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Gastrin-releasing Peptide Receptor-induced Internalization, Down-Regulation, Desensitization, and Growth: Possible Role for Cyclic AMP

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SUMMARY

Stimulation of the gastrin-releasing peptide receptor (GRP-R) in Swiss 3T3 cells resembles that of a number of other recently described G protein-coupled receptors, insofar as both the phospholipase C and adenylyl cyclase signal transduction pathways are activated. GRP-R activation induces numerous alterations in both the cell and the receptor, but because two signal transduction pathways are activated it is difficult to determine the specific contributions of either pathway. We have found that BALB/3T3 fibroblasts transfected with the coding sequence for the GRP-R are pharmacologically indistinguishable from native receptor-expressing cells and activate phospholipase C in a manner similar to that of the native receptor but fail to increase cAMP in response to bombesin; thus, they may be useful cells to explore the role of activation of each pathway in altering cell and receptor function. Swiss 3T3 cells and GRP-R-transfected BALB/3T3 cells expressed identically glycosylated receptors that bound various agonists and antagonists similarly. G protein activation, as determined by evaluation of agonist-induced activation of phospholipase C and by analysis of the effect of guanosine-5′-(β,γ-imido)triphosphate on GRP-R binding affinity, was indistinguishable. Agonist stimulation of GRP-R caused similar receptor changes (internalization and down-regulation) and homologous desensitization in both cell types. Bombesin stimulation of Swiss 3T3 cells that had been preincubated with forskolin increased cAMP levels 9-fold, but no bombesin-specific increase in cAMP levels was detected in transfected cells, even though forskolin and cholera toxin increased cAMP levels in these cells. Quiescent Swiss 3T3 cells treated with bombesin rapidly increased c-*fos* mRNA levels and [^{3}H]thymidine incorporation, whereas both effects were potentiated by forskolin. The specific protein kinase A inhibitor H-89 blocked increases in c-*fos* levels and [^{3}H]thymidine incorporation induced by low concentrations of bombesin. GRP-R-transfected BALB/3T3 cells increased c-*fos* mRNA levels and [^{3}H]thymidine incorporation with the addition of serum but not bombesin. These data suggest that bombesin-stimulated increases in cellular levels of cAMP appear not to be an important mediator of GRP-R internalization, down-regulation, or desensitization but do play an important role in bombesin-induced mitogenesis.

GRP is the mammalian homologue of the amphibian tetradecapeptide bombesin and has numerous biological activities, including thermoregulation (1), chemotaxis (2), stimulation of the release of numerous gastrointestinal peptides (3), smooth muscle contraction (4), satiety (5), and stimulation of pancreatic enzyme secretion (6). GRP and other bombesin-related peptides also have been found to be involved as growth factors in various normal (7) and tumorigenic (8) cell lines, as well as being involved as autocrine growth factors in small cell lung cancer cells (8). The GRP-R has been cloned and, by hydropathy analysis, has been found to be a member of the seven-transmembrane domain G protein-coupled receptor superfamily (9).

GRP-related peptides interact with high affinity with the GRP-R on various tissues such as Swiss 3T3 fibroblasts (10). Similarly to a few other G protein-linked receptors (11–13), GRP-R occupation by agonist in Swiss 3T3 cells results in activation of adenylyl cyclase as well as of phospholipase C (14). In various cells GRP-R activation results in receptor internalization (15), homologous receptor down-regulation (16, 17), desensitization (16, 17), increased proto-oncogene expression including that of c-*fos* (18), and cell proliferation (7, 8, 18). Studies using GRP in Swiss 3T3 cells (and by analogy to other receptors in other cell systems that activate these intracellular cascades) (11, 19–21) raise the possibility that activation of

ABBREVIATIONS: GRP, gastrin-releasing peptide; GRP-R, gastrin-releasing peptide receptor(s); [Ca^{2+}]$_i$, intracellular calcium concentration; DSS, disuccinimidyl suberate; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; PGNase F, peptide-N^+-(*N*-acetyl-β-glucosaminyl)asparagine amidase; NMB, neuromedin B; PACAP, pituitary adenylyl cyclase-activating protein; βARK, β-adrenergic receptor kinase; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PKC, protein kinase C.

both intracellular signal transduction mechanisms could be involved in mediating the effects of GRP on receptor regulation and cell function. Evidence suggests that activation of either intracellular pathway may be involved in receptor down-regulation, desensitization, and perhaps internalization. Indeed, after activation the extensively studied β_2-adrenergic receptor undergoes desensitization (22), sequestration likely due to internalization via coated pits (23), and, later, receptor down-regulation (24). Multiple intracellular pathways mediate these processes, including activation of protein kinase A (19, 21), βARKs (19, 20), and phospholipase C (19). With other receptors that activate adenylyl cyclase, such as the dopamine D_1 receptor, adenylyl cyclase is involved in mediating down-regulation but not desensitization (25). In contrast, other receptors, such as the M_1, M_3, and M_5 muscarinic cholinergic receptors (26), the substance P receptor (27), and the cholecystokinin receptor (28), increase only phospholipase C but also undergo down-regulation, desensitization, and internalization. Similarly, activation of either the adenylyl cyclase or phospholipase C pathway may be involved in mediating the growth effects of GRP. In Swiss 3T3 cells, numerous studies demonstrate that activation of the adenylyl cyclase or phospholipase C cascades can result in proto-oncogene activation and induce cell growth and that concomitant activation of these two pathways has a synergistic effect (18). With GRP-R, however, and similarly to other receptors that activate multiple signal transduction pathways, it has been difficult to determine the specific role of either adenylyl cyclase or phospholipase C activation in regulating receptor function or altering cell behavior.

Recently we have found that BALB/3T3 fibroblasts transfected with the coding sequence for the receptor for the GRP-related peptide NMB, which has >50% homology with the GRP-R, functioned in a fashion identical to that of the natively expressed receptor (15). Similarly, when GRP-R was transfected into the same cell line, agonist exposure resulted in activation of phospholipase C; however, it failed to activate adenylyl cyclase and did not increase cAMP. The availability of GRP-R-transfected BALB/3T3 cells, therefore, raises the possibility of exploring the participation of activation of adenylyl cyclase, either alone or with activation of phospholipase C, in mediating the various receptor and cellular changes induced by GRP-R stimulation. In this study we demonstrate that GRP-R on BALB/3T3-transfected cells, compared with the native receptors on Swiss 3T3 cells, were indistinguishable in terms of stoichiometry and binding kinetics, general coupling with G proteins, activation of phospholipase C, and their ability to increase intracellular inositol phosphate levels and $[Ca^{2+}]_i$. Consequent to receptor activation, both transfected cells and Swiss 3T3 cells internalize and down-regulate GRP-R similarly and exhibit similar patterns of homologous desensitization. However, bombesin failed to activate adenylyl cylase in GRP-R-transfected cells, failed to increase c-*fos*, and failed to induce cell proliferation. Furthermore, inhibition of the ability of bombesin to activate protein kinase A in Swiss 3T3 cells resulted in a decreased ability to activate c-*fos* and induce cell proliferation. These findings suggest that activation of adenylyl cyclase by bombesin, leading to increased cAMP levels, appears not to be an important mediator of GRP-R internalization, homologous down-regulation, and desensitization but may play an important role in bombesin-induced activation of proto-oncogenes and mitogenesis.

Experimental Procedures

Materials. BALB/3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD), whereas Swiss 3T3 cells were a gift from Dr. John Taylor (Biomeasure Inc., Hopkinton, MA). DMEM, fetal calf serum, and aminoglycoside G-418 were from GIBCO (Waltham, MA). BSA (fraction V) and HEPES were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); soybean trypsin inhibitor, EGTA, trypsin, and bacitracin were obtained from Sigma Chemical Co. (St. Louis, MO); glutamine was from the Media Section, National Institutes of Health (Bethesda, MD); bombesin, GRP, and NMB were obtained from Peninsula Laboratories (Belmont, CA); Gpp(NH)p tetralithium salt was from Fluka Chemical Co. (Ronkonkoma, NY); $Na^{125}I$ was from Amersham Co. (Arlington Heights, IL); *myo*-[2-^{3}H]inositol (16–20 Ci/mmol), [*methyl*-^{3}H]thymidine, and cAMP radioimmunoassay reagents were from New England Nuclear (Boston, MA); Dowex AG1-X8 anion exchange resin (100–200 mesh, formate form) was from Bio-Rad (Richmond, CA); Hydro-Fluor scintillation fluid, methanol, and hydrochloric acid were from the J.T. Baker Chemical Co. (Phillipsburg, NJ); fura-2/acetoxymethyl ester was from Molecular Probes (Eugene, OR); PGNase F was from Genzyme (Cambridge, MA); cholera toxin and forskolin were from Calbiochem (San Diego, CA); and DSS was from Pierce (Rockford, IL). Standard buffer consisted of 98 mM NaCl, 6 mM KCl, 25 mM HEPES, 5 mM pyruvate, 5 mM fumarate, 5 mM glutamate, and 0.1% soybean trypsin inhibitor.

