0026-895X/96/051355-09$3.00/0

MOLECULAR PHARMACOLOGY, 50:1355–1363 (1996).

Discovery of High Affinity Bombesin Receptor Subtype 3 Agonists

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Received April 8, 1996; Accepted July 15, 1996

SUMMARY

Human bombesin receptor subtype 3 (BRS-3) was cloned based on its homology to the human gastrin-releasing peptide (GRP) receptor and neuromedin B (NMB) receptor. Some bombesin-like peptides were shown to activate BRS-3 expressed in *Xenopus laevis* oocytes, but only at relatively high concentrations, which suggests that BRS-3 is an orphan receptor. To study the pharmacology of BRS-3 in the context of a mammalian cell, we used BR2 cells, which are Balb/3T3 fibroblasts transfected with BRS-3 cDNA. A number of bombesin-like peptides found in mammals and amphibians stimulated calcium mobilization in BR2 cells but exhibited no effect on nontransfected parental Balb/3T3 cells. Of these peptides, NMB (EC_{50} ~ 1–10 μM) was the most active for stimulation of calcium mobilization. Testing of a series of NMB analogs truncated at the amino terminus and carboxyl terminus indicated that the minimal size of NMB required for retention of full activity was Ac-NMB(3–10). Systematically replacing each residue with alanine, or changing its chirality, demonstrated that the carboxyl-terminal residues His8, Phe9, and Met10 of NMB are important for optimal activity. We also tested whether a number of bombesin (BN) analogs that are potent pure or partial antagonists of the GRP receptor can activate BRS-3 in BR2 cells. One such analog, D-Phe^6-BN(6–13) propyl amide, activated BRS-3-mediated calcium mobilization with an EC_{50} level of 84 nM. Through additional synthesis, we generated a significantly more potent analog, D-Phe^6-Phe^{13}-BN(6–13) propyl amide, which displayed an EC_{50} level of 5 nM for activation of BRS-3. Taken together, our data show that the core portions of bombesin-like peptides required for activation of BRS-3 are similar to those necessary for activation of the GRP and NMB receptors and thus provide pharmacological evidence that BRS-3 is in the BN receptor family. Furthermore, we have identified an agonist of BRS-3, namely D-Phe^6-Phe^{13}-BN(6–13) propyl amide, which is roughly 1000-fold more potent than BRS-3 agonists described previously.

BRS-3 is an orphan receptor containing a putative seven-transmembrane spanning domain that was cloned from human SCLC cells and guinea pig uterus and is homologous to GRP and NMB receptors (1, 2). Human BRS-3 has 47% and 51% amino acid identity to the human NMB and GRP receptors, respectively (3). BLPs are a family of carboxyl-terminally amidated peptides with pleiotropic biological effects. Whereas 14 BLPs have been identified in amphibian species, sharing extensive homology over the carboxyl-terminal 8–10 amino acid residues (4, 5), only two family members, GRP and NMB, have been found in mammalian tissues. The high degree of homology between BRS-3 and NMB and GRP receptors is consistent with BRS-3 belonging to a family of BN receptor subtypes. Although relatively high concentrations of several BLPs have been shown to activate BRS-3 expressed in *Xenopus laevis* oocytes (1), an agonist of BRS-3 potent enough to activate the receptor under physiological conditions has not been identified.

In voltage-clamped *X. laevis* oocytes injected with BRS-3 mRNA, the BLPs BN, GRP, NMB, Phe8-phyllolitorin, and ranatensin evoked a chloride current (1). Activity was consistently observed, however, only when BLPs were applied at 10 μM. In contrast, at concentrations roughly 3 orders of magnitude lower, BLPs can activate human GRP and NMB receptor-mediated chloride currents in this system (3). These experiments indicate that BRS-3 has a relatively low affinity for known members of the BLP family and that the physiological activator of BRS-3 may be a novel BLP structure. An alternate possibility is that, unlike the human GRP and NMB receptors, human BRS-3 couples poorly to chloride currents in voltage-clamped *X. laevis* oocytes and may display higher sensitivity to BLPs when expressed in a mammalian cell. It may also be possible that BRS-3, although structurally related to the NMB and GRP receptors, has a physiological activator that is unrelated to GRP or NMB.

GRP and NMB, as well as other BLPs, have varied biological activities in mammalian systems (6), including the stimulation of hormone release, smooth muscle contraction, and

ABBREVIATIONS: BRS-3, bombesin receptor subtype 3; NMB, neuromedin B; BN, bombesin; BLP, bombesin-like peptide; $[Ca^{2+}]_i$, intracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GRP, gastrin-releasing peptide; NMB, neuromedin B.

cell proliferation and the regulation of appetite, behavior, and homeostasis. BLPs also serve as autocrine growth factors in some SCLC cell lines (7, 8) and have been postulated to play a role in regulating the growth of other cancers, including those of the breast (9), colon (10), prostate (11), and pancreas (12). The expression of BRS-3 mRNA is more restricted than GRP receptor mRNA (1), which is widely expressed in gut, brain, and neuroendocrine tissues, or NMB receptor mRNA, which is expressed in brain and esophageal smooth muscle tissue (3). BRS-3 mRNA has been detected in some SCLC cell lines and normal bronchial epithelial cells (13), which suggests that BRS-3 may play a role in regulating the growth of SCLC. BRS-3 mRNA is also expressed in pregnant human and guinea pig uteri (2, 14) and in rat testes (1), which suggests a possible role of BRS-3 in reproduction.

To further establish the functional relationship between BRS-3 and the NMB or GRP receptors and to gain insight into its natural ligand, it is critical to characterize the pharmacology of BRS-3 in the context of a mammalian cell. However, BRS-3-expressing cell lines have not been identified that do not also express the NMB receptor and GRP receptor, which would interfere in analysis of the activity of BLPs for BRS-3. Consequently, we have prepared BLP-receptor-negative Balb/3T3 cell lines stably transfected with BRS-3 cDNA. NMB, at micromolar doses, stimulated calcium mobilization in these cells, which depended on the expression of BRS-3. NMB, in combination with insulin, also promoted quiescent BR2 cells to enter the S phase of the cell cycle (15). These results indicate that BRS-3 can be activated by NMB, as was found with BRS-3 expressed in *X. laevis* oocyte cells. Furthermore, the coupling of BRS-3 to calcium mobilization in these cells provided a suitable assay to characterize the pharmacology of BLPs and analogs with respect to BRS-3 expressed in a mammalian cell.

