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Molecular Mechanisms of G Protein - Receptor Coupling

Janna Eugenievna Slessareva

**Dissertation submitted to the School of Medicine at West Virginia University in partial
fulfillment of the requirements for the degree of**

**Doctor of Philosophy
In
Pharmacology and Toxicology**

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ABSTRACT

Molecular Mechanisms of G Protein - Receptor Coupling

Janna Eugenievna Slessareva

A big diversity in heterotrimeric G proteins and their cognate receptors raises many questions about the mechanisms that regulate the specificity of signaling along G protein-mediated pathways. The major goal of this project was to understand how the selectivity of G protein-receptor coupling is achieved at the level of the G protein-receptor interface. Additional goals of this project were to assess the generality of molecular mechanisms controlling G protein-receptor coupling selectivity and to investigate how agonist may regulate G protein-receptor interactions. Molecular determinants of G protein-receptor coupling were studied by reconstitution of exogenously supplied, purified G protein heterotrimers with receptors of interest expressed in Sf9 cell membranes. The receptor coupling activity of native G proteins was compared with that of chimeric G proteins, containing chimeric G α subunits composed from different regions of Gi1 α , Gq α and Gt α . We found a prominent role for the N- and C- terminus and α 4 helix- α 4/ β 6 loop domain of G α for receptor coupling and speculate that the N- and C-termini cooperate to couple receptors. Data demonstrate that multiple and distinct determinants of selectivity exist for individual receptor families and suggest that receptors recognize specific patterns formed by the amino acids of G α on the receptor recognition surface. We also show that individual receptors distinguish themselves by the EC₅₀ with which they interact with Gi1, indicating that G protein concentration may regulate G protein-receptor coupling. We obtained evidence that agonist concentration can regulate the selectivity of G protein-receptor coupling by demonstrating that chimeric G proteins unable to couple receptors at agonist concentrations used for native G proteins, were able to form the ternary complex at higher agonist concentration and, with some exceptions, exchange guanine nucleotides. Additionally, results suggest that G proteins play a more active role in receptor coupling than previously thought. Furthermore, results supporting the existence of multiple active receptor states indicate that the two-state receptor activation model needs to be changed. This project presents interesting findings regarding the molecular mechanisms that regulate G protein-receptor coupling and contributes to better understanding of GPCRs function.

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CHAPTER I. INTRODUCTION

1. INTRODUCTION AND SIGNIFICANCE

G protein coupled receptors (GPCRs) can be activated by a variety of extracellular signals including hormones, photons and odorants. G proteins, in turn, couple GPCRs to different intracellular effectors such as enzymes and ion channels (Gilman, 1987). G proteins are heterotrimers composed of α , β and γ subunits. There are 20 α subunits, 6 β subunits and 12 γ subunits which can produce many different combinations. G proteins are classified by the nature of their α subunits. They are divided into 4 different families based on their amino acid sequence homology, which include Gs, Gi/o, Gq/11 and G12/13. G proteins are active in their GTP bound form and inactive in GDP bound form. Ligand activated receptors promote GDP/GTP exchange on G protein α subunits, thereby activating the G protein and dissociating α and $\beta\gamma$ subunits. Then α and $\beta\gamma$ subunits modulate their effectors. Activation is terminated by the intrinsic GTPase activity of α subunits (Neer, 1995; Gilman, 1987). GPCRs are grouped into four classes based on the amino acid sequence similarity. These include Class I. Rhodopsin-type receptors, Class II. Secretin/glucagon receptors, Class III. Metabotropic glutamate/calcium sensor receptor and Class IV. “Gi-vomeronasal” pheromone receptors (Wess, 1998). The total number of human GPCRs is close to 750 (Vassilatis *et al.*, 2003). Given such a big number of GPCRs and G proteins, it is important to know how the selectivity of G protein-receptor coupling is achieved (Wess, 1998). As it will be described later, there are different mechanisms in living cells that regulate G protein-receptor coupling. At the molecular level the selectivity of G protein-receptor coupling is determined by the amino acid sequences of both receptors and G proteins. However,

the individual amino acids of G proteins and receptors controlling G protein-receptor coupling selectivity remain to be defined.

G proteins and GPCRs are involved in numerous cell signaling pathways (Gudermann *et al.*, 2000; Offermanns, 2001). Dysfunctions of G proteins and receptors are implicated in a growing number of diseases (Iiri *et al.*, 1998; Farfel *et al.*, 1999; Levine, 1999; Spiegel, 1999). Therefore, studying the mechanisms of G protein-receptor interactions may suggest new approaches in treating disease states and may help to develop new drugs that act at the G protein-receptor interface. One strategy involves blocking multiple receptors coupled to the same G protein without the use of multiple antagonists. For example, enhanced Gq signaling over time produces cardiac hypertrophy and apoptotic heart failure (Zolk *et al.*, 2000). Interestingly, Akhter *et al.* demonstrated that it is possible to inhibit myocardial hypertrophy *in vivo* by blocking the response from heart Gq coupled receptors using a peptide corresponding to the C terminus of Gq α (Akhter *et al.*, 1998). Using Gq/11 knock-out mice Wettschureck *et al.* demonstrated recently that Gq/11-mediated pathways are essential for cardiac hypertrophy induced by pressure overload, making it an attractive drug therapy target (Wettschureck *et al.*, 2001). There is also the possibility of developing a strategy to specifically block G protein coupling with a receptor of interest. In addition, chimeric G α subunits may serve as a tool for novel drug discovery (Milligan and Rees, 1999). Overall, this project will contribute to our understanding of the mechanisms underlying selectivity in G protein-receptor interactions.

2. MUSCARINIC RECEPTORS

Muscarinic receptor subtypes. Molecular cloning has identified five molecularly distinct members of the human muscarinic receptor family that are designated M1-M5 (Bonner *et al.*,

1987; Hulme *et al.*, 1990). Muscarinic receptor subtypes share high sequence homology, especially in the transmembrane domains, where there is about 90% sequence conservation (Bonner, 1989). M1, M3 and M5 receptors preferentially couple to G proteins from the Gq/11 class which leads to activation of phospholipase C β (PLC β) and, in turn, to breakdown of membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). M2 and M4 receptors preferentially couple to G proteins from the Gi/o class, which leads to inhibition of adenylyl cyclase and to activation of K⁺ channels, which is a $\beta\gamma$ -mediated response (Wess, 1996). Besides these well characterized responses muscarinic receptors regulate other signaling processes, including regulation of additional ion channels (Hulme *et al.*, 1990; Hosey *et al.*, 1999) and stimulation of MAP kinase pathways (Gutkind, 1998).

Tissue distribution and functional role. Techniques ranging from subtype-selective antagonist binding, northern blot analysis, in situ hybridization and subtype-specific antibodies have demonstrated that muscarinic receptors are present in many peripheral tissues including heart, smooth muscle and exocrine glands and also in many regions of the CNS (Hulme *et al.*, 1990; Wess, 1996). Peripheral muscarinic receptors mediate the action of acetylcholine on parasympathetically innervated tissues while central muscarinic receptors are involved in regulation of higher cognitive functions and extrapyramidal motor function (Wess, 1996). Although many tissues express several different muscarinic receptors, some of the muscarinic responses are thought to be mediated by a particular receptor subtype. For example, the M2 receptor is the predominant form of muscarinic receptors in the heart where it mediates negative chronotropic and ionotropic effects. M3 receptors mediate smooth muscle contraction and glandular secretion (Caulfield, 1993). M1 receptors in the cerebral cortex and hippocampus are

thought to be involved in memory and learning. M4 receptors in striatum play a role in regulation of motor function (Levey *et al.*, 1991). The function of M5 receptor, which is expressed at low levels in CNS and in some peripheral tissues, is not clear yet. However, a recent study suggests that M5 receptors play a role in facilitating dopamine release in striatum and in the dilation of cerebral blood vessels (Yamada *et al.*, 2001).

Structure of the GPCRs. Muscarinic receptors belong to the rhodopsin-like family of the GPCR superfamily and share a common overall structure, consisting of 7 transmembrane helices connected by the extracellular (o2-o4) and intracellular (i1-i3) loops. The N terminus of the receptor (o1) is located at the extracellular surface while the C terminus is located at the intracellular surface (Wess, 1998). The fourth intracellular loop (i4) is formed when the palmitoylated C terminus attaches to membrane. Ligands bind to receptors from different classes in different modes depending on their nature. For example, biogenic amines and nucleotides bind to the receptor core within the plane of the bilayer, small peptides (≤ 40 amino acids) bind to both the core and extracellular loops, polypeptides (≤ 90 amino acids) bind to the extracellular loops and the N terminus and glycoproteins (≥ 30 kD) bind to the N terminus (Ji *et al.*, 1998). Muscarinic receptor ligands bind to transmembrane helices in the receptor core (Wess, 1996). The intracellular receptor surface is involved in G protein coupling. The seven transmembrane helices are arranged in a helical bundle. Before the crystal structure of the first GPCR became available, a model of arrangement of transmembrane helices had been proposed by Baldwin (Baldwin, 1993; Baldwin, 1994; Unger *et al.*, 1997). This “Baldwin model” predicts an anticlockwise connectivity of transmembrane helices as viewed from the extracellular side. Recently a crystal structure of rhodopsin, a prototypical GPCR, has been determined at 2.8 Å resolution (Palczewski *et al.*, 2000) supporting the anticlockwise connectivity of transmembrane

helices as predicted by the “Baldwin model”. This first detailed model of a GPCR allowed us to view receptor mutagenesis data in the context of the three-dimensional structure and provided structural information for the modeling of the G protein–receptor interactions.

Muscarinic receptors have several potential N-glycosylation sites (Asn-X-Ser/Thr) at the N terminus. However mutational studies indicate that glycosylation is not required for ligand binding or G protein coupling (Ohara *et al.*, 1990; van Koppen and Nathanson, 1990). Two conserved cysteine residues in o1 and o2 loops of muscarinic receptors form a disulfide bond, which is important for receptor folding but not required for ligand binding (Hulme *et al.*, 1990). Acetylcholine binds to the receptor in a cleft between transmembrane helices about 10-15 Å from membrane surface. A conserved Asp residue in TM III is thought to be important for interactions with the positively charged ammonium/amino head of muscarinic receptor ligands. Other amino acids shown to be important for ligand binding include Tyr in TM VII, Thr in TM IV and Thr in TM V (Wess, 1996).

Muscarinic receptor agonists. An extensive review of muscarinic receptor agonists can be found in Goodman and Gilman’s “The Pharmacological Basis of the Therapeutics” (Goodman & Gilman's, 2001) Briefly, muscarinic receptor agonists are divided into naturally occurring alkaloids (pilocarpine, muscarine, arecoline) and choline esters (acetylcholine, methacholine, carbachol, bethanechol). Muscarinic agonists are used in ophthalmology (pilocarpine for glaucoma), for treatment of gastrointestinal and urinary bladder disorders (bethanechol) and for treatment of xerostomia (pilocarpine). Methacholine is useful for diagnosis of bronchial hyperreactivity and asthmatic conditions. There are also other synthetic muscarinic receptor agonists available. Oxotremorine is the pharmacologically active metabolite of the synthetic agent tremorine. Oxotremorine is a selective muscarinic receptor agonist equipotent to

acetylcholine. Because of the high amino acid sequence homology between muscarinic receptor subtypes and especially because the amino acids that are thought to be involved in agonist binding are identical, selective muscarinic agonists are not available at present. McN-A-343 was proposed to be a selective M1 agonist, however this selectivity appeared to be functional as opposed to subtype-selective.

