0026-895X/00/061490-12$3.00/0
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 58:1490–1501, 2000

Vol. 58, No. 6
247/864834
Printed in U.S.A.

Glycosylation of the Gastrin-Releasing Peptide Receptor and Its Effect on Expression, G Protein Coupling, and Receptor Modulatory Processes

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Received April 28, 2000; accepted August 17, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Many gastrointestinal G protein-coupled receptors are glycosylated; however, which potential glycosylation sites are actually glycosylated and their role in receptor transduction or receptor modulation (internalization, down-regulation, desensitization) is largely unknown. We used site-directed mutagenesis to address these issues with the gastrin-releasing peptide receptor (GRP-R). Each of the four potential glycosylation sites was mutated by converting the Asn (N) to Gln (Q). Transient expression in CHOP cells demonstrated that changing Asn^{24} or Asn^{191} inhibited GRP-R cell surface expression, whereas elimination of Asn^{5} and Asn^{20} had no effect. Using ligand cross-linking studies in stable mutants expressed in Balb 3T3 cells, all four potential extracellular sites were glycosylated with carbohydrate residues of approximately 13 kDa on Asn^{5}, 10 kDa on Asn^{20}, 5 kDa on Asn^{24}, and 9 kDa on Asn^{191}. Removal of three glycosylation sites (N5,20,24,Q mutant) did not alter receptor affinity or G protein coupling; therefore, it could be speculated that deglycosylation at Asn^{191} might be responsible for the altered G protein coupling seen with complete enzymatic deglycosylation of the native receptor previously reported. Removal of any single glycosylation site did not interfere with GRP-R induced chronic desensitization or down-regulation. However, elimination of all three NH_2-terminal sites (N5,20,24) markedly attenuated both processes, with no effect on acute homologous desensitization and with only a minimal alteration of GRP-R internalization, supporting the findings of other studies that suggest that chronic desensitization and down-regulation are functionally coupled, distinct from acute desensitization and distinct from internalization. These data show that separate and specific glycosylation sites are important for GRP-R trafficking to the cell surface, ligand binding, G protein coupling, chronic desensitization, and down-regulation.

The tetradecapeptide bombesin and its family of related peptides have diverse physiological effects in humans including the regulation of circadian rhythm and body temperature and are involved in stimulating pancreatic secretion, inducing the release of many gastrointestinal hormones (Tache et al., 1988), and mediating smooth muscle contraction, satiety, and chemotaxis (Jensen et al., 1988; Tache et al., 1988). Bombesin-related peptides also generate growth effects in normal and tumorous tissues (Tache et al., 1988), and these peptides can act as autocrine growth factors in some human small cell lung cancer cell lines (Tache et al., 1988). Two mammalian bombesin receptors were cloned and include the gastrin-releasing peptide receptor (GRP-R) and the neuromedin B receptor (NMB-R) (Battey and Wada, 1991). These phospholipase C-activating receptors are widely distributed in the central nervous system and gastrointestinal tract and are closely related proteins, sharing 56% homology at the amino acid level (Battey and Wada, 1991). However, these receptors differ in their pharmacology, expression patterns, and abilities to alter the biological activities of different tissues (Jensen and Coy, 1991; Kroog et al., 1995). The GRP-R and the NMB-R also vary in the extent of their glycosylation (Kusui et al., 1994).

Glycosylation is a common post-translational modification of seven transmembrane-spanning, G protein-coupled receptors. Although glycosylation generally has been shown to be important for protein folding, trafficking, and targeting of the receptor to the cell membrane (Rands et al., 1990; Giovannelli et al., 1991; Kuwano et al., 1991; Russo et al., 1991;

This work was supported partially by National Institutes of Health Grant DK51168 and a Veterans Affairs Merit Review (to R.V.B.).

ABBREVIATIONS: GRP-R, gastrin-releasing peptide receptor; NMB-R, neuromedin B receptor; FSH, follicle-stimulating hormone; LH, luteinizing hormone; DMEM, Dulbecco's modified essential medium; Gpp(NH)p, guanosine 5-($\beta_1\gamma$-imido)-triphosphate tetralithium salt; [^{3}H]IP, [^{3}H] inositol phosphates (total); VIP, vasoactive intestinal peptide; TSH, thyroid-stimulating hormone; DSS, disuccinimidyl suberate; PNGase F, peptide *N*-glycosidase; β_2-AR, β_2-adrenergic receptor.

Petaja-Repo et al., 1993; Lanctot et al., 1999; Ray et al., 1999; Wheatley and Hawtin, 1999), its role in other receptor functions is still unclear. In some receptors, glycosylation is reported to be important in agonist affinity and receptor G protein coupling (Wheatley and Hawtin, 1999). However, no consistent pattern has emerged to define the role of receptor carbohydrate in regulating these processes. For example, glycosylation of the M1, M2, and M4 muscarinic cholinergic receptors (Ohara et al., 1990; van Koppen and Nathanson, 1990), the angiotensin$_1$ receptor (Lanctot et al., 1999), and the β_2-adrenergic receptor (Rands et al., 1990) is not necessary for high-affinity agonist binding or G protein coupling; one or both of these processes are regulated by glycosylation of the receptors for VIP (el Battari et al., 1991), human calcitonin (Ho et al., 1999), and various growth factors (Soderquist and Carpenter, 1984). Furthermore, in some receptors, specific carbohydrate residues are found to be critical, such as with the TSH receptor (Russo et al., 1991), where alteration of only one of the six amino-terminal glycosylation consensus sequences was found to regulate high-affinity agonist binding.

In contrast to the effects of receptor glycosylation on receptor affinity and G protein coupling, which have been well studied, little is known about the role of these carbohydrate residues in modulating the receptor consequent to its activation by agonist. Indeed, the role of glycosylation in modulating receptor desensitization, internalization, and down-regulation is almost completely unknown. The importance of glycosylation in mediating down-regulation has not been studied and for modulating internalization and desensitization has been studied for only one of the seven transmembrane-spanning, G protein-coupled receptors. This study, which used chemical methods to alter receptor carbohydrate, suggested a role for glycosylation in mediating internalization of the vasopressin V2 receptor (Jans et al., 1992). A study (Giovannelli et al., 1991) on the nicotinic cholinergic receptor demonstrated that using chemical methods to inhibit receptor glycosylation also altered their ability to undergo desensitization. Studies relying on inhibitors of glycosylation need to be interpreted with caution, however, because one study suggests that tunicamycin, an agent commonly used to inhibit receptor glycosylation during its post-translational modification in the Golgi, alters the desensitization profile of the muscle cholinergic receptor independently of its ability to modify receptor carbohydrate (Nishizaki and Sumikawa, 1992).

Although the receptors for neurotransmitters, such as adrenergic and cholinergic agents (Ohara et al., 1990; Rands et al., 1990; Giovannelli et al., 1991), and the receptors for classical hormones including LH and TSH (Ji et al., 1990; Russo et al., 1991; Zhang et al., 1991; Liu et al., 1993; Petaja-Repo et al., 1993), have been extensively studied using both traditional pharmacological and molecular biological techniques, relatively little is known about the nature or importance of carbohydrate for the receptors for gastrointestinal hormones including the GRP-R. From cross-linking studies and timed PNGase F digestions, it was proposed that the GRP-R possesses four separate extracellular carbohydrate residues (Kusui et al., 1994) and that the glycosylation of this receptor was of critical importance for maintaining high-affinity binding and mediating G protein coupling (Kusui et al., 1994). However, no direct data were provided as to whether each glycosylation consensus sequence was in fact glycosylated, the exact amount of carbohydrate attached to each consensus sequence, or whether these alterations in binding and G protein coupling consequent to receptor deglycosylation could be attributed to glycosylation of any particular consensus sequence. This earlier nonmolecular study (Kusui et al., 1994) could not investigate the role of GRP-R glycosylation at various sites in regulating this receptor's trafficking to the cell membrane, nor could it examine the role of glycosylation at various sites in regulating internalization, desensitization, and down-regulation. In the present study, therefore, we used site-directed mutagenesis to examine the contributions of each of the four extracellular glycosylation consensus sequences present in the GRP-R to mediating receptor expression at the cell surface, agonist binding, and G protein coupling and to modulating this receptor's regulation consequent to agonist activation, including internalization, down-regulation, and chronic desensitization.

Experimental Procedures

Materials. Balb-3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and were then subject to clonal expansion to identify a line devoid of NMB-R or GRP-R as determined by binding and RNase protection assays. All peptides were obtained from Peninsula Laboratories (Belmont, CA), guanosine 5′-(β,γ-imido)-triphosphate tetralithium salt [Gpp(NH)p] was from Fluka Chemical Co. (Ronkonkoma, NY), Iodo-Gen was from Pierce (Rockford, IL), Na^{125}I was from Amersham (Arlington Heights, IL), and *myo*-[2-^{3}H]inositol (16–20 Ci/mmol) was from New England Nuclear (Boston, MA). Dulbecco's modified essential medium (DMEM), fetal bovine serum, and aminoglycoside G-418 were all from Life Technologies (Waltham, MA); cell culture dishware was from Costar (Cambridge, MA). Bovine serum albumin (fraction V) and HEPES were obtained from Boehringer Mannheim Biochemical (Indianapolis, IN); soybean trypsin inhibitor, EGTA, trypsin, and bacitracin were obtained from Sigma (St. Louis, MO); glutamine was from the Media Section, National Institutes of Health (Bethesda, MD); Dowex AG 1-X8 anion exchange resin (100–200 mesh, formate form) was from Bio-Rad (Richmond, VA); Hydro-Fluor scintillation fluid was from J.T. Baker Co. (Phillipsburg, NJ); PNGase F was from Genzyme (Cambridge, MA); and disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL). Standard buffer was comprised 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.