In this study, we used BRS-3-mediated calcium mobilization to assay the activity of a number of BLPs and NMB analogs on BRS-3. These data reveal similarities in the pharmacology of BRS-3 and the GRP and NMB receptors, which provide functional evidence of the homology of these receptor forms. Furthermore, through screening of BN analogs for BRS-3 agonistic activity and through optimization, we obtained a potent BRS-3 agonist, D-Phe^6-Phe^{13}-BN(6–13) propyl amide. This analog will facilitate additional studies to identify the biological function of BRS-3 and to discover potent and selective receptor antagonists.

Experimental Procedures

Materials. GRP(1–27), NMB, alytesin, litorin, BW-10 [deamino-Phe^6-His^7-D-Ala^{11}-D-Pro^{13}-ψ(CH$_2$NH)Phe^{14}-BN(6–14)], Leu^{13}-ψ(CH$_2$NH)-Leu^{14}-BN, ranatensin, substance P, and gastrin were purchased from Bachem California (Torrance, CA). D-Phe^6-BN(6–13) methyl ester was a gift from Dr. David Coy (Tulane University, New Orleans, LA), and BIM-26226 (F$_5$-D-Phe^6-D-Ala^{11}-BN(6–13) methyl ester) was a gift from Biomeasure (Milford, MA). Fluo-3 acetoxymethyl ester and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Hygromycin B was purchased from Calbiochem (La Jolla, CA). All other cell culture media, supplements, and fetal calf serum were purchased from BioWhittaker (Walkersville, MD). Bovine serum albumin (fatty acid free), calcium ionophore A23187, and HEPES were purchased from Sigma Chemical (St. Louis, MO). All other chemicals used were of reagent grade.

Synthesis of peptides. The des-methionine BN analogs D-Phe^6-Leu^7-BN(6–13) propyl amide, D-Phe^6-Phe^{13}-BN(6–13) propyl amide, D-Phe^6-Leu^7-Phe^{13}-BN(6–13) propyl amide, D-Phe^6-Leu^7-Thr^{10}-BN(6–13) propyl amide, D-Phe^6-Thr^{10}-BN(6–13) propyl amide, D-Phe^6-Leu^7-Thr^{10}-Phe^{13}-BN(6–13) propyl amide, D-Phe^6-Thr^{10}-Phe^{13}-BN(6–13) propyl amide, and D-Phe^6-BN(6–13) propyl amide were synthesized by Peninsula Laboratories (Belmont, CA). 4-Pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-CO-CH$_2$CH$_2$Ph-BN(7–13) methyl amide and NMB deletion peptides [NMB(1–9), Ac-NMB(2–10), Ac-NMB(3–10), Ac-NMB(4–10), and Ac-NMB(5–10)] were custom synthesized by Multiple Peptide Systems (San Diego, CA). The remaining peptides used in our study, namely analogs of NMB, were synthesized by solid-phase peptide synthesis methods, using 9-fluorenylmethoxycarbonyl chemistry in BIOSEARCH 9500 and 9600 instruments (Milligen/Biosearch, Navato, CA). The peptides were cleaved from the resin in trifluoroacetic acid and preparatively purified by reverse- phase high performance liquid chromatography on a C-18 column using a H$_2$O/CH$_3$CN/0.01% trifluoroacetic acid solvent gradient. Identities of all peptides were confirmed by mass spectroscopy, and purity was determined to be >95% by analytical reverse-phase high performance liquid chromatography.

Culture of BR2 transfectants. Balb/3T3 fibroblasts were obtained from the American Type Culture Collection and cultured under their recommended conditions. The open reading frame for BRS-3 was cloned into pBBS70, and the cell transfections were performed as described previously (15). Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 4.5 g of glucose/liter, 10% fetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate, 2 mM glutamine, and the selection reagent hygromycin B (100 μg/ml) at 37°, in a humidified atmosphere containing 10% CO_2.

Analysis of intracellular calcium mobilization. The calcium-chelating dye Fluo-3 acetoxymethyl ester can be loaded into intact cells and trapped by cleavage of the ester moiety by intracellular esterases. In the presence of free intracellular calcium, Fluo-3 fluorescence increases, and relative changes in calcium concentration can be monitored using the appropriate analytical instrumentation (16, 17).

To prepare cells for analysis, flasks of cells grown to confluence were rinsed twice with phosphate-buffered saline and 2 mM EDTA. Cells were dislodged from the flasks by agitation after incubation with phosphate-buffered saline + 2 mM EDTA + 1% glucose at 37° for 10–15 min. Cells were pelleted at 800 × *g* for 10 min and resuspended in loading medium (Roswell Park Memorial Institute 1640 culture medium containing 25 mM HEPES, pH 7.5, and 0.1% bovine serum albumin) at a density of 3–5 × 10^6 cells/ml. Fluo-3 acetoxymethyl ester and Pluronic F-127 were added to cells to give final concentrations of 10 μM and 0.05% (w/v), respectively. After an incubation for 1 hr in the dark at 37°, extracellular dye was removed by washing the cells three times with loading medium. Cells were finally resuspended at a density of 2–5 × 10^5 cells/ml in assay medium (Dulbecco's modified Eagle's medium containing 25 mM HEPES, pH 7.5, and 1.0% fetal calf serum) and used within several hours for measurements of $[Ca^{2+}]_i$ concentration.

Cell fluorescence was measured using a fluorescence-activated cell sorter (FACScan equipped with argon laser; Becton Dickinson, San Jose, CA), which minimizes the number of cells as well as the time required to perform an analysis. The volume of cell suspension used per assay was 0.5 ml (1–3 × 10^5 cells). All peptide additions to cells were performed at ambient temperatures (20–23°). Untreated or vehicle-treated cells were sampled for 20 sec to establish the basal fluorescence. Both conditions gave the same values. Measurements on test compounds were initiated within 5 sec of compound addition to a fresh tube of cells. Only gated data from viable cells were used, as reflected in their side and forward light-scattering characteristics, and their average Ca^{2+}-dependent fluorescence was measured using a fluorescein filter. Cells loaded with Fluo-3 increase their fluorescence when treated with 10 μM of the calcium ionophore A23187, a signal that represents the maximal fluorescence attainable for each experiment. The fluorescent signal increases from 2- to 3-fold over the basal fluorescence when calcium ionophore is added, and this

treatment was measured in each experiment, to verify cell loading of Fluo-3. The maximum A23187 signal varied from experiment to experiment, depending on the efficiency of Fluo-3 loading. Data for basal and stimulated cell fluorescence, analyzed using LYSYS II software (Becton Dickinson, San Jose, CA), represent the mean fluorescence of cells over the relevant sampling period (first 20 sec of untreated and 30 sec after addition of test solutions). Because calcium responses were induced very rapidly and measurements were completed within 30 sec, it was unlikely that the results were affected significantly by peptide degradation. Data points are the average ± standard error of two or more replicates within each experiment, and plots and bar graphs represent at least two or more independent experiments.

