The Bombesin Receptor Subtypes Have Distinct G Protein Specificities*

Xiaoying Jian‡, Eduardo Sainz‡, William A. Clark§, Robert T. Jensen¶, James F. Battey‡, and John K. Northup§

From the §Laboratory of Molecular Biology, National Institute on Deafness and Other Communication Disorders, Rockville, Maryland 20850, ¶Digestive Diseases Section, NIDDKD, National Institutes of Health, Bethesda, Maryland 20892, and ¶Laboratory of Cellular Biology, National Institute on Deafness and Other Communication Disorders, Rockville, Maryland 20850

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We used an in situ reconstitution assay to examine the receptor coupling to purified G protein α subunits by the bombesin receptor family, including gastrin-releasing peptide receptor (GRP-R), neuromedin B receptor (NMB-R), and bombesin receptor subtype 3 (BRS-3). Cells expressing GRP-R or NMB-R catalyzed the activation of squid retinal Gαq and mouse Gαq, but not bovine retinal Gαo or bovine brain Gαo. The GRP-R- and NMB-R-catalyzed activations of Gαq were dependent upon and enhanced by different βγ dimers in the same rank order as follows: bovine brain βγ > βγ γ > βγ γ. Despite these qualitative similarities, GRP-R and NMB-R had distinct kinetic properties in receptor-G protein coupling. GRP-R had higher affinities for bovine brain βγ, βγ γ, and βγ γ and squid retinal Gαq. In addition, GRP-R showed higher catalytic activity on squid Gαq. Like GRP-R and NMB-R, BRS-3 did not catalyze GTPγS binding to Gαq, or Gαo. However, BRS-3 showed little, if any, coupling with squid Gαo, but clearly activated mouse Gαo. GRP-R and NMB-R catalyzed GTPγS binding to both squid and mouse Gαo with GRP-R activating squid Gαq more effectively, and NMB-R also showed slight preference for squid Gαq. These studies reveal that the structurally similar bombesin receptor subtypes, in particular BRS-3, possess distinct coupling preferences among members of the Gαq family.

Mammalian bombesin-like peptides, gastrin-releasing peptide (GRP)1 and neuromedin B (NMB), are widely distributed in the nervous system and the gut. They regulate various physiological processes such as secretion, growth, muscle contraction, and neuromodulation through high affinity receptors (1, 2). Three pharmacologically and structurally distinct bombesin receptor subtypes have been cloned and characterized in mammals as follows: the GRP-prefering receptor (GRP-R), the neuromedin B-prefering receptor (NMB-R), and bombesin receptor subtype 3 (BRS-3) which has a structure related to GRP-R and NMB-R but for which no high affinity, naturally occurring ligand has been identified as yet (2). Comparison of the predicted amino acid sequences (2) of the bombesin receptor subtypes shows all three to be structurally related members of the G protein-coupled receptor superfamily with pairwise sequence identity ranging from 48 to 54% (see Fig. 1). Upon agonist binding, G protein-coupled receptors activate specific heterotrimeric G proteins, which in turn regulate a variety of intracellular effectors such as adenyl cyclase, phospholipase C, ion channels, and cGMP-phosphodiesterase (3).

Heterotrimeric G proteins are composed of three polypeptides as follows: an α subunit and a βγ dimer that acts as a functional monomer. Ligand-activated G protein-coupled receptors catalyze the exchange of GTP for GDP bound to the Gα subunit, resulting in dissociation of the GTP-activated α subunit from both its cognate Gβγ dimer and the receptor. The GTP-activated α subunit as well as dissociated Gβγ dimer in turn regulate intracellular effectors. At least 20 different α subunits, 5 β subunits, and 12 γ subunits have been identified to date. The Gα subunits have been divided into four groups based upon sequence homology and intracellular effector regulation (4, 5). The Gαq subfamily, which includes Gαq, Go11, Gα14, and Gα15, stimulates phosphoinositide hydrolysis by activating phospholipase C-β (6–10). In addition, Gβγ subunits can also stimulate phospholipase C-βs in concert with Gαq (11, 12).

Given that the seven transmembrane domain receptor superfamily consists of thousands of distinct receptors, and the family of heterotrimeric G proteins involved in receptor coupling is also very diverse, a central issue in receptor signaling is how these protein families contribute to the diversity of receptor/G protein-mediated responses while conserving the specificity of each response. One level of specificity is likely to be determined by the thermodynamics of protein-protein interactions between subunits of the heterotrimeric G protein and the receptor. An in situ reconstitution procedure has been used successfully to study receptor-G protein interactions for baculovirus-infected Sf9 cell membranes expressing the 5-HT2α receptor (13), and for mouse fibroblast cell membranes expressing stably transfected GRP-R (14). This technique utilizes chaotrope-extracted membrane fractions in which endogenous GTP-binding proteins as well as other extrinsic membrane proteins are removed or inactivated by urea, while leaving uncoupled receptors fully functional when reconstituted with agonist and purified G protein subunits.

