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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

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SUMMARY

The receptor that interacts with the mammalian bombesin-related peptide neuromedin B (NMB) is ubiquitous in the gastrointestinal tract and central nervous system. However, little is known regarding its cellular mechanisms of action. This receptor has been recently cloned, sequenced, and stably transfected into BALB 3T3 fibroblasts, permitting detailed study of the pharmacology and coupled biological activities of this receptor. In the present study, we compare the ability of transfected receptors to alter cell function with that of receptors natively expressed in small numbers by the rat glioblastoma cell line C6. NMB inhibited binding of ^{125}I-[D-Tyr^{0}]NMB with high affinity in transfected cells (K_i = 3.08 ± 0.14 nM) and in C6 cells (K_i = 1.90 ± 1.10 nM), whereas the bombesin-related agonists gastrin-releasing peptide (GRP) and [D-Phe^{6}, D-Ala^{11}, Leu^{14}]bombesin(6–16) (GRP analogue) had 100- and 300-fold lower affinities, respectively, for NMB receptors in either cell type. For both cell systems, maximal binding was observed between 5 and 15 min at 22°. Both cell types internalized NMB at similar rates, with >70% of bound ligand being internalized by 60 min at 22°. The nonhydrolyzable guanosine analogue guanosine 5′-(β,γ-imido)triphosphate was equipotent in causing a decrease in binding of ^{125}I-[D-Tyr^{0}]NMB due to decreased receptor affinity in both cell types, without a change in receptor number, demonstrating that the NMB receptor remained coupled to a guanine nucleotide-binding protein in both native and transfected cells. In both cell systems, NMB increased inositol monophosphate, inositol bisphosphate, and inositol trisphosphate in a time-dependent fashion. Inositol phosphates were increased in a dose-dependent fashion, with similar half-maximal values being obtained for NMB in both cell types (transfected, 1.01 ± 0.09 nM; C6, 2.09 ± 0.15 nM) and for the GRP analogue (transfected, 1855 ± 140 nM; C6, 2129 ± 250 nM). NMB mobilized intracellular Ca^{2+} in both cell systems, and the dose-response curves were superimposible (EC_{50} for transfected, 0.10 ± 0.08 nM; C6, 0.11 ± 0.02 nM). These data demonstrate that activation of the receptor for NMB stimulates phospholipase C and increases intracellular Ca^{2+}. These results also demonstrate that transfected and native NMB receptors behave similarly, suggesting that the transfected cell line will be useful in future studies investigating ligand-receptor interactions, as well as in molecular biological studies of the structure-function relationship of the receptor.

Two classes of receptors for mammalian bombesin-related peptides have been cloned and sequenced (1–3). The receptor for GRP has been extensively studied using Swiss 3T3 cells, pancreatic acinar cells, small cell lung cancer cells, and other tumor cell lines (4–8), whereas relatively little is known about the receptor for NMB. Hydropathy analysis of the sequence for the NMB receptor suggests it has seven membrane-spanning regions, as observed for other G protein-coupled receptors (3). Recent investigations demonstrate that agonist stimulation of the NMB receptor causes generation of inositol phosphates (9), suggesting that this receptor activates phopholipase C. However, little else is known regarding the ability of this receptor to interact with ligands or to alter cell function.

Receptors for related peptides often coexist on the same tissues, thereby interfering with investigations attempting to study one receptor subtype in detail. Such studies are facilitated

ABBREVIATIONS: GRP, gastrin-releasing peptide; NMB, neuromedin B; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCK-8, Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; HPLC, high performance liquid chromatography; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; BSA, bovine serum albumin; IBMX, isobutylmethylxanthine; $[Ca^{2+}]_i$, intracellular calcium concentration; SP, substance P; G protein, guanine nucleotide-binding protein.

when a cloned receptor gene construct is transfected and expressed in a neutral cell system. This approach, however, assumes that the behavior observed in the transfected cell is identical to that which occurs in cells normally expressing the receptor. This assumption has been challenged by recent data. Certain muscarinic cholinergic receptors, such as the M2 and M4 receptors, have been shown to inhibit adenylate cyclase, stimulate phospholipase C, or do both, depending on the cell type used for transfection (10, 11). Similarly, agonist stimulation of the dopaminergic D_2 receptor also can result in differential inhibition of adenylate cyclase or stimulation of phospholipase C, depending on the transfected cell system studied (12). Consequently, it is important to establish that the transfected receptor being studied behaves similarly to the same receptor expressed natively.

In this study, we describe both the pharmacology and the ability to alter cell function of stably transfected NMB receptors, and we compare our results with those obtained using a cell system that natively expresses the NMB receptor. C6 rat glioblastoma cells express only NMB receptors (9), albeit in low numbers, permitting this comparative analysis.

Experimental Procedures

Materials

Rat glioblastoma C6 cells were obtained from the American Type Culture Collection (Rockville, MD), Dulbecco's modified essential medium, fetal bovine serum, and Geneticin (aminoglycoside G-418) were from GIBCO (Waltham, MA), and cell culture flasks and 24-well plates were obtained from Costar Co. (Cambridge, MA).

Bovine serum albumin (fraction V) and HEPES were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); soybean trypsin inhibitor, EGTA, trypsin, bacitracin, bestatin, phosphoramidon, leupeptin, chymostatin, and amastatin were from Sigma Chemical Co. (St. Louis, MO); glutamine was from the Media Section, National Institutes of Health (Bethesda, MD); [D-Pro^4,D-Trp^{7,9,10}]SP(4–11), NMB, bombesin, GRP, gastrin-17-I, CCK-8, vasoactive intestinal peptide, and SP were from Peninsula Laboratories (Belmont, CA); Gpp(NH)p tetralithium salt was from Fluka Chemical Corp. (Ronkonkoma, NY); Na^{125}I was from Amersham Co. (Arlington Heights, IL); *myo*-[2-^{3}H]inositol (16–20 Ci/mmol) and ^{45}Ca (20 mCi/mg) were from New England Nuclear (Boston, MA); Dowex AG 1-X8 anion exchange resin (100–200 mesh, formate form) was from Bio-Rad (Richmond, CA); Hydro-Fluor scintillation fluid, methanol (absolute), and hydrochloric acid were from the J. T. Baker Chemical Co. (Phillipsburg, NJ); and fura-2/acetoxylmethyl ester was from Molecular Probes (Eugene, OR).

Methods

Transfection and maintenance of cell lines. As described previously (3), BALB 3T3 cells expressing a stably transfected rat NMB receptor were obtained using calcium phosphate precipitation of a full length NMB-preferring bombesin receptor clone generated from rat esophagus and subcloned into a modified version of the pCD2 plasmid. Cells were passaged every 3–4 days at confluence, using 0.1% trypsin in 1 mM EDTA. Rat glioblastoma C6 tumor cells were maintained similarly and were passaged weekly at confluence. Both cell lines were cultured at 37° in a 5% CO_2 atmosphere.

Preparation of peptides. [D-Phe^6]Bombesin(6–13)ethyl ester, [D-Tyr^4,D-Phe^{12}]bombesin, [D-Tyr^0]NMB, [D-Phe^6]bombesin(6–13)propylamide, and [D-Phe^6,D-Ala^{11},Leu^{14}]bombesin(6–14) (GRP analogue) were synthesized using solid phase methods as described previously (13, 14). The alkylamide analogue was synthesized on a standard leucine-*O*-polystyrene resin using tosyl group protection for the imidazole group of histidine. Peptides were purified on a 2.5- × 90-cm Sephadex G-25 column, followed by elution with linear gradients of acetonitrile and 0.1% trifluoroacetic acid using an Eldex Chromatrol gradient controller (flow rate, ~1 ml/min) and 1.5- × 50-cm Vydac C_{18} silica (10–15 μm) columns. Peptides were further purified by rechromatography on the same column, with slight modifications to the gradient conditions when necessary, to >97% purity.