Results

BLPs activate BRS-3 expressed in Balb/3T3 cells. Previously, we observed that NMB triggered a rapid mobilization of intracellular calcium in BRS-3-transfected Balb/3T3 cells (15) but had no effect on nontransfected parental cells, consistent with the lack of mRNA for BLP receptors in parental Balb/3T3 cells. The EC_{50} value of NMB in this assay was between 1 and 10 μM. This low potency suggests that NMB is not a physiological activator of BRS-3, as was also suggested from studies in which BRS-3 was expressed in *X. laevis* oocytes (1).

To potentially discover agonists with higher potency for BRS-3, we screened a number of other members of the BLP family, shown in Table 1, for BRS-3-mediated calcium mobilization activity. For these experiments, we used Balb/3T3 cells stably expressing BRS-3 cDNA under the control of the myeloproliferative sarcoma virus promoter (15) (termed BR2 cells). Previously, we found that GRP and NMB receptor cDNAs expressed in the same manner in Balb/3T3 cells efficiently coupled to several signaling pathways, including activation of phospholipase C and calcium mobilization, and could induce quiescent cells to enter the S phase of the cell cycle. To conveniently monitor changes in $[Ca^{2+}]_i$ in BR2 cells, we used a fluorescence-based assay using the calcium-chelating dye Fluo-3, which was loaded into the cells.

As shown in Fig. 1, several BLPs, added at a concentration of 1 μM, promoted calcium mobilization in BR2 cells. The most active peptides were NMB, followed by litorin and ranatensin. None of the peptides were able to stimulate Ca^{2+} mobilization in untransfected Balb/3T3 cells or in cells transfected with a control vector (data not shown), which demonstrates that their effects on BR2 cells resulted from activation of BRS-3. The three BLPs that activated BRS-3 (i.e., litorin, NMB, and ranatensin) have a common carboxyl-terminal sequence (. . . -Gly-His-Phe-Met-amidated) (Table 1). In contrast, GRP, BN, and alytesin, which have a leucine

TABLE 1

BLPs share extensive sequence homology at their carboxyl-terminal ends, as indicated by the underlined regions. BLPs are also carboxyl-terminally amidated.

Peptide	Sequence
Alytesin	pEGRLGTQWAVGHLM-NH_2
Gastrin-releasing peptide	VPLPAGGGTVLTKMYPRGNHWAVGHLM-NH_2
Bombesin	pEQRLGNQWAVGHLM-NH_2
Neuromedin B	GNLWATGHFM-NH_2
Litorin	pEQWAVGHFM-NH_2
Ranatensin	pEVPQWAVGHFM-NH_2
Phyllolitorin	pELWAVGFM-NH_2

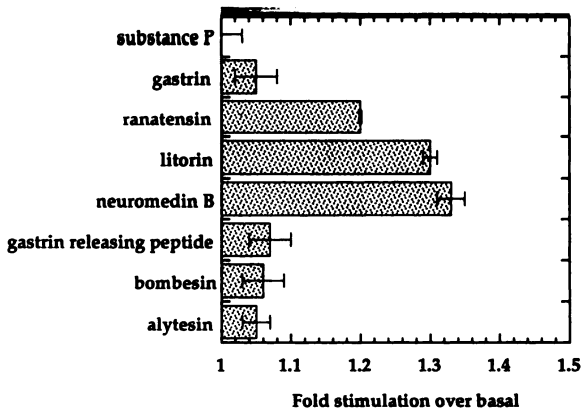

Fig. 1. BLPs stimulate Ca^{2+} mobilization in BR2 cells. Cells were loaded with Fluo-3 as described in Experimental Procedures. Peptides were added at a final concentration of 1 μM to the cells, and fluorescence was measured, gated, and averaged as described in Experimental Procedures. Data represent the average ± standard error fold-stimulation of two or more determinations and are representative of two or more experiments.

instead of a phenylalanine at the penultimate position, promoted very little calcium mobilization at a concentration of 1 μM. At a 10-fold higher concentration (10 μM), GRP and BN were able to promote a significant increase in Ca^{2+} mobilization (data not shown). Taken together, these data indicate that a phenylalanine at the penultimate position significantly enhances BRS-3 agonistic activity.

Leu^8-phyllolitorin, which has a serine instead of a histidine at position 7 (Table 1), has been shown to be inactive in experiments using *X. laevis* oocytes expressing BRS-3, whereas phyllolitorin, like bombesin, GRP, NMB, and ranatensin, promoted a response at 10 μM (1). We also found that an analog of NMB, which was extended at its carboxyl terminus by a glycine residue, has significantly less activity than NMB (data not shown). Such an extended NMB form could potentially exist *in vivo* as an intermediate in the posttranslational proteolysis and carboxyl-terminal amidation of prepro-NMB. Other peptides tested, which were not in the BLP family, namely substance P and gastrin, also did not promote an elevation of Ca^{2+} mobilization in BR2 transfectants.

A number of additional experiments supported our conclusion that the calcium mobilization responses promoted by BLPs in BR2 cells, discussed above, were mediated by BRS-3. First, NMB promoted an increase in Ca^{2+} mobilization by two independent Balb/3T3 transfectants expressing BRS-3, in addition to BR2 cells (data not shown). The EC_{50} values for these responses were roughly 1 μM, although it should be noted that these values represent minimal estimates because of the low potency of NMB and the uncertainty in establishing the saturation point for the response. Even though Balb/3T3 cells do not express GRP or NMB receptors, it has been found that the expression of receptors in parental cells and stable transfectants can vary. A comparison of the potency of BLPs for stimulating calcium mobilization through GRP and NMB receptors with their activity on BR2 cells, however, rules out the possibility that BR2 cells express functionally significant levels of GRP or NMB receptors. NMB and GRP stimulated an elevation of Ca^{2+} mobilization in Balb/3T3 cells expressing either the human NMB receptor or GRP receptor (measured by Fluo-3 fluorescence) with an EC_{50}

value of about 1–5 nM (data not shown). By comparison, roughly 3 orders of magnitude higher concentrations of NMB (or GRP) were required to stimulate calcium mobilization in BR2 cells.

