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Pharmacology

Selective reconstitution of gastrin-releasing peptide receptor with $G\alpha_q$

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ABSTRACT **Identification of the molecular mechanisms that determine specificity of coupling interactions between gastrin-releasing peptide receptors (GRPrs) and their cognate heterotrimeric GTP-binding proteins is a fundamental step in understanding the signal transduction cascade initiated by receptor–ligand interaction. To explore these mechanisms in greater detail, we have developed an *in situ* reconstitution assay in chaotrope-extracted membranes from mouse fibroblasts expressing the GRPr, and we have used it to measure GRPr-catalyzed binding of GTPγS to purified G protein α subunits. Binding studies with ^{125}I-labeled [D-Tyr^6]bombesin(6–13) methyl ester (^{125}I-Tyr-ME), a GRPr specific antagonist, show a single binding site with a K_d = 1.4 nM ± 0.4 (mean ± SD, n = 3) and capacity of 15–22 pmol of receptor per mg of protein in the extracted membrane preparations, representing a 2- to 3-fold enrichment of binding sites compared with the membranes before extraction. Quantitative ligand displacement analysis using various unlabeled GRPr agonists shows a rank order of potency characteristic of the GRPr: bombesin ≥ GRP ≫ neuromedin B. Reconstitution of urea extracted membranes with a purified $G\alpha_q$ showed that receptor-catalyzed binding of GTPγS was dependent on agonist (GRP) and G$\beta\gamma$ subunits. The EC_{50} for GRP was 3.5 nM, which correlates well with the reported K_d of 3.1 nM for GRP binding to GRPr expressed in mouse fibroblasts [Benya, R. V., *et al.* (1994) *Mol. Pharmacol.* 46, 235–245]. The apparent K_d for bovine brain G$\beta\gamma$ in this assay was 60 nM, and the K_m for squid retinal $G\alpha_q$ was 90 nM. The GRPr-catalyzed binding of GTPγS is selective for $G\alpha_q$, since we did not detect receptor-catalyzed exchange using either $G\alpha_{i/o}$ or $G\alpha_t$. These data demonstrate that GRPr can functionally couple to $G\alpha_q$ but not to the pertussis toxin-sensitive $G\alpha_{i/o}$ or retinal specific $G\alpha_t$. This *in situ* receptor reconstitution method will allow molecular characterization of G protein coupling to other heptahelical receptors.**

Gastrin-releasing peptide (GRP) and its amphibian homolog bombesin (Bn) elicit a broad spectrum of biological responses in mammals. These responses include: secretion of gastrointestinal hormones (e.g., gastrin, neurotensin, cholecystokinin, somatostatin, and enteroglucagon), regulation of smooth muscle contractility, modulation of neuronal activity, and growth regulation of normal and neoplastic tissues (for review see ref. 1). In the central nervous system, these peptides play a role in the regulation of homeostasis, thermoregulation, metabolism, and behavior (reviewed in ref. 2). *In vitro*, GRP and/or Bn stimulate the growth of Swiss 3T3 murine embryonic fibroblasts (3) and several human cancer cell lines, including the gastrinoma line SIIA (4) and the prostate cancer cell line PC-3 (5). *In vivo*, Bn stimulates the growth of a human pancreatic gastrinoma xenograft in nude mice (6) and inhibits growth of a human pancreatic adenocarcinoma xenograft (7).

Three Bn receptor subtypes with distinct pharmacological and structural properties have been cloned and characterized in mammals: the GRP-preferring receptor (GRPr, or bb2; refs. 8–10); the neuromedin B-preferring receptor (NMBr, or bb1; refs. 10 and 11); and Bn receptor subtype 3 (BRS-3, or bb3; refs. 12 and 13), a receptor structurally similar to GRPr and NMBr but for which no high-affinity ligand has been identified. G protein-coupled receptors, including the three Bn receptor subtypes, transmit extracellular signals across membranes by activating specific signal-transducing heterotrimeric G proteins, which in turn regulate a variety of intracellular effectors such as adenylyl cyclase, phospholipase C (PLC), ion channels, and cGMP-phosphodiesterase (14).

G proteins involved in receptor coupling are heterotrimeric structures composed of the products of three gene families encoding α, β, and γ subunits. Ligand activation of a G protein-coupled receptor catalyzes the exchange of GTP for GDP bound to the Gα subunit, as well as release of the GTP-activated Gα subunit from its cognate G$\beta\gamma$ dimer subunit. The GTP-activated α subunit in turn regulates intracellular effectors as does the dissociated G$\beta\gamma$ dimer. At least 20 distinct mammalian Gα subunits have been identified and have been subclassified into four groups based upon sequence homology and intracellular effector regulation (15, 16). At least four Gα subunit proteins have been found to stimulate phosphoinositide hydrolysis by activating PLC-β (17); these are designated as the $G\alpha_q$ subfamily (18–21). In addition, $\beta\gamma$ subunits can activate PLC-βs (22, 23). G proteins in the $G\alpha_i$ family can regulate the activity of PLC, presumably via the release of the $\beta\gamma$ subunit. Hence, *in vivo* data for the regulation of PLC can be ambiguous as to the G protein mediating the response.

