Pharmacology and Cell Biology of the Bombesin Receptor Subtype 4 (BB_4-R)†

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Received January 27, 1999; Revised Manuscript Received April 9, 1999

ABSTRACT: Recently, a fourth member of the bombesin (Bn) receptor family (fBB_4-R) was isolated from a cDNA library from the brain of the frog, *Bombina orientalis*. Its pharmacology and cell biology are largely unknown, and no known natural cell lines or tissues possess sufficient numbers of fBB_4-R's to allow either of these to be determined. To address these issues, we have used three different strategies. fBB_4-R expression in cells widely used for other Bn receptor subtypes was unsuccessful as was expression in two frog cell lines. However, stable fBB_4-R cell lines were obtained in CHO-K1 cells which were shown to faithfully demonstrate the correct pharmacology of the related Bn receptor, the GRP receptor, when expressed in these cells. [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) was found to have high affinity (K_i = 0.4 nM) for the fBB_4 receptor and ^{125}I-[DTyr^6,βala^{11},Phe^{13},Nle^{14}]Bn(6−14) to be an excellent ligand for this receptor. The fBB_4-R had a unique pharmacology for naturally occurring Bn-related agonists, with the presence of a penultimate phenylalanine being critical for high-affinity interaction. It also had a unique profile for six classes of Bn antagonists. The fBB_4-R was coupled to phospholipase C with activation increasing [^{3}H]inositol phosphates and mobilizing Ca^{2+} almost entirely from cellular sources. There was a close correlation between agonist the receptor occupation and the receptor activation. Three of the five classes of Bn receptor antagonists that interacted with higher affinity with the fBB_4-R functioned as fBB_4-R antagonists and two as partial agonists. fBB_4-R activation stimulated increases in phospholipase D (PLD) over the same range of concentrations at which it activated phospholipase C. These results demonstrate that the fBB_4 receptor has a unique pharmacology for agonists and antagonists and is coupled to phospholipase C and D. The availability of these cell lines, this novel ligand, and the identification of three classes of antagonists that can be used as lead compounds should facilitate the further investigation of the pharmacology and cell biology of the BB_4 receptor.

Recently (*1*), a fourth member of the bombesin receptor family (fBB_4 receptor) was isolated from a *Bombina orientalis* brain cDNA library. The BB_4 receptor is unusual in that it has a high degree (>55% at the amino acid level) (*1*) of homology with all three mammalian bombesin receptors, the gastrin-releasing peptide receptor (GRP[1] receptor), neuromedin B receptor (NMB receptor), and the bombesin receptor subtype-3 receptor (BRS-3 receptor). At present the mammalian equivalent of the fBB_4 receptor has not been identified. In amphibians the fBB_4 receptor is expressed in brain (*1*), occurring in high density in cortical and forebrain regions. Transient transfection studies show that the fBB_4 receptor has high affinity for [Phe^{13}]bombesin, a natural occurring bombesin-like peptide in some amphibians including *Bombina orientalis* (*1*, *2*). However, at present the pharmacology of the fBB_4 receptor is largely unknown. In addition, little is known about the cellular basis of action of the fBB_4 except that in one study (*1*) when it was expressed in *Xenopus oocytes* (*1*), the frog BB_4 receptor (fBB_4) coupled to phospholipase C. The ability to establish the cell biology and pharmacology of the fBB_4 receptor is limited by the lack of a cell containing sufficient native fBB_4 receptors to allow cell biology studies.

To deal with this latter issue in the present study, we have used three different approaches to attempt to produce cell lines expressing the fBB_4 receptor whose pharmacology and coupling will likely resemble that of the native fBB_4 receptor. One approach, the stable transfection of the fBB_4 receptor into Chinese hamster ovary cells (CHO-K1 cells), yielded fBB_4 receptor stably expressed cell lines that were used to characterize the pharmacology and cell biology of this receptor. A unique synthetic Bn analogue, [DPhe^6,βAla^{11},-

† This work was partially supported by NIH grant NS35580 (E.R.S.).

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[1] Abbreviations: BN, bombesin; fBB_4-R, fourth member of the bombesin receptor family; GRP, gastrin-releasing peptide; NMB, neuromedin B; NMC, neuromedin C; CHO-K1 cells, Chinese hamster ovary cells; BRS-3, bombesin receptor subtype 3; PLD, phospholipase D; IP, inositol phosphate; $[Ca^{2+}]_i$, cytosolic calcium concentration; PETH, phosphatidylethanol; CHOP cells, polyoma large T antigen Chinese hamster ovary cell line; ψ13−14-pseudopeptide bond between amino acids in position 13 and 14 of Bn; SP, substance P.

10.1021/bi990204w CCC: $18.00
Published on Web 05/08/1999

Phe[13],Nle[14]]Bn(6−14), was identified that binds to this receptor with high affinity ($K_i = 0.4$ nM), and using the high-affinity radioligand, ^{125}I-[DTyr[6],βAla,Phe[13],Nle[14]]Bn(6−14), the fBB_4 receptor was found to possess a unique affinity for bombesin-related agonists and antagonists and to activate both phospholipase C and phospholipase D.

EXPERIMENTAL PROCEDURES

Materials

The mammalian expression vector, pcDNA3, was from Invitrogen (Carlsbad, CA). Oligonucleotides were from Midland Certified Reagent Company (Midland, TX). Restriction endonucleases (*Bam*HI, *Hin*dIII, and *Xba*I), T4 DNA Ligase, Dulbecco's modified eagle medium (D-MEM), RPMI medium 1640 with L-glutamine, F-12 nutrient mixture (HAM) with L-glutamine, minimum essential medium (MEM) with Earle's salts and nonessential amino acids without L-glutamine, Leibovitz's L-15 Medium with L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, LIPOFECTAMINE reagent, OPTI-MEM I reduced serum medium, GENETICIN selective antibiotic (G418 Sulfate), Dulbecco's phosphate-buffered saline (D-PBS), and trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na) were from Life Technologies (Gaithersburg, MD). BALB/3T3 cells (Mouse embryo), FT cells (Bullfrog tongue, ATCC CCL-41), ICR 134 cells (Grass frog embryo, ATCC CCL-128), and CHO-K1 cells (Chinese hamster ovary, ATCC CCL-61) were from American Type Culture Collection (Rockville, MD). The non-small cell lung cancer cell line, NCI-H1299 cells, was a gift from Herb Oie of the NCI-Navy Medical Oncology Branch, Naval Medical Center (Bethesda, MD). Swiss 3T3 cells were a gift from Dr. John Taylor (Biomeasure Inc., Milford MA). CHOP cells (Polyoma large T antigen-expressing Chinese hamster ovary cells) were a gift from James W. Dennis (Samuel Lunenfeld Research Institute, Toronto, Canada). Petri dishes (100 × 20 mm) (Falcon 3003) were from Becton Dickinson (Plymouth, England). Twenty four-well cell culture dishes were from Corning Costar Corp. (Cambridge, MA). 4-Methylbenzhydrylamine resin was from Advanced Chem. Technology (Louisville, KY). [9,10(n)-^{3}H]-Palmitic acid (53 Ci/mmol) and Na^{125}I (2,200 Ci/mmol) were from Amersham Life Science Inc. (Arlington Heights, IL). 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (IODO-GEN) and dithiothreitol (DTT) were from Pierce Chemical Co., (Rockford, IL). Tris HCl was from Bethesda Research Labs (Gaithersburg, MD). Bovine serum albumin fraction V (BSA) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from ICN Pharmaceutical Inc. (Aurora, OH). Soybean trypsin inhibitor type I−S and bacitracin were from Sigma (St. Louis, MO). Nyosil M20 oil (specific gravity 1.0337) was from Nye Lubricants Inc. (New Bedford, MA). Monobasic sodium phosphate was from Mallinckrodt Inc. (Paris, KY). Phosphatidylethanol (PETH) was from Avanti Polar Lipids (Birmingham, AL). Silica gel G thin-layer chromatography (TLC) plates (LK6D) were from Whatman (Clifton, NJ). Solvents for TLC were from Fisher (Pittsburgh, PA). Fura-2/AM was from Molecular Probes (Eugene, OR). *Myo*-[2-^{3}H] inositol was from Dupont NEN (Boston, MA). AG 1-X8 anion-exchange resin was from BIO-RAD (Hercules, CA). Formic acid, ammonium formate, disodium tetraborate, ethylenediaminetetraacetic acid (EDTA), and ethylene glycol-bis (β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were from Sigma (St. Louis, MO). Hydrofluor scintillation cocktail was from National Diagnostics (Atlanta, GA).

Bombesin (Bn), gastrin-releasing peptide (GRP), neuromedin B (NMB), neuromedin C (NMC), rhodei-litorin, phyllolitorin, [Leu[8]]phyllolitorin, alytesin, ranatensin, pGlu-Gly-Gly-Gly-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2 (Pseudophryne guntheri, PG-L) (*3*), and litorin were from Bachem (Torrence, CA). [DArg[1],DTrp[7,9],Leu[11]]substance P ([DArg[1],DTrp[7,9],Leu[11]]SP) and [DPro[4],DTrp[7,9,10]]substance P(4−11) ([DPro[4],DTrp[7,9,10]]SP(4−11)) were from Peninsula Laboratories (Belmont, CA). [Phe[13]]Bn, [Ser[19]]GRP(18−27) (frog-GRP-10) (*1*, *4*), [Ser[3],Arg[9],Phe[13]]Bn (SAP−Bn) (*2*), [DPhe[6],βAla[11],Phe[13],Nle[14]]Bn(6−14), and [DTyr[6],βAla[11],-Phe[13],Nle[14]]Bn(6−14) were gifts from John Taylor of Biomeasure Inc. (Milford, MA). All other chemicals were of the highest purity commercially available.

Methods

Construction of Plasmid. A full-length cDNA encoding the frog BB_4 receptor (fBB_4-R) originally isolated from a *Bombina orientalis* brain cDNA library was used (*1*). A DNA fragment containing the coding region of fBB_4-R was subcloned into the *Bam*HI site of pcDNA3 after the polymerase chain reaction (PCR) with the following primers: 5′ AAGTTA**GGATCC**AAAATGCCTGAAGGTTTTCAGTCAC 3′ (sense) and 3′ GAATGCTACATTTCTTTCTCACTGTC**CCTAGG**ATTGAA 5′ (anti-sense) where the bold letters refer to the recognition sequence of *Bam*HI and the underlined letters refer to the first or the last coding region of fBB_4-R. cDNA of the mouse GRPR (mGRPR) was identical to that described previously (*5*) and was subcloned between the *Hin*dIII site and *Xba*I site of pcDNA3. Nucleotide sequence analysis of the entire coding region was performed on an automated DNA sequencer (ABI 373A DNA sequencer or ABI PRISM 377 DNA sequencer: Applied Biosystems Inc., Foster City, CA).

Cell Maintenance. BALB 3T3 cells (Mouse embryo) and Swiss 3T3 cells were maintained in DMEM containing 10% (v/v) FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. NCI-H1299 cells (non-small cell lung cancer cells) were maintained in RPMI 1640 containing 10% (v/v) FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. CHO-K1 cells (Chinese hamster ovary cells) were maintained in HAM containing 10% (v/v) FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin. CHOP cells were maintained in HAM containing 10% (v/v) FBS, units/mL penicillin, 50 μg/mL streptomycin, and 200 μg/mL G418. Each mammalian cell line was incubated at 37 °C and 5% CO_2 atmosphere and was passaged every 3−4 days at confluence after detaching the cells with trypsin-EDTA.

