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Importance of Amino Acids of the Central Portion of the 2nd Intracellular Loop of the Gastrin-Releasing Peptide Receptor for Phospholipase C Activation, Internalization and Chronic Down-regulation

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Running title: GRP-R i2 loop-structure-function

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Tables 2

Figures 6

References 56

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Abstract 249

Introduction 525

Discussion 2,663

Abbreviations: DR – down-regulation; Bn – bombesin; i2 loop – 2nd intracellular loop; GRP-R – gastrin-releasing peptide receptor; PLC – phospholipase C; NMB – neuromedin B; CNS – central nervous system; IP – inositol phosphate; GPCR – G protein-coupled receptor

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Abstract

Little is known about the function of the central portion of the 2nd intracellular loop (i2 loop) of peptide receptors in activation of downstream pathways and receptor modulatory processes like receptor internalization or chronic down-regulation (DR). Recent data suggest a role for i2 loop hydrophobic amino acids in these processes. We used site-directed mutagenesis to address these issues with the gastrin-releasing peptide receptor (GRP-R). Each i2 loop residue from #142 to #148 was mutated and the receptors were expressed in Balb 3T3 cells. Two mutants showed a minimal (<2-fold) decrease in affinity. Five mutants showed decreased efficacy for activating phospholipase C (PLC). Two double mutants (IM$^{143.147}$AA and VM$^{144.147}$AA) showed a minimal decrease in affinity, but had a decreased ability to fully activate PLC. Only the IM double mutation had decreased maximal internalization, whereas the R^{145}A single mutant showed an increase, suggesting a tonic inhibitory role for Arg-145 in internalization. Three single and both double mutants showed decreases in receptor DR. There was a weak correlation between the extent of GRP-R internalization and the maximal PLC activation, whereas changes in the maximal PLC activation were significantly (p=0.008) coupled to receptor DR. This study shows that amino acids of the i2 loop of the GRP-R are important in activation of PLC, internalization and down-regulation, but not for affinity. Our results support the proposal that internalization and chronic down-regulation have differing dependence on PLC and are largely independent processes, since some mutants showed no changes in internalization, but significant alterations in down-regulation.

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Receptors for the mammalian bombesin (Bn)-related peptides GRP and neuromedin B are responsible for a number of physiological and pathological processes (Tache et al., 1988; Bunnett 1994). Physiological effects in the CNS include the regulation of circadian rhythm, body temperature and satiety, and in the GI system include the release of many gastrointestinal hormones, trophic effects, and the regulation of gallbladder and smooth muscle contractility (Tache et al., 1988; Bunnett 1994). Pathological effects include stimulation of the growth of various human tumors including small cell lung cancer, prostate cancer and breast cancer (Tache et al., 1988; Bunnett 1994). Cloning and pharmacologic studies have described two mammalian Bn receptors, the gastrin-releasing receptor (GRP-R) with high affinity for GRP, and the neuromedin B (NMB) receptor with high affinity for NMB (Kroog et al., 1995a). Both mammalian Bn receptors are members of the G protein-coupled receptor superfamily and are coupled to phospholipase C (PLC) with activation resulting in an increase in IP, mobilization of intracellular Ca^{2+} and activation of protein kinase C (Jensen 1994; Kroog et al., 1995a). Both receptors on activation by agonists undergo receptor modulating processes including internalization, down-regulation and desensitization (Kroog et al., 1999; Benya et al., 1994b; Kroog et al., 1995a; Benya et al., 1994c).

GRP receptors are widely distributed in the brain, especially in the hypothalamus, the olfactory tract, dendate gyrus and cortex. Peripherally, GRP receptors are expressed on smooth muscle cells of the intestine, stomach and bladder and on hormone-secreting cells, as well as pancreatic acinar cells (Kroog et al., 1995a; Bunnett 1994). Previous structure-function studies in the GRP-R reveal a role for the 3rd intracellular loop (i3 loop) and the COOH-terminus of the receptor in coupling to phospholipase C and receptor modulatory processes (Benya et al., 1993; Benya et al., 1995). However, little is known about the role of the receptor domains toward the N-terminal of the GRPR from the i3 loop. Only one study has analyzed a GRP-R domain N-terminal to the 3rd intracellular loop and identified the importance of the i2 loop-DRY motif in G-protein coupling and receptor internalization (Benya et al., 1994a). In studies on muscarinic cholinergic receptors hydrophobic residues in the central portion of the i2 loop were described to be important

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for G protein-coupling and internalization (Moro et al., 1993; Moro et al., 1994). However, nothing is known about the function of the central portion of the i2 loop in the GRP-R or most gastrointestinal peptide hormone/neurotransmitter receptors. An amino acid sequence alignment shows conservation of the hydrophobic i2 loop residues between the Bn receptor family members and other G protein-coupled receptors (Fig. 1). Furthermore, a second recent study demonstrates that i2 loop residues in muscarinic receptors that are located between the DRY motif and the central hydrophobic residues show further evidence for a role of the i2 loop in G protein activation (Burstein et al., 1998; Spalding and Burstein, 2001). Therefore, in this study we have investigated, using mutagenesis, the importance of the central portion of the GRP-R i2 loop for receptor affinity, activation of PLC, GRP-R internalization and chronic down-regulation.

Our results demonstrate that the central portion of the GRP-R i2 loop is important for coupling to PLC and the regulation of the two receptor modulatory processes, receptor internalization and chronic receptor down-regulation, but not for receptor affinity. Using these i2 loop mutants we found that chronic receptor down-regulation correlates well with the mutant GRP-R's ability to activate PLC, whereas internalization does not, supporting the proposal that these two GRP receptor modulating processes are independent processes and have different cellular mechanisms.

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Materials and Methods

Materials

pcDNA3, oligonucleotides, LipofectAMINE™ reagent, and LipofectAMINE™ Plus reagent were from Invitrogen (Carlsbad, CA). QuikChange™ Site-Directed Mutagenesis Kit was from Stratgene (La Jolla, CA). Restriction endonucleases (HindIII, XbaI, and SmaI) were from New England BioLabs (Beverly, MA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, 100x, trypsin-EDTA, and Dulbecco's phosphate-buffered saline (PBS) were from Biofluids (Rockville, MD). The aminoglycoside G418 sulfate was from Mediatech, Inc. (Herndon, VA). Balb 3T3 cells were from American Type Culture Collection (Rockville, MD). Bn and leupeptin were from Bachem Biosciences (King of Prussia, PA). $Na^{125}I$ (2,200 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ). 1,3,4,6-Tetrachloro-3α,6α-diphenylglucouril (IODO-GEN™) and dithiothreitol were from Pierce Chemical (Rockford, IL). Bovine serum albumin fraction V was from ICN Pharmaceutical Inc. (Aurora, OH). Bacitracin and 4-(2-aminoethyl)-benzenesulfonyl-flouride (AEBSF) were from Sigma (St. Louis, MO). Myo-[2-^{3}H(N)]inositol was from Perkin Elmer Life Sciences (Boston, MA). Dowex AG1-X8 anion exchange resin (100-200 mesh formate form) was from BioRad (Richmond, CA). Hydro-Fluor scintillation fluid was from National Diagnostics (Atlanta, GA). KELL for Windows, version 6.0 was from Biosoft (Ferguson, MO), and GraphPad Prism 3 was from GraphPad Software Inc., (San Diego, CA).

Construction of Mutant Receptors

The pcDNA3-mouse GRPR construct used has been described before (Tokita et al., 2001). Mouse GRP-R mutants were made by using the QuikChange™ site-directed mutagenesis kit, following the manufacturer's instruction except that the annealing temperature was 60ºC and the *DpnI* digestion was for 2 hours. For verification of the correct nucleotide sequence the entire coding region of the GRP-R mutants was sequenced using an automated DNA sequencer (ABI PRISM™ 377 DNA sequencer, Applied Biosystems Inc., Foster City, CA).

