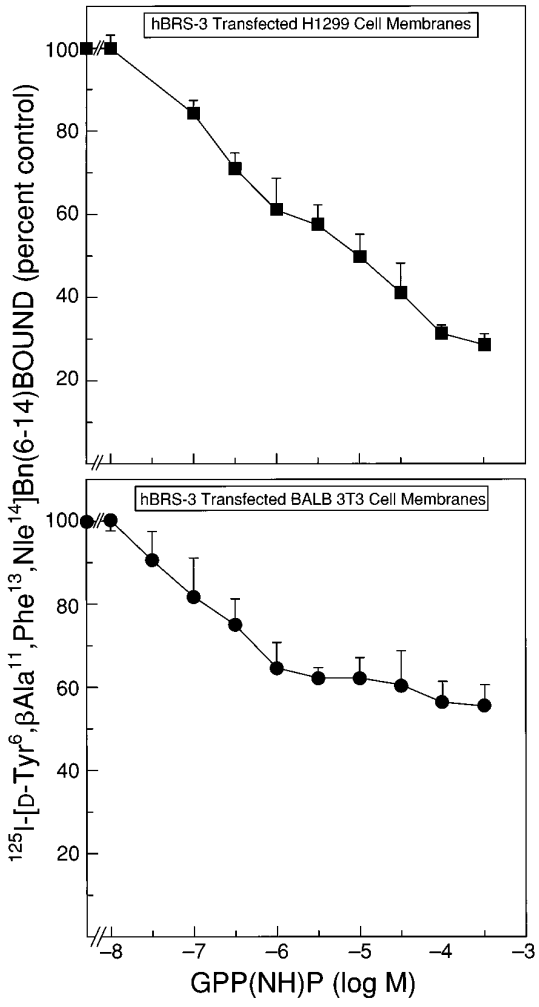

FIG. 8. **Ability of Gpp(NH)p to inhibit binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to transfected H1299 (*top*) or Balb 3T3 (*bottom*) cell membranes.** Cell membranes (200 μg protein/ml) were incubated with 50 pM [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) for 45 min at 25 °C with the indicated concentration of Gpp(NH)p. Results are expressed as the percentage of the saturable binding seen with no Gpp(NH)p added. Results are the mean ± S.E. from four experiments, and in each experiment each point was determined in duplicate.