Mutant Receptor Construction and Expression. All mutants were constructed using the Altered Sites in vitro Mutagenesis System (Promega, Madison, WI) using murine GRP-R as the template (Benya et al., 1993). All constructs converted the Asn (N) of the glycosylation consensus sequence N-*X*-S/T to Gln (Q). Specific constructs mutated the Asn at amino acid position 5 (construct N5Q); at positions 4 and 5 (construct N4,5Q); at position 20 (construct N20Q); at position 24 (construct N24Q); at positions 5, 20, and 24 (construct N5, 20, 24Q), thus eliminating all NH_2 terminus glycosylation sites; and at amino acid position 191 (construct N191Q) resident in the second extracellular loop (Fig. 1). In all instances, the correct sequence was confirmed by dideoxy sequencing. The mutant cDNA was subcloned into the mammalian expression vector pcDNA-3 (Stratagene, La Jolla, CA) and purified by CsCl density gradient banding.

Transient expression of wild-type and mutant cDNA was performed by transfecting CHOP cells, which are Chinese hamster ovary fibroblasts modified to express papovavirus large T antigen (Heffernan and Dennis, 1990) (gift of Dr. James W. Dennis, Samuel Lunenfeld Research Institute, Toronto, Canada) using calcium phosphate precipitation. Binding assays were performed 48 h later. Stably transfected cell lines were established by transfecting Balb 3T3

fibroblasts by calcium phosphate precipitation, 48 h later cells were exposed to 800 μg/ml aminoglycoside G-418 for 4 to 6 weeks, and stable clones were selected. For each mutant, up to 50 clones were screened by binding studies, and two to six clones with binding similar to wild type were isolated and characterized by detailed binding studies and an assessment of the ability to activate phospholipase C. In all instances, the clones selected for study were those with similar amounts of expressed receptor protein or those with the highest number of receptors as determined by binding studies. For the N5,20,24Q mutant, because of the distinct differences from the wild-type receptor found, four clones were fully characterized in all studies.

Binding Studies. ^{125}I-[Tyr^4]Bombesin (2200 Ci/mmol), ^{125}I-[D-Tyr^6]bombesin (methyl ester), and ^{125}I-GRP were prepared using Iodo-Gen and purified by high-performance liquid chromatography as previously described (Benya et al., 1993). Disaggregated cells were resuspended in binding buffer, comprising standard buffer additionally containing 1.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.2 mM $KHPO_4$, 2 mM glutamine, 11 mM glucose, 0.2% bovine serum albumin, and 0.1% bacitracin. Incubation of 3×10^6 cells/ml with 75 pM ^{125}I-[Tyr^4]bombesin and variable concentrations of bombesin for 30 min at 37°C was performed, with nonsaturable binding of ^{125}I-[Tyr^4]bombesin being the amount of radioactivity associated with cells when the incubation mixture contained 1 μM bombesin. Nonsaturable binding was always <15% of total binding. Analysis of the binding data was performed using the least squares curve-fitting program LIGAND, and all values in this article are reported as saturable binding.

Receptor down-regulation using the ligand ^{125}I-[Tyr^4]bombesin, which assesses the number of cell surface receptors, was determined as described previously (Benya et al., 1995a). Briefly, to plated cells 3 nM bombesin in DMEM was added for up to 24 h, whereas the matched control cells had their media replaced with DMEM alone; in neither situation was fetal bovine serum present. Analysis of the binding data using the least squares curve-fitting program LIGAND permitted comparisons in mathematically derived receptor number (B_{max}) and affinity (K_i) between bombesin pretreated and control cells. Down-regulation was expressed as the percentage of control receptor number present on bombesin-pretreated cells compared with untreated control cells, which were processed in parallel.

Internalization of wild-type and mutant GRP-R was measured as that percentage of ^{125}I-[Tyr^4]bombesin resistant to acid wash as described previously (Benya et al., 1993). Disaggregated cells were incubated with 75 pM ^{125}I-[Tyr^4]bombesin for various times at 37°C in binding buffer. After incubation, cell samples were added to 10 volumes of 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl for 5 min at 4°C or to a similar volume of binding buffer. 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 saturably bound ^{125}I-[Tyr^4]bombesin that was internalized.

Cell Membrane Preparation. Disaggregated confluent cells were resuspended in homogenization buffer (50 mM Tris, pH 7.4, 0.2 mg/ml soybean trypsin inhibitor, 0.2 mg/ml benzamidine). Cells were homogenized on ice using a Polytron (Beckman Instruments, Palo Alto, CA) at speed 6 for 30 s. The homogenate was centrifuged at

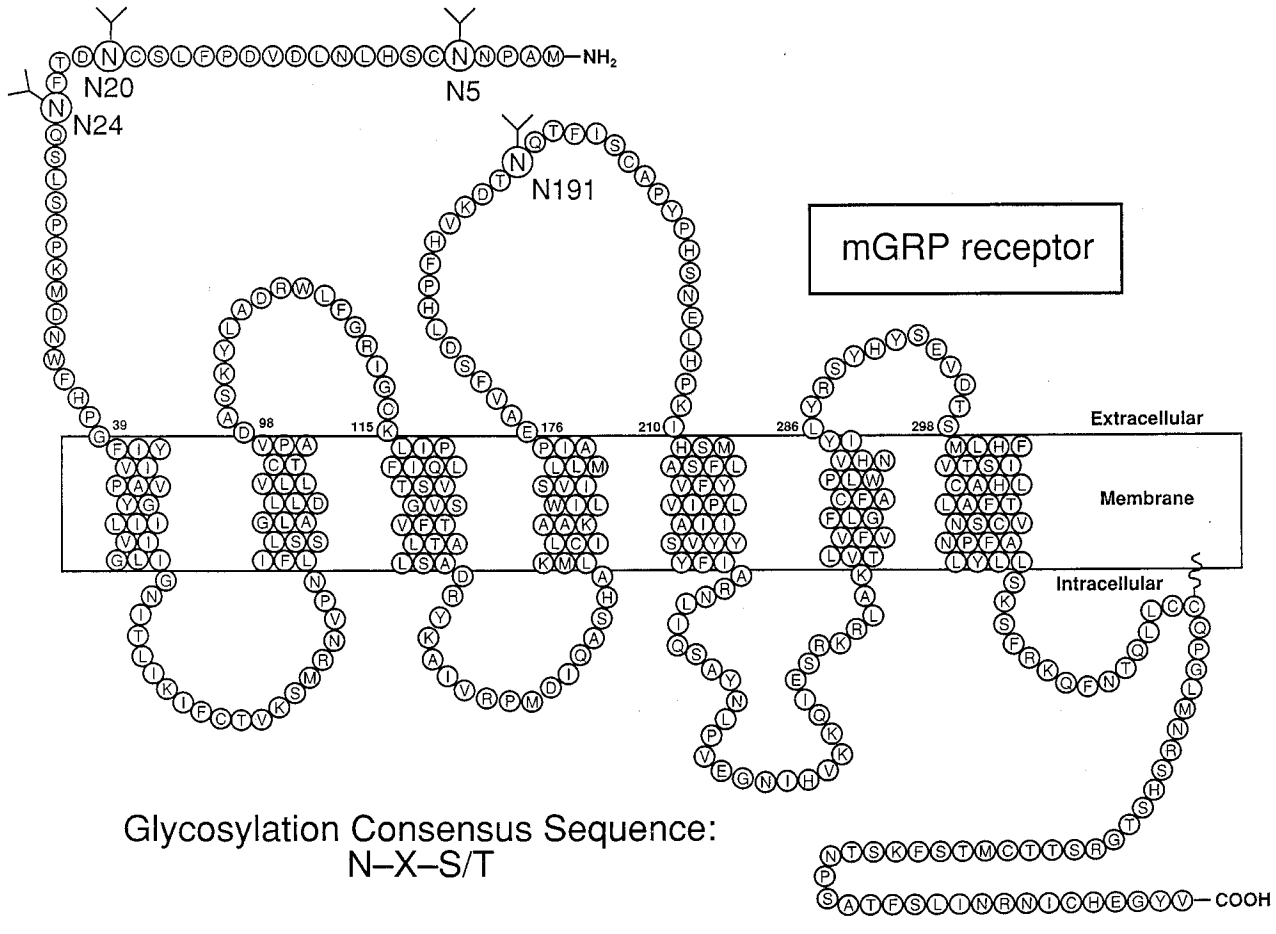

Fig. 1. Amino acid sequence of the murine GRP-R (mGRP-R) aligned according to its putative seven transmembrane-spanning topology. The four glycosylation consensus sequences (N-*X*-S/T) of the GRP-R are shown, three on the amino terminus (Asn at positions 5, 20, and 24) and one on the second extracellular loop (Asn at position 191). In all instances, glycosylation mutant GRP-Rs were generated by replacing the relevant Asn with Gln. All amino acids are shown in single-letter code.

1500 rpm for 10 min in a Sorvall RC-5B Superspeed centrifuge (DuPont Corp., Wilmington, DE), and the supernatant was removed and recentrifuged at 20,000 rpm for 20 min. The pellet was resuspended in homogenization buffer and stored at −40°C.

Molecular Mass Determination of Wild-Type and Mutant GRP-R. Cell membranes (0.25 mg of protein/ml) in binding buffer were used for cross-linking studies performed as described previously (Kusui et al., 1994; Benya et al., 1995b). Briefly, aliquots (500 μl) were preincubated with 0.5 nM ^{125}I-GRP at 22°C in 1.6-ml polypropylene tubes. After 15 min of incubation, the reaction mixture was centrifuged at 10,000*g* for 3 min. The pellet was washed twice in 1 ml of PBS (4°C) and resuspended in 200 μl of cross-linking buffer [50 mM HEPES (pH 7.5) 5 mM $MgCl_2$] containing 1 mM DSS as the cross-linking agent. After cross-linking at 22°C for 30 min, the reaction was stopped by adding 25 μl of 1 M glycine. After 10 min on ice, the sample was centrifuged at 10,000*g* for 3 min. Cross-linked membranes were solubilized by adding 25 μl of gel loading buffer [0.4 M Tris-HCl, (pH 6.8), 20% (w/v) SDS, 50% (v/v) glycerol, 0.05% (w/v) bromphenol blue, and 0.5 M dithiothreitol] at 22°C for 60 min. After adjusting the protein concentration, 10 μg of protein/lane of cell membranes was applied to a 10% (v/v) acrylamide 0.1% (w/v) SDS separating gel. Solubilized membranes were electrophoresed using the Laemmli buffer system as previously described (Kusui et al., 1994). Electrophoresis was carried out at 40 mA using 25 mM Tris, 0.2 M glycine, and 0.1% (w/v) SDS. Dried gels were exposed to storage phosphor screens for 3 days at 22°C and processed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Using the PhosphorImager, the intensity of each band was analyzed, and the molecular mass of the maximal intensity was calculated.