Muscarinic receptor antagonists. An extensive review of muscarinic receptor antagonists can be found in Goodman and Gilman's "The Pharmacological Basis of the Therapeutics" (Goodman & Gilman's, 2001). Briefly, muscarinic receptor antagonists are divided into naturally occurring alkaloids (atropine, scopolamine), semisynthetic derivatives of these alkaloids (homatropine, tropicamide, ipratropium) and synthetic agents (pirenzepine, tolterodine). Several antagonists have been developed that show some degree of subtype selectivity, however no agent is completely selective. For example, pirenzepine is selective for M1 receptors in comparison to M2 and M3 receptors but has comparable affinity for M4 receptor. Telenzepine is a higher potency analog of pirenzepine. AF-DX116 and triptamine are selective for M2 receptors. Hexahydro-sila-difenidol and darifenacin are selective for M3 receptors. Also mamba toxins have shown selectivity for M1 and M4 receptor subtypes (Caulfield and Birdsall, 1998). Muscarinic receptor antagonists are used to treat gastrointestinal tract disorders (pirenzepine, belladonna alkaloids), chronic obstructive pulmonary disease (ipratropium), motion sickness (scopolamine), anticholinesterase and mushroom poisoning (atropine). They are also useful in ophthalmology (atropine, cyclopentolate).

Muscarinic receptor domains involved in G protein coupling. Receptor domains involved in G protein coupling have been extensively investigated using mutagenesis (loss of function and gain of function approaches) and were found to be located at the intracellular side

of the receptor. Using hybrid M1/M2 receptors Kubo *et al* first demonstrated that the i3 loop plays an important role in G protein coupling (Kubo *et al.*, 1988). Later it was demonstrated that only 8-15 N and C terminal amino acids of i3 (Ni3 and Ci3) are critical for G protein coupling (Wess, 1998; Strader *et al.*, 1994). Mutagenesis studies by different investigators precisely identified amino acids in both Ni3 and Ci3 regions involved in G protein coupling and demonstrated that Ni3 residues involved in coupling selectivity are primarily hydrophobic or uncharged and lie on the hydrophobic side of an amphiphilic α helix (Wess, 1998). Detailed analysis of Ni3 identified Tyr254 of M3 receptor as residue important for Gq coupling (Blum *et al.*, 1994a; Blum *et al.*, 1994b). Gain of function studies with M2 and M3 receptors identified 4 conserved amino acids in Ci3 important for M1, M2 and M5 receptor coupling as Ala488, Ala489, Leu492 and Ser493 (residue numbers as in M3 sequence), which are known as “AALS motif” (Blin *et al.*, 1995). Corresponding amino acids in M2 and M4 receptors have been identified as Val385, Thr386, Ile389 and Leu390 (residue numbers as in M2 sequence) and known as “VTIL motif” (Liu *et al.*, 1995; Kostenis *et al.*, 1997a). These amino acids are located in a helically arranged i3/TM VI junction and together with amino acids from Ni3 define hydrophobic surfaces important for G protein coupling. However because Ni3 and Ci3 are thought to be spatially close they may form one G protein recognition surface (Wess, 1998). Burstein *et al* similarly identified amino acids of M5 receptor in Ni3 and Ci3 regions and proposed that these residues form a G protein binding pocket with a hydrophobic core and positively charged lip (Burstein *et al.*, 1998). Although the i3 loop is important for G protein coupling, it is not the only determinant of coupling selectivity. Blin *et al* demonstrated that the i2 loop of M3 receptor is also important for proper Gq recognition (Blin *et al.*, 1995). Residues within i2 that have been shown to be important for G protein coupling include the “DRY” motif,

which is conserved among most GPCRs (Fraser *et al.*, 1989; Zhu *et al.*, 1994; Jones *et al.*, 1995) and a Pro-Leu motif (Moro *et al.*, 1993). Gain of function studies by Blin *et al.* (Blin *et al.*, 1995) identified amino acids of M3 receptor in the i2 loop important for Gq coupling selectivity as Ser168, Arg171, Arg176 and Arg183. The C terminus of muscarinic receptor does not play an important role in G protein coupling selectivity, although 8-16 amino acids of i4 proximal to the membrane may play a role in G protein coupling. The i1 loop also does not contribute significantly to G protein coupling selectivity (Wess, 1998). Because the length and amino acid sequence of the i1 loop are conserved in the muscarinic receptor family, the i1 loop mostly plays a role in receptor structural integrity (Moro *et al.*, 1994). In summary, receptor regions important for G protein coupling selectivity are i2, Ni3 and Ci3. Studies by different groups suggest that these regions cooperate to achieve efficient G protein coupling and activation (Wess, 1998).

3. HETEROTRIMERIC G PROTEINS

G α subunits. More than 20 different G α subunits encoded by 17 distinct genes have been identified. On the basis of their amino acid sequence homology G α subunits are divided into four families: Gs, which includes several splice variants of Gs α and Golf α , Gi/o, which includes Gi1 α , Gi2 α , Gi3 α and splice variants; Go1 α , Go2 α ; Gt1 α , Gt2 α ; Ggust α ; Gz α , Gq/11, which includes Gq α , G11 α , G14 α , G15 α , G16 α and G12/13, which includes G12 α and G13 α (Neer, 1995; Hamm, 1998). Among the well characterized activities of G α subunits are activation of adenylyl cyclase by Gs α , inhibition of adenylyl cyclase by Gi α , activation of cGMP phosphodiesterase by Gt α and activation of phospholipase C by Gq α . In addition, Gq α can directly stimulate the activity of Bruton's tyrosine kinase *in vitro* and *in vivo* (Bence *et al.*, 1997). G12 α and G13 α can couple to thrombin, thromboxane and angiotensin receptors (Barr *et*

al., 1997) and have been shown to regulate Na^+/H^+ exchange (Voyno-Yasenetskaya *et al.*, 1994). G13 α has also been shown to activate radixin (Vaiskunaite *et al.*, 2000).

Several crystal structures of G α subunits in both active and inactive conformations and as complexes with G $\beta\gamma$ subunits have been solved. These include Gt α :GTP γ S·Mg²⁺ (Noel *et al.*, 1993), Gt α :GDP·Mg²⁺ (Lambright *et al.*, 1994), Gi1 α :GTP γ S·Mg²⁺ (Coleman *et al.*, 1994), Gi1 α :GDP (Mixon *et al.*, 1995), Gi1 α :GDPG $\beta_1\gamma_2$ (Wall *et al.*, 1995) and Gt α /Gi1 α :GDPGt $\beta\gamma$ (Lambright *et al.*, 1996). G α subunits contain two domains: the ras-like GTPase domain, which is involved in the guanine nucleotide binding and GTP hydrolysis; and the helical domain with yet unclear function. The α helical domain is inserted into the GTPase domain. The domains are connected by two flexible linkers. The GTPase domain consists of a six-stranded β -sheet (β_1 - β_6) surrounded by six helices (α_1 - α_5 and α_G). The helical domain consists of a central helix (α_A) surrounded by five shorter helices (α_B - α_F). The guanine nucleotide binds in the cleft between the GTPase and the helical domains. In the GTPase domain the guanine nucleotide-binding site is formed by five polypeptide loops (G-1 through G-5), which are highly conserved and define the G protein superfamily, which include heterotrimeric G proteins, Ras and its homologs, and elongation factors Tu and G.

Most G α subunits (except Gt α) are S-palmitoylated at a cysteine (or cysteines) at the N-terminus and others (Go α , Gi α , Gz α , Gt α) are also N-myristoylated at Gly-2. These lipid modifications help target G α subunits to the plasma membrane to which they are loosely attached. In addition, palmitoylation is a dynamic process which may be involved in the trafficking of G α between membrane and cytosol (Milligan *et al.*, 1995; Wedegaertner *et al.*, 1995). The Gi1 α family members (except Gz α) can be ADP-ribosylated by pertussis toxin at a

cysteine residue, four amino acids from the C terminus, thus inhibiting receptor coupling (Gilman, 1987). Gs α can be ADP-ribosylated at Arg-201 by cholera toxin, which allows GTP binding but inhibits GTPase activity of Gs α (Gilman, 1987) resulting in constitutively active Gs α .

The Gi1 α is a 40.3 kD protein, which is widely expressed in different tissues (Jones and Reed, 1987). Gi1 α is both myristoylated (at glycine through an amide linkage) and palmitoylated (at cysteine through thioester linkage) at the N terminus and attached to the membrane surface. Dissociation of GDP from Gi1 α is slow (0.03 min^{-1}) (Linder *et al.*, 1990) and is almost undetectable in the heterotrimer.

There are two isoforms of Gt α (transducin) – one present in the rod outer segments (Gt1 α) and the other present in cones (Gt2 α). Both of them activate cGMP phosphodiesterase by coupling to the light activated rhodopsin (Gt1 α) or cone-opsins (Gt2 α) (Gilman, 1987; Chabre and Deterre, 1989). Gt α has a very low basal rate of GDP/GTP exchange which is required for the low noise signaling in the visual system. Gt α is activated by rhodopsin very rapidly and one molecule of activated rhodopsin may activate several hundred Gt α subunits, resulting in a dramatic signal amplification (Chabre and Deterre, 1989).

The Gq α is a 43 kD protein which stimulates phospholipase C β (Smrcka *et al.*, 1991; Taylor *et al.*, 1991; Hepler *et al.*, 1993). It is expressed in many tissues, with tissues of particular interest being the heart, as Gq α can be a novel target for heart failure (Wettschureck *et al.*, 2001) and platelets, because a defect in platelet activation is seen in Gq α null mice (Shenker *et al.*, 1991; Offermanns, 2001). The GDP dissociation rate from Gq α is unusually slow. In addition, Gq α does not readily bind GTP γ S so that concentrations of GTP γ S as high as 30 μ M are required

for half-maximal activity (Hepler *et al.*, 1993). These properties delayed identification and purification of Gq α in a functionally active state and pose difficulty for reconstitution experiments.

The conformational changes that occur in G α when it goes from the GTP bound form to the GDP bound form are mostly localized to so-called switch regions. Switch I corresponds to the linker 2 (α F- β 2 loop), switch II is the β 3/ α 2 loop and α 2 helix in the GTPase domain, switch III is a β 4/ α 3 turn in the GTPase domain and switch IV is α B/ α C loop in the helical domain (Mixon *et al.*, 1995).

In the Gi1 α ·GTP γ S·Mg²⁺ conformation, switches II and III are ordered but 32 N terminal and 10 C terminal residues are disordered (Coleman *et al.*, 1994). In the Gi1 α ·GDP·Mg²⁺ conformation switches II and III are disordered but N terminal (amino acids 8-32 in the structure) and C terminal (amino acids 344-354) residues fold into a compact microdomain (Mixon *et al.*, 1995). In the heterotrimer, N terminal residues (N terminus and α N helix) of Gi1 α unfold to interact with G β . Thus the N terminus is stabilized by contacts with G β . In contrast, the C terminus is disordered in the heterotrimer as a result of the loss of interactions with the N terminus (Wall *et al.*, 1995). The C terminus, which is a major G α receptor interaction domain, is stabilized upon receptor interaction (Kisselev *et al.*, 1998). In the heterotrimer, G α makes two contact surfaces with G β . The first contact surface is in β 2, β 3, α 2 region, including switch II. The second contact surface is the interface between N terminus of G α and the side of the β propeller of G β (Wall *et al.*, 1995).

