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# Internalization of the Gastrin-Releasing Peptide Receptor Is Mediated by Both Phospholipase C-Dependent and -Independent Processes

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## SUMMARY

Consequent to agonist exposure, many G protein-coupled receptors undergo sequestration or internalization. Results with receptors linked to adenylate cyclase, such as the $\beta_2$-adrenergic receptor, or receptors linked to phospholipase C (PLC) have provided conflicting results regarding the role of second messenger-dependent (i.e., protein kinase A or C) and -independent (i.e., $\beta$-adrenergic receptor kinase) kinases in mediating this process. Recent results for truncated and mutated gastrin-releasing peptide (GRP) receptors (GRP-R), as well as muscarinic cholinergic receptors, suggest that activation of protein kinase C may be needed for full receptor internalization. Nearly all G protein-coupled receptors studied to date, including the GRP-R, possess two highly conserved amino acids that are important in mediating receptor-G protein coupling to second messengers, i.e., arginine in the proximal second intracellular loop and alanine in the distal third intracellular loop. We selectively mutated each of these residues in the GRP-R to determine their importance for activation of PLC. Site-directed mutagenesis was performed to change arginine at position 139 to glycine (R139G mutant) and alanine at position 263 to glutamate (A263E mutant), with stable cell lines being created by transfection of the wild-type or mutated receptor cDNA into BALB/3T3 fibroblasts. Both R139G ($K_d$ = 12.0 ± 1.6 nM) and A263E ($K_d$ = 12.2 ± 1.7 nM) had a lower affinity for bombesin than did wild-type GRP-R ($K_d$ = 1.4 ± 0.4 nM); however, characteristic stoichiometries for the binding of agonists to this receptor were maintained equally in all three cell lines (bombesin > GRP ≫ neuromedin B). The wild-type GRP-R exposed to bombesin increased [$^3$H]inositol phosphates (a measure of PLC activation) approximately 4-fold, with an $EC_{50}$ of 5.1 ± 2.2 nM. In contrast, [$^3$H]inositol phosphates were not significantly increased in cells expressing R139G or A263E receptors, demonstrating that Arg$^{139}$ and Ala$^{263}$ are required for GRP-R activation of PLC. However, when receptor internalization at 37° was assessed by ligand acid-stripping studies, 53 ± 2% of A263E receptors were internalized at 90 min, compared with 85 ± 5% of wild-type GRP-R, whereas only 10 ± 3% of R139G receptors were internalized. Preincubation of either mutant cell line with 100 nM 12-*O*-tetradecanoylphorbol-13-acetate markedly increased internalization rates, such that at 90 min 62 ± 2% of R139G receptors and 82 ± 1% of A263E receptors were internalized. To assess receptor-G protein coupling, membranes from all three cell lines were incubated with $^{125}$I-[Tyr$^4$]bombesin and guanosine-5′-($\beta,\gamma$-imido)triphosphate, a nonhydrolyzable guanine nucleotide analogue. Increasing concentrations of guanosine-5′-($\beta,\gamma$-imido)triphosphate decreased $^{125}$I-[Tyr$^4$]bombesin binding to wild-type GRP-R and A263E, but there was little effect on binding to R139G. Thus, internalization of the GRP-R requires intact receptor-G protein coupling, whereas activation of PLC is not essential. These results, coupled with previous studies showing less than complete internalization when a protein kinase C site in the GRP-R tail was mutated, suggest that both PLC-dependent and -independent pathways are important in mediating GRP-R internalization.

Agonist interaction with most G protein-coupled receptors results in their immediate loss from the cell surface, a process variously described as sequestration or internalization. Internalization is one of the mechanisms whereby G protein-coupled receptors attenuate effector coupling in the face of continued exposure to agonist. Although certain structural determinants within the receptor have been found to be necessary for internalization to occur (1), the role of intracellular kinases (both second messenger dependent and independent) in mediating internalization has not been widely studied or clearly defined. Initial studies with the $\beta_2$AR suggested that, for efficient internalization to occur, receptor-G protein coupling with activation of adenylate cyclase was necessary (2, 3). Other investigators, however, demonstrated that $\beta_2$AR internalization could proceed

**ABBREVIATIONS:** $\beta_2$AR, $\beta_2$-adrenergic receptor(s); $\beta$ARK, $\beta$-adrenergic receptor kinase; [$Ca^{2+}$]$_i$, intracellular calcium concentration; EGF-R, epidermal growth factor receptor(s); GRP-R, gastrin-releasing peptide receptor(s); Gpp(NH)p, guanosine-5′-($\beta,\gamma$-imido)triphosphate; PKC, protein kinase C; PLC, phospholipase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GRP, gastrin-releasing peptide; AM, acetoxymethyl ester.