FT cells (Bullfrog tongue) were maintained in MEM containing 10% (v/v) FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin at 25 °C, 5% CO_2 atmosphere. ICR 134 cells (Grass frog embryo) were maintained in Leibovitz's L-15 medium containing 10% (v/v) FBS, 40% (v/v) distilled water, 50 units/mL penicillin, and 50 μg/mL streptomycin at 25 °C, normal atmosphere. Each amphibian cell line was subcultivated 1:2 to 1:3 after detaching the cells with trypsin-EDTA.

Cell Transfection. BALB/3T3 cells, FT cells, ICR 134 cells, NCI-H1299 cells, CHOP cells, or CHO-K1 cells were transfected with 10 μg of plasmid DNA (fBB_4-R or mGRP-R) by a cationic lipid-mediated method using 25 μL of LIPOFECTAMINE reagent and 5 mL of OPTI-MEM I reduced serum medium in 100 × 20 mm Petri dishes. Selection for fBB_4-R or mGRP-R-transfected CHO-K1 cells with G418 sulfate (800 μg/mL) was started 24 h after transfection. Approximately three weeks after the transfection, individual clones were obtained by single cell cloning and screened for fBB_4-R or mGRP-R expression by assessing saturable binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) in 24-well cell culture dishes (*6*, *7*). CHO-K1 cells stably expressing fBB_4-R or mGRP-R were maintained in F-12 nutrient mixture (HAM) containing 10% (v/v) FBS, 50 units/mL penicillin, 50 μg/mL streptomycin, and 300 μg/mL G418 sulfate (the standard growth medium) at 37 °C and 5% CO_2 atmosphere and were passaged every 3−4 days at confluence after detaching the cells with trypsin-EDTA.

Preparation of Peptides. The peptides were synthesized using solid-phase methods (*8*−*10*). Briefly, introduction of the reduced peptide bond was carried out by the standard method described previously (*8*) on 4-methylbenzhydrylamine resin. Alkylamide analogues were synthesized in a standard Leu-*O*-polystyrene resin by using tosyl group protection for the imidazole group of His as described previously. Peptide esters were prepared by standard, automated solid-phase techniques on Advanced Chem. Technology ACT200 machines with Merrifield Leu-*O*-polystyrene resin and α-Boc protection for all amino acids and both the α and imidazole nitrogen of His in position 12 as described previously (*9*). Free peptides were then cleaved from the resin by transesterification with 10% triethylamine/methanol at 40 °C (2 days). Peptides were purified on a column (2.5 × 90 cm) of Sephadex G-25 followed by elution with linear gradients of acetonitrile in 0.1% trifluoroacetic acid with a Rainin preparative HPLC system (flow rate ∼5 mL/min) and columns (1.5 × 50 cm) of Vydac C_{18} silica (10−15 μm). Peptides were further purified by rechromatography on the same column with slight modifications to the gradient conditions when necessary to greater than 97% purity. Peptides were characterized by amino acid analysis and matrix-assisted laser desorption mass spectroscopy (Finnegan, Hemel Hemstead, UK).

Iodination of [Tyr^4]Bn and [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6−14). ^{125}I-[Tyr^4]bombesin (^{125}I-[Tyr^4]Bn) and ^{125}I-[DTyr^6,-βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) at a specific activity of 2200 Ci/mmol were prepared by a modification of the methods described previously (*6*, *11*). Briefly, 0.8 μg of IODO-GEN (in 0.01 μg/mL chloroform) was transferred to a vial, dried under a stream of nitrogen, and washed with 100 μL of KH_2PO_4 (pH 7.4). To this vial was added 20 μL of KH_2PO_4 (pH 7.4), 8 μg of peptide in 4 μL of water, and 2 mCi (20 μL) Na ^{125}I, which were mixed gently and incubated at room temperature for 6 min. The incubation was stopped by the addition of 100 μL of distilled water and 300 μL of 1.5 M DTT. The iodination mixture of ^{125}I-[Tyr^4]Bn was incubated 80 °C for 60 min.

The reaction mixture was applied to a Sep-Pak (Waters Assoc., Milford, MA), and free ^{125}I was eluted with 5 mL of water followed by 5 mL of 0.1% trifluoroacetic acid (TFA). The radiolabeled peptides were eluted with 200 μL sequential elutions (×10) with 60% acetonitrile in 0.1% TFA. The two or three fractions containing the highest radioactivity were combined and purified on a reverse-phase, high-performance liquid chromatography with a μBondaPak column (0.46 × 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% TFA (v/v) from 16% to 60% acetonitrile in 60 min. One milliliter fractions were collected and checked for radioactivity and receptor binding. The pH of the fractions were adjusted to 7.0 using 0.2 M Tris (pH 9.5), and radioligands were stored in aliquots with 0.5% bovine serum albumin (BSA) at −20 °C.

Whole Cell Radioligand Binding Assays. The standard binding buffer (pH 7.4) contained 98.0 mM NaCl, 6.0 mM KCl, 11.5 mM glucose, 5.0 mM sodium fumarate, 5.0 mM sodium glutamate, 5.0 mM sodium pyruvate, 24.5 mM HEPES (pH 7.4), 2.5 mM KH_2PO_4, 1.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.01% (w/v) soybean trypsin inhibitor, 0.2% (v/v) essential amino acid solution, 0.2% (w/v) BSA, and 0.1% (w/v) bacitracin.

Incubations contained 1.0 to 2.0 × 10^6 cells/mL or sufficient cells to bind less than 10% of the added radioactivity and were performed at 22 °C for 60 min unless otherwise stated. Fifty picomolar ^{125}I-[Tyr^4]Bn or ^{125}I-[DTyr^6,βAla^{11},-Phe^{13},Nle^{14}]Bn(6−14) was added alone (total binding) or with (nonsaturable binding) 1 μM unlabeled Bn. The final incubation volume was 250 μL. Bound radioactivity was determined by layering 100 μL of the cell suspension over 250 μL of standard incubation solution in 400 μL microcentrifuge tubes (PGC Scientifics, Gaithersburg, MD) and centrifuging the samples for 3 min at 10000*g* in a Microfuge E (Beckman, Palo Alto, CA). The supernatant was aspirated, and the microcentrifuge tubes were washed twice with the incubation buffer without disturbing the cell pellet. The cell pellet was counted for radioactivity in a gamma counter. One hundred microliter aliquots of the incubation mixture were taken in duplicate to determine the total radioactivity. Binding was expressed as the percentage of total radioactivity that was associated with the cell pellet. All binding values represented saturable binding (i.e., total binding minus nonsaturable binding). Nonsaturable binding was <15% of the total binding in all experiments. Each point was measured in duplicate, and each experiment was replicated three times. The affinity for the radiolabeled ligand was calculated using a least-squares curve-fitting program (LIGAND). The K_i values for other peptides were calculated using the method of Cheng and Prusoff.

Dissociation of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14). The time and temperature dependence of dissociation of saturably bound ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) from stably transfected CHO-K1 cells expressing fBB_4-R was determined after incubation of the ligand with the cells for 45 min at 37 °C. The incubation mixture was diluted 100-fold in incubation buffer at 4 or 37 °C and incubated at various times before filtering the cells on GF/B filters. The filters were washed two times with buffer and counted for saturably bound radioactivity.

Internalization of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14). Internalization of radiolabeled ligand was determined using acid washing as described previously (*11*, *12*). Briefly, cells were disaggregated, washed, and resuspended in binding buffer as described above, and then 1.0 × 10^6 cells/mL were incubated with 100 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn-

(6−14) for various times at 37 °C. After incubation, 100 μL samples were added to 1.0 mL of 0.2 M acetic acid in 0.5 M NaCl, pH 2.5, for 5 min at 4 °C, to remove surface-bound ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14). In all cases, parallel incubations were conducted in the presence of 1 μM [DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) to allow changes in saturable binding only to be assessed.

Measurement of Inositol Phosphates ([^{3}H]IP). Changes in [^{3}H]IP were measured using the method described previously (*13*, *14*). Briefly, stably transfected CHO-K1 cells expressing fBB$_4$-R were subcultured into 24-well cell culture dishes (5×10^4 cells/well) in the standard growth medium. After a 24 h incubation period at 37 °C, the cells were loaded with 3 μCi/ml *myo*-[2-^{3}H]inositol in growth medium supplemented with 2% FBS for 24 h. Prior to assay, the cells were washed and incubated for 10 min at 37 °C with the wash buffer containing 1 mL/well PBS (pH 7.0) and 20 mM lithium chloride. The wash buffer was aspirated and replaced with 500 μL of IP assay buffer/well containing 135 mM sodium chloride, 20 mM HEPES (pH 7.4), 2 mM calcium chloride, 1.2 mM magnesium sulfate, 20 mM lithium chloride, 11.1 mM glucose and 0.05% BSA (v/v) with or without any of the peptides. Experiments were terminated with 1 mL ice-cold hydrochloric acid/methanol (0.1% v/v). After a 30 min extraction period (4 °C), total [^{3}H]IP were isolated by anion-exchange chromatography as described previously (*13*). Briefly, the samples were applied to glass columns containing 500 μL of a 1:1 (v/v) slurry of AG 1-X8 anion-exchange resin:distilled water. The columns were washed with 5 mL distilled water and then washed with 2 mL 5 mM disodium tetraborate and 60 mM sodium formate. The columns were finally eluted with 2 mL 1 mM ammonium formate and 100 mM formic acid. Each of the elutes was collected and mixed with 10 mL Hydrofluor scintillation cocktail and the radioactivity was measured in a scintillation counter.

Measurement of Intracellular Calcium ([Ca^{2+}]$_i$). Cells harvested by scraping with a rubber policeman were resuspended in a calcium assay buffer [24.5 mM HEPES (pH 7.4)], 98 mM sodium chloride, 6 mM potassium chloride, 2.5 mM monobasic sodium phosphate, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 100 mM calcium chloride, 1 mM magnesium chloride, 0.01% soybean trypsin inhibitor, 0.2% (v/v) amino acid mixture, and 0.2% BSA] to a concentration of 5.0×10^6 cells/mL and incubated with 2 μM Fura-2/AM for 30 min at 37 °C. After being washed two times with calcium assay buffer, washed cells were resuspended in calcium assay buffer (5.0×10^6 cells/mL) and 2 mL of cell suspension was placed in a Delta PTI Scan 1 spectrofluorimeter (Photon Technology International, South Brunswick, NJ) equipped with a stir bar and water bath (37 °C). Fluorescence was measured at dual excitation wavelengths of 340 and 380 nm using an emission wavelength of 510 nm. The calcium concentration was calculated using the method described previously (*15*). Autofluorescence was corrected by assaying a sample of unlabeled cells under identical experimental conditions.

Measurement of Phospholipase D (PLD) Activity. PLD was determined by assaying transphosphatidylation activity according to a slight modification of the method of Cook et al. (*16*) described recently. Briefly, cells were grown to confluence, quiescence induced by reducing fetal calf serum in the medium to 2% (v/v) for 24 h, and labeled with [^{3}H]-palmitic acid (4 μCi/well). After preincubation in serum-free F-12 (HAM) containing 20 mM hepes (pH 7.4) and 1% (w/v) BSA (PLD buffer) for 30 min at 37 °C, PLD buffer containing 1% (v/v) ethanol was added for 5 min. The incubation was then begun by replacing the medium with fresh PLD buffer containing 1% ethanol and the agent to be tested at the appropriate concentrations and for the appropriate time. The incubation was terminated by the addition of methanol after removal of the medium. The cells were collected and extracted with an equal volume of chloroform at room temperature for 15 min. Phases were separated by the addition of water followed by centrifugation at 2500*g* for 5 min. The organic phase (lower phase) was dried under a stream of nitrogen gas and then redissolved in a mixture of chloroform/methanol (19:1, v/v). A phosphatidylethanol (PETH) standard was added into each sample, and the sample was applied to a Whatman TLC plate which was then developed in the solvent system consisting of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.). The plate was stained with iodine vapor, and [^{3}H]PETH was identified as a band comigrating with an authentic PETH standard. The bands were scraped off the plates, and their radioactivities were determined by scintillation counting in a Packard liquid scintillation counter after adding Hydrofluor scintillation cocktail.

