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Differential Activation of Human Gastrin-Releasing Peptide Receptor-Mediated Responses by Bombesin Analogs

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SUMMARY

To enable the detailed pharmacological characterization of five bombesin (BN) analogs with respect to the human gastrin-releasing peptide (GRP) receptor, we ectopically expressed the receptor in BALB/3T3 cells. In such cells (termed GR1 cells), GRP stimulated DNA synthesis and Ca^{2+} mobilization. Two of these analogs, D-Phe^6-BN(6–13) methyl ester (K_i = 1.38 ± 0.07 nM) and 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-COCH$_2$CH$_2$-phenyl-BN(7–13) methyl amide (K_i = 2.17 ± 0.05 nM), were pure antagonists of GRP-stimulated DNA synthesis in GR1 cells (IC_{50} = 14 ± 8.5 nM and 5.1 ± 2.0 nM, respectively), whereas three analogs, Leu^{13}-ψ-Leu^{14}-BN (K_i = 21.6 ± 2.2 nM), D-Phe^6-BN(6–13) ethyl amide (K_i = 5.17 ± 0.64 nM), and D-Phe^6-BN(6–13) propyl amide (K_i = 0.68 ± 0.01 nM), displayed significant partial agonistic activity. Although three analogs promoted mitogenesis in GR1 cells, none of the analogs stimulated calcium mobilization in GR1 cells. This dichotomy was not limited to transfected cells, because the same result was obtained for D-Phe^6-BN(6–13) propyl amide using human fetal lung cells, which naturally express the GRP receptor. We also assessed the effect of BN analogs on calcium mobilization in transfected GR9 cells expressing about 30 times higher levels of the GRP receptor, compared with GR1 cells. D-Phe^6-BN(6–13) ethyl amide, D-Phe^6-BN(6–13) propyl amide, and Leu^{13}-ψ-Leu^{14}-BN were partial agonists of the intracellular Ca^{2+} mobilization response of GR9 cells. One conclusion consistent with our data is that GRP-stimulated DNA synthesis requires the activation of far fewer receptors than does GRP-stimulated calcium mobilization. Thus, analogs with a small amount of agonist activity can trigger a mitogenic response but not an intracellular Ca^{2+} mobilization response, unless cells express a high level of receptors. These studies also provide evidence that the promotion of DNA synthesis in quiescent GR1 or human fetal lung cells via the GRP receptor does not require mobilization of intracellular Ca^{2+}.

BN is a tetradecapeptide isolated from the skin of the frog *Bombina bombina* (1). As many as 13 peptides with structural and functional homology to BN, referred to as BLPs, have been isolated from different species of amphibians (for review, see Ref. 2). Two BLPs, NMB and GRP, are expressed in mammals and have been shown to have diverse biological effects. In the central nervous system BLPs cause alterations in behavior and homeostasis, whereas in the gastrointestinal tract they stimulate gastrin release and smooth muscle contraction (2). In Swiss 3T3 fibroblasts, GRP stimulates the mobilization of intracellular calcium and promotes additional intracellular signals triggering the entry of quiescent cells into S phase of the cell cycle (3). BLPs have also been shown to stimulate the proliferation of breast (4), colon (5), and prostate (6) cancer cells *in vitro* and have been implicated as autocrine growth factors in small cell lung cancer (7–10).

The physiological effects of BLPs in mammals are mediated by at least two distinct receptors. cDNAs encoding two BLP receptors, with binding preference for either NMB or GRP, have been cloned from rodent species (11, 12) and humans (13). These receptors are members of the seven-transmembrane domain receptor superfamily and are coupled to signaling pathways via heterotrimeric G proteins. Recently, a third receptor, with extensive homology to the NMB and GRP receptors (termed BLP receptor subtype 3), was cloned (14, 15); it binds only weakly to known BLPs, suggesting that its physiological ligand is a novel peptide in the BLP family. Further elucidation of the physiological functions of BLPs, including their role in regulating the growth of human cancers, will be aided by the design of potent and specific BLP receptor blockers.

A large number of peptide analogs of BLPs have been developed and act as GRP antagonists. The most effective analogs either contain a reduced peptide bond between the carboxyl-terminal two amino acids (16) or have various moieties substituted for the carboxyl-terminal amidated methionine (17–19). The potency and antagonistic activity exhibited by many of these analogs vary when measured using different assay systems or GRP receptor homologs from dif-

ABBREVIATIONS: BN, bombesin; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GRP, gastrin-releasing peptide; HFL, human fetal lung; NMB, neuromedin B; PBS, phosphate-buffered saline; SV-40, simian virus 40; BLP, bombesin-like peptide; Ph, phenyl.

ferent species. For example, markedly different amounts of receptor agonism were exhibited by several analogs when they were assayed for promotion of amylase secretion using rat versus guinea pig pancreatic acinar cells (20). This observation is consistent with the large pharmacological differences frequently observed among peptide receptors from different species. Furthermore, some BN analogs, such as D-Phe^6-BN(6–13) propyl amide, completely blocked GRP-stimulated Ca^{2+} mobilization in human small cell lung carcinoma cell lines (21) but were activators of gastrin release from human G cells (22). It is not clear whether these pharmacological variations reflect differences in receptor coupling among the different systems used or are due to the effects of other BLP receptor subtypes that may be expressed.

The availability of cDNAs encoding BLP receptors makes it possible to use cells that ectopically express these receptors to study the pharmacology of BN analogs. Such a model system ensures that a homogeneous population of a single receptor subtype is studied and potentially allows the measurement of several different receptor-mediated responses. Of particular interest was the analysis of BN analogs with respect to the mitogenic response elicited by the human GRP receptor. Because we have not identified a suitable human cell line lacking the GRP receptor, we ectopically expressed the human GRP receptor in murine BALB/3T3 cells. BALB/3T3 cells neither display detectable ^{125}I-GRP high affinity binding sites nor express mRNA encoding BLP receptor subtypes detectable by Northern blotting. When stably transfected with rodent BLP receptors, this cell line was found by us (data not shown) and others (23, 24) to display a wide range of GRP-mediated responses, including the stimulation of inositol phosphate formation, the mobilization of intracellular Ca^{2+}, and the promotion of mitogenesis.

In this paper, we report that activation of the human GRP receptor, ectopically expressed in quiescent and contact-inhibited BALB/3T3 fibroblast cells, results in a stimulation of DNA synthesis and the mobilization of intracellular calcium. The pharmacology of a collection of BLP analogs was characterized using these mitogenic and calcium responses of GRP receptor-transfected BALB/3T3 cells. The results corresponded to those obtained using HFL cells, which are nontransformed fibroblasts naturally expressing the GRP receptor. A novel finding of our study is that several BN analogs stimulated DNA synthesis in HFL cells or transfected BALB/3T3 cells expressing moderate levels of the human GRP receptor, without significantly activating the mobilization of intracellular Ca^{2+}.

Experimental Procedures

Materials. GRP(1–27), D-Phe^6-BN(6–13) ethyl amide, D-Phe^6-BN(6–13) propyl amide, and Leu^{13}-ψ-Leu^{14}-BN were purchased from either Peninsula Laboratories (Belmont, CA) or Bachem California (Torrance, CA). 4-Pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-COCH$_2$CH$_2$Ph-BN(7–13) methyl amide was custom synthesized by Multiple Peptide Systems (San Diego, CA). D-Phe^6-BN(6–13) methyl ester was obtained from Dr. David Coy (Tulane University, New Orleans, LA). All peptides were shown to be >96% pure by high performance liquid chromatography, and their identities were confirmed by either amino acid analysis or mass spectroscopy. Insulin/transferrin/selenium medium supplement was obtained from Collaborative Research (Bedford, MA). Geneticin (G418) was purchased from GIBCO-BRL and hygromycin B from Calbiochem (La Jolla, CA). All other cell culture media, supplements, and fetal calf serum were purchased from Whittaker Biochemicals (Walkersville, MD). Bovine serum albumin (fatty acid free), sulfinpyrazone, bacitracin, polyethyleneimine, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Fluo-3/acetoxymethyl ester, fura-2/acetoxymethyl ester, and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). ^{125}I-GRP (~2000 Ci/mmol) was purchased from Amersham, and [*methyl*-^{3}H]thymidine (2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals used were of reagent-grade quality.

Expression plasmids. pGRP3 was derived by cloning the *Xba*I fragment containing the human GRP receptor open reading frame (13) into the *Xba*I site of a slightly modified version of pCD2 (25), so that the GRP receptor gene was oriented in the same direction as the SV40 early promoter. pGRP100 is an expression vector that was obtained by cloning the human GRP receptor open reading frame from pGRP3 into pBBS70, so that the GRP receptor open reading frame was downstream of a myeloproliferative sarcoma virus promoter (26) and upstream of a cytomegalovirus enhancer and the SV40 late poly(A)$^+$ addition site. pBBS70 has three dominant selection markers, i.e., the puromycin-*N*-acetyltransferase gene and the dihydrofolate reductase gene expressed via the SV40 early promoter and SV40 early poly(A)$^+$ addition site and the hygromycin B gene expressed using the thymidine kinase promoter and the thymidine kinase poly(A)$^+$ addition site. Two SV40 origins of replication are also carried on the plasmid.