Transfection and maintenance of cell lines. BALB/3T3 cells devoid of GRP-R were selected by clonal expansion after assaying for GRP-R by RNase protection and binding studies. These BALB/3T3 cells were stably transfected using a full length bombesin-preferring GRP-R clone generated from Swiss 3T3 cells (9). The receptor was subcloned into a modified version of the pCD2 plasmid and transfected using calcium phosphate precipitation. Stable transfectants were isolated in the presence of 800 μg/ml aminoglycoside G-418. Transfected cells and Swiss 3T3 cells were maintained identically by culturing in DMEM containing 10% fetal calf serum (plus 270 μg/ml G-418 for stable transfectants), at 37° in a 5% CO_2 atmosphere. Cells were passaged every 3–4 days at confluence, using 0.1% trypsin in 1 mM EDTA.

Preparation of peptides and ^{125}I-labeled ligands. [D-Tyr^6]Bombesin(6–13) methyl ester, [D-Phe^6]bombesin(6–13) propylamide, and [ψ13–14,Leu^{14}]bombesin were synthesized using solid-phase methods, as described previously (29). ^{125}I-[Tyr^4]Bombesin (2200 Ci/mmol) and ^{125}I-GRP were prepared using Iodo-Gen, by adding 0.4 μg of Iodo-Gen, as described previously (29,30). ^{125}I-[Tyr^4]Bombesin was stored with 1% (w/v) BSA at −20°, was stable for at least 6 weeks, and was used for all binding studies, whereas ^{125}I-GRP was used for cross-linking studies and was stored without added BSA.

Binding of ^{125}I-[Tyr^4]bombesin to Swiss 3T3 cells and GRP-R-transfected BALB/3T3 cells. Confluent cells were mechanically disaggregated, washed in binding buffer (standard buffer additionally containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.2 mM KH_2PO_4, 2 mM glutamine, 11 mM glucose, 0.1% bacitracin, and 0.2%, w/v, BSA, pH 7.4), and suspended at a concentration of 3×10^6 cells/ml. Incubations contained 75 pM ^{125}I-[Tyr^4]bombesin and proceeded for 60 min at 22°. Nonsaturable binding of ^{125}I-[Tyr^4]bombesin was the amount of radioactivity associated with GRP-R-transfected or Swiss 3T3 cells when the incubation mixture contained 1 μM bombesin. Nonsaturable binding was <15% of total binding in all experiments, with all values reported in this paper being saturable binding (i.e., total minus nonsaturable binding).

Cross-linking of GRP-R. Cells from two 175-cm^2 flasks were washed twice with standard buffer (4°) and resuspended in 10 ml of homogenization buffer. Cells were homogenized as described above and resuspended at a concentration of 0.25 or 0.5 mg of protein/ml of binding buffer for GRP-R-transfected BALB/3T3 and Swiss 3T3 cells, respectively. Aliquots (500 μl) were incubated with 0.5 nM ^{125}I-[Tyr^4]bombesin for 15 min and then centrifuged at 10,000 × *g* for 3 min. The

pellet was washed twice with 1 ml of PBS (4°) and resuspended in 200 μl of cross-linking buffer (50 mM HEPES, pH 7.5, 5 mM $MgCl_2$) containing 1 mM DSS. After cross-linking for 30 min at 25°, the reaction was stopped by the addition of 25 μl of 1 M glycine. After 10 min (4°), the sample was centrifuged at 10,000 × *g* for 3 min. The supernatant was aspirated and the pellet was resuspended in 100 μl of 120 mM Tris·HCl, pH 6.8. Cross-linked membranes were solubilized by the addition of 25 μl of 5× gel loading buffer (0.4 M Tris·HCl, pH 6.8, 20%, v/v, SDS, 0.05%, w/v, bromphenol blue, 0.5 M dithiothreitol) and incubated at 25° for 1 hr. Solubilized membranes were subjected to SDS-polyacrylamide gel electrophoresis using the Laemmli buffer system, as described previously (31), with a 3% (v/v) acrylamide/0.1% (w/v) SDS stacking gel and a 10% (v/v) acrylamide/0.1% (w/v) SDS separating gel. For GRP-R-transfected cells 10 μg of protein and for Swiss 3T3 cells 20 μg of protein were added to each lane. Gels were stained with 0.1% (w/v) Coomassie blue R-250 in 40% (v/v) ethanol/10% (v/v) acetic acid, destained with 10% (v/v) ethanol/7.5% (v/v) acetic acid, equilibrated in 45% (v/v) ethanol/5% (v/v) glycerol for 30 min, and dried in a gel-slab dryer (model SE-540; Hoefer Scientific, San Francisco, CA). Dried gels were exposed to a storage phosphor screen for 3 days and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Measurement of cAMP. Cells were mechanically disaggregated and washed twice in binding buffer containing 0.1% bacitracin. Cells (5×10^6/ml) were incubated with various peptides for 60 min at 37°, after which cAMP was solubilized by the addition of 2 volumes of ice-cold ethanol. Peptide effects were measured in the presence of 30 μM forskolin or 100 ng/ml cholera toxin (14). When forskolin was used cells were preincubated for 30 min with forskolin, and when cholera toxin was used cells were preincubated for 60 min with cholera toxin before exposure to peptide. cAMP was measured by radioimmunoassay as described previously (32, 33), with all samples diluted so that the values remained in the linear portion of the standard curve.

Measurement of inositol phosphates. Total inositol phosphates were determined in both cell types as described previously (15). Briefly, 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 DMEM with 2% fetal calf serum, at 37° for 48 hr. Inositol phosphates were isolated using a Dowex anion exchange column.

Measurement of $[Ca^{2+}]_i$ using fura-2. Changes in $[Ca^{2+}]_i$ were determined using fura-2, as described previously (15). Fluorescence was measured at 500 nm after excitation at 340 nm (F_{340}) and at 380 nm (F_{380}), and $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* (34).

GRP-R internalization. Confluent cells were mechanically disaggregated, washed, and then suspended at a concentration of 3×10^6 cells/ml in binding buffer with 0.1% bacitracin. Cells were incubated with 75 pM ^{125}I-[Tyr^4]bombesin for various times, at various temperatures. After incubation, 100-μl samples were added to 0.2 M acetic acid, pH 2.5, in 0.5 M NaCl, for 5 min at 4° to remove surface radioligand, as described previously (15). In all cases, parallel incubations were conducted in the presence of 1 μM unlabeled bombesin to determine changes in nonsaturable binding. Results are expressed as the percentage of saturable ^{125}I-[Tyr^4]bombesin added that is internalized (i.e., not removed by acid treatment).

GRP-R down-regulation and desensitization. Cells were split 1:2 and 48 hr later were washed once in PBS. One half of the cells were resuspended in DMEM alone, whereas the other half were resuspended in DMEM containing 6 nM bombesin. At various time points, cells were harvested for binding experiments and $[Ca^{2+}]_i$ was determined. Analysis of the binding data using the least-squares, curve-fitting program LIGAND (35) permitted comparisons of mathematically derived receptor numbers (B_{max}) and affinities (K_d) between bombesin-pretreated and control cells. Down-regulation was expressed as the percentage of control receptor number (B_{max}) present on bombesin-pretreated cells, compared with untreated cells processed in parallel. Desensitization was defined as the decrease in the ability of peptide to alter cell activity and increase $[Ca^{2+}]_i$ at various time points after preincubation with 6 nM bombesin and was expressed as the percentage of control response obtained using cells processed in parallel that had not been exposed to bombesin preincubation.

Mitogenic assays. The mitogenic ability of bombesin was assayed by measuring [*methyl*-^{3}H]thymidine incorporation, as described previously (36). Briefly, cells were plated on 96-well trays at confluence density and maintained for 3–4 days until the medium became acidic. Medium was replaced with DMEM without serum for 24 hr, after which cells were continuously exposed to various peptides and 1 μCi/ml [*methyl*-^{3}H]thymidine for an additional 24 hr. Cell monolayers were washed three times with ice-cold PBS and detached using 0.1% trypsin in 1 mM EDTA, and the radioactivity of DNA precipitable with 10% trichloroacetic acid was determined.