Preparation of ^{125}I-[D-Tyr^0]NMB. ^{125}I-[D-Tyr^0]NMB (2200 Ci/mmol) was prepared using Iodo-Gen. In brief, 0.4 μg of Iodo-Gen was added to 8.0 μg of [D-Tyr^0]NMB with 2 mCi of Na^{125}I in 20 μl of 0.5 M KPO_4 buffer (pH 7.4). After incubation at 22° for 6 min, 300 μl of 1.5 M dithiothreitol were added and the reaction mixture was incubated at 80° for 60 min. Free ^{125}I was separated by applying the reaction mixture to a Sep-Pak cartridge (Waters Associates, Milford, MA), which had been prepared by washing with 5 ml of methanol, 5 ml of 0.1% trifluoroacetic acid, and 5 ml of water. Free ^{125}I was eluted with 5 ml of 0.1% trifluoroacetic acid, and the radiolabeled peptide was eluted with 200-μl sequential elutions (10 times) of 60% acetonitrile/0.1% trifluoroacetic acid. Radiolabeled peptide was separated from unlabeled peptide by combining the three elutions with the highest radioactivity and applying them to a reverse phase high performance liquid chromatograph (Waters Associates model 204, with a Rheodyne injector), using a 0.46- × 25-cm μ BondaPak column. The column was eluted with a linear gradient of acetonitrile and 0.1% trifluoroacetic acid (v/v) from 16 to 64% acetonitrile in 60 min, with a flow rate of 1.0 ml/min. ^{125}I-[D-Tyr^0]NMB was stored with 1% (w/v) BSA at −20° and was stable for at least 6 weeks.

Binding of ^{125}I-[D-Tyr^0]NMB to C6 and NMB receptor-transfected cells. Binding studies using rat glioblastoma C6 cells or NMB receptor-transfected cells were performed by suspending disaggregated cells in binding buffer, which was composed of standard buffer (130 mM NaCl, 7.7 mM KCl, 1.0 mM EGTA, 0.02% soybean trypsin inhibitor) additionally containing 50 mM HEPES, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 2.2 mM $KHPO_4$, 0.015% glutamine, and 0.2% BSA (w/v) (pH 7.4). Incubations contained 75 pM ^{125}I-[D-Tyr^0]NMB and 15 × 10^6 C6 cells/ml or 5 × 10^5 NMB receptor-transfected cells/ml, for 60 min at 22°. Nonsaturable binding of ^{125}I-[D-Tyr^0]NMB was the amount of radioactivity associated with C6 cells or NMB receptor-transfected cells when the incubation mixture contained 1 μM NMB. Nonsaturable binding was <15% of total binding in all experiments; all values in this paper are reported as saturable binding (i.e., total minus nonsaturable binding).

Ligand degradation studies. ^{125}I-[D-Tyr^0]NMB (50 pM) was incubated with either NMB receptor-transfected cells or C6 cells for 60 min at 37° either in the presence or in the absence of 0.1% bacitracin. Cell suspensions were centrifuged in a Beckman Microfuge B (10,000 × *g*) and the supernatant was separated. Using HPLC as described for preparation of ^{125}I-[D-Tyr^0]NMB, aliquots (400 μl, 250,000 cpm) were separated using a linear gradient of 16 to 64% acetonitrile in 0.1% trifluoroacetic acid. As a control, ^{125}I-[D-Tyr^0]NMB in buffer only was incubated and centrifuged and the supernatants were applied to the HPLC column. Results are expressed as the percent difference in ^{125}I-[D-Tyr^0]NMB detectable with and without 0.1% bacitracin present in the buffer.

Stripping of surface-bound ^{125}I-[D-Tyr^0]NMB. Cells were disaggregated, washed, and resuspended in binding buffer as described above. C6 cells (15 × 10^6 cells/ml) or NMB receptor-transfected cells (5 × 10^5 cells/ml) were incubated with 75 pM ^{125}I-[D-Tyr^0]NMB for various times and temperatures. After incubation, 100-μl samples were added to 0.2 M acetic acid (pH 2.5), containing 0.5 M NaCl, for 5 min at 4° to remove surface-bound radioligand as described previously (15). In all cases, parallel incubations were conducted in the presence of 1 μM unlabeled NMB to determine changes in nonsaturable binding. Results are expressed as the percentage of saturable ^{125}I-[D-Tyr^0]NMB added that is surface bound (acid stripped) or internalized (not acid stripped).

Cell membrane preparation. C6 cells or NMB receptor-transfected cells were grown to confluence, mechanically disaggregated as

described above, washed once in binding buffer to remove excess medium, and resuspended in homogenization buffer (50 mM Tris, pH 7.4, 0.2 mg/ml soybean trypsin inhibitor, 0.2 mg/ml benzamidine). Cells were then homogenized on ice using a Polytron (Beckman Instruments) at speed 6 for 30 sec. The homogenate was then centrifuged at 1500 rpm for 10 min in a Sorvall RC-5B Superspeed centrifuge (DuPont Corp); the supernatant was removed and recentrifuged at 20,000 rpm for 20 min. The pellet was resuspended in homogenization buffer and stored at −40°.

Measurement of $[Ca^{2+}]_i$ using fura-2. Rat glioblastoma C6 cells were mechanically disaggregated and then resuspended in binding buffer containing 5×10^6 cells and 2 μM fura-2 for 45 min at 37°. Fura-2-loaded cells were then washed three times in binding buffer. For measurement of $[Ca^{2+}]_i$, 2.0-ml samples were placed in quartz cuvettes in a Delta PTI Scan 1 spectrophotomer (PTI Instruments, Gaithersburg, MD). This instrument was modified so as to maintain an incubation temperature of 37° while the cuvette contents were continuously mixed by means of a magnetic stirrer. Fluorescence was measured at 500 nm after excitation at 340 nm (F_{340}) and at 380 nm (F_{380}). Autofluorescence of the unloaded cells was subtracted from all measurements. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* (16), using the formula $[Ca^{2+}]_i = K_d \times (R - R_{min})(R_{max} - R) \times S_f/S_b$, where K_d is the affinity of fura-2 for Ca^{2+}, determined to be 225 nM in our experiments, R is F_{340}/F_{380} or the ratio of the fluorescence of the two excitation wavelengths, R_{max} is F_{340}/F_{380} in a saturated Ca^{2+} environment after the addition of 0.1% Triton X-100, R_{min} is the fluorescence ratio at virtually zero calcium after the addition of 25 mM EGTA, S_f is the F_{380} at zero calcium, and S_b is the F_{380} at saturated $[Ca^{2+}]_i$.

Measurement of changes in $[Ca^{2+}]_i$ using ^{45}Ca efflux. ^{45}Ca efflux from cells cultured to confluence was performed as follows. At the time of regular cell passage 5×10^4 cells/well were plated in 24-well plates. At confluence 1–3 days later the medium was removed and the cells were washed with phosphate-free buffer (standard buffer additionally containing 0.015% glutamine, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 0.2% BSA, pH 7.4) for 15 min. Cells were then exposed to the same buffer but containing 5 μCi/ml ^{45}Ca for 90 min at 37° in a 5% CO_2 atmosphere. Immediately before the assay, cells were rapidly washed once in phosphate-free buffer and then incubated at 22° in buffer containing appropriate concentration of peptide. After 5 min the supernatant was removed and discarded; the cells were lysed immediately in 1% HCl in methanol, and the cell-associated radioactivity was determined in a liquid scintillation counter. Calcium efflux was expressed as the percentage of the cellular ^{45}Ca that became extracellular during the incubation.

Measurement of phosphoinositides. Total phosphoinositides in C6 and in NMB receptor-transfected cells were determined as described previously, with minor modifications (16, 17). Cells were grown to confluence in 24-well plates in regular medium and then loaded with 100 μCi/ml *myo*-[2-^{3}H]inositol in Dulbecco's modified essential medium with 2% fetal bovine serum at 37° for 48 hr. Cells were washed and incubated in phosphoinositide buffer (standard buffer additionally containing 10 mM LiCl, 20 mM HEPES, 2 mM $CaCl_2$, 2% BSA, and 1.2 mM $MgSO_4$) for 15 min and then for 60 min at 37° with agonists at various concentrations or for variable times with 1 μM NMB.

