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Comparison of the peptide structural requirements for high affinity interaction with bombesin receptors

Jaw-Town Lin [a], David H. Coy [b], Samuel A. Mantey [a], Robert T. Jensen [a,*]

[a] *Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-1804, USA*

[b] *Peptide Research Laboratories, Tulane University Medical Center, New Orleans, LA 70112, USA*

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Abstract

Recently it has been established that both a gastrin-releasing peptide (GRP)-preferring bombesin receptor and a neuromedin B-preferring bombesin receptor mediate the mammalian actions of bombesin-related peptides. Because many tissues used for studies of the structure-activity relationship of these peptides possess both receptor subtypes and none possess only the neuromedin B-preferring subtype, there is minimal information on the peptide structural features determining receptor selectivity and it is unknown whether the determinants of agonism at both bombesin receptor subtypes are similar. In the present study we have used native cells either possessing only one bombesin receptor subtype or stably transfected with one subtype to study in detail the peptide structural requirements for interacting and activating each receptor subtype. For the naturally occurring agonists, at the GRP-preferring bombesin receptor the relative affinities were litorin = ranatensin = bombesin > GRP ≫ neuromedin B, phyllolitorin and at the neuromedin B-preferring bombesin receptor were litorin = neuromedin B = ranatensin > bombesin, phyllolitorin ≫ GRP. For the GRP-preferring bombesin receptor the heptapeptide and for the neuromedin B-preferring bombesin receptor the octapeptide was the minimal carboxyl fragment interacting with the receptor/or causing biologic activity, and the nonapeptide and full decapeptide, respectively, were the minimal required for full affinity. Making neuromedin B more bombesin- or GRP-like by replacing amino acids in position 3, 6, and 9 demonstrated that position 3 was the most important, followed by position 9 for receptor subtype selectivity. A conformationally restricted GRP analogue, [D-Cys^{6},D-Ala^{11},Cys^{14}]bombesin-(6–14) had a significantly higher affinity for GRP-preferring bombesin receptor than NMB receptor. These results demonstrate that: (1) the structure-function relations for the two mammalian bombesin receptors have important differences; (2) suggest that the active conformation of neuromedin B must differ markedly from the β-sheet model proposed for GRP; and (3) suggest that one important function of the NH_2 terminus of GRP and neuromedin B is determining receptor subtype selectivity.

Keywords: Gastrin-releasing peptide; Neuromedin B; Bombesin; Structure-function study

1. Introduction

The mammalian bombesin-related peptides, gastrin-releasing peptide (GRP) and neuromedin B are thought to play a physiological or pathological role in numerous processes (Tache et al., 1988). These include diverse effects in the central nervous system (maintenance of circadian rhythm, thermoregulation, satiety, regulation of thyrotropin release) (Brown et al., 1988; Albers et al., 1991; McCoy and Avery, 1990; Rettori et al., 1992); potent developmental effects (stimulation of chrondocytes, lung maturation) (Hill and McDonald, 1992; Sunday et al., 1993); effects in the gastrointestinal tract (stimulation of the release of many gastrointestinal hormones, pancreatic secretion, regulation of motility) (Ghatei et al., 1982; Severi et al., 1991; Jensen et al., 1988; Jensen, 1994; Kawai et al., 1988); and effects on the immune system (stimulation of macrophages, antibody secretion, natural killer cell cy-

* Corresponding author. NIH/NIDDK/DDB, Building 10/9C103, 10 Center Dr MSC 1804, Bethesda, MD 20892-1804, USA. Tel.: (301) 496-4201; fax: (301) 402-0600.

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toxicity (Ruff et al., 1985; DelaFuente et al., 1993). GRP and related peptides have potent growth effects on both normal tissues and tumors (Tache et al., 1988; Rozengurt, 1988; Moody et al., 1992) and function as autocrine growth factors in small cell lung cancer cells (Cuttitta et al., 1985). Binding studies, pharmacological studies and recent cloning studies have provided evidence that these peptides exert their biologic effects by interacting with two different subtypes of bombesin receptors, a GRP-preferring bombesin receptor and a neuromedin B-preferring bombesin receptor (Battey et al., 1991; Spindel et al., 1990; Wada et al., 1991; Von Schrenck et al., 1989; Falconieri-Erspamer et al., 1988; Jensen and Coy, 1991). Each bombesin receptor subtype is a member of the G protein-coupled 7 transmembrane superfamily, activation of which in all cells examined, stimulates increases in phospholipase C, mobilization of cellular Ca^{2+} and generation of inositol phosphates (Wang et al., 1992; Rozengurt, 1988; Benya et al., 1992). However, the two receptor subtypes differ in the affinities for GRP and neuromedin B (Battey et al., 1991; Jensen and Coy, 1991; Marki et al., 1981; Wang et al., 1993; Rozengurt, 1988) as well as for different classes of bombesin receptor antagonists (Jensen and Coy, 1991).

In the past there have been numerous structure-function studies on bombesin-related peptides attempting to define the peptide binding domain and requirements for receptor activation (Ghatei et al., 1982; Falconieri-Erspamer et al., 1988; Heimbrook et al., 1988; Marki et al., 1981; Rivier and Brown, 1978; Broccardo et al., 1976; Jensen, 1994; Jensen and Coy, 1991; Jensen et al., 1988; Guo et al., 1987; Amiot et al., 1993; Girard et al., 1984; Mukai et al., 1991; Mazzanti et al., 1982; Gargosky et al., 1987; Girard et al., 1983). Whereas these studies demonstrated that the carboxyl terminus is the biologically active portion of the molecule, the detailed structure-function information is limited in a number of ways. When most of these studies were performed, only a GRP-preferring bombesin receptor was clearly established and it was not known that two receptor subtypes mediated the actions of this family of peptides. It is now known that many of these studies were performed on tissues which likely possess mixtures of the two bombesin receptor subtypes. For example, most structure-function studies used various smooth muscle preparations, whereas other studies have examined various in vivo central nervous system effects of these peptides, each of which are now known to possess both bombesin receptor subtypes, hence the structure-function results could represent variable interaction with both bombesin receptor subtypes (Falconieri-Erspamer et al., 1988; Rivier and Brown, 1978; Broccardo et al., 1976; Marki et al., 1981; Jensen, 1994). Recent studies demonstrate some tissues used for structure-function studies such as Swiss 3T3 cells or pancreatic acinar cells possess only GRP-preferring bombesin receptors (Jensen, 1994), however none of the interactions with these various tissues, or functions measured are now known to be due to occupation of neuromedin B-preferring bombesin receptors. Therefore almost no peptide structural information is available for affinity or efficacy at the recently described neuromedin B-preferring bombesin receptor. Furthermore, there is almost no information on which are the important amino acids of the peptides for determining receptor specificity for one bombesin receptor subtype or the other. The result of this limited information is that minimal information exists to design selective agonists.

To address these issues in the present study we have systematically examined the effects of peptide length and amino acid differences in the GRP and neuromedin B carboxyl termini on the affinity for and biologic activity at GRP- and neuromedin B-preferring bombesin receptors. To be certain these results reflected interaction only with a specific bombesin receptor subtype, cells known to possess only one bombesin receptor subtype were used.

2. Materials and methods

2.1. Materials

Male Sprague-Dawley rats (100–200 g) were obtained from Taconic Farm, Germantown, NY. Leupeptin, bovine serum albumin (fraction V) (BSA) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes) were from Boehringer Mannheim Biochemicals, Indianapolis, IN; purified collagenase (CLSPA, 440 units/mg) from Worthington Biochemicals, Freehold, NJ; soybean trypsin inhibitor, ethylene glycol-bis-*N*,*N'*-tetraacetic acid (EGTA), chymotrypsin, and bacitracin from Sigma Chemical Co., St. Louis, MO; essential vitamin mixture (100 × concentrated) from Microbiological Associates, Bethesda, MD; glutamine was from the Media Section, National Institutes of Health, Bethesda, MD; bombesin, [Tyr^4]bombesin, neuromedin B, gastrin-releasing peptide (GRP), ranatensin, phyllolitorin and litorin were from Peninsula Laboratories, Belmont, CA; Na ^{125}I was from Amersham Co., Arlington Heights, IL; myo-[2-^{3}H]inositol was from New England Nuclear, Boston, MA; Dowex AF 1-X8 anion exchange resin (100–200 mesh, formate form) from Bio-Rad, Richmond, CA; and methanol (absolute) from J.T. Baker Chemical, Phillipsburg, NJ. Standard buffer consisted of 98 mM NaCl, 6 mM KCl, 25 mM Hepes, 5 mM pyruvate, 5 mM fumarate, 5 mM glutamate and 0.1% soybean trypsin inhibitor.