Growth and stable transfection of cells. HFL-1 cells (CCL 153) were obtained from the American Type Culture Collection (Rockville, MD) and were grown in F-12K medium, supplemented with 15% fetal calf serum and 2 mM glutamine, at 37° in a humidified atmosphere containing 5% CO_2. BALB/3T3 cells, obtained from the American Type Culture Collection, were grown in DMEM, supplemented with 4.5 g/liter glucose, 10% fetal calf serum, 1% nonessential amino acids, 1% sodium pyruvate, and 2 mM glutamine, at 37° in a humidified atmosphere containing 10% CO_2. Stable gene expression was achieved by transfection of cells using a calcium phosphate precipitation procedure and selection with either G418 (pGRP3) or hygromycin B (pGRP100) (27). Cells transfected with pGRP3, growing under selective conditions, were screened for the presence of high affinity ^{125}I-GRP binding sites. Four receptor-positive clones tested for GRP-stimulated intracellular Ca^{2+} mobilization showed a response. A representative clone chosen for further study, GR1, expressed 0.057 pmol of GRP receptor/mg of membrane protein (K_d = 0.035 nM), based on a nonlinear regression analysis of binding data using the computer program LIGAND. Similar ligand binding studies using intact GR1 cells indicated that these cells express 46,000 ± 2,200 receptors/cell (K_d = 0.010 ± 0.0012 nM).

To generate transfected BALB/3T3 cells expressing higher levels of receptor, cells were transfected with pGRP100. One clone, selected because of its high level of expression of ^{125}I-GRP binding sites, was used for further studies. GR9 expressed 1.00 pmol of receptor mg of membrane protein (K_d = 0.030 nM). Assays of intact cells indicated that GR9 expressed 1,340,000 ± 130,000 receptors/cell (K_d = 0.067 ± 0.0049 nM).

^{125}I-GRP(1–27) binding to cell membranes. GR9 membranes (2 μg) or Swiss 3T3 membranes (18 μg), prepared as described previously (28), were suspended in 0.5 ml of ice-cold binding medium (50 mM HEPES, pH 7.5, 2 mM EDTA, 0.13 M NaCl, 5 mM $MgSO_4$, 5 mM KCl, 10 mg/ml bovine serum albumin, 30 μg/ml bacitracin). For studies of BN analog binding, unlabeled GRP or BN analogs were added to the medium at the concentrations indicated in the figures. Binding reactions were initiated by the addition of 0.02 nM ^{125}I-GRP and were carried out at 37° for 1 hr. Binding was terminated by cooling of the reaction mixtures on ice and rapid filtration through polyethyleneimine-treated Whatman GF/B glass fiber filters, followed by four washes (4 ml each) with ice-cold 50 mM Tris·HCl, pH

7.5. Radioactivity on the filters was determined using a γ counter. Total bound counts ranged from 7 to 9% of the total counts added, whereas nonspecifically bound counts accounted for <1% of the total counts added. Data from competitive displacement experiments were used to calculate K_d and K_i values, using the computer program LIGAND (29).

Analysis of intracellular calcium. Flasks of cells grown to confluence were rinsed twice with PBS plus 2 mM EDTA. Cells were dislodged from the flasks by agitation after incubation with PBS plus 2 mM EDTA and 1% glucose at 37° for 10–15 min. Cells were pelleted at 800 × *g* for 10 min and resuspended, at a density of 1 × 10^6 cells/ml, in loading medium (RPMI 1640 medium, 25 mM HEPES, pH 7.5, 0.1% bovine serum albumin). This medium contained 2 mM $CaCl_2$. Cells were then slowly combined, with gentle agitation, with an equal volume of loading medium containing 5 μM fura-2/acetoxymethyl ester. Sulfinpyrazone (final concentration, 0.25 mM), an anion transport inhibitor that inhibits leakage of intracellular fura-2 (30), slightly enhanced the signal and was added in the experiments reported in this paper. After incubation for 45 min at 37° in the dark, cells were washed twice with loading medium and once with assay buffer (50 mM HEPES, pH 7.5, 2 mM EDTA, 5 mM $MgSO_4$, 5 mM KCl, 0.13 M NaCl, 2 mM $CaCl_2$, 0.1% bovine serum albumin). Cells were finally suspended at a density of 2 × 10^5 cells/ml in assay buffer supplemented with sulfinpyrazone (0.25 mM) and were incubated for an additional 15–20 min at 37°. The cellular fluorescence was analyzed at 37° with a PTI Deltascan 4000 fluorimeter (Photon Technology, South Brunswick, NJ) equipped with dual-excitation monochromators. All fluorescence measurements were obtained at an emission wavelength of 510 nm. Intracellular calcium concentration was calculated using the formula $[Ca^{2+}] = K_d[[(R - R_{min})(Sf_2/Sb_2)]/(R_{max} - R)]$, where K_d is the measured dissociation constant of the complex of fura-2 and Ca^{2+} (215 nM), R is the ratio of the fluorescence measured using excitation wavelengths of 340 nm and 380 nm, R_{max} is R measured in the presence of Triton X-100, R_{min} is R measured in the presence of Triton X-100 and EGTA, Sf_2 is the fluorescence measured in the presence of Triton X-100 using an excitation wavelength of 380 nm, and Sb_2 is the fluorescence measured in the presence of Triton X-100 and EGTA using an excitation wavelength of 380 nm.

The final medium Ca^{2+} concentration in the studies shown in Figs. 4 and 7 ranged from 50 to 100 μM, as determined for each experiment from the fluorescence of fura-2 after the dye was released from cells by treatment with Triton X-100. Intracellular Ca^{2+} mobilization stimulated by GRP was not significantly changed when the medium Ca^{2+} concentration was increased by an additional 2 mM. Furthermore, at either calcium concentration, addition of EGTA to the medium had no significant effect (<20%) on measured intracellular Ca^{2+} mobilization, indicating that the source of Ca^{2+} was mostly from intracellular stores.

To assess the dose-response relationship for inhibition of GRP-mediated calcium mobilization (see Fig. 5), we monitored changes in the relative fluorescence of cells loaded with fluo-3. Cells at a density of 3–5 × 10^6 cells/ml in loading medium were obtained as described for experiments with fura-2. 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 incubation for 1 hr at 37° in the dark, extracellular dye was removed by washing the cells three times with loading medium. Cells were finally resuspended at a density of 3–5 × 10^5 cells/ml in assay medium (DMEM with 25 mM HEPES, pH 7.5, and 1.0% fetal calf serum) and were used within several hours for measurements of intracellular Ca^{2+} concentration, using a FACScan fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA). Experiments were performed at 25°. Only 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. Measurements were initiated within 5 sec after addition of test compounds. To determine the effects of BN analogs on GRP-stimulated Ca^{2+} mobilization, analogs were added first, followed by the addition of GRP 5 sec later, followed by measurement of fluorescence 5 sec later. Data for basal and stimulated cell fluorescence, analyzed using LYSYS II software, represent the mean fluorescence of cells over a sampling period of the first 5–30 sec after addition of test solutions. Although the basal fluorescence was stable within an experiment, it varied among experiments from 5 to 12 relative units, due to variations in dye loading. Control values for the stimulation of fluo-3 fluorescence by GRP in different experiments ranged from 10 to 30 relative units. The stimulation by GRP in each experiment (stimulated/basal fluorescence) ranged from 2- to 3-fold. The results are reported as the average of two or more independent experiments.

Mitogenesis assays. Cells (2 × 10^4/well) were grown in 24-well plates, as described above, for 4–5 days, at which time they were confluent. The medium was then changed to assay medium (DMEM/Waymouth's medium, 1:1, with 1% glutamine and 1% insulin/transferrin/selenium medium supplement), and the cells were incubated for 24 hr at 37° in an atmosphere containing 5% CO_2. Peptide stocks (100×), prepared in assay medium supplemented with antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamicin), were added to the cells to yield the final concentrations specified in the figures. After 16 hr, cells were incubated for 2 hr with [*methyl*-^{3}H]thymidine (5 μCi/well) at 37° in an atmosphere containing 5% CO_2. The medium was then aspirated, the cells were detached from the plate by trypsinization, and the contents of the wells were collected onto glass fiber filters using a PhD cell harvester (Cambridge Technologies, Watertown, MA). Washes from the wells were also passed over the filters. In all, three 1-ml washes each of PBS, 10% (w/v) trichloroacetic acid, and 95% ethanol, all chilled on ice, were collected. Radioactivity on the filters was then determined by liquid scintillation counting. Incorporated counts were <2% of the total counts added. All treatments were performed in triplicate for each experiment, and the data are presented as the averages of at least three independent experiments, unless indicated otherwise in the figure legends.

Data analysis. Dose-response curves shown and IC_{50} and EC_{50} values reported in this paper were obtained by fitting the experimental data to a four-parameter logistic equation, using the software package Kaleidagraph (Synergy Software, Reading, PA).

Results

Binding of BN analogs to human and murine forms of the GRP receptor. Table 1 shows the structures of five BN analogs, whose binding affinities for the human GRP receptor are shown in Table 2. Analogs with des-methionine alkyl ester or amide motifs displayed the highest affinity for the receptor, exhibiting K_i values in the range of 0.68–5.2 nM (Fig. 1; Table 2). Analog **V**, Leu^{13}-ψ-Leu^{14}-BN, was about 1 order of magnitude less potent. The five BN analogs displayed similar binding affinities for the murine and human forms of the GRP receptor (Fig. 1; Table 2), with the notable exception of 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2$Ph-BN(7–13) methyl amide (**IV**). This compound displayed 30 times greater potency for the murine GRP receptor (K_i = 0.070 ± 0.001 nM) than for the human GRP receptor (K_i = 2.2 ± 0.047 nM), suggesting that the bulky hydrophobic moiety at position 12 of analog **IV** fit better in the binding pocket of the murine receptor.