Minimal sequence of NMB required for activation of BRS-3. To determine the importance of carboxyl-terminal and amino-terminal residues of NMB for agonism of BRS-3, we prepared a series of truncated NMB peptides (Table 2) and tested their ability to promote Ca^{2+} mobilization in BR2 cells. The deletion of the carboxyl-terminal methionine residue ([desMet]NMB), which leaves an amidated phenylalanine at the carboxyl terminus, resulted in complete loss of activity when assayed at a concentration of 1 μM. We also found that [desMet]NMB, tested at 10 μM, failed to block a calcium response promoted by NMB, indicating that it does not function as an antagonist and likely fails to bind significantly to BRS-3 under our assay conditions (data not shown).

Four additional NMB analogs that had progressive deletions from the amino terminus of NMB were synthesized and tested. To reduce effects in a charged amino terminus in truncated peptides, the amino terminus was acetylated. As shown in Table 2, up to two residues could be deleted from the amino terminus, yielding Ac-NMB(3–10), without loss of activity. In fact, Ac-NMB(3–10) displayed an EC_{50} value of 219 nM (Fig. 2) and thus was significantly more potent than NMB (EC_{50} ~ 1–10 μM). Additional deletions from the amino terminus resulted in significant loss of activity. For instance, Ac-NMB(4–10) and Ac-NMB(5–10) displayed 55% and 24% of the NMB response, respectively. Taken together, these studies indicate that the minimal length of the NMB sequence retaining full agonistic activity for BRS-3 was eight residues [i.e., Ac-NMB(3–10)].

Contributions of amino acid side chain groups on the activity of NMB. To further delineate the structural and functional relationships that are important for activation of BRS-3, we systematically switched the chirality of each residue in NMB. Furthermore, we replaced each residue in NMB with alanine. The structures of these peptides and their ability to promote calcium mobilization in BR2 cells, normalized to the activity of the parent molecule, NMB, are shown in Table 3. A large loss of activity occurred if one of three carboxyl-terminal residues, His8, Phe9, or Met10, was replaced with alanine, yielding 27%, 26%, or 12% of the activity of NMB, respectively. Our finding, discussed above, that deletion of Met10 also results in a large loss of activity, is consistent with these data. Switching NMB residues His8, Phe9, or Met10 from L to D amino acids also resulted in a large loss of activity, again showing the importance of the three carboxyl-terminal NMB residues in BRS-3 activation.

A relatively conservative change in NMB of Gly7 to either alanine or D-alanine resulted in a moderate loss of activity (68% or 29% of the activity of NMB, respectively). Because the methyl R group of alanine substituted in these peptides restricted the conformational flexibility of the molecule normally afforded by Gly7, a bend of the NMB backbone at

TABLE 2

NMB minimal sequence for activation of BRS-3

The ability of truncated NMB analogs to promote calcium mobilization in BR2 cells was monitored using Fluo-3 fluorescence, as described in Experimental Procedures. Peptides were added at a 1-μM concentration. Average fluorescence values shown are normalized to the stimulus produced by 1 μM NMB. Data represent the mean ± standard error of two or more determinations and are representative of two or more independent experiments.

Analog	Peptide	Sequence	Calcium mobilization
			% of NMB response
I	NMB	GNLWATGHFM-NH_2	100 ± 9
II	NMB(1–9)	GNLWATGHF-NH_2	n.d.[a]
III	Ac-NMB(2–10)	Ac-NLWATGHFM-NH_2	77 ± 20
IV	Ac-NMB(3–10)	Ac-LWATGHFM-NH_2	140 ± 8
V	Ac-NMB(4–10)	Ac-WATGHFM-NH_2	55 ± 5
VI	Ac-NMB(5–10)	Ac-ATGHFM-NH_2	24 ± 1

[a] n.d., not detectable.

TABLE 3

BRS-3 activation by NMB analogs

The ability of NMB analogs shown in the table to activate calcium mobilization in BR2 cells was monitored using Fluo-3 fluorescence, as described in Experimental Procedures. Peptides were tested at a final concentration of 1 μM. Amino acid substitutions are shown in bold; lower case letters denote D-amino acid residues. Average fluorescence values shown are normalized to the stimulus produced by 1 μM NMB.

Analog	Sequence	Calcium mobilization
		% of NMB response
I (NMB)	GNLWATGHFM-NH_2	100 ± 14
VII	**A**NLWATGHFM-NH_2	91 ± 2
VIII	G**A**LWATGHFM-NH_2	196 ± 8
IX	GN**A**WATGHFM-NH_2	86 ± 1
X	GNL**A**ATGHFM-NH_2	45 ± 2
XI	GNLWA**A**GHFM-NH_2	88 ± 9
XII	GNLWAT**A**HFM-NH_2	68 ± 1
XIII	GNLWATG**A**FM-NH_2	27 ± 1
XIV	GNLWATGH**A**M-NH_2	26 ± 6
XV	GNLWATGHF**A**-NH_2	12 ± 2
XVI	G**n**LWATGHFM-NH_2	77 ± 5
XVII	GN**l**WATGHFM-NH_2	61 ± 3
XVIII	GNL**w**ATGHFM-NH_2	102 ± 3
XIX	GNLW**a**TGHFM-NH_2	102 ± 1
XX	GNLWA**t**GHFM-NH_2	126 ± 1
XXI	GNLWAT**a**HFM-NH_2	29 ± 0
XXII	GNLWATG**h**FM-NH_2	5 ± 2
XXIII	GNLWATGH**f**M-NH_2	n.d.[a]
XXIV	GNLWATGHF**m**-NH_2	20 ± 5