Reactions were halted using ice-cold 1% HCl in methanol, and the inositol phosphates were isolated as described previously (17, 18). Briefly, after loading of the anion exchange column, free [^{3}H]inositol was removed by washing with water, and [^{3}H]glycerophosphoryl inositol was removed by washing with 5 mM disodium tetraborate in 60 mM sodium formate. Total [^{3}H]inositol phosphates were then eluted using 100 mM formic acid in 1.0 M ammonium formate. In separate experiments [^{3}H]IP_1, [^{3}H]IP_2, and [^{3}H]IP_3 were isolated, after removal of free [^{3}H]inositol and [^{3}H]glycerophosphoryl inositol, by eluting the columns sequentially with 100 mM formic acid in 200 mM ammonium formate ([^{3}H]IP_1), with 100 mM formic acid in 400 mM ammonium formate ([^{3}H]IP_2), and then with 100 mM formic acid in 1.0 M ammonium formate ([^{3}H]IP_3). Eluates were then assayed for their radioactivity after the addition of Hydro-Fluor scintillation fluid.

Measurement of cAMP. Cells were mechanically disaggregated and washed twice in standard binding buffer. Cells (4×10^6/ml) were incubated with various peptides for 10 min at 37°, and the cAMP was then solubilized in 2 volumes of icecold ethanol. Peptide effect was measured in the presence and absence of 25 μM forskolin, 250 mM IBMX, and 100 ng/ml cholera toxin; cells were preincubated for 60 min with cholera toxin before exposure to peptide. cAMP was measured by radioimmunoassay as described previously (19, 20).

Comparisons were made using the Student t test and values of p of <0.05 were considered significant.

Results

The time and temperature dependences of binding of ^{125}I-[D-Tyr^0]NMB by both transfected and C6 cells were similar (Fig. 1). With both cell types more ligand was bound at 22° than at 4° or 37°. With both cell types at 22° half-maximal binding occurred at 5 min; maximal binding was observed at 30 min for C6 cells and at 60 min for NMB receptor-transfected cells. Binding was rapid at 37°, with maximal binding at this temperature occurring between 10 and 15 min for either cell type. By 90 min, however, there was approximately 50% less ^{125}I-[D-Tyr^0]NMB bound to either NMB receptor-transfected or C-6 cells at 37°. Reducing the incubation temperature to 4° from 22° resulted in a decreased rate and amount of binding with both cell types. Adding 1 μM NMB at 22° reduced binding by ≥80% for both C6 cells and NMB receptor-transfected cells at all temperatures (Fig. 1).

To determine whether cellular degradation of ligand could be contributing to the decrease in binding with time at 37°, the radioactivity in the supernatant after incubation of ^{125}I-[D-Tyr^0]NMB with each cell type was analyzed. For either C6 or NMB receptor-transfected cells, >80% of ^{125}I-[D-Tyr^0]NMB was degraded after a 60-min incubation at 37°, by HPLC analysis (data not shown). In contradistinction, 0.1% bacitracin present under the same incubation conditions resulted in 63-67% of ^{125}I-[D-Tyr^0]NMB remaining intact in C6 and NMB receptor-transfected cells. To gain further insight into the peptidases involved in ligand degradation, a variety of peptidase inhibitors were tested by comparing the amount of ^{125}I-[D-Tyr^0]NMB bound at 60 min with the amount bound at 15 min. Specifically, in C6 cells or transfected cells without protease inhibitors binding at 60 min was only 33 ± 1% and 42 ± 2%, respectively, of that at 15 min. Bacitracin (0.1%) was effective in inhibiting ^{125}I-[D-Tyr^0]NMB degradation (98 ± 2% bound at 60 min to C6 cells versus 73 ± 1% to transfected cells), as was leupeptin (81 ± 5% versus 69 ± 2%). Phosphoramidon (35 μM) (41 ± 2% versus 61 ± 1%) and chymostatin (500 μg/ml) (58 ± 2% versus 61 ± 2%) were less effective but binding was still statistically better ($p < 0.05$) than binding observed without the use of a peptidase inhibitor. In contradistinction, binding in the presence of amastatin (10 μM) (36 ± 1% versus 39 ± 1%) or bestatin (100 μM) (38 ± 1% versus 39 ± 2%) was similar to that observed without any additions. The addition of 0.1% bacitracin to the incubation medium with both cell types altered the kinetics of binding only at 37° (data not shown). At 37° the decrease in binding with both cell types seen in Fig. 1 was markedly attenuated with 0.1% bacitracin present, such that similar maximal binding was seen at 37° and 22° and binding was maximal by 10 min and decreased only 15% in incubations up to 30 min (data not shown).

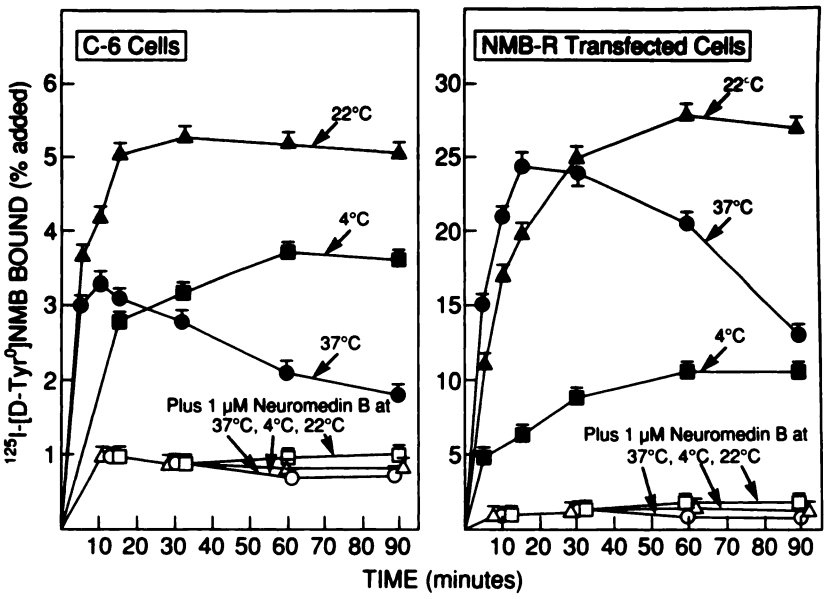

Fig. 1. Time- and temperature-dependent binding of ^{125}I-[D-Tyr^0]NMB to rat glioblastoma C6 tumor cells (*left*) and to BALB 3T3 fibroblasts transfected with the NMB receptor (*right*). C6 cells (15×10^6/ml) and NMB receptor-transfected cells (5×10^5/ml) were incubated with 75 pM ^{125}I-[D-Tyr^0]NMB alone (*closed symbols*) or with 1 μM NMB (*open symbols*) at the indicated temperatures and for the indicated times. Results are expressed as the percentage of added counts bound. In each experiment each value was determined in duplicate, and results are given as the means ± standard errors of at least three separate experiments.

The binding of ^{125}I-[D-Tyr^0]NMB to either native C6 cells or NMB receptor-transfected cells was specific for NMB receptors. Binding of ^{125}I-[D-Tyr^0]NMB was reduced by 97% and 96% by 0.1 μM NMB in transfected and C6 cells, respectively. The structurally related peptides bombesin (0.1 μM) and GRP (0.1 μM) similarly inhibited binding of ^{125}I-[D-Tyr^0]NMB to each cell type but were less potent, causing only 70% inhibition. In contrast, the structurally unrelated peptides CCK-8, gastrin-17-I, vasoactive intestinal peptide, and SP (all at 0.1 μM) had no effect on the binding of ^{125}I-[D-Tyr^0]NMB to either cell type.