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Preparation of Stable Cell Lines Expressing Mutant GRP-Rs

For stable transfection Balb 3T3 cells were seeded on a 10 cm dish at a density of 10^6 cells/dish in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin one day prior to transfection. Cells were maintained at 37ºC with a 5% CO_2 atmosphere. The following morning cells were transfected with 5 µg of plasmid DNA by lipofection (Felgner et al., 1987) using 30 µl of lipofectAMINE reagent and 20 µl of lipofectAMINE Plus reagent in serum-free DMEM for 3 hours at 37ºC. Then the medium was replaced with DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The following day cells were trypsinized and split into two 10 cm dishes, one which was used for a binding assay to check for expression of the GRP-R. The second 10 cm dish was trypsinized again 2 days later. Cells were plated at a range of different densities on 10 cm dishes in Selecting Medium (DMEM containing 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 800 mg/L G418 sulfate). Selecting Medium was changed every 3 days. Approximately 10 days later single colonies growing in selecting medium were picked and transferred to 24 well flasks containing Keeping Medium (DMEM containing 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 300 mg/L G418 sulfate). Clones were expanded to 75 cm^2 flasks in Keeping Medium. 5 to 10 clones were studied using ^{125}I-[Tyr^4]Bn binding studies. Clones that expressed the mutant GRP-R at a similar level as the wild-type GRP-R (reflected by similar Bmax in the binding data analysis) were selected.

Binding of ^{125}I-[Tyr^4]Bn to GRP-R Transfected Cells

^{125}I-[Tyr^4]Bn (2,200 Ci/mmol) was prepared as described previously using iodogen and purified using HPLC (Benya et al., 1994b). Monoiodinated ^{125}I-[Tyr^4]Bn has been shown to be fully biologically active (Singh et al., 1990). Homologous competitive binding studies using the GRP-R-transfected cells were performed as described previously (Benya et al., 1994b). Briefly, cells were seeded on 75 cm^2 flasks and split 1:5 one day prior to the experiment. Mechanically disaggregated cells were suspended for 1 hour at room temperature in 300 µl binding buffer [24.5

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mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 2.2 mM KH_2PO_4, 5 mM theophylline, 2 mM glutamine, 11 mM glucose, 1% (v/v) amino acid mixture, 1% (v/v) essential vitamin mixture, 0.1% (w/v) bacitracin, and 0.2% (w/v) bovine serum albumin] with 50 pM ^{125}I-[Tyr^4]Bn (2,200 Ci/mmol) in the presence of the indicated concentrations of unlabeled Bn. The cell concentration was adjusted to 0.1-1x10^6 cells/ml to assure that no more than 15% of the total added radioactive ligand bound. At the end of the incubation, 100 µl of cell suspension was centrifuged at 10,000 x g for 1 min to separate bound from unbound ligand. The pelleted bound ligand was washed twice with PBS containing 1% of bovine serum albumin and counted in a gamma-counter. Nonspecific binding was the amount of radioactivity associated with GRP-R transfected cells when the incubation mixture contained 1 µM Bn. Nonspecific binding was <15% of total binding in all experiments, and all values reported represent specific binding (i.e., total minus nonspecific binding). Analysis of binding data was performed using the least-square analysis curve-fitting program KELL (Munson and Rodbard, 1980), which permitted comparisons in mathematically derived receptor number (B_{max}) and affinity (K_D) of GRP-R wild-type and mutants.

Chronic Down-regulation of GRP-Rs

Chronic receptor down-regulation was defined as a decrease in cell surface binding after long-term agonist treatment as was assessed as described previously (Benya et al., 1994b). Briefly, cells were plated in Keeping Medium at a density of 4x10^6 cells/185 cm^2 flask. Twenty-four hours later medium was replaced with DMEM containing 2% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 300 mg/L G418 sulfate with or without 10 nM Bn. After incubation at 37°C for 24 hours, cells were washed and down-regulation was measured by assessing the binding of ^{125}I-[Tyr^4]Bn to compare the number of GRP-R per cell in Bn-treated and non-treated cells. Binding dose-inhibition curves of Bn-treated and non-treated cells were analyzed using the curve-fitting program KELL (Munson and Rodbard, 1980). Down-regulation of mutant GRP-R was compared

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to wild-type GRP-R down-regulation and expressed as the percentage of decrease in mathematically derived receptor number (B_{max}).

Internalization of GRP-Rs

Internalization was performed as described previously (Koenig et al., 1997; Benya et al., 1994b; Benya et al., 1994a) with minor modifications. Cells were transfected three days prior to the experiment and split in 24 wells two days prior to the experiment. On the day of the experiment cells were washed with PBS and preincubated for 15 min in binding buffer [24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 2.2 mM KH_2PO_4, 5 mM theophylline, 2 mM glutamine, 11 mM glucose, 1% (v/v) amino acid mixture, 1% (v/v) essential vitamin mixture, and 0.2% (w/v) bovine serum albumin] containing bacitracin (0.1%) and Sigma's protease inhibitor cocktail (1/1000). Cells were incubated with 50 pM ^{125}I-[Tyr^4]Bn in binding buffer for various times at 37ºC. After incubation, the 24 well plates were put on ice. Cells were washed twice with ice-cold PBS containing 1% BSA. One ml of acid-stripping solution (0.2 M acetic acid, 0.5 M NaCl) was added for 10 min at 4ºC. The supernatant after acid stripping was collected into the first set of counting tubes. Then PBS without calcium or magnesium was added and the aspirant saved in a second set of tubes to be counted. Finally, 250 µl of 0.05% trypsin/0.02% EDTA was added per well and after incubation for 15 min, the cells were added to the second set of tubes and both sets were counted using a CobraII Autogamma Counter (Packard, Sterling, VA). All data points were performed in duplicates. In all cases parallel incubations with unlabeled bombesin to determine nonspecific binding were performed. Internalization results are expressed as percentage of the total specific acid-resistant ^{125}I-[Tyr^4]Bn binding. Analysis of internalization data was done using the GraphPad Prism software and the approach of Koenig and Edwardson (Koenig and Edwardson, 1997). Equation #2 from their review paper (Koenig and Edwardson, 1997) was used: $R_s = 1/(k_e+k_r)\ [k_r (R_{s0}+R_{e0}) + (k_eR_{s0} - k_rR_{e0})\ \exp[-(k_e+k_r)t]$ where k_e is the constant of endocytosis, k_r is the constant of recycling, R_s is the number of receptors on the cell surface, R_e is the number of receptors in

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endosomes and t is the time of the incubation. R_{s0} (i.e., the amount of receptors in the endosomes at time t=0) was set to zero. This yielded $R_s = R_{s0}\ \{k_r+k_e \exp[-(k_e+k_r)t]\}/(k_e+k_r)$. Since $R_e = R_{s0} - R_s$ the % internalized receptor = 100 R_e/R_0 = 100 $(1 - R_s/R_{s0})$. This finally ends up in the relationship % internalized receptor = 100 $k_e\ [1-\exp[-(k_e+k_r)t]/(k_e+k_r)$. Nonlinear regression showed for the wild-type GRP-R a k_e of 0.19 ± 0.025/min and a k_r of 0.24 ± 0.040/min, which is in the range of other G protein-coupled receptors (Koenig and Edwardson, 1997). The time needed for 80% of maximal internalization (= t_{80}), expressed in minutes was then derived from the relationship $t_{80} = -\ln(0.2)/(k_e+k_r)$.

Measurement of [^{3}H]Inositol Phosphates

Changes in [^{3}H]IP were performed as described previously (Benya et al., 1994b). Briefly, cells were plated onto 24 well flasks at a density of 10^4 cells/well in Keeping Medium. 24 hours later cells were loaded with 3 µCi/ml myo-[2-^{3}H(N)]inositol in DMEM containing 2% (v/v) FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin for 24 hours. After washing, cells were then incubated with PBS and 20 mM LiCl for 15 minutes at 37ºC. Then cells were incubated with varying concentrations of Bn in IP buffer [135 mM NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 20 mM LiCl, 11.1 mM D-Glucose, 0.5% bovine serum albumin] at 37ºC. After 60 minutes the reaction was stopped with 1% (v/v) HCl in methanol. Total [^{3}H]IP was isolated by anion-exchange chromatography as described previously (Benya et al., 1994b). Briefly, free [^{3}H]inositol was removed by washing with 3 volumes of water. 5 mM disodium tetraborate in 60 mM sodium formate was used to remove [^{3}H]glycerophosphoryl inositol. Total [^{3}H]IP was eluted with 1 M ammonium formate in 100 mM formic acid. Eluates were assayed for their radioactivity after the addition of Hydro-Fluor scintillation fluid. All data points were performed in duplicate.