Activation of PLC by Bn receptors is well established (24). However, the identification of the G protein mediating this response is unclear. In *Xenopus* oocytes, neither $G\alpha_q$ nor $G\alpha_{11}$ antisense phosphothiorate oligonucleotides (S-oligos) had any effect on GRPr signal transduction, as measured by activation of a calcium-sensitive chloride channel (25). In addition, Lach *et al.* (26) have reported a pertussis toxin (PTX) sensitivity of the Bn receptor signal transduction pathway in a guinea pig lung membrane preparation, suggesting that Bn receptors may couple to $G\alpha_i$ and/or $G\alpha_O$. Finally, the GRPr can activate multiple effector pathways within the same cell, including adenylyl cyclase and PLC (27). These data raise the possibility

Abbreviations: GRP, gastrin-releasing peptide; GRPr, GRP-preferring receptor; Bn, bombesin; NMB, neuromedin B; NMBr, NMB-preferring receptor; PLC, phospholipase C; PTX, pertussis toxin.

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that the GRPr, as has been suggested for several other G protein-coupled receptor types, may couple to multiple pathways, using distinct heterotrimeric G proteins. In this study we have adapted a chaotropic membrane extraction procedure (28) to develop an *in situ* reconstitution of G protein coupling to the GRPr. These methods have allowed the examination of G protein selectivity using purified G protein subunits as well as extended our knowledge of the molecular pharmacology of the GRPr.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and aminoglycoside G-418 were from BRL–Life Technologies (Gaithersburg, MD). Protein gel electrophoresis equipment and gels for SDS/PAGE were from NOVEX (San Diego). Prestained molecular mass markers and other SDS/PAGE reagents were purchased from Bio-Rad Laboratories. The GRPr agonists Bn, GRP, and neuromedin B (NMB) were purchased from Peninsula Laboratories. Frozen enucleated squid eyes were obtained from Calamari (Woods Hole, MA). 4-(2-Aminoethyl)-benzenesulfonyl fluoride HCl (AEBSF) was purchased from ICN. GF/F glass fiber filters were purchased from Whatman. Nitrocellulose filters and the vacuum manifold used for binding experiments were from Millipore.

Cell Culture. Cells were cultured in DMEM containing 300 μg/ml G-418 and 10% fetal bovine serum. Before harvesting, cells were grown to confluence at 37°C in 5% CO_2.

Membrane Preparation. Membranes were prepared from the cell line 5ET4, a Balb 3T3 mouse fibroblast cell line expressing a stably transfected mouse GRPr (29). GRPr-enriched membranes were obtained as a P2 fraction from these cells. To obtain a P2 fraction, the cells were washed twice with 10 ml of phosphate-buffered saline (PBS) at room temperature and incubated at 4°C for 15 min in 5 ml of solution A (10 mM Hepes, pH 7.4/1 mM EGTA) fortified with 100 μM AEBSF. The swollen cells were harvested by scraping and homogenized in a Dounce homogenizer (15–20 strokes with the tight pestle), and the nuclei and cell debris were removed by centrifugation at 750 $\times$ *g* for 10 min at 4°C. The postnuclear membrane fraction (P2) was collected from the supernatant by centrifugation at 75,000 $\times$ *g* for 30 min at 4°C.

Chaotropic Extraction of Endogenous GTP Binding Activity. The P2 membrane pellet was resuspended in solution A containing a chaotropic agent (usually 6 M urea), incubated on ice for 30 min and sedimented at 75,000 $\times$ *g* for 30 min at 4°C. After a second extraction and centrifugation, the membrane pellet was washed once with solution A alone. The final pellet was resuspended in solution A supplemented with 12% (wt/vol) sucrose, and aliquots were frozen and stored at −80°C.

