0022-3565/04/3103-1161–1170
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
U.S. Government work not protected by U.S. copyright
JPET 310:1161–1170, 2004

Vol. 310, No. 3
66761/1161856
Printed in U.S.A.

Development of Bombesin Analogs with Conformationally Restricted Amino Acid Substitutions with Enhanced Selectivity for the Orphan Receptor Human Bombesin Receptor Subtype 3

Samuel A. Mantey, David H. Coy, Laurence K. Entsuah, and Robert T. Jensen

Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (S.A.M., L.K.E., R.T.J.); and Peptide Research Laboratories, Department of Medicine, Tulane University Health Sciences Center, New Orleans, Louisiana (D.H.C.)

Received February 9, 2004; accepted April 20, 2004

ABSTRACT

The human bombesin receptor subtype 3 (hBRS-3) orphan receptor, which has a high homology to bombesin (Bn) receptors [gastrin-releasing peptide (GRP) and neuromedin B (NMB) receptors], is widely distributed in the rat central nervous system. Its natural ligand or role in physiology is unknown due to lack of selective ligands. Its target disruption leads to obesity, diabetes, and hypertension. A synthetic high-affinity agonist, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), has been described, but it is nonselective for hBRS-3 over other Bn receptors; however, substitution of (*R*)- or (*S*)-amino-3-phenylpropionic acid (Apa) for β-Ala^{11} resulted in a modestly selective ligand. In the present study, we have attempted to develop a more selective hBRS-3 ligand by using two strategies: substitutions on phenyl ring of Apa^{11} and the substitution of other conformationally restricted amino acids into position 11 of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14). Fifteen analogs were synthesized and affinities were determined for hBRS-3 and Bn receptors (hGRP-R and hNMB-R). Selective analogs were tested for their ability to activate each receptor by stimulating phospholipase C. One analog, [D-Tyr^6,Apa-4Cl,Phe^{13},Nle^{14}]Bn(6-14), retained high affinity for the hBRS-3 (K_i = 8 nM) and had enhanced selectivity (>230-fold) for hBRS-3 over hGRP-R or hNMB-R. This analog specifically interacted with hBRS-3, fully activated hBRS-3 receptors, and was a potent agonist at the hBRS-3 receptor. This enhanced selectivity should allow this analog to be useful for investigating the possible role of hBRS-3 in physiological or pathological processes.

Bombesin receptor subtype 3 (BRS-3) is a G protein-coupled heptahelical orphan receptor that shares 47 to 51% amino acid identities with the mammalian bombesin (Bn) receptors [gastrin-releasing peptide (GRP) and neuromedin B (NMB) receptors] (Gorbulev et al., 1992; Fathi et al., 1993; Ohki-Hamazaki et al., 1997a; Whitley et al., 1999; Liu et al., 2002). BRS-3 is widely expressed in the rat central nervous system and is present in a few peripheral tissues, although its distribution is species-dependent and much more limited than the GRP-R or NMB-R (Tache et al., 1988; Fathi et al., 1993; Ohki-Hamazaki et al., 1997a; Whitley et al., 1999; Liu et al., 2002; Jennings et al., 2003). BRS-3 is reported to occur in human islets (Fleischmann et al., 2000); in the testes of the rat, but not mouse, human, or sheep; and in the guinea pig and human uterus, but not mouse or sheep uterus (Gorbulev et al., 1992, 1994; Fathi et al., 1993; Ohki-Hamazaki et al., 1997a; Whitley et al., 1999; Liu et al., 2002). Recent studies show it is also expressed on small cell lung cancer, lung carcinoid tumors, ovarian, breast, and prostate cancer (Gorbulev et al., 1992; Fathi et al., 1993; Sun et al., 2000a,b; Reubi et al., 2002).

The natural ligand of BRS-3 is unknown and its role in physiological and pathological processes is largely unknown. However, in recent studies, BRS-3 knockout mice developed obesity, diabetes, and hypertension (Ohki-Hamazaki et al., 1997b), suggesting the BRS-3 receptor was involved in regulation of blood pressure, glucose metabolism, and energy balance. This proposal is supported by the recent finding that BRS-3 regulates insulin release (Matsumoto et al., 2003). The elucida-

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.104.066761.

ABBREVIATIONS: BRS-3, bombesin receptor subtype 3; Bn, bombesin; GRP, gastrin-releasing peptide; GRP-R, gastrin-releasing peptide receptor; NMB, neuromedin B; NMB-R, neuromedin B peptide receptor; FBS, fetal bovine serum; CCK, cholecystokinin; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide; Apa, aminopropionic acid; β-Ala, β-alanine; β-Asp, Boc-β-aspartic acid; Cl, chloro; β-AspBzlAmd, Boc-β-aspartic acid benzilamide; Nα-acetyl-Dap, 2-acetylamino,3-aminopropionic acid; Nα-benzoyl-Dap, 2-benzoylamino,3-aminopropionic acid; β-Dap, 2,3,diaminopropionic acid; β-Dap(Pr), 2,3-diaminopropionic acid-proline; Acpb4-Boc(*R*)-3-amino-3-(4-chlorobenzyl)-propionic acid; hBRS-3, human bombesin receptor subtype 3; CHO, Chinese hamster ovary; IP, inositol phosphates; Nle, norleucine.

tion of the role of BRS-3 in various physiological/pathological processes has been limited by the lack of availability of high-affinity ligands that activate or antagonize the action of this receptor. hBRS-3 has a unique pharmacology and despite its high homology to mammalian Bn receptors, none of the naturally occurring high-affinity ligands for these receptors had high affinity for BRS-3 (Gorbulev et al., 1992; Fathi et al., 1993; Wu et al., 1996; Mantey et al., 1997; Ryan et al., 1998a,b). However, we have recently found the synthetic ligand [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) functioned as a high-affinity agonist at the hBRS-3 receptor (Mantey et al., 1997; Pradhan et al., 1998; Ryan et al., 1998a,b). Using this ligand and its derivative, it has been possible to explore the pharmacology as well as the cellular transduction cascades of the hBRS-3 (Mantey et al., 1997; Pradhan et al., 1998; Ryan et al., 1998a,b). These studies confirm the unique pharmacology of hBRS-3 with low affinity for all known naturally occurring agonists for all Bn receptors (GRP-R, NMB-R, and bombesin receptor subtype 4) (Mantey et al., 1997; Pradhan et al., 1998; Ryan et al., 1998a,b) as well as demonstrate that hBRS-3 activation is coupled to a phospholipase C, changes in cytosolic calcium, mitogen-activated protein kinase activation, immediate oncogene activation (e.g., c-fos), and activation of other tyrosine kinases (e.g., p125^{FAK}) (Fathi et al., 1993; Wu et al., 1996; Ryan et al., 1998a,b, 1999; Weber et al., 2001). Unfortunately, the usefulness of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) to study the role of hBRS-3 in various processes is limited because recent studies show it has a high affinity for all human Bn receptor subtypes and is nonselective (Mantey et al., 1997; Pradhan et al., 1998; Ryan et al., 1998a, 1999; Katsuno et al., 1999; Reubi et al., 2002). In a recent study (Mantey et al., 2001), in an attempt to develop more selective hBRS-3 ligands, the β-Ala in [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) was replaced by different conformationally restricted amino acids and two peptides with an (*R*)- or (*S*)-amino-3 phenylpropionic (Apa) substitution for β-Ala^{11} had some selectivity for the hBRS-3. However, this selectivity was modest for differential receptor activation because of the marked difference in receptor spareness for hBRS-3 and the different Bn receptors, limiting their potential utility.

Therefore, the aim of the present study was to attempt to identify a more selective hBRS-3 ligand. Two strategies were used. First, modification of the phenyl ring of the Apa^{11} of the moderately selective ligand [D-Tyr^6,(*R*) or (*S*)Apa^{11},Phe^{13},Nle^{14}]Bn(6-14), and second, replacement of the Apa^{11} with other conformationally restricted amino acids. Using the first approach, we identified a ligand with enhanced selectivity for hBRS-3 that could be useful for exploring its role in various processes.

Materials and Methods

Materials. Balb/c 3T3 and AR42J cells were from American Type Culture Collection (Manassas, VA); Dulbecco's modified Eagle's medium and phosphate-buffered saline were from Biofluids (Rockville, MD); G418 sulfate and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA); iodine-125 (100 mCi/ml), ^{125}I-Bolton-Hunter-labeled CCK-8 (2200 Ci/mmol), ^{125}I-somatostatin-14 (2200 Ci/mmol), ^{125}I-vasoactive intestinal peptide (VIP) (2200 Ci/mmol), ^{125}I-pituitary adenylate cyclase-activating peptide (PACAP) (2200 Ci/mmol), ^{125}I-Bolton-Hunter-labeled substance P (2200 Ci/mmol), and [*N*-methyl-^{3}H]scopolamine methyl chloride (60–85 Ci/mmol) were from Amersham Biosciences Inc. (Piscataway, NJ); myo-(2-^{3}H)inositol (20 Ci/mmol) from PerkinElmer Life and Analytical Sciences (Boston, MA); formic acid, ammonium formate, disodium tetraborate, soybean trypsin inhibitor, and bacitracin were from Sigma-Aldrich (St. Louis, MO); 1,2,4,6-tetrachloro-3α-6α-diphenylglycouril was from Pierce Chemical (Rockford, IL); AG 1-X8 resin was from Bio-Rad (Hercules, CA); and Bn, GRP, NMB, and [Tyr^4]Bn were from Bachem California (Torrence, CA). Standard, protected amino acids and other synthetic reagents were obtained from Bachem Biosciences (King of Prussia, PA). Boc(*R*)-3 amino-3-[4-chlorophenyl] propionic acid (Apa-4Cl), Boc(*R*)-3 amino-3-[3-chlorophenyl]propionic acid (Apa-3Cl), Boc(*R*)-3 amino-3-[2-chlorophenyl]propionic acid (Apa-2Cl), Boc-β-aspartic acid (β-Asp), Boc-β-aspartic acid benzilamide (β-AspBzlAmd)), Boc-2,3-diamino propionic acid (β-Dap), Boc-2-acetylamino, 3-amino propionic acid (*N*α-acetyl-Dap), Boc-2-benzoylamino, and 3-amino propionic acid (*N*α-benzoyl-Dap) (Fig. 1) were purchased from ChiroTech (Cambridge, UK). All chemicals were reagent grade.