Northern blot analysis for c-*fos* or $G_{\alpha s}$. Cells were split 1:6, grown to postconfluence, and then exposed to serum-free DMEM. After 4 hr of incubation with serum-free medium, experimental cells were pretreated with 30 μM forskolin or 20 μM H-89 for 30 min before stimulation with peptide for an additional 30 min. Cells were lysed *in situ* using guanidium isothiocyanate, and total RNA was extracted according to the method of Chomczynski and Sacchi (37). Total RNA was separated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose paper. RNA for c-*fos* was probed using a random-primed *Hind*III-*Eco*RI fragment of pYN3158 containing the human coding sequence for the c-Fos protein. After extensive washing at 80°, the membranes were reprobed for actin using a random-primed fragment of the human actin gene sequence. Northern blot analysis for $G_{\alpha s}$ were performed as described for c-*fos*, except that $G_{\alpha s}$ was probed using an *Eco*RI-*Eco*RI fragment of pGEM containing the rat coding sequence for $G_{\alpha s}$ (gift of Dr. Allen Spiegel, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health).

Western blot analysis for $G_{\alpha s}$. Cell membranes were isolated from Swiss 3T3 cells and transfected cells as described above. Proteins (20 μg/lane) were separated on a 10% SDS-polyacrylamide minigel apparatus (Bio-Rad) according to the method of Laemmli (31); bovine brain served as a positive control. After electrophoresis the protein was transferred to nitrocellulose paper. $G_{\alpha s}$ primary antibody (5 μg/ml; gift of Dr. Allen Spiegel, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) and goat anti-rabbit secondary antibodies were applied; the final conjugation used ^{125}I-Protein A. Gels were developed using a PhosphorImager.

All experiments comparing GRP-R function in Swiss 3T3 cells and GRP-R-transfected cells were performed in parallel on the same day. Consequently all comparisons were made using the Student *t* test, and *p* values of <0.05 were considered to be significant.

Results

Receptor kinetics and stoichiometry. Initial studies were performed to confirm the presence of GRP-R in GRP-R-transfected cells and to establish the structural similarity of the receptors in the two cell types (Fig. 1). In both Swiss 3T3 cells and the GRP-R-transfected cells a single wide protein band of 85 ± 1 kDa was cross-linked using ^{125}I-GRP. Deglycosylation using PGNase F demonstrated a shift in the cross-linked receptor from 85 ± 1 kDa to 41 ± 1 kDa, which was identical in both cell types.

Ligand binding kinetics and stoichiometry for native and transfected GRP-R were determined to establish their similarity. The time and temperature dependencies of binding of ^{125}I-[Tyr^4]bombesin to Swiss 3T3 cells and GRP-R-transfected cells were similar (Table 1). Specifically, binding was more rapid at 37° than at 22°, with maximal binding being observed at 30–45 min for both cell types. Reduction of the temperature to 4° or addition of 1 μM bombesin at any temperature reduced binding by >85% for both cell types. The kinetics of dissociation of

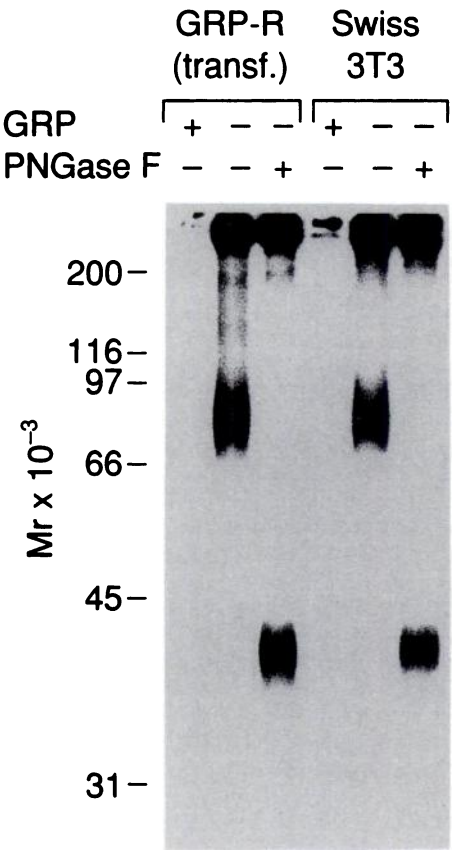

Fig. 1. Affinity cross-linking and deglycosylation of GRP-R-transfected cell membranes (*left*) and Swiss 3T3 cell membranes (*right*). Membranes were prepared as outlined in Experimental Procedures. ^{125}I-GRP was cross-linked using 1 mM DSS, as described in Experimental Procedures, after binding in the presence or absence of 1 μM GRP. After cross-linking, membranes were deglycosylated by incubation with PGNase F (10 units/ml). In Swiss 3T3 cells and GRP-R-transfected BALB/3T3 cells a single cross-linked band was seen, with an apparent molecular mass of 85 ± 1 kDa, and after deglycosylation a single cross-linked protein band was seen, with a molecular mass of 41 ± 1 kDa. This gel is representative of three separate experiments, with the molecular mass given as the mean ± standard error of three separate experiments.

TABLE 1

Time and temperature dependence of ^{125}I-[Tyr^4]bombesin binding

Cells at a concentration of 3 × 10^6 cells/ml were incubated with 75 pM ^{125}I-[Tyr^4]bombesin in binding buffer containing 0.1% bacitracin, 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 the results are given as the mean ± standard error of at least three separate experiments.

	^{125}I-[Tyr^4]Bombesin binding			
	Swiss 3T3 cells		Transfected cells	
	30 min	60 min	30 min	60 min
	%			
4°	1.8 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	2.4 ± 0.2
22°	11.4 ± 0.3	12.9 ± 0.4	12.8 ± 0.4	13.9 ± 0.7
39°	16.0 ± 0.1	16.1 ± 0.1	14.7 ± 0.2	16.1 ± 0.3

bound ^{125}I-[Tyr^4]bombesin were investigated and also found to be similar (data not shown). For both cell types, at 4° dissociation was slowed such that at 60 min >90% of ^{125}I-[Tyr^4]bombesin remained bound. At 37°, however, dissociation of bound ^{125}I-[Tyr^4]bombesin was faster, with 35% of ligand having dissociated at 60 min from both cell types (data not shown). These studies demonstrate that the kinetics of ligand-GRP-R interaction in GRP-R-transfected cells and Swiss 3T3 cells are similar.

To compare the abilities of native and GRP-R-transfected cells to interact with bombesin-related peptides, dose-inhibition curves for various bombesin agonists and antagonists were obtained (Table 2). Bombesin was the most potent at inhibiting binding of ^{125}I-[Tyr^4]bombesin, causing half-maximal inhibition at approximately 1 nM. Analysis of the binding data using the least-squares, curve-fitting program LIGAND (35) demonstrated that, for either cell type, the data were best fit by a single-binding site model. There was no difference in the affinities of bombesin for the native GRP-R expressed by Swiss 3T3 cells and the GRP-R transfected into BALB/3T3 fibroblasts. The affinity of GRP for the GRP-R was similar in the two cell types and was not significantly different from values obtained for bombesin (Table 2). In contrast, the affinity of NMB was approximately 100–150-fold lower than that of bombesin in both cell types, although the affinity was not significantly different between the two cell types (Table 2). Scatchard analysis of the binding data revealed that there were 8-fold more receptors on transfected cells, compared with Swiss 3T3 cells (240 ± 22 fmol/10^6 cells versus 30 ± 2 fmol/10^6 cells, respectively). The GRP-R antagonists [D-Phe^6]bombesin(6–13) propylamide and [D-Tyr^6]bombesin(6–13) methyl ester were slightly but significantly more potent in Swiss 3T3 cells than in GRP-R-transfected cells and were approximately 50-fold more potent than [ψ13–14,Leu^{14}]bombesin (Table 2). These data demonstrate that, in terms of their relative affinities for various GRP-R agonists and antagonists, the GRP-R expressed by transfected BALB/3T3 cells and Swiss 3T3 cells are similar.

Effects of bombesin in altering cellular activity. Previous investigations (14) have demonstrated that agonist binding to natively expressed GRP-R in Swiss 3T3 cells activates both phospholipase C and adenylyl cyclase. We confirmed these findings in Swiss 3T3 cells by first demonstrating the ability of bombesin to increase cellular cAMP levels (Fig. 2). Bombesin alone caused a small but significant increase in cAMP (with bombesin, 36 ± 2 pmol/10^6 cells; basal, 18 ± 2 pmol/10^6 cells;

TABLE 2

Comparison of the ability of various bombesin-related agonists and antagonists to inhibit binding of ^{125}I-[Tyr^4]bombesin to Swiss 3T3 cells and transfected cells

Cells were incubated in binding buffer with 0.1% bacitracin and 75 pM ^{125}I-[Tyr^4]bombesin alone or with the indicated concentrations of peptide. Data are expressed as the affinity constant calculated from analysis of the dose-inhibition curves using LIGAND(35) and represent the concentration causing half-maximal receptor occupation. Results are given as the mean ± standard error of at least three separate experiments, with each value determined in duplicate in each experiment.