Ligand Binding. GRPr ligand binding sites in the membrane preparation were quantitated by analysis of binding to the radiolabeled antagonist ^{125}I-labeled [D-Tyr^6]Bn(6–13) methyl ester (^{125}I-Tyr-ME; ref. 30). ^{125}I-Tyr-ME (2200 Ci/mmol; 1 Ci = 37 GBq) was prepared as described previously (31). The binding reaction contained 20 mM Hepes (pH 7.4), 100 mM NaCl, 0.3% BSA, 3 mM $MgSO_4$, 1 mM EGTA, and 24–300 pM ^{125}I-Tyr-ME. For equilibrium binding experiments, 0.4–1.6 μg of membrane protein (containing about 8–32 fmol of GRPr) was incubated at 30°C for 45 min with ^{125}I-Tyr-ME and other reagents as indicated in a total volume of 50 μl. The binding reaction was terminated by adding 4 ml of ice-cold solution B (20 mM Tris·HCl, pH 8.0/100 mM NaCl/25 mM $MgCl_2$) followed by filtration over GF/F glass fiber filters and washing four times with 4 ml each of ice-cold solution B. ^{125}I-Tyr-ME binding was quantitated on a Wallac 1470 gamma counter. The dissociation constant and binding capacity of ^{125}I-Tyr-ME binding to the membranes were calculated from best fits to a single site binding model using the program GRAFIT. IC_{50} values for the competition of ^{125}I-Tyr-ME by various agonist were calculated using the same program.

Purification of G Proteins. Squid transducin, a member of the $G\alpha_q$ family (32) was purified from squid photoreceptors as described by Hartman and Northup (28). Briefly, a microvillus membrane fraction was isolated by flotation of total retinal membrane fraction from 100 squid retina on 34% sucrose in solution C (10 mM Mops, pH 7.5/3 mM $MgSO_4$/1 mM EGTA/100 mM NaCl). The membranes were then washed and extracted with solution D (20 mM Tris, pH 8/1 mM EDTA/3 mM $MgSO_4$/1 mM DTT) with 1% (wt/vol) sodium cholate. The extract was fortified with $AlCl_3$, $MgCl_2$, and NaF (AMF) to final concentrations of 100 μM, 10 mM, and 10 mM, respectively, and incubated at room temperature for 30 min before chromatography. Homogeneous $G\alpha_q$ was isolated by sequential chromatography over DEAE-Sephacel (Pharmacia) and Ultrogel AcA44 (IBF, Villeneuve-la-Garenne, France).

Bovine brain $G\alpha_{i/o}$ and $G\beta\gamma$ subunits were purified as described previously (33). Bovine retinal $G\alpha_t$ and $G\beta\gamma$ were isolated by modifications of the methods of Kuhn (34) and Fung *et al.* (35) as described (36).

GDP/GTPγS Exchange Assay. The receptor-catalyzed exchange of GDP for GTPγS on $G\alpha_q$ was determined by modification of the procedures described by Fawzi *et al.* (37). Reactions were carried out in 12 $\times$ 75 mm siliconized borosilicate glass test tubes at a total assay volume of 50 μl. Membranes containing the GRPr were mixed with G protein subunits on ice in a total volume of 40 μl. An addition of 10 μl of reaction solution was used to initiate the reactions that contained a final concentration of 20 mM Hepes (pH 7.5), 100 mM NaCl, 3 mM $MgSO_4$, 1 mM DTT, 1 mM EDTA, 1 μM GDP, 0.3% BSA, and [^{35}S]GTPγS (about 4 $\times$ 10^5 cpm). When required, peptide ligands for the GRPr were added to the reaction. Reactions were incubated at 30°C and terminated by the addition of ice-cold solution, followed by filtration over nitrocellulose membranes on a vacuum manifold. Filters were washed 4 times with 4 ml each of ice-cold solution B. The filters were dried and the radioactivity quantitated by liquid scintillation in a Wallac 1219 beta counter.

RESULTS

For the work reported in this manuscript, we utilized a Balb 3T3 mouse fibroblast cell line stably transfected to express a mouse GRPr cDNA containing the sequence for 11 amino acid residues of the *c-myc* gene added to amino terminus of the GRPr. This epitope-tagged receptor construct has been shown to exhibit all the characteristics of the wild-type GRPr and was indistinguishable from the wild-type GRPr when assayed for ligand binding, activation of PLC, internalization, and desensitization (29). We selected the transformed cell line expressing this receptor because of its high level of receptor expression ($\approx$10^6 receptors per cell).

To generate a GRPr membrane preparation that would be useful for studying G protein coupling by *in situ* reconstitution, we adapted urea extraction procedures described previously for rod outer disk membranes from bovine retina (36) and for baculovirus-infected Sf9 cell membranes expressing the 5-HT_{2c} receptor (28). As opposed to these previous reports, urea extraction alone did not produce membranes with a pure population of receptor functionally uncoupled from G proteins. To eliminate any functional coupling in the membrane preparation, we treated the P2 membrane pellet with 100 nM Bn or GRP for 30 min at 25°C before extraction with 6 M urea. This treatment with agonist would be expected to drive dissociation of any G proteins interacting with GRPr in the membranes.