2.2. Preparation of dispersed rat pancreatic acini

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

2.3. Growth of the rat C_6 glioma cells and BALB 3T3 cells transfected with the rNMB receptor (rNMB receptor transfected cells)

Rat C_6 glioma cells were obtained form the American Type Culture Corp., Rockville, MD and were grown in Dulbecco's modified Eagle's medium and 10% fetal bovine serum as described previously (Wang et al., 1992, 1993). Cultures were passaged weekly at subconfluence after trypsinization. Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Grand Island Biological Co., Grand Island, NY. The cells were grown in an atmosphere of 5% CO_2. BALB 3T3 fibroblasts were stably transfected using calcium phosphate precipitation with a full length clone of the rat neuromedin B-preferring bombesin receptor generated from a rat neuromedin B clone isolated from a rat esophagus cDNA library which was subcloned into a modified version of the pCD2 plasmid. Stably transfected cells were selected for resistance to aminoglycoside G-418 as described previously (Benya et al., 1992), and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum supplemented with 218 μg/ml G-418.

2.4. Preparation of peptides

The peptides were synthesized using standard solid phase methods. 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 using a Rainin preparative HPLC 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 re-chromatography 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 description mass spectroscopy (Finnegan).

2.5. Amylase release from rat pancreatic acini

Amylase release was measured using the procedure published previously (Peikin et al., 1978; Jensen et al., 1982). Briefly, dispersed acini from one pancreas were suspended in 100 ml of standard incubation buffer containing standard buffer with 1 mM $MgCl_2$, 2.2 mM $KHPO_4$, 1.5 mM $CaCl_2$, 11 mM glucose, 1% (v/v) amino acid mixture, 1% essential amino acid mixture, and 1% bovine serum albumin. Incubations contained 0.5 ml of cell suspension and were at 37°C for 30 min. Amylase activity was determined using the Phadebas reagent and was expressed as the percentage of cellular amylase released into the extracellular medium during the incubation. For all peptides with agonist activity, the EC_{50} was calculated, the concentration that gave half-maximal stimulation seen with a maximally effective concentration of bombesin (i.e., 10 nM) using the curve-fitting program Kaleidograph.

2.6. Preparation of [^{125}I][Tyr^4]bombesin and [^{125}I][D-Tyr^0]neuromedin B

[^{125}I][Tyr^4]bombesin (2000 Ci/mmol) was prepared using iodogen and purified by HPLC using the modification (Wang et al., 1993) of the method described previously (Jensen et al., 1978). [^{125}I][D-Tyr^0]neuromedin B (2000 Ci/mol) was prepared by adding 0.4 μg iodogen to 8.0 μg of [D-Tyr^0]neuromedin B with 3 mCi Na^{125}I in 20 μl of 0.5 M KPO_4 buffer (pH 7.4). After incubation at 22°C for 6 min, 300 μl of 1.5 M dithiothreitol was added and the reaction mixture was incubated at 80°C for 60 min. Free ^{125}I was separated by applying the reaction mixture to a Sep-Pak from Waters Associates, Milford, MA, which was prepared by washing with 5 ml of methanol, 5 ml of 0.1% trifluoroacetic acid, and 5 ml of water. Free ^{125}I was eluted by 5 ml of 0.1% trifluoroacetic acid, and the radiolabeled peptide by 200 μl sequential elutions (×10) with 60% acetonitrile in 0.1% trifluoroacetic acid. Radiolabeled peptide was separated from unlabeled peptide by combining the three elutions (0.6 ml) with the highest radioactivity and applying them to a reverse-phase HPLC (Waters Associates, Model 204 with a Rheodyne injector) with a μBondapak column C_{18} (0.46 × 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v) from 24–72% acetonitrile in 60 min with a flow rate of 1 ml/min. [^{125}I][D-Tyr^0]neuromedin B was stored with 1% (w/v) bovine serum albumin at −20°C and was stable for at least 6 weeks.

2.7. Binding of [^{125}I][Tyr^4]bombesin to GRP-preferring bombesin receptors on rat pancreatic acini

Binding was performed as described recently (Mantey et al., 1993) with acini suspended in standard incubation buffer containing 0.1% bacitracin and 0.2% bovine serum albumin (w/v) (pH 7.4). Briefly, incubations contained 50 pM [^{125}I][Tyr^4]bombesin and were for 60 min at 37°C. Nonsaturable binding of [^{125}I][Tyr^4]bombesin was the amount of radioactivity associated with the acini when containing 50 pM [^{125}I][Tyr^4]bombesin plus 1 μM bombesin. Nonsaturable binding was < 10% of total binding in all experiments. All values in this paper are for saturable bind-

ing, i.e., total binding minus nonsaturable binding. K_i values were calculated by the method of Cheng and Prusoff from the dose-inhibition curves (Cheng and Prusoff, 1973).

2.8. Binding of [^{125}I][D-Tyr^0]neuromedin B to C_6 glioma cells and rNMB receptor transfected cells

Rat C_6 glioma cells or rNMB receptor transfected cells were suspended in standard incubation buffer and binding was performed as described recently (Wang et al., 1993; Benya et al., 1995). Incubations contained 75 pM [^{125}I][D-Tyr^0]neuromedin B for C_6 cells (15×10^6 cells/ml) or 50 pM [^{125}I][D-Tyr^0]neuromedin B for rNMB receptor transfected cells (5×10^6 cells/ml) and were for 60 min at 22°C. Nonsaturable binding for [^{125}I][D-Tyr^0]neuromedin B was the amount of radioactivity associated with C_6 cells or rNMB receptor transfected cells when the incubation mixture contained 1 μM neuromedin B. Nonsaturable binding was < 15% of total binding in all experiments. All values in this paper are for saturable binding, i.e., total binding minus nonsaturable binding. K_i values were calculated by the method of Cheng and Prusoff from the dose-inhibition curves (Cheng and Prusoff, 1973).

2.9. Measurement of inositol phosphates in C_6 cells

Subconfluent C_6 cells were trypsinized and cultured in a 24-well cell culture cluster (Costar Co.) at a concentration of 10^5 cells/well and [^{3}H]inositol phosphates was measured as recently described (Wang et al., 1993; Benya et al., 1995). Briefly, 1.5 μCi of myo-[2-^{3}H]inositol was added into each well 24 h later. The cells were incubated at 37°C with 5% CO_2 for 48 h. Prior to incubation with peptides inositol phosphate buffer (standard buffer containing 10 mM LiCl, 2 mM $CaCl_2$, 2% bovine serum albumin and 1.2 mM $MgSO_4$) was added to each well. For peptides with agonist activity, the EC_{50}, the concentration causing half-maximal stimulation seen with a maximally effective concentration of neuromedin B (i.e., 1 μM) was calculated using the curve-fitting program Kaleidograph. The incubation reaction was stopped by adding 1 ml of ice-cold methanol with 1% HCl (v/v) and total inositol phosphates were determined using Dowex anion exchange chromatography using the modification of the method of Berridge et al., as described previously (Qian et al., 1993; Berridge et al., 1983). Briefly, the procedures for the separation of water soluble [^{3}H]inositol phosphates were as follows. The aliquot in each well was collected and applied to a glass column containing 500 μl of 1:1 (v/v) mixture of Dowex AG1-X8 anion exchange resin in distilled water. The columns were first washed with 4 ml distilled water to remove [^{3}H]inositol; 1.25 ml of 5 mM disodium tetraborate and 60 mM sodium formate to remove [^{3}H]glycerophosphorylinositol and then 4 ml of 100 mM formic acid and 1.0 M ammonium formate to remove the total [^{3}H]inositol phosphates. The eluates were then assayed for their radioactivity after the addition of Ready Gel scintillation solution to each vial.