Cell Culture. Balb/c 3T3 cells stably expressing human BRS-3 receptors, human NMB receptors, or human GRP receptors were made as described previously (Mantey et al., 1993, 1997; Ryan et al., 1998a) and grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 300 mg/l G418 sulfate. CHO cells stably expressing human vasoactive intestinal peptide receptor subtype 1 (hVIP$_1$-R) and subtype 2 (hVIP$_2$-R) were made as described previously (Ito et al., 2000; Igarashi et al., 2002a,b) and grown in Ham's F-12K media supplemented with 10% FBS and 300 mg/l G418 sulfate. AR42J cells were grown in Dulbecco's modified Eagle's medium

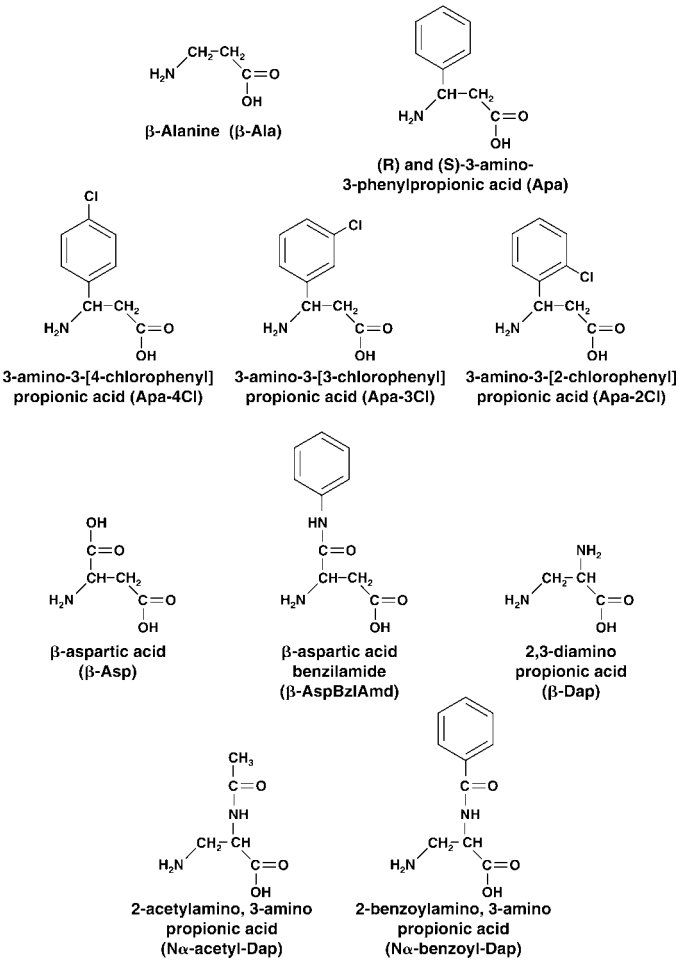

Fig. 1. Structures of various conformationally restricted amino acids used in this study.

supplemented with 10% FBS. All cells were incubated at 37°C in a 5% CO_2 atmosphere.

Preparation of Peptides. Peptides were synthesized with solid-phase methods as described previously (Sasaki and Coy, 1987; Wang et al., 1990; Mantey et al., 2001). Briefly, solid-phase syntheses of peptide amides were carried out using Boc chemistry on methylbenzhydrylamine resin (Advanced ChemTech, Louiville, KY) followed by HF-cleavage of free peptide amides. The crude peptides were purified, and in some cases separated into *R*-isomers and *S*-isomers by preparative high-pressure liquid chromatography on columns (2.5 × 50 cm) of Vydac C18 silica (10 μm), which was eluted with linear gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid. Homogeneity of the peptides was assessed by analytical reverse-phase high-pressure liquid chromatography, and purity was usually 97% or higher. Amino acid analysis (only amino acids with primary amino acid groups were quantitated) gave the expected amino acid ratios. Peptide molecular masses were obtained by matrix-assisted laser desorption mass spectrometry (Lasermat; Thermo Finnigan, San Jose, CA), and all corresponded well with calculated values.

Preparation of ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), ^{125}I-[Tyr^4]Bn, and ^{125}I-[D-Tyr^0]NMB. These radioligands, with specific activities of 2200 Ci/mmol, were prepared as described previously (Mantey et al., 1993, 1997, 2001). Briefly, 0.8 μg of 1,2,4,6-tetrachloro-3α-6α-diphenylglycouril solution (0.02 μg/μl in chloroform) was added to a 5-ml plastic test tube, dried under nitrogen, and washed with 100 μl of 0.5 M potassium phosphate solution (pH 7.4). To this tube 20 μl of potassium phosphate solution (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 water and heated with 300 μl of 1 M dithiothreitol for 60 min at 80°C. I^{125}-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), which does not have a COOH-terminal methionine group, was not incubated with the dithiothreitol. The radiolabeled peptides were separated using a Sep-Pak (Waters, Milford, MA) and further purified by reverse-phase high-performance liquid chromatography, as described previously (Mantey et al., 1993, 1997). The fractions with the highest radioactivity and binding were neutralized with 0.2 M Tris buffer (pH 9.5) and stored with 0.5% bovine serum albumin (w/v) at −20°C.

Binding of ^{125}I-Labeled Peptides to Balb/c 3T3 Cells Transfected with hBRS-3, hGRP-R, or hNMB-R. Binding studies to Balb/c 3T3 cells transfected with human bombesin receptor subtypes were carried out as described previously (Benya et al., 1994, 1995; Mantey et al., 1997, 2001). Briefly, the standard incubation 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 $MgCl_2$, 0.01% (w/v) soybean trypsin inhibitor, 0.2% (w/v) bovine serum albumin, and 0.05% (w/v) bacitracin. Balb/c 3T3 cells transfected with hBRS-3 (0.5 × 10^6/ml), hGRP-R (0.2 × 10^6/ml), or hNMB-R (0.03 × 10^6/ml) were incubated with 50 pM ^{125}I-labeled ligand at 22°C for 60 min. Aliquots (100 μl) were removed and centrifuged through 300 μl of 4°C incubation buffer in 400-μl Microfuge tubes at 10,000*g* for 1 min using a Microfuge B (Beckman Coulter Inc., Fullerton, CA). The pellets were washed twice with buffer and counted for radioactivity in a gamma counter. The nonsaturable binding was the amount of radioactivity associated with cells in incubations containing 50 pM of radioligand (2200 Ci/mmol) and 1 μM unlabeled ligand. Nonsaturable binding was <10% of total binding in all the experiments. Receptor affinities of ligands were determined using a least-square curve-fitting program (LIGAND) (Westendorf and Schonbrunn, 1983) and the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Binding of ^{125}I-Ligands to Dispersed Rat Pancreatic Acini, AR42J Cells, and hVPAC$_1$-R- and hVPAC$_2$-R-transfected CHO Cells. Dispersed rat pancreatic acini (1 pancreas/10 ml), AR42J cells (1 × 10^6/ml), hVPAC$_1$-R transfected into CHO cells (1.5 × 10^6/ml) (Ito et al., 2000; Igarashi et al., 2002a,b), and hVPAC$_2$-R transfected into CHO cells (0.2 × 10^6/ml) (Ito et al., 2000; Igarashi et al., 2002a,b) were suspended in standard incubation solution with 0.05% (w/v) bacitracin. Dispersed acini from rat pancreas were prepared using collagenase digestion as described previously (Jensen et al., 1982; Garcia et al., 1997). Incubations with ^{125}I-somatostatin and ^{125}I-BH-substance P contained phosphoramidon (10 mM), leupeptin (25 μg/ml), 4-(2-aminoethyl)-benzenesulfonyl fluoride (10 μM), and amastatin (10 mM). Incubations with the other ligands (^{125}I-BH-CCK-8 and ^{125}I-PACAP) were at 37°C for 60 min with rat pancreatic acini and 23°C with AR42J cells or hVPAC-R-transfected cells. Binding was performed as described previously (Mantey et al., 1993, 1997, 2001; Ito et al., 2001; Igarashi et al., 2002a,b). Briefly, 50 pM radioligand was incubated with the cells alone or with 1 μM unlabeled other peptides at the specified concentrations or with 1 μM unlabeled ligand to determine saturable binding. Bound ligand was

TABLE 1

Affinities of bombesin, neuromedin B, and various synthetic bombesin analogs with position 11 conformationally restricted substitutions for human BRS-3, GRP, and NMB receptors

Data are calculated from dose-inhibition curves in Figs 2 to 4. The affinities of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), Bn, and NMB were calculated by a least-squares curve-fitting program (LIGAND). The remaining affinities were calculated using the Cheng-Prusoff equation. All values are means ± S.E.M. from at least three experiments. The structures of these conformationally restricted analogs are shown in Fig. 1.