Effect of BN analogs on DNA synthesis in GRP receptor-transfected BALB/3T3 cells. Activation of the GRP receptor in quiescent Swiss 3T3 cells results in the stimulation of [^{3}H]thymidine incorporation in the absence of other growth factors (3). As shown in Fig. 2, GRP also promoted a dose-dependent increase in the incorporation of [^{3}H]thymi-

TABLE 1

BN, GRP, and GRP receptor antagonists

Analog	Peptide	Structure
	BN	-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
	GRP	-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
I	D-Phe^6-BN(6–13) methyl ester	D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-O-CH_3
II	D-Phe^6-BN(6–13) ethyl amide	D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-$NHCH_2CH_3$
III	D-Phe^6-BN(6–13) propyl amide	D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-$NHCH_2CH_2CH_3$
IV	4-Pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2Ph$-BN(7–13) methyl amide	4-Pyridyl-CO-His-Trp-Ala-Val-D-Ala-Lys-Leu-$NHCH_3$ (with $COCH_2CH_2Ph$ on Lys)
V	Leu^{13}-ψ-(CH_2NH)-Leu^{14}-BN	-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-ψ-Leu-NH_2

TABLE 2

Affinities of BN analogs for the human and mouse GRP receptors and functional activities of analogs with respect to mitogenesis of GR1 cells ectopically expressing the human GRP receptor

Analog	^{125}I-GRP binding (K_i or K_d)		Agonism of mitogenesis		Antagonism of mitogenesis		Biological effect
	Mouse GRP receptor[a]	Human GRP receptor[b]	Effect[c]	EC_{50}[d]	Inhibition (1.0 nM GRP)[e]	IC_{50}	
	nM			% of maximum	nM	% of maximum	nM
GRP	0.12 ± 0.03	0.03 ± 0.01	100	0.18 ± 0.04[f]			Agonist
I	0.37 ± 0.03	1.38 ± 0.07	ND[g]		91	14 ± 8.5	Antagonist
II	2.09 ± 0.25	5.17 ± 0.64	31	1.2 ± 0.5	80	55 ± 14.2	Partial antagonist
III	0.44 ± 0.05	0.68 ± 0.01	38	0.2 ± 0.1	39	2.5 ± 1.7	Partial antagonist
IV	0.07 ± 0.01	2.17 ± 0.05	ND		79	5.1 ± 2.0	Antagonist
V	30.2 ± 0.05	21.6 ± 2.2	20	4.1 ± 1.3	69	182 ± 66	Partial antagonist

[a] Determined as described in Experimental Procedures, using membranes prepared from Swiss 3T3 cells. Values represent the average ± standard error of duplicate experiments.
[b] Determined using GR9 cell membranes. Values represent the average ± standard error of duplicate experiments.
[c] Maximal stimulation of [^{3}H]thymidine incorporation by analogs added alone to quiescent GR1 cells, relative to a maximal dose (100 nM) of GRP.
[d] Values represent the averages ± standard errors of three experiments.
[e] Maximal inhibition of [^{3}H]thymidine incorporation into quiescent GR1 cells triggered by 1.0 nM GRP.
[f] Fit of data from 13 independent experiments.
[g] ND, not detectable (<10% of maximal stimulation).

dine into the DNA of quiescent GRP receptor-transfected BALB/3T3 cells (GR1 cells), producing a maximal stimulation of about 3–5-fold over basal levels (EC_{50} = 0.18 ± 0.04 nM). Under the same conditions, GRP had no effect on the incorporation of [^{3}H]thymidine into BALB/3T3 cells stably transfected with a control expression vector not containing a GRP receptor cDNA insert. Furthermore, untransfected BALB/3T3 cells were not mitogenically stimulated by GRP (data not shown). These data establish that the mitogenic effect of GRP on quiescent GR1 cells is mediated through activation of ectopically expressed GRP receptors.

To assess the activity of analogs I-V with respect to mitogenesis triggered by the human form of the GRP receptor, we determined their effects on the incorporation of [^{3}H]thymidine by quiescent GR1 cells. We found that three of the five BN analogs analyzed, namely D-Phe^6-BN(6–13) ethyl amide (**II**), D-Phe^6-BN(6–13) propyl amide (**III**), and Leu^{13}-ψ-Leu^{14}-BN (**V**), were partial activators of GRP receptor-mediated mitogenesis. Analogs **II**, **III**, and **V** stimulated [^{3}H]thymidine incorporation into GR1 cells in a dose-dependent manner (Fig. 2), producing maximal responses that were 31%, 38%, and 20%, respectively, of the maximal effect elicited by a saturating dose of GRP (Table 2). When added without GRP, D-Phe^6-BN(6–13) propyl amide (**III**) had no effect on the incorporation of [^{3}H]thymidine by untransfected BALB/3T3 cells, indicating that the responses seen in GR1 cells were mediated by ectopically expressed GRP receptors (data not shown). As shown in Fig. 3, analogs **II**, **III**, and **V** were also incomplete inhibitors of GRP-stimulated [^{3}H]thymidine incorporation into quiescent GR1 cells. These results are consistent with their partial agonistic effects on mitogenesis. The rank order of potency of the three agonistic BN analogs correlates well with their relative binding affinities for the GRP receptor in GR9 cell membranes (Table 2).

In contrast to the partial agonistic activity of analogs **II**, **III**, and **V** described above, the two analogs with a desmethionine methyl ester or methyl amide motif, namely D-Phe^6-BN(6–13) methyl ester (**I**) and 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2Ph$-BN(7–13) methyl amide (**IV**), acted as pure GRP antagonists with respect to the stimulation of [^{3}H]thymidine incorporation in GR1 cells. At the highest concentration tested (1 μM), both analogs inhibited the mitogenic response elicited by 1 nM GRP by roughly 80–90% (Fig. 3; Table 2) and were not able to significantly stimulate [^{3}H]thymidine incorporation activity when added to quiescent GR1 cells in the absence of GRP (Fig. 2; Table 2).

Effect of BN analogs on Ca^{2+} mobilization in GRP receptor-transfected BALB/3T3 cells. To further assess the pharmacological properties of the human GRP receptor, we tested the ability of BN analogs to trigger the release of intracellular Ca^{2+} into the cytoplasm of GR1 cells, a response known to be promoted by activation of the GRP receptor in other cells (3). The intracellular Ca^{2+} concentration was monitored by fluorescence measurements in cells loaded with the Ca^{2+}-chelating dye fura-2. As shown in Fig. 4, GRP promoted a rapid rise in intracellular Ca^{2+} concentration. Control BALB/3T3 cells not expressing the GRP receptor

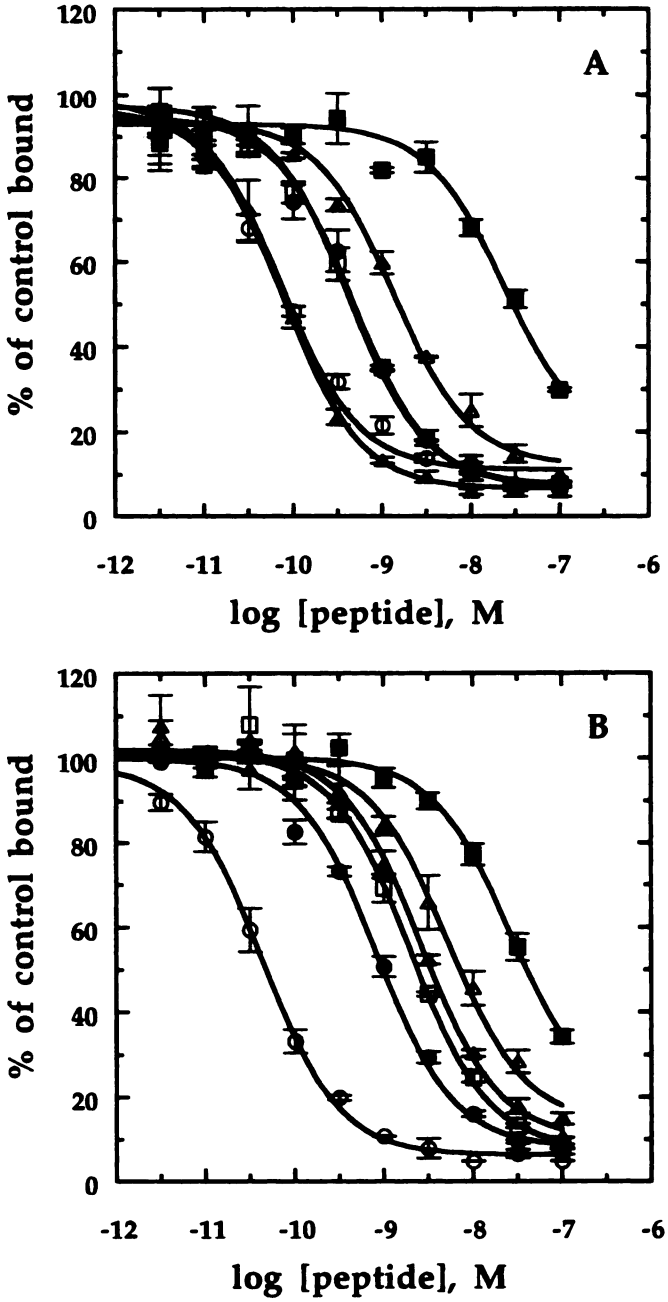

Fig. 1. Affinity of BN analogs for the mouse and human forms of the GRP receptor. Increasing doses of BN analogs were assayed for their ability to inhibit ^{125}I-GRP binding to membranes prepared from murine Swiss 3T3 cells (A) or GR9 cells (B), which ectopically express the human GRP receptor, as described in Experimental Procedures. Peptides tested were GRP (○), D-Phe^6-BN(6–13) methyl ester (□), D-Phe^6-BN(6–13) ethyl amide (△), D-Phe^6-BN(6–13) propyl amide (●), 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-COCH$_2$CH$_2$Ph-BN(7–13) methyl amide (▲), and Leu^{13}-ψ-(CH$_2$NH)-Leu^{14}-BN (■). Data represent the mean ± standard error from two independent assays.