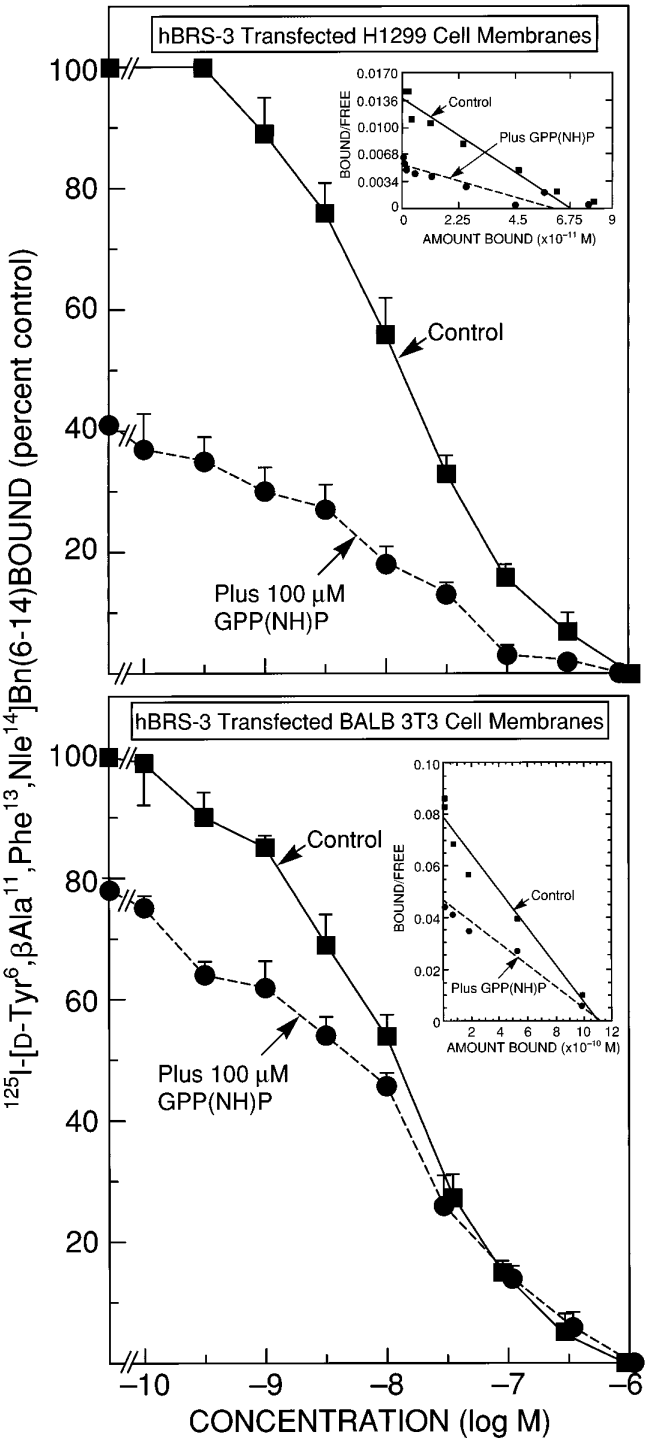

FIG. 9. **Effects of Gpp(NH)p on the ability of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to inhibit binding of [^{125}I-D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) to hBRS-3 receptor-transfected H1299 (*top*) or Balb 3T3 cells (*bottom*).** The experimental conditions were the same as for Fig. 7 except that the membranes (200 μg/ml) were incubated with or without 100 μM Gpp(NH)p and the indicated concentrations of unlabeled [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14). Results are expressed as the percentage of saturable binding with no Gpp(NH)p present and are the mean ± S.E. from four experiments with each point determined in duplicate. In the *inset*, the data are plotted in the form of Scatchard. A comparison is shown of the affinity of naturally occurring peptides and various synthetic GRP and NMB agonists and antagonists for the hBRS-3, the GRP receptor, and the NMB receptor.

members of each of these different classes of antagonists were examined, and none were found to interact with the hBRS-3 with affinity above the micromolar range.

Our results have some similarities and differences from the three previous studies that have provided some information on the pharmacology of the hBRS-3 receptor (1, 2, 4). Our finding that the hBRS-3 receptor has a low affinity for NMB, GRP, Bn, [Phe^8]phyllolitorin, and ranatensin is consistent with the findings that when the hBRS-3 was expressed in *Xenopus* oocytes, 100-fold higher concentrations of NMB, GRP and bombesin were needed to activate the hBRS-3 than to activate the GRP or the NMB receptor expressed in the same system (1). Furthermore, [Phe^8]phyllolitorin or ranatensin even at very high concentrations (10 μM) did not activate the hBRS-3 expressed in *Xenopus* oocytes (1). Similarly, when the guinea pig BRS-3 receptor was expressed in LLK-PK$_1$ cells, it had a low affinity for GRP, NMC, and NMB in a binding assay using ^{125}I-bombesin (2). Our results are consistent with some findings in a recent study (4) examining the effects of various naturally occurring bombesin-related peptides that cause changes in $[Ca^{2+}]_i$ in Balb 3T3 cells transfected with the hBRS-3. This study concluded that hBRS-3 probably had a higher affinity for the NMB, litorin, and ranatensin than GRP or bombesin. Our results demonstrate that although each of these peptides has a low affinity for the hBRS-3, NMB, ranatensin, and litorin will

interact with the BRS-3 in the micromolar range; however, even with concentrations as high as 10 μM bombesin, GRP, or NMC have no affinity for this receptor. Our results differ from this latter study (4) in that the synthetic analogue [D-Phe^6]Bn-(6–13) propylamide was reported to have a relatively high affinity (EC_{50} ~84 nM) for stimulating changes in $[Ca^{2+}]_i$ in hBRS-3-transfected Balb 3T3 cells (4); however, we found this analogue to have a low affinity. At present, the explanation for the differences from the study on $[Ca^{2+}]_i$ (4) is unclear. The differences are not due to inactivity of the peptide in our study, because it had a high affinity for the GRP-R, as reported previously (13, 29). It remains possible that, in contrast to the GRP-R and NMB-R, there could be large receptor spareness in hBRS-3 receptors such that minimal changes in receptor occupation cause marked changes in $[Ca^{2+}]_i$, and therefore the biologic activity dose-response curve for agonist-induced changes in $[Ca^{2+}]_i$ is far to the left of the receptor occupation curve.

Detailed structure-function studies of the unique ligand [Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn-(6–14) were not performed; however, our data provides some insights into the important structural components that might contribute to the unique ability of this bombesin-related peptide to interact with the hBRS-3 with high affinity. First, the deletion of the five NH_2 amino acids of bombesin or the insertion of D-phenylalanine in position 6 of bombesin in this ligand is unlikely to be responsible for its high affinity for hBRS-3. This can be concluded because neither [D-Phe^6]Bn-(6–14), litorin, nor [D-Phe^1,Nle^9]litorin, which all lack the first five NH_2-terminal amino acids of bombesin and two of the analogues that have a D-phenylalanine in the equivalent position to the sixth position of bombesin, have high affinity for the hBRS-3. Second, the norleucine in position 14 of this unique ligand is unlikely to be responsible for its high affinity, because [D-Phe^1,Nle^9]litorin, which has a D-phenylalanine and norleucine in equivalent positions to the hBRS-3 ligand, had low affinity. Third, it is also unlikely the phenylalanine *per se* in position 13 is a major factor in the high affinity of this ligand because [Phe^{13}]bombesin, PG-L, litorin, ranatensin, NMB, PLL, and rohdei-litorin, all of which possess a phenylalanine in the penultimate position, had low affinity for the BRS-3. However, the presence of a penultimate phenylalanine could play an important role in combination with alterations in the other locations, because, in general, the peptides with this substitution had a higher affinity for the hBRS-3 than those with a leucine in this position (see Tables III and IV). These data suggest that the important substitution in this unique ligand is the presence of the β alanine in the eleventh position of bombesin. It remains at present unclear whether the principal effect of this substitution is only an extension of the length of the peptide backbone or if there are other factors such as the side chain modification. This question will need to be explored in future studies, and the answer may provide important insights into the possible structure of the natural ligand for this receptor.

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