Measurement of Inositol Phosphates. Confluent cells were loaded with 100 μCi/ml *myo*-[2-^{3}H]inositol in DMEM supplemented with 2% fetal bovine serum at 37°C for 24 h. Cells were washed and incubated with phosphoinositol buffer (standard buffer additionally containing 10 mM $LiCl_2$, 2 mM $CaCl_2$, 2% bovine serum albumin, and 1.2 mM $MgSO_4$) for 15 min and then for 60 min at 37°C with varying concentrations of bombesin. Reactions were halted by using 1% HCl in methanol, and total [^{3}H]inositol phosphates were isolated by anion exchange chromatography as described previously (Benya et al., 1995a).

Chronic desensitization was measured as described previously (Benya et al., 1994a, 1995a). The extent of desensitization was defined as that decrease in the ability of 1 μM bombesin to increase total cellular inositol phosphates at variable time points after preincubation with 3 nM bombesin compared with untreated control cells.

Northern Blot Analysis for Wild-Type and Mutant GRP-R Message. CHOP cells transfected with the coding sequence for either the wild-type GRP-R or for mutant N191Q were harvested 48 h later by lysing with guanidium isothiocyanate in situ, and total RNA was extracted using cesium chloride ultracentrifugation. Total RNA was separated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose paper. RNA for wild-type or mutant GRP-R was probed using a random-primed murine GRP-R cDNA probe encompassing the entire open reading frame of the receptor. All image analysis was performed using a PhosphorImager (Molecular Dynamics).

Acute Desensitization Using Microphysiometry. Acute desensitization was assessed by measuring metabolic activity of wild-type GRP-R and the N5,20,24Q mutant using the cytosensor microphysiometer system (Molecular Devices, Sunnyvale, CA) (McConnell et al., 1992). This system uses a light-addressable potentiometric sensor to continuously detect pH changes in the extracellular fluid (McConnell et al., 1992). Briefly, the two cell types were harvested by centrifugation and resuspended to a concentration of 2×10^7 cells/ml in assay medium [bicarbonate-free DMEM (pH 7.4) supplemented with 44 mM sodium chloride and 0.17% (w/v) BSA]. The cell solution was mixed 1:1 with agarose entrapment medium (Molecular Devices, Sunnyvale, CA), and 10 μl of this solution was seeded into 12-mm capsule cups and placed into the cytosensor. The assembly was equilibrated in assay medium at a perfusion rate of 100 μl/min. The cells were exposed to bombesin or no additions for up to 30 min, and the acidification rates were determined. The cells were then washed and re-exposed to bombesin or bradykinin, and the acidification rate was continuously measured. A temperature of 37°C was maintained throughout the equilibrium and experimental periods.

Results

To assess the relative abilities of the wild-type and mutant GRP-Rs to be expressed at the cell surface, 5 μg of cDNA was used to transiently transfect CHOP fibroblasts. Cell surface receptor expression was determined by assessing receptor number using the antagonist [D-Tyr^6]bombesin(6-13) methyl ester to displace the binding of ^{125}I-[D-Tyr^6]bombesin(6-13) methyl ester, a ligand that is not internalized (Mantey et al., 1993) and therefore only measures the density of cell surface receptors (Fig. 2). Wild-type GRP-R was readily expressed by CHOP cells (B_{max} = 78 ± 12 fmol/10^6 cells) and bound antagonist with high affinity (K_i = 3.6 ± 0.8 nM). Elimination

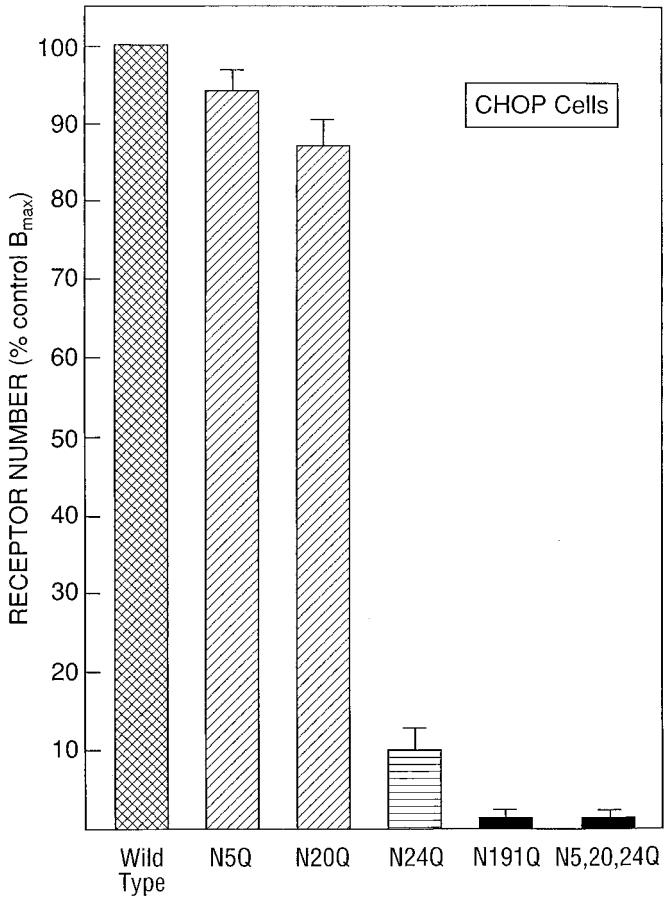

Fig. 2. Cell surface receptor number per cell for wild-type and glycosylation mutant GRP-R expressed by CHOP cells. Subconfluent CHOP cells were transfected with 5 μg of cDNA wild-type or glycosylation mutant GRP-R by calcium phosphate precipitation as described under *Experimental Procedures* and used in binding studies 48 h later. Receptor number (B_{max}) was determined by Scatchard analysis of the data obtained measuring the ability of [D-Tyr^6]bombesin methyl ester to inhibit binding of the radiolabeled antagonist, ^{125}I-[D-Tyr^6]bombesin methyl ester. Data were normalized to that observed for wild-type GRP-R. Receptor number for CHOP cells expressing wild-type receptor was 78 ± 12 fmol/10^6 cells. For each experiment, each value was determined in duplicate, with each point representing the mean ± S.E.M. of at least three separate experiments.

of glycosylation of the Asn at amino acid positions 5 or 20 had little effect on GRP-R expression or antagonist binding affinity (Fig. 2; Table 1). In contrast, elimination of the NH_2 terminus glycosylation consensus sequence located at Asn^{24} markedly reduced mutant receptor expression in the cell membrane by 97% but without altering receptor affinity for antagonist (Table 1). Similarly, elimination of the second extracellular loop glycosylation consensus sequence at Asn^{191}, or elimination of all NH_2 terminus glycosylation sites (i.e., mutant 5, 20, 24), resulted in a mutant GRP-R that completely failed to be expressed at the cell surface of CHOP cells (Fig. 2; Table 1). To confirm that the cDNA for mutant N191Q could be transcribed similarly as wild-type GRP-R, CHOP cells were transiently transfected with cDNA both for the wild-type GRP-R and for construct N191Q, and Northern blot analysis was performed. mRNA levels for mutant N191Q were similar to those seen for wild-type GRP-R (data not shown).

All subsequent studies were performed on receptors stably expressed by Balb 3T3 fibroblasts. This cell line previously has been shown to stably express wild-type GRP-R, which behave similarly to natively expressed receptors found on Swiss 3T3 fibroblasts with respect to ligand binding, receptor glycosylation, coupling to phospholipase C, and ability to undergo agonist-induced receptor modulation (internalization, down-regulation, and desensitization) (Benya et al., 1994a). Stable cell lines expressing approximately equal numbers of wild-type GRP-R, as well as the Asn^5, Asn^{20}, Asn^{24}, and $Asn^{5,20,24}$ mutants, were identified (Table 1). In the case of the Asn^{24} mutant, which was poorly expressed, 40 clones had to be screened to find two clones with receptor numbers similar to the wild-type GRP-R. No stable cell lines expressing Asn^{191} mutated to Gln could be identified. All stable cell lines bound agonist with similar high affinity (K_i = 2.4–5.3 nM) (Table 1). Initial studies were performed to determine the extent of GRP-R glycosylation at each potential glycosylation consensus sequence by cross-linking to the wild-type and various mutant receptors (Fig. 3). The wild-type GRP-R had an apparent molecular mass of 83 ± 1 kDa, similar to that reported for the GRP-R natively expressed by Swiss 3T3 fibroblasts (Kusui et al., 1994). Replacement of the Asn at amino acid position 5 (N5Q) or at positions 4 and 5 (N4, 5Q) resulted in clones with apparent identical molecular masses of 70 ± 1 kDa (Fig. 3; Table 1). This finding demonstrates that only Asn^5 and not Asn^4 is glycosylated and that the carbohydrate residue at this particular location accounts for approximately 13 kDa of the GRP-R's molecular mass. Similarly, replacement of Asn^{20} with Gln (N20Q) resulted in a mutant receptor with an apparent molecular mass of 73 ± 1 kDa, whereas replacement of Asn^{24} (N24Q) resulted in a receptor with an apparent molecular mass of 78 ± 1 kDa, demonstrating that the glycosylation of these particular residues accounted for approximately 10 ± 1 and 5 ± 1 kDa of wild-type GRP-R receptor mass, respectively (Fig. 3; Table 1).

After screening over 30 clones generated by three separate transfections, we were unable to isolate cells expressing GRP-R with Asn^{191} mutated to Glu (N191Q), a finding in keeping with the transient transfection results that showed that this residue was likely essential for sorting to obtain cell surface expression. By examining Balb 3T3 cells devoid of all NH_2 terminus glycosylation consensus sequences (N5,20,24Q), it was possible to obtain an estimate of the extent of glycosylation of Asn^{191}. The apparent molecular mass of mutant N5,20,24Q by cross-linking was 52 ± 1 kDa (Fig. 3; Table 1), whereas complete deglycosylation of the wild-type GRP-R using PNGase F resulted in a receptor with an apparent molecular mass of 43 ± 1 kDa. This suggests that the carbohydrate residue at Asn^{191} accounts for approximately 9 kDa of total wild-type receptor mass (Fig. 3).

