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Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes

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Abstract

Four subtypes of bombesin receptors are identified (gastrin-releasing peptide receptor, neuromedin B receptor, the orphan receptor bombesin receptor subtype 3 (BB_3 or BRS-3) and bombesin receptor subtype 4 (BB_4)), however, only the pharmacology of the gastrin-releasing peptide receptor has been well studied. This lack of data is due in part to the absence of a general ligand. Recently we have discovered a ligand, ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) that binds to BRS-3 receptors. In this study we investigate its ability to interact with all four bombesin receptor subtypes. In rat pancreatic acini containing only gastrin-releasing peptide receptor and in BB_4 transfected BALB cells, this ligand and ^{125}I-[Tyr^4]bombesin, the conventional gastrin-releasing peptide receptor ligand, gave similar results for receptor number, affinity for bombesin and affinity for the unlabeled ligand. In neuromedin B receptor transfected BALB cells, this ligand and ^{125}I-[D-Tyr^0]neuromedin B, the generally used neuromedin B receptor ligand, gave similar results for receptor number, neuromedin B affinity or the unlabeled ligand affinity. Lastly, in BRS-3 transfected BALB cells, only this ligand had high affinity. For all four bombesin receptors this ligand had an affinity of 1–8 nM and was equal or greater in affinity than any other specific ligands for any receptor. The unlabeled ligand is specific for gastrin-releasing peptide receptors on rat pancreatic acini and did not inhibit binding of ^{125}I-cholecystokinin octapeptide (^{125}I-CCK-8), ^{125}I-vasoactive intestinal peptide (^{125}I-VIP) or ^{125}I-endothelin to their receptors. The unlabeled ligand was an agonist only at the gastrin-releasing peptide receptor in rat acini and did not interact with CCK_A receptors or muscarinic M_3 acetylcholine receptors to increase [^{3}H]inositol phosphates. These results demonstrate ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) is a unique ligand with high affinity for all subtypes of bombesin receptors. Because of the specificity for bombesin receptors, this ligand will be a valuable addition for such pharmacological studies as screening for bombesin receptor agonists or antagonists and, in particular, for investigating BRS-3 cell biology, a receptor for which no ligand currently exists. Published by Elsevier Science B.V.

Keywords: Bombesin; Neuromedin B; Gastrin-releasing peptide receptor; Neuropeptide

1. Introduction

Recently three different subclasses of bombesin receptors have been described including two identified in mammals (gastrin-releasing peptide receptor (Spindel et al., 1990; Battey et al., 1991; Anonymous, 1996) and neuromedin B receptor (Von Schrenck et al., 1989; Wada et al., 1991)) and one identified in frog brain (bombesin receptor subtype 4 (BB_4 receptor) (Nagalla et al., 1995)), but not yet identified in mammals. A possible fourth subtype has been proposed which is a 399 amino acid orphan receptor which, because of its high degree of homology to mammalian bombesin receptors (51–52% for the gastrin-releasing peptide receptor and 47% for the neuromedin B receptor (Gorbulev et al., 1992; Fathi et al., 1993)), was named BRS-3 for bombesin receptor subtype-3

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in one study (Fathi et al., 1993) and BB_3 in one classification (Anonymous, 1996). After expression in Xenopus oocytes, BRS-3 receptors could be activated by bombesin, albeit at concentrations several orders of magnitude higher than those required to activate the gastrin-releasing peptide receptor or neuromedin B receptor in the same assay (Fathi et al., 1993). All four receptors are members of the heptahelical superfamily of G protein-coupled receptors (Spindel et al., 1990; Battey et al., 1991; Gorbulev et al., 1992; Nagalla et al., 1995).

Whereas the pharmacology and cell biology of the gastrin-releasing peptide receptor has been extensively studied (Kroog et al., 1995), much less is known about the neuromedin B receptor and only a few studies have dealt with these aspects of the BRS-3 receptor (Gorbulev et al., 1992; Wu et al., 1996) or the BB_4 receptor (Nagalla et al., 1995). In the case of the BRS-3 receptor this lack of information is because of a lack of a high affinity ligand, with the BB_4 receptor it is because this receptor was only recently described and with the neuromedin B receptor it is in part due to the fact that its ligand (^{125}I-[D-Tyr^0]neuromedin B or ^{125}I-Bolton–Hunter-neuromedin B (Von Schrenck et al., 1989; Wang et al., 1993; Lin et al., 1996) is not generally available. In the process of screening synthetic bombesin analogues that could interact with the hBRS-3 receptor, we recently discovered that [D-Phe^6, βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) [D-Phe^6,βAla^{11}, Phe^{13},Nle^{14}]bombesin-(6–14) had high affinity for the hBRS-3 receptor (Mantey et al., 1997). In the non-small cell lung cancer cell H1299 we found that this peptide also interacted with human gastrin-releasing peptide receptor cells natively expressed in low numbers in these cells (Corjay et al., 1991). In the present study we have investigated the ability of this peptide and an analogue which can be radiolabeled ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]-bombesin-(6–14) to interact with all four subtypes of bombesin receptors. We find this ligand is unique in that it has selective high affinity for all four bombesin receptor subtypes, but does not interact with receptors mediating the action of unrelated peptides.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats weighing 80–100 g were purchased from Taconic Farms of New York. *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES) and albumin bovine fraction V were from ICN Biomedical (Aurora, OH); soybean trypsin inhibitor type 1-S, bacitracin, atropine sulfate and carbachol were from Sigma (St. Louis, MO); basal medium Eagle vitamin and basal medium Eagle amino acids solutions (100-times concentrated), Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle medium (DMEM), F-12 nutrient mixture, fetal bovine serum, 0.05% trypsin in 0.53 mM EDTA and G418 sulfate (Geneticin) were from Gibco BRL (Grand Island, NY); glutamine was from Media Section, National Institutes of Health (Bethesda, MD); Na ^{125}I (2200 Ci/mmol) was from Amersham Life Science (Arlington Heights, IL); ^{125}I-vasoactive intestinal peptide ([^{125}I]-VIP), [^{125}I]-Bolton–Hunter cholecystokinin octapeptide CCK-8 (^{125}I-CCK-8), [^{125}I]-Tyr^{13} endothelin-1 (all 2200 Ci/mmol) and myo-[2-^{3}H(N)]inositol (20.5 Ci/mmol) were purchased from DuPont/New England Nuclear (Boston, MA); 1,2,4,6-tetrachloro-3α-6α-diphenylglycouril (IODO-GEN) and dithiothreitol were from Pierce Chemical Co. (Rockford, IL); collagenase (1,492 U/mg) was from Worthington Biochemical (Freehold, NJ); Nyosil M20 oil was from Nye Lubricants (New Bedford, MA); bombesin, neuromedin B and cholecystokinin octapeptide (CCK-8) were from Peninsula Laboratories (Belmont, CA); L-364,718 was a gift from Dr. Paul Anderson and Dr. Ben Evans, Merck, Sharp and Dohme (West Point, PA); NCI-H1299 cells were a gift from Herb Oie of NCI-Navy Medical Oncology Branch, Bethesda Naval Medical Center (Bethesda, MD) and BALB 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). All other chemicals were of the highest purity commercially available.