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independently of receptor-mediated alterations in adenylate cyclase activity (4, 5) and also without G protein coupling (4, 6). Similarly, recent evidence has suggested that receptor activation (but not necessarily second messenger stimulation) is necessary for internalization of the human Hm1 muscarinic cholinergic receptor (7). Other studies also suggest that, although changes in muscarinic cholinergic receptor internalization and stimulation of inositol phosphates are not closely coupled (8), involvement of a G protein is required (9). These studies therefore suggest that the role of signal transduction activation in mediating receptor internalization remains unclear.

GRP is the mammalian homologue of the amphibian tetradecapeptide bombesin and is widely distributed in the central nervous system (10, 11) and gastrointestinal tract (12, 13). GRP and other bombesin-like peptides are involved in numerous diverse functions, including acting as a growth factor in normal (14, 15) and neoplastic (16, 17) cells and regulating thermoregulation (18), circadian rhythm (19), and certain aspects of behavior (20); they have also been implicated in regulating the release of gastrointestinal peptides (21, 22) and altering immunologic function (23). Agonist interaction with the GRP-R results in activation of PLC and receptor internalization (24–26), down-regulation (25–29), and desensitization (27–32). The GRP-R has been cloned and sequenced (33, 34), thereby facilitating studies investigating the relationship between the structure of this receptor and its biological function. Recent investigations have demonstrated that multiple serines and threonines in the carboxyl terminus of the GRP-R are necessary for efficient internalization but do not alter the ability of this receptor to couple to PLC (35). However, those studies also demonstrated that an alteration in a PKC consensus sequence located in the GRP-R carboxyl terminus decreased internalization (35), suggesting that PLC-induced activation of PKC may be partially responsible for mediating internalization. However, the role of PKC in mediating GRP-R internalization remains uncertain, for the following reasons. First, three separate PKC consensus sequences exist within the intracellular domains of the GRP-R, but because only one site was mutated previously the full extent of the involvement of this kinase could not be precisely determined. Second, not all consensus sequences are necessarily phosphorylated, so the aforementioned mutation of the PKC consensus sequence may have altered internalization by a mechanism independent of this second messenger pathway. Third, it may be that some other kinase causing phosphorylation, not mediated by PKC but occurring at this consensus sequence, was altered. One way to test the hypothesis that GRP-R internalization is regulated by PKC phosphorylation might be to construct a mutant with no PKC consensus sequences; however, without a receptor antibody to exclude PKC-mediated phosphorylation it still would not be possible to exclude a PKC effect at an atypical site. One way to attempt to clearly define the role of PLC activation, with consequent elevation of PKC activity, in internalization would be to identify receptor mutants that selectively failed to activate PLC when exposed to agonists.

Amino acid sequence alignment and comparisons of the GRP-R with many other G protein-coupled receptors reveal that two amino acids are highly conserved (Fig. 1), i.e., arginine in the proximal second intracellular loop and alanine in the distal third intracellular loop. The alanine in the third intracellular loop is nearly ubiquitous in all seven-transmembrane domain receptors, whereas the arginine in the second intracellular loop is not present in some G protein-linked receptors, including those for secretin and vasoactive intestinal peptide (Fig. 1). These two conserved amino acids have been shown to be important in mediating receptor-G protein coupling, and their absence modulates the activation of various receptor-activated second messengers. Specifically, mutation of this arginine in the second intracellular loop to glutamine in the prototypical rhodopsin receptor eliminates rhodopsin-transducin interaction (36), whereas changing the distal third intracellular loop alanine selectively prevents the thyrotropin-stimulating hormone receptor from activating PLC, without altering its ability to activate adenylate cyclase (37). In this study, therefore, we mutated either of these critical amino acids to determine their importance for GRP-R-induced activation of PLC and the ability of these mutants to internalize ligand upon agonist exposure.