	K_i	
	Swiss 3T3 cells	Transfected cells
	nM	
Agonists		
Bombesin	0.9 ± 0.1	0.9 ± 0.3
GRP	1.4 ± 1.0	3.1 ± 1.4
NMB	105 ± 22	174 ± 44
Antagonists		
[D-Tyr^6]Bombesin(6-13) methyl ester	1.7 ± 0.2[a]	6.1 ± 1.1
[D-Phe^6]Bombesin(6-13) propylamide	1.7 ± 0.2[a]	6.8 ± 0.9
[ψ13-14,Leu^{14}]Bombesin	65 ± 7	87 ± 11

[a] Results significantly different between transfected cells and Swiss 3T3 cells ($p < 0.05$).

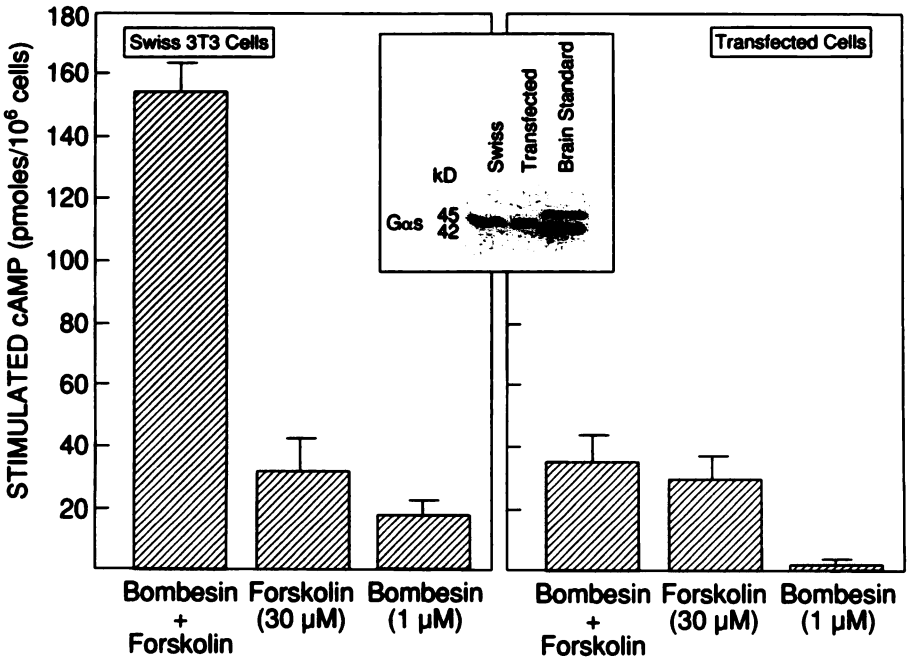

Fig. 2. Ability of bombesin or forskolin to increase cAMP levels in Swiss 3T3 (*left*) or GRP-R-transfected BALB/3T3 (*right*) cells. Cells (5×10^6/ml) were incubated for 30 min with bombesin alone or after a 1-hr preincubation with forskolin. cAMP was measured by a radioimmunoassay as described in Experimental Procedures. In Swiss 3T3 cells and GRP-R-transfected BALB/3T3 cells basal values were 18 ± 2 pmol/10^6 cells and 21 ± 2 pmol/10^6 cells, respectively. cAMP is expressed as the stimulated increase over the basal value. Each data point represents the mean ± standard error of at least four separate experiments, with each point determined in triplicate. *Inset:* Similar quantities of $G_{\alpha s}$ protein were detected by Western blotting in membranes from the two cell types when 20 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis, as described in Experimental Procedures.

$p < 0.05$). Incubation with cholera toxin increased cAMP to 41 ± 8 pmol/10^6 cells, whereas incubation with the adenylyl cyclase activator forskolin alone increased cellular cAMP to 53 ± 7 pmol/10^6 cells (Fig. 2). Coincubation with bombesin and forskolin caused a supra-additive increase in cellular cAMP levels, with levels increasing to 188 ± 18 pmol/10^6 cells (Fig. 2). In contrast, bombesin alone had no effect in increasing cAMP levels in GRP-R-transfected cells, whereas forskolin alone increased cAMP to 51 pmol/10^6 cells, levels similar to those seen with Swiss 3T3 cells (Fig. 2). Coincubation of GRP-R-transfected cells with maximal concentrations of bombesin and forskolin together failed to increase cAMP levels beyond those seen with forskolin alone (Fig. 2). Similarly, Swiss 3T3 cells preincubated with cholera toxin additionally increased cellular cAMP levels to 58 ± 4 pmol/10^6 cells after stimulation with 1 μM bombesin ($p < 0.05$), whereas no additional increase in cAMP levels over that obtained with cholera toxin alone was recorded in GRP-R-transfected cells (data not shown). To confirm that Swiss 3T3 and GRP-R-transfected cells both made the necessary G protein for adenylyl cyclase activation, Northern and Western blot analyses were performed on both cell types. Copious amounts of $G_{\alpha s}$ mRNA (data not shown) and $G_{\alpha s}$ protein of the same predominant isoform (Fig. 2, *inset*) were detected in both cell types. These data demonstrate that, although natively expressed GRP-R are capable of increasing cellular cAMP in response to stimulation with bombesin, activation of GRP-R transfected into BALB/3T3 cells does not significantly alter intracellular cAMP concentrations. This difference also cannot be accounted for on the basis of $G_{\alpha s}$ availability between the two cell types or the lack of adenylyl cyclase able to be activated in either cell type, because the two cell types showed equal increases with forskolin and cholera toxin.

To determine whether the failure of bombesin to increase cAMP in GRP-R-transfected cells could be attributed to a general defect in receptor-G protein coupling, the effect of increasing concentrations of the nonhydrolyzable guanine analogue Gpp(NH)p on ligand binding to cell membranes from the two cell types was assessed (Fig. 3, *insets*). Gpp(NH)p caused a concentration-dependent decrease in binding of ^{125}I-[Tyr^4]bombesin to cell membranes from either cell type. For membranes derived from either cell type, half-maximal inhibition of ^{125}I-[Tyr^4]bombesin binding occurred with 0.1 μM Gpp(NH)p (Fig. 3, *insets*). The basis for this Gpp(NH)p-induced decrease in ^{125}I-[Tyr^4]bombesin binding was determined by analyzing the effect of a fixed concentration of Gpp(NH)p (0.1 μM) on the bombesin dose-inhibition curve (Fig. 3). The decrease in binding produced by Gpp(NH)p was due to a decrease in receptor affinity in both Swiss 3T3 cells [K_i of 1.5 ± 0.1 nM versus 2.4 ± 0.2 nM, without and with Gpp(NH)p, respectively; $p < 0.05$] and GRP-R-transfected cells [K_i of 1.3 ± 0.12 nM versus 2.8 ± 0.4 nM, without and with Gpp(NH)p, respectively; $p < 0.05$]. Receptor numbers (B_{max}) in either Swiss 3T3 cells (150 ± 10 fmol/mg of protein) or GRP-R-transfected cells (129 ± 20 fmol/mg of protein), however, were not significantly different in the presence of 0.1 μM Gpp(NH)p (160 ± 10 fmol/mg of protein and 152 ± 42 fmol/mg of protein, respectively; $p > 0.05$). These data demonstrate that there is not a general defect in GRP-R coupling to G proteins in GRP-R-transfected BALB/3T3 cells.