Unless otherwise stated the standard incubation solution contained the following (in mM): 24.5 HEPES, 98 NaCl, 6 KCl, 2.1 KH_2PO_4, 1.5 $CaCl_2$, 0.8 $MgCl_2$, 5 sodium pyruvate, 5 sodium fumarate, 5 sodium glutamate, 11.5 glucose, 2 glutamine, 0.1% (w/v) albumin, 0.1% (w/v) trypsin inhibitor, 1% (v/v) essential vitamin mixture and 1% (v/v) essential amino acid mixture. The pH of the incubation solution was 7.4 and all incubations with rat acinar cells were performed with 100% O_2 as the gas phase.

2.2. Preparation of peptides

The peptides were synthesized with solid-phase methods as described before (Sasaki and Coy, 1987; Coy et al., 1988). Peptide esters were prepared by standard, automated solid-phase techniques on Advanced Chem Tech ACT200 machines with Merrifield Leu-O-polystyrene resin and a α-Boc protection for all amino acids and both the α and imidazole nitrogen of His in position 12 as described previously (Wang et al., 1990). Free peptides were then cleaved from the resin by transesterification with 10% triethylamine/methanol at 40°C (2 days). Peptides were purified on a column (2.5×90 cm) of Sephadex G-25 followed by elution with linear gradients of acetonitrile in 0.1% trifluoroacetic acid with a Rainin preparative high performance liquid chromatography system (flow rate ca. 5 ml/min) and columns (1.5×50 cm) of Vydac C_{18} silica (10–15 μm). Peptides were further purified by rechromatography on the same column with slight modifications to the gradient conditions when necessary to greater than

97% purity. Peptides were characterized by amino acid analysis and matrix-assisted laser desorption mass spectroscopy (Finnegan, Hemel Hemstead, UK).

2.3. Growth and maintenance of cells

Bombesin receptor subtype four (BB_4) transfected cells were grown in F-12 nutrient mixture with L-glutamine. rNMB-R transfected BALB 3T3 cells (Kroog et al., 1995), hBRS-3 transfected non-small cell lung cancer cell line 1299 (Mantey et al., 1997) and hBRS-3 transfected BALB 3T3 cells (Mantey et al., 1997) were grown in DMEM. All cell media were supplemented with 10% (v/v) fetal bovine serum (GIBCO/BRL), penicillin (50 U/ml) and streptomycin 50 μg/ml (GIBCO/BRL) plus 300 μg/ml Geneticin. 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 trypsin–EDTA.

2.4. Construction of plasmids

The frog BB_4 receptor cDNA was a gift from Srinivasa R. Nagalla and Eliot R. Spindel and was cloned into the *Bam*HI site of the mammalian expression vector pcDNA 3 (Invitrogen; San Diego, CA). The correct DNA sequence of the insert in the expression plasmids was verified by automated sequencing on both strands using gene-specific primers (Model A373, Applied Biosystems, Perkin Elmer; Foster City, CA).

2.5. Stable cell transfection

Chinese hamster ovary-K1 cells (CHO-K1 cells) (American Type Culture Collection; Rockville, MD) were transfected with the plasmid containing the BB_4 receptor DNA (10 μg DNA) using 25 μl LipofecTAMINE™ (Life Technologies; Gaithersburg, MD) in one 100×20 mm petri dish type Falcon® 3003 (Becton Dickinson; Plymouth, England) according to the recommendations by the supplier. 24 h after transfection, cells were exposed to F-12 nutrient mixture containing 10% (v/v) fetal bovine serum, 1% (v/v) penicillin–streptomycin and 800 μg/ml of Geneticin. Approximately three weeks after transfection, individual clones were expanded and screened for BB4 receptor expression using a ^{125}I-[Tyr^4]bombesin binding assay. Stable cell lines were maintained in F-12 nutrient mixture (HAM) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin–streptomycin and 300 μg/ml Geneticin.

2.6. Acini preparation

Dispersed acini from rat pancreas were prepared by the modification of the methods of Peikin et al. (1978) described previously (Jensen et al., 1982).

2.7. Binding studies

Binding studies with rat dispersed acini were performed by incubating the cells at 37°C for 30 min in standard incubation solution plus bacitracin (0.1%, w/v) unless otherwise stated, as described previously (Jensen et al., 1978; Lin et al., 1996). Binding studies with all transfected cells were performed by incubating the cells at room temperature for 45 min in standard incubation solution containing 0.1% (w/v) bacitracin. ^{125}I-labeled peptides were added at 50 pM without (total binding) or with 1 μM unlabeled peptides (nonsaturable binding) unless otherwise stated. The final incubation volume was 0.30 ml. Bound radioactivity was determined by layering 100 μl of the cell suspension over 300 μl of standard incubation solution containing 1% (w/v) albumin and 0.1% (w/v) bacitracin in microfuge tubes and centrifuging the sample for 2 min in a Microfuge B at $10{,}000 \times g$ (Beckman; Palo Alto, CA). The supernatant was aspirated and the microfuge tubes were washed twice with incubation buffer without disturbing the cell pellet. The cell pellet was counted for radioactivity in a gamma counter. 100 μl aliquots of the incubation mixture were taken in duplicate to determine the total radioactivity. Binding was expressed as the percentage of total radioactivity that was associated with the cell pellet. All binding values, unless otherwise stated, represented saturable binding (i.e. total binding minus nonsaturable binding). In all cases nonsaturable binding was $< 15\%$ of total binding.

2.8. Iodination of [D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), [Tyr^4]bombesin and [D-Tyr^{0}neuromedin B]

The radioligands, at specific activity of 2000 Ci/mmol, were prepared by a modification of the methods described previously (Mantey et al., 1993; Wang et al., 1993). Briefly, 0.8 μg of IODO-GEN (in chloroform) was transferred to a vial, dried under a stream of nitrogen and washed with 100 μl of KH_2PO_4 (pH 7.4). To this vial, 20 μl of KH_2PO_4 (pH 7.4), 8 μg peptide in 4 μl water and 2 mCi (20 μl) Na ^{125}I were added, mixed gently 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 dithiotreitol was added. The iodination mixture was incubated at 80°C for 60 min. ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), which does not have a COOH terminal methionine, did not undergo the incubation with dithiotreitol. The reaction mixture was applied to a Sep-Pak (Waters Assoc., Milford, MA) and the free ^{125}I was eluted with 5 ml water followed by 5 ml 0.1% trifluoroacetic acid. The radiolabeled peptides were eluted with 200 μl sequential elutions ($\times 10$) with 60% acetonitrile in 0.1% trifluoroacetic acid. The two or three fractions with the highest radioactivity were combined and purified on a reverse-phase, high performance liquid chro-

matography with a μBondaPak column (0.46 × 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v) from 16 to 60% acetonitrile in 60 min. 1 ml fractions were collected and checked for radioactivity and receptor binding. The pH of the fractions were adjusted to 7 using 0.2 M Tris (pH 9.5), and radioligands were stored in aliquots with 0.5% bovine serum albumin (BSA) at −20°C.