Peptide No.	Peptide Structure	K_i		
		hBRS-3/Balb/c 3T3 Cells	hGRP-R/Balb/c 3T3 Cells	hNMB-R/Balb/c 3T3 Cells
			nM	
1	[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14)	0.82 ± 0.1	0.5 ± 0.04	5.9 ± 0.4
2	[D-Tyr^6,(*R*)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14)	2.8 ± 0.1	151 ± 6	2400 ± 96
3	[D-Tyr^6,(*S*)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14)	8.2 ± 1.1	110 ± 5	4370 ± 150
4	[D-Tyr^6,(*S*)-Apa^{11}-2Cl,Phe^{13},Nle^{14}]Bn(6-14)	85 ± 7	398 ± 28	5890 ± 385
5	[D-Tyr^6,(*R*)-Apa^{11}-2Cl,Phe^{13},Nle^{14}]Bn(6-14)	24 ± 1	229 ± 28	6920 ± 350
6	[D-Tyr^6,(*S*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14)	776 ± 134	741 ± 85	1950 ± 337
7	[D-Tyr^6,(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14)	8.2 ± 1.1	1860 ± 69	7200 ± 840
8	[D-Tyr^6,(*S*)-Apa^{11}-3Cl,Phe^{13},Nle^{14}]Bn(6-14)	15.8 ± 1.1	110 ± 17	831 ± 192
9	[D-Tyr^6,(*R*)-Apa^{11}-3Cl,Phe^{13},Nle^{14}]Bn(6-14)	107 ± 4	2510 ± 290	>10,000
10	[D-Tyr^6,β-Asp^{11},Phe^{13},Nle^{14}]Bn(6-14)	490 ± 50	396 ± 44	427 ± 54
11	[D-Tyr^6,β-Asp(BzlAmd)^{11},Phe^{13},Nle^{14}]Bn(6-14)	708 ± 61	397 ± 51	646 ± 59
12	[D-Tyr^6,β-Dap^{11},Phe^{13},Nle^{14}]Bn(6-14)	2.3 ± 0.1	1.0 ± 0.1	43 ± 5
13	[D-Tyr^6,Nα-acetyl-Dap^{11},Phe^{13},Nle^{14}]Bn(6-14)	15.8 ± 2.9	1.7 ± 0.2	89 ± 4
14	[D-Tyr^6,Nα-benzoyl-Dap^{11},Phe^{13},Nle^{14}]Bn(6-14)	12.3 ± 1.3	3.1 ± 0.5	55 ± 3
15	[D-Tyr^6,β-Ala^{11},Phe^{13},β-Dap(Pr)^{14}]Bn(6-14)	2400 ± 138	85 ± 17	>10,000
Bn	Bombesin	>10,000	0.36 ± 0.07	22 ± 0.9
NMB	Neuromedin B	>10,000	97 ± 11	0.55 ± 0.03

separated from free ligand by centrifugation. Tubes were washed twice with 2% (w/v) bovine serum albumin in standard incubation solution and radioactivity was counted. Nonsaturable binding was less than 30% of total binding.

Measurement of [^{3}H]IP. Changes in total [^{3}H]inositol phosphates ([^{3}H]IP) were measured as described previously (Benya et al., 1992, 1994; Ryan et al., 1998a; Mantey et al., 2001). Briefly, hBRS-3, hGRP-R-, or hNMB-R-transfected Balb/c 3T3 cells were subcultured into 24-well plates (5×10^4 cells/well) in regular propagation media and then incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The cells were then incubated with 3 μCi/ml of myo-[2-^{2}H]inositol in growth media supplemented with 2% FBS for an additional 24 h. Before assay, the 24-well plates were washed by incubating for 30 min at 37°C with 1 ml/well of phosphate-buffered saline (pH 7.0) containing 20 mM lithium chloride. The wash buffer was aspirated and replaced with 500 μl of inositol phosphate assay buffer containing 135 mM sodium chloride, 20 mM HEPES (pH 7.4), 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 20 mM LiCl, 11.1 mM glucose, and 0.05% bovine serum albumin (w/v) and incubated with or without any of the peptides studied. After 60 min of incubation at 37°C, the experiments were terminated by the addition of 1 ml of ice-cold 1% (v/v) hydrochloric acid in methanol. Total [^{3}H]IP was isolated by anion exchange chromatography as described previously (Benya et al., 1992, 1994; Ryan et al., 1998a; Mantey et al., 2001). Briefly, samples were loaded onto Dowex AG1-X8 anion exchange resin columns, washed with 5 ml of distilled water to remove free [^{3}H]inositol, and then washed with 2 ml of 5 mM disodium tetraborate/60 mM sodium formate solution to remove [^{3}H]glycerophosphorylinositol. Two milliliters of 1 mM ammonium formate/100 mM formic acid solution was added to the columns to elute total [^{3}H]IP. Each eluate was mixed with scintillation cocktail and measured for radioactivity in a scintillation counter.

Results

In this study, we sought to develop hBRS-3 ligands with greater selectivity for the hBRS-3 receptor using, as our reference peptide, [D-Tyr^6,(*R*)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 2). To accomplish this, we synthesized 11 peptides by making alterations primarily at position 11. These studies were performed only in human bombesin receptors (hBRS-3, hGRP-R, and hNMB-R) because recent studies demonstrate that rat and mouse BRS-3 receptors (Liu et al., 2002), but not avian (Iwabuchi et al., 2003), have low affinity for β-Ala^{11} analogs of bombesin. At present, no selective or high-affinity ligands have been identified for the rat or mouse BRS-3 receptor. We first examined six analogs of [D-Tyr^6,Apa^{11},Phe^{13},Nle^{14}]Bn(6-14) with various position 11 chloro-substitutions (Table 1, peptides 4–9; Fig. 2). This strategy was performed because previous studies in a number of G protein-coupled receptors demonstrated that insertions of electron-withdrawing groups, such as chloro groups in aromatic rings in critical residues of various ligands, can have important steric or electronic effects resulting in significant changes in receptor affinity or selectivity (Jensen et al., 1985; Li et al., 1998; Perrone et al., 1998; Ananthan et al., 2003). As was seen in previous studies (Pradhan et al., 1998; Katsuno et al., 1999) [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 1) bound to all three human Bn receptor subtypes with high affinity (Fig. 2; Table 1). The replacement of the β-Ala at position 11 by chloro-substituted 3-amino-3-phenylpropionic acid moieties (Fig. 2; Table 2, peptides 4–9) resulted in peptides that when compared with peptide 1, had from 10- to 800-fold less affinity for the hBRS-3 but in general had increased selectivity for the hBRS-3 because they

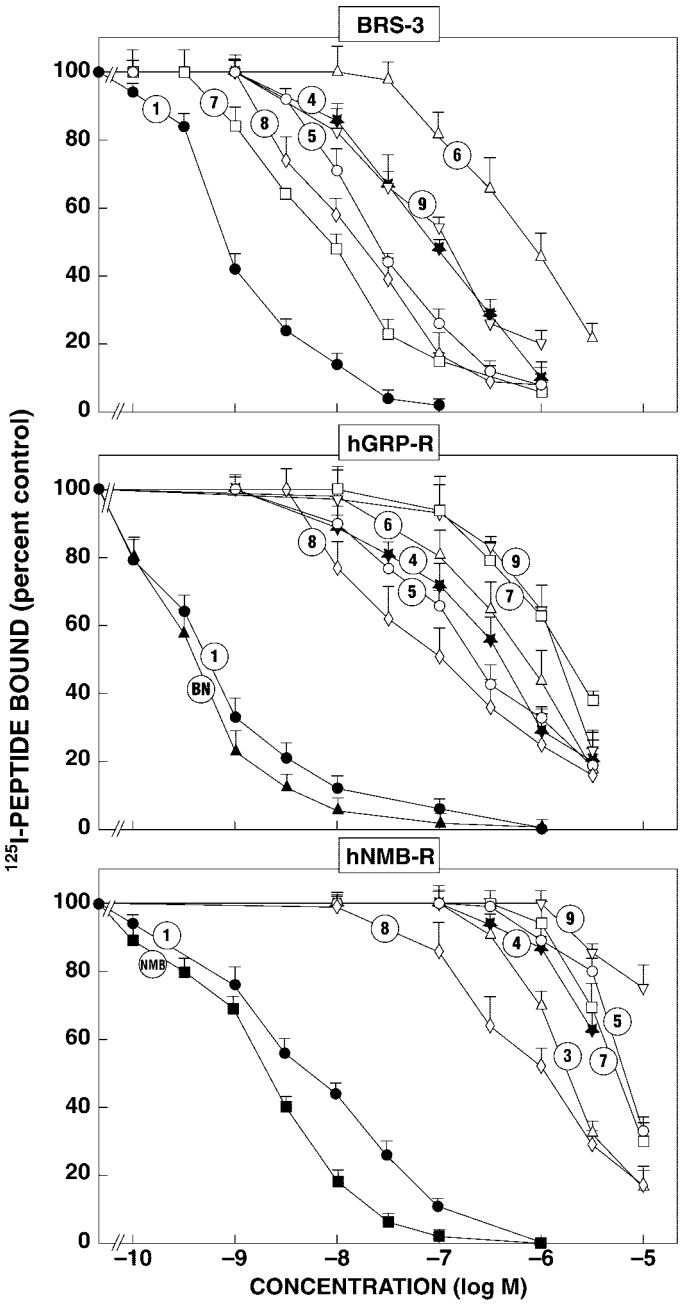

Fig. 2. Ability of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) and various analogs of D-Tyr^6,Apa^{11},Phe^{13},Nle^{14}]Bn(6-14) with position 11 chloro-substitutions to inhibit binding at the hBRS-3 (top), hGRP-R (middle), or hNMB-R (bottom). Balb/c 3T3 cells stably transfected with hBRS-3 (0.5×10^6 cells/ml), hGRP-R (0.3×10^6 cell/ml), or hNMB-R (0.03×10^6 cells/ml) were incubated for 60 min at 22°C with 50 pM I^{125}-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), ^{125}I-[Tyr^4]Bn, or ^{125}I-[D-Tyr^0]NMB, respectively, with or without the indicated concentrations of the various peptides added. Results are expressed as the percentage of saturable binding without unlabeled peptide added (percentage of control). Results are the mean ± S.E.M. from at least three experiments, and in each experiment the data points were determined in duplicate. Numbers refer to the peptide number in Table 1. For description of abbreviations, see Fig. 1 and abbreviations list.