Preparation of Cell Membranes

For Gpp(NH)p studies cell membranes of Balb 3T3 cells transiently transfected with wild-type or mutant GRP-R constructs one day prior to membrane preparation were used.

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Disaggregated cells were resuspended in homogenization buffer (50 mM Tris, pH 7.4, 0.2 mg/ml soybean trypsin inhibitor, 2 mg/ml benzamidine and 1 mg/ml bacitracin). Cells were homogenized on ice using a Polytron (Beckman Instruments, Palo Alto, CA) at speed 6 for 30 seconds. The homogenate was centrifuged at 1500 rpm for 10 minutes in a Beckman GS-6KR centrifuge. The supernatant was recentrifuged in a Sorvall RC-5B Plus (SS-34 rotor) at 15,000 rpm for 20 minutes and the pellet was resuspended in homogenization buffer at a concentration of 0.5×10^6 cells/ml and stored at –20°C.

Binding of ^{125}I-[Tyr^4]Bn to Balb 3T3 Cell Membranes

To analyze the effect of the non-hydrolyzable guanine analogue Gpp(NH)p on binding of ^{125}I-[Tyr^4]Bn, cell membranes were incubated with 50 pM ^{125}I-[Tyr^4]Bn either alone or with 0.1 mM Gpp(NH)p in binding buffer (50 mM Hepes, 5 mM $MgCl_2 x6H_2O$, 1 mg/ml bacitracin and 1% BSA) for 60 minutes at room temperature. At the end of the incubation, 100 µl of cell suspension were added to 3 ml of cold PBS containing 1% BSA and immediately applied to a wet Whatman glass microfiber filter (GF/C) on a 12-well manifold (Millipore, Bedford, MA). The filter was washed twice with cold PBS containing 1% BSA and counted in a gamma-counter.

Statistics

Comparison of receptor affinity and receptor number between wild-type and mutant receptors were analyzed with the nonparametric Mann-Whitney U test. Comparisons of potency and efficacy for [^{3}H]IP generation between wild-type and mutant receptors were made using the Student's 2-tailed, paired *t*-test. Regression lines, correlation coefficients and their significance were calculated using a least-squares analysis. The time-course of internalization was analyzed using a curve-fitting program in GraphPadPrism 3 (San Diego, CA). The K_D and B_{max} of all receptors was calculated using a least-squares curve-fitting program KELL. P values smaller than 0.05 were considered significant.

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Results

The proximal part of the 2nd intracellular loop of all members of the Bn receptors contains a DRY motif (Fig. 1) which has been extensively studied (Benya et al., 1994a; Benya et al., 1995). Immediately distal to the DRY motif the central part of the 2nd intracellular loop (i2 loop) of the mouse gastrin-releasing peptide receptor (amino acids 142-148 of the GRP-R, Fig. 1) is well preserved among receptors of the Bn receptor family and a number of other G protein-coupled receptors (Fig. 1). This region of the i2 loop includes the conserved DRYXXV(I)XXPL motif (Fig. 1) (X is any amino acid and L is leucine or another bulky, hydrophobic amino acid) that has been shown to be important for coupling and internalization (Moro et al., 1993). To study the relevance of the amino acid residues of the central IC2 region of these receptors for receptor affinity, activation and receptor modulatory processes (internalization and chronic down-regulation) seven point mutants were made starting at alanine-142 of the GRP-R. Each individual amino acid was mutated to an alanine, except alanine-142 in the native GRP-R which was mutated to a serine.

Three different wild-type GRP-R clones that bound different amounts of radioligand were studied in detail. Affinity constants (K_D) for all 3 clones for Bn were close to 1 nM and results for the two clones with the lowest and highest receptor number are shown in Fig. 2 and Table 1. The receptor number (B_{max}) ranged between 353 and 1760 fmol/10^6 cells. Wild-type GRP-R clones #10 and #9 expressing 353 and 1760 fmol/10^6 cells, respectively, were used for further studies (Table 1). Both clones demonstrated similar potencies for Bn (EC_{50} 0.22-0.31 nM, Table 1) and stimulation by Bn resulted in similar efficacies for [^{3}H]IP generation, with each receptor showing a 9-10 fold increase (Table 2). These results suggest that differences in receptor number in this range do not result in alterations in either agonist affinity, potency or efficacy for activating phospholipase C. For each GRP-R mutation three to four stable clones were studied by binding studies and at least one stable clone for each was identified that expressed the GRP-R point mutation near the range of the two wild-type GRP-Rs (Table 1). Five of the seven single GRP-R mutants had a similar affinity to the wild-type GRP-Rs (i.e. 0.9-1.2 nM), however $I^{143}A$ and $M^{147}A$

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had a slight, approximately 2-fold decrease in affinity for GRP as assessed by binding of ^{125}I-[Tyr^4]Bn (1.7-1.8 nM, Table 1, Fig. 2).

Each of the single mutant GRP-Rs activated phospholipase C as assessed by their abilities to generate inositol phosphates (Table 1, Fig. 2). Six of the seven single amino acid GRP-R mutants had a similar potency to the wild-type GRP-R for stimulating an increase in [^{3}H]IP (EC_{50} 0.24-0.35 nM, Table 1), however the I^{143}A mutant demonstrated an approximate 2-fold decrease in potency (EC_{50} 0.7 nM, Table 1). Even though the GRP-R mutants demonstrated similar basal levels of [^{3}H]IP accumulation, the different single mutants showed a marked variation in efficacy for stimulating [^{3}H]IP (Table 2). Instead of the 10-fold increase in [^{3}H]IP caused by the wild-type GRP-Rs, less than a 3-fold increase was seen with the I^{143}A, V^{144}A, and the M^{147}A receptor mutants (Fig. 2, Table 2). Maximal [^{3}H]IP generation with the A^{142}S and the D^{148}A mutant was reduced to approximately 50% of that seen with the wild-type GRP-R receptor cells (Table 2).

Because of the effect of alterations of I^{143}A and M^{147}A on receptor affinity (Table 1) as well as their effects on maximal phospholipase C activation as well as that of V^{144}A, we made two double mutants, IM$^{143.147}$AA and VM$^{144.147}$AA. Each of the double mutants demonstrated only a 2-fold decrease in receptor affinity (Fig. 2, Table 1). However, the potency of the IM double mutant for stimulating an increase in [^{3}H]IP was more than 300-times lower than the wild-type GRP-R, whereas the potency of the VM double mutant was not altered (Fig. 2, Table 1). The efficacy for activating phospholipase C was markedly reduced in both GRP-R double mutants. The VM double mutant stimulated about 20% and the IM double mutant only 10% of the maximal IP increase seen with the wild-type GRP-R (Table 2).

To determine whether a difference in receptor-G protein-coupling contributed to the reduced ability of the GRP-R mutants to activate phospholipase C, we studied the effect of the non-hydrolzyable guanine analogue Gpp(NH)p on binding of ^{125}I-[Tyr^4]Bn to cell membranes of Balb 3T3 cells that transiently expressed either wild-type or mutant GRP receptors (Fig. 3). Increasing concentrations of Gpp(NH)p decreased ^{125}I-[Tyr^4]Bn binding to cell membranes of GRP-R

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expressing cells dose-dependently with a half-maximal effect at approximately 1 µM Gpp(NH)p and a maximal inhibition at 0.1 mM Gpp(NH)p (data not shown). Similar decreases of ^{125}I-[Tyr^4]Bn binding to the wild-type GRP-R were seen with membranes obtained from cells expressing the R145A mutant (Fig. 3). In contrast, the ^{125}I-[Tyr^4]Bn binding to membranes expressing either the I^{143}A, the M^{147}A or the IM double mutant was not (I^{143}A and IM double mutant) or significantly less (M^{147}A) inhibited by 0.1 mM Gpp(NH)p (Fig. 3).