[a] n.d., not detectable

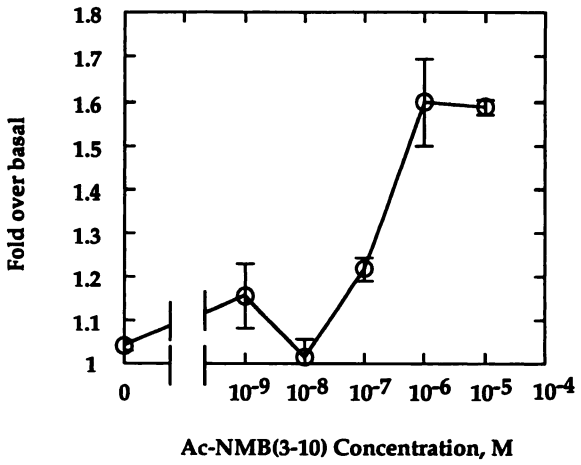

Fig. 2. Dose responses of Ac-NMB (3–10) for promotion of BRS-3-stimulated calcium mobilization in BR2 cells. BRS-3-mediated increases in $[Ca^{2+}]_i$ were determined using Fluo-3, as described in Experimental Procedures. Fluo-3 loaded cells were treated with various doses of Ac-NMB(3–10) as indicated. Data represent the average fold-stimulations over basal ± standard error of two or more determinations and are representative of three experiments.

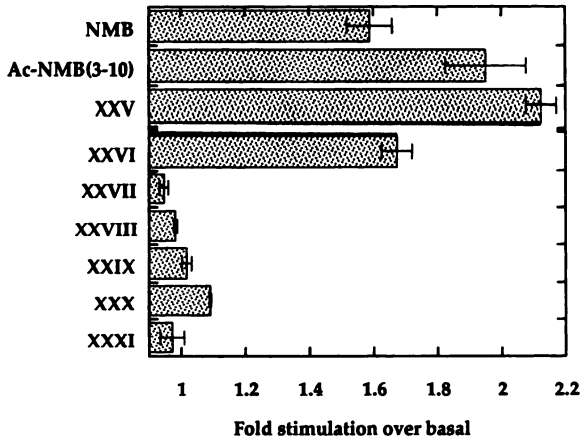

Fig. 3. BN analogs promote BRS-3-mediated calcium mobilization in BR2 cells. BRS-3 mediated increases in $[Ca^{2+}]_i$ were determined using Fluo-3 as described in Experimental Procedures. Fluo-3-loaded cells were treated with 1-μM doses of the analogs specified. In addition to NMB and Ac-NMB(3–10), the following bombesin analogs were assayed: *XXV*, D-Phe^6-BN(6–13) propyl amide; *XXVI*, D-Phe^6-BN(6–13) ethyl amide; *XXVII*, 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-CO-$(CH_2)_2$Ph-BN(7–13) methyl amide; *XXVIII*, D-Phe^6-BN(6–13) methyl ester; *XXIX*, F_5-D-Phe^6-D-Ala^{11}-BN(6–13) methyl ester; *XXX*, Leu$^{13}\psi(CH_2NH)$Leu^{14}-BN; and *XXXI*, deamino-Phe^6-His^7-D-Ala^{11}-D-Pro^{13}-$\psi(CH_2NH)$Phe^{14}-BN(6–14). Data represent average fold-stimulations over basal ± standard error of two or more determinations and are representative of two or more experiments.

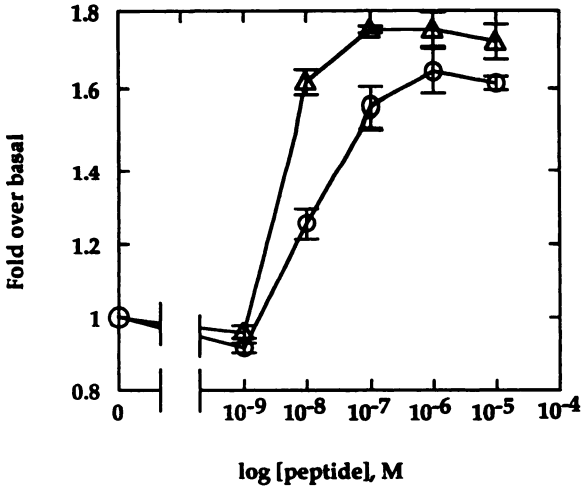

Fig. 4. Dose response of D-Phe^6-BN(6–13) propyl amide and D-Phe^6-Phe^{13}-BN(6–13) propyl amide for activation of BRS-3 in BR2 cells. BRS-3-mediated increases in $[Ca^{2+}]_i$ were determined using Fluo-3 as described in Experimental Procedures. Fluo-3-loaded cells were treated with various doses of D-Phe^6-bombesin(6–13) propyl amide (○) or D-Phe^6-Phe^{13}-bombesin(6–13) propyl amide (△). Data represent the average ± standard error fold-stimulation of two or more determinations and are representative of three experiments.

position 7 may be important for BRS-3 agonistic activity. Replacement of Trp4 with alanine also had an moderate effect on BRS-3 agonistic activity, giving 45% of the activity of NMB. In contrast, replacement of Asn2 with alanine resulted in a molecule that promoted nearly twice the calcium mobilization response promoted by NMB. Indeed, the EC_{50} value of [Ala^2]NMB was 575 nM compared with an EC_{50} value of about 1–10 μM for NMB. As discussed above, deletions of the amino-terminal glycine and asparagine residues also resulted in an analog with increased potency relative to NMB. These data suggest that the amide side-chain group of L-asparagine has a negative effect on NMB agonistic activity.

Agonistic effects of BN analogs on BRS-3. A large number of BN analogs have been described that have potent antagonist activity with respect to the GRP receptor. Among the analogs that display the highest GRP-receptor-binding affinity are desMet BN analogs with carboxyl-terminal alkyl amide or ester moieties (18–21) and BN analogs with reduced peptide (ψ) bonds between the two carboxyl-terminal residues (22, 23). These analogs characteristically have much lower affinities for the NMB receptor subtype, however (24, 25).[1] Some of these analogs also had significant amounts of partial agonistic activity on GRP and NMB receptors (16, 20, 26).

To determine whether a number of representative bombesin analogs shown can activate BRS-3, we measured their effects on BRS-3-mediated Ca^{2+} mobilization (Fig. 3). When tested at a concentration of 1 μM, two of the peptides tested, D-Phe^6-BN(6–13) propyl amide (XXV) and D-Phe^6-BN(6–13) ethyl amide (XXVI) (27), activated BRS-3, promoting 118% and 71%, respectively, of the calcium response stimulated by 1 μM Ac-NMB(3–10). Dose-response experiments indicated that D-Phe^6-BN(6–13) propyl amide promoted calcium mobilization in BR2 cells with an EC_{50} of 84 nM (Fig. 4), compared with a value of 219 nM for Ac-NMB(3–10) (Fig. 2). Within the error of our assay, we found that D-Phe^6-BN(6–13) propyl amide and Ac-NMB(3–10) maximally stimulated calcium mobilization in BR2 cells to the same extent. Thus, D-Phe^6-BN(6–13) propyl amide seems to function as a pure agonist of BRS-3 in our assay system.