To investigate the role of glycosylation on the ability of the GRP-R to couple to G proteins, we next determined the effect of the nonhydrolyzable guanine nucleotide analog Gpp(NH)p on binding of ^{125}I-[Tyr^4]bombesin to membranes expressing wild-type or mutant N5,20,24Q receptor (Fig. 4). Increasing concentrations of Gpp(NH)p decreased ^{125}I-[Tyr^4]bombesin binding to cell membranes expressing wild-type GRP-R, with half-maximal inhibition observed with approximately 10 nM and maximal displacement observed with 100 μM Gpp(NH)p. This effect of Gpp(NH)p was similar for membranes expressing glycosylation mutant N5,20,24Q (Fig. 4), suggesting that amino terminus glycosylation is not involved in regulating GRP-R G protein coupling. A previous study using partial digestion of the GRP-R with PNGase F, which removed three of the four sites of glycosylation (Kusui et al., 1994), demonstrated that one of the four glycosylation sites in the GRP-R is involved in regulating the ability of the GRP-R to couple to G proteins. Our present result would support the speculation that the carbohydrate attached to the second extracellular loop on Asn^{191} must be the critical moiety necessary for this coupling because glycosylation at N5, N20, and N24 in the

TABLE 1

Pharmacological parameters for wild-type and glycosylated mutant GRP-R transiently expressed by CHOP cells and stably expressed by Balb 3T3 cells

Data are expressed as the mean ± S.E. of at least three separate experiments. Receptor affinity (K_i) and number (B_{max}) were calculated using the least squares curve-fitting program LIGAND. For CHOP cells, 5 μg of cDNA was used for $CaPO_4$ precipitation as described under *Experimental Procedures*, and binding parameters were determined using the bombesin receptor-specific antagonist ^{125}I-[D-Tyr^6]bombesin methyl ester. Data are mean ± 1 S.E.M. of at least three separate experiments.

Mutant	CHOP Cells		Balb 3T3 Cells				
				Binding		[^{3}H]IP	
	K_i	B_{max}	Molecular Mass	K_i	B_{max}	EC_{50}	Fold Increase
	nM	*fmol/10^6 cells*	*kDa*	*nM*	*fmol/10^6 cells*	*nM*	
Wild type	3.6 ± 0.8	78 ± 12	83 ± 1	2.4 ± 0.4	523 ± 31	2.9 ± 0.9	6.1
N5Q	2.8 ± 0.2	74 ± 12	70 ± 1	5.3 ± 1.5	620 ± 22	4.9 ± 0.8	4.5
N20Q	3.4 ± 0.3	68 ± 9	73 ± 1	2.9 ± 0.7	433 ± 65	3.8 ± 0.5	6.0
N24Q	2.9 ± 0.1	2.6 ± 0.1	78 ± 1	4.2 ± 1.1	457 ± 61	5.3 ± 1.1	6.2
N5,20,24Q	N.D.	N.D.	52 ± 1	3.9 ± 1.9	592 ± 47	5.1 ± 0.9	4.6

EC_{50}, concentration necessary for half-maximal increases in [^{3}H]IP; N.D., not determinable.

amino terminus can be removed without an effect on G protein coupling.

All glycosylation mutant GRP-Rs studied were able to activate phospholipase C, as determined by measuring increases in total cellular [^{3}H]inositol phosphates consequent to stimulation with bombesin (Table 1). All stably transfected cell lines generated similar basal levels of [^{3}H]IP (GRP-R wild type = 5500 ± 400 dpm; N5Q = 5400 ± 250 dpm; N20Q = 3900 ± 130 dpm; N24Q = 4200 ± 230 dpm; N5,20,24Q = 3300 ± 350 dpm), and the fold increase in [^{3}H]IP generation consequent to stimulation with 1 μM bombesin varied from 4.5-fold for cells expressing mutant N5Q to 6.2-fold for cells expressing mutant N24Q (Table 1). Finally, similar concentrations of agonist caused half-maximal increases in cellular [^{3}H]IP formation (EC_{50}) (Table 1).

Consequent to stimulation with agonist, the GRP-R undergoes chronic homologous desensitization, a process that some (Benya et al., 1995a) but not all (Swope and Schonbrunn, 1990) investigators believe is associated with receptor internalization and/or down-regulation. To determine whether any specific GRP-R glycosylation site was primarily responsible for these processes, the ability of the wild-type and various glycosylation mutants to undergo chronic desensitization, down-regulation, and internalization were compared. As we have previously demonstrated, maximal desensitization of wild-type GRP-R occurs after exposure to 3 nM bombesin for 24 h (Benya et al., 1994a). In separate experiments, cells expressing wild-type GRP-R increased [^{3}H]IP over 10-fold after exposure to 1 μM bombesin (from 2,080 ± 250 to 22,140 ± 410 dpm), whereas preincubation with 3 nM bombesin for 24 h caused cellular [^{3}H]IP to increase only 3-fold (from 3,380 ± 300 to 9,040 ± 600 dpm) (Fig. 5) and thus was only 28% of the control response observed in untreated cells. Elimination of any single amino terminus glycosylation consensus sequence did not significantly alter the observed desensitization response, with each mutant decreasing the response to 1 μM bombesin to 24 to 32% of that of the untreated cells (Table 2). However, removal of all amino terminus carbohydrate resulted in significant attenuation of mutant N5,20,24Q's ability to undergo chronic desensitization, with 73 ± 3% of the [^{3}H]IP response observed after preincubating with 3 nM bombesin for 24 h compared with control cells processed in parallel (control N5,20,24Q basal = 4,930 ± 320 dpm, 1 μM bombesin stimulated = 22,570 ± 4,100 dpm; pretreated with 3 nM bombesin for 24 h basal = 3,500 ± 1,150 dpm, then 1 μM bombesin stimulated = 16,330 ± 800 dpm) (Fig. 5; Table 1). To confirm that this difference from the wild type was not due to an alteration in the particular N5,20,24 cell line used, the ability of three other stably expressed N5,20,24 cell lines to undergo chronic desensitization was determined. Each of these three N5,20,24 cell lines also demonstrated impaired desensitization, with the [^{3}H]IP response to 1 μM bombesin demonstrating 68 ± 4, 64 ± 5, and 64 ± 4% of the control [^{3}H]IP response after preincubation with 3 nM bombesin for 24 h. In contrast, the wild-type cells demonstrated a decrease to 26 ± 3% of the control response with 3 nM bombesin preincubation processed in parallel with each of the three 5,20,24N mutant cell lines. The chronic desensitization with the GRP-R cell lines was due to a change in agonist efficacy only because there was no change in agonist potency (data not shown).

To investigate whether altered down-regulation of glyco-

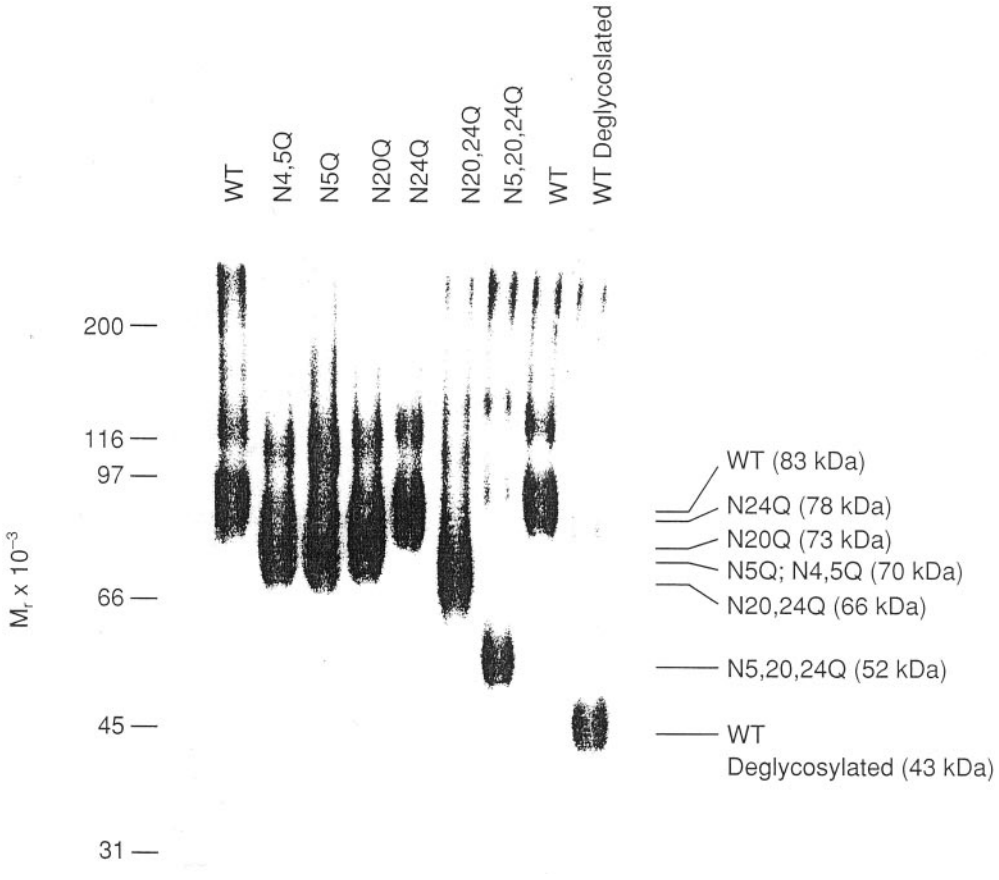

Fig. 3. Affinity cross-linking of stably transfected Balb 3T3 cell membranes expressing wild-type or glycosylation mutant GRP-R. Membranes were prepared as detailed under *Experimental Procedures*. ^{125}I-GRP was cross-linked to wild-type and GRP-R mutants using 1 mM DSS. Postcross-linking to determine the molecular mass of the fully glycosylated receptor, membranes from the wild-type GRP-R (WT) underwent deglycosylation by incubating with PNGase F (10 U/ml) as described under *Experimental Procedures*. This gel is representative of three separate experiments, with the molecular masses given as the means ± S.E.M.

sylation mutant receptors also might be occurring, binding studies with ^{125}I-[Tyr^4]bombesin were performed under conditions similar to those causing desensitization. In cells with wild-type GRP-R after 24 h of exposure to 3 nM bombesin, 28 ± 3% of GRP receptors remained (Table 2). Mutant receptors N5Q and N24Q underwent similar degrees of receptor down-regulation (Fig. 6; Table 2), whereas the down-regulation of mutant N20Q was attenuated (48 ± 3% of receptors remained). Down-regulation of mutant N5,20,24Q was even further attenuated, with 62 ± 4% of receptors remaining (Fig. 6; Table 2). In three other N5,20,24 cell lines, down-regulation was also attenuated with 60 ± 10, 69 ± 9, and 80 ± 9% of receptors remaining after a 24-h incubation with 3 nM bombesin, compared with only 17 to 29% with the wild-type GRP-R in parallel experiments.

