Selective Role of G Protein γ Subunits in Receptor Interaction*

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Receptor stimulation of nucleotide exchange in a heterotrimeric G protein (αβγ) is the primary event-modulating signaling by G proteins. The molecular mechanisms at the basis of this event and the role of the G protein subunits, especially the βγ complex, in receptor activation are unclear. In a reconstituted system, a purified muscarinic receptor, M2, activates G protein heterotrimers αi2β1γ5 and αi2β1γ7 with equal efficacy. However, when the α subunit type is substituted with αo, αoβ1γ7 shows a 100% increase in M2-stimulated GTP hydrolysis compared with αoβ1γ5. Using a sensitive assay based on βγ complex stimulation of phospholipase C activity, we show that both β1γ5 and β1γ7 form heterotrimers equally well with αo and αi. These results indicate that the γ subunit interaction with a receptor is critical for modulating nucleotide exchange and is influenced by the subunit-type composition of the heterotrimer.

The G protein cycle is primarily regulated by the interaction of heterotrimeric G protein and cell surface receptors. Both α and βγ subunits are required for interaction with receptor (1–5). The G protein α subunit has been demonstrated to interact with and selectively couple to receptors, especially muscarinic receptors (4). The role of the βγ complex in interaction with the receptor is, however, less well understood. There is evidence for interaction of the G protein γ subunit with receptors (5). There are also indications for specificity in this interaction. Results from experiments in pituitary GH3 cells using antisense oligonucleotides specific to different β or γ subunit types indicated that signaling stimulated by different receptors can be specifically inhibited (6). The γ1 subunit type allowed effective coupling of Gt with rhodocin in contrast to γ2 and γ3 (7). In superior cervical ganglion (SCG) neurons peptides specific to the γ5 subunit type disrupted signaling from the M2/M4 muscarinic receptors, whereas peptides specific to γ7 and γ12 had no effect (8). Because this implied that the M2 muscarinic receptor selectively interacts with a G protein containing γ5 but not γ7, we tested the ability of the M2 receptor to activate G proteins containing these two subunits. Earlier studies addressing the question of G protein specificity for receptors used whole cells or crude membranes from cells. Experiments with intact cells do not definitively allow identification of the site at which the disruption in signaling occurs. Crude membranes contain endogenous G proteins, receptors, and other components that may affect analysis of specificity in receptor-G protein interactions. To more rigorously and directly examine the effect of G protein subunit constitution on receptor-G protein coupling, we reconstituted a purified muscarinic receptor, M2, in lipids and measured its ability to activate G proteins containing different γ subunits. The M2 receptor is known to couple to members of the G/o family but not Gq (9). To examine whether the subunit-type constitution of a heterotrimer influenced receptor interaction we tested different combinations of the α and γ subunit types αoγ5, αoγ1γ7, αi2β1γ5, and αi2β1γ7. Recombinant G protein subunits were purified from insect cells, and heterotrimers constituting different combinations of α and γ subunits were assembled. We measured M2-stimulated GTPγS binding and GTPase activity using defined G protein heterotrimers. GTPγS binding assays were performed at a ratio of the G protein α subunit to receptor of 100:1 (1 nm receptor). Because subtle differences in receptor activation could be missed under these conditions, we developed GTPase assays to measure receptor activation of a G protein at a ratio of G protein to receptor approaching 1:1 (1 nm receptor). These assays detected consistent and significant differences in the ability of the M2 receptor to activate αoβ1γ5 compared with αoβ1γ7. In contrast, when the α subunit type was substituted with α2 there was no difference in receptor-stimulated GTPase activity between αi2β1γ5 and αi2β1γ7. The difference in receptor-stimulated activity between αoβ1γ5 and αoβ1γ7 could be due to differential heterotrimer formation between αo and these βγ complexes. To test this possibility we developed a novel phospholipase C (PLC)-based assay to measure heterotrimer formation. This assay indicated that the differences in GTPase activity between αoγ5 and αoγ7 arose as a result of differential receptor coupling rather than differential heterotrimer formation.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant G Protein Subunits—G protein βγ subunits were expressed in the baculovirus Sf9 cell system. The purification was essentially performed according to the procedures described before (10). The purity and quantity of these βγ proteins were assessed by separating by SDS gel electrophoresis, staining with Coomassie Blue, scanning with a laser densitometer, and comparing with protein standards. G proteins αo and α2 were purified from Escherichia coli using published methods (11). RGS4 was a gift from Dr. M. Linder, Washington University.