2.9. Cellular inositol phosphate ([^{3}H]IP) studies

Changes in [^{3}H]IP were measured using the method described by Rowley et al., (Rowley et al., 1990). Briefly, dispersed rat acini were incubated with 100 μCi/ml *myo*-[2-^{3}H]inositol for 2 h at 37°C. After washing, pancreatic acini were resuspended in standard incubation buffer with 10 mM LiCl and incubated alone or with the indicated peptides for 30 min at 37°C. [^{3}H]IP was separated using Dowex AGI-X8 anion exchange chromatography as outlined previously (Rowley et al., 1990; Benya et al., 1992).

2.10. Statistical analyses

Binding curves were analyzed using a non-linear curve-fitting program (LIGAND) (Munson and Rodbard, 1980). Hill plots (Levitzki, 1984) and Scatchard plots (Scatchard, 1949) were construed and Hill coefficients (Levitzki, 1984) calculated by a least-squares analysis (Biosoft, Ferguson, MO). All data were expressed as means ± S.E.M. Statistical significance of differences between the group means was determined using the Student's *t*-test. Values with $P < 0.05$ were considered significant.

3. Results

The ability of peptides to interact with the gastrin-releasing peptide receptor has been characterized using either the radiolabeled agonists [^{125}I-Tyr^4]bombesin or ^{125}I-gastrin-releasing peptide, or the radiolabeled antagonist ^{125}I-[D-Phe^6]bombesin-(6–13)methyl ester (Ladenheim et al., 1991; Vigna and Mantyh, 1991; Mantey et al., 1993; Shapira et al., 1993). To investigate the ability of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to interact with this receptor, we compared the abilities of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) (Fig. 1, left panel) and ^{125}I-[Tyr^4]-bombesin (Fig. 1, right panel) to rat pancreatic acinar cells which possess gastrin-releasing peptide receptors (Zhu et al., 1991; Jensen, 1994; Lin et al., 1996). Both [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin inhibited the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and ^{125}I-[Tyr^4]bombesin, re-

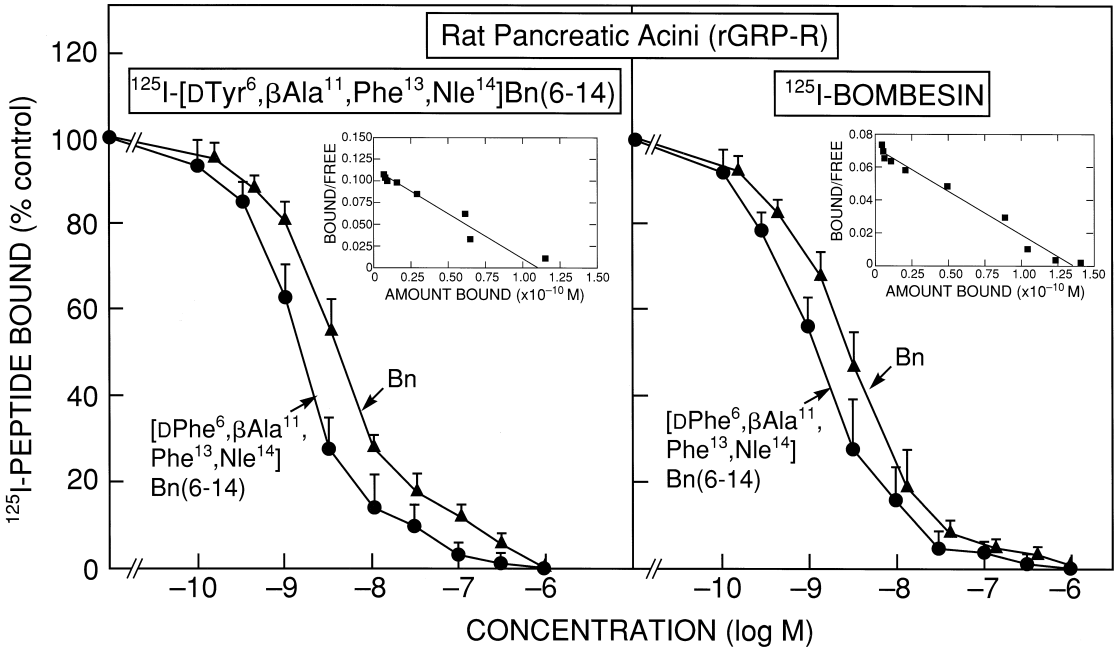

Fig. 1. Comparison of the abilities of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) (left panel) and ^{125}I-[Tyr^4]bombesin (right panel) to rat pancratic acinar cells (rGRP-R). Rat pancreatic acinar cells were incubated for 30 min at 37°C with 75 pM ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) or ^{125}I-[Tyr^4]bombesin plus the concentration of unlabeled peptide indicated. Binding was expressed as the percentage of radioactive peptide that was saturably bound in the absence of any nonradioactive peptide (i.e. percent control). In each experiment, each value was determined in duplicate and the results given are means ± S.E.M. of at least three separate experiments. Inserts show the ability of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) (left panel, insert) or of bombesin to inhibit the binding of ^{125}I-[Tyr^4]bombesin (right panel, insert) plotted in the form of Scatchard (Scatchard, 1949). The data are the mean of at least three experiments.