RESULTS

To study the fBB$_4$ receptor, we first attempted to transiently express the fBB$_4$ receptor in murine BALB/3T3 cells. This cell line has been used extensively for studies of other Bn receptors (GRP receptor, NMB receptor, and BRS-3 receptor), and when the other Bn receptors were expressed in these cells, their cell biology, pharmacology, and receptor modulatory activity (internalization, down-regulation, desensitization) were similar to those of native cells containing these receptors (*6*, *14*, *17*−*19*). Undetectable or very low levels of saturable binding were obtained using either ^{125}I-[Tyr^4]Bn or ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14), both of which are reported to have high affinity for fBB$_4$ receptors (*1*, *7*). Similar results were obtained with a human non-small cell lung cancer cell line (NCI-H1299 cells), a Bullfrog tongue cell line (FT cells), or a Grass frog embryo cell line (ICR 134 cells). In a previous study (*1*), the fBB$_4$ receptor was transiently expressed in a polyoma large T antigen-expressing Chinese hamster ovary cell line (CHOP cells). We confirmed moderate levels of binding to fBB$_4$-R transiently transfected CHOP cells. To be able to study the cell biology and binding, we constructed and isolated a number of CHO-K1 cell lines that were stably expressing fBB$_4$-R as described in Methods. Prior to studying the fBB$_4$-R-transfected CHO-K1 cells, we first attempted to determine whether members of the bombesin receptor family maintain their correct pharmacology when stably expressed in CHO-K1 cells. Because no cells natively containing fBB$_4$ receptors have been described, we stably transfected CHO-K1 cells with the murine gastrin-releasing peptide receptor (mGRP-R) which has 56% amino acid identity with the fBB$_4$ receptor (*1*) and compared its pharmacology in these cells for four Bn-related peptides to that when expressed in BALB/3T3 cells or natively expressed in Swiss 3T3 cells (Table 1). We

Table 1: Comparison of the Affinities of Bombesin-Related Peptides for mGRP-R-Transfected CHO-K1 Cells, mGRP-R-Transfected BALB 3T3 Cells, and Swiss 3T3 Cells Natively Possessing mGRP Receptors[a]

	K_i (nM)			
	CHO-K1 Cells			
peptides added	clone no. 5	clone no. 8	BALB 3T3 cells	Swiss 3T3 cells
Bombesin	1.32 ± 0.54	1.76 ± 0.40	1.24 ± 0.36	1.31 ± 0.11
GRP	1.24 ± 0.07	1.74 ± 0.21	2.50 ± 0.4	1.52 ± 0.50
NMB	127 ± 36	134 ± 58	174 ± 44	100 ± 3
[DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14)	0.19 ± 0.01	0.11 ± 0.01	0.26 ± 0.01	0.24 ± 0.02

[a] Two clones of CHO-K1 cells (clone nos. 5 and 8) or one of BALB 3T3 cells (1×10^6 cells/mL) stably transfected with the mGRP receptor were incubated with 100 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) for 45 min at 22 °C. Swiss 3T3 cells (3×10^6 cells/mL) were incubated with 75 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) for 60 min at 22 °C. Increasing concentrations of unlabeled peptides {Bn, GRP, NMB, or [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14)} were added, and dose−response curves were analyzed using a least-squares, curve-fitting program (LIGAND). K_i values were calculated using the method of Cheng and Prusoff. Values are means ±sem from at least three experiments, and each point was determined in duplicate. Abbreviations: βAla, β-alanine; Nle, norleucine; GRP, gastrin-releasing peptide; NMB, neuromedin B.

assessed two mGRP-R-expressing clones of CHO-K1 expressing different receptor numbers (clone no. 5, receptor density 143 ± 46 fmol/10^6 cells and clone no. 8, receptor density 58 ± 20 fmol/10^6 cells) and a mGRP-R-expressing clone of BALB/3T3 cells (receptor density 70 ± 24 fmol/10^6 cells) and compared the results to those in Swiss 3T3 cells (30 ± 2 fmol/10^6 cells), which natively express the mGRP receptor. The affinities of the mGRP-R-transfected CHO-K1 cells clone no. 5 and clone no. 8 for the four peptides {Bn, GRP, NMB, or [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6−14)} were almost identical with each other and were similar to those of mGRP-R-transfected BALB/3T3 cells and Swiss 3T3 cells for each of the four peptides (Table 1). These results demonstrated that the pharmacology of the mGRP receptor in mGRP-R-transfected CHO-K1 cells was not significantly different from that in mGRP-R-transfected BALB/3T3 cells or the native mGRP receptor expressed in Swiss 3T3 cells, suggesting that other closely related members of the Bn-receptor family would likely maintain their pharmacology in these cells. Therefore, five different stable clones of fBB_4-R-transfected CHO-K1 cells were identified by binding studies and characterized. Under identical binding conditions with 100 pM ^{125}I-[DTyr^6,βAla^{11},-Phe^{13},Nle^{14}]Bn(6−14), the five clones bound different amounts of ligand (clone no. 8, 44 ± 1 fmol/10^9 cells; clone no. 13, 108 ± 7 fmol/10^9 cells; clone no. 3, 209 ± 12 fmol/10^9 cells; clone no. 17, 301 ± 12 fmol/10^9 cells; and clone no. 18, 874 + 34 fmol/10^9 cells). Each of the five different fBB_4-R-transfected cell lines had a similar affinity for [DPhe^6,-βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) (range 0.15−0.39 nM) and for bombesin (0.6−1.8 nM). Clone no. 18, with the highest receptor density and affinity of 0.39 ± 0.07 nM for [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14), was selected for further studies.

The time course of binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},-Nle^{14}]Bn(6−14) to fBB_4-R-transfected CHO-K1 cells at 22 and 37 °C was rapid (Figure 1). At 22 °C and 37 °C, binding was maximal after 30 min and remained constant for an additional 30 min (Figure 1). Adding 3 μM [DTyr^6,βAla^{11},-Phe^{13},Nle^{14}]Bn(6−14) at 22 and 37 °C reduced binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) by greater than 70% (Figure 1). Reducing the incubation temperature to 4 °C decreased the rate of binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},-Nle^{14}]Bn(6−14) such that, after 60 min of incubation, binding had reached only 40% of the maximal binding at 37 °C (Figure 1).

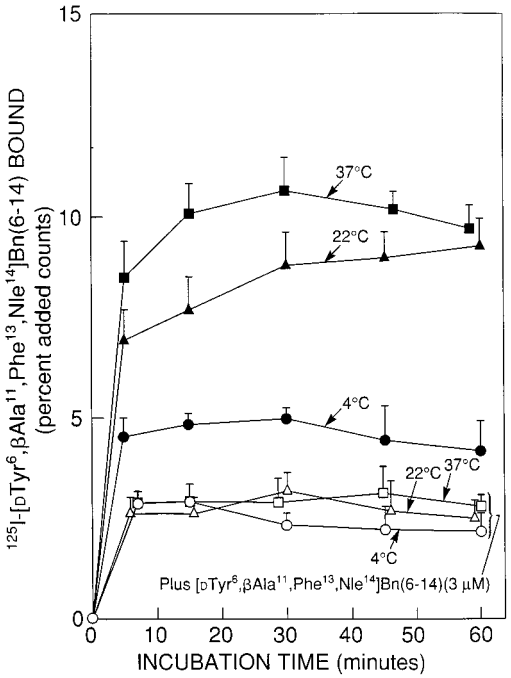

FIGURE 1: Time course of binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},-Nle^{14}]Bn(6−14) to fBB_4-R-transfected CHO-K1 cells. fBB_4-R-transfected CHO-K1 cells (1.0×10^6 cells/ml) were incubated with 100 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) for the times indicated at 4, 22, or 37 °C alone or with 3 μM [DTyr^6,βAla^{11},-Phe^{13},Nle^{14}]Bn(6−14). Results are the percentage of the added radioactivity bound at the times indicated. Results are the mean ±sem from at least three experiments, and each point was determined in duplicate.

To examine the reversibility of binding of ^{125}I-[DTyr^6,-βAla^{11},Phe^{13},Nle^{14}]Bn(6−14), we preincubated fBB_4-R-transfected CHO-K1 cells with 100 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},-Nle^{14}]-Bn(6−14), diluted 100-fold with fresh incubation solution, and then the amounts of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},-Nle^{14}]Bn(6−14) saturably bound at different times were determined. At 37 °C, dissociation of ^{125}I-[DTyr^6,βAla^{11},-Phe^{13},Nle^{14}]Bn(6−14) was relatively slow, with 40% of the bound tracer dissociating by 45 min of incubation (Figure 2). Reducing the temperature from 37 to 4 °C slowed the dissociation to <5% of that originally bound in 45 min (Figure 2).

Because of the slow dissociation rate, the possibility that internalization of ligand was occurring was explored by performing acid-stripping experiments to remove surface-

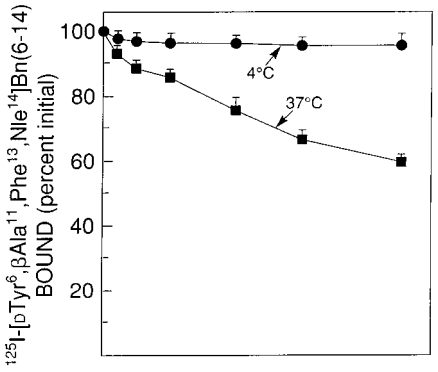

FIGURE 2: Dissociation of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) from fBB$_4$-R-transfected CHO-K1 cells. fBB$_4$-R-transfected CHO-K1 cells (1.0×10^6 cells/mL) were preincubated for 45 min at 37 °C with 100 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) and incubated at 4 or 37 °C for the incubated times. Values are the percentage of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) bound at the beginning of the incubation. Results are the mean ±sem from at least three experiments, and each point was determined in duplicate.

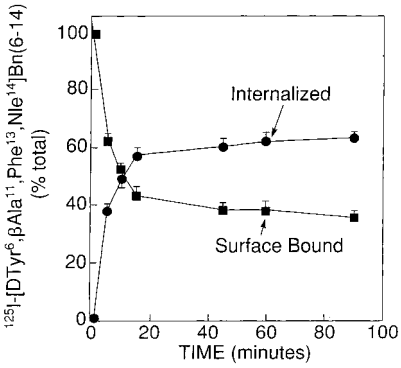

FIGURE 3: Time course of internalization of the fBB$_4$-R in fBB$_4$-R-transfected CHO-K1 cells. fBB$_4$-R-transfected CHO-K1 cells (1.0×10^6 cells/mL) were incubated at 37 °C with 100 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) for the indicated time. Surface-bound and internalized ligand were determined using acid stripping of surface ligand as described in "Methods". Surface-bound ligand was the proportion of saturably bound counts removed by acid stripping, whereas the internalized ligand was the proportion not removed. Results are expressed as the percentage of total saturable ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) bound. Results are means ±sem from at least three experiments, and each point was determined in duplicate.

bound ligand at various times and temperatures (*11*, *12*) (Figure 3). After a 5 min incubation, 38% ± 1% of bound radiolabel was internalized by fBB$_4$-R-transfected CHO-K1 cells. Maximal internalization at 37 °C representing 60% of the saturably bound was achieved by approximately 60−90 min in fBB$_4$-R-transfected CHO-K1 cells (Figure 3).