G $\beta\gamma$ subunits. Six different β and twelve different γ subunits have been described (Clapham and Neer, 1997). The β and γ subunits bind very tightly and can only be separated by

denaturation. Thus the G $\beta\gamma$ dimer can be viewed as a functional monomer. In theory, 72 different $\beta\gamma$ combinations can be formed, however there is evidence suggesting that not all combinations are possible (Clapham and Neer, 1997). The functional importance of such diversity is not clear because most of the $\beta\gamma$ complexes have similar functional properties. The β subunits share a high sequence homology while the γ subunits are more diverse.

The G β is composed of two types of structural regions: N terminal amphipathic α helix followed by the 7-membered β -propeller structure (Wall *et al.*, 1995; Lambright *et al.*, 1996; Sondek *et al.*, 1996). Each of the 7 β -sheets contains 4 antiparallel strands which radiate from a central core. Amino acid sequence of G β contains 7 WD repeats. The WD motif is about 40 amino acids long, contains conserved amino acids, and is frequently terminated by a Trp-Asp dipeptide (WD). The WD repeat in $\beta\gamma$ begins at the turn between the 3rd and 4th strands of a β -sheet and ends at the same position in the next β -sheet (Sondek *et al.*, 1996). Thus a WD repeat does not correspond to a defined folded domain. WD repeats are not unique to G β subunits and occur in about 40 other proteins with diverse functions (Neer *et al.*, 1994).

G γ is mostly helical. The N terminus of G γ forms a coiled-coil with the N terminal helix of G β . The rest of G γ extends across G β contacting propeller blades 5, 6 and 7. Thus G γ forms extensive interactions with G β , binding to G β in the extended conformation and making no contacts with itself (Sondek *et al.*, 1996). The C terminus of G γ contains the CAAX motif. This motif directs the G γ prenylation – either farnesylation or geranylgeranylation (Casey, 1994). Lipid modifications are attached to the cysteine via a thioester bond and serve for membrane attachment of G $\beta\gamma$ (Simonds *et al.*, 1991).

Gβγ greatly enhances Gα binding to the receptor (Heithier *et al.*, 1992; Phillips and Cerione, 1992). Although Gα is the main determinant of receptor coupling, both Gβ and Gγ subunits have been shown to interact directly with the receptor (Phillips and Cerione, 1992; Heithier *et al.*, 1992; Kisselev *et al.*, 1994; Taylor *et al.*, 1996; Azpiazu *et al.*, 1999; Ernst *et al.*, 2000). Gα prevents Gβγ from interaction with effectors. Upon dissociation from Gα, Gβγ can interact with a great number of downstream targets (Clapham and Neer, 1997). Gβγ has been shown to interact with phospholipases β2 and β3 (Morris and Scarlata, 1997; Rhee and Bae, 1997), adenylyl cyclases AC II, AC IV, AC VII (activation) and AC I (inhibition) (Sunahara *et al.*, 1996), K⁺ and Ca²⁺ ion channels (Logothetis *et al.*, 1987; Schneider *et al.*, 1997), G protein-coupled receptor kinases (Pitcher *et al.*, 1992; Pitcher *et al.*, 1998) and phosphoinositide 3-kinases (p110γ) (Tang and Downes, 1997; Vanhaesebroeck *et al.*, 1997). Other potential Gβγ effectors include Raf1 protein kinase (Pumiglia *et al.*, 1995) and Btk and Tsk nonreceptor tyrosine kinases (Langhans-Rajasekaran *et al.*, 1995).

The G protein cycle. G proteins cycle between GDP-bound inactive conformation and GTP-bound active conformation. The details of this cycle have been described in several review papers (Gilman, 1987; Neer, 1995; Hamm, 1998). When GDP is bound to an α subunit, it associates with a βγ subunit to form a heterotrimer which can interact with receptors. Agonist binding to receptor causes conformational changes in the receptor structure which allows it to interact with G protein heterotrimer. Activated receptor then promotes release of GDP from Gα. This leads to the formation of the ternary complex of the agonist, receptor and G protein in the guanine-nucleotide free state. This so-called “empty pocket” conformation of Gα has high affinity for activated receptor and it is able to stabilize the high affinity state of the receptor for agonist. Affinity shift assay (described below) is able to detect the formation of the ternary

complex as an enhanced level of agonist binding. Because the concentration of GTP in cells is much higher than the concentration of GDP, GTP binds to $G\alpha$, which leads to dissociation of $G\alpha$ from $G\beta\gamma$ and from receptor. Then both $G\alpha$ and $G\beta\gamma$ are free to interact with their target effectors. $G\alpha$ has an intrinsic GTPase activity so it hydrolyzes GTP to GDP and inorganic phosphate which leads to the dissociation of $G\alpha$ from the effector. The GDP-bound $G\alpha$ has a high affinity for $G\beta\gamma$ so it associates with $G\beta\gamma$ thus terminating $G\beta\gamma$ activity. The GDP-bound G protein heterotrimer is then able to enter a new activation cycle.

4. G PROTEIN-RECEPTOR INTERACTIONS

Affinity Shift Assay. Receptors can exist in at least two affinity states for agonist – a low affinity state, which represents uncoupled receptors and a high affinity state, which represents receptors coupled to an appropriate G protein, or receptors in the active conformation (spontaneous isomerisation). As described above, the ternary complex of agonist, receptor and guanine nucleotide-free G protein represents the high affinity receptor state. The ability of a specific G protein to interact with a receptor of interest and stabilize the high affinity state of the receptor can be studied in the affinity shift activity assay. This assay is based on early work with native receptors (Asano *et al.*, 1985; Pobiner *et al.*, 1991) and has been completely described for several recombinant receptors (Figler *et al.*, 1996; Clawges *et al.*, 1997; Cabrera-Vera *et al.*, 2002). In the affinity shift assay, partially purified membranes, containing the expressed receptor, are reconstituted with saturating amounts of purified, exogenous, G proteins. A radioligand binding assay with a low concentration of agonist (near the K_D for the high affinity state) is used to determine the fold- enhancement (affinity shift) in agonist binding by reconstitution with G protein. Affinity shift assay allows us to measure the G protein-receptor

interaction directly and eliminates interference from other proteins which may modulate the activity of downstream effectors. In addition, this reconstitution approach allows control of the stoichiometry of receptors and G proteins, which is hard to achieve in transfection experiments.

GPCR models. There are several receptor models that describe ligand-receptor interactions (Kenakin, 1996). The extended ternary complex model (Figure A) remains the most widely accepted model of GPCR activation (Samama *et al.*, 1993). According to this model, receptors exist in equilibrium between inactive R and active R* conformations. The R* conformation is stabilized by guanine-nucleotide free G protein heterotrimers and binds agonists with the highest affinity. Thus agonists shift the equilibrium toward the accumulation of R*. However, receptors are able to undergo spontaneous isomerisation from the R to R* in the absence of agonists. This is described as a constitutive receptor activity. Constitutive receptor activity leads to agonist-independent activation of G proteins and downstream signaling cascades. Inverse agonists are able to destabilize active receptor complexes and prevent agonist-independent signaling.

To characterize the effects of ligands in more detail the cubic ternary complex model, which takes into consideration all the previous models, has been described (Kenakin, 1996; Weiss *et al.*, 1996). The first face of the cube represents the effect of ligand on receptor activation. The second face describes the interactions between receptors and G proteins. The third face describes the effect of ligand on G protein-receptor interactions. The cube is completed by microreversibility with the appropriate constants. Despite the fact that this model has too many constants and is not very useful for the modeling of data, it is complete and useful in describing and predicting receptor behavior.

Interestingly, new experimental data suggest that the two-state receptor activation model ($R \leftrightarrow R^*$) cannot be applied in all cases (Chidiac, 1998) and that the conformation of the HR^*G complex may depend on the identity of the agonist (Krumins and Barber, 1997). In addition, the possible existence of receptor-specific multiple G protein conformation states (Wenzel-Seifert and Seifert, 2000) may further complicate the model. It is also important to note that kinetic considerations are not included in these models. Shea *et al.* recently proposed the cubic ternary complex activation model which modifies the cubic ternary model by including G protein activation and deactivation steps (Shea *et al.*, 2000).

FIGURE A. The extended ternary complex model

Structural considerations. In recent years several G protein-receptor interaction models have been proposed (Lichtarge *et al.*, 1996; Oliveira *et al.*, 1999; Grishina and Berlot, 2000). When the high resolution structure of rhodopsin in the ground state became available (Palczewski *et al.*, 2000), modeling of the G protein-receptor complex was raised to a new level (Hamm, 2001). However, because the structure was solved for the inactive state of rhodopsin, many questions remain unanswered. The guanine nucleotide is buried in the cleft between GTPase and α helical domains of $G\alpha$, and thought to be approximately 30 Å away from the membrane surface. Because the intracellular receptor loops are not long enough to directly contact the GDP in this cleft, the receptor must act at a distance to promote nucleotide release. The most widely accepted view is that receptor interaction with the C terminus (and probably amino acids in the $\alpha 5$ helix and $\alpha 4/\beta 6$ loop) of $G\alpha$ leads to the conformational changes that are propagated to the $\beta 6/\alpha 5$ loop. The $\beta 6/\alpha 5$ loop (TCAT motif) is known to contact the guanine nucleotide directly. Thus the receptor can promote GDP release by weakening the binding interactions (Wess, 1998). Recently Kisselev *et al.* proposed a two-site sequential fit model for the signal transfer from the activated receptor to the G protein (Kisselev *et al.*, 1999). According to this model, sites with distinct conformations on R^* can be specifically recognized by the C terminus of $G\alpha$ and the farnesylated C terminus of $G\gamma$. Upon binding of the first matching pair of domains, conformational changes in the R^* and/or G protein occur that lead to the subsequent binding of the second matching pair of domains.

5. SELECTIVITY OF G PROTEIN-RECEPTOR COUPLING

The question of selectivity. Many different heterotrimers can be produced from the 20 α subunits, 6 β subunits and 12 γ subunits. Many GPCRs are known to date and most of them

include multiple subtypes. Therefore, the question of selectivity in G protein-receptor coupling arises (Gudermann *et al.*, 1996; Wess, 1998). There are examples of both promiscuous and highly selective interactions between receptors and G proteins. For example, the thyrotropin-releasing hormone receptor has been shown to couple with G proteins from all 4 classes (Laugwitz *et al.*, 1996). In contrast, gonadotropin-releasing hormone receptor has been shown to mediate multiple signaling pathways by coupling only to G_{q/11} proteins (Gudermann *et al.*, 2000). An even higher degree of selectivity has been seen in studies utilizing antisense nucleotides *in vivo*. One example demonstrates that the G₁₃ heterotrimer composed of $\alpha_{13}\beta_1\gamma_3$ subunits couples angiotensin AT_{1A} receptor to increase intracellular Ca²⁺ in rat portal vein myocytes (Macrez-Lepretre *et al.*, 1997). However, this level of specificity is usually not seen *in vitro*.

Most GPCRs couple only with a distinct subset of G proteins expressed in the cell and therefore are described as G_s, G_{i/o} or G_q coupled receptors. Interestingly, G protein coupling preferences of individual GPCRs is often relative rather than absolute because the receptor can couple with other G proteins but with reduced efficiency. In general, functionally important amino acids are very conserved. However, if they mutate, they often do so in groups to maintain a common function. Correlated mutational behavior is described as a tendency of sequence patterns to remain conserved or to mutate pair-wise. Using such correlated mutation analysis, which is a sequence-only based technique, Horn *et al.* have come to the conclusion that such low efficiency coupling is a result of a recent divergent evolutionary process (Horn *et al.*, 2000). Because the molecular mechanism of G protein-receptor coupling is not completely understood, at present it is impossible to predict the G protein coupling preferences of a newly cloned GPCR based only on its sequence.