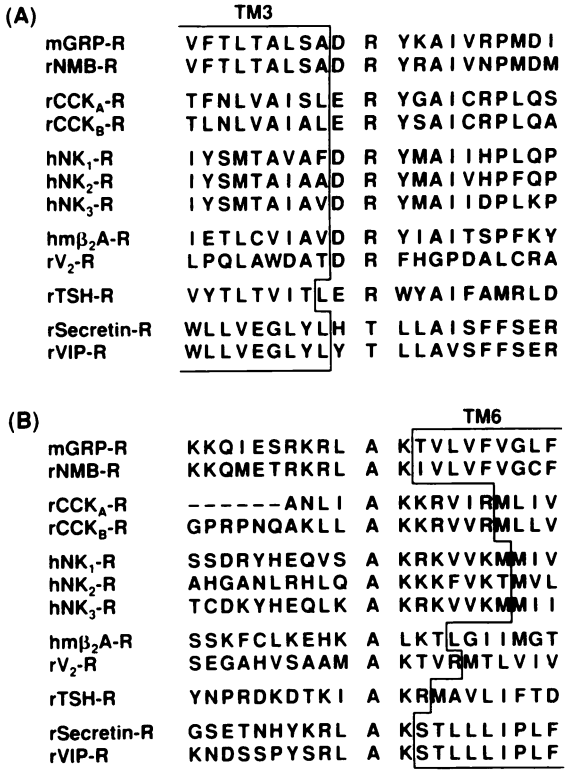


**Fig. 1.** Amino acid sequence alignment of various G protein-coupled receptors in the regions of the proximal second intracellular loop (A) and the distal third intracellular loop (B). A, The highly conserved arginine at amino acid position 139 for the GRP-R is ubiquitous except for the secretin-vasoactive intestinal peptide family of receptors. B, Alanine at position 263 for the GRP-R is likewise present in nearly all G protein-coupled receptors studied. *TM*, transmembrane region; *r*, rat; *m*, mouse; *hm*, hamster; *h*, human; *NMB*, neuromedin B; *CCK*, cholecystokinin; *NK*, neurokinin; *V*, vasopressin; *TSH*, thyrotropin-stimulating hormone; *VIP*, vasoactive intestinal peptide; *R*, receptor. Amino acids are shown in single-letter code.

We herein demonstrate that neither amino acid is required for agonist binding to the GRP-R but both are required for GRP-R-induced activation of PLC. However, the resultant abrogation of PLC activation does not necessarily eliminate the ability of the GRP-R to undergo internalization, thus demonstrating that PLC activation followed by activation of PKC only partially mediates internalization.

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## Experimental Procedures

**Materials.** BALB/3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and were subjected to clonal expansion to identify a line devoid of neuromedin B receptors or GRP-R, as determined by binding and RNase protection assays. Bombesin, GRP, and neuromedin B were obtained from Peninsula Laboratories (Belmont, CA); Gpp(NH)p tetralithium salt was from Fluka Chemical Co. (Ronkonkoma, NY); Iodo-Gen was from Pierce (Rockford, IL); $Na^{125}I$ was from Amersham (Arlington Heights, IL); *myo*-[2-$^3$H]inositol (16–20 Ci/mmol) was from New England Nuclear (Boston, MA); fura-2/AM was from Molecular Probes (Eugene, OR); and TPA was from Calbiochem (La Jolla, CA).

**Mutant receptor construction and expression.** Mutant cDNAs were constructed by using mouse GRP-R cDNA (33) as a template for site-directed mutagenesis (Altered Sites *in vitro* mutagenesis system; Promega, Madison, WI). The arginine at position 139 of the GRP-R was mutated to a glycine by changing the corresponding ATT of the wild-type cDNA to GTT (final construct R139G), whereas the alanine at position 263 was mutated to a glutamate by converting the corresponding GCC of the wild-type cDNA to GAA (final construct A263E). The structure of the mutated GRP-R cDNA was confirmed by dideoxy sequencing (38). All cDNA constructs were subcloned into a modified version of the mammalian expression vector pCD2 (39). Stable transfection into BALB fibroblasts was accomplished by calcium phosphate precipitation (40) using 15 μg of recombinant pCD2 plasmid; selection using the aminoglycoside G418 (800 μg/ml) was started 48 hr later and continued for 4–6 weeks. Clonal cell lines were obtained by amplifying single cells (41) and screening for $^{125}$I-[Tyr$^4$]bombesin binding as described previously (42). Only the clones expressing the greatest numbers of receptors were maintained for the experiments discussed herein. Cells were maintained at 37° in a 5% $CO_2$ atmosphere, in Dulbecco's modified essential medium supplemented with 10% fetal calf serum and 270 μg/ml G418.