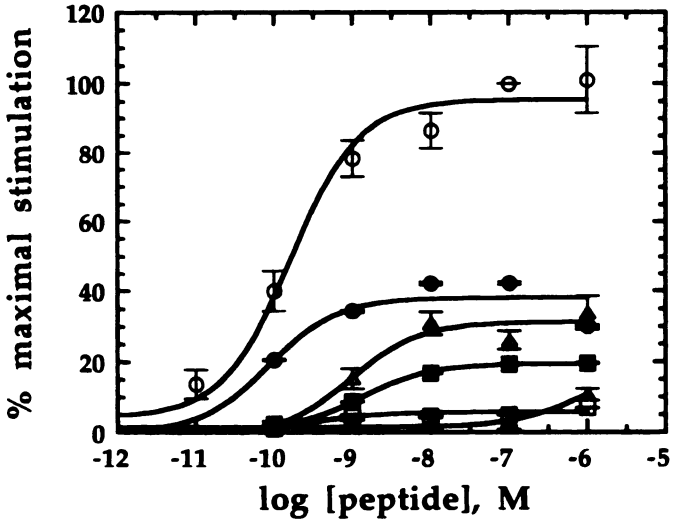

Fig. 2. Ability of BN analogs to stimulate [^{3}H]thymidine incorporation in quiescent GR1 cells. Stimulation of [^{3}H]thymidine incorporation into confluent GR1 cells, expressing the human GRP receptor, was assessed as described in Experimental Procedures. Increasing doses of the following peptides were analyzed: GRP (○), D-Phe^6-BN(6–13) methyl ester (□), D-Phe^6-BN(6–13) ethyl amide (△), D-Phe^6-BN(6–13) propyl amide (●), 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-COCH$_2$CH$_2$Ph-BN(7–13) methyl amide (▲), and Leu^{13}-ψ-(CH$_2$NH)-Leu^{14}-BN (■). In the experiments reported, the basal rate of [^{3}H]thymidine incorporation ranged from 5000 to 9000 cpm, whereas the incorporation achieved at a saturating dose of GRP (1 nM) ranged from 27,000 to 50,000 cpm. The stimulation by GRP ranged from 3- to 5-fold. After subtraction of the basal incorporation of [^{3}H]thymidine, the data were normalized to the incorporation observed at a saturating dose of GRP (1 nM). Results shown represent the averages ± standard errors of three independent experiments.

showed no GRP-dependent increases in intracellular Ca^{2+} concentration (data not shown). Interestingly, D-Phe^6-BN(6–13) ethyl amide (**II**) and D-Phe^6-BN(6–13) propyl amide (**III**) completely blocked GRP-mediated Ca^{2+} mobilization in GR1 cells (Fig. 4). The failure of these analogs to trigger a significant mobilization of calcium in GR1 cells contrasts with their ability to partially activate [^{3}H]thymidine incorporation in these cells, as discussed above. The same result was obtained with Leu^{13}-ψ-Leu^{14}-BN (**V**) (data not shown).

We further examined the effect of BN analogs on Ca^{2+} mobilization in GR1 cells by measuring the fluorescence of cells loaded with the dye fluo-3. None of the five BN analogs examined (at 1 μM) was able to trigger a significant mobilization of calcium in GR1 cells, as assessed with fluo-3 (data not shown). Further testing of the effects of analogs **I**, **II**, **IV**, and **V** on calcium mobilization by GR1 cells at various doses ranging from 10 nM to 10 μM revealed no agonistic effects. In contrast, all five BN analogs effectively inhibited, in a concentration-dependent manner, the Ca^{2+} mobilization response of GR1 cells stimulated by a saturating dose of GRP (10 nM) (Fig. 5). At the highest concentration of each analog tested (1 μM), the response was inhibited by >80%. As shown in Table 3, the rank order of potency for antagonism of calcium mobilization in GR1 cells was the same as their relative affinities for the human GRP receptor (Table 2), with the exception of a minor variation with respect to analogs **I** and **IV**. These analogs displayed similar K_i values for receptor binding and IC_{50} values for the antagonism of [^{3}H]thymidine incorporation by GR1 cells. These results demonstrate that analogs **I-V** act as pure antagonists of GRP-stimulated Ca^{2+} mobilization in GR1 cells.

Effect of D-Phe^6-BN(6–13) propyl amide (III) on intracellular Ca^{2+} mobilization and DNA synthesis in HFL cells. Considering our finding that some BN analogs displayed differential effects on calcium mobilization and DNA synthesis responses of GR1 cells, it was important to deter-

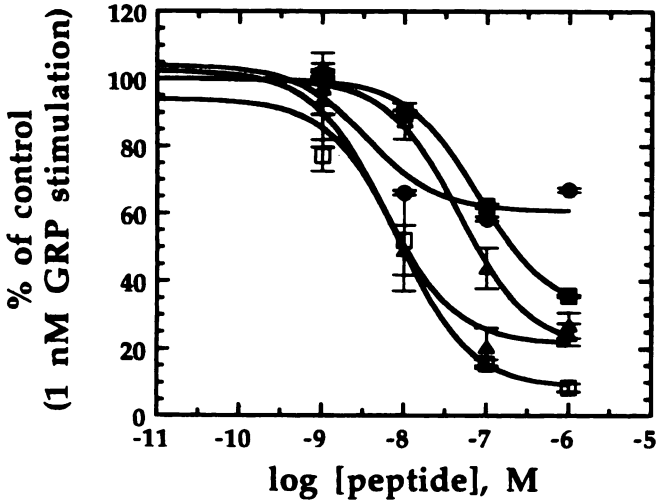

Fig. 3. Ability of BN analogs to inhibit GRP-stimulated [^{3}H]thymidine incorporation in quiescent GR1 cells. Confluent GR1 cells were assayed for [^{3}H]thymidine incorporation as described in Experimental Procedures. Increasing doses of the following analogs were assayed in the presence of GRP (1.0 nM): D-Phe^6-BN(6–13) methyl ester (□), D-Phe^6-BN(6–13) ethyl amide (△), D-Phe^6-BN(6–13) propyl amide (●), 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-COCH$_2$CH$_2$Ph-BN(7–13) methyl amide (▲), and Leu^{13}-ψ-(CH$_2$NH)-Leu^{14}-BN (■). In the experiments reported, the basal rate of [^{3}H]thymidine incorporation ranged from 5000 to 9000 cpm, whereas the incorporation achieved at a saturating dose of GRP (1 nM) ranged from 27,000 to 50,000 cpm. The stimulation by GRP ranged from 3- to 5-fold. After subtraction of the basal incorporation of [^{3}H]thymidine, the data were normalized to the maximal incorporation stimulated by GRP (1 nM). Results shown represent the average ± standard error of three independent experiments.

mine whether BN analogs have similar effects on a nontransformed human cell line naturally expressing GRP receptors. For these studies, we chose HFL cells, which were shown previously to express high affinity binding sites for ^{125}I-GRP (31). From competitive displacement ^{125}I-GRP binding studies, we found that membranes prepared from HFL cells expressed 0.084 pmol of GRP receptor/mg of membrane protein (K_d = 0.043 nM).

GRP promoted a roughly 2-fold increase in [^{3}H]thymidine incorporation by confluent and serum-starved HFL cells ($p < 0.01$) (Fig. 6). D-Phe^6-BN(6–13) propyl amide (**III**) and D-Phe^6-BN(6–13) ethyl amide (**II**), at a saturating dose (1 μM), partially stimulated [^{3}H]thymidine incorporation by quiescent HFL cells (Fig. 6A). Conversely, analogs **II** and **III** partially inhibited DNA synthesis by quiescent HFL cells stimulated with 1 nM GRP (Fig. 6B). In contrast, D-Phe^6-BN(6–13) methyl ester (**I**) displayed no agonistic activity and was able to completely block the GRP response. Using polymerase chain reaction amplification methods, we found that mRNA for both NMB and GRP receptors is expressed in HFL cells.[1] Whereas NMB promoted DNA synthesis by HFL cells, the response could be completely blocked by D-Phe^6-BN(6–13) methyl ester (**I**) (data not shown), which is a selective analog for the GRP receptor. These results indicate that the mitogenic responses we observed in HFL cells were promoted by activation of the GRP receptor subtype.

GRP also triggered a rapid increase in the intracellular calcium concentration in HFL cells, as determined from the fluorescence of cells loaded with fura-2 (Fig. 7A). In spite of their ability to partially activate DNA synthesis by HFL cells, analogs **II** and **III** did not stimulate an intracellular Ca^{2+} mobilization response in HFL cells (Fig. 7, B and C) and were able to completely inhibit intracellular Ca^{2+} mobilization stimulated by 10 nM GRP. Therefore, the activities of BN analogs with respect to mitogenic and intracellular Ca^{2+} mobilization responses in HFL cells and GR1 cells were very similar, demonstrating a correspondence in the pharmacology of BN analogs determined with GRP receptor-transfected BALB/3T3 cells and a natural human cell line.