Mechanisms controlling selectivity of coupling. There are many mechanisms that control the G protein-receptor coupling. Cells can regulate the selectivity of G protein-receptor coupling by localizing certain components of the signal transduction pathway into microdomains so that there is no mixing of components between different compartments (Neubig, 1994; Albert and Robillard, 2002). There are also examples of polarized cells that distribute receptors and G proteins asymmetrically (von Zastrow *et al.*, 1993; Keefer *et al.*, 1994). The expression level of specific G proteins or receptors in the cell can also dictate the selectivity of coupling (Gudermann *et al.*, 1996). Accessory proteins have also been shown to regulate G protein-receptor coupling. For example, Natochin *et al.* had demonstrated that AGS3 (Activator of G protein Signaling 3) inhibits GDP dissociation from Gi1 α (Natochin *et al.*, 2000a). In addition, once a G protein is activated, the selectivity of downstream pathway activation can be regulated by the intrinsic rate of GTP hydrolysis of the specific G α (Neer, 1995) which in turn can be regulated by RGS (Regulators of G protein Signaling) proteins (Hepler, 1999), which act as GTPase-activating proteins for G α (Hepler, 1999; Wieland and Chen, 1999).

It is often the case that receptors, which mediate functionally related responses, couple to the same G protein. For example, pancreatic acinar cells respond to acetylcholine, bombesin and cholecystokinin by activating phospholipase C through G_q (Xu *et al.*, 1998; Xu *et al.*, 1999). Activation of PLC results in mobilization of Ca²⁺ from intracellular stores, which ultimately leads to exocytotic release of digestive enzymes from pancreatic acini. Interestingly, Xu *et al.* demonstrated that each agonist produced different pattern of Ca²⁺ wave propagation, which was shown to be modulated by RGS proteins (Xu *et al.*, 1999). The factors that influence G protein-receptor coupling selectivity *in vitro* include receptor density, G protein and agonist concentrations (Wess, 1998).

Gα domains involved in receptor coupling selectivity. Gα is thought to be the major receptor contact subunit in the G protein heterotrimer. Several Gα domains have been implicated in receptor coupling selectivity. Although the distinction has to be made between the Gα domains required for general G protein coupling and the Gα domains involved in the regulation of coupling selectivity, these receptor interaction domains often overlap.

The C terminus of Gα has been shown to interact with receptors using different experimental approaches (West, Jr. *et al.*, 1985; Hamm *et al.*, 1988; Conklin *et al.*, 1993; Natochin *et al.*, 2000b). With respect to selectivity, it has been demonstrated that substitution of the last five C terminal residues of Gqα with corresponding sequences of Giα, Goα or Gzα allowed PLCβ stimulation by receptors normally coupled to Gi/o such as A1 adenosine, D2 dopamine and M2 muscarinic receptors (Conklin *et al.*, 1993; Liu *et al.*, 1995). Other studies with different receptors verified that the C terminus of Gα is involved in direction of receptor coupling selectivity, which is determined by the C terminus amino acid composition (Conklin *et al.*, 1996; Kostenis *et al.*, 1997a; Kostenis *et al.*, 1997b). The C terminal amino acids of Giα important for receptor coupling are -4 cysteine, -3 glycine and -1 phenylalanine, while the C terminal amino acids of Gqα involved in receptor coupling selectivity are -5 glutamate and -3 asparagine (“-“ represents amino acid position counting from the C terminus) (Wess, 1998). Although the C terminus of Gα is important for receptor coupling, in some cases it is not sufficient when present in the context of an inappropriate Gα (Ma *et al.*, 2000; Slessareva and Graber, 2000). However, reconstitution studies by Natochin *et al.* indicate that five C- terminal amino acids of transducin are sufficient for rhodopsin coupling (Natochin *et al.*, 2000b). These differences support the idea that molecular mechanisms of G protein-receptor coupling selectivity vary among receptors.

The N terminus of G α has also been shown in a variety of experiments to be a determinant of G protein-receptor coupling selectivity. Early studies indicated that G α N terminal regions interact with rhodopsin (Hamm *et al.*, 1988), mastoporan (a wasp venom mimicking the active receptor state) (Higashijima and Ross, 1991) and α 2 adrenergic receptors (Taylor *et al.*, 1994). Bae *et al.* demonstrated that the replacement of amino acids 1-210 of Gi1 α with the corresponding amino acids of Gt α impaired, but did not prevent, 5-HT_{1B} receptor coupling (Bae *et al.*, 1997). Hepler *et al.* showed that cysteines at positions 9 and 10 of Gq α are important but not critical for receptor coupling (Hepler *et al.*, 1996). Kostenis *et al.* demonstrated the importance of the N terminus of Gq α in constraining receptor coupling selectivity. They showed that deletion of six N- terminal amino acids of Gq α , which are unique for the Gq family, led to promiscuous coupling so that several Gi/o and Gs coupled receptors gained the ability to productively couple with this Gq α mutant (Kostenis *et al.*, 1997c). The N terminal α N helix of Gi2 has been shown to be important for PAR1 thrombin receptor coupling and transferring the signal between receptor and G protein (Swift *et al.*, 2000).

Other domains important for receptor coupling include the α 2 helix- α 2/ β 4 loop domain (Lee *et al.*, 1995), the α 3/ β 5 loop (Grishina and Berlot, 2000), 20 amino acids in the α helical domain (Krieger-Brauer *et al.*, 1999) and the α N/ β 1 loop (Blahos *et al.*, 2001). The α 4 helix- α 4/ β 6 loop region of Gi1 α , which is a known effector interaction domain, has been shown to be absolutely essential for 5-HT_{1B} serotonergic receptor coupling (Bae *et al.*, 1997). Recently it has been demonstrated that two amino acids (Gln304 and Glu308) within the α 4 helix mediate 5-HT_{1B} receptor coupling (Bae *et al.*, 1999). The work of Natochin *et al.* supports the role of α 4/ β 6 loop as a receptor interacting domain and indicates that Arg310 and Asp311 are involved in the

interaction with transducin (Natochin *et al.*, 1999). Recent evidence supports the role of $\alpha 4/\beta 6$ loop in receptor coupling (Mody *et al.*, 2000). Data from the Graber laboratory demonstrate that multiple regions on G protein α subunits including C terminus, $\alpha 4$ helix- $\alpha 4/\beta 6$ loop region and N terminal part are involved in functional coupling with Gi/o-coupled receptors. It has been demonstrated that four distinct Gi-coupled receptors (5-HT_{1A} and 5-HT_{1B} serotonergic, A1 adenosine and M2 muscarinic receptors) use slightly different domains on Gi1 α for functional interactions (Ma *et al.*, 2000). Figure B shows the spacefilling model of Gi1 $\alpha\beta_1\gamma_2$ heterotrimer. The regions important for receptor coupling are exposed, located on the common G α surface and thus are available for receptor interaction.

Taken together, these reports indicate that multiple and different regions on G protein heterotrimer are intimately involved in selective receptor coupling.

FIGURE B. Spacefilling representation of the Gαi1β1γ2 heterotrimer. This model was generated from the crystal structure of Gαi1β1γ2 (Wall *et al.*, 1995)(PDB ID is 1GP2) and colored using RasMol. The α subunit is shown in blue, β in green and γ in yellow. The C and N termini of Gαi1 are shown in light blue and orange, respectively. The regions that are important for receptor coupling are described in the text (Section 5).

6. SUMMARY AND GOALS

The molecular mechanisms of G protein-receptor coupling are not very clear at present. The major goal of this project was to understand how the selectivity of G protein-receptor coupling is achieved at the level of the G protein-receptor interface. The specific aims of this project were to determine how the selectivity of Gq-M1 muscarinic receptor coupling and Gi1-M2 muscarinic receptor coupling is achieved, to assess the generality of molecular mechanisms underlying G protein-receptor coupling selectivity and to understand how the selectivity of G protein-receptor coupling is regulated by agonist. Fulfillment of these goals will help us to gain more understanding of how GPCRs function, and may be helpful for the development of drugs that act at the G protein-receptor interface.

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CHAPTER II. MATERIALS AND METHODS

1. Materials. [^3H]-Oxotremorine-M Acetate ([^3H]-OXO-M) (85.8 Ci/mmol), [^3H]-N-Methyl Scopolamine (70 Ci/mmol) and [^{35}S]-GTP γ S (1250 Ci/mmol) were from New England Nuclear Life Science Products, Inc. (Boston, MA). Atropine Sulfate and Oxotremorine-M (OXO-M) were from Sigma-Aldrich Corporation (St. Louis, MO). Molecular biology enzymes were from New England Biolabs (Beverly, MA). Wild type baculovirus DNA and lipofectin were from Invitrogen (Carlsbad, CA). Anti-G $\alpha_{i1/2}$ C-terminal antibodies were from Calbiochem-Novabiochem Corporation (San Diego, CA). Anti-G α_{i1} internal antibodies and anti-G $\alpha_q/11$ C-terminal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Pertussis Toxin was from Calbiochem-Novabiochem Corporation (San Diego, CA). The ProtoBlot Western Blot reagents were from Promega (Madison, WI). The BCA Protein Assay reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO) or Calbiochem-Novabiochem Corporation (San Diego, CA).

2. Sf9 cell culture. Recombinant receptors and G proteins were produced in *Spodoptera frugiperda* (Sf9) insect cells using the baculovirus expression system (Richardson, 1995; Summers and Smith, 1987). Sf9 cells were grown in suspension in the complete insect cell FMNH media (Grace's insect cell culture media (Gibco BRL, Rockville, MD) + 0.35 g/L NaHCO₃, 3.33 g/L lactalbumin, 3.33 g/L TC-yeastolate) supplemented with 10% fetal bovine serum, at 27°C with constant stirring at 50-60 rpm in the incubator with a gas mixture of 50% O₂/50% air. Cells were seeded at a density 1.25x10⁶ cell/ml and were subcultured every third day. Only cells with at least 95% viability were used.

3. Preparation of Sf9 cell membranes containing recombinant receptors. To produce Sf9 cell membranes containing recombinant receptors Sf9 insect cells were infected at a density of 3.0×10^6 c/ml with a recombinant baculovirus expressing receptors of interest at a 2-fold multiplicity of infection. M1 and M2 muscarinic receptor baculoviruses were the kind gift of Dr. Elliot Ross (University of Texas Southwestern Medical Center, Dallas, Texas). Cells were harvested 48-60 hrs postinfection depending on cell density and viability. Cells were harvested at 4°C by centrifugation at 170xg (IEC Centrifuge) for 7.5 minutes. Then cells were washed three times in ice-cold IPBS (7.3 mM NaH₂PO₄, 60 mM KCl, 47 mM NaCl, 2.0 mM CaCl₂, pH 6.2), resuspended in SGHB1 buffer (10 mM Tris/HCl, 25 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, pH 8.0) with protease inhibitors (2 mg/ml aprotinin, 20 mg/ml benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin and 0.1 mM PMSF) at 0.25 - 0.5 g/ml and snap-frozen in liquid nitrogen. To prepare membranes cell pellets were thawed in 15x their wet weight of ice-cold SGHB1 buffer with protein inhibitors and burst by nitrogen cavitation (600 psi, 20 minutes). Cavitated cells were centrifuged at 4°C for 10 minutes at 500xg to remove the unbroken nuclei and cell debris. The supernatant from the low speed spin was centrifuged at 4°C for 30 min at 28,000xg. The supernatant was discarded and the pellets were resuspended in 35 ml of HE buffer (5 mM Hepes, 1 mM EDTA, pH 7.5) with protein inhibitors at concentrations indicated above for each pellet. The membranes were then washed twice, resuspended in the HE buffer at 1-3 mg protein/ml, snap frozen in liquid nitrogen and stored at -70°C.