**Binding studies.** $^{125}$I-[Tyr$^4$]Bombesin (2200 Ci/mmol) was prepared using Iodo-Gen and was purified by high pressure liquid chromatography as described previously (43). Disaggregated cells were resuspended in binding buffer, composed of standard buffer (98 mM NaCl, 6 mM KCl, 25 mM HEPES, 5 mM pyruvate, 5 mM fumarate, 5 mM glutamate, 0.1% soybean trypsin inhibitor) 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 $3 \times 10^6$ cells/ml with 75 pM $^{125}$I-[Tyr$^4$]bombesin and varying concentrations of bombesin for 30 min at 37° 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 <10% of total binding. Analysis of the binding data was performed using the least-squares curve-fitting program LIGAND (44), and all values in this paper are reported as saturable binding. To assess internalization of the radioligand, acid stripping was performed as described previously (35). In this study we do not distinguish between receptor internalization and sequestration because we do not have a hydrophilic and cell-permeable ligand to differentiate cell surface receptor number from total receptor number. Internalization refers to the proportion of radiolabeled agonist that is not acid strippable and therefore resides within the cell. To assess the contribution of PKC activation to mutant receptor internalization, disaggregated cells were preincubated for 30 min at 37° with 100 nM TPA and solvent (0.01% dimethylsulfoxide) or with solvent alone.

**Measurement of inositol phosphates.** Confluent cells were loaded with 100 μCi/ml *myo*-[2-$^3$H]inositol, in Dulbecco's modified essential medium supplemented with 2% fetal calf serum, at 37° for 24 hr. Cells were washed and incubated for 15 min with phosphatidylinositol buffer (standard buffer additionally containing 10 mM $LiCl_2$, 2 mM $CaCl_2$, 2% bovine serum albumin, and 1.2 mM $MgSO_4$) and were then incubated with varying concentrations of bombesin for 60 min at 37°. Reactions were halted by using 1% HCl in methanol, and total [$^3$H]inositol phosphates were isolated by anion exchange chromatography, as described previously (45).

**Measurement of $[Ca^{2+}]_i$.** Cells were mechanically disaggregated, resuspended in binding buffer at a concentration of $2 \times 10^6$ cells/ml with 2 μM fura-2/AM, and incubated at 22° for 45 min. After loading with fura-2, cells were washed three times with binding buffer. For measurement of $[Ca^{2+}]_i$, 2-ml samples were placed in quartz cuvettes in a Delta PTI Scan-1 spectrophotometer (PTI Instruments, Gaithersburg, MD). Fluorescence was measured at 500 nm after excitation at 340 nm and 380 nm. Autofluorescence of the unloaded cells was subtracted from all measurements, and $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz *et al.* (46).

**Cell membrane preparation.** Confluent cells were mechanically disaggregated, washed in binding buffer, and resuspended in homogenization buffer (50 mM Tris, pH 7.4, 0.2 mg/ml soybean trypsin inhibitor, 0.2 mg/ml benzamidine). Cells were homogenized on ice using a Polytron homogenizer (Beckman Instruments), at speed 6, for 30 sec. The homogenate was centrifuged at 1500 rpm for 10 min in a Sorvall RC-5B Superspeed centrifuge (DuPont), 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°.

**Statistics.** All comparisons were made using the Student *t* test, and values of $p < 0.05$ were considered significant.

## Results

Both R139G ($K_d$ = 12.0 ± 1.6 nM) and A263E ($K_d$ = 12.2 ± 1.7 nM) had lower affinity for bombesin than did the wild-type GRP-R ($K_d$ = 1.4 ± 0.4 nM); however, the characteristic stoichiometric relationships for the binding of agonists to these receptors were retained (Fig. 2). For all three receptors, in comparison to bombesin, GRP was approximately 2.5–4.3-fold less potent and neuromedin B was 120–150-fold less potent at inhibiting the binding of $^{125}$I-[Tyr$^4$]bombesin (Fig. 2). All three cell types expressed high concentrations of receptors; Scatchard analysis of the binding data obtained from cells expressing the wild-type GRP-R generated a $B_{max}$ of 1023 ± 128 fmol/$10^6$ cells, whereas for the A263E cell line the $B_{max}$ was 1495 ± 195 fmol/$10^6$ cells and for the R139G cell line the $B_{max}$ was 2251 ± 235 fmol/$10^6$ cells.