A number of different naturally occurring Bn-related peptides have been described (*2*, *4*, *20*, *21*), and 15 different peptides were tested for their abilities to inhibit binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) to fBB$_4$-R-transfected CHO-K1 cells (Figure 4 and Table 2). [Phe^{13}]-bombesin and litorin had the highest affinity (K_i = 1 nM), with PG-L also having a high affinity (K_i = 5 nM). Phyllolitorin, NMB, Xenopus NMB, bombesin, ranatensin, alytesin, [Leu^8]phyllolitorin, GRP, and rhodei-litorin had moderately high affinities (K_i = 10−100 nM). Frog GRP-10, neuromedin C, and SAP bombesin ([Ser^3,Arg^9,Phe^{13}]Bn) had low affinities (K_i >100 nM) (Figure 4 and Table 2).

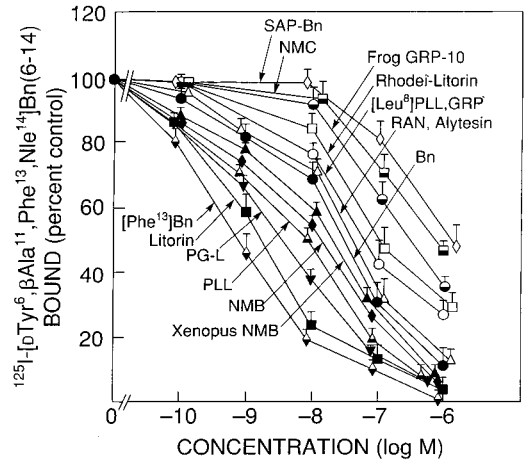

FIGURE 4: Affinity of various naturally occurring Bn-related peptides for fBB$_4$-R. fBB$_4$-R-transfected CHO-K1 cells ($1.0-2.0 \times 10^6$/mL) were incubated with 100 pM ^{125}I-[DTyr^6,βAla^{11},Phe^{13},-Nle^{14}]Bn(6−14) for 45 min at 22 °C with or without the indicated concentrations of unlabeled peptides. Results are expressed as the percentage of the ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) saturably bound with none of the indicated unlabeled peptides present. Results are means ±sem from at least three experiments, and in each experiment each value was determined in duplicate. Abbreviations: PLL, phyllolitorin; Leu-PLL, [Leu^8]phyllolitorin; NMC, neuromedin C; Bn, bombesin; RAN, ranatensin; GRP, gastrin-releasing peptide; SAP-Bn, [Ser^3,Arg^9,Phe^{13}]bombesin (*2*); PGL, pseudophryne guntheri (*3*); Xenopus NMB, Xenopus neuromedin B-[Gln^3,Ile^6]NMB (*21*).

The synthetic Bn-related agonist, [DPhe^6,βAla^{11},Phe^{13},-Nle^{14}]Bn(6−14), had very high affinity for the fBB$_4$ receptor (K_i = 0.4 nM), which was 2.5-fold higher than the highest affinity of the naturally occurring peptide, [Phe^{13}]bombesin (Figure 4, Table 2). Elimination of the 5 amino acids from the NH$_2$ terminus of Bn did not decrease its affinity for the fBB$_4$ receptor because [DPhe^6]Bn(6−14) retained high affinity (K_i = 5 nM, Table 2). The dose−inhibition curve for the ability of [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) to inhibit binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) to fBB$_4$-R-transfected CHO-K1 cells was analyzed by a least-squares, curve-fitting program and found to be best fit by a single-site model (data not shown). The fBB$_4$ receptor had an affinity of 0.41 ± 0.06 nM for [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]-Bn(6−14), and the transfected cell line studied (clone no. 18) had a receptor density of 1160 ± 160 fmol/mg of protein.

Six different classes of bombesin receptor antagonists have been described (*10*, *22*), and representative members from each of these classes were tested for their abilities to inhibit binding of ^{125}I-[DTyr^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) to fBB$_4$-R-transfected CHO-K1 cells (Figure 5 and Table 3). [DPhe^6]-Bn(6−13)hexylamide had the highest affinity (K_i = 18 ± 3 nM, Figure 5 and Table 3). [DPhe^6]Bn(6−13)propylamide, [(3-Ph-Pr^6),His^7,DAla^{11},DPro^{13},ψ13−14,Phe^{14}]Bn(6−14), [DTyr^6,DAla^{11}]Bn(6−13)butylamide, [DPhe^6]Bn(6−13)-methyl ester, and DNal,Cys,Tyr,DTrp,Lys,Val,Cys,Nal-NH$_2$ had moderate affinities (K_i = 60−150 nM). [DPro^4,DTrp^{7,9,10}]-substance P(4−11), [DArg^1,DTrp^{7,9},Leu^{11}]substance P, [DPhe^6]-Bn(6−13)NH$_2$, [Leu^{13},ψ(CH$_2$NH),Leu^{14}]Bn, [DPhe^6]Bn-(6−13)NHN(ME)$_2$, [DPhe^6,Leu^{13},ψ(CH$_2$NH)Cpa^{14}]Bn-(6−14), [DPhe^6]Bn(6−13)hydrazide, and [DPhe^{6,12},Leu^{14}]Bn had low affinities (K_i = 800−2500 nM). [DPhe^{12}]Bn and [DPhe^6]Bn(1−13)NH$_2$ had very low affinities (K_i >10 000 nM) (Figure 5 and Table 3).

Table 2: Comparison of the Affinity of Naturally Occurring and Synthetic Bombesin-Related Peptides for the fBB_4, rGRP, rNMB, and hBRS-3 Receptors[a]

peptides added	K_i (nM)			
	fBB_4-R-transfected CHO-K1 Cells	rat pancreatic acini (GRP−R)	rNMB-R-transfected BALB 3T3 cells	hBRS-3-transfected BALB 3T3 cells
Naturally Occurring Bn-Related Peptides				
Bombesin	14 ± 1	4 ± 1	34 ± 2	>10000
GRP	79 ± 12	18 ± 5	440 ± 70	>10000
NMB	11 ± 1	248 ± 5	4 ± 1	4800 ± 400
Ranatensin	22 ± 5	2 ± 1	13 ± 2	6900 ± 2000
Litorin	1.2 ± 0.3	6 ± 1	7 ± 3	4100 ± 400
Alytesin	32 ± 9	62 ± 7	460 ± 70	3600 ± 1200
[Leu[8]]phyllolitorin	77 ± 15	420 ± 45	>10000[b]	5400 ± 1900
Phyllolitorin	10 ± 1	240 ± 50	47 ± 3	2100 ± 200
PG-L	5 ± 1	3 ± 1	210 ± 20	5300 ± 1400
Rhodei-Litorin	89 ± 9	31 ± 4	460 ± 20	3100 ± 700
Neuromedin C	884 ± 95	20 ± 12	140 ± 10	>10000
Xenopus NMB	11 ± 4	180 ± 30	340 ± 60	7100 ± 2500
SAP bombesin	1010 ± 180	3200 ± 400	>10000(1)	7100 ± 950
[Phe[13]]bombesin	0.96 ± 0.17	0.77 ± 0.15	6 ± 1	6600 ± 1750
Frog GRP-10	128 ± 18	130 ± 7	>10000	>10000
Synthetic Bn-Related Peptides				
[DPhe[6],βAla[11],Phe[13],Nle[14]]Bn(6−14)	0.41 ± 0.06	0.99 ± 0.16	0.36 ± 0.06	8.9 ± 0.7
[DPhe[6]]Bn(6−14)	5.4 ± 0.18	2 ± 0.1	14 ± 2	>10000

[a] Cells ((1−2) × 10^6/mL) were incubated with 50 pM ^{125}I-[DTyr[6],βAla[11],Phe[13],Nle[14]]Bn(6−14) (hBRS-3-transfected cells) or 100 pM ^{125}I-[DTyr[6],βAla[11],Phe[13],Nle[14]]Bn(6−14) (fBB_4-R-transfected CHO-K1 cells), 50 pM ^{125}I-[Tyr[4]]Bn (rat pancreatic acini) or 50 pM ^{125}I-[DTyr[0]]NMB (rNMB−R-transfected cells) for 45 min at 22 °C (transfected cells) or 30 min at 37 °C (pancreatic acini). Increasing concentrations of unlabeled peptide were added, and dose−response curves were analyzed using a least-squares, curve-fitting program (LIGAND). K_i values were calculated using the method of Cheng and Prusoff. Values are means ±sem from at least four experiments. Abbreviations: GRP, gastrin-releasing peptide; NMB, neuromedin B; PG-L, pGlu-Gly-Gly-Gly-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-Met-NH_2 (*4*); Xenopus NMB, [Gln[3],Ile[6]]NMB (*36*); SAP bombesin, [Ser[3],Arg[9],Phe[13]]bombesin (*2*); frog GRP-10, [Ser[19]]GRP(18−27) (*5*); Bn, bombesin. [b] >10000 means the affinity was greater than 10000 nM.

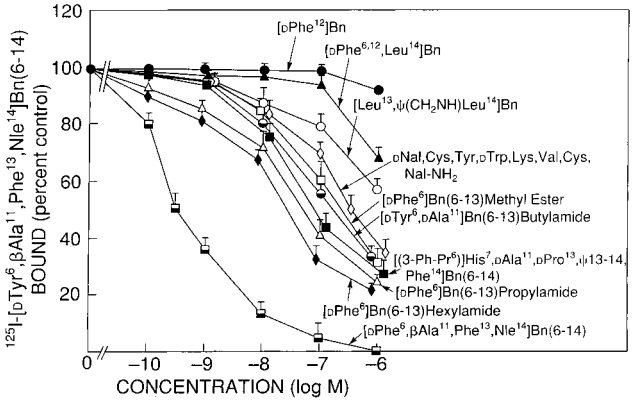

FIGURE 5: Affinity of various synthetic Bn-receptor agonists and antagonists for the fBB_4-R. fBB_4-R-transfected CHO-K1 cells (1.0−2.0 × 10^6 cells/mL) were incubated with 100 pM ^{125}I-[DTyr[6],βAla[11],Phe[13],Nle[14]]Bn(6−14) for 45 min at 22 °C with or without the indicated concentrations of unlabeled peptides. Results are expressed as the percentage of the ^{125}I-[DTyr[6],βAla[11],Phe[13],Nle[14]]Bn(6−14) saturably bound with none of the indicated unlabeled peptides present. Results are means ±sem from at least three experiments, and in each experiment each value was determined in duplicate. Abbreviations: Bn, bombesin; DNal, naphthyl-D-alanine; ψ(CH_2-NH) indicates insertion of a reduced peptide bond in which −CO− was changed to $-CH_2-$ at the indicated location; 3-Ph-Pr, phenylpropanolamine.