4. Urea stripping of Sf9 membranes expressing M1 muscarinic receptors. Sf9 cell membranes expressing M1 receptors require treatment with 6 M urea in order to couple with recombinant Gq. The successful use of this method has been reported before (Hartman and Northup, 1996; Hellmich *et al.*, 1997; Lindorfer *et al.*, 1998). However the role of urea in this

process is not clear. One of the possible mechanisms of urea action is inactivation of endogenous G proteins which interfere with receptor coupling. Another possible mechanism is inactivation of other membrane proteins which regulate G protein-receptor coupling. For urea stripping, membranes were thawed, resuspended at about 0.15 $\mu\text{g}/\mu\text{l}$ in binding buffer (50 mM Tris/HCl, pH 7.5, 5 mM MgCl_2 , 0.5 mM EDTA) with 120 μM OXO-M and 6 μM $\text{GTP}\gamma\text{S}$ and incubated for 30 min at 25°C. Then membranes were centrifuged at 288,000xg for 30 min at 4°C, pellets were resuspended at approximately 0.6 $\mu\text{g}/\mu\text{l}$ in stripping buffer (HE + 6M Urea, added fresh) and extracted on ice for 30 minutes. The extract was centrifuged at 142,000xg for 30 min and 4°C and pellets were washed once in HE buffer. Membranes were resuspended in HE buffer at concentration of about 2 $\mu\text{g}/\mu\text{l}$ and snap frozen in liquid nitrogen. Table I shows the examples of M1 receptor preparations. After urea treatment about 60% of protein was lost and receptor number increased. Urea stripping significantly reduced agonist binding by control membranes (Figure 1). In many cases agonist binding by control membranes was not significantly different from non-specific binding. Urea treatment significantly improved M1-Gq reconstitution and was used routinely for the production of M1 receptors. Importantly, the EC_{50} for Gq interaction with M1 receptor was 66 ± 23.5 nM (see Results Part I), which is comparable with EC_{50} of Gi1-M2 interactions (47 ± 4.3 nM) and experimentally useful.

5. Urea stripping of Sf9 membranes expressing M2 muscarinic receptors. Urea stripping is not required for M2 receptor coupling and it was not used routinely. After urea stripping about 34% of membrane protein was lost and receptor number did not increase (25.1 pmol/mg before urea treatment and 25.7 pmol/mg after urea treatment for one preparation). The K_D for antagonist NMS also did not significantly change after urea stripping (0.78 nM before and 0.56 nM after). The basal binding to control membranes after urea treatment decreased to the

level of nonspecific binding resulting in the bigger affinity shift (described in section 12). Figure 3 shows the effect of urea treatment on agonist OXO-M binding to Sf9 cell membranes expressing M2 muscarinic receptors. Interestingly, our results demonstrate that urea stripping markedly changes the EC_{50} of M2-Gi1 interactions making it harder to saturate the receptor (Figure 3). The EC_{50} for interaction between Gi1 and unstripped M2 receptor is 47 ± 4.3 nM (n=8), while EC_{50} for the urea stripped M2 receptor is 412 ± 5.5 nM (n=2).

Prep Number	Before Urea Stripping		After Urea Stripping		% Protein Lost
	B _{MAX}	K _D	B _{MAX}	K _D	
JS-86	5.8 pmol/mg	0.1 nM	7.8 pmol/mg	0.15 nM	63 %
JS-100	5.3 pmol/mg	0.1 nM	6.1 pmol/mg	0.15 nM	69 %
JS-118	5.3 pmol/mg	0.1 nM	6.4 pmol/mg	0.12 nM	65 %
JS-184	N/D	N/D	6.3 pmol/mg	0.17 nM	60 %
JS-229	N/A	N/A	16.4 pmol/mg	0.2 nM	N/A
JS-298	10.9 pmol/mg	0.2 nM	18 pmol/mg	0.3 nM	59 %
JS-336	10.1 pmol/mg	0.2 nM	23 pmol/mg	0.35 nM	66 %

TABLE I. Urea stripping of M1 Muscarinic Receptors. Sf9 cell membranes expressing M1 muscarinic receptors (before urea stripping) were treated with 6M Urea as described in section 4 (Urea stripping of Sf9 membranes expressing M1 muscarinic receptors) and characterized (after urea stripping). Receptor number (B_{MAX}) and antagonist affinity (K_D) were determined as described in section 10 (Receptor number) using radiolabeled antagonist [³H]-NMS. Protein concentration was determined using BCA protein assay with BSA as a standard. N/A – data not available because receptor preparation and urea stripping were done in one step. N/D – not done.

M1 receptors

FIGURE 1. Effect of urea stripping on agonist binding to M1 receptors. Agonist binding to the Sf9 cell membranes expressing M1 muscarinic receptors (JS-86 before stripping and JS-100 after stripping in Table I) was measured in the presence of 5 nM [^{3} H]-OXO-M. 0.18 pmol of unstripped receptor (expression level 5.8 pmol/mg) and 0.07 pmol of stripped receptor (expression level 6.1 pmol/mg) in the final volume of 150 μ l was used. Data are mean $\pm$ SEM from 3-4 experiments where Gq was present in 150 molar excess over receptors. Agonist binding to control membranes after urea stripping was significantly ($p < 0.01$, t test) lower than agonist binding to unstripped membranes.

FIGURE 2. Effect of urea stripping on agonist binding to M2 receptors. Agonist binding to the Sf9 cell membranes expressing M2 muscarinic receptors was measured in the presence of 4.3 nM [^3H]-OXO-M. 0.27 pmol of unstripped receptor (expression level 25.1 pmol/mg) and 0.25 pmol of stripped receptor (expression level 25.7 pmol/mg) in the final volume of 150 μl was used. Gi1 is present in 70 fold molar excess over receptors. Agonist binding to stripped membranes was significantly ($p < 0.001$, t test) lower than agonist binding to unstripped membranes. Data are mean $\pm$ SD from one experiment performed in triplicates.

FIGURE 3. Effect of urea stripping on M2-Gi1 reconstitution. Membranes expressing M2 receptors were reconstituted with increasing amounts of Gi1 heterotrimers and high affinity agonist binding was determined as described in section 11 (G α saturation experiments). EC₅₀ for M2-Gi1 interactions was determined from the data fit to a single-site interaction model between receptor and G protein. Data points represent the mean $\pm$ SD of triplicate determinations from a representative experiment. The concentrations of [³H]-OXO-M were 4.5-6 nM in all experiments. **A. Unstripped M2 receptors.** Apparent affinity between unstripped M2 receptor and Gi1 is 47.0 $\pm$ 4.3 nM (mean $\pm$ SEM, n=8). The final concentration of unstripped receptor in representative experiment shown here was 0.82 nM in a final volume of 150 μ l. The highest concentration of G proteins represents a 407 fold molar excess over unstripped receptors. **B. Stripped M2 receptors.** Apparent affinity between stripped M2 receptor and Gi1 is 412 $\pm$ 5 nM (mean $\pm$ SEM, n=2). The final concentration of stripped receptor was 2 nM in a final volume of 150 μ l. The highest concentration of G proteins represents a 390 fold molar excess over stripped receptors.

A Unstripped M2 receptors

B Stripped M2 receptors

6. Expression and purification of G protein α and $\beta\gamma$ subunits. G protein $G_{i1}\alpha$ and $G\beta_1\gamma_2$ subunits were expressed in Sf9 insect cells and purified using a Waters advanced purification system as described before (Graber *et al.*, 1992a; Graber *et al.*, 1992b). The major difference in $G_{i1}\alpha$ purification from Gq purification described below is that $G_{i1}\alpha$ was purified from the supernatant obtained after the first high-speed centrifugation and Gq was purified as a detergent extracted $Gq\alpha\beta_1\gamma_{2HIS}$ heterotrimer.

To produce Gq, Sf9 cells were infected with $Gq\alpha$, $G\beta_1$ and $G\gamma_{2HIS}$ baculoviruses at a multiplicity of infection 1:1:1, as described by Kozasa and Gilman (Kozasa and Gilman, 1995). Cells were harvested about 72 hrs postinfection using the same procedure as described for receptor harvest except 10 μ M GDP was added to the final resuspension buffer. To prepare Gq, cell pellets were thawed in 15x their wet weight of ice-cold homogenization buffer (50 mM Tris/HCl, pH 8.0, 10 μ M GDP, 2 mg/ml aprotinin, 20 mg/ml benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin and 0.1 mM PMSF), burst by nitrogen cavitation (600 psi, 20 minutes) and centrifuged at 100,000xg for 60 min and 4°C. The pellets were extracted for 1 hr in extract buffer (50 mM Tris/HCl, pH 8.0, 10 μ M GDP, 2 mg/ml aprotinin, 20 mg/ml benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin, 0.1 mM PMSF, 5mM BME, 1% CHAPS) and centrifuged at 100,000xg for 45 min and 4°C. Gq was purified from the detergent extract using a Waters Advanced Purification System with all steps done at 4°C. Purification traces are shown in Figure 4. The supernatant was filtered and loaded onto a 30 ml AP-2 DEAE anion exchange column. DEAE buffers contained 50 mM Tris, pH 8.0, 0.7% CHAPS, 5 mM BME and 10 μ M GDP. Proteins were eluted with 4 column volumes of a linear gradient from 0-400 mM NaCl (Figure 4A). Fractions containing G proteins (as indicated by anti $Gq\alpha$ immunoblotting in pilot experiments, fractions 2-17 in Figure 4A) were pooled and mixed with 2 ml of Ni-NTA

Superflow resin (4 ml of 50% slurry). The resin was incubated for at least 1hr and loaded into a 15 ml column. The resin was washed with buffer A (20 mM Hepes, pH 8.0, 5 mM Imidazole, 1 mM MgCl_2 , 100 mM NaCl, 5mM BME, 10 μM GDP, 0.7% CHAPS), then buffer B (the same as buffer A but with 300 mM NaCl), then buffer A. Each step continued until the baseline was flat for at least 10 ml. Bound proteins were eluted with buffer C (20 mM Hepes, pH 8.0, 150 mM Imidazole, 1mM MgCl_2 , 25 mM NaCl, 5 mM BME, 0.7 % CHAPS) (Figure 4B). Eluate was diluted 3 times with Q buffer A (20 mM Hepes, pH 8.0, 2 mM MgCl_2 , 1mM DTT, 0.7% CHAPS) and loaded onto a 8 ml AP-1 QHR15 anion exchange column. Fractions were eluted with 4 volumes of linear NaCl gradient from 0-400 mM. Free $\beta_1\gamma_{2\text{HIS}}$ subunits were eluted at 100-125 mM NaCl and $\text{G}\alpha\text{q}\beta_1\gamma_{2\text{HIS}}$ heterotrimers were eluted at 200-220 mM NaCl (Figure 4C). Fractions were concentrated using Amicon-30 concentrators, snap-frozen in liquid nitrogen and stored at -70°C . Fractions were resolved by SDS-PAGE and immunoblotted using anti- αq and anti- β_1 antibodies. Protein concentration was determined using the BCA method with BSA as a standard. Both $\text{G}\alpha\text{q}\beta_1\gamma_{2\text{HIS}}$ heterotrimers and $\beta_1\gamma_{2\text{HIS}}$ subunits were used in the reconstitution studies. Figure 5 shows Coomassie Blue stained $\text{G}\alpha\text{q}\beta_1\gamma_{2\text{HIS}}$ heterotrimers (lane 9) and $\beta_1\gamma_{2\text{HIS}}$ subunits (lane 8) resolved on a 12% SDS-polyacrylamide gel. $\text{G}\alpha$ was purified from Gq heterotrimers by elution from Ni-NTA Superflow resin with 10 mM NaF/50 μM AlCl_3 (20 mM Hepes, pH 8.0, 10 mM MgCl_2 , 100 mM NaCl, 5 mM BME, 0.7 % CHAPS, 10 μM GDP, 10 mM NaF/50 mM AlCl_3) at room temperature. In addition, before 10 mM NaF/50 μM AlCl_3 elution Ni-NTA resin was washed with 10 μM $\text{GTP}\gamma\text{S}$ to elute endogenous $\text{G}\alpha$ proteins. Ni-NTA eluate was diluted 3 times with Q buffer A (20 mM Hepes, pH 8.0, 2 mM MgCl_2 , 1mM DTT, 0.7% CHAPS) and loaded onto a 8 ml AP-1 QHR15 anion exchange column. Fractions were eluted

with 4 volumes of linear NaCl gradient from 0-400 mM. Gq α subunits were eluted at 80-100 mM NaCl. Coomassie Blue stained Gq α is shown in Results section Part I Figure 1C.