Effect of BN analogs on intracellular Ca^{2+} mobilization in transfected BALB/3T3 cells expressing high levels of the GRP receptor. It is clear from the results presented above that some BN analogs display more agonistic activity with respect to the [^{3}H]thymidine incorporation response of transfected GR1 cells or HFL cells than to the Ca^{2+} mobilization response of these cells. This phenomenon might be explained by the fact that maximal triggering of [^{3}H]thymidine incorporation requires the activation of fewer GRP receptors than needed to maximally activate Ca^{2+} mobilization.

To test this hypothesis, we examined the effects of BN analogs on Ca^{2+} mobilization by a BALB/3T3 clone (GR9 cells) ectopically expressing about 30 times more human GRP receptor molecules than expressed by GR1 cells. GRP was 6 times more potent in stimulating Ca^{2+} mobilization in GR9 cells than in GR1 cells (EC_{50} = 0.17 ± 0.06 versus 1.0 ± 0.44 nM) (Fig. 8). This finding indicates that the increased level of GRP receptor expression in GR9 cells resulted in the generation of excess receptors with respect to the Ca^{2+} mobilization response. According to our hypothesis, GRP receptor-coupled Ca^{2+} mobilization in GR9 cells should show an increased sensitivity to agonism by analogs **II**, **III**, and **V**. Indeed, each of these analogs was able to significantly stimulate mobilization of Ca^{2+} in GR9 cells (Fig. 9). The response to analogs **II**, **III**, and **V** increased in a dose-dependent manner and plateaued at levels of 48%, 74%, and 42%, respectively, of the response generated by saturating concentrations of GRP (Table 4).

Consistent with our finding that D-Phe^6-BN(6–13) methyl ester (**I**) and 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}COCH$_2$CH$_2$-Ph-BN (7–13) methyl amide (**IV**) were pure antagonists of GR1 cell mitogenesis, these compounds were not able to trigger a detectable Ca^{2+} mobilization response in GR9 cells (Fig. 9). Additional experiments demonstrated that analog **I** [D-Phe^6-BN(6–13) methyl ester] was a potent blocker of Ca^{2+} mobilization in GR9 cells stimulated by treatment with 10 nM GRP (data not shown). These data confirm that D-Phe^6-BN(6–13) methyl ester (**I**) and 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-COCH$_2$CH$_2$Ph-BN(7–13) methyl amide (**IV**) are the purest BN antagonists profiled in our study.

Discussion

A number of studies have demonstrated a potential use for GRP receptor blockers in the treatment of breast (32), colon (33), lung (1, 10), prostate (6, 34), and pancreatic (35) cancers. Although most BN analogs have been extensively characterized using rodent model systems, further characterization of their pharmacology with respect to human BLP receptor subtypes is warranted. In particular, it is important

[1] J. M. Wu, D. O. Hoang, and R. I. Feldman, unpublished observations.

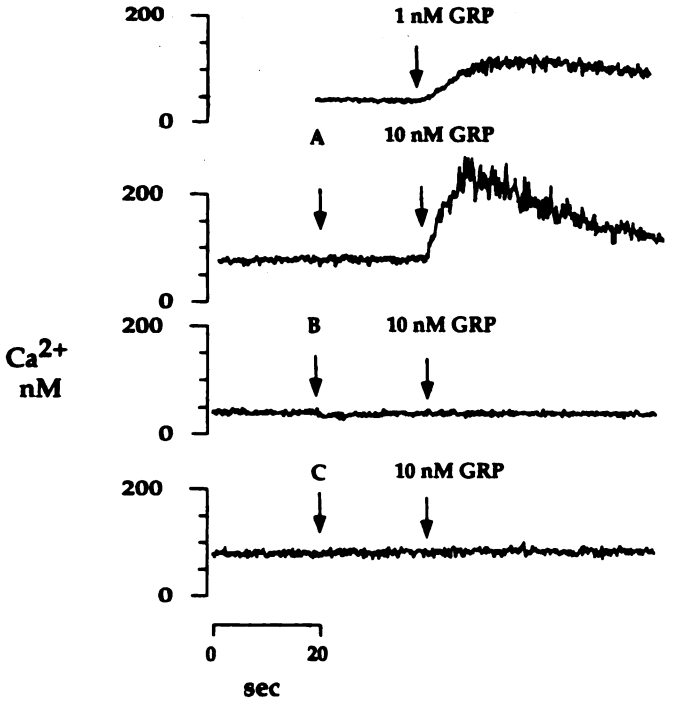

Fig. 4. Effects of GRP and BN analogs on Ca^{2+} mobilization in GR1 cells. Measurement of intracellular Ca^{2+} concentration in GR1 cells was performed as a function of time, using the dye fura-2, as described in Experimental Procedures. *Arrow A*, addition of vehicle; *arrow B*, addition of 1 μM D-Phe^6-BN(6–13) ethyl amide; *arrow C*, addition of 1 μM D-Phe^6-BN(6–13) propyl amide. The addition of GRP at the specified concentration is also indicated.

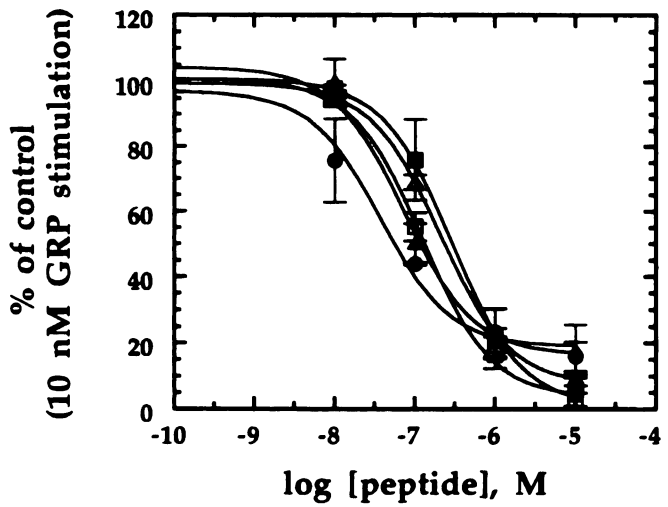

Fig. 5. Ability of BN analogs to inhibit GRP-stimulated Ca^{2+} mobilization in GR1 cells. Changes in intracellular Ca^{2+} concentration were monitored in GR1 cells loaded with the calcium-sensing dye fluo-3, as described in Experimental Procedures. Shown are the effects of increasing doses of the following peptides on the ability of GRP (10 nM) to increase fluo-3 fluorescence: D-Phe^6-BN(6–13) methyl ester (□), D-Phe^6-BN(6–13) ethyl amide (△), D-Phe^6-BN(6–13) propyl amide (●), 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2$Ph-BN(7–13) methyl amide (▲), and Leu^{13}-ψ-(CH_2NH)-Leu^{14}-BN (■). Data were normalized to the maximal increase in fluorescence stimulated by 10 nM GRP and represent the average ± standard error of two independent experiments.

TABLE 3

Ability of BN analogs to inhibit GRP-stimulated Ca^{2+} mobilization in GR1 cells

Analogs were tested for their ability to inhibit Ca^{2+} mobilization stimulated by treatment of GR1 cells, expressing the human GRP receptor, with GRP (10 nM). IC_{50} values represent the mean ± standard error of two independent experiments.

Analog	Peptide	Inhibition (10 nM GRP)	IC_{50}
		% of maximum	*nM*
I	D-Phe^6-BN(6–13) methyl ester	96	120 ± 30
II	D-Phe^6-BN(6–13) ethyl amide	93	220 ± 60
III	D-Phe^6-BN(6–13) propyl amide	81	34 ± 20
IV	4-Pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2$Ph-BN(7–13) methyl amide	85	71 ± 7
V	Leu^{13}-ψ-(CH_2NH)-Leu^{14}-BN	99	300 ± 120

to understand the ability of each analog to activate or to block a mitogenic response mediated by BLP receptors. In this paper, we characterized the pharmacology of the human GRP receptor using five BN analogs that are the among the most potent reported blockers of rodent GRP receptors. We demonstrated that D-Phe^6-BN(6–13) methyl ester (I) and 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2$Ph-BN(7–13) methyl amide (IV) antagonized GRP-stimulated DNA synthesis in quiescent BALB/3T3 cells ectopically expressing the GRP receptor. In contrast, D-Phe^6-BN(6–13) ethyl amide (II), D-Phe^6-BN(6–13) propyl amide (III), and Leu^{13}-ψ-Leu^{14}-BN (V) were partial activators of this response.

An unexpected finding of our study was that, in BALB/3T3 cells ectopically expressing a moderate number of GRP receptors (i.e., GR1 cells), three analogs, namely D-Phe^6-BN(6–13) ethyl amide (II), D-Phe^6-BN(6–13) propyl amide (III), and Leu^{13}-ψ-Leu^{14}-BN (V), stimulated mitogenesis without significantly elevating the level of intracellular Ca^{2+}. The possibility that this phenomenon was limited to transfected BALB/3T3 cells was eliminated by the demonstration of similar results with several compounds using HFL cells, which naturally express the GRP receptor. The correspondence in the pharmacology obtained with GR1 and HFL cells also indicates a high degree of conservation in the signaling pathways of mouse and human fibroblast cells.