Table 1
Comparison of bombesin receptor parameters (affinity, density) assessed by ^{125}I-[D-Phe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6–14) or by other receptor ligands

	Bn receptor examined				
	rGRP-R (rat pancreatic acinar cells)	BB4 transfected CHO K-1 cells	rNMB-R transfected BALB 3T3 cells	hBRS transfected BALB 3T3 cells	hBRS transfected H1299 cells
Receptor density (fmol/mg protein)					
[D-Phe^{6},βAla^{11},Phe^{13},Nle^{14}]-Bn(6–14) versus ^{125}I-BRS-3[a]	620 ± 87	1160 ± 162	51300 ± 10260	2690 ± 180	458 ± 40
Bombesin versus ^{125}I-[Tyr^{4}]Bn	756 ± 60	1050 ± 262			
NMB versus ^{125}I-[D-Tyr^{0}]NMB			58000 ± 16240		
Receptor affinity (nM)					
[D-Phe^{6},βAla^{11},Phe^{13},Nle^{14}]-Bn(6–14) versus ^{125}I-BRS-3[a]	0.99 ± 0.16	0.41 ± 0.06	0.36 ± 0.06	8.9 ± 0.7	4.2 ± 1.0
[D-Phe^{6},βAla^{11},Phe^{13},Nle^{14}]-Bn(6–14) versus ^{125}I-[Tyr^{4}]Bn	1.70 ± 0.65	0.29 ± 0.08			
[D-Phe^{6},βAla^{11},Phe^{13},Nle^{14}]-Bn(6–14) versus ^{125}I-[D-Tyr^{0}]NMB			2.50 ± 0.80		
Bombesin versus ^{125}I-BRS-3[a]	3.8 ± 1.1	8.6 ± 1.8			
Bombesin versus ^{125}I-[Tyr^{4}]Bn	1.88 ± 0.13	1.7 ± 0.5			
NMB versus ^{125}I-BRS-3[a]			0.21 ± 0.06	> 10000	> 10000
NMB versus ^{125}I-[D-Tyr^{0}]NMB			0.71 ± 0.14		

Rat pancreatic acinar cells were incubated for 30 min at 37°C with 75 pM ^{125}I-[D-Tyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6–14) or ^{125}I-[Tyr^{4}]bombesin plus the peptides indicated. BB4 transfected CHO-K1 cells were incubated for 60 min at 25°C with 125 pM ^{125}I-[D-Tyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6–14) or ^{125}I-[Tyr^{4}]bombesin plus the peptides. Neuromedin B receptor transfected BALB 3T3 cells were incubated at 25°C for 45 min with 50 pM ^{125}I-[D-Tyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6–14) or ^{125}I-[D-Tyr^{0}]neuromedin B plus the peptides. hBRS-3 transfected BALB 3T3 cells or hBRS-3 transfected H1299 cells were incubated for 45 min at 25°C with 50 pM ^{125}I-[D-Tyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6–14) plus the peptides. Results of ligand bound were expressed as percentage of radioactive peptide that was saturably bound in the absence of any nonradioactive peptide. Each binding curve was analyzed using a least-squares curve-fitting program (LIGAND; Munson and Rodbard, 1980). Results are ± S.E.M. of at least four separate experiments and in each experiment, each value was determined in duplicate.

[a] ^{125}I-BRS-3 is the abbreviation for ^{125}I-[D-Tyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6–14).

spectively, in a dose–response manner. Detectable inhibition of binding was observed in both cases with 0.1 nM [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin, half-maximal inhibition at 1 nM with [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and at 3 nM with bombesin and complete inhibition at 1 μM with both (Fig. 1). Analysis of the dose–inhibition curve of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) with ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and of bombesin with ^{125}I-[Tyr^4]bombesin using a least-squares, curve-fitting program (LIGAND) (Munson and Rodbard, 1980) indicated that the binding was best fitted by a single binding site model (Fig. 1, insert). The Hill coefficient (n_H) was 0.99 ± 0.01 ($n = 3$) with ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and 1.02 ± 0.04 with ^{125}I-[Tyr^4]bombesin. When ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) was used as the ligand the gastrin-releasing peptide receptor binding capacity was determined to be 620 ± 87 fmol/mg protein which was not significantly different from the binding capacity of 756 ± 60 fmol/mg protein determined when ^{125}I-[Tyr^4]bombesin was used as the radioligand (Table 1, Fig. 1). [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin(6–14) had a similar affinity for the gastrin-releasing peptide receptor, whether determined from analysis of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) or binding of ^{125}I-[Tyr^4]bombesin, as did bombesin itself (Table 1, Fig. 1). Specifically [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) had an affinity of 0.99 ± 0.16 nM with ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and 1.7 ± 0.6 nM with ^{125}I-[Tyr^4]bombesin, whereas bombesin had an affinity of 3.8 ± 1.1 nM with ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and 1.9 ± 0.13 nM with bombesin, respectively.

To examine the comparative ability of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and ^{125}I-[D-Tyr^0]neuromedin B to interact with neuromedin B receptors, we determined the abilities of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and neuromedin B to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) (Fig. 2, left) and ^{125}I-[D-Tyr^0]neuromedin B (Fig. 2, right) to neuromedin B receptor transfected BALB 3T3 cells. Previous studies have demonstrated the neuromedin B receptors transfected in these cells function in an identical manner to the native receptor in terms of binding, coupling to phospholipase C and G protein coupling (Benya et al., 1992). Both neuromedin B and [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) inhibited the binding of ^{125}I-[D-Tyr^0]neuromedin B and ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},