3. Results

For interaction and biologic activity with GRP-preferring bombesin receptors, rat pancreatic acini were used which are known to possess only this bombesin receptor subtype, occupation of which results in pancreatic enzyme secretion (Jensen, 1994). For interaction and biologic activity at neuromedin B-preferring bombesin receptors, rat C_6 glioma cells (Wang et al., 1992, 1993) and BALB 3T3 cells stably transfected with rat esophageal neuromedin B-preferring bombesin receptors and which behave in an identical fashion to the

AMINO ACID SEQUENCE

Peptide	No. from Bombesin NH_2 Terminus													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Bombesin	pGlu -	Gln -	Arg -	Leu -	Gly -	Asn -	Gln -	Trp -	Ala -	Val -	Gly -	His -	Leu -	Met-NH_2
GRP(14-27)	Met -	Tyr -	Pro -	Arg	—	—	His	—	—	—	—	—	—	—
Ranatensin				pGlu -	Val -	Pro	—	—	—	—	—	—	Phe	—
Litorin						pGlu	—	—	—	—	—	—	Phe	—
Phyllolitorin						pGlu	Leu	—	—	—	—	Ser	Phe	—
Neuromedin B					—	—	Leu	—	—	Thr	—	—	Phe	—
					1	2	3	4	5	6	7	8	9	10
					No. from Neuromedin B NH_2 Terminus									

Fig. 1. Amino acid structures of the GRP, neuromedin B and related naturally occurring peptides of the bombesin peptide family. Numbers at the top relate to the position from the amino terminus of bombesin and at the bottom numbered from the amino terminus of neuromedin B. — means amino acid identity with bombesin.

native rat neuromedin B-preferring bombesin receptor (Benya et al., 1992) were used.

The ability of six different naturally occurring bombesin-related peptides (Fig. 1) which differ at one or two amino acids at the carboxyl terminus to interact with GRP- and neuromedin B-preferring bombesin receptors was first examined (Fig. 2; Fig. 3; Table 1). For occupation of GRP-preferring bombesin receptors on rat pancreatic acini assessed by inhibition of binding of [^{125}I][Tyr^4]bombesin, the relative potencies were ranatensin = bombesin = litorin > GRP ≫ neuromedin B, phyllolitorin (Table 1; Fig. 2-top). In contrast, for neuromedin B-preferring bombesin receptors on C_6 cells or stably expressed in BALB 3T3 fibroblasts assessed by inhibition of [^{125}I][D-Tyr^0]neuromedin B binding the relative potencies were litorin = neuromedin B = ranatensin > bombesin, phyllolitorin ≫ GRP (Table 1; Fig. 2-bottom). Therefore, litorin and ranatensin were nonselective, having equipotency for both GRP- and neuromedin B-preferring bombesin receptors, whereas bombesin and GRP had a 4- and 30-fold higher affinity for GRP-preferring bombesin receptors and neuromedin B and phyllolitorin had a 120- and 15-fold higher affinity for neuromedin B-preferring bombesin receptors (Table 1). The biologic activity assessed by amylase release from rat pancreatic acini (Table 1; Fig. 3-top) or stimulation of the accumulation of [^{3}H]inositol phosphates in rat C_6 glioma cells (Table 1; Fig. 3-bottom) showed similar differences in their potencies with each peptide being fully efficacious. Therefore, for naturally occurring peptides, GRP and bombesin have the higher selectivity for the GRP-preferring bombesin receptors and neuromedin B for the neuromedin B-preferring bombesin receptors and the other three naturally occurring peptides are relatively nonselective (Table 1).

Various length carboxyl fragments of bombesin and neuromedin B were compared for their abilities to interact with GRP- and neuromedin B-preferring bombesin receptors (Table 2; Fig. 4). For occupation of GRP-preferring bombesin receptors on rat pancreatic acini, the bombesin hexapeptide [i.e., bombesin-(9–14)] was inactive, the heptapeptide had very low affinity, and the bombesin nonapeptide had equal potency to bombesin (Table 2; Fig. 4-top). For occupation of neuromedin B-preferring bombesin receptors on either C_6 cells or rNMB receptor transfected 3T3 cells, peptides shorter than the neuromedin B octapeptide had low potency and the full neuromedin B decapeptide was required to have an affinity equal to neuromedin B (Table 2; Fig. 4-bottom).

The ability of each carboxyl terminal fragment to cause changes in biologic activity at the GRP-preferring bombesin receptor (Table 2; Fig. 5-top) or neuromedin B-preferring bombesin receptor (Table 2; Fig. 5-bottom) correlated closely with their relative abilities

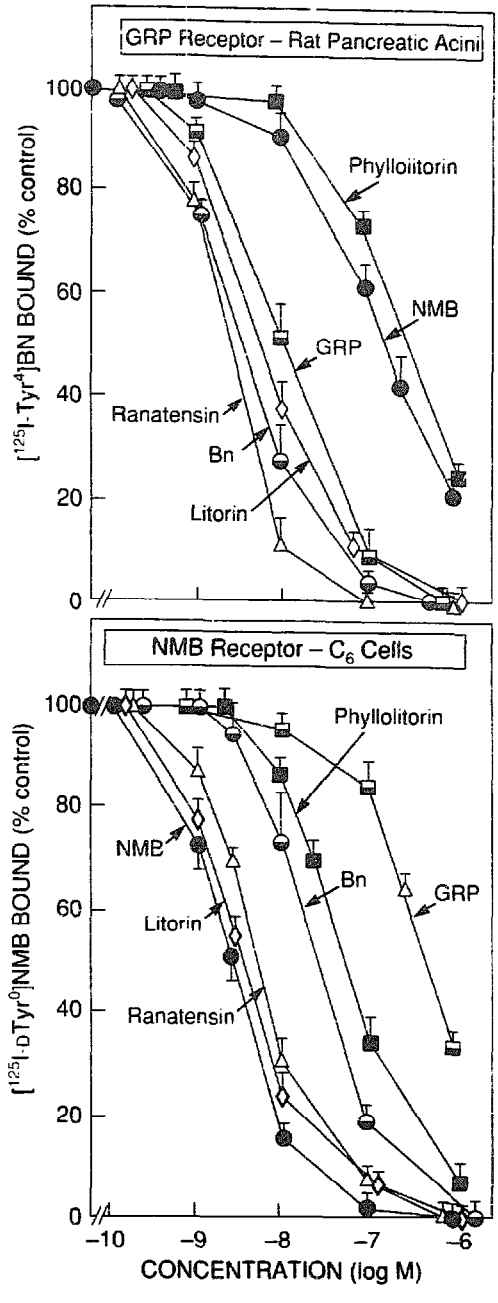

Fig. 2. The ability of bombesin and naturally occurring bombesin analogues to interact with GRP-preferring bombesin receptors on rat pancreatic acini and neuromedin B-preferring bombesin receptors on rat C_6 glioma cells. Top panel: pancreatic acini were incubated with 50 pM [^{125}I][Tyr^4]bombesin either alone or with 1 μM bombesin or the indicated concentrations of bombesin analogues at 37°C for 30 min. Results expressed as the percentage of saturable binding with no analogues present (i.e., percent control). Each point is the mean from four separate experiments and in each experiment each point was determined in duplicate. Vertical lines represent 1 S.E.M. Bottom panel: C_6 glioma cells (15×10^6/ml) were incubated with 75 pM [^{125}I][D-Tyr^0]neuromedin B either alone or with 1 μM neuromedin B or the indicated concentrations of bombesin analogues at 22°C for 60 min. Results are expressed as the percentage of saturable binding with no analogues present (i.e., percent control). Each point is the mean from four separate experiments and in each experiment each point was determined in duplicate. Vertical lines represent 1 S.E.M.