The GRP-R is rapidly internalized after exposure to agonist (Benya et al., 1993; Mantey et al., 1993), so we determined whether alteration of this receptor's glycosylation altered its internalization. Elimination of any single amino terminus glycosylation consensus sequence (i.e., Asn^5, Asn^{20}, or Asn^{24}) did not alter internalization extent or kinetics (Fig. 7; Table 2). Elimination of all three amino terminus glycosylation consensus sequences in mutant N5,20,24Q resulted in modest, but statistically insignificant (P = .15), attenuation of internalization (at t = 90 min, wild type = 83 ± 2% versus N5,20,24Q = 70 ± 2% internalized) (Fig. 7; Table 2).

The GRP-R can also undergo acute desensitization (Walsh et al., 1993). To determine whether altered glycosylation affected acute desensitization, wild-type GRP-R-containing cells or cells containing the N5,20,24Q GRP-R mutant were exposed to bombesin and, after a wash, exposed to bombesin or bradykinin (Fig. 8). Pre-exposure to bombesin (0.3 nM) decreased the subsequent response to a maximal effective

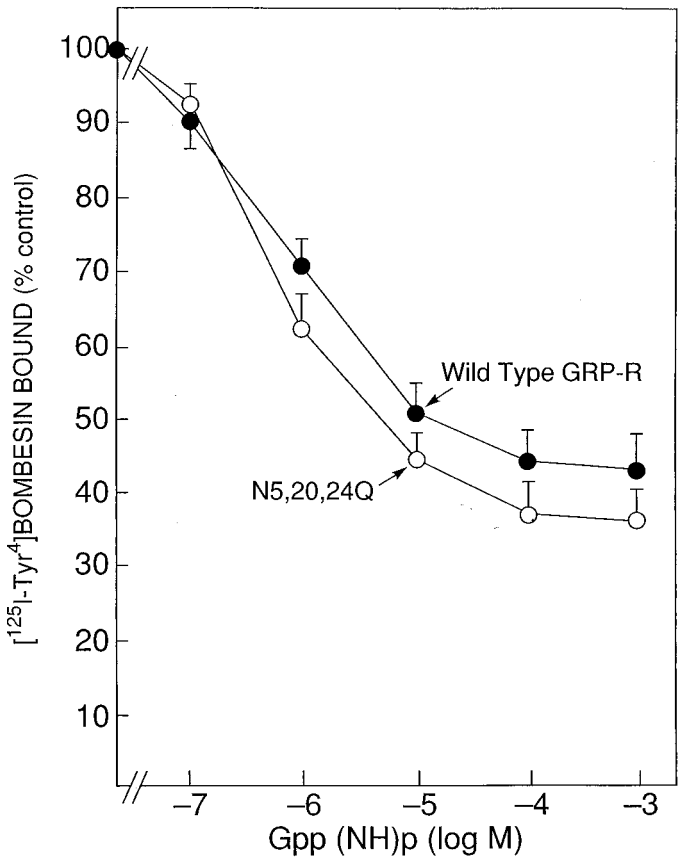

Fig. 4. Effect of the nonhydrolyzable guanine analog Gpp(NH)p on binding of ^{125}I-[Tyr^4]bombesin to Balb 3T3 cell membranes expressing either wild-type GRP-R or mutant N5,20,24Q. Cell membranes were incubated at 22°C in binding buffer with 50 pM ^{125}I-[Tyr^4]bombesin for 60 min either alone or with the indicated concentrations of Gpp(NH)p. Results are expressed as the percentages of saturable binding in the absence of unlabeled peptide. Values represent the mean ± S.E.M. of at least three separate experiments, with each value determined in duplicate for each experiment.

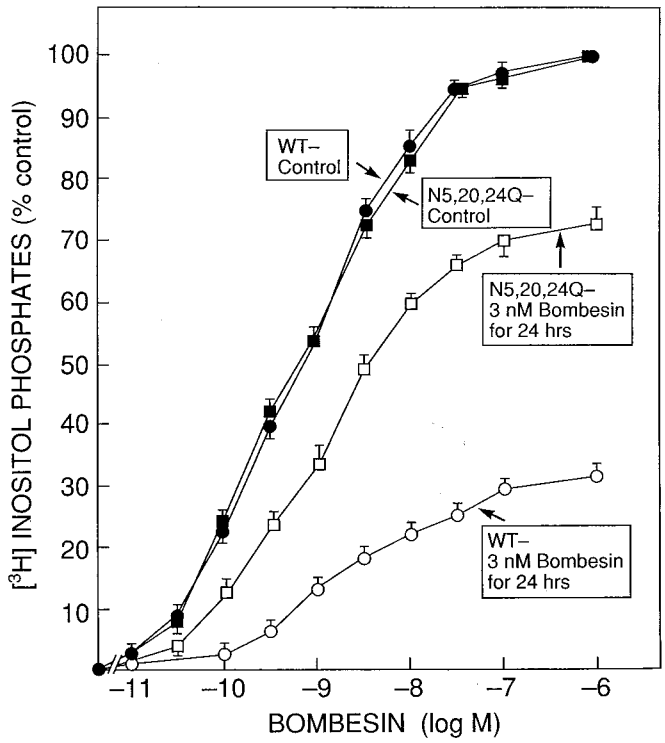

Fig. 5. Effect of preincubation with 3 nM bombesin for 24 h on the dose-response curves for [^{3}H]inositol phosphate generation in Balb 3T3 cells expressing wild-type (WT) GRP-R or mutant GRP-R. Confluent cells were incubated in DMEM supplemented with 2% fetal bovine serum and 100 μCi/ml *myo*-[2-^{3}H]inositol alone or with 3 nM bombesin for 24 h. After washing in IP buffer, cells were exposed to the indicated concentrations of bombesin for 60 min, with the data expressed as the percentages of maximal increase obtained using 1 μM bombesin. Each data point represents the mean ± S.E.M. of a minimum of four separate experiments, with each point determined in duplicate.

TABLE 2

Internalization, down-regulation, and chronic desensitization of wild-type and glycosylation mutant GRP-R stably expressed in Balb 3T3 fibroblasts

Data are expressed as the mean ± S.E. of at least three separate experiments. Internalization was determined as described in Fig. 7 and expressed as the percentage of the saturably bound ligand internalized at 90 min. Down-regulation was determined as described in Fig. 6, with data given as the percentage of receptors (B_{max}) expressed compared with control cells processed in parallel. Chronic desensitization was determined as described in Fig. 5, with data given as the percentage of the maximal increase in [^{3}H]IP with 1 μM bombesin compared with control cells processed in parallel.

Mutant	Internalized (at t = 90 min)	Down-Regulation (% Control of B_{max} at t = 24 h)	Chronic Desensitization (% Control Stimulation with 1 μM)
		%	
Wild type	83 ± 2	28 ± 3	28 ± 4
N5Q	82 ± 1	27 ± 3	24 ± 2
N20Q	85 ± 1	48 ± 3	30 ± 2
N24Q	83 ± 2	30 ± 2	32 ± 3
N5,20,24Q	71 ± 2	62 ± 4	73 ± 3

concentration of bombesin (i.e., 1 μM) by 56 ± 15% after a 5-min wash and 43.1 ± 3.1% after a 15-min wash (n = 4) (Fig. 8, top). However, preincubation with 0.3 nM bombesin had no effect on the subsequent response to bradykinin (100 nM) (Fig. 8, middle), demonstrating that the acute desensitization by bombesin was homologous. The N5,20,24Q mutant GRP-R also underwent acute desensitization with preincubation with 0.3 nM bombesin, and the degree of acute desensitization was similar to that seen with the wild-type GRP-R with a 57.9 ± 3.8% decrease after a 5-min wash and a 40.0 ± 2.8% decrease after a 15-min wash (n = 4) (Fig. 8, bottom).