To compare further the ability of transfected and native NMB receptors to interact with NMB-related peptides, dose-inhibition curves for various NMB and other bombesin receptor agonists (Fig. 2) and bombesin receptor antagonists (Fig. 3) were performed. NMB was the most potent agonist at inhibiting binding of ^{125}I-[D-Tyr^0]NMB, causing detectable inhibition at 0.1 nM, half-maximal inhibition at approximately 2 nM, and complete inhibition at 1 μM for both cell types (Fig. 2). Analysis of the ability of NMB to inhibit binding of ^{125}I-[D-Tyr^0]NMB using a least-squares curve-fitting program (LIGAND) (21) demonstrated that the data with both cell types were best fit by a single binding site model and that there was no significant difference in affinity of NMB either for NMB receptors transfected into BALB 3T3 fibroblasts or for native receptors on C6 cells (3.08 ± 0.14 nM versus 1.90 ± 1.10 nM, $p > 0.05$). Calculated by the method of Cheng and Prusoff (22), the affinity of GRP for the NMB receptor was similar in both cell types, being at least 150-fold lower than that of NMB (K_i = 520 ± 30 nM versus 440 ± 70 nM, respectively); the affinity of the GRP analogue was approximately 300–500-fold lower than that of NMB in the same cell types and the affinity of the GRP analogue did not differ in the two cell types (Table 1). Four different classes of bombesin receptor antagonists have been described for bombesin-related peptides (23, 24), and representative potent members of each class were examined for their abilities to interact with NMB receptors on both cell types. Each of the antagonists inhibited binding of ^{125}I-[D-Tyr^0]NMB to either native receptor on C6 cells or transfected NMB receptors, albeit with relatively low affinities (Fig. 3, Table 1). For either cell type the affinity of a given bombesin receptor antagonist was similar; [D-Phe^6]bombesin(6–13)propylamide was the most potent and was approximately 4–7-fold more potent than [D-Phe^4,D-Trp^{7,9,10}]SP(4–11), [D-Phe^6]bombesin(6–13)ethyl ester, or [Tyr^4,D-Phe^{12}]bombesin (Fig. 3, Table 1).

The kinetics of binding were further examined by investigating the reversibility of bound ^{125}I-[D-Tyr^0]NMB (Fig. 4). Lowering the temperature to 4° slowed the dissociation of bound ^{125}I-[D-Tyr^0]NMB such that by 60 min 80% remained bound to

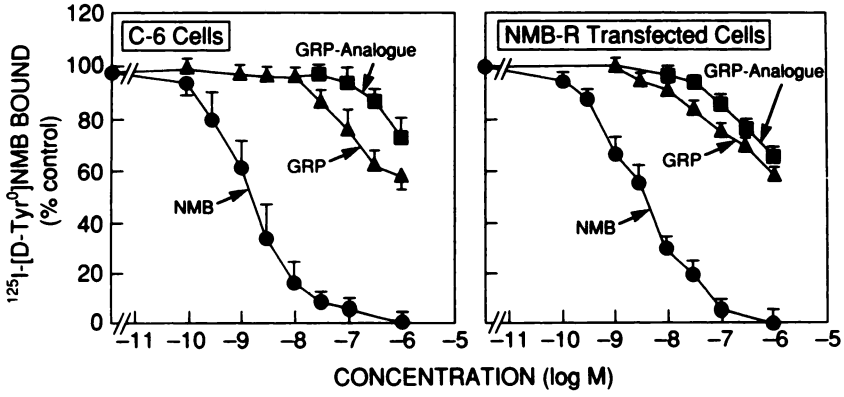

Fig. 2. Comparison of various bombesin-related receptor agonists in inhibiting binding of ^{125}I-[D-Tyr^0]NMB to C6 cells (*left*) or to NMB receptor-transfected cells (*right*). C6 cells or NMB receptor-transfected cells were incubated in binding buffer with 0.1% bacitracin with 75 pM ^{125}I-[D-Tyr^0]NMB alone or with the indicated concentrations of bombesin-related peptide. Data are expressed as the percentage of saturably bound radioactivity in the absence of nonradioactive peptide. For each experiment, each value was determined in duplicate, and results are the means ± standard errors of at least three separate experiments. *GRP-Analogue*, [D-Phe^6,D-Ala^{11},Leu^{14}]bombesin(6–14).

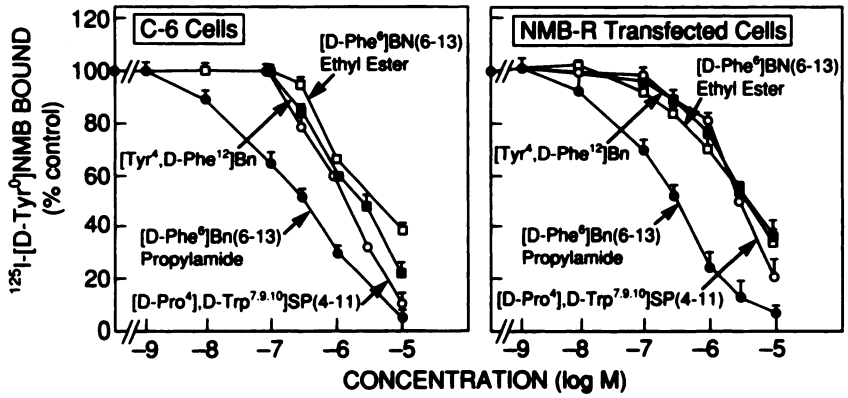

Fig. 3. Ability of various bombesin (*Bn*) receptor antagonists to inhibit binding of ^{125}I-[D-Tyr^0]NMB to C6 cells (*left*) or to NMB receptor-transfected cells (*right*). Experimental conditions were similar to those described in the Fig. 2 legend.

TABLE 1

Comparison of the ability of various bombesin receptor agonists and antagonists to inhibit binding of ^{125}I-[D-Tyr^0]NMB to C6 and NMB receptor-transfected cells

Binding was performed as described in Experimental Procedures, in binding buffer with 0.1% bacitracin. K_i values for binding of the various peptides were calculated by the method of Cheng and Prusoff (22), and each value is the mean ± standard error of at least three separate experiments.

Peptides	K_i	
	C6 cells	NMB receptor-transfected cells
	nM	
Agonists		
NMB	1.9 ± 1.1	3.0 ± 0.1
GRP	520 ± 30	440 ± 70
GRP analogue[a]	1000 ± 150	950 ± 120
Antagonists		
[D-Pro⁴,D-Trp⁷·⁹·¹⁰]SP(4–11)	1410 ± 90	2897 ± 142
[Tyr⁴,D-Phe¹²]Bombesin	3330 ± 240	3046 ± 127
[D-Phe⁶]Bombesin(6–13)propylamide	560 ± 40	440 ± 60
[D-Phe⁶]Bombesin(6–13)ethyl ester	2530 ± 150	3033 ± 220

[a] GRP analogue, [D-Phe⁶,D-Ala¹¹,Leu¹⁴]bombesin(6–14).

C6 cells and 89% remained bound to transfected cells (Fig. 4). With both C6 cells and NMB receptor-transfected cells, at 37° the dissociation of bound ^{125}I-[D-Tyr^0]NMB was rapid, with half-maximal dissociation occurring at 10 min for both cell types. At 30 min, the amount of ^{125}I-[D-Tyr^0]NMB remaining bound was 38% for C6 cells and 36% for transfected cells, with little subsequent change over the final 30 min of incubation (Fig. 4). These observations suggest that a component of the cell-associated radioactivity might be internalized.

To investigate directly the possibility of internalization, acid stripping was performed in order to remove surface-bound ligand at various times at 22°. At 5 min (time of half-maximal binding; Fig. 1), 19% of the bound radioligand on C6 cells and 12% of bound radioligand on transfected cells was internalized (Fig. 5). Maximal internalization was achieved by 60 min, with approximately 70% of bound ligand being internalized by either cell system at 22° (Fig. 5). In both cell systems, NMB receptor internalization rates were temperature dependent. At 4°, >90% of bound ligand after a 60-min incubation was able to be acid stripped (data not shown).