became even less potent at the hGRP-R and the hNMB-R (Fig. 2; Table 1). In this group of six peptides, there were three sets of *R*- and *S*-optical isomers (Table 1, peptides 4 and 5, 6 and 7, 8 and 9; Fig. 2). The *R*-isomers showed more selectivity for hBRS-3 over hGRP-R and hNMB-R than the *S*-isomers. Except for [D-Tyr^6,(*S*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14)

(Table 1, peptide 6), which was equipotent for hBRS-3 and hGRP-R, these peptides had from 5- to 800-fold increase in selectivity for the hBRS-3 over hGRP-R and from 3- to 900-fold increase in selectivity for hBRS-3 over hNMB-R (Fig. 2; Table 1) compared with the original β-Ala^{11} analog (Table 1, peptide 1). The peptide that showed the highest selectivity for hBRS-3 over both hGRP-R and hNMB-R was [D-Tyr^{6},(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 7), which, when compared with the original β-Ala^{11} peptide (Table 1, peptide 1), was 10 times less potent for hBRS-3 (K_i = 8.2 ± 1.1 versus 0.82 ± 0.1 nM); however, it was 227- and 878-fold more selective for hBRS-3 than hGRP-R and hNMB-R, respectively (Fig. 2; Table 1). A 3-chloro-substitution on the (*R*)-Apa^{11} group yielding [D-Tyr^{6},(*R*)-Apa^{11}-3Cl,Phe^{13},Nle^{14}]Bn(6-14) resulted in a peptide (Fig. 2; Table 2, peptide 9) that was very selective for hBRS-3 but is unlikely to be useful, because of its low affinity for hBRS-3 (K_i = 107 ± 4 nM).

Previous studies have shown that the substitution at position 14 can have a pronounced effect on a peptide ligand's affinity for GRP-R and NMB-R, in addition to the position 11 substitution (Saeed et al., 1989; Wang et al., 1990; Lin et al., 1996; Mantey et al., 1997, 2001). We therefore made six peptides with substitutions of different conformationally restricted amino acids of different sizes and polarity at positions 11 or 14 (Table 1; Fig. 3, peptides 10–15). This group of peptides consisted of a 2,3-diaminopropionic acid-proline substitution at position 14 (peptide 15) and five different substitutions at the β-Ala in position 11 of [D-Tyr^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) (Table 1; Fig. 3, peptides 9–14). These alterations produced peptides with a 3- to 3000-fold decrease in affinity for the hBRS-3 receptor compared with the prototype β-Ala^{11} peptide (Table 1, peptide 1) and none demonstrated increased selectivity for hBRS-3 over hGRP-R and hNMB-R. In fact, in general these peptides became less selective for hBRS-3 and more selective for hGRP-R. Specifically, peptides 10 and 11 (Table 1; Fig. 3), which have a –COOH and benzylamide substitution, respectively, added at the β position of β-Ala^{11} (Fig. 1), showed a decrease in affinity (60–75-fold) to all the Bn receptor subtypes (Table 1; Fig. 3). The three peptides in this group with substitution at the α position of the β-Ala^{11} (Table 1, peptides 12–14; Figs. 1 and 3) had a 3- to 18-fold decrease in affinity (K_i = 2.3 ± 0.1, 15.8 ± 2.9, and 12.3 ± 1.3 nM, respectively) for the hBRS-3 receptor but retained high affinity for hGRP-R (K_i = 1.0 ± 0.1–3.1 ± 0.5 nM), making them hGRP-R-selective. The insertion of a substituted Dap moiety in position 14 (Table 1, peptide 15; Fig. 3) resulted in a peptide that had >20-fold selectivity for hGRP-R over hBRS-3 or hNMB-R.

Bn and NMB have very low affinities (K_i > 10,000) for the hBRS-3 (Table 1) compared with the ability of these peptides to interact with hGRP-R (Fig. 3, middle; Table 1) or with the hNMB-R (Fig. 3, bottom; Table 1). These results are consistent with results from previous studies (Fathi et al., 1993; Wu et al., 1996; Mantey et al., 1997, 2001; Pradhan et al., 1998; Ryan et al., 1998a) that show the hBRS-3 receptor is unique among the mammalian Bn receptor family because it interacts with all the naturally known Bn-related peptides with very low affinity.

In previous studies, it was reported that the nonselective prototype ligand [D-Tyr^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 1), and the more hBRS-3-selective ligand [D-Tyr^{6},(*R*)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 2), specifically interact with the Bn family of receptors (Mantey et al., 1997, 2001). To determine whether the addition of a 4-chloro group to the Apa^{11} made the newly discovered hBRS-3-selective peptide [D-Tyr^{6},(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 7) less selective for the Bn group of receptors, we investigated its ability to interact with a number of other receptors involved with the actions of gastrointestinal hormone/neurotransmitters (Table 2). Similar to the nonselective Bn analog peptide 1, the hBRS-3-selective analog peptide 7, at a concentration that completely inhibited binding to the hBRS-3 receptor (i.e., 1 μM) (Fig. 2), did not inhibit binding to receptors for cholecystokinin, gastrin, PACAP, VIP ($VPAC_1$-R and $VPAC_2$-R), somatostatin, muscarinic cholinergic agents, or substance P (Table 2). This lack of activity at these receptors was not due to the fact that either Bn analog is inactivated, because the assays were performed under the same conditions used to assess binding to Bn receptors, and in the different assays using the same cells, both peptides 1 and 7 inhibited binding to GRP receptors on rat pancreatic acini. These results demonstrate that the newly discovered selective peptide, peptide 7, has high selectivity for the Bn family of receptors.

The comparative abilities of the nonselective hBRS-3 li-

TABLE 2

Ability of hBRS-3-selective analogs [D-Tyr^{6},(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14) (peptide 7) and the nonselective ligand [D-Tyr^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) (peptide 1) to interact with various gastrointestinal hormone/neurotransmitter receptors

AR42J cells (1×10^6 cells/ml), dispersed rat acini (1 pancreas/10 ml), and CHO cells that were stably transfected with $hVPAC_1$-R (0.2×10^6 cells/ml) or $hVPAC_2$-R (3.0×10^6 cells/ml), were incubated with 50 pM of the indicated ^{125}I-ligand or 0.6 nM of [*N*-methyl-^{3}H]scopolamine methyl chloride for 60 min as specified under *Materials and Methods*. Results are the percentage of the total saturable binding with or without 1 μM peptide 1 or peptide 7 (Table 1). All results are the means ± S.E.M. from at least four experiments, and in each experiment each value was performed in duplicate.

Cells Used	Ligand Added	^{125}I-Ligand Saturably Bound	
		1 μM Peptide 1	1 μM Peptide 7
		% Control	
AR42J	^{125}I-PACAP-27	104 ± 2	101 ± 3
	^{125}I-BH-CCK-8	109 ± 7	94 ± 1
	^{125}I-Somatostatin-14	83 ± 5	98 ± 6
	^{125}I-BH-Substance P	100 ± 12	104 ± 7
	^{125}I-[Tyr^{4}]Bn	0*	50 ± 3*
Stably transfected CHO cells			
$hVPAC_1$-R	^{125}I-VIP	97 ± 2	96 ± 4
$hVPAC_2$-R	^{125}I-PACAP	109 ± 5	91 ± 5
Rat pancreatic acini	^{125}I-BH-CCK-8	95 ± 6	102 ± 3
	[^{3}H]Methyl scopolamine	100 ± 7	113 ± 7

h, human; VPAC, vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide receptor.
*$P < 0.01$ compared with no additions (i.e., control) (paired Student's *t* test).