Previous studies report that wild-type GRP-R is rapidly internalized (Benya et al., 1994b; Benya et al., 1994a). Studies in other GPCRs show the second intracellular loop amino acids can play an important role in mediating this process (Moro et al., 1994; Arora et al., 1995). Therefore, we investigated the ability of the various GRP-R mutants and the wild-type GRP-Rs to internalize radiolabeled Bn (Table 2, Fig. 4). Both Balb 3T3 cell clones, stably transfected with wild-type GRP-R, showed a maximal internalization of 46-47% of the bound radioligand after 60 minutes of incubation with ligand (Table 2, Fig. 4), with 80% of the maximal internalization seen at 4.6-5.7 minutes (Fig. 4, Table 2). Six of the point mutations did not significantly alter maximal internalization in the stable GRP-R mutants. However, with the R^{145}A point mutant, maximal internalization was significantly increased to 132% of the maximal seen with wild-type GRP-R internalization (Table 2, Fig. 4). All of the mutants demonstrated similar kinetics of internalization except for the M^{147}A mutant, which demonstrated a significant prolongation of the time needed to reach 80% of maximal (i.e. t_{80}), indicating a delayed internalization process (Table 2). The two double mutations demonstrate different effects on GRP-R internalization (Fig. 4, Table 2). Although the time needed to reach 80% of maximal internalization (i.e., t_{80}) of the R^{145} A mutant was not changed, the rate constant of recycling (k_r) (0.11 ± 0.01 vs. 0.24 ± 0.04, p <0.01), but not the rate of constant of endocytosis (k_e) (0.16 ± 0.01 vs. 0.19 ± 0.02, p=0.32), was significantly reduced by more than one-half relative to the k_r of the wild-type GRP-R. This result points to an alteration of the recycling process in this mutant as the actual cause of the overall increased maximal receptor uptake. I^{143} A and M^{147} A demonstrated a significant prolongation of their t_{80}

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values, indicating a delayed internalization process (Table 2). The two double mutations demonstrate different effects onGRP-R internalization (Fig. 4, Table 2). Both double mutants demonstrate a decrease in the maximal internalization compared to the wild-type GRP-R (Table 2, Fig. 4). The time to reach 80% of maximal internalization was significantly prolonged with both double mutants compared with the wild-type GRP-R (Table 2). In all of the GRP-R mutants with a prolonged t_{80} the k_e rate constant was significantly decreased relative to wild-type levels. Interestingly, in two of the kinetically altered GRP-R mutants (I^{143} A and M^{147} A) the k_r rate constant was also significantly decreased compared to the wild-type GRP-R (0.13 ± 0.01 and 0.10 ± 0.02 vs. 0.23 ± 0.04, p <0.015).

To ascertain that the changes in internalization were not artifacts of the selection process used to isolate stable clones, we repeated the experiments in Balb 3T3 cells transiently transfected with the wild-type GRP-R or with the selected mutants which had altered internalization in the stably transfected cells. Balb 3T3 cells that were transiently transfected with the wild-type GRP-R construct showed a maximal internalization of 31±3.5% of the radioligand at 120 minutes (Fig. 4, inset). The stimulatory effect on maximal internalization seen with the stable GRP-R mutants (Fig. 4) by the R145A mutation was confirmed in the transient GRP-R transfected cells (39±4% at 120 minutes) (Fig. 4, inset). The inhibitory effect of the IM double mutant (21±5.4% at 120 minutes) was also confirmed. No other single or double GRP-R mutant showed a significant effect on maximal internalization.

Previous studies demonstrate the wild-type GRP-R can undergo chronic down-regulation, however the molecular mechanism is largely unknown. To determine the possible importance of the GRP-R i2 loop in activating processes involved in mediating chronic down-regulation we determined the effect of these GRP-R mutations on chronic GRP-R down-regulation (Fig. 5). To assess down-regulation, after preincubation with Bn, homologous competitive binding assays were performed to assess remaining cell surface receptors. Similar to a previous study with the GRP-R (Benya et al., 1994b), no significant change in the affinity (K_D) of the receptor for bombesin was

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seen during down-regulation, demonstrating that the down-regulation reflects loss in cell membrane receptor protein. Wild-type GRP-R was down-regulated by 61.3±3.4% after a 24 hour incubation with Bn (Table 2, Fig. 5). The magnitude of the chronic down-regulation was altered in three of the seven single GRP-R mutants and in both double mutants (Table 2). In contrast to wild-type GRP-R, the $A^{142}S$, the $I^{143}A$ and the $M^{147}A$ GRP-R mutants showed a significant decrease (40-60% of control) in maximal down-regulation (Fig. 5, Table 2). Down-regulation of the VM double mutant also was decreased by 38% and the IM double mutation completely inhibited all down-regulation (Fig. 5, Table 2).

Previous studies (Benya et al., 1994a; Benya et al., 1993) have demonstrated that GRP-R internalization and down-regulation are at least partially dependent on phospholipase C activation. To analyze this relationship in more detail, we correlated the magnitude of maximal phospholipase C activation with the wild-type GRP-R and different GRP-R mutants as assessed by maximal stimulation of [^{3}H]IP with the extent of maximal internalization (Fig. 6, top panel) and extent of maximal receptor down-regulation (Fig. 6, bottom panel). There was only a weak (p=0.04) association between the maximal increase in [^{3}H]IP and the degree of internalization. This conclusion was further supported by the result that almost maximal internalization occurred with minimal changes in [^{3}H]IP, which was reflected in the low slope (i.e., 0.3 x) of the regression equation (Fig. 6, top panel). In contrast, the extent of maximal activation of phospholipase C showed a significant (p=0.008) correlation with the maximal degree of chronic GRP-R down-regulation (Fig. 6, bottom panel). This latter conclusion was further supported by the correlation of the different GRP-R mutants to stimulate maximal changes in these two processes which showed a proportional increase in [^{3}H]IP with increasing down-regulation (Fig. 6, bottom panel), resulting in a slope of 0.8 for the regression equation, which was not significantly different from unity.

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Discussion

The GRP-R is a G-protein coupled receptor that is a member of the mammalian Bn receptor family (Kroog et al., 1995a). Activation of the GRP-R, like other members of the Bn receptor family stimulates phospholipase C (PLC), phospholipase D and tyrosine kinases (Kroog et al., 1995a; Bunnett 1994; Rozengurt, 1988; Tsuda et al., 1997; Sinnett-Smith et al., 1993), as well as various receptor modulatory processes (receptor desensitization, internalization, down-regulation) (Benya et al., 1994c; Benya et al., 1994b; Benya et al., 1994a). Despite the importance of the GRP-R in many physiological and pathological processes (Tache et al., 1988; Bunnett 1994), little is known about receptor structural domains mediating the changes in intracellular mediators or receptor modulatory processes. Limited data exist from previous studies supporting the conclusion that the carboxyl terminus of the GRP-R, similar to a number of other GPCRs, is important in activating processes mediating internalization, but not down-regulation, chronic desensitizaton or activation of PLC (Benya et al., 1995; Benya et al., 1993). Furthermore, alterations in the 3rd intracellular loop (i3 loop) have been shown to have important effects on Bn receptor activation and both receptor internalization and down-regulation (Lameh et al., 1992; Tseng et al., 1995; Benya et al., 1995; Benya et al., 1994a). In other GPCRs the 2nd intracellular loop (i2 loop) of the receptor is involved not only in mediating receptor activation, but also in receptor internalization (Wong et al., 1990; McClue et al., 1994; Thompson et al., 1998; Schreiber et al., 1994). Studies of the GRPR in this region have mainly focused on the i2 loop's N-terminal DRY motif which is present in many GPCRs and which is shown to be important for receptor activation and internalization in a number of G-protein coupled receptors (Fraser et al., 1988; Fraser et al., 1989; Wang et al., 1991; Jones et al., 1995; Benya et al., 1994a). However, a few recent studies reveal that hydrophobic residues in the central part of the i2 loop can be important for some GPCRs for G-protein coupling and to stimulate receptor internalization (Moro et al., 1993; Modrall et al., 2001; Arora et al., 1995; Moro et al., 1994). Nothing is known of the importance of this region in GRP receptors. Therefore, the aim of the present study was to examine the importance of the central

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portion of the i2 loop of the GRP-R for mediating activation of PLC, receptor internalization and chronic receptor down-regulation, using receptor mutagenesis.