Since mammalian bombesin receptors stimulate phosho-
in situ reconstitution of GRP-R with purified G protein subunits shows explicitly that GRP-R activates a Go as but not Goq or Gbg (14). Such observations raise a possibility that the ambiguity in the antisense oligonucleotide experiments could be due to a difference in the relative affinity or activity of GRP-R for Goq as compared with that of NMB-R for Goq. The assessment of this possibility requires a quantitative comparison of GRP-R and NMB-R coupling with purified G protein subunits in vitro.

In this report we compare the receptor-G protein interactions within the structurally related bombesin receptor family using the in situ reconstitution assay. We have quantitatively examined the G protein activation by these related receptor structures using homogeneous preparations of defined G protein subunits. Our studies revealed that whereas GRP-R and NMB-R selectively coupled with squid and mouse Goq, Goa, or Gbg, respectively, the structurally related bombesin receptor family using in situ reconstitution of GRP-R with purified G protein subunits shows explicitly that GRP-R activates a Go as but not Goq or Gbg (14). 

EXPERIMENTAL PROCEDURES

Membrane Preparation—Membranes were prepared from Balb 3T3 mouse fibroblast cells expressing mouse GRP-R (19), rat NMB-R (20), or recombinant mouse GRP-R (22) or BRS-3 were also prepared. Receptor-enriched membranes were obtained as a 2% fraction from these cells as described previously (14).

Formation of Recombinant BRS-3 Baculovirus—A cDNA fragment encoding the open reading frame of the human BRS-3 (hBRS-3) is flanked by FLAG epitope tag at the 5' end was cloned into the RI site of a transfer vector pBacPAK5 (CLONTECH). The sequence at the 5' end is identical to that of the original hBRS-3 clone inserted into an EcoRI site (23). Insect cell line Sf9 cells expressing recombinant mouse GRP-R, rat NMB-R, and human BRS-3 (hBRS3) are presented as the best sequence alignment. The seven transmembrane (TM) helical domains predicted by hydrophathy plots are represented by TM-I to TM-VII with darker underlines. The predicted four extracellular domains (e1 to e4) and four intracellular domains (i1 to i4) are designated. Bold amino acid residues (indicated with *) are the positions mutated in 4BRS3 to their counterparts in NMB-R and GRP-R.

Purification of G Protein Subunits—G proteins were isolated from squid retina, bovine brain, bovine retina, and baculovirus-infected Sf9 cells expressing recombinant mouse Goa or Goq. Squid retinal Goq was purified as described by Hartman and Northup (13). Bovine brain Goa and Gbg (26), bovine retina Goa and Gbg (27–29), and recombinant mouse Goq (30) and Goa (31) expressed in Sf9 cells were purified using previously published protocols. Bovine brain Goa preparations were further purified by additional chromatography over phenyl-Sepharose to remove GTPyS binding activity (13).

GDP/GTPyS Exchange Assay—The receptor-catalyzed GTPyS exchange on Goa was determined essentially as described previously (32) with the addition of 2 μM GDP to compete for unbound GTPyS binding (14). Receptor-containing membranes were mixed with G protein subunits and with or without agonist on ice in a total volume of 30 μl. An addition of 20 μl of reaction solution was used to initiate the reactions. The reactions contained a final concentration of 50 mM MOPS, pH 7.5, 100 mM NaCl, 1 mM EDTA, 3 mM MgSO4, 1 mM dithiothreitol, 3 mg/ml bovine serum albumin, 2 μM GDP, and [35S]GTPyS (about 4–8 × 106 cpm) which were incubated at 30 °C for 10 or 15 min, terminated by adding 2 ml of ice-cold solution B (20 mM Tris/HCl, pH 8.0, 25 mM MgCl2, 100 mM NaCl), and filtered over nitrocellulose membranes on a vacuum manifold. The filters were washed four times with 2 ml each of ice-cold solution B and dried, and the bound radioactivity was counted by liquid scintillation.

RESULTS

Urea-extracted cell membranes containing different heptahelical receptors have been successfully reconstituted with pu-
rified G protein subunits. These receptor membranes include rod outer segment disc membranes of bovine retina (29), baculovirus-infected Sf9 cell membranes containing the 5-HT2c receptor (13), and stably transfected fibroblast cell membranes containing GRP-R (14). We applied this in situ receptor reconstitution technique to the bombesin receptor family to compare the G protein coupling properties of bombesin receptor subtypes, which share 48 to 54% amino acid homology (Fig. 1).