Scatchard analysis of these 6 M urea-extracted membranes shows a single high affinity antagonist binding site with a K_d

of 1.4 nM ± 0.4 (mean ± SD; $n = 3$) and a capacity of 15–22 pmol of receptor per mg of protein in the extracted membrane preparations, a 2- to 3-fold enrichment of receptor when compared with the unextracted P2 membrane pellets (data not shown). Fig. 1 presents the results of experiments analyzing the ligand specificity of the GRPr for peptide agonists of the receptor in these membranes. Competition for the binding of ^{125}I-Tyr-ME by various GRPr agonists shows a rank order of potency characteristic of the GRPr (Fig. 1*B*): Bn ≥ GRP ≫ NMB. However, the values for the relative affinities of each of these compounds is ≈10-fold lower than those reported for the GRPr receptor in whole cells (K_i = 3.1 ± 1.4 nM (mean ± SE) vs. 46 ± 18 nM (mean ± SD) for GRP; 174 ± 4 nM (mean ± SD) vs. 1326 ± 226 nM (mean ± SD) for NMB; ref. 27]. These shifts to lower affinity are consistent with the shifts observed when binding is performed in the presence of a high concentration of guanine nucleotide and are indicative of the uncoupled receptor (27).

Since the extracted P2 membranes contained an uncoupled GRPr, we tested the capacity of these receptors to activate purified G proteins. First, we examined a squid retinal $G\alpha_q$, which had previously been found to couple to rat 5-HT$_{2c}$ receptors (28). Fig. 2 shows a comparison of the P2 membranes (Fig. 2 *Left*) to the urea-extracted P2 membranes (Fig. 2 *Right*) after reconstitution with $G\alpha_q$ alone, $\beta\gamma$ alone, or $G\alpha_q$ together with $\beta\gamma$. Four conclusions can be drawn from this experiment: (*i*) due to endogenous GTP-binding activity in the unextracted P2 membrane fraction, very little agonist stimulated exchange of GDP for GTPγS on exogenously added $G\alpha_q$ can be measured (Fig. 2 *Left*); (*ii*) extraction of the membranes dramatically decreases the endogenous GTP binding (Fig. 2 *Right*); (*iii*) the GRPr can catalyze the exchange of GDP for GTPγS on squid retinal $G\alpha_q$ subunit added to the assay; and (*iv*) the exchange is dependent on both agonist and addition of $\beta\gamma$ subunits to the assay. The $\beta\gamma$ dependence of GRPr catalyzed GTPγS-binding is similar to that observed with both the 5-HT$_{2c}$ receptor (28) and bovine rhodopsin (37).

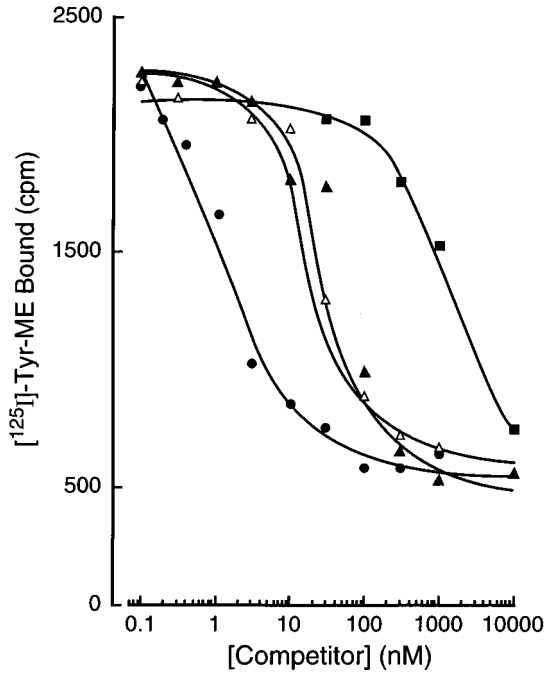

FIG. 1. Competition of binding of the antagonist ^{125}I-Tyr-ME to membranes pretreated with agonist and extracted with 6 M urea. The relative potency of different Bn receptor agonists (Bn, ▲; GRP, △; NMB, ■) and a GRPr-specific antagonist (ME, ●) were compared by competition binding to membranes pretreated with agonist before extraction with 6 M urea. The binding reaction was initiated by adding 25 μl of membranes (0.41 μg of protein) to 25 μl of binding solution containing ^{125}I-Tyr-ME (87 pM final concentration) and various concentrations of unlabeled competitor. Binding proceeded for 45 min at 30°C, and bound radioligand was measured as described.