One explanation for the fact that DNA synthesis was more sensitive to agonism by BN analogs than was intracellular Ca^{2+} mobilization is that mitogenesis is stimulated by the activation of a far fewer number of receptors. Thus, if the mitogenic response of a GR1 cell was maximally triggered by the activation of a small percentage of its pool of available GRP receptors, then BN analogs retaining the ability to activate a few percent of the receptor molecules could still trigger a significant response. In contrast, the intracellular Ca^{2+} mobilization response, whose size may be more proportional to the total percentage of receptors activated, might not be activated at a detectable level.

Such an hypothesis is supported by the fact that GRP was 6 times more potent in triggering a mitogenic response in GR1 cells than a calcium mobilization response (EC_{50} = 0.18 ± 0.04 versus 1.0 ± 0.44 nM). Fewer activated GRP receptors were therefore required to generate a maximal mitogenic response. The hypothesis is further supported by the fact that analogs II, III, and V became partial agonists of Ca^{2+} mobilization in a BALB/3T3 transfectant expressing much higher levels of the GRP receptor, compared with GR1 cells.

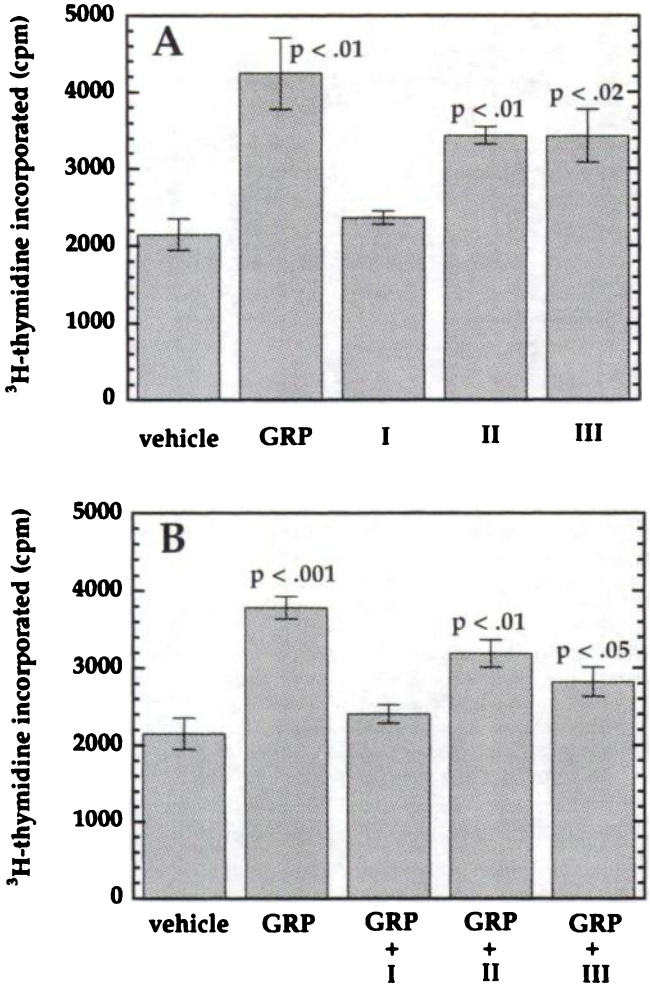

Fig. 6. Effects of GRP and BN analogs on [^{3}H]thymidine incorporation in quiescent HFL cells. Stimulation of [^{3}H]thymidine incorporation into confluent HFL cells was assayed as described in Experimental Procedures. A, The agonistic effects of GRP (1 μM) and a 1 μM dose of analogs D-Phe^6-BN(6–13) methyl ester (I), D-Phe^6-BN(6–13) ethyl amide (II), or D-Phe^6-BN(6–13) propyl amide (III) are shown. B, The ability of analogs I, II, and II (1 μM) to inhibit [^{3}H]thymidine incorporation stimulated by 1 nM GRP is shown. Experiments were performed at least three times, and representative data are shown.

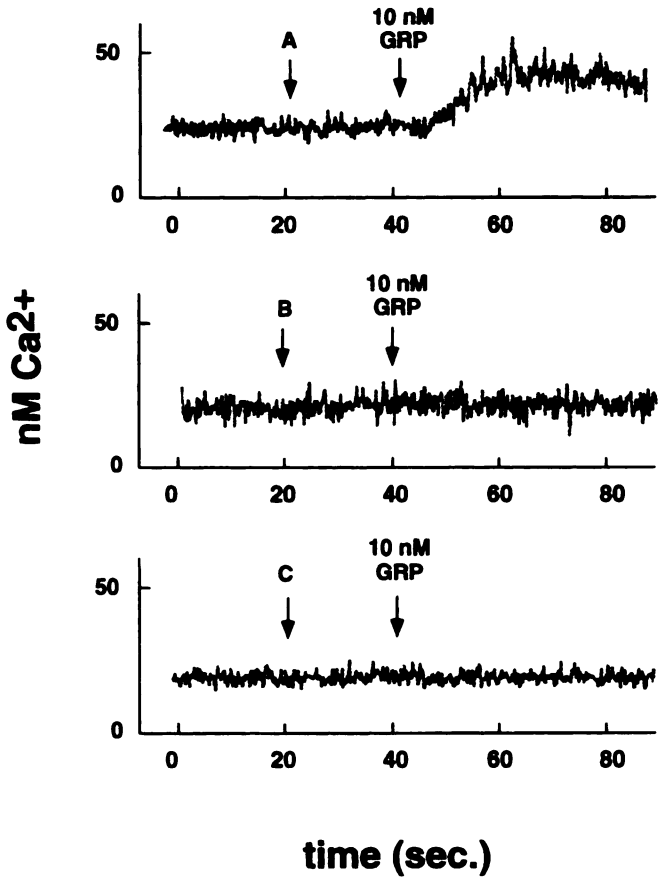

Fig. 7. Effects of GRP and BN analogs on Ca^{2+} mobilization in HFL cells. Measurements of intracellular Ca^{2+} concentration in HFL cells were performed as a function of time, using the dye fura-2, as described in Experimental Procedures. *Arrow A*, addition of vehicle; *arrow B*, addition of 1 μM D-Phe^6-BN(6–13) ethyl amide (II); *arrow C*, addition of 1 μM D-Phe^6-BN(6–13) propyl amide (III). The addition of GRP at the specified concentration is also indicated.

In that clone (GR9 cells), spare receptors for the intracellular Ca^{2+} mobilization response appeared to be present, because we observed that the level of Ca^{2+} mobilization (measured by fura-2 fluorescence) elicited by a saturating dose of GRP was not greater in GR9 cells than in GR1 cells (data not shown). The fact that GRP was 6 times more potent in eliciting an intracellular Ca^{2+} mobilization response in GR9 cells than in GR1 cells (EC_{50} = 0.17 ± 0.06 versus 1.0 ± 0.44 nM) (Fig. 8) is also consistent with the presence of a greater number of spare receptors in GR9 cells than in GR1 cells. There is an alternative explanation for our data, which cannot be completely ruled out, if Ca^{2+} mobilization and mitogenic responses are triggered by the GRP receptor through the coupling of distinct G proteins. In this case, analog binding to the receptor could conceivably activate one of these G proteins while not activating the other.

Stimulation of Ca^{2+} mobilization by GRP in murine Swiss 3T3 cells is thought to be mediated by inositol-1,4,5-triphosphate produced by the action of phospholipase C-β (36). This enzyme appears to activate the receptor via a G protein of the G_q family (37). In contrast, the requisite signals leading to a mitogenic response have not been clearly elucidated but could involve a number of known receptor-mediated responses (2, 3, 38–40), including the activation of mitogen-activated protein kinase and p125 focal adhesion kinase, the stimulation of arachidonic acid formation, and the elevation of c-*fos* and c-*myc* expression. Seckl and Rozengurt (41) found that tyrphostin [[(3,4,5-trihydroxypenyl)-methylene]propanedinitrile] blocks the mitogenic effects of GRP on Swiss 3T3 cells without inhibiting Ca^{2+} mobilization or protein kinase C activation, underscoring the potential role of tyrosine kinases in the mitogenic response. By triggering these and possibly other amplifying events, a small amount of receptor activation could generate a large mitogenic signal. It has been difficult to definitively address the role played by Ca^{2+} mobilization in the mitogenic effects of many growth factors, due to the difficulty of dissociating these two responses. It is therefore significant that the BN analogs D-Phe^6-BN(6–13) ethyl amide (II), D-Phe^6-BN(6–13) propyl amide (III), and Leu^{13}-ψ-Leu^{14}-BN (V) were able to stimulate mitogenesis in GR1 cells without significant activation of intracellular Ca^{2+} mobilization. These results provide evidence that Ca^{2+}

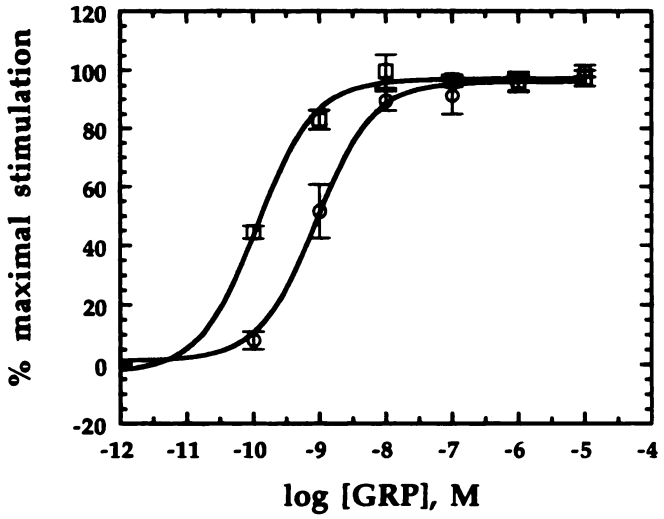

Fig. 8. Dose-response relationship for GRP effects on Ca^{2+} mobilization in GR1 and GR9 cells. Changes in the concentration of intracellular Ca^{2+} in GR1 (○) or GR9 (□) cells induced by various concentrations of GRP were determined as described in Experimental Procedures. Data were normalized to the maximal increase in intracellular Ca^{2+} concentration stimulated by GRP and represent the average ± standard error of three independent experiments. For GR1 cells the EC_{50} value was 1.0 ± 0.40 nM and for GR9 cells the EC_{50} value was 0.17 ± 0.6 nM.