Measuring PLC Activity—The βγ-stimulated PLC assay was performed using a procedure as stated previously (12).

Purification, Reconstitution, and Functional Characterization of Recombinant M2—His-tagged M2 was expressed in Sf9 cells and purified using a CoCl2 affinity column (13). Purified M2 was reconstituted into dylinositol-4,5-biphosphate; DTT, dithiothreitol; IP3, inositol 1,4,5-trisphosphate.
2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 0.5 mg/ml BSA. This enzyme activity as described above.

Reconstituted M2 was then added and incubated for an additional 30 min. The M2-G protein complex was incubated at room temperature for 60 min in a binding buffer containing 20 mM sodium phosphate (pH 7.4) and 10 mM MgCl₂. The reactions were terminated by filtration through Whatman GF/B membranes, and the filters were then washed with ice-cold binding buffer before counting the radioactivity.

**RESULTS AND DISCUSSION**

**Purification and Functional Characterization of Recombinant G Protein Subunits and M2—βγ complexes purified as described above were over 95% pure (Fig. 1A).** When PLC β3 isozyme stimulation by β1γ5 or β1γ7 complexes was measured, the similar levels of PLC β3 activity by β1γ5 or β1γ7 complexes indicated that the functional proportion of each βγ complex was the same (Fig. 1B). In addition, we ensured that the concentration of detergent in the purified β1γ5 and β1γ7 samples were identical, using thin layer chromatography and detergents at various concentrations as standards (data not shown). His-M2 has been shown to possess similar properties to native M2 after reconstitution into lipids (13). His-M2 receptor was purified as described earlier. The purified M2 was reconstituted as described earlier. The reconstituted receptor was bound to various concentrations of [3H]NMS. Specific binding of M2 for NMS was calculated to be 0.25 ± 0.06 nm, similar to that of native M2 as reported previously. The experiment was performed twice.

Brain lipids (Folch type VII, Sigma) and characterized by binding to antagonist, [3H]-[3H]methylscopolamine (NMS). The receptor (50 μg) was incubated with various concentrations of [3H]NMS at room temperature for 60 min in a binding buffer containing 20 mM sodium phosphate (pH 7.4) and 10 mM MgCl₂. The reactions were terminated by filtration through Whatman GF/B membranes, and the filters were then washed with ice-cold binding buffer before counting the radioactivity.

**GTPγS Binding and GTP Hydrolysis—**The formation of heterotrimeric Go protein, the M2-G protein complex, GTPγS binding, and GTP hydrolysis assays are described in the legends for Figs. 2 and 3.

**Measuring Heterotrimer Formation—**To form the G protein heterotrimer, 360 nM βγ complex was initially incubated with increasing concentrations of α subunit in ice for 30 min to obtain a wide range of α:βγ ratios in a buffer containing 20 mM Hepes (pH 8.0), 100 mM NaCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 0.5 mg/ml BSA. This mixture was then diluted 10-fold in a buffer containing 50 mM Hepes (pH 7.2), 3 mM EGTA, 1 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, and 1 mM DTT. 10 μl of these diluted samples containing βγ and various concentrations of α subunit was then added to 50 μl of PLC reaction buffer containing [3H]PIP₂ substrate and PLC β3 for determining enzyme activity as described above.

**RESULTS AND DISCUSSION**

**Purification and Functional Characterization of Recombinant G Protein Subunits and M2—**βγ complexes purified as described above were over 95% pure (Fig. 1A). When PLC β3 isozyme stimulation by β1γ5 or β1γ7 complexes was measured, the similar levels of PLC β3 activity by β1γ5 or β1γ7 complexes indicated that the functional proportion of each βγ complex was the same (Fig. 1B). In addition, we ensured that the concentration of detergent in the purified β1γ5 and β1γ7 samples were identical, using thin layer chromatography and detergents at various concentrations as standards (data not shown). His-M2 has been shown to possess similar properties to native M2 after reconstitution into lipids (13). His-M2 receptor was purified as described earlier. The purified M2 was ~90% pure as assessed by the methods used to quantify the G protein subunits. Reconstituted M2 had a Kᵦ for an antagonist, NMS, similar to native M2 (14) (Fig. 1C).