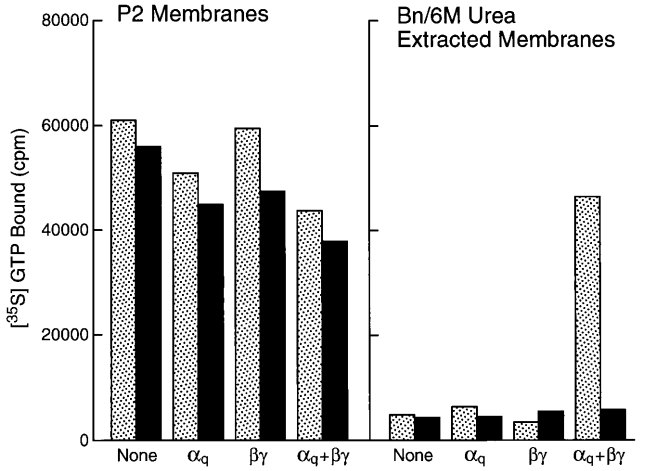

FIG. 2. Reconstitution of GRPr membranes with $G\alpha_q$ and/or $\beta\gamma$. A P2 GRPr membrane fraction was assayed for agonist-catalyzed GTPγS binding directly (*Left*), or after the membranes were pretreated with agonist and extracted with 6 M urea (*Right*). Either GRPr containing membranes alone (None) or membranes reconstituted with either $G\alpha_q$ or $\beta\gamma$ alone, or $G\alpha_q$ and $\beta\gamma$ were assayed with (stippled bars) or without (solid bars) 1 μM GRP. The GTPγS binding assay proceeded for 10 min at 30°C as described.

These experiments indicate that the urea-extracted membranes display all the appropriate features of GRPr found in intact cells. Since we have assayed the first biochemical process subsequent to ligand binding to the receptor, it is predicted that this assay should display saturation of G protein activation that is identical to the binding of ligand. The experiment presented in Fig. 3 examines the GRP saturation of GRPr-catalyzed GDP/GTPγS exchange on $G\alpha_q$. These data conform

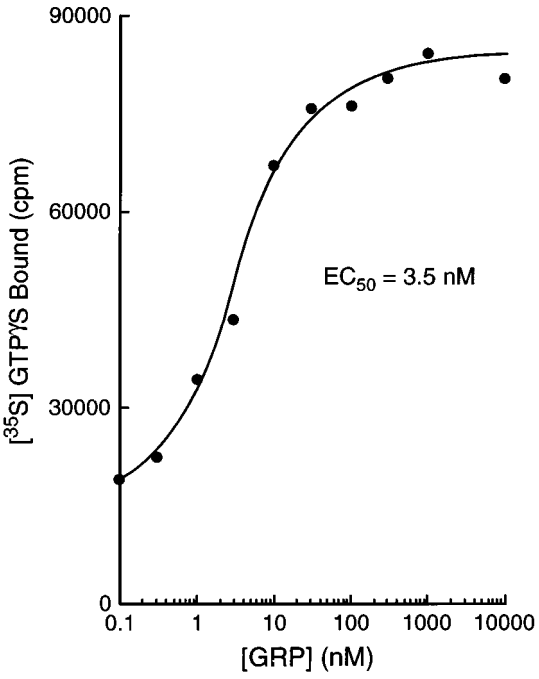

FIG. 3. GRP saturation of GRPr-catalyzed exchange of GTPγS for GDP. GTPγS binding was measured in a reaction containing 3 nM GRPr, 1 μM $\beta\gamma$, 280 nM $G\alpha_q$, and the indicated amounts of GRP. Binding reactions proceeded for 3.5 min at 30°C as described.

well to a single-site model with a $K_{0.5}$ of 3.5 nM, which agrees well with the reported K_d of 3.1 ± 1.4 nM for the GRPr expressed in Balb 3T3 cells (27).

The *in situ* reconstitution assay allows an examination of carefully controlled protein interactions between receptor and G protein unavailable when using intact cells. The experiments presented in Fig. 4 examine the saturation of the GTPγS exchange reaction with the G protein subunits. In Fig. 4*A* we analyzed the saturation of the exchange reaction catalyzed by GRPr with saturating Gβγ. The initial velocities conformed to a single-site model with a K_m of 87 nM for $G\alpha_q$. In Fig. 4*B* we investigated the saturation of the catalysis with Gβγ at nearly saturating $G\alpha_q$. These data also are well fit as a single-site interaction with a $K_{0.5}$ of 57 nM. In additional experiments we have analyzed the variation of the α and βγ saturations with GRPr concentration. While the K_m for $G\alpha_q$ did not vary, the apparent affinity for βγ increased with increasing GRPr (data not shown).