Discussion

In contrast to the receptors for neurotransmitters, including adrenergic and cholinergic agents, and receptors for classical hormones, including those for LH and FSH, relatively little is known about the extent and functional significance of gastrointestinal hormone receptor glycosylation. The cloning of the GRP-R (Battey and Wada, 1991), however, has now permitted this gastrointestinal hormone receptor to be systematically studied. Before its being cloned, limited data derived from cross-linking and lectin binding studies had shown that the GRP-R was variably glycosylated, with carbohydrate accounting for 30% of receptor mass in human cells to approximately 50% of receptor mass in mouse Swiss 3T3 cells (Benya et al., 1995b). Since cloning of the GRP-R, we have confirmed that the wild-type GRP-R stably expressed in Balb 3T3 fibroblasts, the same cell line used in the present study to investigate the properties of the various GRP-R glycosylation mutants, is glycosylated identically to those natively expressed by Swiss 3T3 fibroblasts (Benya et al., 1994a; Kusui et al., 1994) and also behaves in a fashion similar to the native receptor (Benya et al., 1993, 1994b, 1995a). In a study using these stably transfected Balb 3T3 fibroblasts, it was proposed from analyzing the changes in molecular mass of affinity cross-linked GRP-R incubated for various times with PNGase F that four separate *N*-linked carbohydrate residues are attached to the GRP-R (Kusui et al., 1994), consistent with the four glycosylation consensus sequences shown to exist by analysis of the cloned receptor's primary structure (Battey and Wada, 1991). Furthermore, this same study demonstrated that GRP-R glycosylation on at least one site was important in mediating high-affinity agonist binding and in regulating G protein coupling, whereas with the closely related NMB-R expressed in the same cells, glycosylation had no effect on these parameters (Kusui et al., 1994). In that study no data was provided to directly demonstrate that each glycosylation consensus sequence was in fact glycosylated, nor was data provided regarding the amount of carbohydrate present at each such consensus sequence nor to which glycosylation site the aforementioned alteration in GRP-R binding and G protein coupling could be attributed. Furthermore, no information could be provided in such a study relying on chemical deglycosyla-

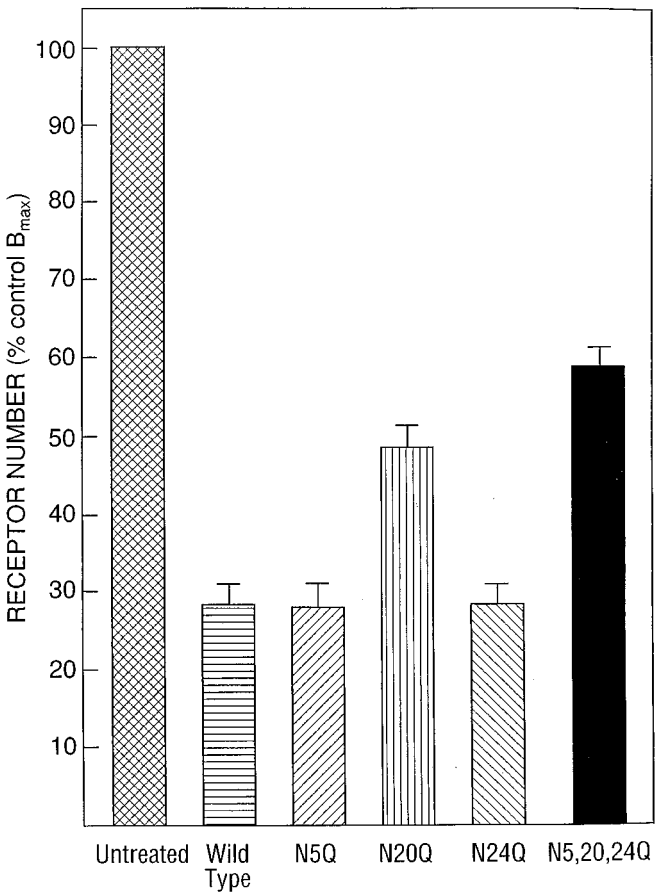

Fig. 6. Down-regulation of wild-type and glycosylation mutant GRP-R expressed by Balb 3T3 cells after exposure to 3 nM bombesin for 24 h. Confluent Balb 3T3 cells stably expressing wild-type or mutant GRP-R were incubated with 3 nM bombesin in DMEM for 24 h and were then resuspended in binding buffer containing ^{125}I-[Tyr^4]bombesin alone or varying concentrations of unlabeled bombesin. Scatchard analysis of the competitive binding data demonstrated no change in receptor affinity.

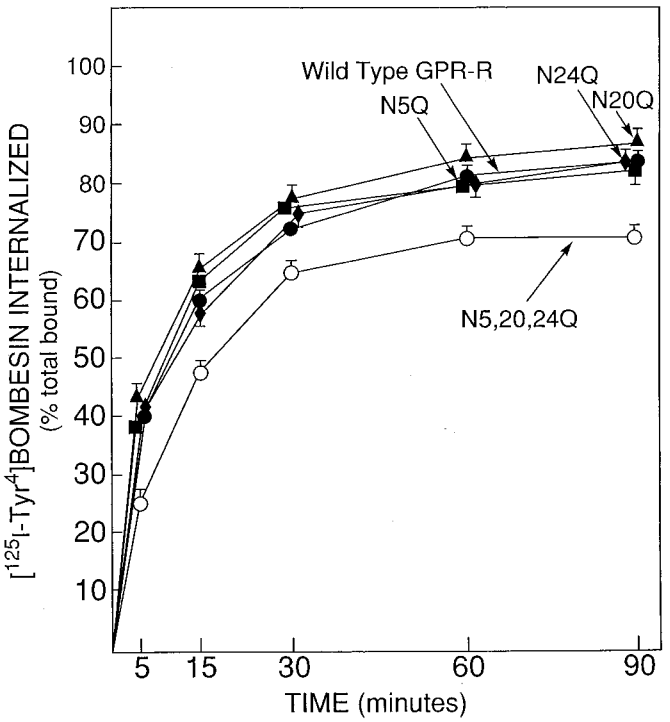

Fig. 7. Internalization rates for wild-type and glycosylation mutant GRP-R expressed by Balb 3T3 cells. The internalized ligand was the proportion not removed by acid stripping. Results are expressed as the proportion of total saturably bound ligand at any time point that was not acid strippable. For each experiment, each value was determined in triplicate, with each point representing the mean ± S.E.M. of at least three separate experiments.

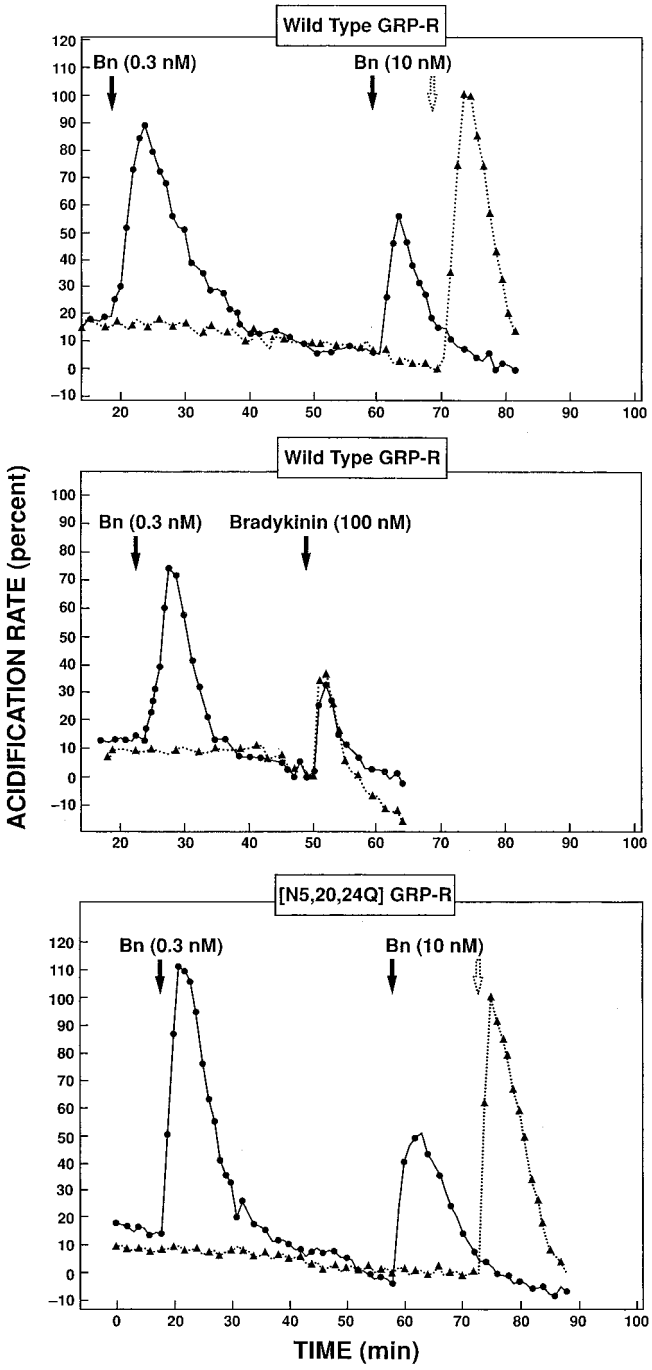

Fig. 8. Abilities of wild-type and N5,20,24Q glycosylation mutant GRP-R to undergo acute homologous desensitization. Cells were prepared as described under *Experimental Procedures* and the metabolic response measure using a Cytosensor. The acidification rate was monitored continuously and expressed as the percentage of the maximal increase caused by bombesin (Bn, 10 nM), a maximally effective concentration. Cells were treated initially with bombesin (0.3 nM) or no additions. After the acidification rate had returned to control levels, the cells were washed and bombesin (10 nM) (top and bottom) or bradykinin (100 nM) (middle) was added. These results are representative of four independent experiments. The wild-type GRP-R response to bombesin (10 nM) is reduced by 46% after the preincubation with 0.3 nM bombesin (top), whereas the preincubation has no effect on the response to bradykinin (100 nM) (middle). The [N5,20,24Q] GRP-R mutant's response to bombesin (10 nM) is reduced by 48% after preincubation with 0.3 nM bombesin, similar to the wild-type GRP-R.

tion as to the contributions of each carbohydrate residue to GRP-R trafficking, internalization, down-regulation, or desensitization.

Although most extracellular receptor glycosylation consensus sequences are glycosylated, this is not invariably the case. For the recently described human calcium receptor, a G protein-coupled receptor, eight of the 11 potential *N*-linked glycosylation sites are glycosylated (Lanctot et al., 1999); whereas for both the FSH receptor (Davis et al., 1995) and gonadotropin-releasing hormone receptor (Davidson et al., 1995), only two of the three potential extracellular *N*-linked glycosylation sites are actually glycosylated. It was previously concluded by analyzing the time course of change in receptor molecular mass with PNGase digestion that each of the potential *N*-linked glycosylation sites in the murine GRP-R are glycosylated and each carbohydrate moiety was likely of approximate equal mass (Kusui et al., 1994). In the present study we provide evidence from cross-linking studies to the various mutant GRP-Rs that all four of the extracellular GRP-R glycosylation consensus sequences are indeed glycosylated but with different amounts of carbohydrate attached at each site. We estimate that approximately 13 kDa of carbohydrate is attached at Asn^{5}, 10 kDa is attached at Asn^{20}, 5 kDa is attached at Asn^{24}, and the GRP-R has approximately 9 kDa attached to Asn^{191} in the second extracellular loop. However, given the nonlinearity of glycosylated proteins in SDS gels these can only be considered estimates at present.