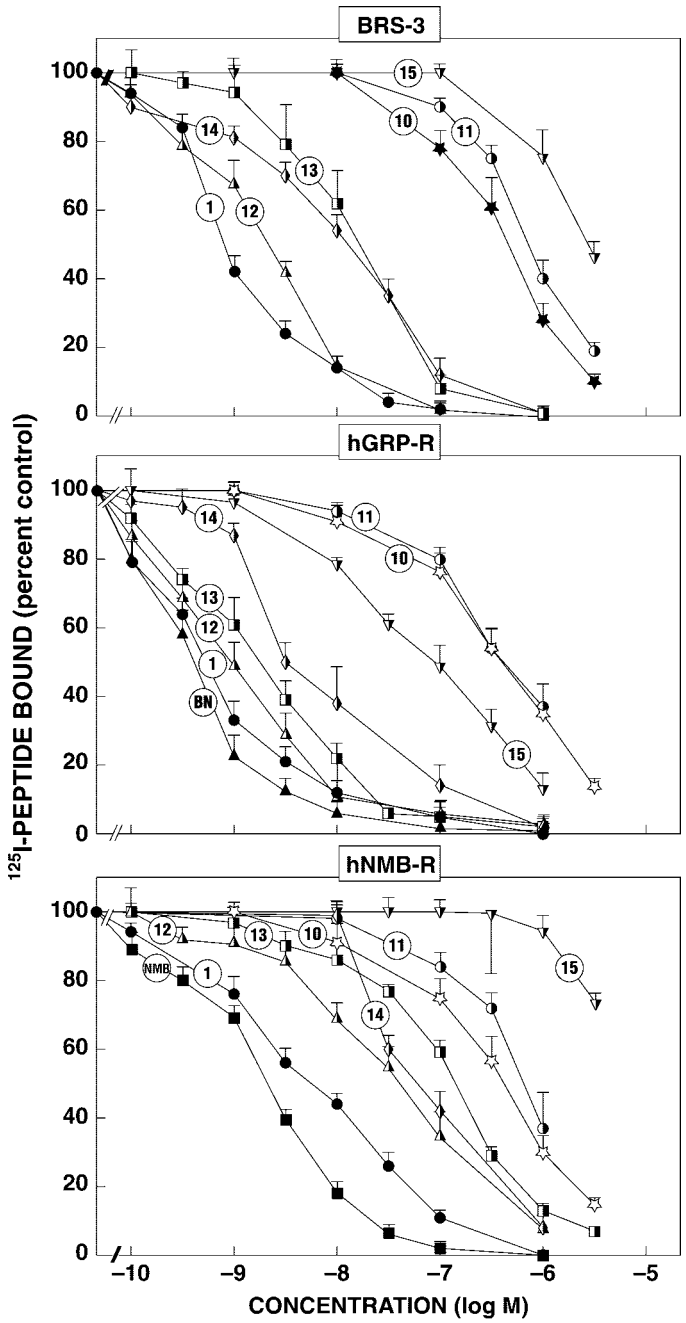

Fig. 3. Ability of Bn, NMB, and analogs of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) with various position 11 or 14 conformationally restrictive substitutions to inhibit binding at hBRS-3 (top), hGRP-R (middle), or hNMB-R (bottom). The experimental conditions were similar to those outlined in the Fig. 2 legend. The results are expressed as the percentage of saturable binding without unlabeled peptide added (percentage of control) and are means ± S.E.M. from at least three experiments with each data point determined in duplicate. Numbers refer to peptide numbers in Fig. 1 and legend of Table 1. For description of abbreviations, see Fig. 1 and abbreviations list.

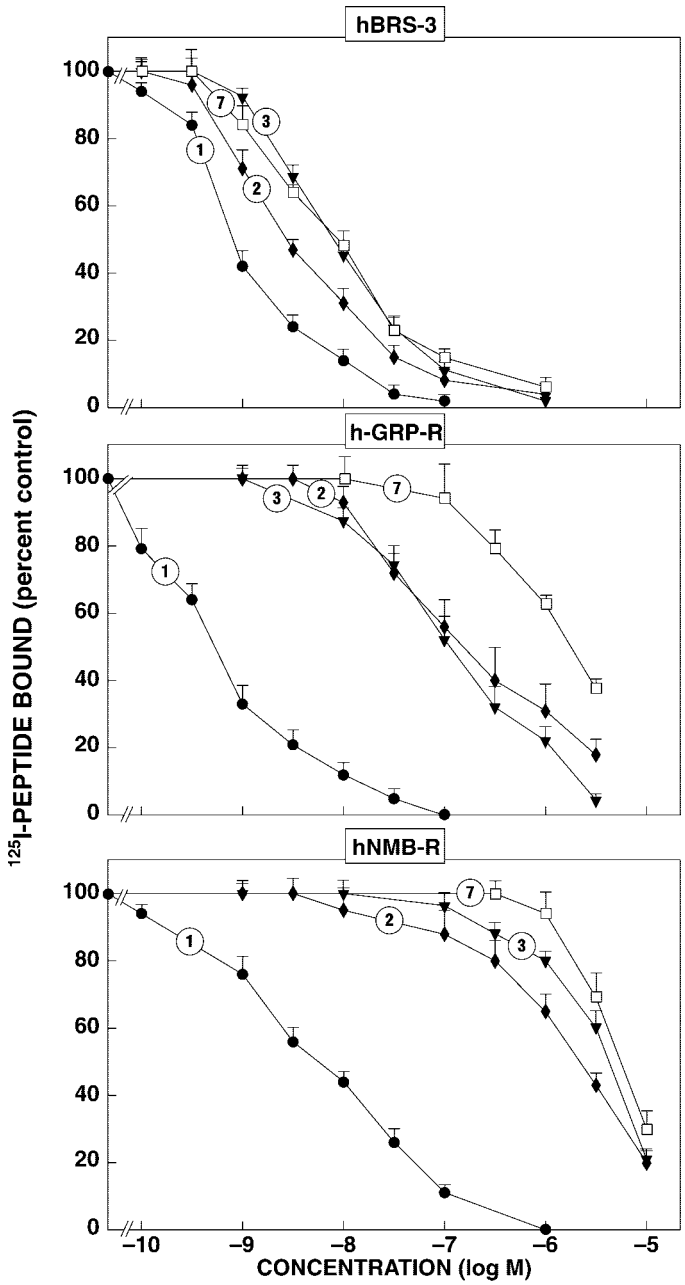

Fig. 4. Comparison of the ability of [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) and various hBRS-3-selective ligands to inhibit binding to hBRS-3 (top), hGRP-R (middle), or hNMB-R (bottom). The experimental conditions are the same as outlined in the legend to Fig. 2. The results are expressed as the percentage of saturable binding without unlabeled peptide added (percentage of control). Results are the means ± S.E.M. of at least three experiments and in each experiment the data points were determined in duplicate. Numbers refer to peptide numbers in Table 1. For description of abbreviations, see legend of Table 1 and Fig. 1. Peptide 2 (Table 1) and peptide 3 (Table 1) were reported to be hBRS-3-selective ligands (Mantey et al., 2001).

gand [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), the two hBRS-3-selective ligands from our previous study (Mantey et al., 2001) (Table 1, peptides 2 and 3), and the most selective hBRS-3 ligand found in the present study (Table 1, peptide 7) to interact with the three mammalian Bn receptor subtypes are shown in Fig. 4 and Table 1. Each of the three selective peptides retained high affinity for hBRS-3 receptor (nanomolar range), but were from 3- to 10-fold less potent than the nonselective prototype peptide (Table 1, peptide 1). However, each of these three hBRS-3 peptides became more hBRS-3-selective because their affinities for hGRP-R and hNMB-R were diminished substantially compared with peptide 1, the prototype peptide (Table 1; Fig. 4). Specifically, similar to what has been reported previously (Mantey et al., 2001), the *R*-isomer of Apa^{11}, [D-Tyr^6,(*R*)Apa^{11},Phe^{13},Nle^{14}]Bn(6-14)] (Table 1, peptide 2) was more hBRS-3 receptor-selective than

the *S*-isomer [D-Tyr^6,(*S*)Apa^{11},Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 3). The 4-chloro-substitution on the (*R*)-Apa^{11} (Table 1, peptide 7; Fig. 4) yielded a peptide that was significantly more selective for hBRS-3 over hGRP-R (227-fold over hGRP-R) compared with the hBRS-3 selectivity of peptide 2 described in a previous study (Mantey et al., 2001) (54-fold over hGRP-R). Both peptide 7 and peptide 2 (Table 2) were more than 800-fold selective for hBRS-3 over hNMB-R (Table 1; Fig. 4)

Activation of hBRS-3, hGRP-R, and hNMB-R results in the stimulation of phospholipase C activity with increases in inositol phosphates (Benya et al., 1992, 1994, 1995; Mantey et al., 2001). To assess whether the newly discovered hBRS-3 receptor-selective peptide [D-Tyr^6,(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14) (Table 1, peptide 7) is an agonist at this receptor and whether its selectivity in binding to hBRS-3 is mirrored in its ability to activate the receptor, we determined its ability compared with those of peptides 1, 2, 3, Bn, and NMB to stimulate [^{3}H]IP release in cells transfected with hBRS-3, hGRP-R, and hNMB-R (Table 3; Fig. 5). All peptides stimulated increases in [^{3}H]IP in a concentration-dependent manner at each of the three Bn receptor subtypes (Fig. 5). At a concentration of up to 10 μM, each peptide functioned as a full agonist (Fig. 5). The nonselective peptide (Table 1, peptide 1) [D-Tyr^6,β-Ala^{11},Phe^{13},Nle14]Bn(6-14) stimulated increases in [^{3}H]IP in hBRS-3, hGRP-R, and hNMB-R with high potencies (EC_{50} = 0.17–1 nM) (Table 3; Fig. 5). Both Bn and NMB had very low potencies for stimulation at the hBRS-3. NMB had a high potency for activating hNMB-R (EC_{50} = 1.2 ± 0.1) but lower potency for the hGRP-R (EC_{50} = 530 ± 23), whereas Bn had a high potency for stimulating both hGRP-R and hNMB-R (0.4 ± 0.06 and 2.5 ± 0.1, respectively). Similar to previous results (Mantey et al., 2001), peptides 2 and 3, which are Apa^{11}-substituted compounds (Table 1), had ≤6-fold selectivity for activating hBRS-3 over hGRP-R and hNMB-R. The most selective hBRS-3 receptor ligand identified in the present study, peptide 7, [D-Tyr^6,(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14), had >20-fold selectivity for activating the hBRS-3 receptor over hGRP-R or hNMB-R. Therefore, peptide 7 was 10- and 4-fold more selective for activating the hBRS-3 receptor over the hGRP-R and hNMB-R, respectively, than peptide 2, [D-Tyr^6,(*R*)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14), the most hBRS-3-selective peptide described previously (Mantey et al., 2001).