Table 1
Comparison of the affinities of bombesin and various natural occurring bombesin-related peptides for GRP-and neuromedin B-preferring bombesin receptors

Peptide	GRP-preferring Bn receptor		NMB-preferring Bn receptor		
	[^{125}I][Tyr^4]Bn binding to pancreatic acini	Bn-stimulated amylase release	[^{125}I][D-Tyr^0]NMB binding to cell lines		NMB-stimulated [^{3}H]inositol phosphate release from C_6 cells
			C_6 cells	rNMB receptor transfected cells	
	K_i (nM)	EC_{50} (nM)	K_i (nM)	K_i (nM)	EC_{50} (nM)
Bombesin	4 ± 1	0.2 ± 0.1	21 ± 7	34 ± 2	8.8 ± 1.4
GRP	15.0 ± 5.1	0.4 ± 0.1	400 ± 60	440 ± 70	150 ± 10
Neuromedin B	250 ± 5	4.7 ± 0.5	3.3 ± 0.8	4.2 ± 0.6	1.1 ± 0.2
Litorin	5.7 ± 1.3	0.4 ± 0.1	3.1 ± 0.3	6.9 ± 1.0	0.65 ± 0.06
Ranatensin	2.4 ± 0.6	0.18 ± 0.02	8 ± 1	13 ± 2	1.8 ± 0.5
Phyllolitorin	240 ± 46	3.7 ± 0.3	15 ± 4	47 ± 3	2.4 ± 0.2

K_i values for binding of various bombesin analogues to rat pancreatic acini and rat C_6 glioma cells were calculated by the method of Cheng and Prusoff (1973), and were from data shown in Fig. 2. K_i values for binding of various bombesin analogues to BALB 3T3 cells transfected with the NMB receptor from rat esophageal mucosa (rNMB receptor transfected cells) were also calculated by the method of Cheng and Prusoff (1973). The measurement of bombesin-stimulated amylase release and NMB-stimulated [^{3}H]inositol phosphate accumulation was as described Methods. EC_{50} values represent the concentrations of peptides causing half-maximal amylase release caused by 10 nM bombesin, a maximally effective concentration, or causing half-maximal [^{3}H]inositol phosphate accumulation caused by 1 μM NMB and were calculated from the data shown in Fig. 3 as described in Methods. Each value is the mean ± S.E.M. of a minimum of four separate experiments. Abbreviations: GRP = gastrin-releasing peptide; Bn = bombesin; NMB = neuromedin B.

to alter binding to these receptors. Specifically, at the GRP-preferring bombesin receptor the bombesin hexapeptide was inactive, whereas the heptapeptide had low potency but was fully efficacious (Table 2; Fig. 5-top). In contrast, at the neuromedin B-preferring bombesin receptor the neuromedin B heptapeptide was inactive whereas the octapeptide was fully efficacious but had low affinity (Table 1; Fig. 5-bottom). Similar to binding, at the GRP-preferring bombesin receptor the nonapeptide (Table 2; Fig. 5-top), and at the neuromedin B-preferring bombesin receptor, the complete neuromedin B sequence (Table 1; Fig. 5-bottom) was required for full affinity. Because the biologic activity closely correlated with the changes in binding and no partial agonists were seen with any carboxyl terminal bombesin or neuromedin B analogue, these results suggest that it is unlikely that carboxyl peptide fragments for either receptor subtype will function as receptor antagonists.

To assess the importance of each of three amino acid differences in the carboxyl terminus between GRP or bombesin and neuromedin B in determining affinity

Table 2
The affinities of bombesin or NMB carboxyl terminal fragments for GRP- and NMB-preferring bombesin receptors

Peptide	GRP-preferring Bn receptor		NMB-preferring receptor		
	[^{125}I][Tyr^4]Bn binding to pancreatic acini	Bn-stimulated amylase release	[^{125}I][D-Tyr^0]NMB binding to cell lines		NMB-stimulated [^{3}H]inositol phosphate release from C_6 cells
			C_6 cells	rNMB receptor transfected cells	
	K_i (nM)	EC_{50} (nM)	K_i (nM)	K_i (nM)	EC_{50} (nM)
NMB	250 ± 1	4.7 ± 0.5	3.3 ± 0.8	4.2 ± 0.6	1.1 ± 0.2
NMB-(2–10)	190 ± 30	18 ± 3	14 ± 4	64 ± 4	18 ± 3
NMB-(3–10)	7680 ± 1380	287 ± 47	3100 ± 790	4440 ± 90	770 ± 90
NMB-(4–10)	> 10000	3110 ± 570	inactive	inactive	inactive
Bn	4 ± 1	0.2 ± 0.1	21 ± 7	34 ± 2	8.8 ± 1.4
Bn-(6–14)	6.4 ± 0.9	0.2 ± 0.1	49 ± 9	65 ± 22	5.1 ± 1.2
Bn-(8–14)	> 10000	1159 ± 319	> 10000	> 10000	> 10000
Bn-(9–14)	> 10000	inactive	inactive	inactive	inactive

K_i values for binding studies were calculated by the method described in the legend to Table 1 and were from data shown in Fig. 4. EC_{50} values of bombesin-stimulated amylase release and NMB-stimulated [^{3}H]inositol phosphate accumulation were calculated by the method described in the legend to Table 1 and were calculated from the data shown in Fig. 5. Inactive means the indicated fragment caused no inhibition of binding or alteration in biologic activity at concentrations up to 10 μM. Abbreviations: Bn = bombesin; NMB = neuromedin B.

for GRP- or neuromedin B-preferring bombesin receptors, various neuromedin B analogues with a single amino acid substitution in position 3, 6, or 9 by the comparable amino acid in that position of GRP or bombesin were synthesized (Table 3; Fig. 6). Making neuromedin B more GRP-like by the substitution of histidine in position 3 of neuromedin B caused a 180-fold decrease of binding affinity for neuromedin B-preferring bombesin receptors (Table 3; Fig. 6-bottom) with a minimal reduction of binding affinity to GRP-preferring bombesin receptors (Table 3; Fig. 6-top). In contrast, making neuromedin B more bombesin-like by the substitution of glutamine in position 3 of neuromedin B resulted in a 3-fold increase of the binding affinity to GRP-preferring bombesin receptors (Table 3; Fig. 6-top) with a minimal effect on the binding affinity for neuromedin B-preferring bombesin receptors (Table 3; Fig. 6-bottom). Making neuromedin B more bombesin- or GRP-like by the substitution of valine in position 6 of neuromedin B increased the affinity for GRP-preferring bombesin receptors 2-fold (Table 3; Fig. 6-top) and reduced the binding affinity for neuromedin B-preferring bombesin receptors 11-fold (Table 3; Fig. 6-bottom). Similarly, making neuromedin B more bombesin- or GRP-like by the substitution of a leucine in position 9 of neuromedin B caused an 87-fold decrease of binding affinity to neuromedin B-preferring bombesin receptors (Table 3; Fig. 6-bottom) and a 3-fold decreased affinity to GRP-preferring bombesin receptors (Table 3; Fig. 6-top). In contrast, the substitution of leucine in position 10 of neuromedin B caused a similar decrease in binding affinity to the substitution of Leu^9 (i.e., 2.5-fold) for GRP-preferring bombesin receptors (Table 3; Fig. 6-top) but caused a significantly smaller decrease than the [Leu^9] substitution in the affinity for neuromedin B-preferring bombesin receptors (i.e., 20-fold decrease) (Table 3; Fig. 6-bottom).

Previous studies showed that with GRP-preferring bombesin receptors, D-Ala^{11} could be inserted in bombesin, a Leu^{14} replacement made, or a D-Phe^6 inserted into bombesin with an amino terminal truncation and each substitution had minimal effects on potency (Wang et al., 1990; Coy et al., 1989; Rivier and Brown, 1978; Marki et al., 1981; Saeed et al., 1989); however, the effects of such alterations on neuromedin B-preferring bombesin receptors are unknown. To as-