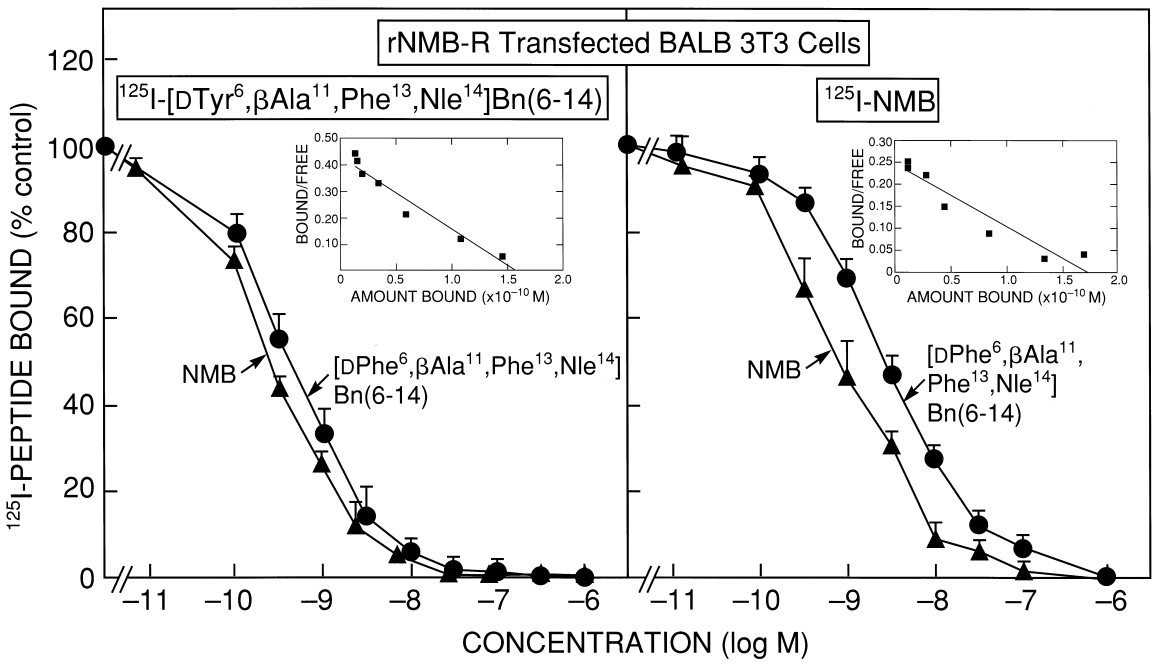

Fig. 2. Comparison of the abilities of neuromedin B and [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) (left panel) and of ^{125}I-[D-Tyr^0]Neuromedin B (right panel) to neuromedin B receptor transfected BALB 3T3 cells. The data are the mean of at least three separate experiments. Neuromedin B receptor transfected BALB 3T3 cells (0.1×10^6 cells/ml) were incubated for 45 min at 25°C with 50 pM ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) or ^{125}I-[D-Tyr^0]neuromedin B plus the indicated concentration of unlabeled peptide. Binding was expressed as the percentage of radioactive peptide that was saturably bound in the absence of any nonradioactive peptide (i.e. percent control). In each experiment, each value was determined in duplicate and results given are ±S.E.M. of at least three separate experiments. Inserts show the ability of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to inhibit binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) (left panel, inset) or of neuromedin B to inhibit binding of ^{125}I-[D-Tyr^0]neuromedin B (right panel, inset) plotted in the form of Scatchard (Scatchard, 1949). Results are the means of at least three separate experiments.

Table 2
Ability of various receptor antagonists to inhibit [^{3}H]IP generation by various agonists in rat pancreatic acini

Agonist added	DPM (mean ± S.E.M.)			
	alone	plus [D-Phe^{6}Bn(6–13)ME (1 μM)	plus L-364,718 (1 μM)	plus atropine (1 μM)
None	1691 ± 169	1480 ± 148	1450 ± 270	1473 ± 282
Carbachol (100 μM)	11685 ± 753	11924 ± 306	11196 ± 610	1547 ± 126[c]
CCK-8 (0.3 μM)	13904 ± 473	13939 ± 526	2190 ± 298[c]	13266 ± 949
[D-Phe^6, βAla^{11},Phe^{13},Nle^{14}]Bn(6–14) (0.3 μM)	5021 ± 234	3573 ± 537[a]	4987 ± 304	4968 ± 474
Bombesin (0.3 μM)	4619 ± 397	2506 ± 51[b]	4393 ± 228	4436 ± 195

[a] Indicate significant differences from the agonist alone ($P < 0.05$).
[b] Indicate significant differences from the agonist alone ($P < 0.01$).
[c] Indicate significant differences from the agonist alone ($P < 0.001$).
Rat pancreatic acinar cells were incubated with 100 μCi/ml [^{3}H]*myo*-inositol for 2 h at 37°C and washed three times with incubation buffer to remove excess [^{3}H]*myo*-inositol. After subsequent incubation of cells with 10 mM LiCl at room temperature for 15 min, 250 μl aliquots were treated with and without the indicated agonists and antagonists. [^{3}H]IP was measured using the method of Rowley et al. (1990). Values are means ± S.E.M. from at least three separate experiments and in each experiment, each value was determined in duplicate.

Nle[14]]bombesin-(6–14) respectively, in a dose–response manner (Fig. 2). For both, detectable inhibition of binding was observed with 0.1 nM [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and neuromedin B, half-maximal inhibition at 0.3 nM for [D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and at 0.2 nM for neuromedin B with ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14), at 2.5 nM for [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and 0.7 nM for neuromedin B with ^{125}I-[D-Tyr[0]]neuromedin B and complete inhibition at 1 μM for both. Analysis of dose–inhibition curves for [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and neuromedin B with ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and ^{125}I-[D-Tyr[0]]neuromedin B indicated that the binding was best fit by a single site model (Fig. 2, inserts). The n_H was 0.96 ± 0.10 for ^{125}I-[D-Tyr[0]]neuromedin B and 1.05 ± 0.05 for ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14). Using ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) as the ligand, the neuromedin B receptor had an affinity of 0.36 ± 0.06 nM for [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and 0.21 ± 0.06 nM for neuromedin B whereas using ^{125}I-[D-Tyr[0]]neuromedin B as the ligand, the neuromedin B receptor had an affinity of 2.5 ± 0.8 nM for [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and 0.71 ± 0.14 nM for neuromedin B. Both ligands gave similar binding capacities. Specifically, analysis of the ability of [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) to inhibit binding of ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) gave a receptor density of 51,300 ± 10,260 fmol/mg protein (1670 ± 334 fmol/10^6 cells), whereas analysis of the ability of neuromedin B to inhibit binding of ^{125}I-[D-Tyr[0]]neuromedin B gave a receptor density of 58,000 ± 16,240 fmol/mg protein (1890 ± 529 fmol/10^6 cells) for the neuromedin B receptor on neuromedin B receptor transfected cells (Table 2).

In order to examine the comparative ability of ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and ^{125}I-[Tyr[4]]bombesin to interact with the newly described BB_4 receptor originally isolated from a cDNA library from frog skin (Nagalla et al., 1995), we stably transfected BB_4 receptors into CHO-K1 cells and investigated the abilities of [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and bombesin to inhibit the binding of ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and ^{125}I-[Tyr[4]]bombesin to these cells. A previous study demonstrated this receptor has high affinity for bombesin, therefore ^{125}I-[Tyr[4]]bombesin can be used as a ligand (Nagalla et al., 1995). Both [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and bombesin inhibited the binding of ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) (Fig. 3, left) and ^{125}I-[Tyr[4]]bombesin (Fig. 3, right) in a dose–response