To determine whether agonist occupation of the transfected receptors resulted in activation of intracellular processes, we measured the ability of bombesin to activate PLC and increase $[Ca^{2+}]_i$. Cells transfected with the wild-type GRP-R rapidly increased [$^3$H]inositol phosphates from 5,500 ± 400 dpm to 21,200 ± 2,200 dpm when maximally stimulated with 1 μM bombesin, whereas half-maximal stimulation was observed with 5.1 ± 2.2 nM bombesin (Fig. 3, *left*). In contrast, [$^3$H]inositol phosphates were not significantly increased in transfected cells expressing the mutant receptor R139G (9000 ± 2100 to 9600 ± 1900 dpm) or A263E (6500 ± 500 to 5300 ± 800 dpm) (Fig. 3, *left*). Because recent evidence suggests that certain cell membrane receptors can increase $[Ca^{2+}]_i$ from inositol phosphate-insensitive calcium pools (47), we also determined agonist-induced alterations in $[Ca^{2+}]_i$ in cells equilibrated with fura-2/AM. Although with the addition of 1 μM bombesin $[Ca^{2+}]_i$ increased from 103 ± 4 nM to 299 ± 23 nM in cells expressing the wild-type GRP-R, there was no detectable increase in $[Ca^{2+}]_i$ in the R139G cell line (98 ± 5 to 95 ± 3 nM) or in the A263E cell line (101 ± 10 to 104 ± 6 nM) (data not shown). These data confirm that Arg$^{139}$ and Ala$^{263}$ are necessary for the GRP-R to activate PLC and increase $[Ca^{2+}]_i$.

To investigate whether wild-type GRP-R or the mutant receptors are internalized, acid stripping to remove surface-

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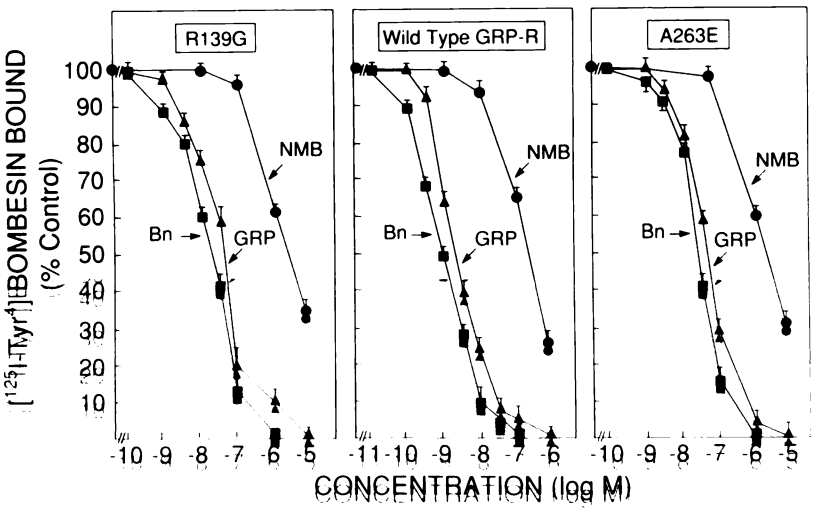


**Fig. 2.** Ability of bombesin and related peptides to inhibit binding of $^{125}$I-[Tyr$^4$]bombesin to BALB fibroblasts stably transfected with the cDNA for wild-type GRP-R (*center*), mutant R139G (*left*), or mutant A263E (*right*). Cell lines stably expressing wild-type or mutant GRP-R were incubated in binding buffer with 75 pM $^{125}$I-[Tyr$^4$]bombesin alone or with the indicated concentration of peptide. Data are expressed as the percentage of saturably bound radioactivity in the absence of nonradioactive peptide. For each experiment, each value was determined in duplicate, and the results are the means ± standard errors of at least three separate experiments. *Bn*, bombesin; *NMB*, neuromedin B.