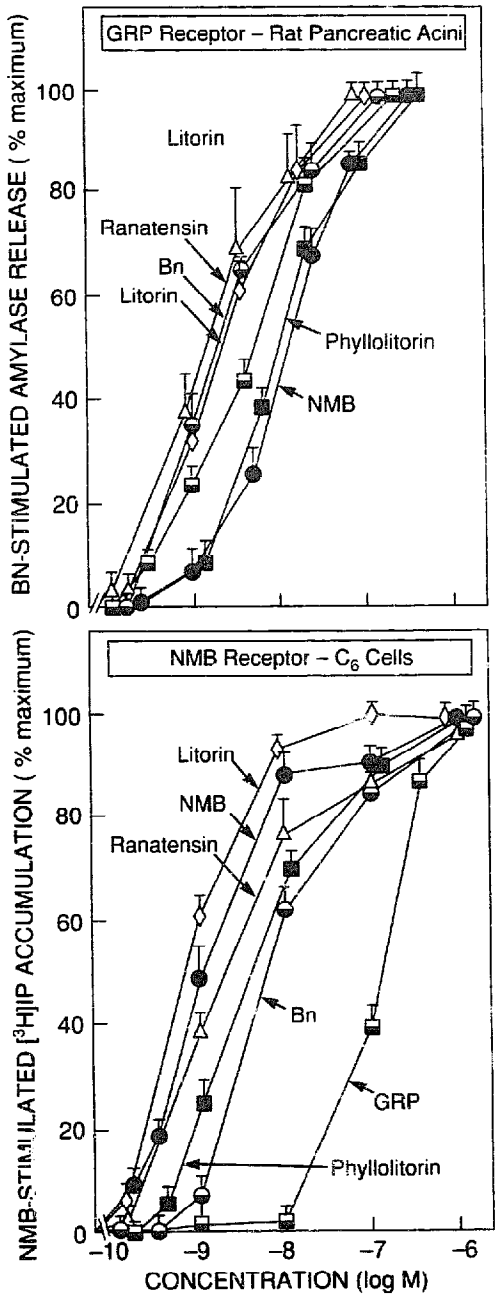

Fig. 3. The ability of bombesin and naturally occurring bombesin analogues to alter cellular function in cells with GRP- and neuromedin B-preferring bombesin receptors. Top panel: rat pancreatic acini were incubated either alone or with 10 nM bombesin or with the indicated peptides for 30 min at 37°C and amylase release was determined. Results are expressed as the percentage of the stimulated amylase release caused by 10 nM bombesin (i.e., percent maximal). Basal and 10 nM bombesin-stimulated amylase release were 2.7±0.3% and 15.8±1.3% of total cellular amylase release during the incubation ($n = 4$). Results are means from four separate experiments and in each experiment each value was determined in duplicate. Vertical bars represent 1 S.E.M. Bottom panel: C_6 glioma cells (10^5 cells/well) were cultured with 1.5 μCi/well of myo-[2-^{3}H]inositol at 37°C for 48 h. Cells were then incubated with 1 μM neuromedin B or with the indicated concentration of the various bombesin-related peptides at 22°C for 30 min. [^{3}H]Inositol phosphates were separated by ion exchange chromatography as described in Methods. Basal and 1 μM neuromedin B stimulated [^{3}H]inositol phosphate accumulation were 7300±2000 dpm and 58000±10000 dpm, respectively ($n = 4$). Results are expressed as the percentage of the accumulation caused by 1 μM neuromedin B (i.e., percent maximum). Results are means from four separate experiments and in each experiment each value was determined in duplicate. Vertical bars represent 1 S.E.M.

sess the effects of such substitutions on both bombesin receptor subtype, these substitutions were individually made and their effects on affinity and biologic activity determined. For bombesin, the substitution of leucine in position 14 caused a 5- and 25-fold decrease of binding affinity for GRP- and neuromedin B-preferring bombesin receptors, respectively (Table 3; Fig. 6, top and bottom). The substitution of a [D-Ala^{11}] in bombesin caused a 13-fold decrease in binding affinity for neuromedin B-preferring bombesin receptors (Table 3) without affecting its binding affinity for GRP-preferring bombesin receptors (Table 3). Substitution of D-Phe in position 6 of the bombesin-(6–14) fragment caused a 2- to 4-fold increase of binding affinities to

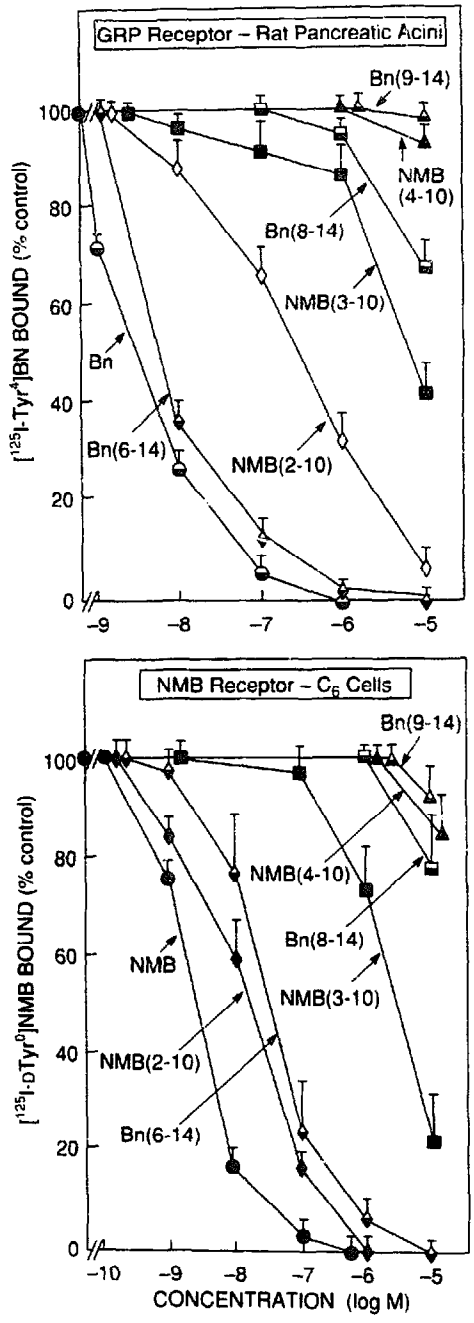

Fig. 4. The ability of various bombesin or neuromedin B carboxyl terminal fragments to interact with GRP-preferring bombesin receptors on rat pancreatic acini and neuromedin B-preferring bombesin receptors on C_6 glioma cells. The effect of the various fragments on binding of [^{125}I][Tyr^4]bombesin to rat pancreatic acini (top panel) or binding of [^{125}I][D-Tyr^0]neuromedin B to C_6 glioma cells (bottom panel) was determined and expressed as outlined in the legend to Fig. 2. Results are means from four separate experiments and in each experiment each point was determined in duplicate.

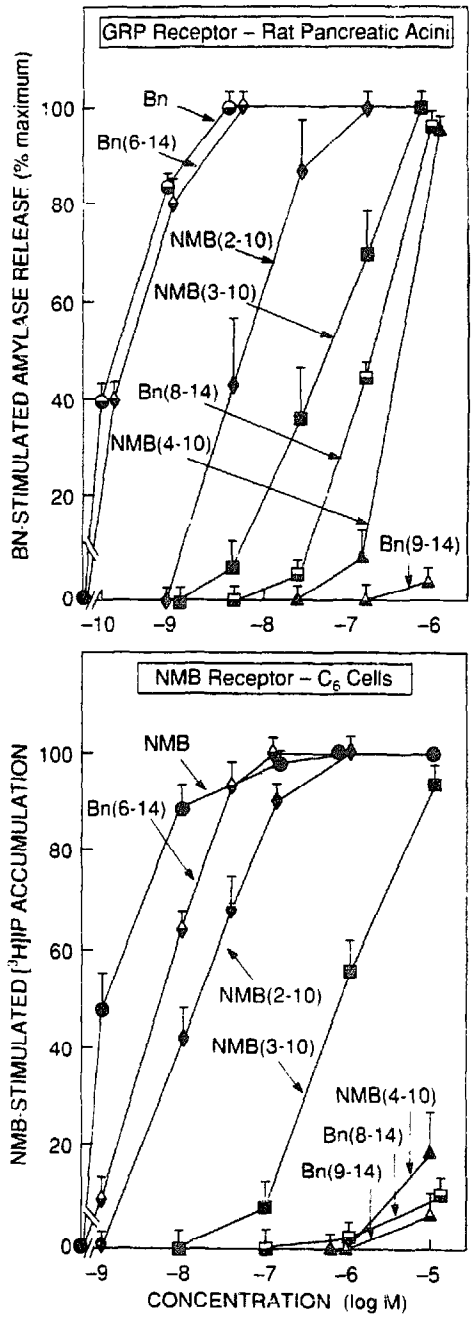

Fig. 5. The ability of various bombesin or neuromedin B carboxyl terminal fragments to alter cellular function in cells containing GRP- or neuromedin B-preferring bombesin receptors. The ability of the various fragments of bombesin or neuromedin B to stimulate amylase release from rat pancreatic acini (top panel) or [^{3}H]inositol phosphate accumulation in rat C_6 glioma cells (bottom panel) were determined and expressed as described in the legend to Fig. 3. Results are means from four separate experiments and in each experiment each point was determined in duplicate.

both bombesin receptor subtypes (Table 3; Fig. 6-top and bottom). For altering cellular function, the potencies of these various single amino-substituted analogues of bombesin and neuromedin B for stimulating amylase release through interaction with GRP-preferring bombesin receptors on rat pancreatic acini (Table 3; Fig. 7-top) or stimulated [^{3}H]inositol phosphates accumulation through interaction with neuromedin B-preferring bombesin receptors on C_6 cells (Table 3; Fig. 7-bottom) were almost identical to the relative potencies from binding studies to the GRP- or neuromedin B-preferring bombesin receptors (Table 3). Furthermore, each single amino-substituted bombesin or neuromedin B analogue was fully efficacious to either bombesin or neuromedin B, demonstrating none of these analogues functioned as partial agonists and would likely not function as a bombesin receptor antagonist (Fig. 7, top and bottom).