To further investigate G protein coupling, we next determined whether agonist activation of the GRP-R in both cell types could activate phospholipase C and increase cellular inositol phosphate levels (Fig. 4) or increase $[Ca^{2+}]_i$ (data not shown). GRP caused a 4-fold increase in [^{3}H]inositol phosphate formation in both cell types. The dose-response curves for bombesin or NMB increasing total [^{3}H]inositol phosphates were similar in Swiss 3T3 cells and in GRP-R-transfected cells (Fig. 4). For either cell type, detectable stimulation occurred with 0.1 nM bombesin, half-maximal stimulation occurred at 1 nM, and maximal stimulation occurred at 1 μM. For both cell types, NMB was approximately 20-fold less potent (Fig. 4). For Swiss 3T3 cells and GRP-R-transfected cells, the concentrations of either bombesin (EC_{50} of 1.03 ± 0.34 nM versus 0.94 ± 1.01 nM, respectively) or NMB (EC_{50} of 23 ± 8 nM versus 18 ±

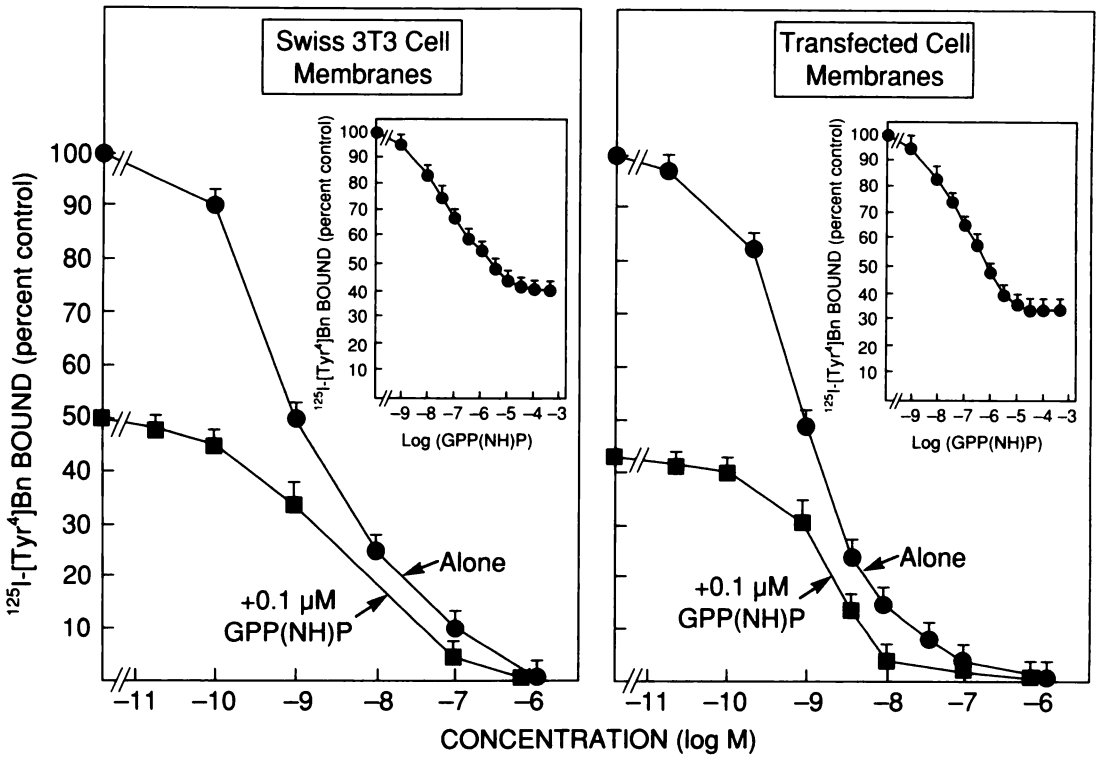

Fig. 3. Effect of the nonhydrolyzable guanine analogue Gpp(NH)p on the GRP dose-response curve for ^{125}I-[Tyr^4]bombesin (*^{125}I-[Tyr^4]Bn*) binding to Swiss 3T3 (*left*) or GRP-R-transfected BALB/3T3 (*right*) cells. Cells were incubated at 22° for 60 min with 75 pM ^{125}I-[Tyr^4]bombesin in binding buffer with 0.1% bacitracin, in the absence of unlabeled peptide, with or without 0.1 μM Gpp(NH)p. Half-maximal inhibition of ^{125}I-[Tyr^4]bombesin binding was observed using 0.1 μM Gpp(NH)p with cell membranes from both Swiss 3T3 cells (*left*, *inset*) and GRP-R-transfected cells (*right*, *inset*). Results are expressed as the percentage of saturable binding in the absence of unlabeled peptide. Each data point represents the mean ± standard error of at least three separate experiments, with each experimental value measured in duplicate.

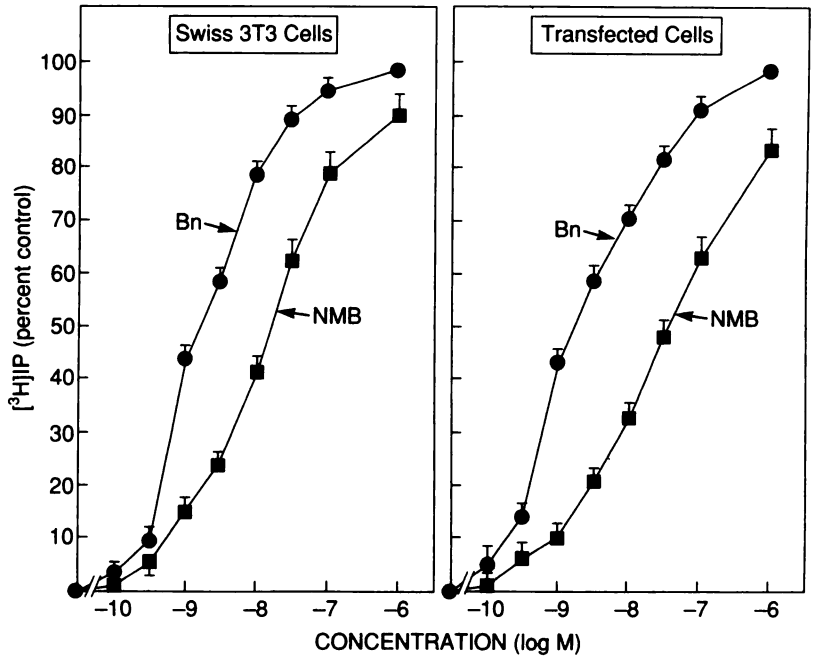

Fig. 4. Ability of bombesin and of NMB to stimulate [^{3}H] inositol phosphate (*[^{3}H]IP*) generation in Swiss 3T3 (*left*) or GRP-R-transfected BALB/3T3 (*right*) cells. Confluent cells were incubated with 100 μCi/ml *myo*-[2-^{3}H]inositol for 48 hr, washed, and incubated with the indicated concentration of agonist for 60 min. Total [^{3}H]inositol phosphates were determined using anion exchange chromatography, as described in Experimental Procedures. In Swiss 3T3 cells and GRP-R-transfected cells, 1 μM bombesin (*Bn*) stimulated [^{3}H]inositol phosphates from 3,600 ± 700 dpm to 17,000 ± 2,900 dpm and from 5,500 ± 400 dpm to 21,200 ± 2,200 dpm, respectively. Data are expressed as the percentage of maximal increase obtained using 1 μM bombesin. Each data point represents the mean ± standard error of a minimum of four separate experiments, with each point determined in duplicate.

5 nM) required to produce a half-maximal increase in total [^{3}H] inositol phosphates were not significantly different (Fig. 4).

Similarly, both bombesin and NMB stimulated increases in $[Ca^{2+}]_i$ in both cell types (data not shown). Detectable increases in $[Ca^{2+}]_i$ were observed with 10 pM bombesin, half-maximal increases were observed at 1 nM, and maximal increases were observed at 10 μM for both cell types. NMB was approximately 10-fold less potent in both cell types. To determine whether the increases in [^{3}H]inositol phosphate levels represented primary activation of phospholipase C or were secondary to an increase in $[Ca^{2+}]_i$, we stimulated both cell types with 1 μM thapsigargin or 1 μM 4-bromo-A23187, agents that directly

increase $[Ca^{2+}]_i$. Both agents increased $[Ca^{2+}]_i$ to a similar extent as did bombesin; however, neither agent was effective in increasing [^{3}H]inositol phosphates in either cell type (data not shown), suggesting that secondary activation of phospholipase C by increased $[Ca^{2+}]_i$ is unlikely to explain the increases caused by bombesin-related peptides.

Bombesin-specific effects on GRP-R processing and mitogenesis. GRP-R occupation by agonists has been reported to cause a number of changes subsequent to phospholipase C and adenylyl cyclase activation, although the role of these agents in mediating these events is unclear. Consequent to acute stimulation with agonist, GRP-R undergo rapid heterologous desensitization followed by homologous desensitization, a process that some (38) but not all (39) investigators believe is associated with receptor internalization and down-regulation. Homologous and heterologous desensitization was assessed by determining alterations in $[Ca^{2+}]_i$ in response to either bombesin or the structurally unrelated peptide bradykinin (Fig. 5, *insets*), with both Swiss 3T3 and GRP-R-transfected cells. Five minutes after the addition of 6 nM bombesin, the further addition of 1 μM bombesin or bradykinin failed to elicit any further increase in $[Ca^{2+}]_i$, reflecting an acute decrease in GRP-R responsiveness to additional agonist exposure (Fig. 5, *insets*). Six hours later, however, 1 μM bradykinin stimulation increased $[Ca^{2+}]_i$ to >90% of that seen before the addition of bombesin, whereas stimulation with 1 μM bombesin failed to increase $[Ca^{2+}]_i$ to >50% of that observed before the addition of bombesin (Fig. 5, *insets*). These results demonstrate that with both cell types homologous desensitization occurs with prolonged bombesin exposure (i.e., hours).