We modified the previously published urea extraction procedure to obtain more consistent receptor recovery and G protein depletion (see “Experimental Procedures”). Table I summarizes the effects of the modified procedure on receptor-binding sites and GTP\(\gamma\)S binding activity of GRP-R- and NMB-R-containing membranes. Compared with the 6 M urea extraction procedure used previously, 7 M urea required only one instead of two or three extractions, removed more endogenous GTP\(\gamma\)S binding activity (94–96% versus 92%), while consistently maintaining high recovery of ligand-binding sites. The GRP-R-binding site abundance was actually enriched more than 3-fold by 7M urea extraction, since 100% of the antagonist binding activity was recovered, whereas 71% of the membrane protein was removed. Furthermore, for both GRP-R and NMB-R, agonist-stimulated GTP\(\gamma\)S binding in the absence of exogenous G proteins was also abolished more thoroughly by 7 M urea extraction than by 6 M urea extraction used previously (data not shown), suggesting 7 M urea treatment resulted in a more homogeneous population.

| Receptor | Assay                  | Pre-7 M urea extraction | Post-7 M urea extraction | Recovery |
|----------|------------------------|-------------------------|-------------------------|----------|
|          | fmol                   | %                       |                         |          |
| GRP-R    | \(^{125}\)I-697 (agonist)-binding sites | 13.1 ± 2.9              | ND\(^a\)                 | ND       |
|          | \(^{125}\)I-ME (antagonist)-binding sites | 11.8 ± 2.3              | 12.5 ± 3.1               | 106      |
|          | \[^{35}\]S\)GTP\(\gamma\)S binding | 123.2 ± 4.5             | 4.5 ± 0.4                | 4        |
| NMB-R    | \(^{125}\)I-697 (agonist)-binding sites | 13.9 ± 4.0              | ND                       | ND       |
|          | \[^{35}\]S\)GTP\(\gamma\)S binding | 71.1 ± 4.2              | 4.2 ± 0.4                | 6        |

\(^a\) ND, not determined. The agonist-binding sites for 7 M urea-extracted GRP-R and NMB-R could not be determined due to the decreased affinities of uncoupled receptors for agonists.
proximately equal catalytic activities of the two receptor types at \( K_{\text{on}} \) concentrations of \( \alpha_{\text{a}} \) and near-saturating \( \beta_{\gamma} \). Thus we are directly comparing both the success of reconstitution and the catalytic properties of the receptors. These experiments demonstrated that (i) very little agonist-stimulated exchange of GDP for GTP\(\gamma\)S on exogenously added Go\(\alpha_{\text{a}}\) was detected for unextracted GRP-R or NMB-R (Fig. 2, A and C); (ii) both 7 M urea-extracted GRP-R and 7 M urea-extracted NMB-R coupled with squid Go\(\alpha_{3}\) (Fig. 2, B and D); (iii) like GRP-R, NMB-R-catalyzed activation of Go\(\alpha_{4}\) was also dependent on both agonist and \( \beta_{\gamma} \) subunits (Fig. 2, B and D). Despite the qualitative similarities between GRP-R and NMB-R in G protein coupling, they were clearly different in the ratio of agonist-independent (basal) to agonist-stimulated activity (Fig. 2, B and D).

In order to obtain initial rate estimates for the receptor-catalyzed GTP\(\gamma\)S binding, we performed the progress analyses for GRP-R- and NMB-R-catalyzed reactions shown in Fig. 3. For both 7 M urea-extracted GRP-R and NMB-R and G protein alone, the binding of GTP\(\gamma\)S progressed at a very low rate; G protein-reconstituted receptors without agonist showed an increased rate of binding, whereas the addition of agonist increased the reaction rate to the highest values. Moreover, for all of these conditions the GTP\(\gamma\)S binding was approximately linear with time for the initial 10 min of the reaction. Therefore we have used 10 min as a fixed time point in the GDP/GTP\(\gamma\)S exchange assay to measure the initial velocity of the receptor-catalyzed activation in all subsequent experiments. The greatly accelerated initial rates of GTP\(\gamma\)S binding in the presence of agonist represent our measure of receptor-catalyzed G protein activation. The GRP-R, NMB-R, and BRS-3 were expressed at widely varying abundance in the Balb 3T3 fibroblasts. Therefore, we have used the modified GTP\(\gamma\)S binding procedures (14) including trace \(^{35}\text{S}\)GTP\(\gamma\)S and 2 \(\mu\)M GDP to suppress residual nucleotide binding activity of the urea-extracted membranes rather than our initial procedures that utilize 1 \(\mu\)M GTP\(\gamma\)S with no competing nucleotide (13). Our modified procedure also accommodates the comparison of the family of Go proteins that differ in spontaneous binding exchange rates. Because the chemical concentration of GTP\(\gamma\)S (4–8 nM) limits the binding reactions, the plateau values obtained in these experiments are not stoichiometric binding of GTP\(\gamma\)S to the Go\(\alpha_{\text{a}}\). Rather, they represent consumption of the \(^{35}\text{S}\)GTP\(\gamma\)S trace in the binding reactions. That these receptors are indeed catalytic was demonstrated by additional experiments using 1 \(\mu\)M GTP\(\gamma\)S without competing GDP in which 1 \(\text{nM} \) GRP-R or NMB-R activated the entire 100 \(\text{nM} \) Go\(\alpha_{\text{a}}\) in about 40 min (data not shown).