In general, a number of our findings support the conclusion that alterations in the central portion of the GRP-R i2 loop have a minimal effect on receptor affinity, however this receptor region was important for activation of intracellular mediators. First, of the 7 receptor mutations of the central portion of the GRP-R i2 loop examined only two showed a small decrease (i.e. <2-fold) in receptor affinity and/or potency for activating the receptor and stimulating PLC activity. Second, even two double mutants of the two amino acids showing a small decrease in receptor affinity when present alone, had a minimal effect on receptor affinity. Third, one double mutant [$IM^{143.147}AA$], however, demonstrated a greater than 300-fold decrease in potency for activating PLC demonstrated these amino acids in concert can play an important role in coupling of the GRP receptor to PLC. Fourth, five of the single mutants and each of the double mutants showed a decreased efficacy for activating PLC, demonstrating the importance of this receptor region for maximal coupling and activation of this intracellular receptor cascade. Of note, it was shown for all the GRP receptors studied, that the likely cause for the decreased coupling to PLC is altered receptor-G protein coupling. This conclusion is supported by the comparison of the effect of Gpp(NH)p on the different mutants with the change in binding affinity. Each of the GRP-R mutants examined showing a decrease in affinity had defective G-protein coupling, whereas the GRP-R mutants without altered affinity, retained G-protein coupling.

Our results of effects of mutations in the GRP-R's i2 loop on binding to Bn and on the potency have both similarities and differences to data coming from the literature. The findings that the single mutants $I^{143}A$ and $M^{147}A$ [GRP-R] and both double mutants reveal not only a slight but significant shift in affinity, but also are uncoupled from the G protein and thereby from PLC, corresponds well with Lefkowitz and Costa's ternary complex model (Lefkowitz et al., 1993). This model predicts a diminished affinity of the receptor to the agonist for the case of a decreased G-protein coupling ability of the receptor. These data also agree with results on corresponding

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mutations in the histamine H2 receptor (Smit et al., 1996) and the angiotensin II receptor (Modrall et al., 2001) that show a decrease in affinity to agonist binding as well as a decreased ability to stimulate PLC. In contrast to our result, with the $M^{147}A$ mutant in the GnRH receptor (Arora et al., 1995), the thromboxane A2 receptor (Zhou et al., 1999), and the angiotensin II receptor (Modrall et al., 2001), a corresponding mutation to our $M^{147}A$ caused either no change or a small decrease in affinity relative to the wild-type receptor. There are no data in the literature to compare our finding that two of our mutants, the $I^{143}A$[GRP-R] and the $IM^{143.147}AA$ double mutant, cause a shift in the potency for receptor activation. However, there are studies on corresponding mutations to $M^{147}A$[GRP-R] in the m1 muscarinic cholinergic receptor (Shockley et al., 1997) and in the histamine H2 receptor (Smit et al., 1996) that both found decreases in potency for activation of PLC and adenylyl cyclase, respectively. In contrast, we did not find a significant alteration in potency for activating PLC with this mutation in the GRP-R. Several studies in other GPCRs have investigated the effect of i2 loop mutations on the efficacy of the ligand-stimulated 2nd messenger production. Our findings are in agreement with results of studies on corresponding mutations in the histamine H2, GnRH, thromboxane A2, angiotensin II and the human m5 muscarinic cholinergic receptor (Smit et al., 1996; Arora et al., 1995; Zhou et al., 1999; Modrall et al., 2001; Burstein et al., 1998). From studies of the m1 muscarinic and β2-adrenergic receptor mutants, Moro et al., (Moro et al., 1993; Moro et al., 1994) proposed a new motif for G-protein coupling of these receptors [DRYXXV(I)XXPL, where X is any amino acid and L is leucine or another bulky, hydrophobic amino acid]. In the GRP-R the Ile-143 and Met-147 correspond to the V(I) and the L in this motif. Our findings that the $I^{143}A$ and $M^{147}A$ single mutants as well as the IM double mutant have markedly reduced efficacy for stimulation of PLC are in agreement with this model. There is only one study (Blin et al., 1995) that has investigated residues next to the hydrophobic residues discussed above, such as Ala-142, Val-144 and Asp-148, which we found are also important for maximal efficacy for PLC activation. In this study (Blin et al., 1995) corresponding mutant to our $A^{142}S$[GRP-R] in the m3 muscarinic cholinergic receptor suggested a role for this residue in G-

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protein coupling. Interestingly, in our study Pro-146, which is well conserved in this position in a number of GPCRs (Fig. 1), did not show any changes in receptor affinity or activation when mutated to alanine. This is in agreement with data from studies on the m1 muscarinic cholinergic receptor (Moro et al., 1993), but is different from data on the m5 muscarinic cholinergic receptor (Burstein et al., 1998). The notion that several adjacent residues of the i2 loop have a function in coupling to signaling processes downstream of the GRP receptor, agrees with the recent findings on the protein structure of GPCRs in which it was discussed that the i3 loop interacts with G proteins and that the i2 loop is in close contact to the i3 loop in the ground state of the GPCR and loops away once the receptor is activated (Spalding and Burstein, 2001; Palczewski et al., 2000; Zeng et al., 1999). Mutations in the i2 loop could, therefore, interfere with the dynamic actions of the i2 loop and thereby decrease the GPCR's capability to interact properly with the G protein. This proposal is further supported by our preliminary 3-D modeling data of the GRP-R using the rhodopsin 3D structure as a template (data not shown). Residues Ile-143 and Val-144 of the i2 loop are located close to the N-terminal part of the 3rd intracellular loop at a distance that could allow interactions to occur.

After activation, wild-type GRP-R similar to other GPCRs undergoes internalization (Benya et al., 1994b; Pandol et al., 1982; Benya et al., 1994a), down-regulation (Benya et al., 1994b; Benya et al., 1995; Benya et al., 2000) and desensitization (Benya et al., 1994b; Benya et al., 1995; Benya et al., 2000). Our results show that amino acid residues of the central portion of the i2 loop play an important role in mediating both internalization and down-regulation of the GRP-R. Alterations of 3 residues (R^{145}A, M^{147}A, $IM^{143.147}$AA) resulted in changes in internalization rate and/or magnitude. Whereas no single alteration of a residue decreased the magnitude of maximal internalization the alteration of one residue (Arg-145) caused an increase in maximal internalization suggesting that the presence of Arg-145 was having a restraining effect on internalization in the wild-type receptor. Interestingly, computer fitting of the internalization data of the R^{145} A mutant using the models of Koenig et al. (Koenig and Edwardson, 1997) revealed that the rate constant of

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recycling (k_r) but not the rate constant of endocytosis (k_e) was significantly reduced by more than one-half relative to the k_r of the wild-type GRP-R. This result points to an alteration of the recycling process in this mutant as possibly contributing to the overall increased maximal receptor internalization seen. There are no data in the literature about an effect of a corresponding mutation in another receptor on internalization. However, using the receptor selection and amplification technology (R-SAT) on the i2 loop of the m5 muscarinic cholinergic receptor, Burstein et al., suggested a pivotal role of the corresponding residue to Arg-145 in switching between active and inactive conformations of this receptor (Burstein et al., 1998). Since in their hands substituting this residue by any amino acid rendered the m5 cholinergic receptor constitutively active, they hypothesized that the native arginine residue in this position is important for "switching off" the receptor. We show that Arg-145 of mutated GRP-R is neither changed in the potency nor the efficacy for PLC activation. Nevertheless, our finding that this residue inhibits internalization in combination with data of Burstein et al., (Burstein et al., 1998) is of interest in the light of recent findings that the process of receptor endocytosis by itself modulates the activation of intracellular growth cascades (Pierce and Lefkowitz, 2001). The inhibitory effect of the mutations on the Ile-143 and the Met-147 – seen in both single and double mutants – corresponds well with studies on the m1 muscarinic cholinergic, the β2-adrenergic and the GnRH receptor (Moro et al., 1994; Shockley et al., 1997; Arora et al., 1995). In contrast, a study on the angiotensin II receptor showed no effect on internalization (Modrall et al., 2001). The greater effect on internalization of the Ile-143 and the Met-147 mutations together, than either alone on internalization rate and magnitude, suggests a synergistic role of these amino acid residues and is in agreement with data on the m1 muscarinic cholinergic receptor (Moro et al., 1994). The changed kinetics of the internalization process in these mutants (i.e., the prolonged t_{80} values) correlated well with significantly decreased endocytic rate constants (k_e) in all of these mutants. Interestingly, the single mutants also showed receptor constants (k_r) that apparently had a smaller impact on the t_{80} time than the decreased k_e constants.