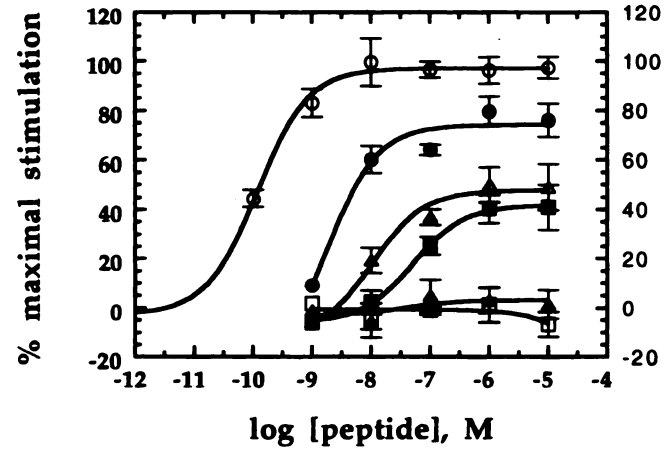

Fig. 9. Stimulation of intracellular Ca^{2+} mobilization by BN analogs in GR9 cells. Ca^{2+} concentration was measured in GR9 cells using the dye fluo-3, as described in Experimental Procedures. Shown are the effects of increasing doses of the following peptides: GRP (○), D-Phe^6-BN(6–13) methyl ester (□), D-Phe^6-BN(6–13) ethyl amide (△), D-Phe^6-BN(6–13) propyl amide (●), 4-pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2$-Ph-BN(7–13) methyl amide (▲), and Leu^{13}-ψ-(CH_2NH)-Leu^{14}-BN (■). Data were normalized to the maximal increase in intracellular Ca^{2+} concentration stimulated by GRP and represent the average ± standard error of three independent experiments.

mobilization is not required to achieve activation of cellular proliferation via the GRP receptor.

A number of other studies have also addressed the functional activities of the BN analogs characterized in this paper. Buchan *et al.* (22) found that D-Phe^6-BN(6–13) propyl amide (III) exhibited partial agonistic/antagonistic activity at GRP receptors in human G cells, as assessed by measurement of the GRP receptor-mediated secretion of gastrin. D-Phe^6-BN(6–13) methyl ester (I) displayed a purely antagonistic profile in this system. These results agree with the findings presented in this paper. They also indicate that the

TABLE 4

Ability of BN analogs to stimulate Ca^{2+} mobilization in GR9 cells

The ability of BN analogs to stimulate Ca^{2+} mobilization in the absence of GRP was assessed in GR9 cells. The data represent the mean ± standard error of three independent experiments.

Analog	Peptide	Stimulation[a]	EC_{50}
		% of maximum	*nM*
	GRP	100	0.17 ± 0.06
I	D-Phe^6-BN(6–13) methyl ester	ND[b]	
II	D-Phe^6-BN(6–13) ethyl amide	48	21.4 ± 16.2
III	D-Phe^6-BN(6–13) propyl amide	74	2.0 ± 1.3
IV	4-Pyridyl-CO-His^7-D-Ala^{11}-Lys^{12}-$COCH_2CH_2$Ph-BN(7–13) methyl amide	ND	
V	Leu^{13}-ψ-(CH_2NH)-Leu^{14}-BN	42	71.6 ± 24.4

[a] Normalized to the maximal stimulation produced by GRP.
[b] ND, not detectable (<10%).

secretory response of human G cells has a sensitivity to partial agonistic effects of BN analogs similar to that of the mitogenic response of GR1 cells. This phenomenon may result from a relatively small number of activated receptors being required to generate this response or the presence of a large number of GRP receptors on these cells. Using the Ca^{2+} mobilization response in human small cell lung carcinoma cells, which express relatively few BLP receptors (7), Staley *et al.* (21) demonstrated that D-Phe^6-BN(6–13) propyl amide (III) is a complete antagonist. This result is consistent with our finding that this compound does not stimulate Ca^{2+} mobilization in GR1 cells. Although D-Phe^6-BN(6–13) propyl amide (III) was able to partially activate a mitogenic response in GR1 cells, it is not clear whether it would also promote growth in cells that express fewer GRP receptors, such as small cell lung carcinoma cell lines (7), or cells that potentially exhibit differences in their coupling of GRP receptors to proliferative responses.

One compound with a reduced peptide bond motif was characterized in our study, i.e., Leu^{13}-ψ-Leu^{14}-BN (V), which also displayed significant partial agonistic/antagonistic activity with respect to the mitogenesis of GR1 cells. A similar profile has been found for this compound in human G cells (42) and rat acinar cells (20), whereas the compound behaved as a complete antagonist in canine G cells (43), guinea pig acinar cells, and murine 3T3 cells (20). A large number of BN analogs with a reduced peptide bond (-CH_2-NH-) between residues 13 and 14 have been generated (16), and further work is warranted to assess their activities against the human GRP receptor.

We have demonstrated that cells stably transfected with BLP receptor cDNAs are useful for the characterization of compound pharmacology. Such a model system provides a number of advantages over available nontransfected human cell lines for the pharmacological characterization of potential BLP receptor blockers. In particular, transfected cells can be used in place of cells that poorly express a receptor of interest or cells that express related receptors that may respond to test compounds. The ability to express different receptors in the same host also facilitates a direct comparison of the pharmacology of different receptor variants. In this regard, we have used BALB/3T3 cells ectopically expressing the human NMB receptor and BLP receptor subtype 3 to

characterize the activity of BLP analogs.[2] Others have also ectopically expressed the rat NMB receptor in BALB/3T3 cells and found that it behaves very similarly to the NMB receptor naturally expressed in C_6 rat glioma cells or rat esophageal muscle (24). Finally, BALB/3T3 cells can be engineered to express receptors at different expression levels. As demonstrated in this report, the effects of partial receptor activators on intracellular signaling pathways can vary greatly, depending upon the parameter of receptor number.

Acknowledgments

We thank Elaina Mann for the construction of cell lines and her initial studies of their [^{3}H]thymidine incorporation, Sarah Fried for her expert technical assistance, Joanne Cordova for her secretarial support, Dr. John Morser and Dr. Anthony Johns for their comments on the manuscript, and Dr. David Coy for his generous gift of D-Phe^6-BN(6–13) methyl ester.