Our previous study has shown selective coupling of GRP-R with Go\(\alpha_{\text{a}}\), but not Go\(\alpha_{\text{G}}\) or Go\(\alpha_{4}\) (14). In order to know whether the other members of the bombesin receptor family share the same selectivity for Go\(\alpha_{\text{a}}\), we tested the ability of urea-extracted membranes to catalyze exchange of GDP for GTP\(\gamma\)S on squid retinal Go\(\alpha_{4}\), bovine retinal Go\(\alpha_{3}\), or bovine brain Go\(\alpha_{4}\), in the presence of bovine brain G\(\beta\gamma\). Both GRP-R (Fig. 4A) and NMB-R (Fig. 4B) selectively catalyze the exchange reaction on squid Go\(\alpha_{4}\) in an agonist-dependent manner. However, BRS-3 did not activate any of these G protein preparations using the universal bombesin receptor agonist 697 (Fig. 4C). In order to understand why BRS-3 failed to catalyze nucleotide exchange on all tested G proteins, we have attempted to exclude the possibility that peptide 697 is only a partial agonist of the BRS-3. We tested a mutated BRS-3 receptor in which four amino acid residues critical for ligand selectivity were replaced with their counterparts in NMB-R and GRP-R (R127Q, S205P, H294R, and S315A, see Fig. 1). This mutant, 4ABRS-3, displays 2 and 3 orders of magnitude increase in affinities for GRP (21) and NMB (33), respectively. Fibroblasts expressing 4ABRS-3

![Diagram of GRP-R and NMB-R binding](image)

**Fig. 2.** Kinetics of GRP-R- and NMB-R-catalyzed GTP\(\gamma\)S binding to Go\(\alpha_{\text{a}}\). Urea-extracted membranes providing final concentrations of 0.5 nM GRP-R (A) or 0.85 nM NMB-R (B) were assayed alone (○) or reconstituted with 840 nM bovine brain \( \beta_{\gamma} \) and 150 nM squid retinal Go\(\alpha_{4}\). Reconstituted membranes were assayed in the absence (○) or presence (●) of 1 \(\mu\)M GRP or NMB. The binding of GTP\(\gamma\)S to the G protein subunits in the absence of membranes was also determined (×). For all conditions, the reaction volumes were scaled up to 150 \(\mu\)L; the binding reaction was conducted at 30 °C, and 10-\(\mu\)L aliquots were removed at the indicated times for the determination of GTP\(\gamma\)S binding as described under “Experimental Procedures.” The lines drawn for G protein-reconstituted samples are the best-fit simple exponential curves using “Grafit.”

Table 1 also shows that \(^{125}\text{I}-697\) (universal bombesin receptor agonist) and \(^{125}\text{I}-\text{ME}\) (GRP-R-specific antagonist) measured identical binding site abundance on membranes before urea extraction, indicating that we could use \(^{125}\text{I}-697\) to determine the receptor concentration for NMB-R and BRS-3, for which a radiolabeled high affinity antagonist is not available. For all conditions, the reaction volumes were scaled up to 150 \(\mu\)L; the binding reaction was conducted at 30 °C, and 10-\(\mu\)L aliquots were removed at the indicated times for the determination of GTP\(\gamma\)S binding as described under “Experimental Procedures.” The lines drawn for G protein-reconstituted samples are the best-fit simple exponential curves using “Grafit.”

of uncoupled receptors.

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Since 7 M urea extraction removed most endogenous GTP\(\gamma\)S binding from GRP-R-containing membranes, we tested whether these uncoupled receptors could couple with purified squid retinal Go\(\alpha_{4}\) and bovine brain G\(\beta_{\gamma}\) as was shown previously for 6 M urea-treated GRP-R (14). Fig. 2 shows the results for reconstitution of membranes containing GRP-R and NMB-R either untreated or 7 M urea-extracted. To facilitate the comparison of the efficiency of reconstitution, we have tested ap-
show NMB-stimulated inositol phosphate increases (33). As shown in Fig. 4D, 4ΔBRS-3 did not catalyze GTPγS binding on any of the tested Ga subunits in the presence of NMB. The enhanced GTPγS binding on Goαq in the presence of all of the bombesin receptors seems to reflect a nonspecific interaction independent of receptors, because the level of GTPγS binding was proportional to total membrane protein concentration instead of receptor concentration (data not shown).