A

B

C

FIGURE 4. $Gq\alpha\beta_1\gamma_{2HIS}$ column purification traces. $Gq\alpha\beta_1\gamma_{2HIS}$ and $G\beta_1\gamma_{2HIS}$ were purified as described in Section 6. **A.** DEAE AP-2 column (30 ml), **B.** Ni-NTA column (2 ml), **C.** QHR15 AP-1 column (8 ml).

FIGURE 5. Gq $\alpha\beta_1\gamma_{2HIS}$ purification. Protein samples (10 μ g for lanes 1-7) from different stages of Gq purification were separated on 12% SDS-polyacrylamide gel and visualized with Coomassie Blue stain. Lane 1 – homogenate, lane 2 – supernatant from the first 100,000xg centrifugation, lane 3 – pellet from the first 100,000xg centrifugation in 1% CHAPS, lane 4 – pellet from the second 100,000xg centrifugation, lane 5 – supernatant from the second 100,000xg centrifugation (1% CHAPS extract), lane 6 – DEAE column pool, lane 7 – Ni-NTA column pool, lane 8 – QHR15 column $\beta_1\gamma_{2HIS}$ (5 μ g), lane 9 – QHR15 column Gq $\alpha\beta_1\gamma_{2HIS}$ (10 μ g).

The chimeric Gt α /Gi1 α and C terminal Gi1 α /Gq α subunits were constructed, expressed in *E.coli* and purified in the laboratory of Dr. Heidi Hamm at Northwestern University (currently at Vanderbilt University Medical Center, Nashville, TN) (Bae *et al.*, 1997; Bae *et al.*, 1999; Skiba *et al.*, 1996). To construct Gt α /Gi1 α and Gi1 α /Gq α chimeric genes, *E.coli* expression vectors pHis₆Gi1 α , pHis₆Gt α or pHis₆Gq α (contain Gi1 α , Gt α or Gq α cDNA respectively, preceded by a nucleotide sequence encoding a hexahistidine tag under the control of a T7 promoter) were used. Chimeric genes were constructed by insertion of unique restriction enzyme sites into Gi1 α or Gt α cDNA using PCR amplification with corresponding oligonucleotide primers followed by replacement of Gi1 α cDNA fragments with corresponding Gt α cDNA or Gq α cDNA fragments or vice versa. Insertion of new restriction sites has not changed protein sequences. The hexahistidine tag was used to facilitate purification. Chimeric α subunits were expressed in *E.coli* BL21(DE) cells and purified from cell lysates by sequential chromatography on Ni-NTA resin (His-bond, Novagen) followed by AP-1 QHR15 anion exchange column (Waters Chromatography). Protein purity was verified by SDS-PAGE and protein concentrations were determined using the Coomassie Blue method. All the chimeras were active as indicated by AlF⁴⁻-dependent conformational change of α subunits in a Fluorescence Assay and GTP γ S binding (Bae *et al.*, 1997; Bae *et al.*, 1999; Skiba *et al.*, 1996).

The Gqi5C recombinant baculovirus was constructed using a plasmid kindly provided by Dr. Bruce Conklin (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco). Gqi5C (~1kb) was cut out of pcDNA1 vector using Bam HI and Nsi I and subcloned into Bam HI/Pst I sites of pVL1393 baculovirus transfer vector (~9.6 kb). This was possible because Nsi I and Pst I have compatible cohesive ends. After ligation Bam HI site was preserved and Pst I site was converted to Bgl II site (unique in pVL1393). The pVL1393qi5C construct was

verified by sequencing. The Gqi5C construct could be cut out of pVL1393 using Bam HI and Bgl II. However because Gqi5C has an internal Bgl II site, pVL1393qi5C (~10.6 kb) digestion with Bam HI and Bgl II resulted in production of 3 fragments (~9.6 kb, 834 kb and 258 kb) as shown in Figure 6A. For production of recombinant virus by homologous recombination pVL1393Gqi5C was co-transfected with Bac-N-Blue^{TM} DNA (Invitrogen) into Sf9 insect cells. Recombinant qi5C baculovirus was plaque purified and analyzed by PCR to verify the presence of insert and to confirm the isolation of pure recombinant plaque. PCR of qi5C viral DNA is shown in Figure 6B. As expected, PCR of both pVL1393qi5C (used as a positive control) and qi5C viral DNA resulted in the ~1378 bp product. Restriction of the PCR products from both pVL1393qi5C and qi5C viral DNA with Bam HI, Bgl II and Afl II (unique to qi5C) resulted in the production of the same size fragments, further confirming the production of qi5C baculovirus (Figure 6B). Gqi5 was expressed together with $\beta 1\gamma 2_{\text{HIS}}$ in SF9 cell membranes and purified by the same protocol as Gq. The construction and expression of the q6N and q6N35C chimeric G α subunits is described in the Chapter III Part I.

FIGURE 6. Gqi5C construct visualized on 1% TAE agarose gel stained with ethidium bromide. A. pVL1393qi5C. Lane 1 – 1kb ladder, lane 2 – uncut pVL1393qi5C, lane 3 – pVL1393qi5C cut with Bam HI and Bgl II, lane 3 – 100 bp ladder. **B. PCR analysis of recombinant qi5C baculovirus.** 0.5 μ l from PCR reactions of pVL1393qi5C (lanes 2-5) or qi5C baculoviral DNA (lanes 8-10 and 12) were loaded on the gel. PVLF and PVLR primers (Invitrogen) were used. Lane 1 – 1kb ladder, lane 2 - uncut pVL1393qi5C, lane 3 - pVL1393qi5C cut with Bam HI, lane 4 - pVL1393qi5C cut with Bgl II, lane 5 - pVL1393qi5C cut with Afl II, lanes 6, 7 – empty, lane 8 – qi5C baculovirus, lane 9 - qi5C baculovirus cut with Bam HI, lane 10 - qi5C baculovirus cut with Bgl II, lane 11 – empty, lane 12 - qi5C baculovirus cut with Afl II, lane 13 – 100 bp ladder.

7. Fluorescence assay. To measure the folded state of chimeric G α and to test the ability of G α to undergo conformational change indicative of activity, intrinsic fluorescence was measured with a QM-2000-4 spectrofluorimeter (PTI fluorescence system hardware and Felix software) at room temperature in a buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM MgCl₂ and 1 μ M GDP. The AlF⁴⁻-dependent conformational changes of activated G α subunits were monitored by intrinsic tryptophan fluorescence changes with excitation at 280 nm and emission at 340 nm. The relative increase in fluorescence of 400 nM G α subunits was determined from absorbance readings before and after addition of 10 mM NaF and 20 μ M AlCl₃ in a total volume of 100 μ l. The increase in fluorescence was calculated as $\Delta F\% = (F - F_0)/F_0 \times 100\%$. Gi1 α contains tryptophans at the positions W131, W211, W258; Gt α at the positions W127, W207; Gq α at the positions W136, W216, W263. The relative change in intrinsic fluorescence for individual G α subunits is consistent with the literature (Skiba *et al.*, 1996) and as follows: Gi1 48.3 $\pm$ 3.2 (n=11), q6N 45 $\pm$ 12.2 (n=8), q35C 56.7 $\pm$ 5.2 (n=3), q6N35C 54.5 $\pm$ 15.6 (n=4), qi5C 47.7 $\pm$ 8.4 (n=3), Gq 50 $\pm$ 22.9 (n=4). Figure 7 shows an example of the fluorescence spectra of Gi1 α .

FIGURE 7. Intrinsic tryptophan fluorescence of Gi1 α . The relative increase in fluorescence of 400 nM Gi1 α was measured before and after the addition of 10 mM NaF and 20 μ M AlCl₃ in a total volume of 100 μ l. Excitation wavelength was 288 nm, emission wavelength was 341 nm. Fo = 711.6 counts/sec, F = 913 counts/sec, Δ F% = 28%.

8. Reconstitution of receptors with G proteins. Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge at 12,000 rpm for 10 minutes and resuspended at 5-10 mg/ml in the reconstitution buffer (5 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.08% CHAPS). To produce G protein heterotrimers α and $\beta\gamma$ subunits were mixed at the ratio 1 α to 1.3 $\beta\gamma$ and diluted in the reconstitution buffer in order to keep reconstitution volume the same for each sample. G protein heterotrimers were added to the membranes at the 60–200 fold molar excess over the recombinant receptors as indicated by saturation experiments. The mixture was incubated at 25°C for 20 minutes and held on ice until the start of the binding assay.

9. Radioligand binding assay. Just prior to the start of the binding assay the reconstitution mixture was diluted 10-12 fold with binding assay buffer appropriate to the receptor of interest such that the desired amount of membranes (5-25 μ g/assay tube) were contained in 10-50 μ l. Binding buffer was 50 mM Tris, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.5. Agonist binding was determined in the presence of the 5 nM [³H]-OXO-M. Antagonist binding was determined in the presence of [³H]-NMS. Non-specific binding was determined by addition of 1000-fold excess of unlabeled ligand atropine sulfate. Incubations were for times sufficient to achieve equilibrium in a temperature controlled shaker (1 hr) and were terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed thrice with 4 ml ice cold 50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% NaAzide, pH 7.5 at 4 °C, placed in 4.5 ml CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter.

10. Receptor number. Muscarinic receptor number was determined in the presence of antagonist [³H]-NMS at concentrations 0.02-8 nM of free [³H]-NMS in a total volume of 500 μ l. Because antagonists do not distinguish between high and low receptor affinity states,

reconstitution of receptors with G proteins was not required to determine receptor number. Receptor number (B_{MAX}) and antagonist affinity constant (K_D) were derived from the data fit to the one-site interaction model between receptor and ligand as shown in Figure 8.

FIGURE 8. Antagonist saturation binding isotherm for M1 and M2 muscarinic receptors.

Data points represent the mean $\pm$ SD of triplicate determinations from a representative experiment.

Data fit to a single-site interaction model between receptor and ligand. Receptor number and K_D

for [^{3} H]-NMS were determined from nonlinear regression analysis. **A. M1 receptors.** M1

receptor number was determined in the presence of [^{3} H]-NMS at concentrations 0.02-7.3 nM of

free [^{3} H]-NMS in a total volume 500 μ l. 11.3 μ g of membrane protein/tube was used. **B. M2**

receptors. M2 receptor number was determined in the presence of [^{3} H]-NMS at concentrations

0.02-7.8 nM of free [^{3} H]-NMS in a total volume 500 μ l. 11 μ g of membrane protein/tube was

used.