Discussion

In previous studies (Mantey et al., 1997; Ryan et al., 1998a,b), we reported the discovery of a synthetic analog of Bn, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), which functioned as a potent agonist for the human G protein-coupled orphan receptor BRS-3, a receptor that is structurally related to mammalian Bn receptors (Fathi et al., 1993; Gorbulev et al., 1994). In subsequent studies (Pradhan et al., 1998; Reubi et al., 2002), this Bn analog was also found to have the unique property of having high affinity for all known mammalian Bn receptors (i.e., GRP-R and NMB-R), as well as the amphibian Bn receptor bombesin receptor subtype 4 (Nagalla et al., 1995; Katsuno et al., 1999), except for the BRS-3 receptor in rat and mouse (Liu et al., 2002). Whereas this finding made this Bn analog valuable as a universal ligand for identifying Bn receptor subtypes (Pradhan et al., 1998; Reubi et al., 2002), its lack of selectivity for the BRS-3 receptor decreased its usefulness for investigating the role of hBRS-3 only in physiological or pathological processes. In previous studies, none of the naturally occurring Bn-related peptides, nor more than 30 synthetic Bn analogs interacted with high affinity or selectivity with the hBRS-3 receptor (Fathi et al., 1993; Wu et al., 1996; Mantey et al., 1997, 2001; Ryan et al., 1998a,b; Katsuno et al., 1999; Liu et al., 2002). However, in a recent study (Mantey et al., 2001) investigating replacement of β-Ala^{11} of the nonselective high-affinity hBRS-3 ligand, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), with conformationally restricted amino acids, two peptides with an (*R*)- or (*S*)-Apa substitution for β-Ala^{11} were reported with some selectivity for the hBRS-3 receptor. However, this selectivity was relatively modest for their differential potencies for receptor activation of hBRS and the Bn receptors hGRP-R and hNMB-R, because of different degrees of receptor spareness in these different receptors (Mantey et al., 2001). Specifically in the present study, the (*R*)-Apa^{11} and (*S*)-Apa^{11} analogs had 50- and 13-fold selectivity for the hBRS-3 over the hGRP-R or hNMB-R by binding studies, but only a 7- and 3-fold selectivity in potency for activating hBRS-3 over the hGRP-R and

TABLE 3

Potency of bombesin, neuromedin B, and various hBRS-3-selective ligands for stimulating phospholipase C activity in hBRS-3, hGRP-R, and hNMB-R-containing cells

Balb/c 3T3 cells stably transfected with hBRS-3, hGRP-R, or hNMB-R were incubated with [^{3}H]inositol and total [^{3}H]IP determined as described under *Materials and Methods*. For each peptide, a dose-response curve was performed with concentrations from 0.01 nM to 1 μM (Fig. 5). Results were expressed as the concentration causing one-half the maximal increase, EC_{50}, seen with 1 μM peptide calculated from the dose-response curves in Fig. 5 using the curve-fitting program KaleidaGraph. Each value is a mean ± S.E.M. from at least three experiments. For hBRS-3/Balb/c 3T3 cells, the control and 1 μM [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) values were 10,624 ± 571 dpm and 37,660 ± 4106 dpm, respectively. For hGRP-R/Balb/c 3T3 cells, the control and 1 μM [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) values were 9231 ± 2260 dpm and 43,060 ± 9137 dpm, respectively. With hNMB-R/Balb/c 3T3 cells, the control and 1 μM [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) values were 2020 ± 166 dpm and 40,667 ± 2371 dpm, respectively.

Peptide No.	Peptide Structure	EC_{50}		
		hBRS-3/Balb/c 3T3 Cells	hGRP-R/Balb/c 3T3 Cells	hNMB-R/Balb/c 3T3 Cells
			nM	
1	[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14)	1.4 ± 0.1	0.18 ± 0.01	0.17 ± 0.01
2	[D-Tyr^6,(*R*)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14)	6.0 ± 0.2	53.7 ± 0.8	38.0 ± 0.3
3	[D-Tyr^6,(*S*)-Apa^{11},Phe^{13},Nle^{14}]Bn(6-14)	7.1 ± 0.8	56.2 ± 1.8	21.9 ± 0.5
7	[D-Tyr^6,(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14)	3.5 ± 0.2	320 ± 20	71.0 ± 1.9
Bn	Bombesin	>10,000	0.45 ± 0.06	2.5 ± 0.1
NMB	Neuromedin B	>10,000	530 ± 23	1.2 ± 0.1

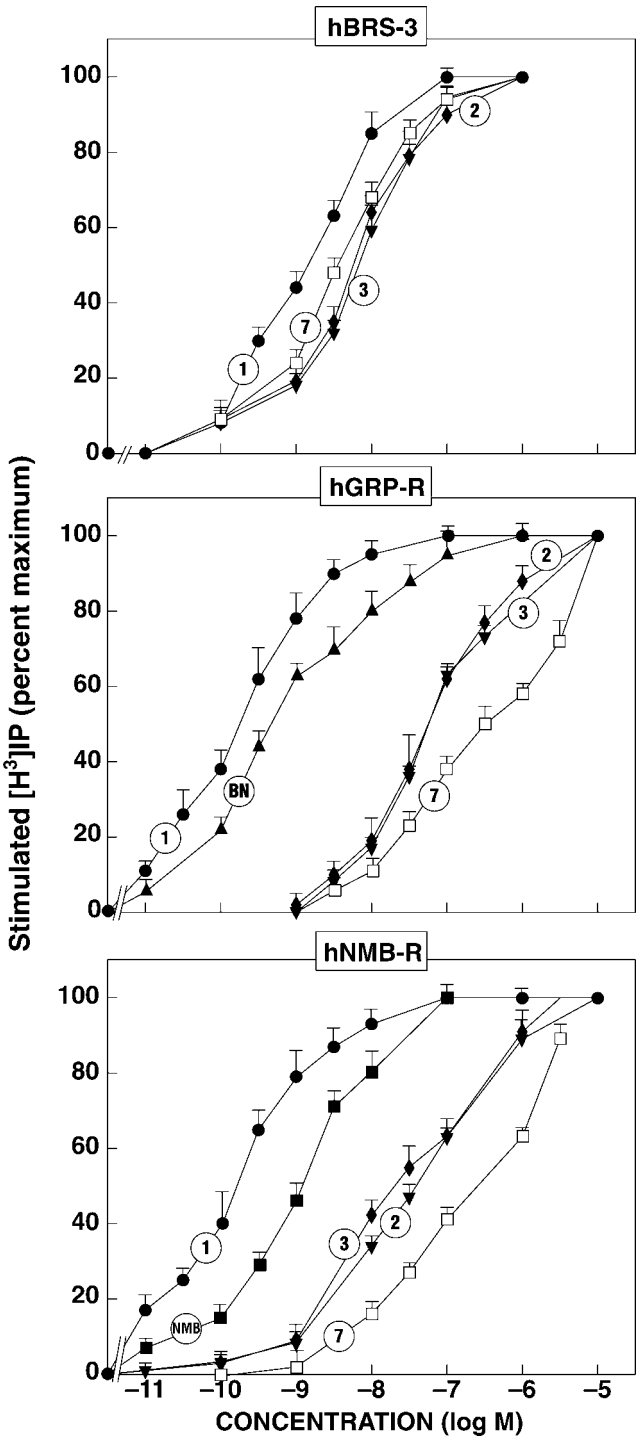

Fig. 5. Ability of NMB, Bn, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), or various BRS-3-selective ligands to stimulate [^{3}H]IP formation in Balb/c 3T3 cells transfected with hBRS-3 (top), hGRP-R (middle), or hNMB-R (bottom). Balb/c 3T3 cells transfected with hBRS-3, hGRP-R, or hNMB-R were subcultured and preincubated for 24 h at 37°C with 3 μCi/ml myo-[2-^{3}H]inositol. The cells were then incubated with NMB, GRP, [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), or the BRS-3-selective ligands at the concentrations indicated for 60 min at 37°C. Values expressed are a percentage of total [^{3}H]IP release stimulated by 1 μM [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) and are mean ± S.E.M. from at least three experiments. The values for basal (control) and 1 μM [D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) stimulated accumulation of [^{3}H]IP were as follows: hBRS-3/Balb/c 3T3 cells, 9624 ± 807 and 39,659 ± 5806 dpm, respectively; hGRP-R/Balb/c 3T3 cells, 6392 ± 536 and 29,800 ± 4789 dpm, respectively; and hNMB-R/Balb/c 3T3 cells, 2020 ± 166 and 40,667 ± 2371 dpm, respectively.

hNMB-R, and for stimulating phospholipase C activity. The aim of the present study was to attempt to identify additional synthetic Bn analogs with enhanced hBRS-3 selectivity.