Recent studies have reported conformationally restricted bombesin analogues which interact with GRP-preferring bombesin receptors (Coy et al., 1991; Knight et al., 1990). To assess whether a similar conformation might interact with both bombesin receptor subtypes, a conformation-restricted analogue of bombesin, which has a high affinity for both receptor subtypes, was synthesized. Cyclization of [Cys^6,Cys^{14}]bombesin-(6–14) between positions 6 and 14 via a disulfide bridge with a substitution of D-Ala in position 11 with the formation of [D-Cys^6,D-Ala^{11},Cys^{14}]bombesin-(6–14), caused a marked decrease of binding affinity of 3600-fold compared to its linear counterpart, [D-Phe^6]bombesin-(6–14), for neuromedin B-preferring bombesin receptor (Table 3; Fig. 6-top and bottom). This cyclized analogue was also 40 times less potent compared to its linear counterpart, [D-Phe^6]bombesin-(6–14), for the GRP-preferring bombesin receptors (Table 3). This marked decrease was due to the cyclization, not the D-Ala^{11} replacement because [D-Phe^6,D-Ala^{11},Leu^{14}]bombesin-(6–14) was equipotent to [Leu^{14}]bombesin at the GRP-preferring bombesin receptor and only 25-fold less potent at the neuromedin B-preferring bombesin receptor (data not shown). For altering cellular activity the cyclic bombesin analogue was fully efficacious to bombesin or neuromedin B, and had similar relative potencies to those found on binding studies for stimulating amylase release by interacting with GRP-preferring bombesin receptors on rat pancreatic acini or stimulating [^{3}H]inositol phosphate accumulation through the neuromedin B-preferring bombesin receptor on C_6 cells (Table 3; Fig. 6, top and bottom).

4. Discussion

Similar to many families of closely related gastrointestinal/central nervous system hormones and transmitters, more than one subtype of receptor mediates the actions of the two mammalian bombesin-related peptides, GRP and neuromedin B (Jensen, 1994). At present, the structure-function information on these peptides is limited. This has occurred because of the recent recognition that not only GRP-preferring, but also neuromedin B-preferring bombesin receptors, mediate the actions of these two peptides (Von Schrenck

Table 3
The affinities of various bombesin-or NMB-related peptides with a single amino acid substitution or a cyclic analogue of bombesin for GRP-and NMB-preferring bombesin receptors

Peptide	GRP-preferring Bn receptor		NMB-preferring Bn receptor		
	[^{125}I][Tyr^4]Bn binding to pancreatic acini	Bn-stimulated amylase release	[^{125}I][D-Tyr^0]NMB binding to cell lines		NMB-stimulated [^{3}H]inositol phosphate release from C_6 cells
			C_6 cells	rNMB receptor transfected cells	
	K_i (nM)	EC_{50} (nM)	K_i (nM)	K_i (nM)	EC_{50} (nM)
NMB	250 ± 1	4.7 ± 0.5	3.3 ± 0.1	4.2 ± 0.6	1.1 ± 0.2
[His^3]NMB	360 ± 35	1.4 ± 0.5	600 ± 66	960 ± 120	220 ± 35
[Gln^3]NMB	78 ± 33	2.1 ± 0.6	4.6 ± 0.2	4.5 ± 1.4	2.7 ± 0.6
[Val^6]NMB	115 ± 7	3.9 ± 1.2	44 ± 10	21 ± 3	11.2 ± 2.7
[Leu^9]NMB	800 ± 120	5.5 ± 0.5	290 ± 20	360 ± 80	146 ± 17
[Leu^{10}]NMB	700 ± 80	36 ± 4	59 ± 15	90 ± 12	26 ± 4
Bn	4 ± 1	0.2 ± 0.1	21 ± 7	34 ± 2	8.8 ± 1.4
[Leu^{14}]Bn	21 ± 4	0.8 ± 0.2	500 ± 169	600 ± 42	970 ± 110
[D-Ala^{11}]Bn	6 ± 1	0.2 ± 0.1	275 ± 60	330 ± 80	22 ± 3
[D-Phe^6]Bn-(6–14)	1.8 ± 0.1	0.6 ± 0.1	4.5 ± 1.1	14 ± 2	1.6 ± 0.3
[D-Cys^6,D-Ala^{11}-Cys^{14}]Bn-(6–14)	690 ± 90	67 ± 6	16500 ± 5500	21700 ± 2670	> 30000

K_i values for binding studies were calculated by the method described in the legend to Table 1 and were from data shown in Fig. 6. EC_{50} values of bombesin-stimulated amylase release and NMB-stimulated [^{3}H]inositol phosphate accumulation were calculated by the method described in the legend to Table 1 and were calculated from the data shown in Fig. 7. Abbreviations: Bn = bombesin; NMB = neuromedin B.

et al., 1989; Battey and Wada, 1991) and because recent studies demonstrate that many of the tissue preparations used for previous pharmacological studies with this family of peptides, such as various smooth muscle preparations or assessments of the central nervous system effects of these peptides are now known to

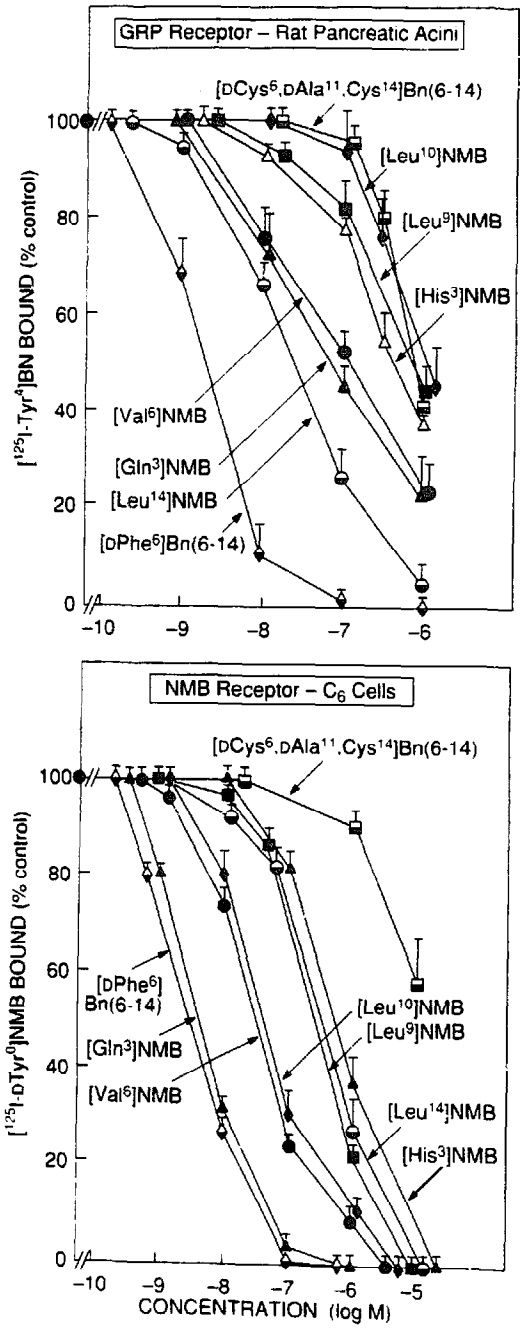

Fig. 6. The ability of bombesin- or neuromedin B-related peptides with a single amino acid substitution or of a cyclic bombesin analogue to interact with GRP-preferring bombesin receptors on rat pancreatic acini or neuromedin B-preferring bombesin receptors on rat C_6 glioma cells. The analogue's effect on binding of [^{125}I][Tyr^4]bombesin to rat pancreatic acini (top panel), or [^{125}I][D-Tyr^0]neuromedin B to C_6 glioma cells (bottom panel) were determined as described and expressed in the legend to Fig. 2. Results are means from four separate experiments and in each experiment each point was determined in duplicate.