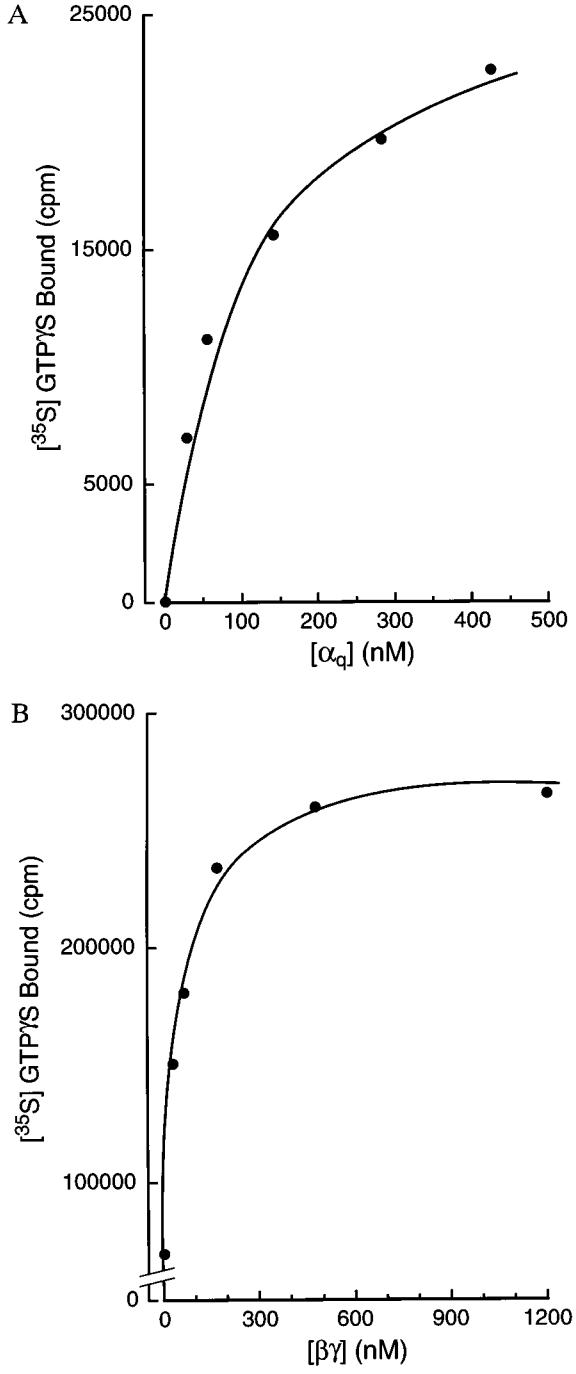

FIG. 4. Saturation of the rate of agonist-stimulated, GRPr-catalyzed GDP/GTPγS exchange by $G\alpha_q$ and Gβγ. (*A*) The concentrations of GRP (1 μM), GRPr (0.4 nM), and βγ (1.2 μM) were fixed, and the concentration of $G\alpha_q$ varied from 28 nM to 426 nM as indicated. (*B*) The concentration of GRP (1 μM), GRPr (3 nM), and $G\alpha_q$ (284 nM) were fixed, and the concentration of βγ was varied from 34 nM to 1200 nM. Binding reactions proceeded for 15 min at 30°C, and the binding was determined as described.

Finally, to address the question of whether GRPr can couple to PTX-sensitive G proteins and to investigate the selective nature of the reconstitution assay, we tested the ability of extracted membranes to catalyze exchange of GDP for GTPγS using G proteins other than $G\alpha_q$, including either $G\alpha_{i/o}$ and $G\alpha_t$ (Fig. 5). For these experiments we used preparations of G proteins from native tissue sources to assure appropriate posttranslational modifications that may be essential for receptor interactions. We chose bovine brain G_o/G_i fractions as an abundant source of at least four identified PTX substrate G proteins ($\alpha_{i1\text{-}3}$ and α_o) and the bovine retinal $G\alpha_t$, which is the other abundant member of the G_i family. While both bovine G protein preparations have been found to functionally couple to appropriate receptors, the experiments summarized in Fig. 5 show that the GRPr cannot catalyze the exchange reaction on either $G\alpha_{i/o}$ or $G\alpha_t$, even at subunit concentrations of 1 μM. These data show that this reconstitution methodology is useful for the evaluation of receptor-G protein selectivity and show that GRPr cannot couple functionally to the PTX-sensitive $G\alpha_{i/o}$ or $G\alpha_t$.

DISCUSSION

In this study we describe the preparation and characterization of membranes from mouse fibroblast cells expressing a recombinant GRPr that have been stripped of endogenous GTP