The lack of coupling of BRS-3 and 4ΔBRS-3 with the G protein subunits tested could be due to the low receptor abundance in Balb 3T3 fibroblast cells (0.26 and 0.33 pmol of receptor/mg of membrane protein for BRS-3 and 4ΔBRS-3, respectively, versus 3.7 and 2.2 pmol/mg for GRP-R and NMB-R) or the absence of essential G protein subunits. To achieve high receptor abundance, SF9 cells were used to express recombinant BRS-3 encoded by a baculovirus. To test the possibility that BRS-3 can couple with a mammalian Goα, rather than squid Goαq, recombinant mouse Goα was purified from baculovirus-infected SF9 cells and used in the reconstitution assays. GRP-R expressed in SF9 cells was also compared with that expressed in fibroblast cells in order to establish that the receptors expressed in these different cells have the same coupling properties. As shown in Fig. 5, GRP-R expressed in mouse fibroblast cells and insect SF9 cells behaved the same way. They activated both mouse Goα and squid Goαq but with higher catalytic activity for the latter. Although BRS-3 expressed in fibroblast cells failed to show agonist-stimulated activity with either Goαq (most likely due to the low receptor abundance), SF9 cell-expressed BRS-3 clearly showed coupling with mouse Goαq, but little if any coupling with squid Goαq. Another member of the bombesin receptor family, NMB-R, falls in between GRP-R and BRS-3 in selectivity for mouse and squid Goαq. NMB-R showed slightly more efficient coupling with squid Goαq than with mouse Goαq.

One of the unique advantages of this in situ reconstitution technique is that it allows a quantitative assessment of receptor-G protein coupling. To determine how well a receptor couples with a G protein as well as to compare coupling efficiency between different receptors, we have performed saturation analysis of the receptor-catalyzed GTPγS exchange with the G protein subunits. Fig. 6A shows the saturation of the exchange reaction catalyzed by GRP-R and NMB-R with squid Goαq. The initial velocities conformed to a single-site model with Km values of 58 nM for GRP-R and 112 nM for NMB-R. The catalytic activities of GRP-R and NMB-R were also different, with Vmax values of 8.5 × 10−3 μmol GTPγS bound per receptor per second for GRP-R and 4.1 × 10−4 μmol GTPγS bound per receptor per second for NMB-R. Fig. 6B shows the saturation of the catalysis with bovine brain Gβγ. These data also fit well to a single-site model with a K1/2 of 115

2 These values almost certainly underestimate the catalytic constants of GRP-R and NMB-R for G protein activation. Our GTPγS binding reactions included 2 μM GDP to suppress the receptor-independent binding to Ga. Since the reactions included carrier-free [35S]GTPγS at 4–8 nM, the rates for GTPγS binding in the absence of competing GDP would be much higher.
The differences between GRP-R and NMB-R in affinity and catalytic activity for G protein subunits were statistically significant, as summarized in Table II.

Given the diversity of Gβγ dimers, the receptor-G protein coupling selectivity is unlikely to be restricted to the Gα subunit alone. To address the question of whether the bombesin receptors also have selectivity for βγ dimers, we tested the ability of GRP-R (Fig. 7A) and NMB-R (Fig. 7B) to activate Gαq with different βγ dimers, including bovine brain βγ, bovine brain βγ, and squ...
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retinal $\beta\gamma$ ($\beta_1\gamma_1$) and $\beta_2\gamma_2$. When tested at a concentration of 0.25 $\mu$M, bovine brain $\beta\gamma$ showed the greatest enhancement of GRP-R- or NMB-R-catalyzed exchange reaction, $\beta_1\gamma_1$ the second highest, whereas $\beta_1\gamma_1$ hardly affected the binding of GTP$\gamma$S to Gao. At a concentration of 1 $\mu$M, $\beta_1\gamma_1$ also enhanced the receptor-catalyzed GTP$\gamma$S binding but incompletely, whereas 0.74 $\mu$M of $\beta_1\gamma_2$ produced the greatest enhancement.

To compare the affinities of GRP-R and NMB-R for G$\beta\gamma$ dimers, we performed saturation analysis of the receptor-catalyzed GTP$\gamma$S binding with $\beta_1\gamma_1$ and $\beta_1\gamma_2$. As shown in Fig. 8 and summarized in Table II, GRP-R consistently showed higher affinity for the G$\beta\gamma$ dimers that we tested. For a given G$\beta\gamma$ preparation, the ratio of $K_{m}$ of NMB-R and GRP-R ranged from 2.4- to 4.7-fold. Despite the quantitative differences between GRP-R and NMB-R, they showed the same rank order of preference among the three $\beta\gamma$ preparations: bovine brain $\beta\gamma > \beta_1\gamma_2 > \beta_1\gamma_1$.