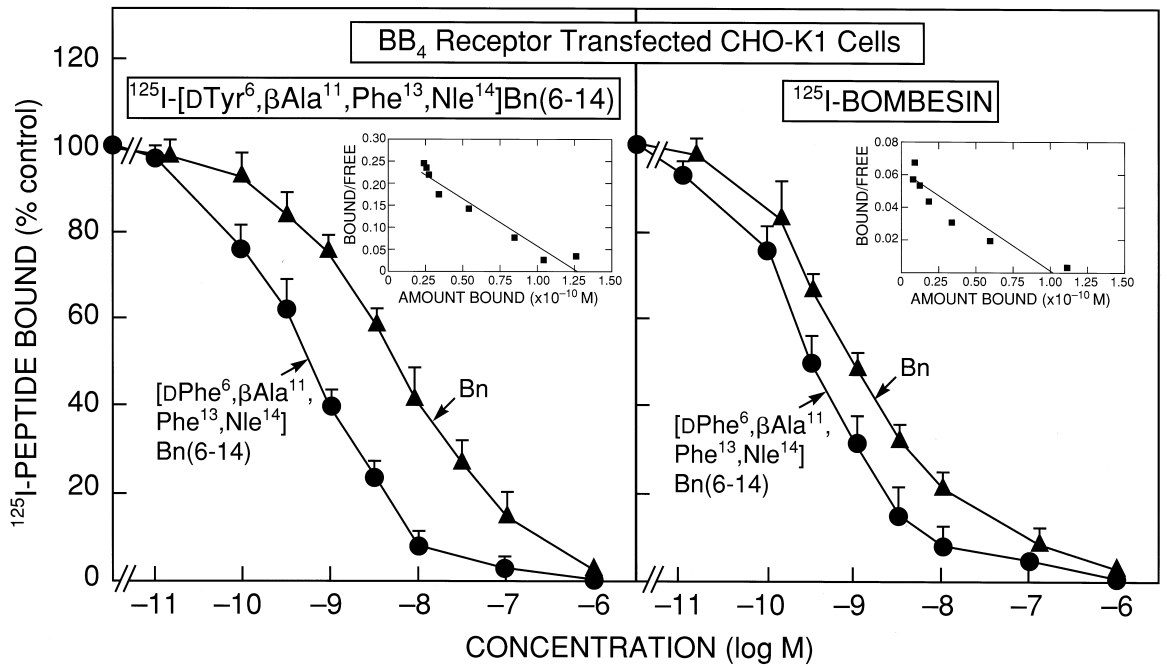

Fig. 3. Comparison of the abilities of [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) and bombesin to inhibit the binding of ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) (left panel) and ^{125}I-[Tyr[4]]bombesin (right panel) to BB_4 receptor transfected CHO-K1 cells. BB_4 receptor transfected CHO-K1 cells (4.8×10^6 cells/ml) were incubated for 60 min at 25°C with 125 pM ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) or ^{125}I-[Tyr[4]]bombesin plus indicated concentration of unlabeled peptide. Binding was expressed as percentage of radioactive peptide that was saturably bound in the absence of any nonradioactive peptide (i.e. percent control). Results are means ± S.E.M. of at least three separate experiments and in each experiment, each value was determined in duplicate. Inserts show the ability of [D-Phe[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) to inhibit binding of ^{125}I-[D-Tyr[6],βAla[11],Phe[13],Nle[14]]bombesin-(6–14) (left panel, insert) or of bombesin to inhibit binding of ^{125}I-[Tyr[4]]bombesin (right panel, insert) plotted in the form of Scatchard (Scatchard, 1949). Results are the means of at least three separate experiments.

manner. For both ^{125}I-[D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin, detectable inhibition of binding was at 0.01 nM, half-maximal inhibition at 0.3 nM and complete inhibition at 1 μM for [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin. Analysis of dose–inhibition curves for [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) with ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and for bombesin with ^{125}I-[Tyr^4]bombesin using a least-squares, curve-fitting program (Munson and Rodbard, 1980) indicated that the binding was fitted best by a single site model (Fig. 3, inserts). The n_H for ^{125}I-[Tyr^4]bombesin binding was 0.97 ± 0.06 and for binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) 0.95 ± 0.06. Using ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) as the receptor ligand, the BB_4 receptor had an affinity of 0.41 ± 0.06 nM for [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and 8.6 ± 1.8 nM for bombesin, whereas using ^{125}I-[Tyr^4]bombesin as the ligand, the BB_4 receptor had an affinity of 0.29 ± 0.08 nM for [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and 1.7 ± 0.5 nM for bombesin. Analysis of the ability of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to inhibit binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) demonstrated a BB_4 receptor density of 1160 ± 162 fmol/mg protein (23.8 ± 3.3 fmol/10^6 cells) which was not significantly different from the value of 1050 ± 262 fmol/mg protein (21.4 ± 5.3 fmol/10^6 cells) obtained from analysis of ^{125}I-[Tyr^4]bombesin binding (Table 2).

Previous studies demonstrate the hBRS-3 receptor has low affinity for bombesin, neuromedin B and the gastrin-releasing peptide receptor antagonist, [D-Phe^6]bombesin-(6–13)methyl ester; therefore, radiolabeled analogues of these demonstrate either no or very low saturable binding to this receptor (Mantey et al., 1997; Gorbulev et al., 1992; Fathi et al., 1993). However, [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) has a relatively high affinity for the hBRS-3 receptor (Mantey et al., 1997). We therefore determined the abilities of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to hBRS-3 transfected BALB 3T3 cells or hBRS-3 transfected H1299 cells. [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) inhibited the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) in a dose–response fashion for both hBRS-3 transfected H1299 cells (Fig. 4, left panel) and hBRS-3 transfected BALB 3T3 cells (Fig. 4, right panel). bombesin did not inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to these cells, even up to a concentration of 1 μM. For [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), detectable inhibition of binding was observed at 0.1 nM, half-maximal inhibition at 5.0 nM with hBRS-3 transfected H1299 cells