To investigate whether receptor down-regulation may occur and may contribute to the desensitization, binding studies with ^{125}I-[Tyr^4]bombesin were done under conditions similar to those causing desensitization. After 3 hr of exposure to 6 nM bombesin, 45% of bombesin receptors expressed by Swiss 3T3 cells remained, whereas this decrease in receptor number (B_{max}) plateaued to approximately 21% of the original number of receptors by 6 hr (Fig. 5, *left*). Similar findings were observed in GRP-R-transfected BALB/3T3 cells (Fig. 5, *right*). At no time did exposure to GRP-R cause a change in GRP-R affinity on either Swiss 3T3 cells or BALB/3T3 cells transfected with GRP-R.

Similarly, internalization of GRP-R was most rapid at 37°, with half-maximal values being observed for Swiss 3T3 cells and GRP-R-transfected cells at 15 min and 6 min, respectively (Fig. 6). By 90 min, approximately 90% of GRP-R were internalized at 37° in both cell types. Internalization was slightly slower at 27° than at 37° in both cell types possessing GRP-R (Fig. 6). In contrast, <5% of receptors were internalized at 4° for either cell type (Fig. 6).

Prolonged incubation of Swiss 3T3 cells with bombesin results in mitogenesis and growth (8, 10, 38). Bombesin caused a dose-dependent increase in [*methyl*-^{3}H]thymidine incorporation in Swiss 3T3 cells (Fig. 7). Concomitant incubation with 30 μM forskolin potentiated this bombesin effect (Fig. 7) in Swiss 3T3 cells. Half-maximal increases in [*methyl*-^{3}H]thymidine incorporation were significantly lower in Swiss 3T3 cells with 30 μM forskolin present (50 ± 12 pM), compared with values obtained when forskolin was absent (821 ± 34 pM, $p < 0.01$), whereas forskolin alone did not significantly affect

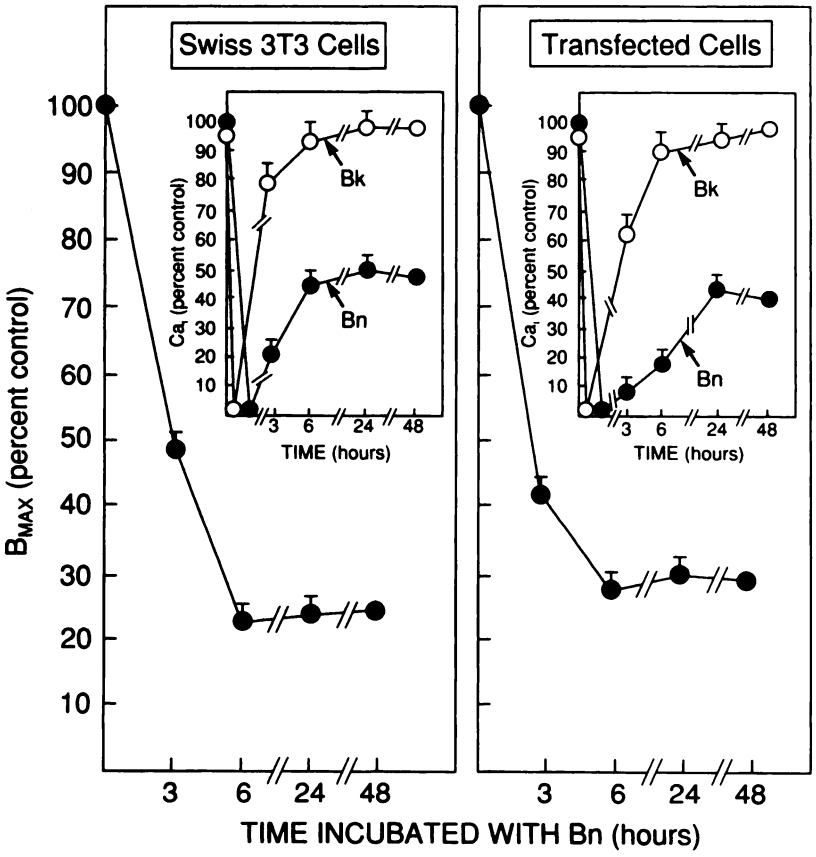

Fig. 5. Effect of bombesin preincubation on GRP-R number (B_{max}) expressed by Swiss 3T3 (*left*) or GRP-R-transfected BALB/3T3 (*right*) cells. Cells grown to confluence in F-175 flasks were exposed to 6 nM bombesin for the indicated times and subjected to bombesin competitive binding experiments with ^{125}I-[Tyr^4]bombesin. B_{max} and K_i values were determined using the least-squares, curve-fitting program LIGAND (35). There was no change in GRP-R K_i for agonist with bombesin preincubation. Corresponding changes in $[Ca^{2+}]_i$ measured by monitoring fura-2 fluorescence in Swiss 3T3 cells (*left*, *inset*) or GRP-R-transfected cells (*right*, *inset*) in response to either 1 μM bradykinin (*Bk*) or 1 μM bombesin (*Bn*) were determined in cells that had been preincubated with 6 nM bombesin. In Swiss 3T3 cells basal $[Ca^{2+}]_i$ values were 101 ± 11 nM and increased to 322 ± 31 nM with 1 μM bombesin and to 243 ± 21 nM with 1 μM bradykinin, whereas in GRP-R-transfected cells basal $[Ca^{2+}]_i$ values were 102 ± 12 nM and increased to 245 ± 28 nM with 1 μM bombesin and to 229 ± 17 nM with 1 μM bradykinin. Data are expressed as the percentage of either GRP-R present or fluorescence measured, compared with that of control cells processed in parallel. Each data point represents the mean ± standard error of at least three separate experiments.

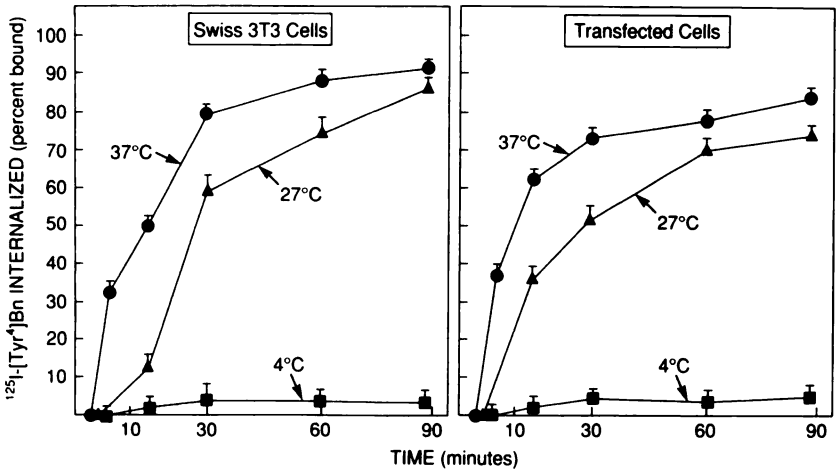

Fig. 6. Time and temperature dependence of internalization of ^{125}I-[Tyr^4]bombesin in Swiss 3T3 (*left*) or GRP-R-transfected (*right*) cells. Swiss 3T3 cells or GRP-R-transfected cells were incubated with 75 pM ^{125}I-[Tyr^4]bombesin (^{125}*I-[Tyr^4]Bn*) for the indicated times and then aliquots were exposed to 0.2 M acetic acid in 0.5 M NaCl, pH 2.5, to remove surface-bound ligand. Surface-bound ligand was that proportion of saturably bound counts removed by exposure to acid wash, whereas internalized ligand was the proportion not removed. Results are expressed as the proportion of total, saturably bound ligand at any time point that was not removed by acid treatment. For each experiment, each value was determined in triplicate, with each point representing the mean ± standard error of at least three separate experiments.