To determine the ability of the various peptides to function as fBB_4 receptor agonists or antagonists and their possible cellular basis of action, we first investigated the ability of [Phe[13]]Bn, the natural occurring Bn-related peptide with the highest affinity (Figure 4 and Table 2) and [DPhe[6],βAla[11],-Phe[13],Nle[14]]Bn(6−14), the synthetic peptide with the highest affinity (Figure 5 and Table 2) to stimulate [^{3}H]IP formation in fBB_4-transfected CHO-K1 cells (Figure 6). In fBB_4-R-transfected CHO-K1 cells, [Phe[13]]Bn stimulated a concentration-dependent increase in [^{3}H]IP with a detectable effect at 0.1 nM and a maximal effect at 10 nM and had an EC_{50} of 1.3 ± 0.2 nM (Figure 6). [DPhe[6],βAla[11],Phe[13],Nle[14]]Bn-(6−14) also functioned as a full agonist and was 2-fold more potent than [Phe[13]]Bn, having an EC_{50} of 0.6 ± 0.1 nM (Figure 6).

To examine the ability of the fBB_4-R activation to cause changes in cytosolic calcium $[Ca^{2+}]_i$, we examined the ability of [Phe[13]]Bn to alter $[Ca^{2+}]_i$ in Fura-2/AM loaded fBB_4-R-transfected CHO-K1 cells (Figure 7). One hundred nanomolar [Phe[13]]Bn stimulated a 3.9- or 2.9-fold increase in $[Ca^{2+}]_i$ with or without extracellular Ca^{2+} present, respectively (Figure 7, left panel). With or without extracellular Ca^{2+} present, the cells demonstrated a rapid increase in $[Ca^{2+}]_i$ and a subsequent decrease over the next 60 s (Figure 7, left and center panels). The high-affinity GRP receptor antagonist, [(3-Ph-Pr[6]),His[7],DAla[11],DPro[13],ψ13−14,-Phe[14]]Bn(6−14), at the concentration of 1 μM which causes 75% inhibition of binding of ^{125}I-[DTyr[6],βAla[11],Phe[13],-Nle[14]]Bn(6−14) to fBB_4-R-containing cells (Figure 5) did not alter $[Ca^{2+}]_i$ when present alone in fBB_4-R-transfected cells (Figure 7, right panel); however, 1 μM [(3-Ph-Pr[6]),-His[7],DAla[11],DPro[13],ψ13−14,Phe[14]]Bn(6−14) inhibited the ability of 100 nM [Phe[13]]Bn to increase $[Ca^{2+}]_i$ by 72% in fBB_4-R-transfected CHO-K1 cells (Figure 7, right panel).

We next explored the ability of the various Bn receptor antagonists with the highest affinity for the fBB_4 receptor from binding studies (Table 3) to function as receptor agonists or antagonists in fBB_4-R-transfected CHO-K1 cells

Table 3: Comparison of the Affinity of Various Synthetic Bn-Receptor Antagonists for the fBB_4, the rGRP, the rNMB, and the hBRS-3 Receptors[a]

peptides added	K_i (nM)			
	fBB_4-R-transfected CHO-K1 cells	rat pancreatic acini (rGRP−R)	rNMB−R-transfected BALB 3T3 cells	hBRS-3-transfected BALB 3T3 cells
[DPhe^{12}]Bn	>10000[b]	>10000	1900 ± 100	>10000
[Leu^{13},ψ(CH_2NH),Leu^{14}]Bn	1096 ± 92	430 ± 60	>10000	>10000
[DPhe^{6,12},Leu^{14}]Bn	2506 ± 732	430 ± 60	2300 ± 300	>10000
[DPhe^{6},Leu^{13},ψ(CH_2NH),Cpa^{14}]Bn(6−14)	1285 ± 298	42 ± 5	2700 ± 200	>10000
[(3-Ph-Pr^{6})His^{7},DAla^{11},DPro^{13}, ψ13−14,Phe^{14}]Bn(6−14)	67 ± 13	0.74 ± 0.04	>10000	6800 ± 900
[DPhe^{6}]Bn(6−13)NH_2	974 ± 91	27 ± 6	>10000	>10000
[DPhe^{6}]Bn(1−13)NH_2	>10000	450 ± 90	>10000	>10000
[DPhe^{6}]Bn(6−13)propylamide	62 ± 11	6 ± 1	4600 ± 600	1900 ± 300
[DPhe^{6}]Bn(6−13)methyl ester	122 ± 10	10 ± 1	7700 ± 1100	5300 ± 2000
[DPhe^{6}]Bn(6−13)hexylamide	18 ± 3	100 ± 10	>10000	3200 ± 100
[DPhe^{6}]Bn(6−13)hydrazide	2243 ± 236	1200 ± 400	>10000	>10000
[DPhe^{6}]Bn(6−13)NHN$(ME)_2$	1187 ± 300	3200 ± 400	>10000	>10000
[DTyr^{6},DAla^{11}]Bn(6−13)butylamide	98 ± 11	6 ± 1	2300 ± 560	5300 ± 1400
DNal,Cys,Tyr,DTrp,Lys,Val,Cys,Nal-NH_2	130 ± 25	>10000	220 ± 40	2800 ± 200
[DArg^{1},DTrp^{7,9},Leu^{11}]substance P	955 ± 123	11300 ± 1800	4100 ± 800	>10000
[DPro^{4},DTrp^{7,9,10}]substance P(4−11)	846 ± 2196	>10000	2500 ± 600	2300 ± 400

[a] Cells ((1−2) × 10^6/mL) were incubated with 50 pM ^{125}I-[DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) (hBRS-3-transfected cells), or 100 pM ^{125}I-[DTyr^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) (fBB_4-R-transfected CHO-K1 cells), 50 pM ^{125}I-[Tyr^{4}]Bn (rat pancreatic acini) or 50 pM ^{125}I-[DTyr^{0}]NMB (rNMB-R-transfected cells) for 45 min at 22 °C (transfected cells) or 30 min at 37 °C (pancreatic acini). Increasing concentrations of unlabeled peptide were added, and dose−response curves were analyzed using a least-squares, curve-fitting program (LIGAND). K_i values were calculated using the method of Cheng and Prusoff. Values are means ±sem from at least four experiments. For abbreviations, see the footnotes for Tables 1 and 2; ψ indicates the insert of a pseudopeptide bond in which −CHO− is replaced by $-CH_2$-; 3-Ph-Pr, (4′-hydroxy)-3-phenylpropanoyl; ME_2, dimethyl; Nal, naphthylalanine; Cpa, chlorophenylalanine. [b] >10000 means the affinity was greater than 10000 nM.

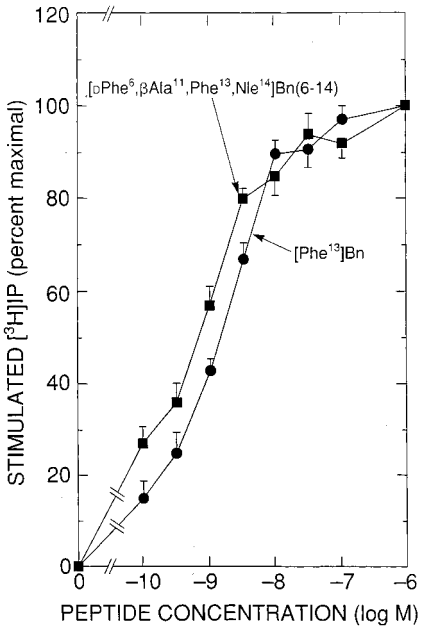

FIGURE 6: Ability of [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) or [Phe^{13}]Bn to stimulate increases in [^{3}H]IP formation in fBB_4-R-transfected CHO-K1 cells. fBB_4-R-transfected CHO-K1 cells (5.0 × 10^4 cells/well) were loaded with *myo*-[2-^{3}H]inositol as described in "Methods", washed, and incubated (5.0 × 10^4 cells/well) with the indicated concentrations of [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn-(6−14) or [Phe^{13}]Bn for 45 min at 37 °C. Values are expressed as the percent of the total [^{3}H]IP release stimulated by 1 μM [Phe^{13}]-Bn. Results are the means ±sem from at least three experiments, and each point was determined in duplicate. The basal and stimulated values for 1 μM [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) are 1610 ± 120 and 2390 ± 110 DPM, respectively.

by examining their abilities to alter basal or [Phe^{13}]Bn-stimulated changes in [^{3}H]IP (Figure 8, Table 4). [(3-Ph-Pr^{6}),His^{7},DAla^{11},DPro^{13},ψ13−14,Phe^{14}]- Bn(6−14),

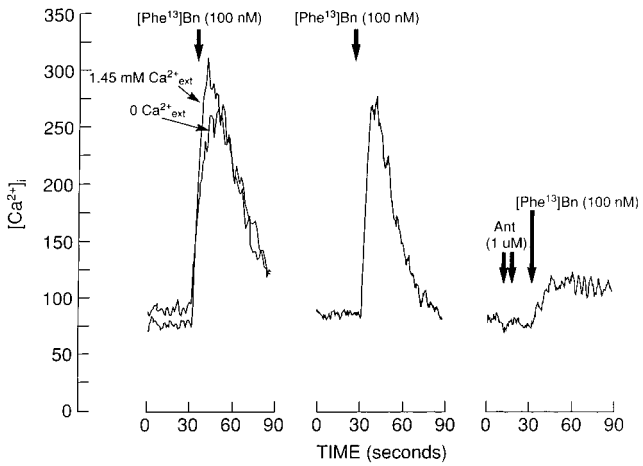

FIGURE 7: Effect of the presence or absence of extracellular calcium ($[Ca^{2+}]_{ext}$) or the Bn receptor antagonist [(3-Ph-Pr^{6}),His^{7},DAla^{11},-DPro^{13},ψ13−14,Phe^{14}]Bn(6−14) on the ability of [Phe^{13}]Bn to alter intracellular calcium ($[Ca^{2+}]_i$) in fBB_4-R-transfected CHO-K1 cells. fBB 4-R-transfected CHO-K1 cells (5.0 × 10^6 cells/mL) were loaded with 2 μM Fura-2/AM as described in "Methods" and incubated with or without extracellular calcium present (left panel), with 100 nM [Phe^{13}]Bn alone, or after the addition of 1 μM antagonist, [(3-Ph-Pr^{6}),His^{7},DAla^{11},DPro^{13},ψ13−14,Phe^{14}]Bn-(6−14) (Ant) (right panel, double short arrows). Results are from a single experiment which was representative of three others. Abbreviations: Bn, bombesin; ANT, [(3-Ph-Pr^{6}),His^{7},DAla^{11},-DPro^{13},ψ13−14,Phe^{14}]Bn(6−14); 0 Ca^{2+}_{ext}, calcium-free incubation medium; 1.45 mM Ca^{2+}_{ext}, 1.45 mM calcium added to incubation medium.