Two strategies were used to attempt to identify more selective hBRS-3 receptor ligands. The first strategy involved inserting additional substitutions on the phenyl ring of Apa in position 11 of the *R*- and *S*-isomers of the selective hBRS ligand [D-Tyr^6,Apa^{11},Phe^{13},Nle^{14}]Bn(6-14), which might change either its steric or electronic properties. This strategy was taken because molecular modeling studies (Mantey et al., 2001) using a β-bend template, a conformation that has been proposed for the Val^{10}-Gly^{11}-His^{12}-Phe^{13} COOH terminal region of Bn (Coy et al., 1991; Mantey et al., 2001), demonstrated these two hBRS-3-selective Bn analogs had a unique orientation of the position 11 β-amino acid that likely contributed to their selectivity for BRS-3. Therefore, further alteration of the conformation might enhance hBRS-3 selectivity. The second approach was to substitute additional conformationally restricted amino acids in the place of Apa at primarily position 11. Because of the previous molecular modeling results (Mantey et al., 2001), which suggested the importance of orientation of the backbone substitution of the conformationally restricted amino acid in position 11, suggesting steric factors were important in determining hBRS-3 selectivity, chloro substitutions were made on 2, 3, and 4 positions of the phenyl ring of Apa^{11} in the *R*- and *S*-isomers. Previous studies on ligands for the CCK receptor (Jensen et al., 1985), A3 adenosine receptor (Li et al., 1998), D4 dopamine receptor (Perrone et al., 1998), and opioid receptors (Ananthan et al., 2003) have demonstrated that insertion of electron-withdrawing groups such as chloro substitutions in the phenyl ring can have important effects on receptor selectivity and affinity by either altering steric or electronic properties of the ligand. Our results demonstrate that the insertion of a chloro group in the ortho, meta, or para position of the phenyl ring in relation to the Apa group had a markedly different effect on the Bn receptor selectivity. Furthermore, the effect of the chloro insertion in the different phenyl ring positions had markedly differing effects on the absolute and relative potencies of Apa^{11} Bn analog containing either the (*R*)- or (*S*)-Apa isomer. The latter result might be expected from the molecular modeling result (Mantey et al., 2001) reported earlier if the insertion of the chloro group was having primarily a steric effect, because in these models the (*R*)- and (*S*)-Apa^{11} analogs had a unique conformation that differed from the other constrained position 11 analogs tested; however, their conformations were not identical; therefore, insertion of a steric group in the different phenyl ring position could have a differing effect. Of the different chloro Apa^{11} analogs tested, the insertion of the chloro in the para position of the phenyl ring in the (*R*)-Apa^{11} analog resulted in an analog that had significantly improved selectivity for hBRS-3 and that retained sufficiently high affinity for hBRS-3 to be likely useful to explore these receptors' roles in various processes. Specifically, [D-Tyr^6,(*R*)-Apa^{11}-4Cl,Phe^{13},Nle^{14}]Bn(6-14) had a 227- and 880-fold selectivity for hBRS-3 over hGRP-R and hNMB-R by binding studies, and a 90- and 20-fold greater potency for activating the hBRS-3, respectively. Therefore, the four chloro Apa^{11} analog has 20- and 5-fold greater

selectivity for the hBRS-3 over the hGRP-R by binding studies than the previously reported selective (*S*)- and (*R*)-Apa[11] analogs (Mantey et al., 2001). Furthermore, in terms of relative potencies for activating hBRS-3 and the two Bn receptor subtypes, the 4 chloro (*R*)-Apa[11] analog had 10- and 4-fold greater selectivity than the previously reported selective (*S*)- and (*R*)-Apa[11] analogs (Mantey et al., 2001). In a previous study (Mantey et al., 2001), replacement of the phenyl ring of the Apa[11] analog with an (*R*)-4 chlorobenzyl group resulted in a Bn analog (Acpb4, compound VIII; Mantey et al., 2001) with a 100-fold less affinity for hBRS-3 and almost no selectivity for hBRS-3 over hGRP-R (3-fold) or hNMB-R (9-fold) (Mantey et al., 2001). These findings, in comparison with the present results with the 4 chloro-phenyl substitution in Ala[11] resulting in retained high affinity for hBRS-3 and increased selectivity, demonstrate the importance of the size of the β substitution in the aminopropionic acid in position 11 for determining both affinity and selectivity.

Whereas the first strategy of insertion of chloro groups on the Apa[11] phenyl ring of [D-Tyr[6],(*R*)-Apa[11],Phe[13],Nle[14]]Bn(6-14) resulted in one potentially useful compound with enhanced BRS-3 selectivity, the second strategy of substitution of other conformationally restricted amino acids for Apa[11] or in position 14 for Nle, did not result in any Bn analogs with enhanced hBRS-3 selectivity. The results using this strategy did, however, provide some interesting insights into the importance of backbone substitutions on the aminopropionic acid moiety in the 11 position of the [D-Tyr[6],β-Ala[11],Phe[13],Nle[14]]Bn(6-14) that are important in determining receptor selectivity and/or affinity. In a previous study (Mantey et al., 2001), the effect of various aliphatic and aromatic substitutions at the α and β position of aminopropionic acid in [D-Tyr[6],Apa[11],Phe[13],Nle[14]]Bn(6-14) were investigated and shown to have a marked effect on affinity and selectivity for hBRS-3, hGRP-R, and hNMB-R. In the present study, the effect of forming a carboxylic acid, an amine or amide with these substitutions on Apa in position 11 was investigated. The formation of aspartic acid by insertion of a β-carboxyl group with or without a benzilamide group markedly decreased affinity for all human Bn receptor subtypes. In contrast, amino or various amide substitutions in the α position, even of different sizes, were well tolerated in contrast to the β position of Apa where backbone size was a critical factor (Mantey et al., 2001). Unfortunately, substitutions in the α position of Apa[11] do not seem to be important for hBRS or Bn receptor selectivity.

In conclusion, using stereospecific substitutions of chloro groups in the hBRS-3-preferring ligands, [D-Tyr[6],(*R*)- or (*S*)-Apa[11],Phe[13],Nle[14]]Bn(6-14), one peptide was identified with enhanced selectivity for the hBRS-3 over the hGRP-R or hNMB-R. The availability of this analog as well as the recently described (Weber et al., 2002, 2003) short bombesin peptide agonists identified from systematic optimization of [D-Phe[6],β-Ala[11],Phe[13],Nle[14]]Bn(6-14), which have high selectivity for the hBRS-3, could be useful for investigating the possible role of hBRS-3 in various physiological or pathological processes.

References

Ananthan S, Khare NK, Saini SK, Davis P, Dersch CM, Porreca F, and Rothman RB (2003) Novel ligands for the opiod receptors: synthesis and structure-activity relationships among 5′-aryl and 5′-heteroaryl 17-cyclopropylmethyl-4,5 alpha-epoxypyridol[2′,3′:6,7]morphinans. *Bioorg Med Chem* **11:**4143–4154.

Benya RV, Fathi Z, Pradhan T, Battey JF, Kusui T, and Jensen RT (1994) Gastrin-releasing peptide receptor-induced internalization, down-regulation, desensitization and growth: possible role of cAMP. *Mol Pharmacol* **46:**235–245.

Benya RV, Kusui T, Pradhan TK, Battey JF, and Jensen RT (1995) Expression and characterization of cloned human bombesin receptors. *Mol Pharmacol* **47:**10–20.

Benya RV, Wada E, Battey JF, Fathi Z, Wang LH, Mantey SA, Coy DH, and Jensen RT (1992) Neuromedin B receptors retain functional expression when transfected into BALB 3T3 fibroblasts: analysis of binding, kinetics, stoichiometry, modulation by guanine nucleotide-binding proteins and signal transduction and comparison with natively expressed receptors. *Mol Pharmacol* **42:**1058–1068.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Coy DH, Jiang NY, Kim SH, Moreau JP, Lin JT, Frucht H, Qian JM, Wang LW, and Jensen RT (1991) Covalently cyclized agonist and antagonist analogues of bombesin and related peptides. *J Biol Chem* **266:**16441–16447.

Fathi Z, Corjay MH, Shapira H, Wada E, Benya R, Jensen R, Viallet J, Sausville EA, and Battey JF (1993) BRS-3: novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. *J Biol Chem* **268:**5979–5984.

Fleischmann A, Laderach U, Friess H, Buechler MW, and Reubi JC (2000) Bombesin receptors in distinct tissue compartments of human pancreatic diseases. *Lab Investig* **80:**1807–1817.

Garcia LJ, Pradhan TK, Weber HC, Moody TW, and Jensen RT (1997) The gastrin-releasing peptide receptor is differentially coupled to adenylate cyclase and phospholipase C in different tissues. *Biochim Biophys Acta* **1356:**343–354.

Gorbulev V, Akhundova A, Buchner H, and Fahrenholz F (1992) Molecular cloning of a new bombesin receptor subtype expressed in uterus during pregnancy. *Eur J Biochem* **208:**405–410.

Gorbulev V, Akhundova A, Grzeschik KH, and Fahrenholz F (1994) Organization and chromosomal localization of the gene for the human bombesin receptor subtype expressed in pregnant uterus. *FEBS Lett* **340:**260–264.

Igarashi H, Ito T, Hou W, Mantey SA, Pradhan TK, Ulrich CD, II, Hocart SJ, Coy DH, and Jensen RT (2002a) Elucidation of vasoactive intestinal peptide pharmacophore for $VPAC_1$ receptors in human, rat and guinea pig. *J Pharmacol Exp Ther* **301:**37–50.

Igarashi H, Ito T, Pradhan TK, Mantey SA, Hou W, Coy DH, and Jensen RT (2002b) Elucidation of the vasoactive intestinal peptide pharmacophore for $VPAC_2$ receptors in human and rat and comparison to the pharmacophore for VPAC1 receptors. *J Pharmacol Exp Ther* **302:**445–460.