Several control experiments confirmed that the activity of D-Phe^6-BN(6–13) propyl amide and D-Phe^6-BN(6–13) ethyl amide was mediated by BRS-3. Neither analog had an effect on calcium mobilization by nontransfected Balb/3T3 cells, which indicates that their effect on BR2 cells was mediated through BRS-3. Furthermore, the fact that D-Phe^6-BN(6–13) propyl amide displayed roughly 2 orders of magnitude greater potency than NMB indicates that NMB receptors did not contribute to the BR2 cell response. In addition, D-Phe^6-BN(6–13) propyl amide is an antagonist of murine GRP receptors (27) and, thus, its activity on BR2 cells could not have been mediated by GRP receptors that could possibly be expressed in BR2 cells.

Five other BN analogs tested [4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-CO-$(CH_2)_2$Ph-BN(7–13) methyl amide (XXVII) (28), D-Phe^6-BN(6–13) methyl ester (XXVIII) (27), F_5-D-Phe^6-D-Ala^{11}-BN(6–13) methyl ester (XXIX) (29), Leu$^{13}\psi(CH_2NH)$-Leu^{14}-BN (XXX) (30) and deamino-Phe^6-His^7-D-Ala^{11}-D-Pro^{13}-$\psi(CH_2NH)$Phe^{14}-BN(6–14) (XXXI) (31)] failed to promote a calcium mobilization response in BR2 cells, when added at a concentration of 1 μM. These analogs include three desMet BN analogs with methyl ester or amide moieties at their carboxyl termini. One significant distinguishing characteristic of these inactive analogs, relative to the BN analogs that promoted calcium mobilization, is a methyl substitution at their carboxyl termini, instead of an ethyl or propyl group. In contrast, the length of this group has not been found to be important for binding to the GRP receptor, although longer alkyl chains can be associated with significantly high amounts of partial agonistic activity.

Two BN analogs of the reduced peptide bond motif, Leu^{13}-

[1] R. Feldman, W. Zheng, J. Wu, manuscript in preparation.

ψ(CH$_2$NH)Leu^{14}-BN and deamino-Phe^6-His^7-D-Ala^{11}-D-Pro^{13}-ψ(CH$_2$NH)Phe^{14}-BN(6–14), did not promote Ca^{2+} mobilization in BR2 cells when added at a concentration of 1 μM. These analogs, added at 10 μM, also did not block the activation of BRS-3 by NMB. Taken together, these results indicate that the lack of activity displayed by the reduced peptide bond analogs tested in our assay was caused by a lack of BRS-3 binding. With respect to human GRP receptors, Leu^{13}-ψ(CH$_2$NH)Leu^{14}-BN displays some partial agonistic activity (16), whereas deamino-Phe^6-His^7-D-Ala^{11}-D-Pro^{13}-ψ(CH$_2$NH)Phe^{14}-BN(6–14) is a pure antagonist.[1] Although Leu^{13}-ψ(CH$_2$NH)Leu^{14}-BN and deamino-Phe^6-His^7-D-Ala^{11}-D-Pro^{13}-ψ(CH$_2$NH)Phe^{14}-BN(6–13) bind to the GRP receptor with K_D values in the low nanomolar or picomolar range (31), these analogs bind significantly more weakly to the human NMB receptor.[1] Therefore, the pharmacology of BRS-3 with respect to the analogs tested gave a pattern more similar to the NMB receptor subtype than to the GRP receptor.

BRS-3 agonistic activity of analogs based on D-Phe^6-BN(6–13) propyl amide. In the studies described above, D-Phe^6-BN(6–13) propyl amide was identified as the most potent agonist of BRS-3 in our peptide collection. To potentially increase the affinity of D-Phe^6-BN(6–13) propyl amide for BRS-3, we prepared a series of additional analogs based on its structure. As shown in Table 4, we varied the peptide independently at three positions, namely Gln7, Val10, and Leu13, to make the molecule more or less NMB-like. The ability of these analogs to promote calcium mobilization in BR2 cells, tested at a concentration of 100 nM, is also shown in Table 4. The most active analog tested was D-Phe^6-Phe^{13}-BN(6–13) propyl amide, which promoted 158% of the calcium response promoted by the parent molecule, D-Phe^6-BN(6–13) propyl amide.

To further define the potencies of these two analogs, dose-response experiments were performed. As shown in Fig. 4, D-Phe^6-Phe^{13}-BN(6–13) propyl amide displayed an EC_{50} value for stimulation of calcium mobilization in BR2 cells of 5 nM, whereas D-Phe^6-BN(6–13) propyl amide was 17-fold less potent, displaying an EC_{50} value of 84 nM. Neither analog stimulated detectable calcium mobilization in nontransfected Balb/3T3 cells or the vector-alone control transfected Balb/3T3 cells (data not shown), which confirmed that their activity was mediated by BRS-3. To further rule out the possibility that expression of NMB or GRP receptors in BR2 cells accounted for the activity of D-Phe^6-Phe^{13}-BN(6–13) propyl amide, an experiment that directly compared its potency with that of NMB and GRP was performed. Although neither GRP nor NMB stimulated a significant increase in Ca^{2+} mobilization in BR2 cells at a concentration of 100 nM, D-Phe^6-Phe^{13}-BN(6–13) propyl amide achieved its maximal response at this concentration (data not shown). Consistent with data presented above, GRP and NMB promoted a 50% and 77% of the maximal response produced by D-Phe^6-Phe^{13}-BN(6–13) propyl amide, respectively, at the higher concentration of 1 μM in this experiment. These data further confirm that the Ca^{2+} mobilization response by BR2 cells promoted by D-Phe^6-Phe^{13}-BN(6–13) propyl amide was mediated by BRS-3 receptors and not other BLP receptor subtypes.