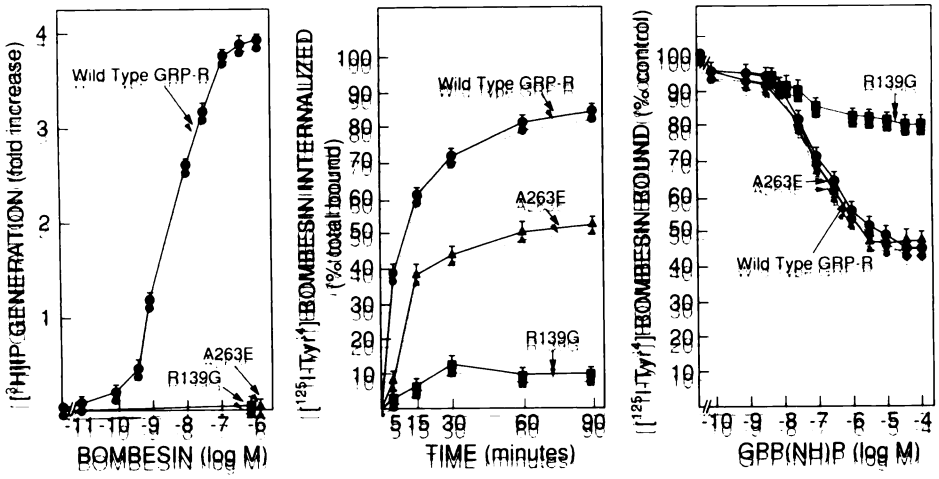


**Fig. 3.** [$^3$H]Inositol phosphate generation (*left*), internalization rates (*center*), and the effect of Gpp(NH)p on the binding of $^{125}$I-[Tyr$^4$]bombesin to membranes (*right*) of wild-type and mutant GRP-R-expressing cells. *Left*, [$^3$H]inositol phosphate ([$^3$H]IP) generation was determined as described in Experimental Procedures, with the data being expressed as the fold increase in dpm. *Center*, internalization is expressed as the proportion of total, saturably bound ligand at each time point that was not removable by exposure to 0.2 M acetic acid in 0.5 M NaCl, pH 2.5. *Right*, the effect of Gpp(NH)p on membranes from wild-type GRP-R-transfected cells ($10 \times 10^6$/ml) or from cells transfected with mutant A263E ($20 \times 10^6$/ml) or mutant R139G ($10 \times 10^6$/ml) was determined by incubation of membranes with 50 pM $^{125}$I-[Tyr$^4$]bombesin alone or with the indicated concentration of the nonhydrolyzable guanine nucleotide analogue. For all experiments, each value was measured in duplicate, with each *point* representing the mean ± standard error of at least three separate experiments.

bound ligand was performed after various periods at 37°. Wild-type GRP-R was internalized rapidly ($t_{½} \equiv 4.8 \pm 0.1$ min), such that by 90 min 85 ± 5% of expressed receptors were internalized (Fig. 3, *center*). However, GRP-R mutant R139G was internalized poorly ($t_{½} \equiv 15.1 \pm 0.9$ min), with only 10 ± 3% of receptors being internalized by 90 min. In contrast to the poor internalization manifested by the mutant receptor R139G, the mutant receptor A263E underwent rapid initial internalization ($t_{½} \equiv 6.9 \pm 0.7$ min), but only 53 ± 2% of receptors were internalized by 90 min. To determine whether activation of PKC could improve internalization of the mutant receptors, R139G-expressing cells and A263E-expressing cells were preincubated with the phorbol ester TPA (100 nM) for 30 min at 37° (Fig. 4). By 90 min, 62 ± 2% of R139G receptors resident on cells that had been preincubated with TPA were internalized, compared with 14 ± 2% on control R139G-expressing cells processed in parallel ($p \leq 0.01$) (Fig. 4). Similarly, TPA preincubation increased internalization of A263E mutant receptors to 82 ± 1% at 90 min, compared with 64 ± 1% internalized in control A263E-expressing cells processed in parallel ($p \leq 0.01$) (Fig. 4); thus, at 90 min the internalization rate for mutant A263E receptors was restored approximately to that exhibited by wild-type GRP-R, whereas the internalization rate for mutant R139G receptors was approximately 75% of that manifested by wild-type GRP-R. The increase in internalization secondary to PKC activation ranged from 31% (A263E) to 52% (R139G) at 15 min and from 22% (A263E) to 58% (R139G) at 90 min. Thus, these data suggest that PKC activation is responsible for approximately 50% of the internalization of the GRP-R consequent to exposure of this receptor to agonist.