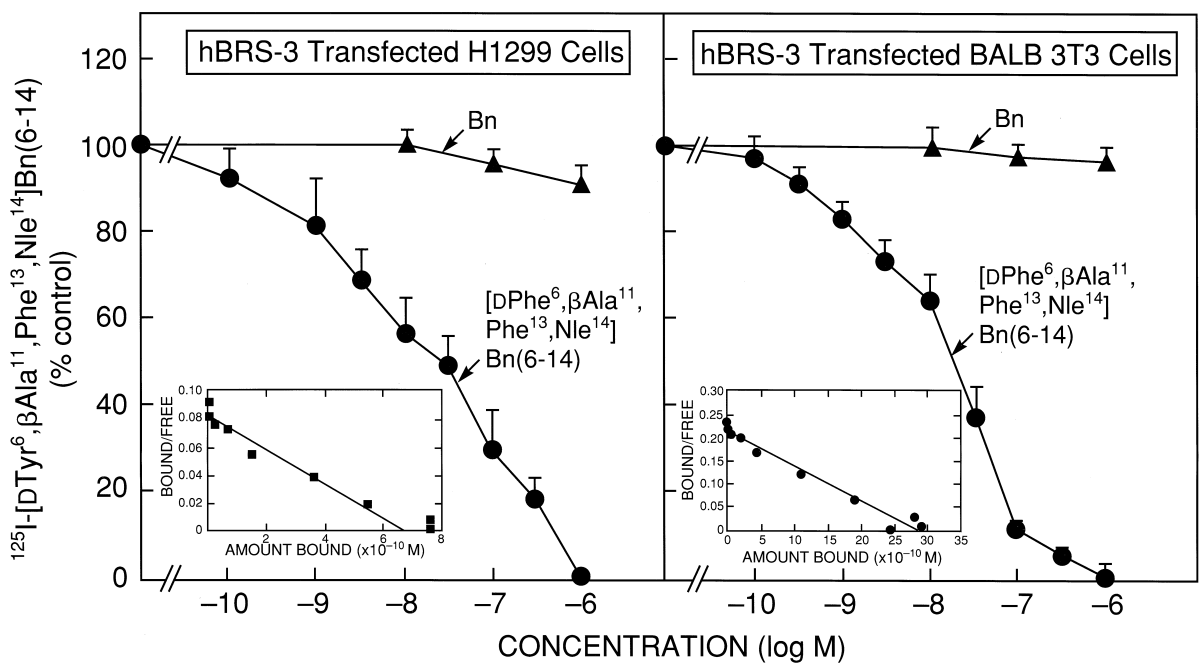

Fig. 4. Comparison of the ability of bombesin and [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to hBRS-3 transfected H1299 cells (left panel) and on hBRS-3 transfected BALB 3T3 cells (right panel). hBRS-3 transfected H1299 cells (1.5 × 10^6 cells/ml) or hBRS-3 transfected BALB 3T3 cells (10^6 cells/ml) were incubated for 45 min at 25°C with 50 pM ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) plus the peptides indicated. Binding was expressed as the percentage of radioactive peptide that was saturably bound in the absence of any nonradioactive peptide (i.e. percent control). In each experiment, each value was determined in duplicate and results given are ± S.E.M. of at least three separate experiments. Inserts show the ability of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to hBRS-3 transfected H1299 cells (left panel, insert) or hBRS-3 transfected BALB 3T3 cells (right panel, insert) plotted in the form of Scatchard (Scatchard, 1949). Results are the means of at least three experiments.

and 9.0 nM with hBRS-3 transfected BALB 3T3 cells and complete inhibition at 1 μM. Computer analysis of the [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) dose–inhibition curve indicated the data was best fit by a single site model (Fig. 4, inserts). For binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to hBRS-3 transfected H1299 cells the n_H was 0.98 ± 0.01 and for binding to hBRS-3 transfected cells it was 1.04 ± 0.05. For [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), hBRS-3 transfected H1299 cells had an affinity of 4.2 ± 1 nM, and for hBRS-3 transfected BALB 3T3 cells, the affinity was of 8.9 ± 0.7 nM (Table 1). hBRS-3 transfected BALB 3T3 cells had a binding capacity of 6.7 ± 0.5 fmol/mg protein (2690 ± 180 fmol/10^6 cells) which was about four-fold higher than the binding capacity of hBRS-3 transfected H1299 cells of 1.52 ± 0.13 fmol/mg protein (458 ± 40 fmol/10^6 cells).

In order to assess the specificity of interaction of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) with bombesin receptors, two different studies were performed. First, the ability of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to interact with rat dispersed pancreatic acinar cells was examined by determining its ability to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), ^{125}I-endothelin-1, ^{125}I-VIP or ^{125}I-BH-CCK-8, because these cells have previously been shown to possess specific receptors for gastrin-releasing peptide, endothelin, VIP and CCK (Jensen, 1994). With ^{125}I-endothelin-1, ^{125}I-VIP or ^{125}I-BH-CCK-8, [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), had no effect on their binding, even up to a concentration of 100 nM (Fig. 5). Detectable inhibition of binding by [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) with these ligands was observed only at 3 μM. However, [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) caused inhibition of binding of ^{125}I-[D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]-bombesin-(6–14) in a dose–response manner, having a half-maximal inhibition at 3 nM and complete inhibition at 1 μM (Fig. 5). This result demonstrates that the [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) in rat pancreatic acinar cells selectively interacts at high affinity with gastrin-releasing peptide receptors. Second, in order to investigate further the specificity of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) for bombesin receptors, we examined in dispersed pancreatic acinar cells from rat, its ability along with other agonists to cause the stimulation of [^{3}H]inositol phosphates ([^{3}H]IP) with and without the addition of various selective receptor antagonists (Table 1). Carbachol, CCK-8, [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]-bombesin-(6–14) or bombesin increased [^{3}H]IP when present alone (Table 2). The bombesin- and [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14)-stimulated [^{3}H]IP increase was inhibited only by [D-Phe^6]bombesin-(6–13)methyl ester (1 μM), a specific gastrin-releasing peptide receptor antagonist (Evans et al., 1986; Jensen et al., 1989; Von Schrenck et al., 1990; Wang et al., 1990).

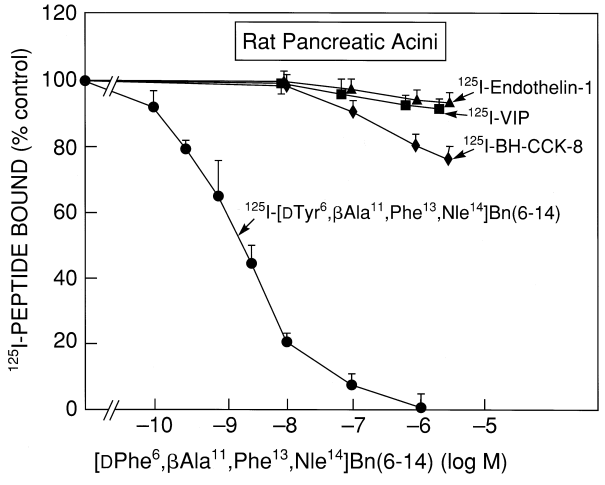

Fig. 5. Comparison of the ability of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to inhibit the binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), ^{125}I-BH-CCK-8, ^{125}I-VIP or ^{125}I-endothelin-1 to rat pancreatic acinar cells. Pancreatic acini were incubated for 30 min at 37°C with 75 pM ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), ^{125}I-BH-CCK-8, ^{125}I-VIP or ^{125}I-endothelin-1 plus the concentration of unlabeled peptide indicated. Binding was expressed as percentage of radioactive peptide that was saturably bound in the absence of any nonradioactive peptide (i.e. percent control). In each experiment, each value was determined in duplicate and results given are ±S.E.M. of at least three separate experiments.