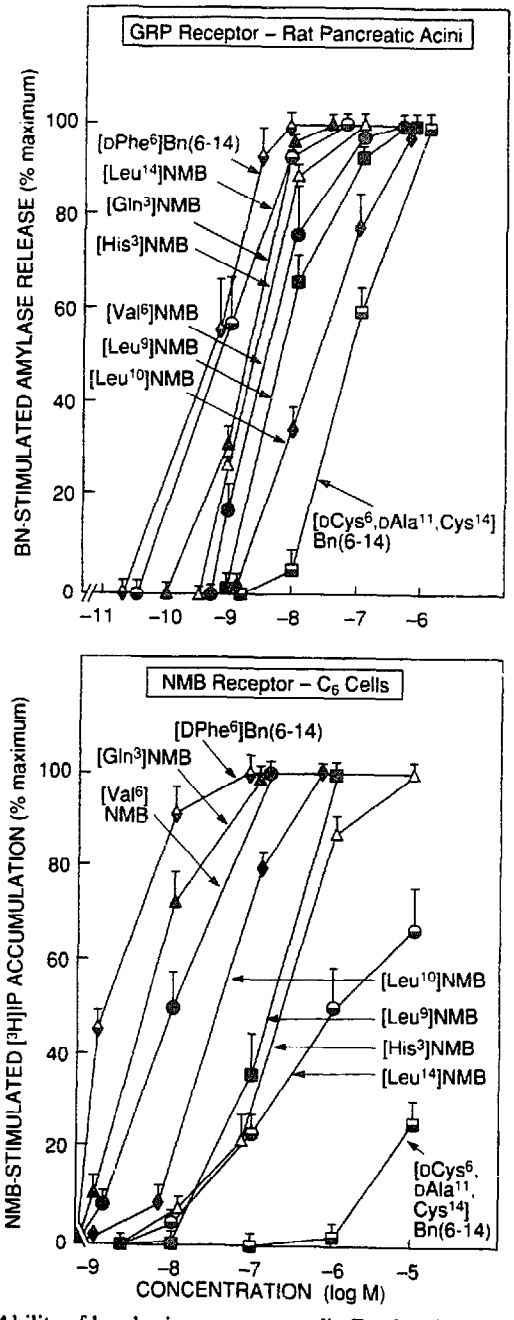

Fig. 7. Ability of bombesin- or neuromedin B-related peptides with a single amino acid substitution or of a cyclic bombesin analogue to alter cellular function in cells containing GRP- or neuromedin B-preferring bombesin receptors. The ability of the various analogues to stimulate amylase release from rat pancreatic acini (top panel) or [^{3}H]inositol phosphate accumulation in rat C_6 glioma cells (bottom panel) was performed and expressed also as described in the legend to Fig. 3.

possess both bombesin receptor subtypes (Ladenheim et al., 1990, 1992; Battey and Wada, 1991, Severi et al., 1991; Falconieri-Erspamer et al., 1988). As a result of this limited peptide structural information, at present it is not clear which domains of bombesin-related peptides are responsible for selectivity for one bombesin receptor subtype over the other. The results presented in the present study provide insights into this area.

Numerous naturally occurring nonmammalian GRP- and neuromedin B-related peptides have been described (Erspamer, 1988; Erspamer and Melchiorri, 1973). In the present study we find that the two bombesin receptor subtypes differed markedly in their relative affinities for different naturally occurring bombesin-related peptides and the results suggest the two bombesin receptor subtypes have markedly different peptide structural requirements for receptor activation. For GRP-preferring bombesin receptors on rat pancreatic acini the relative affinities were: ranatensin = bombesin = litorin > GRP ≫ neuromedin B, phyllolitorin, whereas with neuromedin B-preferring bombesin receptors on both C_6 glioma cells and rat neuromedin B-preferring bombesin receptors from esophageal muscular mucosa stably transfected into BALB 3T3 cells, the relative potencies were: ranatensin = litorin = neuromedin B > bombesin, phyllolitorin ≫ GRP. Therefore, based on their abilities to interact with neuromedin B- and GRP-preferring bombesin receptors, these naturally occurring bombesin-related peptides can be divided into four groups. Ranatensin and litorin have none to minimal selectivity because they have approximately equal high affinities for both bombesin receptor subtypes. GRP has a 30-fold greater and bombesin a 6-fold greater affinity for GRP-preferring bombesin receptors than neuromedin B-preferring bombesin receptors. In contrast, neuromedin B had a 120-fold higher affinity for the neuromedin B-preferring bombesin receptors than for GRP-preferring bombesin receptors. Lastly, phyllolitorin has moderate selectivity for neuromedin B-preferring bombesin receptors, having a 15-fold higher affinity for this receptor than the GRP-preferring bombesin receptor. These peptides vary structurally in the presence of a leucine or phenylalanine in the penultimate position from the carboxyl terminus, a valine or threonine in the 5th position from the carboxyl terminus, either a leucine, glutamine, or histidine in the 8th position from the carboxyl terminus and in the presence or absence of histidine or serine in the 3rd position from the carboxyl terminus (Minamino et al., 1983; McDonald et al., 1979) (Fig. 1). Bombesin has a leucine in the penultimate position from the carboxyl terminus (Anastasia et al., 1971), and litorin and ranatensin have phenylalanine in this position (Erspamer, 1988) (Fig. 1) and yet each has a high affinity for the GRP-preferring bombesin receptor. In contrast, neuromedin B, litorin, phyllolitorin and ranatensin all have a phenylalanine in the penultimate position from the carboxyl terminus and each had high affinity for neuromedin B-preferring bombesin receptors whereas bombesin and GRP, which have a leucine in this position (Fig. 1), have lower affinities for these receptors. These results suggest that the presence of leucine in the penultimate position of the carboxyl terminus alone is not an essential determinant for high affinity interaction with the GRP-preferring bombesin receptor, but that the presence of phenylalanine rather than leucine in this position is important for high affinity for neuromedin B-preferring bombesin receptors. These data also suggest that the presence of either threonine or valine as the 5th amino acid from the carboxyl terminus alone has little influence on the affinity for the neuromedin B-preferring bombesin receptors because although neuromedin B has a threonine, both ranatensin and litorin have valine in this position (Fig. 1) and yet all have high affinity for neuromedin B-preferring bombesin receptors. However, the presence of valine rather than threonine in this position may be somewhat important for high affinity binding to GRP-preferring bombesin receptors since each of the four peptides (bombesin, GRP, litorin and ranatensin) with high affinity for these receptors has a valine in this position. In previous studies (Broccardo et al., 1976; Saeed et al., 1989) the histidine located three amino acids from the carboxyl terminus was thought essential for biologic activity. The results in the present study show that serine can be substituted for histidine as in phyllolitorin, however this results in a marked decrease in affinity for the GRP-preferring bombesin receptor while it has a minimal effect on the neuromedin B-preferring bombesin receptor.