To determine whether a difference in receptor-G protein coupling between the two mutant receptor constructs might partially account for the differences in internalization rates, we analyzed the effect of the nonhydrolyzable guanine nucleotide analogue Gpp(NH)p on $^{125}$I-[Tyr$^4$]bombesin binding to each cell line (Fig. 3, *right*). In membranes derived from cells ex-

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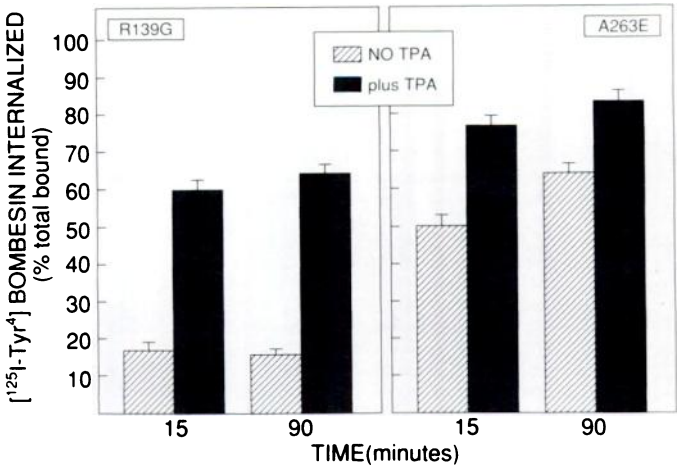


**Fig. 4.** Internalization rates for cells expressing mutants R139G (*left*) and A263E (*right*). Internalization was assessed as described in the legend to Fig. 3. Cells were preincubated for 30 min at 37° with 0.01% dimethylsulfoxide alone (*NO TPA*) or with 100 nM TPA (*plus TPA*). Results are expressed as the proportion of total, saturably bound ligand that was internalized. For each experiment, each value was determined in triplicate, with each *point* representing the mean ± standard error of at least three separate experiments.

pressing the wild-type GRP-R, Gpp(NH)p caused a concentration-dependent decrease in the binding of $^{125}$I-[Tyr$^4$]bombesin. Maximal decreases in binding were observed with approximately 10 μM Gpp(NH)p and half-maximal decreases were observed with 0.1 μM Gpp(NH)p. A similar effect of Gpp(NH)p on the binding of $^{125}$I-[Tyr$^4$]bombesin was seen with membranes obtained from cells expressing mutant A263E (Fig. 3, *right*). In contrast, Gpp(NH)p at concentrations of 10 μM displaced only 18 ± 2% of $^{125}$I-[Tyr$^4$]bombesin bound to membranes obtained from cells expressing mutant R139G. These data suggest that, although mutation of either Ala$^{263}$ or Arg$^{139}$ totally eliminates the ability of the GRP-R to activate PLC, the latter mutation results in a more complete disruption of receptor-G protein coupling.

## Discussion

In the present study we demonstrate that in the GRP-R both Arg$^{139}$ in the proximal second intracellular loop and Ala$^{263}$ in the distal third intracellular loop, amino acids that are highly conserved in many G protein-coupled receptors, are required for activation of PLC. However, internalization is only partially reduced by removal of Ala$^{263}$ from the GRP-R. Because both mutations of the GRP-R studied in this paper completely eliminated PLC activation but did not completely eliminate internalization, activation of PLC-coupled kinases may only partially mediate GRP-R internalization. A previous investigation with the GRP-R suggested that PLC activation could be involved in mediating up to 36% of the internalization observed with this receptor (35). Specifically, the elimination of a PKC consensus sequence located within the carboxyl terminus of the GRP-R decreased receptor internalization to 64% of that observed for the wild-type GRP-R (35). This suggested that components within the PLC-inducible second messenger pathway could account for a proportion of GRP-R internalization. However, the role of PLC and, by extension, that of PKC in regulating GRP-R internalization remain unclear because, although the removal of one PKC consensus sequence attenuated internalization, two additional sites remained. Furthermore, activation of PLC by the GRP-R results in the activation of other second messengers including cAMP (48), as well as increased $[Ca^{2+}]_i$ (15). Thus, removal of a single PKC consensus sequence not only fails to completely address the role of PKC in regulating GRP-R internalization but also fails to address the role of PLC activation in regulating GRP-R internalization. Indeed, because truncation of the GRP-R carboxyl terminus or replacement of all carboxyl-terminal serines and threonines attenuated GRP-R internalization to a greater degree than observed after simple mutation of the PKC concensus sequence, previous evidence also suggests a role for mediators independent of PKC in regulating internalization (35). Because activation of PKC with phorbol esters in this study accelerated the rate of internalization by 22–58% (mean, 41 ± 8%) in cells expressing mutant GRP-R otherwise unable to activate PLC, this study suggests that internalization of the GRP-R is regulated approximately evenly by PLC-dependent and PLC-independent pathways.