4. Discussion

In this study we report for the first time the identification of a unique, specific ligand that interacts with high affinity with each of the four reported subtypes of bombesin receptors. The evidence that the ligand, ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14), is interacting with the bombesin receptors is supported by a number of findings. First, for each of the three subclasses of bombesin receptors (gastrin-releasing peptide receptor, neuromedin B receptor and BB_4 receptor) for which other well-described ligands existed (i.e. ^{125}I-[Tyr^4]bombesin for gastrin-releasing peptide receptor (Ladenheim et al., 1991; Vigna and Mantyh, 1991; Mantey et al., 1993; Shapira et al., 1993), BB_4 receptors (Nagalla et al., 1995) and ^{125}I-[D-Tyr^0]neuromedin B for neuromedin B receptors (Von Schrenck et al., 1989; Benya et al., 1992; Wang et al., 1993)), the results of computer analysis of their binding curves for ligand affinities and receptor densities agreed closely with that obtained from a similar analysis of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) binding. Specifically, there was no significant difference for the gastrin-releasing peptide receptor density on rat pancreatic acini, BB_4 receptor density on BB_4 receptor CHO-K_1 transfected cells, or rat neuromedin B receptor on neuromedin B receptor transfected BALB 3T3 cells with either ligand, suggesting in each case each ligand was

interacting with the same sites. Similarly, the affinities of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) determined with the use of either ligand for each of the three receptors was similar. Second, the relative affinities of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) for each of these three bombesin receptors compared to bombesin for the BB_4 receptor and gastrin-releasing peptide receptor, or neuromedin B for the neuromedin B receptor was generally similar whether obtained from analysis of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) binding or binding of the other well established ligands. Third, previous studies demonstrated that BALB 3T3 cells do not possess the hBRS-3 receptor, and ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) demonstrated no binding to these cells. However, after transfection with the hBRS-3 receptor, we found these cells to demonstrate significant saturable binding with ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14). These results provide strong support for the conclusion that this ligand is binding to the hBRS-3 receptor on these cells.

The conclusion that the interaction of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) is specific for bombesin receptors is supported by a number of results. First, in rat pancreatic acini which possess high affinity specific receptors for gastrin-releasing peptide, endothelins (Et_A and Et_B receptors), vasoactive intestinal peptide (VIP receptors), and cholecystokinin (CCK_A receptors) (Sankaran et al., 1980; Bissonnette et al., 1984; Dehaye et al., 1986; Sekar et al., 1991; Hildebrand et al., 1993; Jensen, 1994; Lin et al., 1996), [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) at concentrations of up to 100 nM, did not inhibit binding of ^{125}I-VIP, ^{125}I-CCK-8, or ^{125}I-endothelin-I, whereas it caused $>90\%$ inhibition of binding of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14). These results demonstrate with different ligands, under identical conditions, in the same cells, [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) was only interacting with gastrin-releasing peptide receptors. Second, the specificity of [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) to interact with the gastrin-releasing peptide receptor and alter cell function was also demonstrated in rat pancreatic acini. In these cells the CCK_A receptor, gastrin-releasing peptide receptor and muscarinic M_3 cholinergic receptor are each coupled to phospholipase C (Rubin et al., 1984; Rowley et al., 1990; Matozaki et al., 1991; Jensen, 1994). When receptor activation of phospholipase C was assessed by determining the generation of [^{3}H]inositol phosphates, only the stimulation caused by [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) and bombesin, a gastrin-releasing peptide receptor agonist, was inhibited by the specific gastrin-releasing peptide receptor antagonist, [D-Phe^6]bombesin-(6–13)methyl ester (Wang et al., 1990; Jensen and Coy, 1991). Furthermore, a specific CCK_A receptor antagonist, L-364,718 (Evans et al., 1986), or atropine which inhibited carbachol-stimulated increases in [^{3}H]IP, had no effect on stimulation caused by [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14). These results provide additional support that [D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) is only interacting with gastrin-releasing peptide receptors to alter cell function.

The conclusion that ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) represents a unique, high affinity ligand is supported by the finding that this ligand had an affinity between 0.4–4.2 nM for each of the four subtypes of bombesin receptors. For the BB_4 receptor it had an affinity of 0.4 nM, which is 5-fold higher than bombesin and only slightly less than [Phe^{13}]bombesin (K_i-0.2 nM), which has the highest affinity of any ligand for this receptor (Nagalla et al., 1995). For the gastrin-releasing peptide receptor, ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) had an affinity of 1 nM which is similar to that of ^{125}I-[Tyr^4]bombesin (Zhu et al., 1991; Lin et al., 1995; Lin et al., 1996) and 3- to 5-fold higher than that of ^{125}I-gastrin-releasing peptide, which are the most common ligands used to identify this receptor (Ladenheim et al., 1991; Vigna and Mantyh, 1991; Mantey et al., 1993; Shapira et al., 1993). For the neuromedin B receptor only two high affinity ligands are generally used (either ^{125}I-Bolton–Hunter labeled neuromedin B or ^{125}I-[D-Tyr^0]neuromedin B (Von Schrenck et al., 1989; Ladenheim et al., 1993; Wang et al., 1993) and ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) was found to have a 3-fold lower affinity for this receptor. Lastly, only ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) binds to the orphan receptor, BRS-3, with high affinity. Even though this receptor has 50–51% amino acid homology with the gastrin-releasing peptide receptor in different species (Fathi et al., 1993; Gorbulev et al., 1992) and 47% with the neuromedin B receptor (Fathi et al., 1993; Gorbulev et al., 1992), its pharmacology has been shown to be unique (Mantey et al., 1997) in that it has a low affinity ($K_d > 300$ nM) for all other known naturally occurring or synthetic bombesin analogues besides [D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]-bombesin-(6–14). The high affinity of ^{125}I-[D-Tyr^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) for each of these four bombesin receptor subtypes is unique because it is the only known ligand with high affinity for each subtype. Radiolabeled bombesin has a high affinity for gastrin-releasing peptide receptors and BB_4 receptors (Nagalla et al., 1995; Lin et al., 1996) but has a 10-fold lower affinity for neuromedin B receptors than ^{125}I-[Tyr^0]neuromedin B (Lin et al., 1996) and does not interact with BRS-3 receptors. Similarly, ^{125}I-gastrin-releasing peptide has a high affinity for gastrin-releasing peptide receptors and BB_4 receptors but has a 20-fold lower affinity for neuromedin B receptors than ^{125}I-[Tyr^0]neuromedin B and does not interact with BRS-3 receptors. Radiolabeled antagonists, ^{125}I-[D-Tyr^6]bombesin-(6–13)methyl ester (Mantey et al., 1993) and ^{125}I-BW1023U90 [^{125}I-[(ph-Pr^6)-D-Ala^{11},Pro^{13},ψ13-14,Phe^{14}]GRP-(20–27) (Moody et al., 1996) are even more specific than the radiolabeled agonists (^{125}I-gastrin-releasing peptide, ^{125}I-

[Tyr^4]bombesin, and [^{125}I-D-Tyr^0]-neuromedin B and are highly selective for gastrin-releasing peptide receptors.

Because ^{125}I-[D-Phe^6,βAla^{11},Phe^{13},Nle^{14}]bombesin-(6–14) has high affinity for all known subtypes of bombesin receptors and yet it specifically interacts only with this class of receptors, its availability should be a valuable addition for pharmacologic studies. It allows ease of screening for high affinity ligands, identification of possible antagonists in receptor structure–function studies and it is the only ligand that allows the pharmacology and cellular basis of action of the orphan receptor, hBRS-3, to be studied. For this receptor its availability will likely facilitate studies of the natural ligand as well as the cellular biology of this receptor.

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