To clearly establish the importance of each of these two amino acids (i.e., the 5th and 2nd amino acids from carboxyl terminus) (Fig. 1) as well as the different amino acids in the 8th position from the carboxyl terminus (Fig. 1) in determining bombesin receptor subtype affinity, selectivity, and ability to alter cellular function, we examined various neuromedin B analogues with a single amino acid replacement in these positions that made them more GRP-like. Substitution of histidine in position 3 of neuromedin B (Fig. 1), which makes it more GRP-like (Fig. 1), caused a 1.5-fold decrease in affinity for the GRP-preferring bombesin receptors and a 180-fold decrease in affinity for the neuromedin B-preferring bombesin receptors. In contrast, substitution of glutamine in this position, therefore making neuromedin B more bombesin-like, resulted in a 3-fold increase in affinity for the GRP-preferring bombesin receptors and caused no change in the affinity for the neuromedin B-preferring bombesin receptors. These results clearly demonstrate that the difference in affinity of bombesin and GRP for the neuromedin B-preferring bombesin receptors is strongly determined by the presence or absence of a glutamine or histidine as the 8th amino acid from the carboxyl terminus, whereas this has only a minimal effect on the affinity for the GRP-preferring bombesin receptors. Because the substitution of leucine in the penultimate position of neuromedin B, such as occurs

in bombesin and GRP, caused a 90-fold decrease of affinity for the neuromedin B-preferring bombesin receptors and a 3-fold decrease for GRP-preferring bombesin receptors, the single amino acid substitution results confirm the conclusion reached using the naturally occurring bombesin-related peptides in demonstrating that the presence of phenylalanine rather than leucine in the penultimate position is only an important determinant of high affinity interaction with neuromedin B-preferring bombesin receptors. Furthermore, because the substitution of valine for threonine in position 6 of neuromedin B (Fig. 1), making it similar to bombesin and GRP, caused a 14-fold decrease of affinity for neuromedin B-preferring bombesin receptors and a 2-fold increase of affinity for GRP-preferring bombesin receptors, the presence of the threonine in position 6 of neuromedin B clearly is an important determinant of selectivity but it is less important than the phenylalanine for leucine substitution in the penultimate amino acid or the leucine substitution in position 3 of neuromedin B for the glutamine in bombesin or histidine in GRP (Fig. 1). These results demonstrate that in contrast to a recent in vivo study of the effects of GRP and neuromedin B analogues on gastrin, insulin and glucagon release in dogs (Mukai et al., 1987) which concluded that the Thr^6 for Val^6 replacement in neuromedin B was particularly important for selective bioactivity, our results demonstrate that the selectivity for bombesin receptor subtypes is determined principally by the amino acid differences at both the amino and carboxyl terminus and to a lesser extent by the Val^6 replacement.

The present study demonstrates that the GRP-preferring bombesin receptors and neuromedin B-preferring bombesin receptors also differ in the minimal peptide fragment necessary for detectable changes in biological activity and for maximal potency. Previous studies have given variable results of the minimal bombesin/GRP carboxyl terminal fragment with biologic activity. In rat stomach strips (Girard et al., 1984), the carboxyl terminal bombesin tetrapeptide has agonist activity and the nonapeptide is the minimal fragment with potency similar to bombesin. In contrast, in the CNS (Guard et al., 1993) for inhibition of binding of [^{125}I][Tyr^4]bombesin to either GRP-preferring bombesin receptors or to neuromedin B-preferring bombesin receptors the heptapeptide was inactive; however, the acetylated heptapeptide had biologic activity. The decapeptide was the minimal fragment required for full biological potency at the GRP- and the neuromedin B-preferring bombesin receptor (Guard et al., 1993). GRP and bombesin require at least the carboxyl terminal heptapeptide for biological activity at GRP-preferring bombesin receptors in smooth muscle strips (Broccardo et al., 1976; Falconieri-Erspamer et al., 1988), for producing hypothermia (Marki et al., 1981; Girard et al., 1983), or for growth effects on murine Swiss 3T3 cells (Heimbrook et al., 1988) and the nonapeptide was required for full potency (Mazzanti et al., 1982). In contrast, the octapeptide was required as the minimal carboxyl fragment for inhibiting binding to Swiss 3T3 cells (Gargosky et al., 1987), which possess GRP-preferring bombesin receptors, and the nonapeptide was required for full potency. These variable results are likely due to differing peptide degradation rates and likely the presence of both GRP- and neuromedin B-preferring bombesin receptors in some of these systems. Our results with rat pancreatic acini which only possess GRP-preferring bombesin receptors (Jensen, 1994) demonstrate that peptides shorter than bombesin heptapeptide are inactive and also demonstrated that the nonapeptide is the minimal fragment required for full potency at the GRP-preferring bombesin receptor subtype. In contrast, for the neuromedin B-preferring bombesin receptors, the heptapeptide is inactive and the neuromedin B octapeptide is the minimal fragment with biological activity. Also in contrast to the GRP-preferring bombesin receptor subtype, the full decapeptide sequence of neuromedin B is required to have full potency at neuromedin B-preferring bombesin receptors. These data demonstrate that amino acids in position 7–10 from the carboxyl terminus of GRP or neuromedin B have markedly different relative importances in determining high affinity receptor interaction with the two different bombesin receptor subtypes. These results, combined with results in the present study demonstrating that the GRP- and neuromedin B-preferring bombesin receptors differ in the importance of the carboxyl terminal methionine in determining receptor affinity, and in the ability of the conformationally restricted bombesin analogue to interact with GRP-preferring and neuromedin B-preferring bombesin receptors, suggest that the active conformations of the peptides may differ markedly for interacting with the two different bombesin receptor subtypes. Previous studies (Saeed et al., 1989; Marki et al., 1981; Kull et al., 1992) have reported that substitution of leucine or norleucine for methionine at the carboxyl terminus of bombesin or GRP has minimal effect on affinity for the GRP-preferring bombesin receptors. We now find in contrast that this substitution has a much greater effect on analogue affinity for neuromedin B-preferring bombesin receptors. Specifically, substitution of leucine for methionine in neuromedin B or of bombesin caused a greater than 20-fold decrease of affinity for neuromedin B-preferring bombesin receptors and but had only a minimal effect on affinity for the GRP-preferring bombesin receptors. It has been proposed (Coy et al., 1988) that the active conformation of bombesin or GRP for interacting with the GRP-preferring bombesin receptor is a β-sheet structure in the carboxyl-terminal

position with a turn at position 10–13 of bombesin and that hydrogen bonds exist between the terminal Leu^{14} amide NH_2 and the Trp^8 C = 0, between Leu^{13} C = 0 and Val^{10} *N*-H and between Leu^{13} *N*-H and Val^{10} C = 0. Support for this model by structure-function studies and computer modeling has recently been reported (Kull et al., 1992). Substitutions of D-Ala in position 11 of bombesin would tend to stabilize this type of folding. The fact that no activity is lost when this substitution is made in bombesin but that much affinity is lost for the neuromedin B-preferring bombesin receptor strongly suggests greatly differing binding conformations. Also, the fact that for the two different bombesin receptor subtypes the insertion of leucine for methionine has such a differing effect on affinity as well as the difference in the minimal length of the biologically active carboxyl fragment length, demonstrating the differing importance of amino acids in positions 7–10 from the carboxyl terminus for each bombesin receptor subtype, suggests the model of the active configuration of bombesin for interacting with the GRP-preferring bombesin receptor is not applicable to the neuromedin B-preferring bombesin receptor. Recent studies (Knight et al., 1990; Coy et al., 1991) have reported various cyclic bombesin analogues with restricted conformations that can interact with GRP-preferring bombesin receptors and function as agonists or antagonists. To provide additional support that the conformation of bombesin-related peptides for activating the two bombesin receptor subtypes may differ significantly, we examined the ability of one such conformationally restricted cyclic bombesin analogue and its linear counterpart to interact with both bombesin receptor subtypes. Whereas the linear peptide, [D-Phe^6]bombesin-(6–14), had no selectivity for the two receptor subtypes, the cyclized analogue, [D-Cys^6,D-Ala^{11},Cys^{14}]bombesin-(6–14), had a 30-fold higher affinity for the GRP-preferring bombesin receptors rather than for neuromedin B-preferring bombesin receptors. These results suggest that the constrained configuration induced by cyclization resembled more an active configuration for interaction with GRP-preferring bombesin receptors than neuromedin B-preferring bombesin receptors and supports the conclusion of a significant difference in peptide conformation required to activate the two bombesin receptor subtypes. In the future this cyclized bombesin analogue may provide an important starting point for the development of even more selective GRP-preferring bombesin receptor agonists.

In conclusion, the present study demonstrates that despite the close structural homology between both the biologically active carboxyl terminus of GRP and neuromedin B and in their receptors, the structure-function relationships for receptor activation are markedly different. Structure-function results suggest that the active conformation of neuromedin B must differ markedly from the proposed β-sheet model for the active conformation of GRP. The structure-function studies demonstrate for the first time that one important function of the amino terminus of the carboxyl decapeptide of both GRP and neuromedin B is in determining receptor subtype selectivity.

Acknowledgements

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