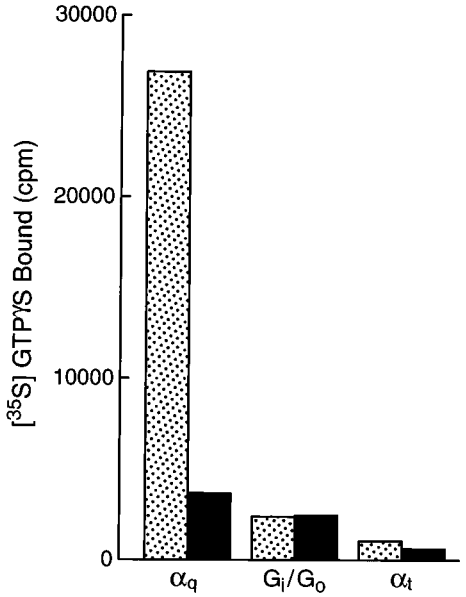

FIG. 5. $G\alpha_q$ coupling selectivity of the GRPr. Membranes containing GRPr (0.4 nM) were mixed with $G\alpha_q$ (280 nM) and βγ (1 μM), $G_{i/o}$ (1 μM), or $G\alpha_t$ (1 μM) and βγ (1 μM). GTPγS exchange for bound GDP in the presence (stippled bars) or absence (solid bars) of 1 μM GRP proceeded for 15 min at 30°C as described. Gα subunit concentrations were determined by GTPγS binding with or without addition of βγ. Membrane-independent background binding of GTPγS was subtracted from total binding to give the values presented in the figure (membrane-independent backgrounds were as follows: 635 cpm for $G\alpha_q$ and βγ; 716 cpm for $G_{i/o}$; and 3173 cpm for $G\alpha_t$ and βγ).

binding proteins by extraction with 6 M urea. A homogenous population of uncoupled receptors is generated by treating the membranes with a GRPr agonist before urea extraction. The membranes generated by this procedure contain functional, but uncoupled, GRPr in a native phospholipid environment. We have shown that this receptor preparation can be reconstituted with purified Gα and G$\beta\gamma$ subunits of heterotrimeric G proteins using an assay designed to measure directly the first event in G protein activation: receptor-catalyzed exchange of GTP for GDP on the Gα subunit.

Using the reconstitution assay we have clarified two issues concerning the GRPr signal transduction pathway. First, we show that the GRPr catalyzes agonist- and $\beta\gamma$ subunit-dependent exchange of GTP for GDP on Gα_q, but not other Gα subunits. This observation helps to resolve the interpretation of previous experiments where GRPr coupling was studied in *Xenopus laevis* oocytes (25). In those experiments, oocytes expressing either NMBr or GRPr were microinjected with antisense phosphothiorate oligonucleotides complementary to specific regions of either *Xenopus* Gα_q or Gα_{11} to deplete selectively the oocyte of either Gα_q or Gα_{11} 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 Gα_q antisense oligonucleotide could inhibit up to 74% of the response of the NMBr, but had no effect on the GRPr response. Gα_{11} antisense oligonucleotides did not affect the response of either receptor. The authors concluded that the NMBr can couple to Gα_q, but the G protein used to couple GRPr to activation of the phosphoinositide-specific PLC remained undefined, since it was not possible to be sure that antisense depletion had reduced G protein to a level that would impair GRPr coupling. The data obtained from the experiments reported here establish with certainty that the GRPr can activate Gα_q. It remains an open question, however, whether the ambiguity in the antisense oligonucleotide experiments is due to a difference in the relative affinity of Gα_q for GRPr compared with Gα_{11} as Shapira *et al.* (25) have hypothesized. The answer to this question will require the use of a pure preparation of Gα_{11} in the reconstitution assay and a comparison of GRPr and NMBr coupling *in vitro*.

The second issue clarified by this report concerns the PTX sensitivity of the Bn receptor signal transduction pathway. Lach *et al.* (26) reported that treatment of the guinea pig lung membranes with PTX, but not cholera toxin, prevented a GTPγS dose-dependent decrease in ^{125}I-Tyr-Bn binding. Letterio *et al.* (38) found that the addition of PTX for 3 h before a 48-h incubation in Bn completely inhibited Bn-stimulated DNA synthesis and cell proliferation in Swiss 3T3 cells expressing GRPr. Zachary *et al.* (39) found PTX inhibits 50% of Bn-induced DNA synthesis, but did not inhibit the Bn-dependent activation of phosphoinositide-specific PLC in Swiss 3T3 fibroblasts. Similarly, Taylor *et al.* (40) showed that Bn-stimulated DNA synthesis was reduced (40%) after pretreatment (65 h) with PTX, whereas total inositol phosphate generation was decreased only by 20% following the same treatment. Profrock *et al.* (41) using photoaffinity labeling by [α^{32}P]GTP-γ-azidoanilide, presented data that showed, in rat pancreatic acinar cell membranes, exposure to Bn increased labeling of proteins with a mass of 40–41 kDa and reduced PTX induced ADP ribosylation of three 40- to 41-kDa proteins, presumably G$\alpha_{i1\text{-}3}$. Taken together, these studies suggest that the GRPr may couple through PTX-sensitive G proteins. In contrast, we have shown that GRPr does not activate GDP/GTP exchange on a purified G$\alpha_{i/o}$ fraction from bovine brain that contains G$\alpha_{i1\text{-}3}$ and Gα_o, the predominant PTX substrates.