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Chronic receptor down-regulation describes the agonist-dependent loss of receptor binding sites on the cell membrane after long-term incubation with the agonist and leads to an attenuation of the signal transduction by this receptor. Different molecular mechanisms in concert account for this process, including intracellular degradation of receptor protein, long-term storage of receptors in intracellular vesicles and various mechanisms of regulation of receptor mRNA levels (von Zastrow, 2001). Studies that examine the structure-function relationship of chronic receptor down-regulation are rare. In our study alterations of 3 residues alone ($A^{142}S$, $I^{143}A$, $M^{147}A$) or in combination ($IM^{143.147}AA$) reduced the ability of the GRP-R to undergo chronic down-regulation. Our results with the M^{147} mutation in the GRP-R differ from those with the angiotensin II receptor (Modrall et al., 2001) and histamine H2 receptor (Smit et al., 1996) where a comparable mutation did not reduce the extent of chronic receptor down-regulation. However, they are similar to results of a comparable $I^{143}A$ mutation, $M^{147}A$ mutation or double mutation ($IM^{143.147}AA$) in the m1 human muscarinic cholinergic receptor (Shockley et al., 1997). These results demonstrate that in addition to serines, threonines and a PKC consensus sequence in the COOH terminus of the GRP-R (Benya et al., 1993), residues in the i2 loop of the GRP-R are important in mediating chronic down-regulation as well as receptor internalization.

The current study of the GRP-R structure-function of the i2 loop provides some insights into the possible relationship between GRP-R internalization and chronic down-regulation as well as the possible role of PLC activation in mediating these two receptor modulatory processes. With the β2-adrenergic receptor chronic receptor down-regulation was proposed to be important in mediating chronic desensitization, whereas both of these processes were proposed to be independent of receptor internalization (Proll et al., 1993). One study proposed that GRP-R internalization mediates chronic receptor down-regulation and desensitization (Pandol et al., 1982). However, other studies involving mutant GRP-Rs, wild-type GRP-R and the closely related NMB-R propose that chronic GRP-R down-regulation is closely linked to chronic desensitization, whereas internalization is likely not coupled to these processes (Benya et al., 2000; Benya et al.,

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1994c). Support is provided in our study for GRP-R internalization and chronic down-regulation not being closely coupled processes because of the different effects of i2 loop mutations on the two receptor modulatory processes. Whereas the magnitude of down-regulation was decreased by various single mutations ($I^{143}A$, $V^{144}A$, $M^{147}A$), the magnitude of internalization was unaltered. Conversely, the $R^{145}A$ mutation augmented internalization, whereas it had no effect on chronic down-regulation. Conflicting results are reported on the role of 2nd messengers and phosphorylation in mediating receptor internalization and down-regulation. Studies on both β_2-adrenergic receptors (Strader et al., 1987; Cheung et al., 1989; Cheung et al., 1990; Campbell et al., 1991) and PLC-linked muscarinic cholinergic receptors (Lameh et al., 1992; Thompson and Fisher, 1990) have provided differing results for their role in mediating internalization, as have studies investigating the relationship between PLC activation and EGF-R internalization (Chen et al., 1989; Chen et al., 1987; Honegger et al., 1987). With β-adrenergic receptor, receptor phosphorylation may play an important role in each of these receptor modulating processes. A previous study showed GRP-Rs deficient in COOH terminal phosphorylation sites have impaired internalization (Benya et al., 1993). Studies on the GRP-R examining agonist-dependent phosphorylation of the receptor revealed a rapid, non-protein kinase C- (PKC) dependent phase of phosphorylation (<10 min) that can be differentiated from a phase that is PKC-triggered late phosphorylation (Kroog et al., 1995b). *In vitro* studies suggested that the best candidate for the rapid phase of phosphorylation is G protein-coupled receptor kinase 2 (GRK2) (Kroog et al., 1999). Since >90% of the internalization process has reached its maximum by 10 min, GRK2 would be a good candidate for the GRP-R phosphorylation that is necessary for the internalization process to occur. On the other hand chronic receptor down-regulation likely is a process that requires PKC-mediated phosphorylation of the GRP receptor since this process has much slower kinetics than from internalization (Benya et al., 1994b), and mutation of the C-terminal PKC consensus site abolished this process (Benya et al., 1995). Studies of the wild-type GRP-R as well as various mutant GRP-Rs have proposed that GRP-R chronic down-regulation and internalization are mediated by

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different intracellular mediators and PLC activation is not equally important in both of these receptor modulatory processes (Benya et al., 1995; Benya et al., 1993; Benya et al., 1994a; Benya et al., 2000). Using GRP-R mutants that do not activate PLC-cascades, GRP-R mutants with PKC consensus site lacking, various COOH-terminal truncation GRP-R mutants and various glycosylation-deficient GRP-R mutants (Benya et al., 1995; Benya et al., 1993; Benya et al., 1994a; Benya et al., 2000) it has been shown that maximal PLC activation is not needed for maximal internalization. The results in the present study provide support for these proposals. In the present study a GRP-R mutant ($IM^{143.147}AA$) with only 11% of the wild-type's ability to activate PLC was able to reach more than 70% of the maximal internalization of the wild-type GRP-R (Benya et al., 1995; Benya et al., 1993; Benya et al., 1994a; Benya et al., 2000). Although there was a strong correlation (r=0.95) between the maximal stimulation of total IP with the rate of endocytosis (rate constant k_e), there was only a weak correlation (p=0.04) between the extent of GRP-R internalization and the maximal extent of phospholipase C activation for the various i2 loop mutants. Furthermore, almost maximal internalization (i.e., 90%) occurred with minimal changes in PLC activity. In contrast, the extent of maximal activation of phospholipase C and the maximal degree of chronic receptor down-regulation demonstrated a highly significant relationship (p=0.008) and the slope of the repression equation was almost unity, demonstrating progressive increases in PLC activity resulted in similar increases in down-regulation.

In conclusion, we show that amino acid residues of the central portion of the i2 loop of the GRP-R are important in coupling the receptor to G proteins and to PLC, in mediating receptor internalization and chronic receptor down-regulation; however, they are not essential for high affinity receptor binding. Our results are consistent with the proposal that maximal activation of the PLC cascade by GRP-R is essential for chronic GRP receptor down-regulation, but not or only minimally required in GRP receptor internalization. They are also consistent with the proposal that chronic GRP receptor down-regulation is not mediated by GRP receptor internalization and these are not coupled processes.

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TABLE 1 Mutant and native GRP-R receptor affinities, receptor numbers, and potencies for stimulation of [^{3}H]IP

GRP-R	Binding		[^{3}H]IP
	K_D [nM]	B_{max} [fmol/10^{6}cells]	EC_{50} [nM]
Wild-type #10	1.0 ± 0.1	353 ± 39	0.31 ± 0.04
Wild-type #9	0.9 ± 0.2	1760 ± 264	0.22 ± 0.02
$A^{142}S$	0.9 ± 0.1	605 ± 55	0.30 ± 0.01
$I^{143}A$	1.8 ± 0.3 **	739 ± 96	0.70 ± 0.03 *
$V^{144}A$	1.0 ± 0.2	177 ± 128	0.25 ± 0.02
$R^{145}A$	1.2 ± 0.2	459 ± 50	0.31 ± 0.01
$P^{146}A$	0.7 ± 0.1	540 ± 76	0.24 ± 0.02
$M^{147}A$	1.7 ± 0.3 **	1530 ± 230	0.31 ± 0.03
$D^{148}A$	1.1 ± 0.2	550 ± 72	0.35 ± 0.01
$IM^{143.147}AA$	1.9 ± 0.2 **	166 ± 12	>100 **
$VM^{144.147}AA$	2.2 ± 0.3 **	458 ± 46	0.45 ± 0.05