To compare the effects of G protein activation on agonist binding to native and transfected NMB receptors, the effect of increasing concentrations of the nonhydrolyzable guanine analogue Gpp(NH)p on ligand binding to cell membranes from both cell types was determined (Fig. 6). Gpp(NH)p caused a concentration-dependent decrease in binding of ^{125}I-[D-Tyr^0]NMB to either C6 or transfected cell membranes. For C6 cell membranes and for transfected cell membranes, half-maximal inhibition of ^{125}I-[D-Tyr^0]NMB binding occurred with 0.1 μM Gpp(NH)p. Maximal inhibition of ^{125}I-[Tyr^0]NMB binding occurred with 100 μM Gpp(NH)p for membranes derived from either cell type (Fig. 6). To determine the basis for the decrease in binding caused by Gpp(NH)p, the effect of a fixed concentration of Gpp(NH)p on the dose-inhibition curve for NMB inhibition of binding of ^{125}I-[D-Tyr^0]NMB was determined (Fig. 7). NMB inhibited binding of ^{125}I-[D-Tyr^0]NMB to cell membranes derived from C6 cells (K_i = 0.7 ± 0.2 nM) or NMB receptor-transfected cells (K_i = 0.72 ± 0.11 nM) in the absence of Gpp(NH)p in a fashion similar to that observed in intact

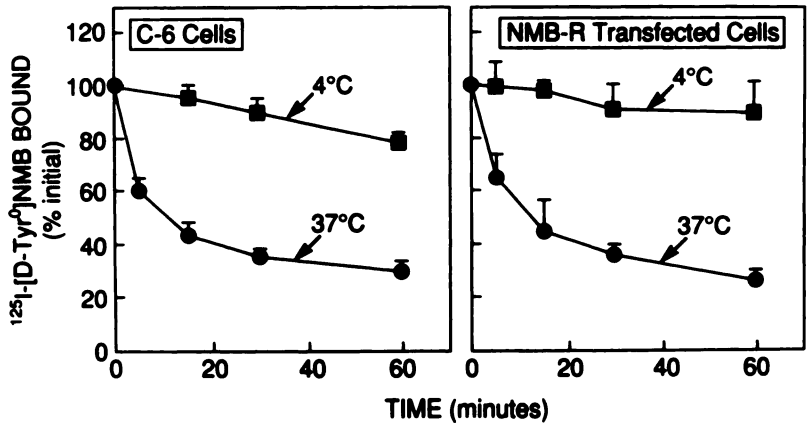

Fig. 4. Dissociation of bound ^{125}I-[D-Tyr^0]NMB from C6 cells (*left*) and from NMB receptor-transfected cells (*right*). C6 cells (15 × 10^6/ml) or NMB receptor-transfected cells (5 × 10^5/ml) were incubated in binding buffer with 0.1% bacitracin with 75 pM ^{125}I-[D-Tyr^0]NMB at 22° for 60 min. At that time, aliquots were diluted 50-fold with standard incubation buffer at 4° or at 37° and were incubated at either temperature for the indicated times. Results are expressed as the percentage of saturable binding at the beginning of the second incubation. In each experiment each value was determined in duplicate, and each point is the mean ± standard error of at least three separate experiments.

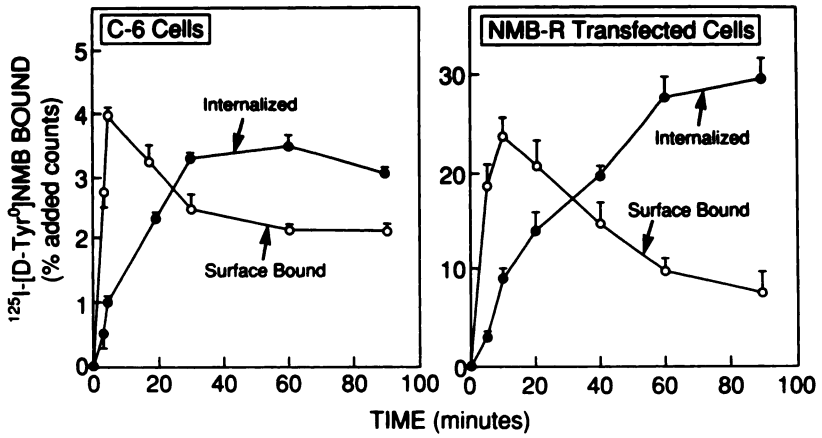

Fig. 5. Time and temperature dependence of surface binding and of internalization of ^{125}I-[D-Tyr^0]NMB in C6 cells (*left*) and in NMB receptor-transfected cells (*right*). Surface-bound ligand was that proportion of the saturably bound counts removed by exposure to 0.2 M acetic acid with 0.5 M NaCl (pH 2.5), as described in Experimental Procedures, whereas the internalized ligand was the proportion not removed. Results for either surface-bound or internalized ligand are expressed as the percentage of ligand added. Each experiment was performed in triplicate, with each point representing the mean ± standard error of at least four separate experiments.

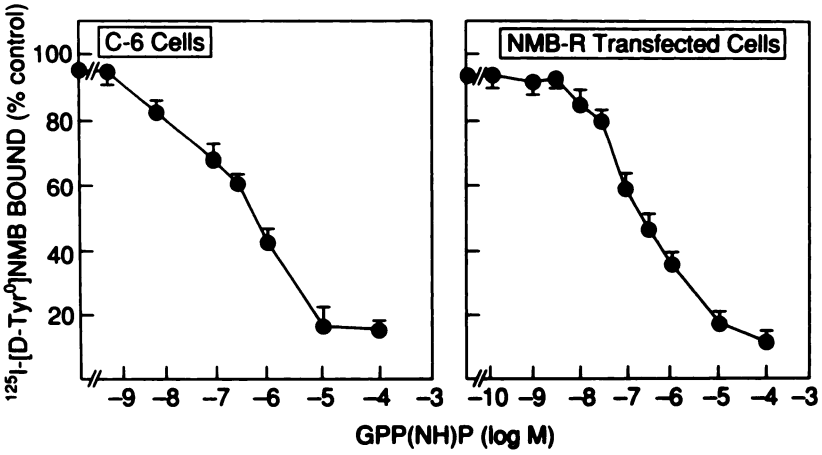

Fig. 6. Effect of the stable guanine nucleotide analogue Gpp(NH)p on the binding of ^{125}I-[D-Tyr^0]NMB to C6 cells (*left*) or NMB receptor-transfected cells (*right*). C6 or NMB receptor-transfected cell membranes were incubated at 22° in binding buffer with 0.1% bacitracin with 50 pM ^{125}I-[D-Tyr^0]NMB for 60 min, either alone or with the indicated concentrations of Gpp(NH)p. Results are expressed as the percentage of saturable binding in the absence of unlabeled peptide. Values represent the mean ± standard error of at least three separate experiments, with each value determined in duplicate in each experiment.

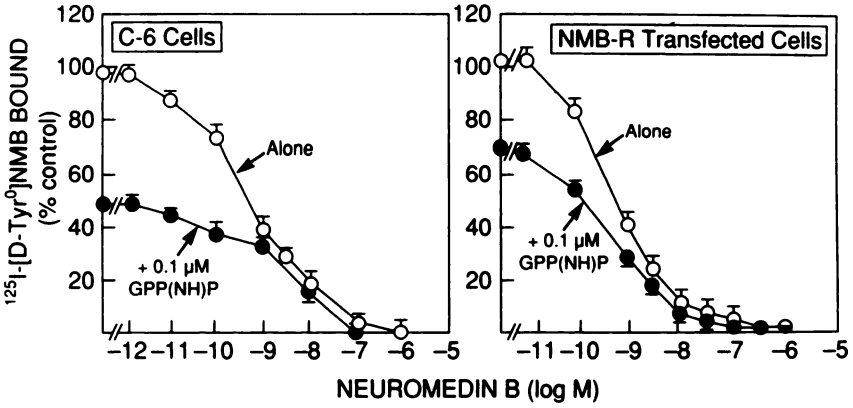

Fig. 7. Effect of the stable guanine nucleotide analogue Gpp(NH)p on the NMB dose-inhibition curve for binding of ^{125}I-[D-Tyr^0]NMB to C6 cells (*left*) or NMB receptor-transfected cells (*right*). Experimental conditions were similar to those in Fig. 6. Results are expressed as the percentage of saturable binding in the absence of unlabeled peptide with or without Gpp(NH)p present. Each data point represents a minimum of three separate experiments, with each value measured in duplicate in each experiment.