[DArg^{1},DTrp^{7,9},Leu^{11}]substance P, [DPro^{4},DTrp^{7,9,10}]substance P(4−11), and the selective NMB receptor antagonist, DNal,Cys,Tyr,DTrp,Lys,Val,Cys,Nal-NH_2 (*10*), had no agonist activity when present alone (1−100 μM) (Table 4). In contrast, [DPhe^{6}]Bn(6−13)hexylamide, [DPhe^{6}]Bn(6−13)-propylamide, and [DPhe^{6}]Bn(6−13)methyl ester all caused

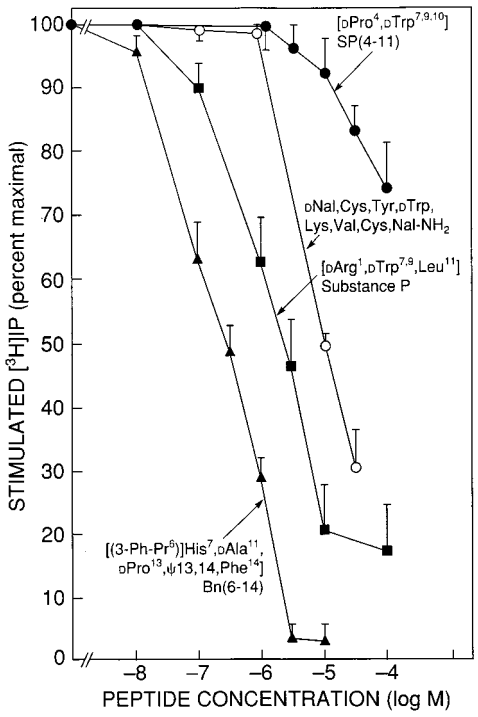

FIGURE 8: Ability of various Bn receptor antagonists to inhibit [Phe^{13}]Bn-stimulated increases in [^{3}H]IP formation in fBB_4-R-transfected CHO-K1 cells. fBB_4-R-transfected CHO-K1 cells (5.0×10^4 cells/well) were loaded with *myo*-[2-^{3}H]inositol as described in Methods, washed, and incubated (5.0×10^4 cells/well) with the indicated concentrations of Bn receptor antagonists in the presence or absence of 30 nM [Phe^{13}]Bn. Results are expressed as the percentage of the increase in [^{3}H]IP caused by 30 nM [Phe^{13}]Bn alone. The basal and 30 nM [Phe^{13}]Bn-stimulated [^{3}H]IP values were 770 ± 190 and 1800 ± 420 DPM, respectively. Values are means ±sem from at least three experiments, and each point was determined in duplicate. For abbreviations, see the caption for Figure 5.

a significant increase in [^{3}H]IP when present at 1 μM (Table 4).

Each of the four Bn-receptor antagonists without agonist activity caused significant inhibition of [Phe^{13}]Bn-stimulated increases in [^{3}H]IP, demonstrating that they functioned as antagonists at the fBB_4-R (Table 4). Each of the four Bn-receptor antagonists without agonist activity at the fBB_4-R was examined for its potency in inhibiting [Phe^{13}]Bn-stimulated increases in [^{3}H]IP in fBB_4-R-transfected CHO-K1 cells (Figure 8). Of these four, [(3-Ph-Pr^6),His^7,DAla^{11},-DPro^{13},ψ13−14,Phe^{14}]Bn(6−14) exhibited the highest potency with half-maximal inhibition at 290 ± 30 nM (Figure 8). [DArg^1,DTrp^{7,9},Leu^{11}]substance P was 7-fold less potent, DNal,Cys,Tyr,DTrp,Lys,Val,Cys,Nal-NH_2 was 34-fold less potent, and [DPro^4,DTrp^{7,9,10}]substance P(4−11) was greater than 3000-fold less potent (Figure 8).

We then examined the effects of various Bn receptor antagonists on the ability of [Phe^{13}]Bn to elicit changes in intracellular calcium $[Ca^{2+}]_i$ in fBB_4-R-transfected CHO-K1 cells (Figure 9). [Phe^{13}]Bn stimulated a 3.6-fold increase in $[Ca^{2+}]_i$ in fBB_4-R-transfected CHO-K1 cells. [(3-Ph-Pr^6),-His^7,DAla^{11},DPro^{13},ψ13−14,Phe^{14}]Bn(6−14), [DPro^4,-DTrp^{7,9,10}]substance P(4−11) and the selective NMB receptor antagonist, DNal,Cys,Tyr,DTrp,Lys,Val,Cys,Nal-NH_2, each caused a concentration-dependent inhibition of [Phe^{13}]Bn stimulation (Figure 9). The most potent antagonist at inhibiting the ability of [Phe^{13}]Bn to stimulate increases in $[Ca^{2+}]_i$ was [(3-Ph-Pr^6),His^7,DAla^{11},DPro^{13},ψ13−14,Phe^{14}]Bn(6−14) with a half-maximal effect at 18 ± 1 nM (Figure 9).

Because a number of peptides which functioned as antagonists for other Bn receptors (*19*, *22*, *23*) had agonist activity in fBB_4-R-transfected cells (Table 4), we explored the ability of one of the peptides, [DPhe^6]Bn(6−13)propylamide, to cause changes in [^{3}H]IP to determine whether it was functioning as a partial agonist (Figure 10, top) in fBB_4-R-transfected CHO-K1 cells. [DPhe^6]Bn(6−13)propylamide alone increased [^{3}H]IP in a concentration-dependent way, causing a maximal effect at 1 μM with an efficacy of 52% of that caused by a maximally effective concentration of [Phe^{13}]Bn (Figure 10, Left). On the other hand, [DPhe^6]-Bn(6−13)propylamide inhibited the increase of [^{3}H]IP by [Phe^{13}]Bn (Figure 10, Left), and the inhibition was dose-dependent (Figure 10). [DPhe^6]Bn(6−13)propylamide (1 μM) inhibited by 43% the stimulation caused by 100 nM [Phe^{13}]Bn, and the stimulation was not significantly different than 1 μM [DPhe^6]Bn(6−13)propylamide alone (Figure 10, Left).

Because different synthetic Bn analogues may vary in their efficacy for stimulating various receptor-activated changes (*9*, *19*), we determined whether similar results were obtained with [Phe^{13}]Bn-stimulated changes in $[Ca^{2+}]_i$. Specifically, the effect of [DPhe^6]Bn(6−13)propylamide on the change of intracellular calcium $[Ca^{2+}]_i$ in fBB_4-R-transfected CHO-K1 cells when present alone or with a Bn-receptor agonist, [Phe^{13}]Bn, was examined (Figure 10, Right). [DPhe^6]Bn-(6−13)propylamide alone stimulated an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner with 1 μM [DPhe^6]Bn-(6−13)propylamide causing a maximal increase which was equal to 40% of the $[Ca^{2+}]_i$ increased by 100 nM [Phe^{13}]Bn alone (Figure 10, Right). On the other hand, [DPhe^6]Bn(6−13)propylamide inhibited the increase of $[Ca^{2+}]_i$ by [Phe^{13}]-Bn (Figure 10, Right), and the inhibition was found to be dose-dependent, with 1 μM [DPhe^6]Bn(6−13)propylamide inhibiting 60% of $[Ca^{2+}]_i$ elicited by 100 nM [Phe^{13}]-Bn alone (Figure 10, Right).

Recent studies demonstrate that the fBB_4-R-related Bn receptors, the GRP receptor, the BRS-3, and the NMB receptor are coupled to both phospholipase D (*16*, *19*) and to phospholipase C. To determine whether fBB_4 receptor activation stimulates increases in phospholipase D activity, we incubated fBB_4-R-transfected CHO-K1 cells with 1 or 100 nM [Phe^{13}]Bn (Figure 11). [Phe^{13}]Bn (1 nM) caused a highly significant ($p < 0.01$) 52% ± 3% increase in PLD activity, and 100 nM [DPhe^{13}]Bn caused a 91% ± 3% increase over basal in PLD activity, demonstrating that activation of this receptor was also coupled to both PLD and PLC activation.

DISCUSSION

Prior to 1995 three classes of receptors were reported that mediated the action of bombesin-related peptides in mammals or nonmammalian species (*1*, *24*). These included a GRP-preferring subtype (the GRP receptor) (*5*, *25*), a NMB-preferring subtype (the NMB receptor) (*26*), and a third subtype with unique pharmacology which interacts with an unknown natural ligand designed bombesin receptor subtype-3 (BRS-3 receptor) (*6*, *18*, *27*, *28*). All of these receptors

Table 4: Ability of Various Bombesin Receptor Antagonists To Stimulate or Inhibit Agonist-Stimulated Increases in [^{3}H]IP Generation in fBB$_4$-R-Transfected CHO-K1 Cells[a]

peptides added	[^{3}H]IP (percent) alone (% maximal)	[^{3}H]IP (percent) plus 30 nM [Phe^{13}]Bn (% control)
[DPhe^6]Bn(6−13)hexylamide (1 μM)	42 ± 6[b]	ND; agonist[c]
[DPhe^6]Bn(6−13)propylamide (1 μM)	52 ± 6[b]	ND; agonist[c]
[DPhe^6]Bn(6−13)methyl ester (1 μM)	29 ± 7[b]	ND; agonist[c]
[(3-Ph-Pr^6)His^7,DAla^{11},DPro^{13},ψ13−14,Phe^{14}]-Bn(6−14)NH$_2$ (1 μM)	4 ± 2	40 ± 6[d]
DNal,Cys,Tyr,DTrp,Lys,Val,Cys,Nal-NH$_2$ (100 μM)	12 ± 4	18 ± 7[d]
[DArg^1,DTrp^{7,9},Lue^{11}]substance P (30 μM)	10 ± 6	31 ± 7[d]
[DPro^4,DTrp^{7,9,10}]substance P(4−11) (100 μM)	12 ± 3	75 ± 8[d]

[a] After loading fBB$_4$-R-transfected CHO-K1 cells with myo-[2-^{3}H]inositol as described in Methods, the cells were incubated with the indicated concentration of reported Bn receptor antagonists either alone or in the presence of 30 or 100 nM [Phe^{13}]Bn. Results with the Bn receptor antagonists alone are expressed as the percentage of the maximal stimulation in [^{3}H]IP caused by 100 nM [Phe^{13}]Bn. The basal and 100 nM [DPhe^{13}]Bn-stimulated [^{3}H]IP values were 1610 ± 120 and 2390 ± 110 dpm, respectively. Results with [Phe^{13}]Bn are expressed as the percentage of the stimulation of [^{3}H]IP caused by 30 nM [Phe^{13}]Bn alone when the indicated concentration of antagonist and 30 nM [Phe^{13}]Bn were incubated simultaneously. The basal and 30 nM [Phe^{13}]Bn-stimulated [^{3}H]IP values were 1616 ± 193 and 2763 ± 428, respectively. Results are the means ±sem of at least four experiments and in each experiment each value was determined in duplicate. For abbreviations, see the footnotes for Tables 1−3. [b] Significantly ($p < 0.05$) greater than basal. [c] "ND; agonist" means that this peptide was not tested as an antagonist with 30 nM [Phe^{13}]Bn because it had agonist activity when tested alone at the indicated concentration. [d] Significantly less than 30 nM [Phe^{13}]Bn alone (control).