Studies were performed as described in Fig. 2 legend. Receptor affinity (K_D) for Bn and receptor number (B_{max}) were calculated using the least squares curve-fitting program KELL (Munson and Rodbard, 1980). EC_{50} is the concentration necessary for half-maximal increases in [^{3}H]inositol phosphates caused by Bn calculated using the curve-fitting program Kaleidograph. Data are expressed as the mean ± SEM of at least three separate experiments. (* $p<0.05$, ** $p<0.01$, compared to wild-type #10)

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TABLE 2 Efficacy for stimulation of [^{3}H]IP, internalization, and down-regulation of wild-type and mutant GRP receptors

GRP-R	[^{3}H]IP	Internalization		Down-regulation
	Fold increase over basal	Maximal % internalized	t_{80} [min]	[% of wild-type]
Wild-type #10	9.6 ± 2.6	47 ± 2	5.7 ± 0.3	100
Wild-type #9	10.1 ± 1.4	46 ± 3	4.6 ± 0.8	100
A^{142}S	6.0 ± 1.1 *	43 ± 2	5.7 ± 0.7	64 ± 5 *
I^{143}A	2.6 ± 0.5 *	45 ± 3	7.0 ± 0.6	52 ± 7 *
V^{144}A	4.2 ± 0.2 *	40 ± 4	5.4 ± 1.2	84 ± 7
R^{145}A	8.6 ± 2.6	62 ± 3 *	6.3 ± 0.1	106 ± 6
P^{146}A	7.3 ± 1.4	48 ± 1	4.7 ± 0.1	80 ± 8
M^{147}A	1.9 ± 0.8 **	42 ± 1	9.5 ± 1.2 *	42 ± 12 **
D^{148}A	5.0 ± 0.6 *	49 ± 2	4.5 ± 0.2	123 ± 23
IM$^{143.147}$AA	1.1 ± 0.3 **	25 ± 3 *	12.6 ± 2.9 *	0 ± 0 **
VM$^{144.147}$AA	2.0 ± 0.9 *	38 ± 4 *	8.5 ± 1.5 *	62 ± 7 **

Stimulation of increases in [^{3}H]IP was determined as described in Fig. 2. The efficacy of [^{3}H]IP stimulation is expressed as the fold increase over basal in total cellular [^{3}H]IP determined as described in Fig. 2 legend. Internalization was assessed as described in Fig. 4 and is expressed as the percentage of total saturable bound ^{125}I-[Tyr^4]Bn that was not removed by acid-stripping. T_{80} is the time in minutes that was needed for internalizing 80% of maximal calculated as described in Methods. Down-regulation is expressed as the percent decrease in the receptor number (*Bmax*) of cells pretreated with 10 nM Bn compared with

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control cells processed in parallel. Wild-type GRP-R were down-regulated 61±3% compared to cells not treated with 10 nM Bn. Data are expressed as the mean ± SEM of at least three separate experiments and each experiment each point was determined in duplicate. (* $p<0.05$, ** $p<0.01$, compared to wild-type GRP-R.)

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FIGURE 1. Alignment of amino acid sequences of the 2nd intracellular loop region of Bn and various G protein-coupled receptors. The proximal and central portions of the second intracellular loop (i2) and adjacent transmembrane domain III (TM III) are shown. Alignment was performed using the GAP algorithm (Needleman and Wunsch, 1970). The amino acid residues in bold letters in the mouse GRP-R were mutated in this study.

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FIGURE 2. Ability of Bn to inhibit binding of [^{125}I-Tyr^{4}]Bn to mutant and wild-type GRP-Rs and stimulate generation of [^{3}H]IP. Results from stably transfected Balb 3T3 cells expressing wild-type and four representative mutant GRP-Rs are shown. **Top panel:** Cells (0.1-1x10^{6}/ml) were incubated with 50 pM [^{125}I-Tyr^{4}]Bn alone or with the indicated concentrations of non-radioactive Bn for 60 minutes at room temperature. Data are expressed as the percentage of specifically bound radioactivity in the absence of non-radioactive peptide. For each experiment, values were determined in duplicate. Results are shown as the mean ± SEM of at least three separate experiments. **Bottom panel:** After loading the cells with [^{3}H]myoinositol (1.5 µCi/well) cells were incubated for 60 minutes at 37°C with the indicated concentrations of Bn and total [^{3}H]IP assessed as described in METHODS. Data are expressed as the mean ± SEM of the fold increase over the basal value in dpm from at least three separate experiments. Two wild-type GRP-Rs (#9, #10) are shown which differ 5-fold in receptor number (see Table 1).

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FIGURE 3. Effect of the non-hydrolyzable guanine analogue Gpp(NH)p on binding of ^{125}I-[Tyr^{4}]Bn to Balb 3T3 cell membranes expressing either wild-type or mutant GRP receptors. Cell membranes of 0.5x10^{6} cells/ml were incubated with 50 pM ^{125}I-[Tyr^{4}]Bn alone or with 0.1 mM Gpp(NH)p for 60 minutes at room temperature. Results were expressed as % change of specific binding to ^{125}I-[Tyr^{4}]Bn in the absence of unlabeled Bn. Data are expressed as the mean ± SEM of five independent experiments and in each experiment each value was determined in duplicate. (* $p<0.01$, compared to wild-type GRP-R.)

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FIGURE 4. Internalization of [^{125}I-Tyr^{4}]Bn by Balb 3T3 cells stably transfected with mutant and wild-type GRP-Rs. Stably or transiently (insert), and transiently (insert) GRP-R transfected cells (0.1-1x10^{6}/ml) were incubated with 50 pM [^{125}I-Tyr^{4}]Bn for the indicated times and the percent of total specific binding internalized determined using acid-stripping as outlined in METHODS. Data are expressed as the mean ± SEM of the percentage of total specifically bound [^{125}I-Tyr^{4}]Bn that was not removed by acid-stripping from four separate experiments and in each experiment each value was determined in duplicate. In the stably transfected cells the best fit of the mean time-course was generated for each GRP-R mutant and the wild-type receptor using GraphPad Prim 3 as described in Methods.

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FIGURE 5. Chronic down-regulation of wild-type GRP-R and mutant GRP-Rs.

Twenty-four hours after plating, cells were incubated with or without 10 nM Bn for an additional 24 hours at 37°C and then subjected to competitive binding experiments with 50 pM [^{125}I-Tyr^{4}]Bn alone or with the indicated concentrations of non-radioactive Bn. Data are expressed as the percentage of maximal specifically bound radioactivity in the absence of non-radioactive Bn in control cells (i.e. not treated). For each experiment, values were determined in duplicate. Results are shown as the mean ± SEM of at least three separate experiments.

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FIGURE 6. Correlation of the magnitude of maximal increase in [^{3}H]IP with the magnitude of maximal internalization and chronic down-regulation for the various GRP-R mutants. For correlation analysis the magnitude of maximal [^{3}H]IP generation, internalization, and down-regulation for each GRP-R mutant was expressed as the percentage of wild-type. The regression equation that best fit the data and the significance of the correlation are shown, which were calculated by least-square analysis using GraphPad Prism software as described in Methods. Data from each of the two wild-type GRP-Rs and the nine GRP-R mutant receptors in Table 1 are shown. [^{3}H]IP, maximal internalization and down-regulation data are from Tables 1 and 2. The equations of the regression equation and *p* values are shown for [^{3}H]IP versus internalization (r=0.64) and for [^{3}H]IP versus down-regulation (r=0.75).