**DISCUSSION**

In this study we adapted a published in situ receptor reconstitution method utilizing membranes from cells expressing recombinant GRP-R, NMB-R, BR5-3, or 4xBR5-3 which have been extracted with 7 M urea to remove endogenous GTP-binding proteins. The urea extraction procedure yielded a homogeneous population of uncoupled receptors with 100% recovery of receptor ligand-binding sites. Such receptor preparations were functional when reconstituted with heterotrimeric G protein subunits as shown by the assay measuring the first biochemical event in G protein activation: receptor-catalyzed exchange of GTP for GDP on a G$\alpha$ subunit. The in situ receptor reconstitution technique has been used successfully in our earlier studies using membranes from baculovirus-infected Sf9 cells expressing 5-HT$\text{_{2a}}$ receptor (13), membranes from stably transfected fibroblast cells expressing GRP-R (14), and now NMB-R and BR5-3. We believe that this method should be applicable to study virtually any receptor-G protein coupling. The major limitation we have observed is the receptor abundance in the membrane fraction.

We found all three bombesin receptors selectively coupled with a G$\alpha_q$ but not G$\alpha_o$ or G$\alpha_o$. However, three similar receptors were different in coupling selectivity toward members of the G$\alpha_q$ family. Although GRP-R and NMB-R coupled to both squid and mouse G$\alpha_q$, GRP-R had a much stronger preference for squid G$\alpha_q$, and NMB-R showed only a slight preference for squid G$\alpha_q$. In contrast to GRP-R and NMB-R, the structurally related BR5-3 did not couple with squid G$\alpha_q$, whereas it clearly coupled with mouse G$\alpha_o$. Given that the differences between squid G$\alpha_q$ and mouse G$\alpha_o$ structures are not much greater than the ones among mouse G$\alpha_o$ subtypes themselves (34–36), it will be interesting to investigate coupling of the bombesin receptors to various G$\alpha_q$ family subtypes within the same species in future studies.

The kinetic analysis of receptor-G protein interactions presented in this report also revealed a quantitative difference between GRP-R and NMB-R. The controlled, independent manipulation of receptor and G protein subunit concentrations required for this analysis is not possible using a whole cell system or prior reconstitution methods using purified, detergent-solubilized receptors and G proteins in phospholipid vesicles. GRP-R and NMB-R, although similar in their selectivity for G$\alpha_o$ and rank-order preference for G$\beta\gamma$ in the receptor-G

![Image](84x279 to 262x658)

**FIG. 6. G protein subunit saturation of GRP-R- and NMB-R-catalyzed GTP$\gamma$S binding.** A presents the G$\alpha_o$ saturation results. Varying concentrations as indicated of squid retinal G$\alpha_q$ were included in reactions containing 0.5 nM GRP-R with 1 $\mu$M GRP ($\bullet$) or 1.0 nM NMB-R with 1 $\mu$M NMB ($\bigcirc$) and 470 nM bovine brain $\beta\gamma$. B presents the G$\beta\gamma$ saturation results. Varying concentrations as indicated of bovine brain $\beta\gamma$ were included in reactions containing 0.5 nM GRP-R with 1 $\mu$M GRP ($\bullet$) or 1.0 nM NMB-R with 1 $\mu$M NMB ($\bigcirc$) and 150 nM G$\alpha_o$. For all conditions the GTP$\gamma$S binding reactions proceeded for 10 min at 30 °C, and bound GTP$\gamma$S was determined as described under “Experimental Procedures.” The values presented are from single determinations. The lines drawn are the best-fit curves for single site saturation using “Grafit.” The results are representative of three to six independent experiments.

**TABLE II**

Summary of GRP-R and NMB-R affinity and catalytic activity for squid retinal G$\alpha_q$ and different G$\beta\gamma$ dimers

| Kinetic parameters | GRP-R | NMB-R |
|--------------------|-------|-------|
| $K_m$ for squid retinal G$\alpha_q$ (nM) | 66 ± 9$^a$, n = 3 | 120 ± 18, n = 3 |
| $V_{max}$ (10$^{-3}$ pmol/mg membrane$^{-1}$s$^{-1}$) | 8.4 ± 0.7$, n = 3$ | 4.3 ± 0.4, n = 3 |
| $K_{m}$ for bovine brain G$\beta\gamma$ (nM) | 93 ± 15$, n = 5$ | 224 ± 20, n = 6 |
| $K_{m}$ for G$\beta_1\gamma_1$ (nM) | 147 ± 12$, n = 3$ | 691 ± 33, n = 4 |
| $K_{m}$ for G$\beta_2\gamma_2$ (nM) | 1163 ± 176$, n = 6$ | 3205 ± 121, n = 4 |

$^a$ All values are the means ± S.D. of values obtained from the indicated number of experiments.

$^b$ Significantly smaller ($p < 0.05$) than NMB-R.

$^c$ Significantly greater ($p < 0.05$) than NMB-R.