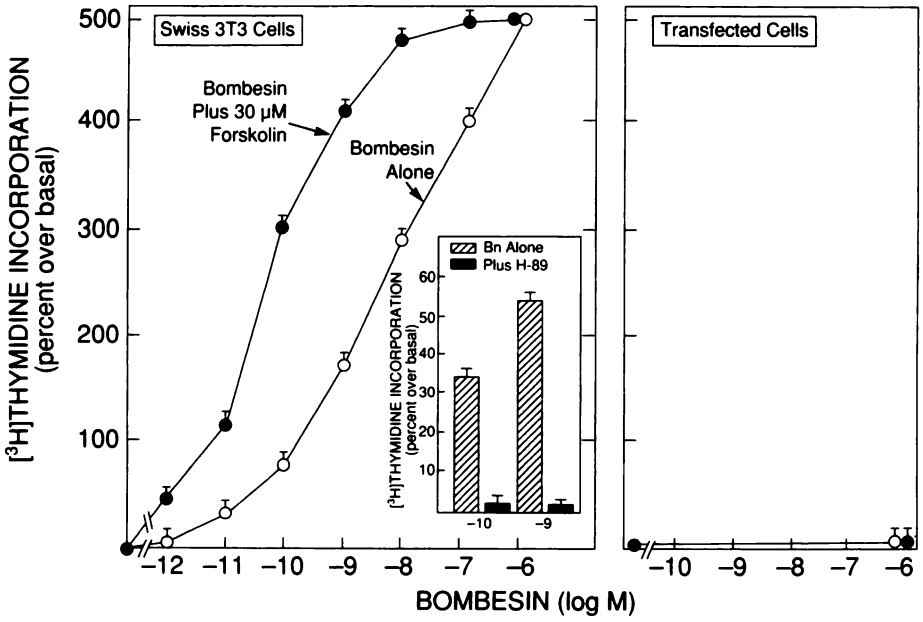

Fig. 7. Ability of bombesin (*Bn*) alone or with forskolin to increase [^{3}H]thymidine incorporation in Swiss 3T3 (*left*) or GRP-R-transfected BALB/3T3 (*right*) cells. Cells were grown to confluence on 96-well plates and growth arrested as described in Experimental Procedures. Cells were then exposed to [*methyl*-^{3}H]thymidine (1 μCi/ml) for 24 hr. Cell monolayers were washed in ice-cold PBS, and the radioactivity of DNA precipitable with 10% trichloroacetic acid was determined. Data are expressed as the percentage increase over basal values. For Swiss 3T3 cells and GRP-R-transfected cells, basal values of [*methyl*-^{3}H]thymidine incorporation were 12,157 ± 1,232 dpm and 14,226 ± 2,089 dpm, respectively. Coincubation with the protein kinase A inhibitor H-89 completely attenuated the ability of bombesin at concentrations of 1 nM or less to increase [*methyl*-^{3}H]thymidine incorporation (*inset*), with data expressed as the percentage of maximal counts obtained with 1 μM bombesin. Each data point represents the mean ± standard error of at least three separate experiments, with each experiment representing the mean of 12 determinations.

[*methyl*-^{3}H]thymidine uptake (basal, 12,157 ± 1,232 dpm; with 30 μM forskolin, 13,329 ± 2,443 dpm). In contrast, GRP-R-transfected cells failed to increase [*methyl*-^{3}H]thymidine incorporation when stimulated with maximal concentrations of bombesin alone (1 μM) or combined with forskolin (30 μM) (Fig. 7). However, addition of 10% serum was able to dramatically increase the ability of GRP-R-transfected cells to increase [*methyl*-^{3}H]thymidine incorporation, with basal levels increasing from 1,340 ± 220 dpm to 144,000 ± 6,700 dpm. To determine whether the bombesin-induced increase in cell proliferation was dependent on the ability of GRP-R activation to increase cAMP, Swiss 3T3 cells underwent coincubation with the specific protein kinase A inhibitor H-89 for the entire period of their exposure to bombesin. H-89 completely attenuated the ability of bombesin to increase [*methyl*-^{3}H]thymidine incorporation when the concentration of bombesin was 1 nM or less (Fig. 7, *inset*).

Previous studies have reported that GRP causes a rapid increase in c-*fos* expression in Swiss 3T3 cells and that this increase in expression is dose dependent (40). When Swiss 3T3 cells were exposed to 1 nM bombesin, a significant increase in c-*fos* expression was detected, compared with unstimulated control cells (Fig. 8). Swiss 3T3 cells exposed only to forskolin failed to show an increase in c-*fos* expression, although the combination of 1 nM bombesin and 30 μM forskolin resulted in a potentiated c-*fos* response (Fig. 8). In contrast, GRP-R-transfected cells did not respond to bombesin either alone or with forskolin (Fig. 8). We attempted to determine whether other G protein-coupled mitogenic receptors could be detected on transfected BALB cells, to determine whether these cells could manifest an increase in [*methyl*-^{3}H]thymidine incorporation or c-*fos* mRNA with their stimulation. We screened the transfected BALB cells by looking for increases in total cellular inositol phosphate levels consequent to stimulation with angiotensin II, bradykinin, carbachol, cholecystokinin-8, epidermal growth factor, endothelin-1, galanin, NMB, neurotensin, PACAP, substance P, and vasopressin. Only bradykinin caused a detectable increase, which was small, with inositol phosphates increasing <2-fold (data not shown). Northern analysis of transfected BALB cells treated with 1 μM bradykinin for 30 min failed to detect any increase in c-*fos*. However, the ability of GRP-R-transfected cells to manifest an increase in c-*fos*

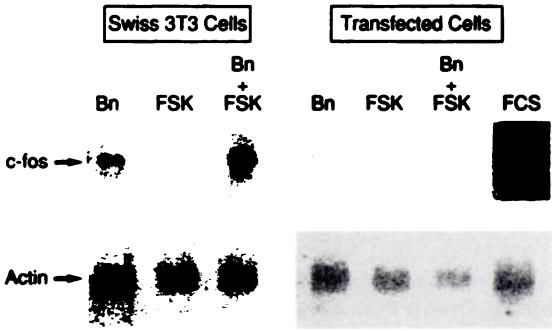

Fig. 8. Northern blot analysis of c-*fos* expression in Swiss 3T3 (*left*) and GRP-R-transfected BALB/3T3 (*right*) cells. Growth-arrested cells grown in F-175 flasks were treated *in situ* with 1 nM bombesin (*Bn*), 30 μM forskolin (*FSK*), or both, whereas growth-arrested GRP-R-transfected cells were additionally treated with 10% fetal calf serum (*FCS*) for 30 min. Cells were lysed *in situ* using guanidium isothiocyanate, as described in Experimental Procedures. Ten micrograms of total RNA were applied in each lane of a 1% agarose-formaldehyde gel and transferred to a nitrocellulose filter. Labeling of the cDNA for human c-*fos* was performed by hexamer priming, to a specific activity of 10^9 cpm/μl. Autoradiography was performed using a PhosphorImager. The bottom bands show hybridization of the same RNA transfer blot with β-actin cDNA, revealing no significant differences in the amounts of loaded RNA. This Northern blot is representative of three separate experiments.

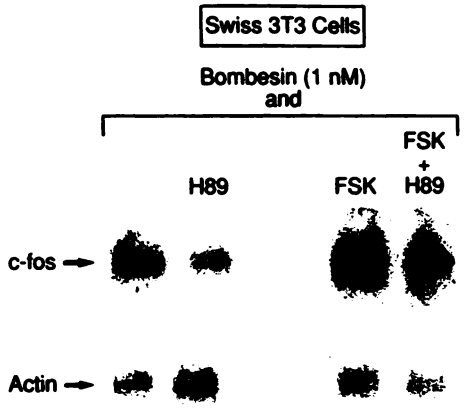

Fig. 9. Northern blot analysis of c-*fos* expression, with and without the protein kinase A inhibitor H-89, in Swiss 3T3 (*left*). Growth-arrested cells were prepared and total RNA was processed as described in the legend to Fig. 8. Cells were preincubated *in situ* for 30 min with 20 μM H-89. This Northern blot is representative of two separate experiments. *FSK*, forskolin.

expression when appropriately stimulated was documented by exposing these serum-starved cells to 10% fetal calf serum (Fig. 8). To attempt to demonstrate that in Swiss 3T3 cells the increase in c-*fos* expression was dependent in part on the ability of GRP-R activation to result in increases in cAMP levels, Swiss 3T3 cells were also pretreated with the cAMP protein kinase inhibitor H-89 (Fig. 9). When Swiss 3T3 cells were treated with either 1 nM bombesin alone or bombesin and 30 μM forskolin, addition of 20 μM H-89 significantly attenuated the increase in c-*fos* expression; the ability of bombesin to increase c-*fos* at concentrations of 100 pM or less was completely eliminated by coincubation with 20 μM H-89 (data not shown).