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Figure 1

		TMIII					i2										
		132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148
GRP	mouse	L	T	A	L	S	A	D	R	Y	K	**A**	**I**	**V**	**R**	**P**	**M**	**D**
GRP	human	L	T	A	L	S	A	D	R	Y	K	A	I	V	R	P	M	D
GRP	rat	L	T	A	L	S	A	D	R	Y	K	A	I	V	R	P	M	D
NMB	rat	L	T	A	L	S	A	D	R	Y	R	A	I	V	N	P	M	D
NMB	human	L	T	A	L	S	A	D	R	Y	R	A	I	V	N	P	M	D
BRS3	human	L	T	I	L	S	A	D	R	Y	K	A	V	V	K	P	L	E
BRS3	guinea pig	L	T	I	L	S	A	D	R	Y	K	A	V	V	K	P	L	E
BB4	frog	L	T	V	L	S	A	D	R	Y	R	A	I	V	K	P	L	Q
muscarinic m1	human	L	L	L	I	S	F	D	R	Y	F	S	V	T	R	P	L	S
muscarinic m3	human	L	L	V	I	S	F	D	R	Y	F	S	I	T	R	P	L	T
beta2-adrenergic	human	L	C	A	I	S	L	D	R	Y	W	A	V	S	R	A	L	E
Histamine H2A	human	L	C	A	I	S	L	D	R	Y	V	A	I	Q	N	P	I	H
Angiotensin II type I	rat	L	T	C	L	S	I	D	R	Y	L	A	I	V	H	P	M	K
GnHR	human	M	V	V	I	S	L	D	R	S	L	A	I	T	R	P	L	A
Thromboxane	human	S	A	A	M	A	S	E	R	Y	L	G	I	T	R	P	F	S

Figure 2

120
100
80
60
40
20
0
[^{125}I-Tyr^{4}]Bn BINDING [% of specific binding]
I143A
IM143.147AA
M147A
VM144.147AA
Wild-type #10
A142S
Wild-type #9
−11
−10
−9
−8
−7
−6

12
10
8
6
4
2
0
[^{3}H]IP GENERATION [fold over basal]
Wild-type #10
Wild-type #9
A142S
I143A
M147A
IM143.147AA
−11
−10
−9
−8
−7
Bn [log M]

Figure 3

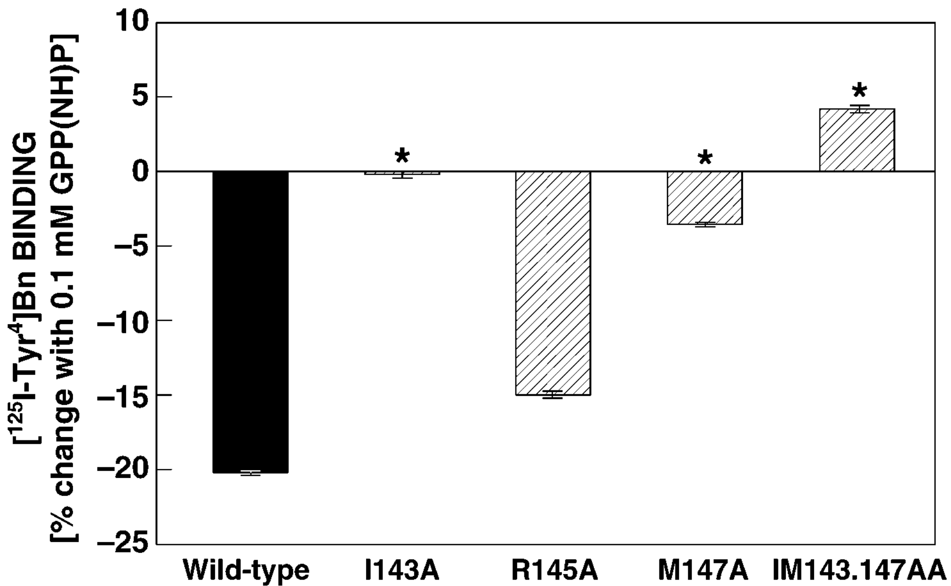

Figure 4

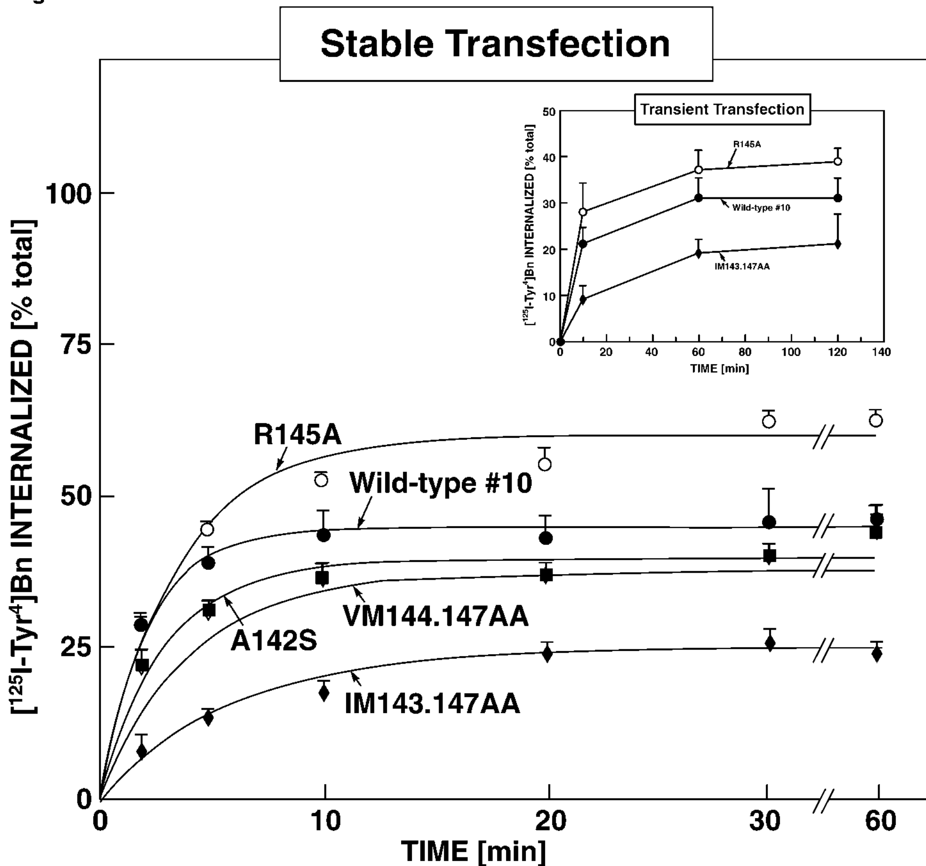

Figure 5

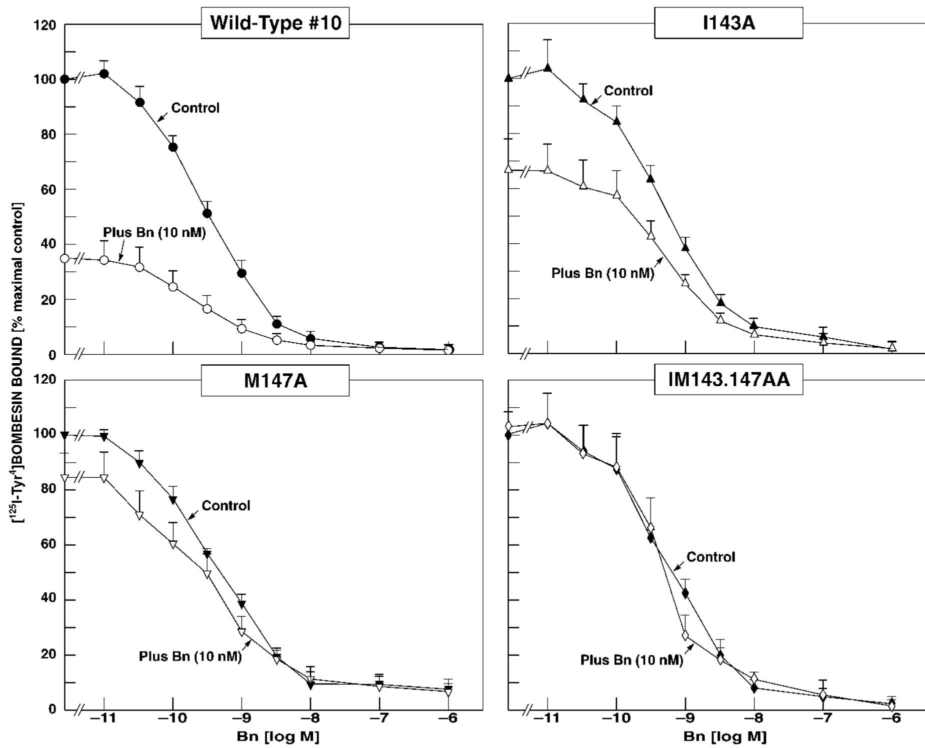

Figure 6