A**M1 receptors** **B****M2 receptors**

11. $G\alpha$ saturation binding experiments. To determine the concentration of $G\alpha$ needed to saturate the affinity shift, increasing amounts of G protein heterotrimers were added to the receptors in the reconstitution and the agonist binding was detected in the binding assay. Typically, 7 different G protein concentrations in triplicates in the range 10-500 nM were used. EC_{50} were generated from nonlinear regression analysis from the data fit to a one site interaction model between receptor and G protein. An example of $G\alpha$ saturation binding experiment is shown in Figure 3.

12. Affinity shift activity assay. M1 and M2 muscarinic receptors exhibit two affinity states for the agonist Oxotremorine-M with the majority of receptors in the low affinity state. For affinity shift assay the Sf9 cell membranes expressing individual receptors were reconstituted with saturating amounts of G protein heterotrimers (60-200 fold molar excess over receptors) as determined by $G\alpha$ saturation binding experiments to achieve the maximal specific binding during the binding assays. For reconstitution of high affinity agonist binding, a single concentration (~5 nM) of [^{3} H]-OXO-M near the high affinity K_D of the receptor was used in a final volume of 150 μ l. Addition of complete heterotrimers (Gq for M1 receptors and Gi1 for M2 receptors) converted the low affinity agonist state to the high affinity state, while the addition of 50 μ M GTP γ S (functional analog of GTP) disrupted G protein-receptor coupling as shown in Figure 9, which is in agreement with the ternary complex model. In order to compare the functional interactions of different G proteins with receptors we express the data as affinity shift activity. We define affinity shift as the fold-enhancement above buffer controls of high affinity agonist binding in membranes expressing recombinant receptors reconstituted with G protein heterotrimers. Therefore subunits that can't stabilize the high affinity state of receptors for agonist (as well as buffer control) will have an affinity shift activity of 1 and the active subunits will have an affinity shift activity

significantly greater than 1. The affinity shift activity depends on the variety of factors including the magnitude of the affinity shift, the actual concentration of agonist used in the assay, the number of expressed receptors coupled with endogenous G proteins and the number of expressed receptors coupled by the addition of exogenous G proteins.

FIGURE 9. Affinity shift activities of M1 and M2 muscarinic receptors. Affinity shift activity represents the –fold enhancement above buffer controls of high affinity [³H]-OXO-M binding in membranes expressing M1 (**A**) or M2 (**B**) muscarinic receptors reconstituted with G protein heterotrimers containing the indicated Gα subunit. The concentrations of [³H]-OXO-M were 4.5-6 nM in all experiments. 50 μM GTPγS was used to disrupt the formation of the ternary complex. Assay volume was 150 μl. **A. M1 receptors.** Data represent the mean±SEM from 2-3 experiments performed in triplicates. Gi1±GTPγS was done once (in triplicates). **B. M2 receptors.** Data represent the mean±SEM from 2-16 experiments performed in triplicates.

A**M1 receptors** **B****M2 receptors**

13. Agonist competition binding experiments. Competition binding experiments with a single concentration of radiolabeled antagonist and the increasing concentrations of non-labeled agonist were performed to determine the extent of receptor coupling with individual G proteins and to find the K_D for the high and low affinity states for agonist. Receptors were reconstituted with G proteins at saturating concentrations. Binding assay was performed in the presence of the agonist OXO-M as a competitor. The 22 different concentrations (in duplicates) of OXO-M were equally spaced on the log scale in the concentration range 10^{-12} - 3×10^{-2} M. Data were analyzed by nonlinear regression. Data reported are the result of the best fit (one-site vs. two-sites inhibition fit) as determined by the F test. The Cheng-Prusoff correction ($K_i = IC_{50}/(1 + [ligand]/K_D)$) was used to calculate K_D values. Figure 10 shows the results of competition binding experiments for M1 receptor control membranes and M1 receptors reconstituted with Gq, and M2 receptors control membranes and M2 receptors reconstituted with Gi1. Agonist competition experiments with control membranes were best fit to a one-site interaction model (low affinity sites), while agonist competition experiments with membranes reconstituted with G proteins were best fit to a two-site interaction model (high and low affinity sites). Table II shows the coupling parameters derived from the agonist competition experiments of M1 and M2 Muscarinic receptors reconstituted with different G proteins described in the latter Chapter III.

FIGURE 10. Agonist competition binding against a fixed concentration of radiolabeled antagonist for M1 (A) and M2 (B) muscarinic receptors. [^3H]-NMS binding to control membranes (no exogenous G proteins) or to membranes reconstituted with 200 fold excess of G proteins over receptors was measured in a final volume of 500 μl . Concentrations of [^3H]-NMS (around 0.8 nM for M1 receptor and 2 nM for M2 receptor) were 4 times the K_D of [^3H]-NMS for a particular receptor preparation. Increasing concentrations of OXO-M (in duplicates) in the range 10^{-12} - 3×10^{-12} M were used. Data were analyzed by nonlinear regression and an F-test was used to compare one-site vs. two-site fits. The Cheng-Prusoff correction was used to calculate K_D values. Agonist competition experiments with control membranes were best fit to a one-site interaction model, while agonist competition experiments with membranes reconstituted with G proteins were best fit to a two-site interaction model. Data points shown here are the results of representative experiments. **A. M1 receptors.** The K_D values in control membranes were $19.1 \pm 2.7 \mu\text{M}$ ($n=2$) which was not different from the low affinity K_D obtained from the 2-site fits with membranes reconstituted with Gq. Gq coupled $18 \pm 2.3\%$ ($n=2$) of M1 receptors with high affinity K_D of $14.1 \pm 12.3 \text{ nM}$ ($n=2$) and the low affinity K_D of $14.3 \pm 2.9 \mu\text{M}$ ($n=2$). **B. M2 receptors.** The K_D values in control membranes were $8.6 \pm 2.2 \mu\text{M}$ ($n=2$) which was not different from the low affinity K_D obtained from the 2-site fits with membranes reconstituted with Gi1. Gi1 coupled $38.5 \pm 5.6\%$ ($n=3$) of M2 receptors with high affinity K_D of $2.6 \pm 2.2 \text{ nM}$ ($n=3$) and the low affinity K_D of $3.3 \pm 0.9 \mu\text{M}$ ($n=2$).

A M1 receptors

B M2 receptors

TABLE II. Coupling parameters of M1 and M2 Muscarinic Receptors with G α Subunits

<i>Gα</i>	<i>M1 Receptors</i>			<i>M2 Receptors</i>		
	% R High	K _D High	K _D Low	% R High	K _D High	K _D Low
Gi1	12.9 $\pm$ 3.9 % (2)	8.9 $\pm$ 8.9 nM (2)	6 $\pm$ 2.1 μ M [*] (2)	38.5 $\pm$ 5.6 % (3)	2.6 $\pm$ 2.2 nM (3)	3.3 $\pm$ 0.9 μ M (3)
q6N	26.6 $\pm$ 1.2 % (2)	30.2 $\pm$ 11.3 nM (2)	4.3 $\pm$ 0.9 μ M [*] (2)	38.3 $\pm$ 4.5 % (2)	1.4 $\pm$ 0.5 nM (2)	2.3 $\pm$ 0.1 μ M (2)
q6N35C	24 $\pm$ 6.3 % (3)	6.5 $\pm$ 6.2 nM (3)	2.6 $\pm$ 1.7 μ M [*] (3)	27.3 $\pm$ 6.8 (5)	240 $\pm$ 128 nM (5)	10.1 $\pm$ 3.1 μ M (5)
Gq	18 $\pm$ 2.3 % (2)	14.1 $\pm$ 12.3 nM (2)	14.3 $\pm$ 2.9 μ M (2)	7.4 $\pm$ 0.2 % (2)	0.04 $\pm$ 0.01 nM (2)	9.6 $\pm$ 1.9 μ M (2)
Control	—	—	19.1 $\pm$ 2.7 μ M (2)	—	—	8.6 $\pm$ 2.2 μ M (2)

Data derived from the competition binding experiments between a single concentration of radiolabeled antagonist [³H]-Scopolamine methyl chloride and increasing concentrations of non-labeled agonist Oxotremorine M. Receptors were reconstituted with saturating amounts of G proteins as indicated by G α Saturation experiments. Agonist concentrations (22 data points in duplicates) were equally spaced on the log scale in the range of 10⁻¹²-3X10⁻² M. Data were analyzed by nonlinear regression and an F test was used to compare one-site vs. two site fits. The Cheng-Prusoff correction was used to calculate K_D values. Agonist competition experiments with control membranes (no exogenous G proteins) were best fit to a one-site interaction model. The low affinity K_D obtained from the 2-site fits with the reconstituted membranes did not differ significantly from the K_D values in control membranes (Tukey's Multiple Comparison Test) with the exception of Gi1, q6N q6N35C and with M1 membranes (* - p<0.05). Data shown are the mean $\pm$ SEM from 2-5 independent experiments.

14. Receptor stimulated [^{35} S]-GTP γ S binding assay. A GTP γ S binding assay was used to analyze receptor-catalyzed GDP/GTP exchange on G protein α subunits. Typically 1.3 pmol of receptors was reconstituted with 80 pmol of G protein heterotrimers (1.3 fold excess of α over $\beta\gamma$) for 15 min at 30°C in reconstitution buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 200 nM GDP, 0.08% CHAPS). After reconstitution the mixture was diluted 10 fold with GTP γ S Binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 25 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA). Reactions were initiated by the addition of 25 μ l of membrane mixture to 25 μ l of GTP γ S with or without agonist. Final assay conditions were 50 nM G α , 0.8 nM receptor, 2 μ M GDP, 100 nM GTP γ S as a mixture of 1nM [^{35} S]-GTP γ S (~125,000 cpm/well) and cold GTP γ S, with or without agonist in a final volume of 50 μ l. In experiments with single agonist concentration 2 μ M Oxotremorine-M was used. Use of MultiScreen 96 well plates (Millipore) allowed collection of samples at 30 second intervals for 4-6 minutes for M2 receptors or 3 minute intervals for 18 minutes for M1 receptors. Filters were rinsed three times with 100 μ l of ice-cold wash buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM MgCl₂). Radioactivity retained on the filters was counted to constant error in a liquid scintillation counter. Greater than 1500 cpm were observed at the shortest time points and ligand depletion was less than 15% at the longest time-points. Data were analyzed by linear regression and agonist driven GTP γ S binding was reported as the ratio of the slopes in the presence and absence of agonist provided the slopes were significantly different ($p < 0.05$). If the slopes did not differ significantly a value of 1.0 was used for the slope ratio. GTP γ S binding in the absence of agonist represented as moles of GTP γ S per mole of receptor.

15. ADP-Ribosylation of $G\alpha$ by pertussis toxin. Pertussis Toxin (1.1 $\mu\text{g}/\mu\text{l}$) was activated by the addition of an equal volume of activation buffer (5 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl_2 , 1 mM EDTA, 0.08% CHAPS, 40 mM DTT, 2 μM GDP) and incubation for 40 min at 32°C. G protein heterotrimers (3 μg α plus 3.6 μg $\beta\gamma$) were ribosylated for 1 hr at 32°C in a total volume of 20 μl containing 10 μl of activated PTX and 400 μM NAD^+ . Ribosylation reactions were reconstituted with membranes under the same conditions described above. At the end of incubation the mixture was diluted 9 fold with binding assay buffer and used in the affinity shift assay.