Ito T, Hou W, Katsuno T, Igarashi H, Pradhan TK, Mantey SA, Coy DH, and Jensen RT (2000) Rat and guinea pig pancreatic acini possess both VIP_1 and VIP_2 receptors, which mediate enzyme secretion. *Am J Physiol* **278:**G64–G74.

Ito T, Igarashi H, Pradhan TK, Hou W, Mantey SA, Taylor JE, Murphy WA, Coy DH, and Jensen RT (2001) GI side-effects of a possible therapeutic GRF analogue in monkeys are likely due to VIP receptor agonist activity. *Peptides* **22:**1139–1151.

Iwabuchi M, Ui-Tei K, Yamada K, Matsuda Y, Sakai Y, Tanaka K, and Ohki-Hamazaki H (2003) Molecular cloning and characterization of avian bombesin-like peptide receptors: new tools for investigating molecular basis for ligand selectivity. *Br J Pharmacol* **139:**555–566.

Jennings CA, Harrison DC, Maycox PR, Crook B, Smart D, and Hervieu GJ (2003) The distribution of the orphan bombesin receptor subtype-3 in the rat CNS. *Neuroscience* **120:**309–324.

Jensen RT, Lemp GF, and Gardner JD (1982) Interaction of COOH-terminal fragments of cholecystokinin with receptors on dispersed acini from guinea pig pancreas. *J Biol Chem* **257:**5554–5559.

Jensen RT, Murphy RB, Trampota M, Schneider LH, Jones SW, Howard JM, and Gardner JD (1985) Proglumide analogues: potent cholecystokinin receptor antagonists. *Am J Physiol* **249:**G214–G220.

Katsuno T, Pradhan TK, Ryan RR, Mantey SA, Hou W, Donohue PJ, Akeson MA, Spindel ER, Battey JF, Coy DH, and Jensen RT (1999) Pharmacology and cell biology of the bombesin receptor subtype 4 (BB4-R). *Biochemistry* **38:**7307–7320.

Li AH, Moro S, Melman N, Ji XD, and Jacobson KA (1998) Structure-activity relationships and molecular modeling of 3,5-diacyl-2,4-dialkylpyridine derivatives as selective A3 adenosine receptor antagonists. *J Med Chem* **41:**3186–3201.

Lin JT, Coy DH, Mantey SA, and Jensen RT (1996) Comparison of the peptide structural requirements for high affinity interaction with bombesin receptors. *Eur J Pharmacol* **294:**55–69.

Liu J, Lao ZJ, Zhang J, Schaeffer MT, Jiang MM, Guan XM, Van der Ploeg LH, and Fong TM (2002) Molecular basis of the pharmacological difference between rat and human bombesin receptor subtype-3 (BRS-3). *Biochemistry* **41:**8954–8960.

Mantey S, Frucht H, Coy DH, and Jensen RT (1993) Characterization of bombesin receptors using a novel, potent, radiolabeled antagonist that distinguishes bombesin receptor subtypes. *Mol Pharmacol* **45:**762–774.

Mantey SA, Coy DH, Pradhan TK, Igarashi H, Rizo IM, Shen L, Hou W, Hocart SJ, and Jensen RT (2001) Rational design of a peptide agonist that interacts selectively with the orphan receptor, bombesin receptor subtype 3. *J Biol Chem* **276:**9219–9229.

Mantey SA, Weber HC, Sainz E, Akeson M, Ryan RR, Pradhan TK, Searles RP, Spindel ER, Battey JF, Coy DH, et al. (1997) Discovery of a high affinity radioligand for the human orphan receptor, bombesin receptor subtype 3, which demonstrates it has a unique pharmacology compared to other mammalian bombesin receptors. *J Biol Chem* **272:**26062–26071.

Matsumoto K, Yamada K, Wada E, Hasegawa T, Usui Y, and Wada K (2003) Bombesin receptor subtype-3 modulates plasma insulin concentration. *Peptides* **24:**83–90.

Nagalla SR, Barry BJ, Creswick KC, Eden P, Taylor JT, and Spindel ER (1995) Cloning of a receptor for amphibian [Phe[13]]bombesin distinct from the receptor for

gastrin-releasing peptide: identification of a fourth bombesin receptor subtype (BB4). *Proc Natl Acad Sci USA* **92:**6205–6209.
Ohki-Hamazaki H, Wada E, Matsui K, and Wada K (1997a) Cloning and expression of the neuromedin B receptor and the third subtype of bombesin receptor genes in the mouse. *Brain Res* **762:**165–172.
Ohki-Hamazaki H, Watase K, Yamamoto K, Ogura H, Yamano M, Yamada K, Maeno H, Imaki J, Kikuyama S, Wada E, et al. (1997b) Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature (Lond)* **390:**165–169.
Perrone R, Berardi F, Colabufo NA, Leopoldo M, and Tortorella V (1998) N-[2-[4-(4-Chlorophenyl)piperazin-1-yl]ethyl]-3-methoxybenzamide: a potent and selective dopamine D4 ligand. *J Med Chem* **41:**4903–4909.
Pradhan TK, Katsuno T, Taylor JE, Kim SH, Ryan RR, Mantey SA, Donohue PJ, Weber HC, Sainz E, Battey JF, et al. (1998) Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes. *Eur J Pharmacol* **343:** 275–287.
Reubi JC, Wenger S, Schumuckli-Maurer J, Schaer JC, and Gugger M (2002) Bombesin receptor subtypes in human cancers: detection with the universal radioligand (125)I-[D-TYR(6), beta-ALA(11),PHE(13), NLE(14)] bombesin(6–14). *Clin Cancer Res* **8:**1139–1146.
Ryan RR, Katsuno T, Mantey SA, Pradhan TP, Weber HC, Battey JF, and Jensen RT (1999) Comparative pharmacology of a nonpeptoid neuromedin B antagonist PD 168368. *J Exp Pharmacol Ther* **290:**1202–1211.
Ryan RR, Weber HC, Hou W, Sainz E, Mantey SA, Battey JF, Coy DH, and Jensen RT (1998a) Ability of various bombesin receptor agonists and antagonists to alter intracellular signaling of the human orphan receptor BRS-3. *J Biol Chem* **273:** 13613–13624.
Ryan RR, Weber HC, Mantey SA, Hou W, Hilburger ME, Pradhan TK, Coy DH, and Jensen RT (1998b) Pharmacology and intracellular signaling mechanisms of the native human orphan receptor BRS-3 in lung cancer cells. *J Pharmacol Exp Ther* **287:**366–380.
Saeed ZA, Huang SC, Coy DH, Jiang NY, Heinz-Erian P, Mantey SA, Gardner JD, and Jensen RT (1989) Effect of substitutions in position 12 of bombesin on antagonist activity. *Peptides* **10:**597–603.
Sasaki Y and Coy DH (1987) Solid phase synthesis of peptides containing the CH2NH peptide bond isostere. *Peptides* **8:**119–121.
Sun B, Halmos G, Schally AV, Wang X, and Martinez M (2000a) Presence of receptors for bombesin/gastrin-releasing peptide and mRNA for three receptor subtypes in human prostate cancers. *Prostate* **42:**295–303.
Sun B, Schally AV, and Halmos G (2000b) The presence of receptors for bombesin/GRP and mRNA for three receptor subtypes in human ovarian epithelial cancers. *Regul Pept* **90:**77–84.
Tache Y, Melchiorri P, and Negri L (1988) Bombesin-like peptides in health and disease. *Ann NY Acad Sci* **547:**1–541.
Wang LH, Coy DH, Taylor JE, Jiang NY, Moreau JP, Huang SC, Frucht H, Haffar BM, and Jensen RT (1990) Des-Met carboxyl-terminally modified analogues of bombesin function as potent bombesin receptor antagonists, partial agonists, or agonists. *J Biol Chem* **265:**15695–15703.
Weber D, Berger C, Eickelmann P, Antel J, and Kessler H (2003) Design of selective peptidomimetic agonists for the human orphan receptor BRS-3. *J Med Chem* **46:**1918–1930.
Weber D, Berger C, Heinrich T, Eickelmann P, Antel J, and Kessler H (2002) Systematic optimization of a lead-structure identities for a selective short peptide agonist for the human orphan receptor BRS-3. *J Pept Sci* **8:**461–475.
Weber HC, Walters J, Leyton J, Casibang M, Purdom S, Jensen RT, Coy DH, Ellis C, Clark G, and Moody TW (2001) A bombesin receptor subtype-3 peptide increases nuclear oncogene expression in a MEK-1 dependent manner in human lung cancer cells. *Eur J Pharmacol* **412:**13–20.
Westendorf JM and Schonbrunn A (1983) Characterization of bombesin receptors in a rat pituitary cell line. *J Biol Chem* **258:**7527–7535.
Whitley JC, Moore C, Giraud AS, and Shulkes A (1999) Molecular cloning, genomic organization and selective expression of bombesin receptor subtype 3 in the sheep hypothalamus and pituitary. *J Mol Endocrinol* **23:**107–116.
Wu JM, Nitecki DE, Biancalana S, and Feldman RI (1996) Discovery of high affinity bombesin receptor subtype 3 agonists. *Mol Pharmacol* **50:**1355–1363.

Address correspondence to: Dr. Robert T. Jensen, Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bldg. 10, Room 9C-103, 10 Center Dr., MSC 1804, Bethesda, MD 20892-1804. E-mail: robertj@bdg10.niddk.nih.gov