cells (compare Fig. 2 with *upper curves* of Fig. 7). The ability of 0.1 μM Gpp(NH)p to alter the dose-inhibition curve for NMB was analyzed using the nonlinear curve-fitting program LIGAND (21), and in all instances the data were best fit using a single-site model. The decrease in ^{125}I-[D-Tyr^0]NMB binding was due to a decrease in the affinity of the NMB receptor for ^{125}I-[D-Tyr^0]NMB in either type of cell membrane. For native NMB receptors expressed on C6 cells, 0.1 μM Gpp(NH)p caused a 6-fold decrease in K_i for NMB inhibition of ^{125}I-[D-Tyr^0]NMB binding (0.7 ± 0.2 nM versus 3.7 ± 0.1 nM, $p < 0.05$). In contradistinction, the receptor number (B_{max}) resident on C6 cell membranes (60 ± 11 fmol/mg of protein) was not significantly different in the presence of 0.1 μM Gpp(NH)p (85 ± 24 fmol/mg of protein, $p > 0.05$). Likewise, transfected cells demonstrated a significantly decreased K_i in response to Gpp(NH)p (0.72 ± 0.11 nM versus 1.51 ± 0.01 nM, $p < 0.05$) without significant alteration in receptor number (429 ± 16 fmol/mg of protein versus 405 ± 6 fmol/mg of protein, $p > 0.05$).

To determine whether agonist occupation of transfected NMB receptors resulted in similar coupling to intracellular processes as seen in native NMB receptors on C6 cells, the ability of NMB and the GRP analogue to activate phospholipase C was determined in both cell systems. This was done by assessing alterations in phosphoinositide breakdown and changes in cellular calcium in response to agonist stimulation. NMB (1 μM) stimulated increases in [^{3}H]IP$_1$, [^{3}H]IP$_2$, and [^{3}H]IP$_3$ accumulation in a time-dependent manner, which was similar in both cell types (Fig. 8). In both C6 cells and trans-

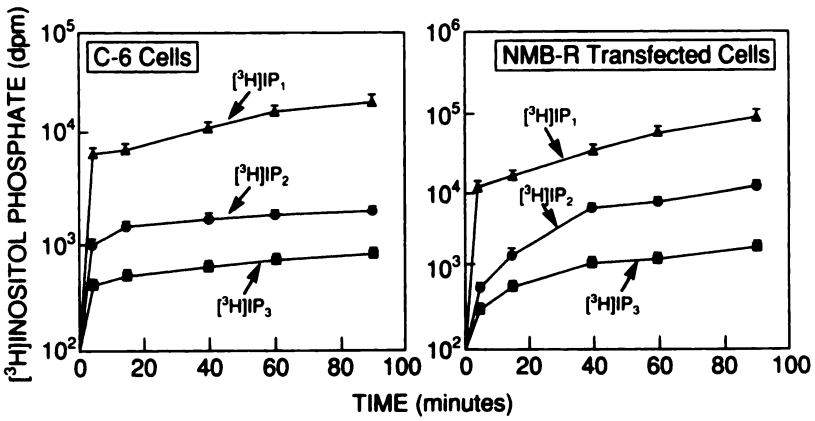

Fig. 8. Time course of the effect of NMB on [^{3}H]IP$_1$, [^{3}H]IP$_2$, and [^{3}H]IP$_3$ generation in C6 cells (*left*) and in NMB receptor-transfected cells (*right*). Confluent cells were incubated with 100 μCi/ml *myo*-[2-^{3}H]inositol for 48 hr, after which they were exposed to 1 μM NMB for the indicated times. Separation of [^{3}H]inositol phosphates was performed as detailed in Experimental Procedures. Each data point represents the mean ± standard error of a minimum of four separate experiments, with each value measured in duplicate in each experiment.

fected cells, the relative increases in [^{3}H]IP$_1$, [^{3}H]IP$_2$, and [^{3}H]IP$_3$ at different times were similar, as was the configuration of the time course (Fig. 8). The dose-response curves for the ability of NMB and of the GRP analogue to increase total [^{3}H]inositol phosphates were nearly identical in both C6 cells and NMB receptor-transfected cells (Fig. 9). With both cell systems detectable stimulation occurred at 0.1 nM, half-maximal stimulation at 1–2 nM, and maximal stimulation at 10 nM. For both the NMB receptor-transfected cells and the C6 cells, the GRP analogue was >200-fold less potent than NMB; the concentrations causing half-maximal stimulation of [^{3}H]inositol phosphates in the two cell types were not significantly different (C6, 2129 ± 250 nM; transfected, 1855 ± 140 nM, $p > 0.05$).

Previous studies (15, 25) have shown with other cell systems that similar dose-response curves for agents that mobilize cellular calcium can be obtained by measuring either ^{45}Ca efflux or changes in $[Ca^{2+}]_i$. Because measuring ^{45}Ca efflux allows greater sample numbers to be analyzed, this method was experimentally easier for us to perform and also allowed ^{45}Ca efflux measurements to be done under conditions identical to those used for measuring changes in phosphoinositides. To validate that similar dose-response curves were obtained using ^{45}Ca efflux and changes in $[Ca^{2+}]_i$, we compared the NMB and GRP analogue dose-response curves in C6 cells obtained using alterations in fura-2 fluorescence and those obtained using ^{45}Ca efflux. NMB in C6 cells caused a detectable increase in $[Ca^{2+}]_i$ at 0.01 nM, a half-maximal increase at 0.10 ± 0.25 nM, and a maximal increase at 10 ± 2 nM. The GRP analogue was 140-fold less potent, causing a half-maximal increase at 14 ± 2 nM. The dose-response curves with ^{45}Ca gave results almost identical to those observed using fura-2 fluourescence, showing a half-maximal increase with NMB at 0.11 ± 0.02 nM and with the GRP analogue at 12 ± 2 nM. BALB 3T3 fibroblasts transfected with the NMB receptor exhibited half-maximal increases in ^{45}Ca efflux with NMB at 0.16 ± 0.08 nM and with the GRP analogue at 14 ± 4 nM (Fig. 10). These values were not significantly different from those generated using C6 cells.

A previous study showed that neither NMB nor GRP increased cAMP in C6 cells (9). To determine whether transfected NMB receptors differed from native NMB receptors in C6 cells in being able to activate adenylate cyclase, the ability of NMB, bombesin, or secretin (which stimulates adenylate cyclase in a number of cell systems) (26, 27) to increase cAMP was determined either alone or in the presence of the phosphodiesterase inhibitor IBMX, cholera toxin, or forskolin (Table 2). IBMX, cholera toxin, and forskolin significantly increased cAMP when present alone, whereas NMB, bombesin, and secretin alone had no effect. Furthermore, the addition of NMB, bombesin, or secretin to IBMX, cholera toxin, or forskolin did not increase cAMP further or inhibit the increase in cAMP produced by these agents (Table 2).