Discussion

Agonist stimulation of GRP-R on Swiss 3T3 cells is unusual and is similar to that of receptors for histamine (H_2) (41), endothelin (12), PACAP (13), thyroid-stimulating hormone (42), and glucagon (11) because it results in activation of both adenylyl cyclase and phospholipase C. In addition to causing changes in cellular function, such as stimulating the proto-oncogenes c-*fos* and c-*myc* (18), as well as cell growth (7, 8, 18), GRP-R activation results in receptor internalization (15), down-regulation (16, 17), and desensitization (16, 17, 38). At present, however, the relative role of either signal transduction pathway in mediating these GRP-R-induced changes in cellular and receptor function is unclear. In a recent study (15), BALB/3T3 cells transfected with the NMB receptor, which is structurally similar to the GRP-R, functioned in terms of ligand interaction and ability to alter cellular activity consequent to receptor activation in a manner indistinguishable from that of the native receptor on rat C-6 glioblastoma cells. When similar experiments were performed by transfecting the GRP-R into the same BALB/3T3 cells, and even though BALB/3T3 cells are closely related to Swiss 3T3 cells, the GRP-R-transfected cells failed to activate adenylyl cyclase and increase cAMP levels in response to bombesin; however, these cells did activate phospholipase C. The difference suggests that these transfected cells might provide a useful model for investigating the specific role that activation of either transduction pathway may play in altering cellular and receptor function consequent to GRP-R activation.

Certain findings suggest that the failure to increase cAMP in the GRP-R-transfected cells was not due to a general defect in G protein coupling. Previous studies have demonstrated in pancreatic acinar cells (6, 43) and Swiss 3T3 cells (43) that binding of agonists, but not radiolabeled antagonists, to the GRP-R is affected by activation of G proteins. In the present study maximal concentrations of the nonhydrolyzable GTP analogue Gpp(NH)p caused a similar 60–65% decrease in binding of ^{125}I-[Tyr^4]bombesin in both cell systems, due to decreased GRP-R affinity independent of alteration in receptor number. Consequent to agonist activation, both GRP-R-transfected BALB/3T3 cells and Swiss 3T3 cells activated phospholipase C in a similar fashion, with agonists showing superimposible dose-response curves for the two cell lines. These results suggest that the failure of GRP to activate adenylyl cyclase in GRP-R-transfected BALB/3T3 cells is not due to a general defect in GRP-R-G protein coupling. Our results do not define the exact reason why GRP fails to activate adenylyl cyclase in the GRP-R-transfected BALB/3T3 cells, but they do provide some insights into where the defect might be. It remains possible that different isoforms of adenylyl cyclase might exist in the two cell types. In the GRP-R-transfected cells abundant G_{α_s} mRNA and protein were present and adenylyl cyclase could be activated by cholera toxin or forskolin to the same extent as in Swiss 3T3 cells. This suggests that the failure to increase cAMP in GRP-R-transfected BALB/3T3 cells is due to a difference proximal to adenylyl cyclase activation. Previous studies using Swiss 3T3 cells demonstrated that activation of PKC results in enhanced cAMP accumulation with agents that activate adenylyl cyclase (18). Because bombesin activates phospholipase C in a similar manner in Swiss 3T3 cells and GRP-R-transfected cells, it is unlikely that a failure to activate PKC explains the failure to activate adenylyl cyclase in the GRP-R-transfected cells. Therefore, the most likely explanation for the loss of agonist-induced adenylyl cyclase activation in the GRP-

R-transfected BALB/3T3 cells is that GRP-R are not coupled adequately to adenylyl cyclase at the G protein level.

Radiolabeled bombesin receptor agonists undergo rapid internalization after binding to GRP-R on Swiss 3T3 cells and AR-42J cells (43) and in some cell lines also undergo GRP-R down-regulation and desensitization (16, 17, 38). Studies using β_2-adrenergic receptors suggest that at least some of these receptor-related changes are mediated by activation of PKC (19), cAMP-dependent kinase (19, 21), or βARKs (βARK-I and βARK-II) (19, 20). A recent study also demonstrated that activation of βARKs may be involved in the desensitization of some G protein-linked receptors coupled to phospholipase C (44). With other adenylyl cyclase-activating receptors, such as the dopamine D_1 receptor, increases in cAMP are involved in mediating down-regulation but not desensitization (25). Our results suggest that activation of adenylyl cyclase and protein kinase A is not involved in GRP-R internalization, down-regulation, or desensitization, because the GRP-R-transfected cells, which do not activate adenylyl cyclase, showed identical results for each of these processes, compared with the native GRP-R expressed by Swiss 3T3 cells. Because the phospholipase C pathway is activated in an indistinguishable manner in the GRP-R-transfected BALB/3T3 cells, these results are consistent with recent studies demonstrating that GRP-R down-regulation and desensitization in some cells (43) are at least partially mediated by PKC. These results also are consistent with a recent study demonstrating that activation of PKC is at least partially responsible for GRP-R internalization (45).

Bombesin-related peptides have been shown to stimulate the expression of the proto-oncogenes c-*fos* and c-*myc* (40), as well as the growth of both normal cells (7) and tumorigenic cell lines (8). In the case of Swiss 3T3 cells, where the effects of bombesin-related peptides on growth have been extensively studied, agents that activate the phospholipase C pathway (such as vasopressin or phorbol esters) (18) or endogenously increase cAMP levels (such as forskolin, vasoactive intestinal peptide, and cholera toxin) (18) can stimulate growth. Additionally, activation of both pathways can have a potentiated effect on growth (18). The contributions of each pathway to the growth effects of bombesin are not clear at present.

A number of results in our study suggest that activation of the cAMP pathway by bombesin stimulation of the GRP-R is an important component of the growth effect of GRP-R activation and that activation of phospholipase C alone by bombesin is not sufficient to maximally stimulate growth. Although in GRP-R-transfected cells the phospholipase C pathway was activated in a manner indistinguishable from that seen with the native GRP-R in Swiss 3T3 cells, bombesin neither stimulated the expression of c-*fos* nor stimulated [^{3}H]thymidine incorporation in the transfected cells. Furthermore, agonist activation of the GRP-R-transfected BALB/3T3 cells did not result in adenylyl cyclase activation, c-*fos* expression, or growth, even though the transfected cells were capable of showing increases in c-*fos* mRNA and [*methyl*-^{3}H]thymidine incorporation with the addition of fetal calf serum. In contrast, agonist stimulation of the native GRP-R on Swiss 3T3 cells did activate adenylyl cyclase, increase c-*fos* expression, and increase [^{3}H]thymidine incorporation. Addition of the highly selective protein kinase A inhibitor H-89 (46) completely blocked the ability of bombesin to increase c-*fos* expression and [^{3}H]thymidine incorporation at concentrations of bombesin up to 100 pM, suggesting that the activation of protein kinase A is an important component of the ability of bombesin to stimulate growth. Indeed, it has been proposed previously that in Swiss 3T3 cells the synergistic interaction of various intracellular signaling pathways potentiates the expression of the proto-oncogenes c-*fos* and c-*myc* and that activation of these signaling pathways results in cell proliferation (18). Our data, therefore, suggest that GRP-R activation of protein kinase A is an important component of the ability of bombesin-related peptides to cause growth. A recent study (47) using a molecular biological approach to determine the importance of cAMP in Swiss 3T3 cell mitogenesis supports our observations made in this paper. By transfecting Swiss 3T3 cells with expression vectors coding for either a defective regulatory subunit of protein kinase A or a low-K_m cAMP phosphodiesterase, a significant decrease in the effect of cAMP-increasing agents was achieved (47). When these altered Swiss 3T3 cells were stimulated with bombesin, a significant reduction in [^{3}H]thymidine incorporation was observed, demonstrating the importance of activation of this pathway in mediating the growth effects of bombesin.

Although our study does not demonstrate the mechanism whereby increases in cellular cAMP levels are achieved in Swiss 3T3 cells, evidence suggests that in this cell line activation of phospholipase C, resulting in activation of PKC, markedly affects the activity of adenylyl cyclase (14). We demonstrated that in the transfected cell line activation of phospholipase C was identical to that observed in Swiss 3T3 cells and, furthermore, that transfected cells expressed the necessary machinery to generate cAMP by traditional means; Western blots demonstrated the presence of $G_{\alpha s}$, whereas stimulation with forskolin increased cAMP. Although the exact pathway used by Swiss 3T3 cells to increase cAMP levels in response to bombesin is not known, our results suggest that the defect in cAMP generation in GRP-R-transfected cells is distal to phospholipase C activation. Regardless of the mechanism whereby GRP-R activation in Swiss 3T3 cells results in cAMP accumulation, we demonstrated that attenuation of the bombesin-induced increase in cAMP levels, either by using the protein kinase A inhibitor H-89 or by using transfected cells that are incapable of increasing cAMP levels in response to bombesin, results in abrogation of the mitogenic response.

Finally, our study suggests that GRP-R-transfected BALB/3T3 cells are a useful model for elucidating the possible roles of adenylyl cyclase and of phospholipase C in mediating the effects of bombesin on cellular and receptor function. A similar approach might be useful for other receptors, such as those for PACAP, endothelin, histamine (H_2), thyroid-stimulating hormone, and glucagon, all of which activate both adenylyl cyclase and phospholipase C.

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