The importance of second messengers in regulating the internalization of other receptors is not clear. Studies on both the $\beta_2$AR (2–5) and PLC-linked muscarinic cholinergic receptors (7, 8) have provided differing results, as have recent studies investigating the relationship between PLC activation and internalization of the EGF-R. Some investigators have demonstrated that intrinsic tyrosine kinase activity resulting in PLC activation is required for EGF-R internalization (49, 50), whereas others, using similarly designed mutant EGF-R, have shown that internalization of this receptor is independent of PLC (51). Recent studies have shown that cellular kinases both dependent upon and independent of PKC can be involved in affecting EGF-R internalization (52–56), whereas others have documented a role for second messengers in regulating internalization of the asialoglycoprotein (57) and transferrin (58, 59) receptors. Taken together, these studies suggest that the role of second messenger activation in mediating receptor internalization is not clear.

In this study we demonstrate that GRP-R internalization can occur independently of PLC activation; therefore, there must be one or more as yet undescribed pathways mediating this phenomenon. A recent study demonstrated that the wild-type GRP-R-expressing cells used in the present study do not activate adenylate cyclase upon stimulation with GRP agonists (60); therefore, the activation of this pathway is unlikely to be involved in mediating GRP-R internalization. However, it is possible that other intracellular kinases may ultimately prove to be responsible for mediating GPR-R internalization. The recent observation that the presence of multiple serines and threonines in the GRP-R carboxyl terminus is necessary for its internalization, independently of PLC activation (35), suggests that GRP-R phosphorylation by kinases distinct from PKC may partially regulate the internalization of this receptor. In contrast, studies of $\beta_2$AR sequestration, a phenomenon proposed to be caused by receptor internalization (1), have suggested that this process is independent of receptor phosphorylation (1). However, the studies investigating $\beta_2$AR sequestration have generated conflicting results. Although elimination of the βARK phosphorylation sites by substitution of the 11 serines and threonines in the carboxyl terminus of the $\beta_2$AR (i.e., Ser$^{355}$ to Ser$^{411}$) did not attenuate receptor internalization (61), another study revealed that substitution of Ser$^{355}$, Ser$^{356}$,

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Thr[360], and Ser[364] with glycine or alanine eliminated internalization of the $\beta_2$AR (62). Consequently, the role of $\beta$ARK-mediated phosphorylation of the $\beta_2$AR in regulating the internalization of this receptor remains unclear. Indeed, recent evidence suggests that a separate internalization consensus sequence exists that includes a tyrosine in the seventh transmembrane region of the $\beta_2$AR and does not appear to be regulated by the currently identified second messenger-dependent and -independent kinases (63). $\beta$ARKs have broad specificity and have been shown to act on PLC-coupled receptors, such as those for substance P (64); however, it is not currently known whether $\beta$ARKs or other G protein-coupled receptor kinases can phosphorylate or otherwise affect GRP-R desensitization or internalization.

In previous studies the role of G protein activation in mediating internalization appeared to vary with different receptors. Whereas evidence suggests that it is not essential for sequestration of the $\beta_2$AR (4), another study (8) suggests that it is essential for sequestration of the $M_3$ muscarinic cholinergic receptor. In the present study internalization of the GRP-R appears to require some interaction with G proteins. Indeed, the ability of membranes derived from cells expressing the mutant A263E, the construct that was partially internalized, to bind $^{125}$I-[Try$^4$]bombesin was affected by Gpp(NH)p similarly, compared with membranes from cells expressing the wild-type GRP-R. In contrast, the ability of membranes derived from cells expressing R139G, the mutant that largely failed to be internalized, to bind $^{125}$I-[Try$^4$]bombesin was relatively unaffected by Gpp(NH)p. This suggests that mutation of Arg[139] results in more complete uncoupling of the GRP-R from G proteins. However, knowledge of the precise nature of GRP-R coupling with G proteins, or of the specific G proteins required for optimal signal transduction, cannot be deduced from this study.

In conclusion, we have shown that Arg[139] and Ala[263] are absolutely necessary for the GRP-R to activate PLC. However, the Ala[263] mutation decreased the ability of the GRP-R to undergo internalization by only 38%, whereas the ability of the Arg[139] mutant was decreased by 86%. The marked decrease seen with the Arg[139] mutant was likely related to the fact that this mutant was not coupled to G proteins, whereas the Ala[263] mutant remained fully coupled, suggesting that internalization also is a G protein-coupled process. These data, combined with the results of a recent study that demonstrates that removal of the PKC consensus sequence from the GRP-R carboxyl terminus attenuates internalization of the receptor (35), lead us to conclude that both PLC-dependent and PLC-independent processes mediate internalization of the GRP-R.

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