Discussion

Bombesin-like peptides are responsible for a wide variety of actions (28–30), including acting as a growth factor in small cell lung cancer cells and a number of other cells (6, 31, 32), regulating temperature control and certain aspects of behavior (33, 34), stimulating exocrine secretion (7, 29), and stimulating the release of numerous other gastrointestinal peptides (35, 36). At present, two distinct receptors that mediate the actions of bombesin-like peptides have been isolated and cloned, the GRP receptor (1, 2) and the NMB receptor (3). These receptors

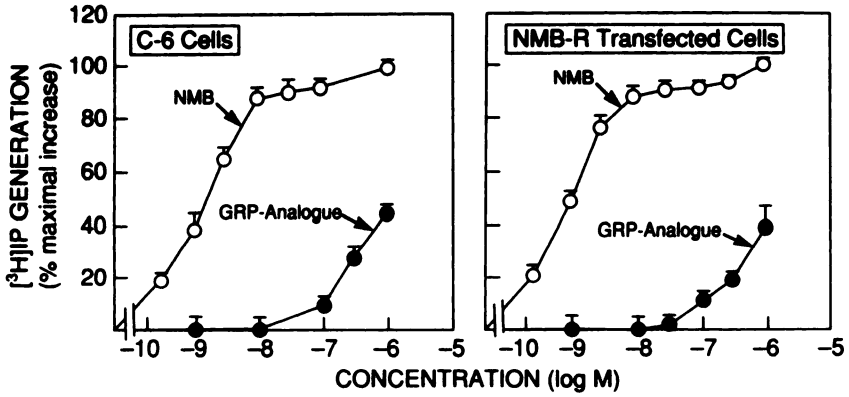

Fig. 9. Ability of NMB and a GRP analogue to stimulate [^{3}H]inositol phosphate generation in C6 cells (*left*) and in NMB receptor-transfected cells (*right*). Experimental conditions were as described in the legend to Fig. 8. Data are expressed as the percentage of maximal increase obtained using 1 μM NMB. In C6 cells NMB (1 μM) increased [^{3}H]inositol phosphates ([^{3}H]IP) from a basal level of 8,250 ± 740 dpm to 68,540 ± 1,390 dpm, and in NMB receptor-transfected cells NMB (1 μM) increased [^{3}H]inositol phosphates from 9,520 ± 540 dpm to 122,800 ± 2,080 dpm. Each data point represents the mean ± standard error of a minimum of four separate experiments, with each value determined in duplicate in each experiment.

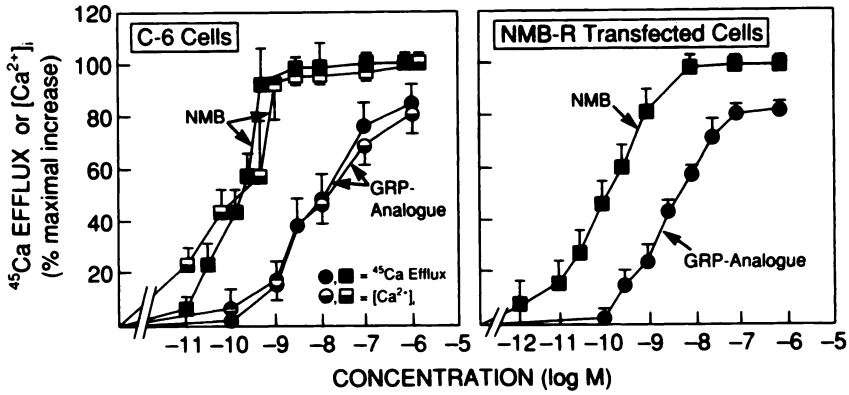

Fig. 10. Ability of NMB and of the GRP analogue to alter cellular calcium in C6 cells (*left*) and in NMB receptor-transfected cells (*right*). Changes in cellular calcium were assessed by determining changes in cytosolic calcium with fura-2 in C6 cells or by measuring ^{45}Ca efflux from C6 cells or transfected cells. Data are expressed as the percentage of maximal increase obtained using 1 μM NMB. In C6 cells 1 μM NMB increased basal efflux from 5,925 ± 590 dpm to 11,180 ± 400 dpm, whereas in transfected cells 1 μM NMB increased basal efflux from 4,760 ± 530 dpm to 175,620 ± 1,250 dpm. Each data point represents the mean ± standard error of at least six separate experiments and each value was determined in duplicate in each experiment. In these experiments basal $[Ca^{2+}]_i$ was 100 ± 10 nM and 1 μM NMB-stimulated $[Ca^{2+}]_i$ was 360 ± 40 nM.

TABLE 2

Ability of NMB, bombesin, or secretin to alter cellular cAMP in the presence or absence of forskolin, IBMX, or cholera toxin

NMB receptor-transfected cells (3 × 10^6 cells/ml) were incubated with no additions, with 100 μM IBMX, or with 25 μM forskolin for 10 min at 37° or were preincubated for 60 min at 37° with 100 ng/ml cholera toxin and then incubated for 10 min with the indicated peptides. Basal levels of cAMP were 83.7 ± 2.34 pmol/10^6 cells. Each value represents four separate experiments performed in triplicate, with mean ± standard error shown.

Additions	cAMP			
	Alone	IBMX	Cholera toxin	Forskolin
	pmol/10^6 cells			
None	1.00	2.06 ± 0.40*	1.46 ± 0.10*	4.04 ± 0.99*
NMB	1.14 ± 0.21	2.73 ± 0.63*	1.62 ± 0.13*	3.83 ± 1.05*
Bombesin	1.08 ± 0.24	2.16 ± 0.38*	1.55 ± 0.26*	4.57 ± 0.61*
Secretin	0.88 ± 0.13	2.02 ± 0.17*	1.77 ± 0.20*	4.17 ± 0.82*

*Significantly different from the value with no additions, $p < 0.05$.

each contain seven putative membrane-spanning regions, as observed in other G protein-linked receptors (1–3). Previously, extensive studies have been done assessing the ability of agonists to interact with GRP receptors and to alter cell function (6–8), whereas relatively little is known about the NMB receptor because it was only recently described and no readily available tissues with large numbers of functional receptors have been described (37). Recent studies show that activation of NMB receptors stimulates phospholipase C, with increased breakdown of phosphoinositides and increases in cytosolic calcium (9); otherwise, little is known regarding their pharmacological properties or their ability to alter cellular function.

The study of receptors mediating the action of bombesin-related peptides, as has been true for other G protein-linked receptors, has depended primarily on pharmacological techniques. Bombesin receptor agonists and antagonists of increasing specificity have been identified and then used to classify and characterize receptor subtypes and their coupled biological activities (23, 24, 37–40). Major limitations to this pharmacologically based analysis are that receptors are often present in small numbers and that multiple receptor subtypes often coexist on the same cell type, as is the case with bombesin receptors on gastrointestinal smooth muscle cells (38). Until recently, therefore, receptor analysis has been limited by the specificity of receptor agonists and antagonists available to the investigator. The recent cloning of these bombesin receptor subtypes, however, raises the possibility that by using molecular biological methods detailed analyses can be performed on transfected cells possessing only one receptor subtype.

Although receptor transfection has provided unparalleled opportunity for their study, an assumption implicit in these particular investigations has hitherto not been addressed, namely, whether transfected wild-type receptors behave similarly to nontransfected receptors expressed by native tissues. The importance of this point is emphasized by recent observations that certain receptors formerly believed to solely activate phospholipase C apparently also affect adenylate cyclase activity in certain transfected cell types. Specifically, tachykinin, α_1-adrenergic, and the muscarinic cholinergic M1, M3, and M5 receptors all are thought to activate phopholipase C in native tissues but in certain transfected cells can also activate or inhibit adenylate cyclase (41–44). These investigators suggest that this degeneracy in G protein coupling reflects a fundamental property of the receptor that is only detectable in transfected cells because of their greatly increased receptor expression. However, work with transfected muscarinic cholinergic M2 and M4 receptors as well as with dopaminergic D_2 receptors clearly demonstrates that this multiplicity in G protein coupling is a function of the cell type used in transfection studies (10–12). It is important, therefore, that studies using transfected receptors establish that these receptors behave similarly to those expressed natively, if they are to be used as an accurate model for the native receptor. In this study we provide insight into the pharmacology of the NMB receptor, as well as determining whether this receptor, when transfected into BALB 3T3 fibroblasts, is functionally similar to the native NMB receptor expressed on C6 rat glioblastoma cells. This comparison is aided by the fact that C6 cells express only NMB receptors (3), although in relatively low amounts, and do not express GRP receptors.[1]

In the present study a number of results demonstrate that pharmacologically the transfected and natively expressed NMB receptors behave similarly, based on the parameters of receptor function measured. Binding of radiolabeled NMB in both instances demonstrates similar kinetics for both association and dissociation, with similar temperature dependence. Stoichiometric relationships with the different bombesin-related agonists and antagonists are similar in both cell systems, with binding affinities for the NMB receptor in either cell type being greatest for NMB (K_i, 1–3 nM), followed by GRP and then the GRP analogue. NMB receptors native to C6 cells or in NMB receptor-transfected cells also manifest similar affinities for a number of different classes of antagonists. With respect to kinetics, stoichiometry, and temperature dependence, there-

[1] J. Battey, unpublished observations.

fore, the NMB receptors are indistinguishable in the native and transfected cell types.