By assessing binding of the GRP-R antagonist, ^{125}I-[D-Tyr^{6}]bombesin(6-13) methyl ester, which was not internalized, we were able to determine the cell surface expression of the various GRP-R glycosylation mutants after their transient expression in CHOP fibroblasts (Fig. 2; Table 1) (Jensen and Coy, 1991; Mantey et al., 1993). Elimination of either the second extracellular (Asn^{191}) or all amino terminus ($Asn^{5,20,24}$) glycosylation consensus sequences resulted in failure of these mutant GRP-R receptors to be detected at the cell surface. Because stable clones retaining only the glycosylation at Asn^{191} (mutant N5,20,24Q) were able to be generated that possessed normal affinity for agonist (Table 1), it does not appear likely that elimination of the amino terminus carbohydrate alone altered GRP-R affinity for ligand, and this resulted in a decreased affinity for the ligand such that its cell surface expression could not be detected. Rather, this suggests that glycosylation of the GRP-R amino terminus, in addition to glycosylation of the Asn^{191}, is required for appropriate trafficking of the receptor to the cell membrane. Furthermore, of the three amino terminus glycosylation residues, the one attached to Asn^{24} is clearly the most critical for proper receptor trafficking to the cell membrane (Fig. 2).

It could be argued that the failure to detect mutant N191Q, either when transiently expressed in CHOP cells (Fig. 2) or when stably expressed by Balb 3T3 cells, is not simply due to a failure in receptor trafficking but could also be due to a crucial role that Asn^{191} may play in ligand binding, or it could also be due to an alteration in the ability of the receptor to be transcribed. An alteration in transcription is unlikely because the CHOP cells transfected with the cDNA expressing the Asn^{191} mutant receptor demonstrated similar mRNA levels as cells transfected with the wild-type GRP-R. However, the result of the study of enzymatic deglycosylation by PNGase F (Kusui et al., 1994) of the GRP-R could be inter-

preted to suggest that the glycosylation of Asn^{191} is important in determining receptor affinity. When the GRP-R is completely deglycosylated using PNGase F the receptor is totally incapable of binding ligand, whereas 75% deglycosylation causes a marked decrease in affinity for ligand (Kusui et al., 1994). PNGase F treatment completely removes *N*-linked Asn residues and the 75% enzymatically deglycosylated GRP-R has one of the four glycosylated sites left fully glycosylated (Kusui et al., 1994). The PNGase F deglycosylation of the other three sites resulted in only a minimal change in affinity (<2-fold decrease), and therefore this 75% deglycosylated receptor behaves similarly to the N5,20,24 mutant in the present study, which is 78% deglycosylated. In the present study the removal of carbohydrates attached to Asn^{5}, Asn^{20}, or Asn^{24} alone or together does not effect GRP-R affinity for agonist because the N5,20,24Q mutant had similar agonist affinity to the wild-type GRP-R. The difference in the fully deglycosylated GRP-R, which does not bind agonist (Kusui et al., 1994), and the N5,20,24Q mutant in the present study, which binds agonist with normal affinity, is the presence of the glycosylation on Asn^{191}. These results support the speculation that the carbohydrate residue attached to Asn^{191} may be responsible for the marked loss of agonist affinity that is seen with full (Kusui et al., 1994) enzymatic deglycosylation of the GRP-R but not with <78% deglycosylated receptor in the N5,20,24Q mutant. Therefore, the data from these two studies support the speculation that Asn^{191} is involved in both receptor trafficking and in the determination of the GRP-R high-affinity state. Evidence for a single glycosylation consensus sequence being involved in the dual role of mediating trafficking and high-affinity agonist binding has been shown to exist for at least one other heptaspanning receptor: Asn^{173} in the amino terminus of the rat LH receptor (Liu et al., 1993). In another study (Ho et al., 1999) involving the human calcitonin receptor, mutation of potential glycosylation sites at position 78 or 83, but not 26, altered receptor affinity and potency. In contrast, other molecularly based studies have failed to demonstrate a role for receptor carbohydrate in mediating agonist affinity for the serotonin (Buck et al., 1991), β_2-adrenergic (Rands et al., 1990), human $angiotensin_1$ receptor (Lanctot et al., 1999), or M2 muscarinic cholinergic (van Koppen and Nathanson, 1990) receptors. Most of these studies did not address the role of glycosylation in regulating receptor trafficking. Earlier studies using chemical or enzymatic means to deglycosylate heptaspanning receptors demonstrated a similar lack of predictability as to the role of receptor carbohydrate in regulating agonist binding affinity. For example, whereas deglycosylation of the somatostatin (Rens-Domiano and Reisine, 1991) and VIP (el Battari et al., 1991) receptors resulted in decreased affinity for agonist, deglycosylation of the FSH receptor (Dattatreyamurty and Reichert, 1992), LH receptor (Liu et al., 1993), and M1, M2, and M4 muscarinic cholinergic receptors (Ohara et al., 1990) had no impact on agonist binding.

In a study using enzymatic deglycosylation, GRP-R carbohydrate was proposed to be involved in regulating this receptor's ability to couple to G proteins (Kusui et al., 1994). This finding is similar to that obtained from studies of the VIP receptor but is in contrast to that obtained for the LH (Ji et al., 1990; Petaja-Repo et al., 1993), somatostatin (Rens-Domiano and Reisine, 1991), and M1, M2, and M4 muscarinic cholinergic receptors (Ohara et al., 1990). Few G protein-coupled, seven transmembrane-spanning receptors have been studied at the molecular level to determine whether glycosylation is involved in G protein activation. Of those studied, glycosylation of the serotonin (Buck et al., 1991) and M2 muscarinic cholinergic (van Koppen and Nathanson, 1990) were not found to play a role in receptor G protein coupling, whereas glycosylation-dependent coupling of the β_2-adrenergic receptor (Rands et al., 1990) depended on the cell type expressing this receptor. In the present study elimination of all GRP-R amino terminus glycosylation sites in mutant N5,20,24Q failed to alter the ability of increasing concentrations of Gpp(NH)p to inhibit binding of ^{125}I-[Tyr^4]bombesin (Fig. 4), a measure of receptor G protein coupling. This suggests that the decrease in GRP-R affinity for Gpp(NH)p observed with enzymatic deglycosylation (Kusui et al., 1994) does not involve carbohydrate residues attached to the amino terminus. By inference, this leads to the speculation that the single carbohydrate residue attached to Asn^{191} is likely responsible for regulating GRP-R G protein coupling.

Consequent to previous exposure to agonist, most receptors become refractory to further stimulation with that same ligand, a process known as desensitization. In the present study we demonstrate that, for complete chronic desensitization to occur, glycosylation of at least one amino-terminal consensus sequence is essential. Although removal of any single amino terminus glycosylation consensus sequence failed to alter the desensitization process, removal of all three such sequences attenuated desensitization by over 65%. Very few studies have investigated the role of heptaspanning receptor carbohydrate moieties in modulating desensitization, with the only molecularly based study showing that acute desensitization as measured in *Xenopus* oocytes was not altered using a deglycosylated rat serotonin receptor (Buck et al., 1991). The limitations of nonmolecular studies are apparent from a study of the mouse muscle acetylcholine receptor, for which it has been shown that tunicamycin, an agent commonly used to prevent receptor glycosylation in the Golgi apparatus, altered the desensitization response independently of any change in receptor glycosylation (Nishizaki and Sumikawa, 1992). Thus, the conclusions that can be drawn from studies using chemical deglycosylation may be limited. In contrast, we clearly show that some GRP-R amino terminus glycosylation is required for the appropriate chronic desensitization response to be manifested. A previous study (Proll et al., 1993) with the β_2-adrenergic receptor (β_2-AR) using a receptor mutagenesis approach demonstrated that chronic β_2-AR involves different intracellular mechanisms than acute β_2-AR desensitization, which involves receptor phosphorylation by protein kinase A and β-adrenergic receptor kinase (Proll et al., 1993). Our results suggest that a similar situation may exist for the GRP-R, because acute homologous GRP-R desensitization was not altered in the N5,20,24Q GRP-R mutant, whereas chronic desensitization was reduced in this mutant, supporting the conclusion that different cellular processes are likely involved.

Receptor internalization and down-regulation are rapid and long-term responses, respectively, to receptor stimulation with agonist. This study is the first to investigate the role of heptaspanning receptor glycosylation in affecting internalization and down-regulation to agonist exposure. Re-

moval of all GRP-R amino terminus glycosylation consensus sequences in mutant N5,20,24Q attenuated receptor down-regulation by 50%, and this attenuation is likely primarily due to glycosylation specifically attached to Asn^{20}. In contrast, removal of all amino terminus carbohydrate reduced the degree of GRP-R internalization in response to stimulation with agonist only 15%, which was not a significant difference ($P = .15$) from wild type, and this phenomenon could not be attributed to any single glycosylation consensus sequence. Recently, from studies of the behavior of mutant GRP-Rs, the wild-type GRP-R, and the closely related NMB-R, it has been proposed that chronic desensitization and down-regulation are closely coupled processes with similar mediators, whereas internalization is likely caused by different intracellular mediators and is not clearly coupled to these processes (Benya et al., 1995a). The relative effect of deglycosylation of Asn^{5}, Asn^{20}, and Asn^{24} of the GRP-R in the present study provide some support for this speculation. Deglycosylation of these three amino-terminal sites resulted in a highly significant 65% decrease in chronic desensitization and a 50% decrease in agonist-induced down-regulation. In contrast, only a minimal effect was seen on internalization with only a 15% decrease. Therefore, chronic GRP-R desensitization and down-regulation were both highly dependent on some amino-terminal GRP-R glycosylation, whereas internalization was not.