Even though earlier experiments showed that NMB and Ac-NMB(3–10) were significantly more potent agonists of BRS-3 than BN (Fig. 1), substitutions making the desMet propyl amide analogs more like NMB did not necessarily result in an increase in activity. Indeed, the most NMB-like analog, D-Phe^6-Leu^7-Thr^{10}-Phe^{13}-BN(6–13) propyl amide, displayed a significantly lower activity than the more BN-like analog, D-Phe^6-Phe^{13}-BN(6–13) propyl amide. Because low nanomolar concentrations of our most potent analog, D-Phe^6-Phe^{13}-BN(6–13) propyl amide, can activate BRS-3, this compound may be of significant value in the elucidation of BRS-3 function and the further development of BRS-3 agonists and antagonists.

Discussion

In this paper, we characterized the pharmacology of a large number of BLPs and analogs with respect to BRS-3 ectopically expressed in a mammalian cell (Balb/3T3 fibroblasts). To assess the activation of BRS-3, BRS-3-mediated calcium mobilization was used as the readout. Taken together, our data demonstrate a functional relationship between human BRS-3 and human GRP and NMB receptors, supplementing previous studies that demonstrated a close relationship between the GRP and NMB receptors and BRS-3 in evolutionary terms. BRS-3 shares 51% amino acid identity with the GRP receptor and 47% identity with the NMB receptor, a level of homology found among subtypes of other seven-transmembrane domain, G protein-coupled receptors. Furthermore, all three receptors also are homologous with respect to the location of their two introns (1). Despite this structural

TABLE 4

Stimulation of BRS-3-mediated calcium mobilization by D-Phe^6-BN(6–13) propyl amide analogs

Calcium mobilization was measured in Fluo-3-loaded BR2 cells as described in Experimental Procedures. D-Phe^6-BN(6–13) propyl amide analogs were added at 100 nM concentrations, and Ca^{+2}-dependent fluorescent signals were measured and normalized to the response produced by D-Phe^6-BN(6–13) propyl amide. The activity of Ac-NMB(3–10) was also measured. Data represent the average ± standard error of two or more determinations and are representative of two or more independent experiments.

No.	Analog	Calcium mobilization
		% of response to XXV
XXV	D-Phe^6-BN(6–13) propyl amide	100 ± 6
IV	Ac-NMB(3–10)	43 ± 6
XXXII	D-Phe^6-Leu^7-BN(6–13) propyl amide	72 ± 8
XXXIII	D-Phe^6-Phe^{13}-BN(6–13) propyl amide	158 ± 8
XXXIV	D-Phe^6-Leu^7-Phe^{13}-BN(6–13) propyl amide	n.d.[a]
XXXV	D-Phe^6-Leu^7-Thr^{10}-BN(6–13) propyl amide	79 ± 1
XXXVI	D-Phe^6-Thr^{10}-BN(6–13) propyl amide	64 ± 1
XXXVII	D-Phe^6-Thr^{10}-Phe^{13}-BN(6–13) propyl amide	83 ± 18
XXXVIII	D-Phe^6-Leu^7-Thr^{10}-Phe^{13}-BN(6–13) propyl amide	10 ± 6

[a] n.d., not detectable.

homology suggesting that BRS-3 is a BLP receptor subtype, a high affinity agonist of BRS-3 had not been identified previously. However, relatively high, nonphysiological concentrations of NMB and other BLPs (1–10 μM) have been shown to activate BRS-3 expressed in *X. laevis* oocytes (1).

In our study, we defined critical residues on BLPs and analogs important for activation of BRS-3 in a mammalian cell and concluded that BRS-3 has a binding site for BLPs that is similar to that on the NMB receptor and, to a lesser degree, the GRP receptor. In particular, we analyzed structural and functional relationships in collections of BLPs and analogs falling into the following five categories: naturally occurring BLPs, NMB analogs with systematic amino-terminal and carboxyl-terminal deletions, NMB analogs in which the chirality of each residue was switched, NMB analogs with alanine substitutions, and BN analogs with unnatural modifications representing several classes of potent full or partial antagonists of the GRP receptor. From this latter group, we discovered the most potent agonist of BRS-3 described to date, D-Phe^6-Phe^{13}-BN(6–13) propyl amide (EC_{50} = 5 nM). This peptide may serve as a useful template in the design of potent and selective BRS-3 antagonists.

A high affinity agonist, such as D-Phe^6-Phe^{13}-BN(6–13) propyl amide, may also be useful to probe the biological function of BRS-3. Several physiological roles for BRS-3 have been suggested (1). BRS-3 is widely expressed in SCLC cell lines (1). Because the growth of SCLC is stimulated by BLPs (7, 8), it is possible that BRS-3 plays a role in the pathogenesis of lung cancer or could be involved in growth regulation of other cancers or normal tissues. We have found that BRS-3, expressed in Balb/3T3 fibroblasts, can promote mitogenic effects in the presence of other growth factors such as insulin (15). BRS-3 expression was prominent in pregnant rodent uteri (2) and in secondary spermatocytes in rodent testes (1) and may, therefore, have a role in reproduction.

To define the importance of individual residues of BLPs in activation of BRS-3, we systematically altered the structure of NMB, which displayed the highest BRS-3 activity among naturally occurring BLPs tested. In our first series of studies, we analyzed truncated forms of NMB for activation of BRS-3. DesMet NMB (carboxyl-terminally amidated) displayed negligible BRS-3 activity. In contrast, a number of residues could be deleted from the amino terminus without greatly diminishing activity. The minimal-size NMB analog retaining full agonistic activity for BRS-3 was Ac-NMB(3–10). Indeed, Ac-NMB(3–10) was actually a more potent agonist of BRS-3 than full-length NMB. Similarly, the minimal size of GRP sufficient for activation of the GRP receptor was the eight-amino-acid carboxyl-terminal portion of the molecule [Ac-GRP(20–27)] (32). Therefore, the binding sites for BLPs to the GRP receptor and BRS-3 both recognize a similarly sized carboxyl-terminal fragment of BLPs, although the interaction of known members of the BLP family to BRS-3 is relatively weak.