Previous studies have demonstrated that cells possessing GRP receptors, such as Swiss 3T3 fibroblasts, pancreatic acinar cells, rat pituitary cells, and the insulinoma cell line HIT-T15, rapidly degrade bombesin-related peptides (45–47). A number of results in the present study demonstrate that in cells natively possessing NMB receptors and in NMB receptor-transfected cells, ligand degradation is also occurring. With both cell systems, the degree of binding of the NMB receptor-specific ligand ^{125}I-[D-Tyr^0]NMB decreases rapidly at 37° with time when no protease inhibitors are present. The fact that this decrease in binding is due to degradation of the radioligand is confirmed by HPLC analysis, which demonstrates that >80% of the radioligand is degraded after a 60-min incubation at 37°. The relative abilities of different protease inhibitors to inhibit this degradation are identical in the C6 cells possessing native NMB receptors and in the NMB receptor-transfected cells. Specifically, this degradation is not significantly impeded by the aminopeptidase inhibitors amastatin and bestatin in either cell type. However, the metalloprotease inhibitor phosphoramidon and the serine protease inhibitor chymostatin slightly inhibit degradation in both cell systems. In both cell systems bacitracin, a broad-spectrum antibiotic peptidase inhibitor of unknown mechanism of action, and leupeptin, an acid protease inhibitor, are the most effective at inhibiting ligand degradation. HPLC studies of cell supernatant samples provide conclusive evidence that these peptidase inhibitors actually retard ^{125}I-[D-Tyr^0]NMB degradation. These results demonstrate that there is no unique or tissue-specific mechanism for degrading this ligand, that cells possessing either the native NMB receptor or the transfected NMB receptor behave similarly in degrading the radioligand, and that similar proteases are involved in both cell systems; however, they do not establish that it is a receptor-mediated event.

Numerous receptor systems internalize ligand, including the GRP receptor, in various tissues (8, 18, 46, 48–50). Similarly, a number of results in the present study demonstrate that internalization of ligand by NMB receptors, expressed in both native and transfected cells, is occurring. Reversibility of bound radioligand demonstrates similar kinetics in both cell systems and is only 60–70% complete at 60 min, thus suggesting internalization of the radioligand. Acid-stripping experiments to remove surface-bound ligand (18, 48, 50–52) demonstrate that internalization rates are time and temperature dependent. Although kinetically similar patterns of receptor internalization are observed for NMB receptor-transfected cells and C6 cells, the absolute amount of radioligand internalized in the transfected cells is 7-fold higher. This difference is likely due to the fact that NMB receptor-transfected cells possess significantly more receptors than are present on C6 cells.

Previous studies suggest that GRP receptors are linked to G proteins (4, 5, 8). Our results suggest that both the native and transfected NMB receptors also are coupled to G proteins. In the present study the nonhydrolyzable guanine nucleotide Gpp(NH)p inhibited NMB binding to receptors present on both cell types. The concentration of Gpp(NH)p causing a 50% reduction in ^{125}I-[D-Tyr^0]NMB binding was identical for native and transfected cells. Furthermore, in both cell types the decrease in binding was secondary to decreased receptor affinity and not to alterations in receptor number. This Gpp(NH)p-induced decrease in receptor affinity, without change in receptor number, is similar to that which has been demonstrated for GRP receptors on 3T3 cells (4) but is different from that observed for GRP receptors on rat pituitary cells or HIT-T15 cells, where changes in both receptor affinity and number have been observed (4, 5, 45). These data demonstrate that the transfected NMB-receptor maintains its coupling to G_p in a fashion similar to that seen in the native tissue.

GRP receptors have been shown to activate phospholipase C (6, 7). A recent study reports that NMB similarly activates phospholipase C (9), and results in our study support this conclusion. To investigate further the coupling of the transfected NMB receptor to intracellular mediators, we assessed the ability of agonists to stimulate inositol phosphate generation and calcium mobilization in each cell type. In both cell systems, NMB generated half-maximal effects in the nanomolar range, whereas the GRP analogue was approximately 100-fold less potent. In both cell types the NMB-stimulated time courses for [^{3}H]IP$_1$, [^{3}H]IP$_2$, and [^{3}H]IP$_3$ accumulation were similar. Although the absolute increase in these inositol phosphates was greater in NMB receptor-transfected cells, perhaps secondary to the greater number of receptors, the relative increases in each of the inositol phosphates were similar in the two cell types. Our data also demonstrate that the potencies of NMB and the GRP analogue were similar for mobilizing cellular calcium in cells possessing either the native or the transfected NMB receptor. In both cell systems, half-maximal values for mobilization of cellular calcium in response to NMB were in the nanomolar range, and NMB was 100-fold more potent than the GRP analogue. In both cell systems, the concentration of NMB causing a half-maximal response in mobilization of cellular calcium was approximately 10-fold lower than that observed for inositol phosphate generation. This suggests that, in both cell systems, nanomolar concentrations of NMB result in half-maximal increases in inositol phosphates but maximal levels of cellular calcium mobilization. This same degree of biological signal amplification was observed in both cell types, demonstrating that receptor-biological activity coupling efficiency is retained by the transfected NMB receptor.

A previous study (53) demonstrated that in 3T3 cells activation of GRP receptors can cause small increases in cAMP. However, a recent study (9) demonstrated that activation of NMB receptors in C6 cells does not increase cAMP. To investigate the specificity of the coupling mechanism in NMB receptor-transfected cells, NMB, bombesin, and secretin (an agent known to increase cAMP in numerous tissues) (19, 26), were specifically evaluated for their ability to increase cAMP. These pepides were further evaluated both with and without forskolin, the phosphodiesterase inhibitor IBMX, and cholera toxin. In neither BALB 3T3 fibroblasts transfected with the NMB receptor nor C-6 cells natively expressing this receptor was cAMP increased by NMB, GRP, bombesin, or secretin. Although incubation with IBMX (a phosphodiesterase inhibitor), cholera toxin (a G_s activator), or forskolin (an adenylate cyclase activator) increased basal levels of cAMP, concomitant incubation with agonist failed to increase cAMP production additionally. Furthermore, neither NMB nor bombesin inhibited the increase in cAMP caused by forskolin, IBMX, or cholera toxin, suggesting that the transfected NMB receptor is not coupled to G_i. Unlike other receptors known to activate phospholipase C, such as the α_1-adrenergic receptor, M1, M3, and M5 mus-

carinic cholinergic receptors, and tachykinin receptors (54–58), transfected NMB receptors did not activate or inhibit adenylate cyclase. With previous demonstrations of phospholipase C-activating receptors influencing adenylate cyclase activity, it was unclear whether the phenomenon was artifactual, was representative of hitherto unappreciated *in vivo* function, or reflected the cell type used for transfection (10–12). In this study, we demonstrate that transfected receptors do not influence adenylate cyclase activity and only stimulate phospholipase C, as has been shown in cells possessing native NMB receptors (9). These findings suggest that transfected NMB receptors couple faithfully and specifically to a phospholipase C-activating G protein. These observations support the contention that these receptors in transfected BALB 3T3 fibroblasts behave similarly to those expressed natively in C6 cells.

In conclusion, we demonstrate that the NMB receptor, when transfected into BALB 3T3 fibroblasts, retains all of its characteristic pharmacological and biological activities, as measured in this study, that are present natively in C6 rat glioblastoma cells. Transfected NMB receptors bind agonist and antagonist, internalize and degrade radiolabeled agonist, and couple to their appropriate signal transduction pathways in a fashion similar to that of natively expressed receptors. This study provides direct evidence that transfected NMB receptors (i.e., wild-type) behave similarly to those receptors expressed by native tissues. NMB receptors transfected into BALB 3T3 fibroblasts, therefore, represent a pharmacologically and physiologically sound model for additional study of this receptor.

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