Our data suggest that the sensitivity of the Bn receptor signal transduction pathway to PTX is due to some other mechanism besides direct activation of Gα_i or Gα_o by GRPr. One possible explanation for the PTX and cross-linking data is that GRPr and G$\alpha_{i/o}$ are capable of physically interacting with each other but that interaction is not productive (e.g., does not result in receptor catalyzed GDP/GTP exchange on the Gα subunit). As presented, our experiment does not differentiate between nonbinding and binding without productive coupling. Alternatively, it has been demonstrated that PTX-catalyzed ADP ribosylation of Gα_i prevents the dissociation of the Gα and G$\beta\gamma$ subunits. Perhaps the effect of PTX on the GRPr signaling pathway is an indirect effect due to changes in the cellular concentration of free G$\beta\gamma$ subunits. Our data demonstrate a marked dependence of GRPr activation of Gα_q on G$\beta\gamma$. Modulation of the free G$\beta\gamma$ content of the plasma membrane either by PTX modification or receptor stimulation of G$_{i/o}$ pathways might thus influence GRPr activation of PLC.

In addition to allowing a more complete characterization of receptor/G protein coupling, the *in situ* reconstitution assay will allow a definitive exploration of the molecular pharmacology of the GRPr. Binding analysis of this receptor preparation shows that the binding constants for agonists and antagonists are consistent with the generally accepted model for receptor-G protein coupling; uncoupled receptors have a decreased affinity for agonists, while the affinity for a pure antagonist is unaffected by the receptor's coupling state. Future studies using this *in situ* reconstitution assay system will include a complete evaluation of Bn receptor agonists and antagonists. Hartman and Northup (28) have demonstrated the utility of the *in situ* receptor reconstitution assay in the identification and characterization of the 5-HT$_{2c}$ receptor inverse agonists mianserin, ketanserin, and mesulergine.

Historically, researchers have used two experimental approaches in an attempt to elucidate the molecular mechanisms regulating receptor-coupled signal transduction: (*i*) reconstitution of purified, detergent-solubilized proteins into artificial phospholipid vesicles (reviewed in ref. 42); and (*ii*) transfection of whole cells with expression constructs that are designed to either augment or inhibit the function or expression of a specific component in the signal transduction pathway (e.g., antisense sequence to a specific Gα subunit; ref. 43). Both methods have advantages and disadvantages. The reconstitution of purified proteins allows careful control of the concentrations of each component which in theory should allow a precise kinetic analysis of the system. The disadvantage of this approach is that the receptors are solubilized in detergent and purified through multiple steps of chromatography that often results in a substantial loss of receptors. Following purification, the receptors are inserted into artificial phospholipid vesicles, which may or may not accurately recreate the native hydrophobic environment necessary for determining receptor-G protein specificity. The artificial nature of the vesicle may partially explain the unexpected coupling observed between the β-adrenergic receptor and members of the G$\alpha_{i/o}$ family of G proteins when the coupling was reconstituted in phospholipid vesicles (44). Others have attempted to determine receptor coupling partners by modulating the levels of G protein α subunit expression using antisense RNA expression constructs transfected into whole cells (43). This analysis has the advantage of studying coupling in the context of an intact cell, minimizing the risk of omitting a key component for functional receptor-G protein interaction, and allows analysis of the receptors in their native membranes. However, there is no obvious way to control the levels of other signal transduction components (e.g., other Gα and G$\beta\gamma$ subunits) that may indirectly influence receptor activation of effector molecules or receptor affinity for ligand, the two parameters typically measured in these experiments. Consequently a detailed kinetic analysis of receptor-G protein coupling is not possible using antisense modulation in whole cells.

In situ receptor reconstitution offers an alternative hybrid method to conventional reconstitution with purified proteins

and whole cell transfection experiments. The receptors are isolated in native membranes, avoiding the need for detergent solubilization and purification. This preserves those potentially important but incompletely characterized elements of the surrounding phospholipid environment that may prove to be important determinants in selectivity of receptor-G protein interactions while allowing the concentrations and character (e.g., mutated receptors or G proteins) of each coupling component to be defined and manipulated. In this study, we demonstrate the practicality of using mammalian cells transfected with a recombinant GRPr as a source of receptor-containing membranes. The GRPr, like other G protein-coupled receptors, is coupled to various effector systems, including: PLC/inositol trisphosphate/calcium; adenylyl cyclase/cAMP; and tyrosine kinase activation. A central problem in cell biology is the elucidation of the contribution of different receptors, G proteins, and effector proteins to the regulation of cellular responsiveness. *In situ* receptor reconstitution appears to be a useful and informative methodology for defining the interactions between individual members of a signal transduction cascade and should facilitate our overall understanding of the molecular mechanisms regulating these processes.

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