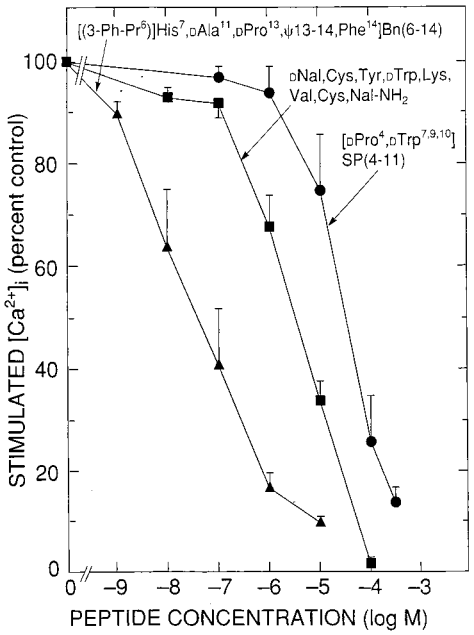

FIGURE 9: The ability of various Bn receptor antagonists to inhibit [Phe^{13}]Bn-stimulated increases in intracellular calcium ([Ca^{2+}]$_i$) in fBB$_4$-R-transfected CHO-K1 cells. fBB$_4$-R-transfected CHO-K1 cells were loaded with 2 μM Fura-2/AM as described in Methods and incubated (5.0×10^6 cells/mL) with the indicated concentrations of antagonists in the presence of 100 nM [Phe^{13}]Bn. Results are expressed as the percentage of the increase in [Ca^{2+}]$_i$ caused by 100 nM [Phe^{13}]Bn alone. The basal [Ca^{2+}]$_i$ value for fBB$_4$-R-transfected CHO-K1 cells was 97 ± 9 nM, and the stimulated value was 344 ± 22 nM. Values are means ±sem from at least three experiments, and each point was determined in duplicate. For abbreviations, see the caption for Figure 5.

are members of the G protein-coupled superfamily with seven transmembrane-spanning regions, each activates phospholipase C to increase inositol 1,4,5 trisphosphate, diacylglycerol, and calcium (*6*, *19*, *24*), and each is coupled to phospholipase D (*6*, *19*). In 1995 a fourth member of the Bn receptor family, the fBB$_4$ receptor, was isolated from a *Bombina orientalis* brain cDNA library, and it showed 56%, 61%, and 70% amino acid identities to the human GRP receptor, NMB receptor, and BRS-3 receptors (*1*). Very little is known of the pharmacology or cell biology of the fBB$_4$ receptor. Only a single study (*1*) exists on this receptor, and it demonstrated that the receptor coupled phospholipase C in *Xenopus oocytes* and, when transiently expressed in CHOP cells, had a high affinity for [Phe^{13}]Bn and lower affinity for GRP and NMB. In the present study we have examined the pharmacology and cell biology of the BB$_4$ receptor in detail. To achieve this it was first necessary for us to prepare stable cell lines expressing the fBB$_4$ receptor that would likely be similar in their receptor pharmacology and cell biology to the native fBB$_4$ receptor. This was necessary because no cell lines or tissues had been identified that contained sufficient numbers of fBB$_4$ receptors to allow studies of direct ligand−receptor interaction or studies of the cell biology. To overcome this problem, we used three different strategies. In the first strategy fBB$_4$ receptors were transfected into two types of amphibian cells (bullfrog tongue FT cells and grass frog embryo ICR 134 cells). However, this was unsuccessful and no saturable binding was obtained. In other studies (*29*) other proteins have been transfected into amphibian cell lines successfully using mammalian expression vectors similar to those we used, and under our culture conditions it was not due to a problem of decreased cell viability, so it is unclear why this approach was unsuccessful. A second approach used was to transfect fBB$_4$ receptors into BALB 3T3 cells or into the non-small cell lung cancer cell line, NCI-H1299, because in previous studies (*14*, *17*−*19*) these cell lines have been extensively used to study transfected BRS-3, GRP, and NMB receptors, which are closely related to the fBB$_4$ receptors (*1*). Specifically, when the human GRP receptor, human NMB receptor, human BRS-3 (*6*, *19*), or rat NMB receptor (*17*) were transfected into either BALB 3T3 cells or NCI-H1299 cells, they behaved in a manner indistinguishable from native cells expressing these receptors in terms of ligand−receptor interactions, G protein-coupling, or receptor modulatory (internalization, down-regulation, desensitization) processes. However, in contrast to the other three related bombesin receptors, transfection of fBB$_4$ receptors into either BALB 3T3 cells or NCI-H1299 cells resulted in such low levels of receptor expression that the cells were not useful for our studies. The third approach used to obtain fBB$_4$-R-containing cells was to stably express the fBB$_4$-R in a

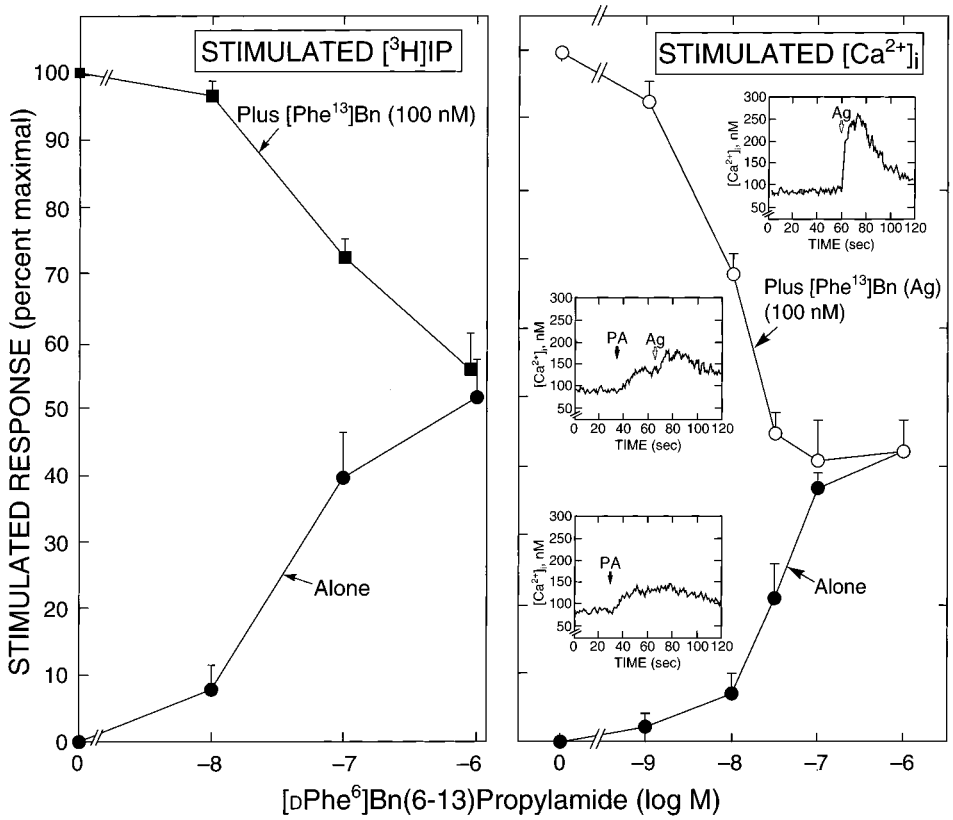

FIGURE 10: Effect of [DPhe^6]Bn(6−13)propylamide and [Phe^{13}]Bn alone or in combination on [^{3}H]IP formation (left panel) or intracellular calcium changes ($[Ca^{2+}]_i$) (bottom panel) in fBB_4-R-transfected CHO-K1 cells. Top panel: fBB_4-R-transfected CHO-K1 cells (5.0×10^4 cells/well) were loaded with *myo*-[2-^{3}H]inositol as described in Methods and incubated (5.0×10^4 cells/well) with the indicated concentrations of [DPhe^6]Bn(6−13)propylamide in the absence or presence of the 100 nM [Phe^{13}]Bn. Results are expressed as the percentage of the increase in [^{3}H]IP caused by 100 nM [Phe^{13}]Bn alone. The basal and 1 μM [Phe^{13}]Bn-stimulated [^{3}H]IP values were 2220 ± 410 and 3670 ± 640 DPM, respectively. Values are means ±sem from at least three experiments, and each point was determined in duplicate. Right panel: fBB_4-R-transfected CHO-K1 cells were loaded with 2 μM Fura-2/AM as described in Methods and incubated (5.0×10^6 cells/mL) with the indicated concentrations of [DPhe^6]Bn(6−13)propylamide (PA) in the absence or presence of the sequential addition of the full agonist, 100 nM [Phe^{13}]Bn (Ag). Results are expressed as the percentage of the maximal increase in $[Ca^{2+}]_i$ caused by 100 nM [Phe^{13}]Bn alone. The basal and 100 nM [Phe^{13}]Bn-stimulated values for $[Ca^{2+}]_i$ were 92 ± 6 and 273 ± 14 nM, respectively. Results from a representative experiment are shown in the insets. The dose−response curves are mean ±sem from at least three experiments, and each point was determined in duplicate. Abbreviations: Bn, bombesin.

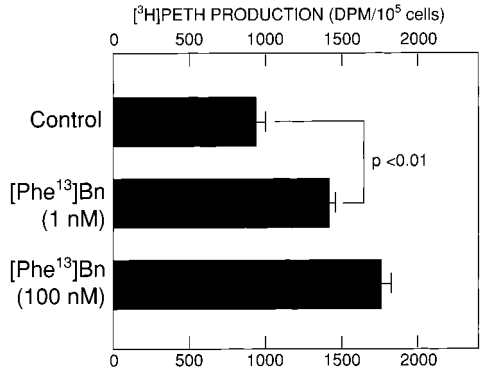

FIGURE 11: Ability of [Phe^{13}]Bn to alter phospholipase D activity in fBB_4-R-transfected CHO-K1 cells. fBB_4-R-transfected K1 cells were incubated for 45 min with the indicated concentrations of [Phe^{13}]Bn. The formation of [^{3}H]PETH was determined using transphosphatidylation and the formation of [^{3}H] phosphatidylethanol ([^{3}H]PETH) as described in Methods. Each experiment was performed in triplicate, and the results are means ±sem of four experiments.

Chinese hamster ovary cell (CHO-K1). This cell line was selected because it did not contain bombesin receptors as do COS-7 cells (*30*). Furthermore, in a previous study, fBB_4 receptors were able to be transiently transfected in the closely related CHOP cells (Chinese hamster ovary fibroblasts modified to express polyoma large T antigen), which we confirmed in the present study. Last, we demonstrated that the fBB_4-R closely related receptor, the mGRP receptor, when stably expressed in CHO-K1 cells, showed pharmacology indistinguishable from that seen when it was stably expressed in BALB 3T3 cells or compared to the native GRP receptor in Swiss 3T3 cells. These results suggested that the bombesin family of receptors would maintain pharmacology similar to that seen in cells containing the native receptor, and therefore the nonmodified CHO-K1 cell line was used to prepare stable fBB_4 receptor-containing cells.

From the ligand binding studies and studies of the effects of fBB_4 receptor activation, a number of results support the conclusion that we are assessing interaction with the fBB_4 receptor, and it likely represents the true pharmacology of this receptor. First, CHO-K1 cells did not possess members of the bombesin receptor family prior to transfection. However, after transient or stable transfection, saturable binding was seen with either ^{125}I-[Tyr^4]Bn which is reported to have high affinity for fBB_4 receptor (*1*), or with the novel ligand ^{125}I-[DTyr^6,βLeu^{11},Phe^{13},Nle^{14}]Bn(6−14) which has been shown to have high affinity for all four subtypes of bombesin receptors including fBB_4 receptors (*6*, *7*). Second,

the interaction of the radioligands with the fBB_4-transfected CHO-K1 cells was characteristic of receptor interaction in that it was saturable, specific, time- and temperature-dependent, and of high affinity. Third, the agonist ligand demonstrated rapid internalization in fBB_4-transfected CHO-K1 cells which is also reported with other specific agonist ligands for other bombesin receptors (*14*, *17*). Fourth, each of five different fBB_4 receptor-transfected CHO-K1 cell lines which had different fBB_4 receptor densities had similar receptor affinities. These results demonstrate that the pharmacology was not a function of the receptor density, which is similar to that reported recently with studies of the effect of receptor density of the structurally related GRP receptor on different cellular functions and its pharmacology (*31*). Therefore, results with fBB_4-transfected cells are likely representative of native cells which may differ in the density of the native fBB_4 receptor compared to the fBB_4-R-transfected cells. Last, there was a close coupling between the ability of agonists to activate the fBB_4-R-transfected CHO-K1 and occupy the fBB_4-R-transfected receptors. Specifically, both the natural agonist [DPhe^{13}]Bn and the high-affinity, synthetic agonist [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn-(6−14) activated phospholipase C over the same concentration ranges that they occupied in the fBB_4 receptors in binding studies. Furthermore, various classes of Bn receptor antagonists that functioned as fBB_4 receptor antagonists did so over the same concentration ranges that caused fBB_4 receptor occupation, demonstrating a close coupling between receptor binding and biologic activity.