**M2-stimulated GTPγS Binding to Go Containing γ5 or γ7—**To allow for accurate titration of various concentrations of G protein subunits with purified receptor, we developed a system in which receptor alone was first reconstituted into lipids, quantified, and then assayed for its ability to activate varying concentrations of added G protein (Fig. 2, see legend). αo was assayed in the presence of two different βγ complexes, β1γ5 and β1γ7. The reconstituted M2 efficiently activates αo in a βγ-dependent manner (Fig. 2A). At an α:βγ:2 ratio of 100:10:1 with 1 mM M2, no differences were noted in the ability of M2 to activate αoβ1γ5 and αoβ1γ7 (Fig. 2A). It was possible that at lower concentrations of αo and an α:2 ratio closer to 1:1, subtle differences would be detected. However, GTPγS binding at lower concentrations of αo (e.g., 10 nM) was not sufficiently above background for use in these assays. Given this constraint, it was possible only to examine the effect of
overcome these problems we developed an alternative ap-

PLC activity is inhibited by the addition of... defined heterotrimers were assayed under these conditions, the receptor-stimulated GTPase activity of... of heterotrimer formation has been measured using pertussis measuring G protein heterotrimer formation. Thus far, efficacy... bg enhancement by the bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg bg b...
exchange in the indirect role at the receptor surface in controlling nucleotide change between the two heterotrimers. It is likely that the difference in the rate of receptor-stimulated nucleotide exchange (100%), whereas the enzyme activity stimulated by a previous conclusion that the α subunit interacts appropriately with the M2 receptor, whereas βγ subunit does not, and the increased GTPase activity is a consequence of more rapid “leaky” nucleotide exchange from the resulting inappropriate configuration of the α subunit with reference to the β1γ7 complex. This interpretation is consistent with a previous conclusion that the βγ complex plays a direct or indirect role at the receptor surface in controlling nucleotide exchange in the α subunit (19).

Strikingly, the difference seen between αβ1γ5 and αβ1γ7 disappears when αo is substituted with αb2. One possibility is that αb2 interacts with a different site on the receptor compared with αo thus changing the overall conformation of the G protein heterotrimer at the receptor surface. There are evidences from the analyses of different receptors for such differential interaction with G protein subtypes (20). The distinctly differential rate of M2-induced Go and Gi GTPase rates (Fig. 3), as well as GTPγS binding (21), are also consistent with this scenario. Regardless of the mechanism, this result indicates that the heterotrimer composition influences receptor-stimulated nucleotide exchange.

Because the G protein subunits are families of proteins it has been thought that particular combinations of α and βγ subtypes may differentially regulate signaling (22). The results here indicate that different α subunit and γ subunit types, through specific interactions with a receptor, can coordinately modulate receptor-stimulated nucleotide exchange resulting in differential signaling kinetics.

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# Table I

| Composition | GTPase activity at various G protein concentrations |
|-------------|-----------------------------------------------|
|             | 1 nM | 2 nM | 4 nM | 6 nM | 10 nM |
| Go-αβ1γ5    | 0.71 ± 0.03 | 1.44 ± 0.05 | 2.81 ± 0.07 | 3.88 ± 0.09 | ND |
| αb1γ7       | 1.3 ± 0.04 | 3.01 ± 0.07 | 5.56 ± 0.17 | 7.40 ± 0.31 | ND |
| Go-αβ1γ7    | ND   | ND   | ND   | 2.82 ± 0.08 | 2.95 ± 0.14 |
| αb2γ7       | ND   | ND   | ND   | 2.82 ± 0.08 | 2.95 ± 0.14 |

* GTPase activity was determined as described before and expressed as μM Pi/minute ± S.E. The data are the mean of five (Go) or four (Gi) time points (similar to Fig. 4). During the time course of the experiment (<10 min) the GTPase activity increases in a linear fashion. Within the Go:M2 ratios of 1:1 to 10:1 (1 nM M2), the Pi production was linear.

* Heterotrimers (αβγ) contain equimolar concentration of each subunit. For instance, 1 nM αβ1γ5 contained 1 nM αo and 1 nM β1γ5.

* The differences in GTPase activity between αβ1γ5 and αβ1γ7 at all concentrations are statistically significant (p < 0.05).

* ND, not determined.