$^d$ Significantly smaller ($p < 0.01$) than NMB-R.
protein coupling, were different in the catalytic activity toward Ga and affinities for G proteins. GRP-R showed higher catalytic activity on squid Ga, and higher affinities for both Ga and Gβγ dimers than NMB-R.

These results may partially explain an ambiguity noted in antisense oligonucleotide experiments in which individual Ga subunits were depleted (18). In those experiments, Xenopus laevis oocytes expressing either GRP-R or NMB-R were micro-injected with antisense phosphorothioate oligonucleotides complementary to specific regions of either Xenopus Ga or Ga11 to deplete selectively Ga or Ga11 protein. Following application of agonist, the activity of the calcium-activated chloride channel was measured under whole cell voltage clamp conditions. These experiments showed that treatment with the Ga antisense oligonucleotides could inhibit up to 74% of the response of the NMB-R but had no effect on the GRP-R response. Ga11 antisense, on the other hand, had little effect on either GRP-R- or NMB-R-mediated responses. The data reported here showed GRP-R coupled more effectively with squid Ga, than with mouse Ga. Squid Ga is 74–78% identical to mouse Ga, Ga11, and Ga14. As the sequence identity between mouse Ga14 and Ga or Ga11 is 80 or 81%, respectively, it is likely that GRP-R couples primarily with Ga14 instead of Ga or Ga11. It is also possible that due to the higher affinity as well as higher catalytic activity for Ga, it would be easier to observe the influence of Ga depletion on NMB-R-regulated response than on GRP-R response. In those experiments, the antisense depletion taking place might simply fail to reduce Ga to a level that would impair GRP-R response.

GRP-R and NMB-R not only showed selective coupling with Ga but also showed a clear discrimination between different βγ dimers. We provide two arguments that this result suggests the different βγ dimers have different affinity and/or efficacy for bombesin receptors, rather than reflecting different affinities of Ga for βγ dimers. First, instead of a uniform difference in Ga for βγ dimers. First, instead of a uniform difference between Gβγ dimers, GRP-R and NMB-R had different K1/2 ratios for βγ and βγ (8-fold versus 4.6-fold). Second, rat 5-HT2c receptor has also been shown to couple with squid retinal Ga, in the in situ reconstruction assay (13). But unlike GRP-R or NMB-R, it has low affinity for both bovine brain βγ (estimated K1/2 is about 600 nM) and bovine retinal βγ, i.e. β1γ1.
Having higher affinity or/and efficacy than 

\[ \text{(13)} \]

Other receptor-G protein coupling studies also supported the notion that receptors can have different affinities for \( \beta y \) dimers. Studies of bovine rhodopsin activation of \( \alpha \) have found differences in apparent affinity among tissue-derived \( \beta y \) dimers of defined compositions or recombinant \( \beta y \) dimers, whereas \( \alpha \) shows essentially no preference for \( \beta y \) dimers (31–32, 37). The fact that both GRP-R and NMB-R preferred bovine brain \( \beta y \) over \( \beta y \) and the diverse composition of bovine brain \( \beta y \) dimers (37, 38) suggest there may be other \( \beta y \) that have higher affinity on/and efficacy than \( \beta y \) in enhancing the catalytic activity of GRP-R and NMB-R on \( \text{G}_{\alpha q} \).

In situ receptor reconstitution has been proven to be a useful methodology for detailed kinetic analysis of receptor-G protein coupling. It allows the identity and concentration of each coupling component to be defined and manipulated, while preserving the receptors in their native phospholipid environment. By using this method, we established a significant quantitative difference between GRP-R and NMB-R for interaction with the same squid \( \text{G}_{\alpha q} \) and \( \text{G}_{\beta y} \) proteins and, in addition, a qualitative difference between those two receptors and BRS-3 which did not interact with that same \( \text{G}_{\alpha q} \). Combining the currently available high expression systems (e.g. baculovirus infection of insect Sf9 cells, transfection of mouse fibroblast cells) with the in situ receptor reconstitution technique, it should be feasible to study functional coupling between any recombinant receptor and G protein subunits, advancing our understanding of the molecular mechanisms governing the signal transduction pathway for G protein-coupled receptors.