Our binding results demonstrate that the fBB_4 receptor has a unique pharmacologic profile for agonists compared to other members of the bombesin family of receptors. Its pharmacology for agonists both differs and is similar in a number of ways from that of the GRP receptor, NMB receptor, or BRS-3 receptor. First, it resembles the GRP and NMB receptors in having a high affinity for the naturally occurring peptide agonists, litorin, ranatensin, [Phe^{13}]-bombesin, and bombesin. However, its affinities for these four peptides differ from the BRS-3 receptor, which shows the greatest structural similarity to fBB_4 receptor [i.e., 70% amino acid identities (*1*)], because the latter receptor has a low affinity (>1 μM) for each of these four peptides. Second, a number of naturally occurring peptides have high affinity only for the GRP receptor (GRP, PG-L, Rhodei-litorin) or the NMB receptor (NMB, phyllolitorin); however, the fBB_4 receptor had high affinity for each of these peptides. This result demonstrates that the fBB_4 receptor is unique in having a combination of the high affinities of the GRP and NMB receptors and, therefore, is not as discriminatory as either of these. Third, a number of naturally occurring bombesin peptides have low affinity for each of the other three bombesin receptors [Leu^{8} (phyllolitorin), Xenopus NMB, SAP bombesin] and in all cases the fBB_4 receptor-demonstrated higher affinities, which in some cases increased from the micromolar to the nanomolar range [Xenopus NMB, Leu^{8} (PLL)]. Fourth, previous studies and the present study demonstrate that the *N*-terminal amino acids of GRP, NMB-32, or neuromedin C or the first five amino acids of bombesin are not essential for high-affinity interaction with GRP or NMB receptors. However, these amino acids contribute to the selectivity of the peptide for one or the other bombesin receptors (*23*). Similar structure−function relationships were found in the present study to exist for the fBB_4 receptor. This conclusion was supported by the finding that bombesin *N*-terminally truncated analogues [[DPhe^{6}]Bn(6−14) or [DPhe^{6},βAla^{11},Phe^{13},Nle^{14}]Bn(6−14)] each had high affinity for the fBB_4 receptor. However, each of these peptides also had high affinity for the GRP and NMB receptor and therefore differed from the *N*-terminally extended forms (NMC, GRP, bombesin) in not having selectivity for one bombesin receptor subtype over the other. An overall analysis of the fBB_4 receptor affinities for agonists demonstrates that the most important determinant of high-affinity interaction with this receptor is the presence of a phenylalanine in the COOH terminal penultimate position. This conclusion was supported by the 14-fold increase in affinity changing bombesin to [Phe^{13}]bombesin or an 8-fold increase in affinity changing [Leu^{8}]phyllolitorin to phyllolitorin. This was a unique structural requirement for the fBB_4 receptor because these latter changes in bombesin or phyllolitorin either decrease affinity for the NMB receptor or resulted in minimal increases with the GRP receptor.

Six different classes of GRP receptor (*22*, *23*), NMB receptor (*10*, *22*, *23*), or BRS receptor (*19*) antagonists have been described, some of which are highly selective. The fBB_4 receptor also had a unique pharmacology compared to the other bombesin receptor subtypes for the different bombesin receptor antagonist classes. It differed from the BRS-3 receptor in possessing affinities higher than 1 μM for eight members of four different classes of antagonists. It differed from the NMB receptor in possessing affinities greater than 1 μM for a number of des met^{14} bombesin alkylamides and esters, as well as two D-amino acid-substituted substance P analogues, [DArg^{1},DTrp^{7,9},Leu^{11}]SP and [DPro^{4},DTrp^{7,9,10}]-SP(4−11), which each have low affinities for the NMB receptor. It differed from the GRP receptor in not possessing high affinities for a number of classes of antagonists which have nanomolar affinity for the GRP receptor {i.e., [(3-Ph-Pr^{6})His^{7},DAla^{11},DPro^{13},ψ13−14, Phe^{14}]Bn(6−14), [DPhe^{6}]-Bn(6−13)propylamide and methylester}. Furthermore, the fBB_4 receptor had a moderate affinity (K_i − 130 nM) for the NMB receptor antagonist, DNal,Cys,Tyr,DTrp,Lys,Val,-Cys,Nal-NH_2 (*10*), an antagonist which had a very low affinity for the GRP receptor (i.e., >18000 nM) (*10*). Previous studies have shown that some peptide antagonists for various GI hormone/neurotransmitter G protein-coupled receptors (CCK, bombesin, gastrin, CGRP) can function as an antagonist at one receptor subtype while also functioning as an agonist at a different receptor subtype, or at the same receptor subtype in a different species (*9*, *32*, *33*). A similar result was found in the present study for three of the most potent putative bombesin receptor antagonists with the fBB_4 receptor. Three members of two classes of potent GRP receptor antagonists (*9*) which had the highest affinity for the fBB_4 receptor (i.e., [DPhe^{6}]Bn(6−13)hexylamide, propylamide, and methylester) (K_i = 18−122 nM), were found not to function as antagonists at the fBB_4 receptor but instead to function as partial agonists at this receptor. This conclusion was supported by the fact that they were only 20−50% as efficacious as the fBB_4 receptor full agonist [Phe^{13}]bombesin for stimulating increases in [^{3}H]IP or $[Ca^{2+}]_i$. Furthermore, increasing concentrations of one of these peptides ([DPhe^{6}]-Bn(6−13)propylamide) inhibited [Phe^{13}]bombesin-stimulated increases in [^{3}H]IP and $[Ca^{2+}]_i$ to the same level of stim-

ulation seen with [DPhe^6]Bn(6−13)propylamide alone, demonstrating that it was functional at the fBB_4 receptor as a partial agonist and not at an unrelated receptor. Members of these three classes of potent GRP receptor antagonists which function as partial fBB_4 receptor agonists have been widely used in vitro and in vivo, including in human studies to determine the role of GRP in various physiological or pathologic processes (*22*, *34*). The results in the present study suggest that if a mammalian form of the fBB_4 receptor exists, its presence and pharmacology will likely limit the usefulness of these compounds as GRP receptor antagonists by confounding the results due to their partial agonist activity at the BB_4 receptor. A number of the remaining classes of bombesin receptor antagonists did function as pure fBB_4 receptor antagonists. Of the six classes of bombesin receptor antagonists, three classes including the Phe^{13} bombesin pseudopeptide analogue [(3-Ph-Pr^6)His^7,DAla^{11},DPro^{13},ψ13−14,-Phe^{14}]Bn(6−14), a potent, highly selective GRP receptor antagonist; a D-amino-substituted somatostatin octapeptide analogue, SS-octa (DNal,Cys,Tyr,DTrp,Lys,Val,Cys,Nal-NH_2), a selective NMB receptor antagonist (*10*); and D-amino acid-substituted substance P (SP) or SP-4−11 analogues which function as GRP and NMB receptor antagonists (*22*, *23*, *35*, *36*) all functioned as fBB_4 receptor antagonists. Each of these compounds inhibited [DPhe^{13}]bombesin-stimulated increases in either [^{3}H]inositol phosphate or $[Ca^{2+}]_i$ over concentration ranges they inhibited binding to the fBB_4 receptor, suggesting that each was functioning as a receptor antagonist. These four antagonists should provide important lead compounds that can be used to identify a more selective fBB_4 receptor antagonist with higher affinity in future studies.

A number of results demonstrate that fBB_4 receptors are coupled to both phospholipase C and phospholipase D activation in the present study, similar to the other three mammalian bombesin receptors (*14*, *16*, *17*, *19*, *24*, *37*, *38*). First, [Phe^{13}]bombesin and [DPhe^6,βAla^{11},Phe^{13},Nle^{14}]Bn(6−14) elicited increases in [^{3}H]IP in fBB_4-transfected CHO-K1 cells under conditions in which no agonist stimulation was seen in untransfected cells. Second, similar stimulation was obtained with increases in $[Ca^{2+}]_i$ in fBB_4-transfected CHO-K1 cells with both peptide agonists, whereas no changes in $[Ca^{2+}]_i$ were seen in CHO-K1 cells not transfected with fBB_4 receptors. Third, the fBB_4 receptor-stimulated increases in $[Ca^{2+}]_i$ were only minimally decreased by the removal of extracellular calcium, demonstrating that calcium release was primarily from IP_3-sensitive intracellular stores. The ability of [Phe^{13}]bombesin to alter $[Ca^{2+}]_i$ in stably transfected fBB_4 CHO-K1 cells is consistent with a previous study with the fBB_4 receptor (*1*). When the fBB_4 receptor was expressed in Xenopus oocytes by injecting fBB_4 receptor RNA and the calcium photoprotein aequorin, the fBB_4 receptor agonist, [Phe^{13}]bombesin, caused an increase in cytosolic calcium and light intensity, whereas GRP or NMB had a minimal effect. Fourth, the fact that the increases in phospholipase C were related to the occupation of the fBB_4 receptor was supported by the failure to see changes in [^{3}H]-IP or $[Ca^{2+}]_i$ with fBB_4 receptor agonists in CHO-K1 cells not transfected with the fBB_4 receptor. This conclusion was further supported by the finding that the relative ability of agonists to cause stimulation of phospholipase C was the same as their relative potency for occupation of the fBB_4 receptor and the fact that various receptor antagonists inhibited increases in phospholipase C activity over the same concentration ranges they occupied in the fBB_4 receptor. Fifth, the ability of fBB_4 receptor activation to stimulate increases in phospholipase D activity was demonstrated by the ability of [Phe^{13}]bombesin to stimulate increases in [^{3}H]-phosphatidylethanol, which is generated selectively through transphosphatidylation when phospholipase D is activated in the presence of ethanol (*39*, *40*). This stimulation occurred over the same concentration range in which [Phe^{13}]bombesin increased phospholipase C activity, which is similar to that described with agonists causing NMB and GRP receptor activation (*16*). These results support the conclusion that activation of both phospholipase C and phospholipase D is likely to be important intracellular cascades for mediating the cellular effects of activation of the fBB_4 receptor.

In conclusion, fBB_4 receptors were stably expressed and functional in CHOK1 cells, whereas fBB_4 receptors were incapable of being functionally expressed in either cells routinely used for study of Bn receptor subtypes, such as BALB 3T3 fibroblasts and NCI-H1299 non-SCLC, or in the amphibian cell lines FT or ICR 134. Successful expression of fBB_4 allowed detailed study of its pharmacology and cell biology, demonstrating that it has a unique pharmacology for natural occurring Bn-related ligands and Bn receptor antagonists and that the presence of a penultimate phenylalanine instead of leucine, characteristic of bombesin and gastrin-releasing peptide, was critically important for high-affinity interaction. Members of three different classes of bombesin receptor antagonists functioned as antagonists at the fBB_4 receptor and could serve as important lead compounds for the development of more potent, selective antagonists in the future. The fBB_4 receptor was found to be coupled to phospholipase D and to phospholipase C, with receptor activation resulting in increases in cytosolic calcium derived almost entirely from intracellular IP_3-sensitive stores. The availability of these fBB_4 receptor cells, the identification of potent agonists, a high-affinity ligand, and three classes of antagonists should prove valuable in further studies of the cell biology of the fBB_4 receptor. Because of the high homology of the fBB_4 receptor to each of the three mammalian bombesin receptors, the elucidation of the receptor structural determinants of its unique pharmacology will likely provide important insights into the basis of selectivity of the three mammalian bombesin receptors for various naturally occurring ligands.

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BI990204W