To further investigate the interaction of NMB with BRS-3 leading to activation, we systematically switched the chirality of each amino acid in the molecule. Furthermore, we also examined NMB analogs in which each amino acid was substituted with L-alanine. Replacement of Asn2 with alanine resulted in an increase in activity, suggesting that Asn2 has a negative effect on NMB binding to BRS-3. This was consistent with our finding that deletion of the amino-terminal Gly-Asn from NMB (yielding Ac-NMB(3–10)) also resulted in increased agonistic activity. The importance of the three carboxyl-terminal residues of NMB for activation of BRS-3 (His8-Phe9-Met10) was also apparent in our NMB analog study. As observed in our deletion studies, our substitution findings are completely analogous to similar studies performed on the GRP receptor. Furthermore, the NMB analogs described in this paper, when assayed in GRP receptor (32) or NMB receptor binding assays, displayed a similar pattern of relative potencies as observed for agonism of BRS-3.[1] These comparative studies, therefore, further indicate that BRS-3 shares many of the features of the ligand binding site with the NMB and GRP receptors.

Another significant finding of our study was that D-Phe^6-Phe^{13}-BN(6–13) propyl amide functions as a potent agonist of BRS-3 (EC_{50} = 5 nM) in a mammalian cell. D-Phe^6-Phe^{13}-BN(6–13) propyl amide displayed nearly three orders of magnitude higher potency for the activation of BRS-3 than agonists of BRS-3 characterized previously. The peptide also maximally stimulated calcium mobilization to a similar extent as Ac-NMB(3–10), which suggests that it acts as a pure agonist of BRS-3 in our assay system. In light of our finding that NMB was a significantly more potent agonist of BRS-3 than BN, we attempted to make D-Phe^6-BN(6–13) propyl amide more NMB-like. This strategy failed, however. In fact, our most NMB-like analog, namely D-Phe^6-Leu^7-Thr^{10}-Phe^{13}-BN(6–13) propyl amide, displayed significantly reduced activity. Of the seven D-Phe^6-BN-propyl amide analogs we tested, D-Phe^6-Phe^{13}-BN(6–13) propyl amide was the most potent activator of BRS-3.

Likewise, among this collection of analogs, D-Phe^6-Phe^{13}-BN(6–13) propyl amide displayed the highest affinity for the human NMB receptor, as determined from competitive displacement, radiolabeled ligand binding studies, although its affinity was significantly lower than NMB. The peptide displayed a K_D value of 2.4 nM for the NMB receptor, whereas NMB displayed a K_D value of 0.024 nM.[1] For the GRP receptor D-Phe^6-Phe^{13}-BN(6–13) propyl amide displayed a K_D value of 8.9 nM. Among the other analogs tested, the most BN-like analog, D-Phe^6-BN(6–13) propyl amide, displayed the highest affinity for the GRP receptor (K_D = 0.56 nM). These data provide further indication that the binding site for BN analogs existing on BLP receptors and BRS-3 is highly conserved, particularly with respect to the NMB receptor, providing further reason to believe that the physiological activator of BRS-3 is highly related to BLPs.

Although D-Phe^6-Phe^{13}-BN(6–13) propyl amide may be extremely useful in studies of BRS-3 function, in systems in which other BLP receptors are functioning, interpretation of the results should be undertaken with caution. We have found that D-Phe^6-Phe^{13}-BN(6–13) propyl amide can partially stimulate Ca^{2+} mobilization in Balb/3T3 cells transfected with the rat NMB receptor with an EC_{50} value of 3 nM.[1] The activity of D-Phe^6-Phe^{13}-BN(6–13) propyl amide with respect to forms of the GRP receptor from different species must be tested; however, we have shown that the analog has relatively high affinity for the human GRP receptor. In addition, the related analog profiled in our study that activated BRS-3 with relatively high potency, namely D-Phe^6-BN(6–13) propyl amide, functions as either a GRP receptor antagonist or partial agonist of the GRP receptor, depending on the species of GRP receptor investigated or the

assay system used (15, 20, 26). For example, D-Phe^6-BN(6–13) propyl amide antagonized GRP-induced [^{3}H]thymidine incorporation by Swiss/3T3 fibroblasts (20), but it acted as a partial agonist of [^{3}H]thymidine incorporation by Balb/3T3 fibroblasts transfected with the human GRP receptor (16). Furthermore, D-Phe^6-BN(6–13) propyl amide was an antagonist of GRP-stimulated amylase release in guinea pig pancreas and acted as a partial antagonist of GRP stimulated amylase release in rat pancreas (20).

It is unclear how the binding of D-Phe^6-Phe^{13}-BN(6–13) propyl amide to BRS-3 relates to the binding of BLP ligands without nonnatural amino acid modifications. D-Phe^6-Phe^{13}-BN(6–13) propyl amide may bind to BRS-3 in a different manner than NMB, because changes in this molecule to make it more NMB-like resulted in decreased activity. Indeed, D-Phe^6-Phe^{13}-BN(6–13) propyl amide displayed significantly higher binding affinity than the more NMB-like analog for both the rat and human NMB receptors.[1] The carboxyl-terminal propyl amide moiety on D-Phe^6-Phe^{13}-BN(6–13) propyl amide may affect the orientation of the entire molecule in the receptor binding site. We have tried several strategies to increase the activity of NMB for BRS-3 without success, including substitution of Met10 with other amino acids and extension of the carboxyl terminus of NMB by a glycine residue. Analogous glycine extended forms of gastrin, which are produced naturally, trigger a high affinity receptor distinct from gastrin receptors (33). It is also possible that simultaneous changes of several residues in the backbone of NMB will combine to result in a large boost in binding affinity for BRS-3.

In summary, we have demonstrated that BRS-3, expressed in Balb/3T3 cells, couples to rapid changes in $[Ca^{2+}]_i$. Among the naturally occurring BLPs we examined, peptides in the ranatensin subclass (carboxyl-terminal sequence -His-Phe-Met-amidated) displayed the greatest BRS-3 activity. Despite the fact that a physiological activator of BRS-3 has not been identified, our structural and functional analyses of BLP analogs provide pharmacological support for categorizing this receptor as a bombesin receptor subtype. Furthermore, we identified an agonist of BRS-3, namely D-Phe^6-Phe^{13}-BN(6–13) propyl amide, that was active in the low nM range. Accordingly, this compound may be used in direct binding assays for BRS-3, if suitably labeled. Such a ligand would greatly facilitate a range of studies on BRS-3. D-Phe^6-Phe^{13}-BN(6–13) propyl amide also may be useful in probing the function of BRS-3 in normal physiology and disease. Finally, D-Phe^6-Phe^{13}-BN(6–13) propyl amide provides a template for the discovery of BRS-3 antagonists or the development of more potent or selective BRS-3 agonists.

Acknowledgments

We thank Sarah Fried for her expert technical assistance.

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