References

1. Anastasi, A., V. Erspamer, and M. Bucci. Isolation and amino acid sequences of alytesin and bombesin, two analogous active tetradecapeptides from the skin of European discoglosid frogs. *Arch. Biochem. Biophys.* **148**:443–446 (1972).
2. Lebacq-Verheyden, A. M., J. Trepel, E. A. Sausville, and J. F. Battey. Bombesin and gastrin-releasing peptide: neuropeptides, secretagogues, and growth factors. *Handb. Exp. Pharmacol.* **95**:71–124 (1990).
3. Zachary, I., J. P. Wolls, and E. Rozengurt. A role for neuropeptides in the control of cell proliferation. *Dev. Biol.* **124**:295–308 (1987).
4. Nelson, J., M. Donnelly, B. Walker, J. Gray, C. Shaw, and R. F. Murphy. Bombesin stimulates proliferation of human breast cancer cells in culture. *Br. J. Cancer.* **63**:933–936 (1991).
5. Narayan, S., Y.-S. Guo, C. M. Townsend, Jr., and P. Singh. Specific binding and growth effects of bombesin-related peptides on mouse colon cancer cells *in vitro*. *Cancer Res.* **50**:6772–6778 (1990).
6. Bologna, M., C. Festuccia, P. Muzi, L. Biordi, and M. Ciomei. Bombesin stimulates growth of human prostatic cancer cells *in vitro*. *Cancer (Phila.)* **63**:1714–1720 (1989).
7. Carney, D. N., F. Cuttitta, T. W. Moody, and J. D. Minna. Selective stimulation of small cell lung cancer clonal growth by bombesin and gastrin-releasing peptide. *Cancer Res.* **47**:821–825 (1987).
8. Mahmoud, S., J. Staley, J. Taylor, A. Bogden, J. P. Moreau, D. Coy, I. Avis, F. Cuttitta, J. L. Mulshine, and T. W. Moody. [*Psi*-13,14] bombesin analogues inhibit growth of small cell lung cancer *in vitro* and *in vivo*. *Cancer Res.* **51**:1798–1802 (1991).
9. Cuttitta, F., D. N. Carney, J. Mulshine, T. W. Moody, J. Fedorko, A. Fischler, and J. D. Minna. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature (Lond.)* **316**:823–826 (1985).
10. Thomas, F., F. Arvelo, E. Antoine, M. Jacrot, and M. F. Poupon. Antitumoral activity of bombesin analogues on small cell lung cancer xenografts: relationship with bombesin receptor expression. *Cancer Res.* **52**:4872–4877 (1992).
11. Battey, J. F., J. Way, M. H. Corjay, H. Shapira, K. Kusano, R. Harkins, J. M. Wu, T. Slattery, E. Mann, and R. I. Feldman. Molecular cloning of the bombesin/gastrin-releasing peptide receptor from Swiss 3T3 cells. *Proc. Natl. Acad. Sci. USA* **88**:395–399 (1991).
12. Wada, E., J. Way, H. Shapira, K. Kusano, A. M. Labacq-verheyden, D. Coy, R. Jenson, and J. Battey. cDNA cloning, characterization, and brain region-specific expression of a neuromedin-B-preferring bombesin receptor. *Neuron* **6**:421–430 (1991).
13. Corjay, M. H., D. J. Dobrzanski, J. M. Way, J. Viallet, H. Shapira, and P. Worland. Two distinct bombesin receptor subtypes are expressed and functional in human lung carcinoma cells. *J. Biol. Chem.* **266**:18771–18779 (1991).
14. Fathi, Z., M. H. Corjay, H. Shapira, E. Wada, R. Benya, R. Jensen, J. Viallet, E. A. Sausville, and J. F. Battey. BRS-3: a novel bombesin receptor subtype selectively expressed in testis and lung caracinoma cells. *J. Biol. Chem.* **268**:5979–5984 (1993).
15. Gorbulev, V., A. Akhuddova, H. Buchner, and F. Fahrenholz. Molecular cloning of a new bombesin receptor subtype expressed in uterus during pregnancy. *Eur. J. Biochem.* **208**:405–410 (1992).
16. Coy, D. H., J. E. Taylor, N.-Y. Jiang, S. H. Kim, L.-H. Wang, S. C. Huang, J.-P. Moreau, J. D. Gardner, and R. T. Jensen. Short-chain pseudopeptide bombesin receptor antagonists with enhanced binding affinities for pancreatic acinar and Swiss 3T3 cells display strong antimitotic activity. *J. Biol. Chem.* **264**:14691–14697 (1989).
17. Wang, L. H., D. H. Coy, J. E. Taylor, H.-Y. Jiang, S. H. Kim, J.-P. Moreau, S. C. Huang, S. A. Mantey, H. Frucht, and R. T. Jensen. Desmethionine alkylamide bombesin analogues: a new class of bombesin receptor antagonists with potent antisecretory activity in pancreatic acini and antimitotic activity in Swiss 3T3 cells. *Biochemistry* **29**:616–622 (1990).
18. Best, J. R., R. Camble, R. Cotton, A. S. Dutta, B. Fleming, A. Garner, J. J. Gormley, C. F. Hayward, P. F. McLachlan, and P. B. Scholes. Design and synthesis of bombesin/gastrin-releasing peptide antagonists. *Biochem. Soc. Trans.* **18**:1294–1296 (1990).
19. Heimbrook, D. C., W. S. Saari, N. L. Balishin, T. W. Fisher, A. Friedman, S. M. Kiefer, N. S. Rotberg, J. W. Wallen, and A. Oliff. Gastrin releasing peptide antagonists with improved protency and stability. *J. Med. Chem.* **34**:2102–2107 (1991).
20. Wang, L. H., D. H. Coy, J. E. Taylor, H.-Y. Jiang, J. P. Moreau, S. C. Huang, H. Frucht, B. M. Haffar, and R. T. Jensen. Des-Met carboxyl-terminally modified analogues of bombesin function as potent bombesin receptor antagonists, partial agonists, or agonists. *J. Biol. Chem.* **265**:15695–15703 (1990).
21. Staley, J., D. H. Coy, J. E. Taylor, S. Kim, and T. W. Moody. [Des-Met^{14}]Bombesin analogues function as small cell lung cancer bombesin receptor antagonists. *Peptides* **12**:145–149 (1991).
22. Buchan, A. M. J., M. Meloche, and D. H. Coy. Inhibition of bombesin-stimulated gastrin release from isolated human G cells by bombesin analogs. *Pharmacology (Basel)* **41**:237–245 (1990).
23. Fathi, Z., R. V. Benya, H. Shapira, R. T. Jensen, and J. F. Battey. The fifth transmembrane segment of the neuromedin B receptor is critical for high affinity neuromedin B binding. *J. Biol. Chem.* **268**:14622–14626 (1993).
24. Benya, R. V., E. Wada, J. F. Battey, Z. Fathi, L. H. Wang, S. A. Mantey, D. H. Coy, and R. T. Jensen. Neuromedin B receptors retain functional expression when transfected into BALB/3T3 fibroblasts: analysis of binding, kinetics, stoichiometry, modulation by guanine nucleotide-binding proteins, and signal transduction and comparison with natively expressed receptors. *Mol. Pharmacol.* **42**:1058–1068 (1992).
25. Wada, K., C. J. Dechesne, S. Shimasaki, R. G. King, K. Kusano, A. Buonanno, D. R. Hampson, C. Banner, R. J. Wenthold, and Y. Nakatani. Sequence and expression of a frog brain complementary DNA encoding a kainate-binding protein. *Nature (Lond.)* **342**:684–689 (1989).
26. Artelt, P., C. Morelle, M. Ausmeier, M. Fitzek, and H. Hauser. Vectors for efficient expression in mammalian fibroblastoid, myeloid and lymphoid cells via transfection or infection. *Gene* **68**:213–219 (1988).
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
28. Feldman, R. I., J. M. Wu, J. C. Jenson, and E. Mann. Purification and characterization of the bombesin/gastrin-releasing peptide receptor from Swiss 3T3 cells. *J. Biol. Chem.* **265**:17364–17372 (1990).
29. Munson, P. J. LIGAND: a computerized analysis of ligand binding data. *Methods Enzymol.* **92**:543–576 (1983).
30. Di Virgillio, R., T. H. Steinberg, and S. C. Silverstein. Inhibitors of fura-2 sequestration and secretion with organic anion transport blockers. *Cell Calcium* **11**:57–62 (1990).
31. Kris, R. M., R. Hazan, J. Villines, T. W. Moody, and J. Schlessinger. Identification of the bombesin receptor on murine and human cells by cross-linking experiments. *J. Biol. Chem.* **262**:11215–11220 (1987).
32. Szepeshazi, K., A. V. Schally, G. Halmos, K. Groot, and S. Radulovic. Growth inhibition of estrogen-dependent MXT mammary cancers in mice by the bombesin and gastrin releasing peptide antagonist RC-3095. *J. Natl. Cancer Inst.* **84**:1915–1922 (1992).
33. Radulovic, S., G. Miller, and A. V. Schally. Inhibition of growth of HT-29 human colon cancer xenografts in nude mice by treatment with bombesin/gastrin releasing peptide antagonist (RC-3095). *Cancer Res.* **51**:6006–6009 (1991).
34. Milovanovic, S. R., S. Radulovic, K. Groot, and A. V. Schally. Inhibition of growth of PC-82 human prostate cancer line xenografts in nude mice by bombesin antagonist RC-3095 or combination of antagonist [D-Trp^6]-luteinizing hormone-releasing hormone and somatostatin analog RC-160. *Prostate* **20**:269–280 (1992).
35. Szepeshazi, K., A. V. Schally, R.-Z. Cai, S. Radulovic, S. Milovanovi, and B. Szoke. Inhibitory effect of bombesin/gastrin-releasing peptide antagonist RC-3095 and high dose of somatostatin analogue RC-160 on nitrosamine-induced pancreatic cancers in hamsters. *Cancer Res.* **51**:5980–5986 (1991).
36. Blank, J. L., A. H. Ross, and J. H. Exton. Purification and characterization of two G-proteins that activate the β_1 isozyme of phosphoinositide-specific phospholipase C. *J. Biol. Chem.* **266**:18206–18216 (1991).
37. Smrcka, A. V., J. R. Hepler, K. O. Brown, and P. C. Sternweis. Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q. *Science (Washington D. C.)* **251**:804–807 (1991).
38. Rozengurt, E. Bombesin stimulation of mitogenesis. *Am. Rev. Respir. Dis.* **142**:S11–S15 (1990).

[2] J. M. Wu, D. O. Hoang, and R. I. Feldman, unpublished observations.

39. Rozengurt, E., and J. Sinnet-Smith. Early signals underlying the induction of the c-*fos* and c-*myc* genes in quiescent fibroblasts: studies with bombesin and other growth factors. *Prog. Nucleic Acid Res. Mol. Biol.* **35**:261–295 (1988).
40. Zachary, I., J. Sinnet-Smith, and E. Rozengurt. Bombesin, vasopressin, and endothelin stimulation of tyrosine phosphorylation in Swiss 3T3 cells. *J. Biol. Chem.* **267**:19031–19034 (1992).
41. Seckl, M., and E. Rozengurt. Tyrphostin inhibits bombesin stimulation of tyrosine phosphorylation, c-*fos* expression, and DNA synthesis in Swiss 3T3 cells. *J. Biol. Chem.* **268**:9548–9554 (1993).
42. Campos, R. V., A. M. J. Buchan, R. M. Meloche, R. A. Pederson, Y. N. Kwok, and D. H. Coy. Gastrin secretion from human antral G cells in culture. *Gastroenterology* **99**:36–44 (1990).
43. Campos, R. V., A. M. J. Buchan, R. A. Pederson, C. H. McIntosh, and D. H. Coy. Inhibtion of bombesin-stimulated gastrin release from isoloated canine G cells by bombesin antagonists. *Can. J. Physiol. Pharmacol.* **67**: 1520–1524 (1989).

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