The GRP-R is unusual insofar as not all the glycosylation consensus sequences are restricted to the receptor's amino terminus (Fig. 1). In contrast to the well studied receptors for muscarinic cholinergic agents, LH, and TSH, the GRP-R belongs to a small group of seven transmembrane-spanning, G protein-coupled receptors possessing a non-amino terminus glycosylation consensus sequence. In this study we have demonstrated that the GRP-R is glycosylated at each of the four extracellular glycosylation consensus sequences with residues of varying mass and that amino terminus glycosylation is necessary for modulating receptor trafficking to the cell membrane, chronic desensitization, and down-regulation but not acute homologous desensitization. Furthermore, the amino-terminal glycosylation plays no role in regulating high-affinity agonist binding or G protein coupling of the GRP-R. In contrast, we show that the uniquely placed carbohydrate on Asn^{191} of the second extracellular loop is critical for receptor trafficking to the cell membrane. Because an earlier study of the GRP-R involving enzymatic deglycosylation of the GRP-R (Kusui et al., 1994) demonstrated a role for one carbohydrate moiety in mediating high-affinity agonist binding and in regulating G protein coupling, and these processes are not affected by elimination of all three amino terminus glycosylation consensus sequences, we also speculate that glycosylation of Asn^{191} may be the glycosylation site necessary for high-affinity GRP-R agonist binding and G protein coupling.

References

Battey J and Wada E (1991) Two distinct receptors for mammalian bombesin-like peptides. *Trends Neurosci* **14:**524–528.

Benya RV, Fathi Z, Battey JF and Jensen RT (1993) Serines and threonines in the gastrin-releasing peptide receptor carboxyl terminus mediate internalization. *J Biol Chem* **268:**20285–20290.

Benya RV, Fathi Z, Pradhan T, Battey JF, Kusui T and Jensen RT (1994a) Gastrin-releasing peptide receptor-induced internalization, down-regulation, desensitization and growth: Possible role of CAMP. *Mol Pharmacol* **46:**235–245.

Benya RV, Kusui T, Battey JF and Jensen RT (1994b) Desensitizaton of neuromedin B receptors (NMB-R) on native and NMB-R transfected cells involves down-regulation and internalization. *J Biol Chem* **269:**11721–11728.

Benya RV, Kusui T, Battey JF and Jensen RT (1995a) Chronic desensitization and down-regulation of the gastrin-releasing peptide receptor are mediated by a protein kinase C-dependent mechanism. *J Biol Chem* **270:**3346–3352.

Benya RV, Kusui T, Pradhan TK, Battey JF and Jensen RT (1995b) Expression and characterization of cloned human bombesin receptors. *Mol Pharmacol* **47:**10–20.

Buck F, Meyerhof W, Werr H and Richter D (1991) Characterization of N- and C-terminal deletion mutants of the rat serotonin HT2 receptor in *Xenopus laevis* oocytes. *Biochem Biophys Res Commun* **178:**1421–1428.

Dattatreyamurty B and Reichert LE Jr (1992) Carbohydrate moiety of follitropin receptor is not required for high affinity hormone-binding or for functional coupling between receptor and guanine nucleotide-binding protein in bovine calf testis membranes. *Endocrinology* **131:**2437–2445.

Davidson JS, Flanagan CA, Zhou W, Becker II, Elario R, Emeran W, Sealfon SC and Millar RP (1995) Identification of *N*-glycosylation sites in the gonadotropin-releasing hormone receptor: Role in receptor expression but not ligand binding. *Mol Cell Endocrinol* **107:**241–245.

Davis D, Liu X and Segaloff DL (1995) Identification of the sites of *N*-linked glycosylation on the follicle-stimulating hormone (FSH) receptor and assessment of their role in FSH receptor function. *Mol Endocrinol* **9:**159–170.

el Battari A, Forget P, Fouchier F and Pic P (1991) Effect of inhibiting *N*-glycosylation or oligosaccharide processing on vasoactive intestinal peptide receptor binding activity and structure. *Biochem J* **278:**527–533.

Giovannelli A, Grassi F, Eusebi F and Miledi R (1991) Tunicamycin increases desensitization of acetylcholine receptors in cultured mouse muscle cells. *Proc Natl Acad Sci USA* **88:**1808–1811.

Heffernan M and Dennis JW (1990) A polyoma and hamster papovavirus large T antigen-mediated replication of expressing shuttle vectors in Chinese hamster ovary cells. *Nucleic Acids Res* **19:**85–92.

Ho HH, Gilbert MT, Nussenzveig DR and Gershengorn MC (1999) Glycosylation is important for binding to human calcitonin receptors. *Biochemistry* **38:**1866–1872.

Jans DA, Jans P, Luzius H and Fahrenholz F (1992) *N*-glycosylation plays a role in biosynthesis and internalization of the adenylate cyclase stimulating vasopressin V2-receptor of LLC-PK1 renal epithelial cells: An effect of concanavalin A on binding and expression. *Arch Biochem Biophys* **294:**64–69.

Jensen RT and Coy DH (1991) Progress in the development of potent bombesin receptor antagonists. *Trends Pharmacol Sci* **12:**13–19.

Jensen RT, Coy DH, Saeed ZA, Heinz-Erian P, Mantey S and Gardner JD (1988) Interaction of bombesin and related peptides with receptors on pancreatic acini. *Ann NY Acad Sci* **547:**138–149.

Ji IH, Slaughter RG and Ji TH (1990) *N*-Linked oligosaccharides are not required for hormone binding of the lutropin receptor in a Leydig tumor cell line and rat granulosa cells. *Endocrinology* **127:**494–496.

Kroog GS, Jensen RT and Battey JF (1995) Mammalian bombesin receptors. *Med Res Rev* **15:**389–417.

Kusui T, Benya RV, Battey JF and Jensen RT (1994) Glycosylation of bombesin receptors: Characterization, effect on binding and G-protein coupling. *Biochemistry* **33:**12968–12980.

Kuwano M, Seguchi T and Ono M (1991) Glycosylation mutations of serine/threonine-linked oligosaccharides in low-density lipoprotein receptor: Indispensable roles of *O*-glycosylation. *J Cell Sci* **98:**131–134.

Lanctot PM, Leclerc PC, Escher E, Leduc R and Guillemette G (1999) Role of *N*-glycosylation in the expression and functional properties of human AT1 receptor. *Biochemistry* **38:**8621–8627.

Liu X, Davis D and Segaloff DL (1993) Disruption of potential sites for *N*-linked glycosylation does not impair hormone binding to the lutropin/choriogonadotropin receptor if Asn-173 is left intact. *J Biol Chem* **268:**1513–1516.

Mantey S, Frucht H, Coy DH and Jensen RT (1993) Characterization of bombesin receptors using a novel, potent, radiolabeled antagonist that distinguishes bombesin receptor subtypes. *Mol Pharmacol* **45:**762–774.

McConnell HM, Owicki JC, Parce JW, Miller DL, Baxter GT, Wada HG and Pitchford S (1992) The cytosensor microphysiometer: Biological applications of silicon technology. *Science (Wash DC)* **257:**1906–1912.

Nishizaki T and Sumikawa K (1992) Tunicamycin increases desensitization of junctional and extrajunctional acetylcholine receptors expressed in *Xenopus* oocytes by a mechanism independent of *N*-glycosylation blocking. *Mol Pharmacol* **42:**152–156.

Ohara K, Uchiyama H, Haga T and Ichiyama A (1990) Interaction of deglycosylated muscarinic receptors with ligands and G proteins. *Eur J Pharmacol* **189:**341–346.

Petaja-Repo UE, Merz WE and Rajaniemi HJ (1993) Significance of the carbohydrate moiety of the rat ovarian luteinizing-hormone/chorionic-gonadotropin receptor for ligand-binding specificity and signal transduction. *Biochem J* **292:**839–844.

Proll MA, Clark RB and Butcher RW (1993) Beta 2-adrenergic receptor mutants reveal structural requirements for the desensitization observed with long-term epinephrine treatment. *Mol Pharmacol* **44:**569–574.

Rands E, Candelore MR, Cheung AH, Hill WS, Strader CD and Dixon RA (1990) Mutational analysis of beta-adrenergic receptor glycosylation. *J Biol Chem* **265:**10759–10764.

Ray K, Clapp P, Goldsmith PK and Spiegel AM (1999) Identification of the sites of *N*-linked glycosylation on the human calcium receptor and assessment of their role in cell surface expression and signal transduction. *J Biol Chem* **273:**34558–34567.

Rens-Domiano S and Reisine T (1991) Structural analysis and functional role of the carbohydrate component of somatostatin receptors. *J Biol Chem* **266:**20094–20102.

Russo D, Chazenbalk GD, Nagayama Y, Wadsworth HL and Rapoport B (1991) Site-directed mutagenesis of the human thyrotropin receptor: Role of asparagine-linked oligosaccharides in the expression of a functional receptor. *Mol Endocrinol* **5:**29–33.

Soderquist AM and Carpenter G (1984) Glycosylation of the epidermal growth factor

receptor in A-431 cells. The contribution of carbohydrate to receptor function. *J Biol Chem* **259:**12586–12594.
Swope SL and Schonbrunn A (1990) Desensitization of islet cells to bombesin involves both down-modulation and inhibition of receptor function. *Mol Pharmacol* **37:**758–766.
Tache Y, Melchiorri P and Negri L (1988) Bombesin-like peptides in health and disease. *Ann NY Acad Sci* **547:**1–541.
van Koppen CJ and Nathanson NM (1990) Site-directed mutagenesis of the M2 muscarinic acetylcholine receptor. Analysis of the role of *N*-glycosylation in receptor expression and function. *J Biol Chem* **265:**20887–20892.
Walsh JH, Bouzyk M and Rozengurt E (1993) Homologous desensitization of bombesin-induced increases in intracellular Ca^{2+} in quiescent Swiss 3T3 cells involves a protein kinase C-independent mechanism. *J Cell Physiol* **156:**333–340.
Wheatley M and Hawtin SR (1999) Glycosylation of G-protein-coupled receptors for hormones central to normal reproductive functioning: Its occurrence and role. *Hum Reprod Update* **5:**356–364.
Zhang R, Tsai-Morris CH, Kitamura M, Buczko E and Dufau ML (1991) Changes in binding activity of luteinizing hormone receptors by site directed mutagenesis of potential glycosylation sites. *Biochem Biophys Res Commun* **181:**804–808.

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