REFERENCES

1. Lebacq-Verheyden, A.-M., Trepel, J., Sausville, E. A., and Battey, J. F. (1990) *Handb. Exp. Pharmacol.* 95, 71–124
2. Kroeg, G., Jensen, R., and Battey, J. (1995) *Med. Res. Rev.* 15, 389–417
3. Gilman, A. G. (1987) *Annu. Rev. Biochem.* 56, 615–649
4. Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) *Science* 252, 802–808
5. Hepler, J. R., and Gilman, A. G. (1992) *Trends Biochem. Sci.* 17, 383–387
6. Majerus, P. W. (1992) *Annu. Rev. Biochem.* 61, 225–250
7. Sternweis, P. C., and Smrcka, A. V. (1992) *Trends Biochem. Sci.* 17, 502–506
8. Kozasa, T., Hepler, J. R., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C., and Gilman, A. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 9176–9180
9. Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweis, P. C. (1991) *Science* 25, 804–807
10. Gutowski, S., Smrcka, A., Nowak, L., Wu, D. G., Simon, M., and Sternweis, P. C. (1991) *J. Biol. Chem.* 266, 20518–20524
11. Park, D., Jhon, D.-Y., Lee, C.-W., Lee, K.-H., and Rhee, S. G. (1993) *J. Biol. Chem.* 268, 4573–4576
12. Smrcka, A. V., and Sternweis, P. C. (1993) *J. Biol. Chem.* 268, 9667–9674
13. Hartman, J. L., IV, and Northup, J. K. (1996) *J. Biol. Chem.* 271, 22591–22597
14. Hellmich, M. R., Battey, J. F., and Northup, J. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 751–756
15. Brown, K. D., Blay, J., Irvine, R. F., Heslop, J. P., and Berridge, M. J. (1984) *Biochem. Biophys. Res. Commun.* 123, 377–384
16. Benya, R., Kusui, T., Pradhan, T., Battey, J., and Jensen, R. (1995) *Mol. Pharmacol.* 47, 10–20
17. Ryan, R. R., Weber, H. C., Hou, W., Sainz, E., Maney, S. A., Battey, J. F., Coy, D. H., and Jensen, R. T. (1998) *J. Biol. Chem.* 273, 13613–13624
18. Shapiro, H., Way, J. M., Lipinsky, D., Oron, Y., and Battey, J. F. (1994) *FEBS Lett.* 348, 89–92
19. Kroeg, G. S., Sainz, E., Worland, P. J., Akeson, M. A., Benya, R. V., Jensen, R. T., and Battey, J. F. (1995) *J. Biol. Chem.* 270, 8217–8224
20. Benya, R., Akeson, M., Mrozinski, J., Jensen, R., and Battey, J. F. (1994) *Mol. Pharmacol.* 46, 495–501
21. Akeson, M., Sainz, E., Maney, S. A., Jensen, R. T., and Battey, J. F. (1997) *J. Biol. Chem.* 272, 17405–17409
22. Kusui, T., Hellmich, M. R., Wang, L.-H., Evans, R. L., Benya, R. V., Battey, J. F., and Jensen, R. T. (1995) *Biochemistry* 34, 8061–8075
23. Fathi, Z., Corjor, J. M., Shapiro, H., Wada, E., Benya, R., Jensen, R., Viallet, J., Sausville, E. A., and Battey, J. F. (1993) *J. Biol. Chem.* 268, 5879–5884
24. Maney, S., Frucht, H., Coy, D. H., and Jensen, R. T. (1993) *Mol. Pharmacol.* 43, 762–774
25. Maney, S. A., Weber, H. C., Sainz, E., Akeson, M., Ryan, R. R., Pradhan, T. K., Searles, R. P., Spinadel, E. R., Battey, J. F., Coy, D. H., and Jensen, R. T. (1996) *Mol. Pharmacol.* 268, 20662–20671
26. Sternweis, P. C., and Robishaw, J. D. (1984) *J. Biol. Chem.* 239, 13806–13813
27. Kuhn, H. (1980) *Nature* 283, 587–589
28. Fung, B. K.-K., Hurley, J. B., and Stryer, L. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 152–156
29. Fawzi, A. B., and Northup, J. K. (1990) *Biochemistry* 29, 3884–3882
30. Kozasa, T., and Gilman, A. G. (1995) *J. Biol. Chem.* 270, 1734–1741
31. Wildman, D. E., Tamir, H., Leberer, E., Northup, J. K., and Dennis, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 90, 794–798
32. Fawzi, A. B., Fay, D. S., Murphy, E. A., Tamir, H., Erdos, J. J., and Northup, J. K. (1991) *J. Biol. Chem.* 266, 12194–12200
33. Sainz, E., Akeson, M., Maney, S. A., Jensen, R. T., and Battey, J. F. (1998) *J. Biol. Chem.* 273, 15927–15932
34. Ryba, N. J., Findlay, J. B., and Reid, J. D. (1993) *Biochem. J.* 302, 1734–1741
35. Strathmann, M., and Simon, M. I. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 9113–9117
36. Wilkie, T. M., Scherle, P. A., Strathmann, M. P., Slepak, V. Z., and Simon, M. I. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 10049–10053
37. Asano, T., Morishita, R., Matsuda, T., Fukuda, Y., Yoshizawa, T., and Kato, K. (1993) *J. Biol. Chem.* 268, 20512–20519
38. Tamir, H., Fawzi, A. B., Tamir, A., Evans, T., and Northup, J. K. (1991) *Biochemistry* 30, 3929–3936