16. Analysis of the data. The data were analyzed using GraphPad Prism software package (GraphPad Software, San Diego, CA). Data were represented as the mean $\pm$ SEM from multiple experiments unless indicated otherwise. One-way ANOVA was used to analyze the differences in activity between G proteins. Curve fitting was done using nonlinear regression analysis.

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CHAPTER III. RESULTS

PART I

Reconstitution Reveals Additional Roles for N- and C- Terminal Domains of G α in Muscarinic Receptor Coupling[†]

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Abbreviations and Textual Footnotes—¹The abbreviations used are: PLC β , phospholipase C β ; PTX, pertussis toxin; GTP γ S, guanosine 5'-3-*O*-(thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; OXO-M, oxotremorine-M; NMS, N-methyl scopolamine.

ABSTRACT

The molecular basis of selectivity of M1 and M2 muscarinic receptor coupling to heterotrimeric G proteins has been studied using receptors expressed in Sf9 cell membranes and reconstituted with purified chimeric G α subunits containing different regions of Gi1 α and Gq α . The abilities of G protein heterotrimers containing chimeric α subunits to stabilize the high affinity state of the receptors for agonist and to undergo receptor stimulated guanine nucleotide exchange was compared with G protein heterotrimers containing either native Gi1 α or Gq α . The data confirm the importance of the proper context of the C- terminus of G α by demonstrating that the C-terminus of Gi1 α , when placed in the context of Gq α , prevents coupling to muscarinic M1 receptors, while the C- terminus of Gq α , when placed in the context of Gi1 α , prevents coupling to muscarinic M2 receptors. However, C- terminal amino acids of Gq α placed in the context of Gi1 α were not sufficient to allow M1 receptor coupling nor were C-terminal amino acids of Gi1 α placed in the context of Gq α sufficient for M2 receptor coupling. The unique six amino acid N- terminal extension of Gq α when added to the N- terminus of Gi1 α neither prevented M2 receptor coupling nor permitted M1 receptor coupling. A Gi1 α based chimera containing both N- and C- terminal regions of Gq α gained the ability to productively couple M1 receptors suggesting that the proper context of both N- and C- termini is required for muscarinic receptor coupling.

INTRODUCTION

Heptahelical receptors transfer a variety of extracellular signals to intracellular effectors by coupling to heterotrimeric guanine nucleotide-binding proteins (G proteins) (Gilman, 1987). The mechanisms responsible for selectivity in G protein mediated signaling pathways remain poorly understood (Wess, 1998). Direct contacts with receptors have been demonstrated for both α and $\beta\gamma$ subunits (Phillips and Cerione, 1992; Heithier *et al.*, 1992; Conklin and Bourne, 1993; Kisselev *et al.*, 1994; Taylor *et al.*, 1996; Azpiazu *et al.*, 1999; Ernst *et al.*, 2000). Although recent evidence suggests both β and γ interact selectively with receptors (Kisselev *et al.*, 1995; Yasuda *et al.*, 1996; Richardson and Robishaw, 1999; Hou *et al.*, 2000), the α subunits appear to play a more prominent role in establishing the selectivity of G protein-receptor coupling. Using different receptor systems and various methodologies the C- terminus and $\alpha 5$ helix (Hamm *et al.*, 1988; Conklin *et al.*, 1993; Gilchrist *et al.*, 1998; Natochin *et al.*, 2000), N- terminus and αN helix (Kostenis *et al.*, 1997a; Swift *et al.*, 2000), $\alpha 4$ helix and $\alpha 4/\beta 6$ loop (Bae *et al.*, 1997), $\alpha 2$ helix and $\alpha 2/\beta 4$ loop (Lee *et al.*, 1995) and $\alpha 3/\beta 5$ loop (Grishina and Berlot, 2000) domains of G α subunits have been shown to be involved in receptor coupling.

Muscarinic receptors are typical Class I heptahelical receptors. When expressed in Sf9 cells they demonstrate structural integrity and display the expected ligand binding properties (Dong *et al.*, 1995; Weill *et al.*, 1997). The family of human muscarinic receptors includes five molecularly distinct members. M1, M3, and M5 are Gq/11 coupled receptors that stimulate phospholipid breakdown through activation of phospholipase C β (PLC β)¹, while M2 and M4 are Gi/o coupled receptors that mediate the inhibition of adenylyl cyclase and activation of K⁺ channels (Wess, 1996; Hosey *et al.*, 1999). Muscarinic receptors also regulate additional

signaling processes, including stimulation of MAP kinase pathways and regulation of other ion channels (Gutkind, 1998; Hosey *et al.*, 1999).

The C- terminus of G α subunits is the most studied determinant of receptor coupling selectivity to date. Numerous studies including transfection experiments (Conklin *et al.*, 1993), synthetic peptide competition experiments (Hamm *et al.*, 1988), ADP-ribosylation of G α by Pertussis toxin (PTX) (West, Jr. *et al.*, 1985) and direct reconstitution studies (Natochin *et al.*, 2000) indicate that the extreme C- terminus of G α interacts with receptors. With respect to selectivity, Conklin, Bourne and Wess demonstrated that substitution of the last 3-5 C- terminal residues of Gq α with the corresponding sequences of Gi α , Go α or Gz α allowed PLC β stimulation by A1 adenosine, D2 dopamine, α 2 adrenergic and M2 muscarinic receptors, which are normally coupled to Gi/o family members (Conklin *et al.*, 1993; Liu *et al.*, 1995). Furthermore, site-directed mutagenesis revealed receptor contact sites for the C- terminus of Gi α and Gq α on M2 and M3 muscarinic receptors respectively (Liu *et al.*, 1995; Kostenis *et al.*, 1997b; Kostenis *et al.*, 1997c). The N- terminus of G α has also been shown to be a determinant of G protein-receptor coupling selectivity (Hepler *et al.*, 1996; Kostenis *et al.*, 1997a; Swift *et al.*, 2000). Importantly, Kostenis *et al.* suggested a role for the unique N- terminal extension of Gq α in constraining receptor coupling selectivity by showing that deletion of the six N- terminal amino acids of Gq α (MTLESI) led to promiscuous coupling of this Gq mutant with several Gi/o and Gs coupled receptors (Kostenis *et al.*, 1997a).

Our study directly examines the interactions of M1 and M2 Muscarinic receptors with Gq and Gi1 proteins in an Sf9 cell membrane based reconstitution system in which the coupling behavior of receptors of interest can be studied by reconstitution with exogenously supplied, purified G protein subunits (Clawges *et al.*, 1997). This system allows the analysis of both the

ability of G proteins to stabilize the high affinity state of receptor for agonist and the ability of the receptors to catalyze guanine nucleotide exchange on $G\alpha$. Our results show that the C-terminus of $G\alpha$ although critical, is not sufficient for M1 or M2 receptor coupling. Furthermore, the six amino acid N- terminal extension of $Gq\alpha$ neither allow M1 receptor coupling nor constrain M2 receptor coupling. In summary, the data suggest that the appropriate context of both N- and C- termini is required for selective receptor coupling.

MATERIALS AND METHODS

Materials. [^3H]-Oxotremorine-M (85.8 Ci/mmol) and [^{35}S]-GTP γ S (1250 Ci/mmol) were from New England Nuclear Life Science Products, Inc. (Boston, MA). Molecular biology enzymes were from New England Biolabs (Beverly, MA). Wild type baculovirus DNA and lipofectin were from Invitrogen (Carlsbad, CA). Anti-G $\alpha_{i1/2}$ C- terminal antibodies were from Calbiochem-Novabiochem Corporation (San Diego, CA). Anti-G α_{i1} internal antibodies and anti-G $\alpha_q/11$ C- terminal antibodies were from Santa Cruz Biotechnology. Pertussis Toxin was from Calbiochem-Novabiochem Corporation (San Diego, CA). The ProtoBlot Western Blot reagents were from Promega (Madison, WI). The BCA Protein Assay reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO) or Calbiochem-Novabiochem Corporation (San Diego, CA).

Construction of G α chimeric Genes. H $_6$ pQE-60-Gi1Q3C and H $_6$ pQE-60-Gi1Q35C plasmids were the kind gift of Dr. Heidi Hamm (Vanderbilt University, Nashville, TN) and are based on H $_6$ pQE-60-Gi1 originally described by Lee *et al* (Lee *et al.*, 1994). The pcDNA1-Gqi5C plasmid was the kind gift of Dr. Bruce Conklin (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco). To construct pVLSG-Gi1Q6N the nucleotides coding for the six N- terminal amino acids of mouse G α_q (MTLESI) were inserted into the BamH1/Nco1 sites of pVLSG-Gi1 (Graber *et al.*, 1992) using synthetic oligonucleotides to create a duplex linker. H $_6$ pQE-60-Gi1Q3C was used to construct both Gi1Q6N3C and Gi1Q6N35C. H $_6$ pQE-60-Gi1Q3C was digested with EcoR1 and Nco1 and recircularized using synthetic oligonucleotides to create a duplex linker encoding the six N- terminal amino acids of mouse G α_q (MTLESI) resulting in the removal of the His $_6$ -tag and creation of the intermediate pQE-60-Gi1Q6N3C. A duplex linker was used to subclone the NcoI/HindIII fragment of pQE-

60-Gi1Q6N3C into NcoI/EcoRI sites of pVLKD. The pVLKD vector was constructed from the commercially available baculovirus expression vector pVL1393 by removing the BamHI site and adding an NcoI site with a duplex linker between the BamHI and XbaI sites of the pVL1393 poly-linker. The BamHI/HindIII fragment of H₆pQE-60-Gi1Q35C was subcloned into the BamHI/EcoRI sites of pVLKD-Gi1Q6N3C using a duplex linker. This resulted in the replacement of the BamHI/HindIII fragment of pVLKD-Gi1Q6N3C with the corresponding fragment from H₆pQE-60-Gi1Q35C creating pVLKD-Gi1Q6N35C. To create pVL1393-Gqi5C, the BamHI/NsiI fragment coding for Gqi5C was cut from pcDNA1 and subcloned into the BamHI/PstI sites of pVL1393. All constructs were verified by sequencing and restriction analysis. PVLSG-Gi1Q6N, pVLKD-Gi1Q6N35C and pVL1393-Gqi5C transfer vectors were used to create recombinant baculoviruses as described (Graber *et al.*, 1994).

Expression and purification of G protein α and $\beta\gamma$ subunits. The chimeric Gi1Q35C α subunit was constructed, purified after expression in *E.coli*, and kindly provided by Dr. Heidi Hamm of Vanderbilt University. While bacterially expressed subunits lack the N- terminal acylation of native and baculovirus expressed subunits, and contain a HIS₆ tag at the extreme N-terminus, their EC₅₀ values for receptor interactions are indistinguishable from those of native or baculovirus expressed subunits (Bae *et al.*, 1997). Gq was purified as detergent extracted G $\alpha_q\beta_1\gamma_{2HIS}$ heterotrimer from Sf9 cells infected with Gq α , β_1 and γ_{2HIS} baculoviruses as described by Kozasa and Gilman (Kozasa and Gilman, 1995) using sequential chromatography on DEAE anion exchange, Ni-NTA Superflow resin and QHR15 anion exchange columns. Free $\beta_1\gamma_{2HIS}$ subunits were eluted at 100-125 mM NaCl and G $\alpha_q\beta_1\gamma_{2HIS}$ heterotrimers were eluted at 200-220 mM NaCl from the QHR15 column. Gqi5 was purified in the same manner as Gq. The HA-epitope tag present between residues 125 and 130 of G α_{qi5C} has been shown not to affect

functionality (Conklin *et al.*, 1993). G α q and G α qi5C were purified from heterotrimers by elution from Ni-NTA Superflow resin with 10 mM NaF/50 μ M AlCl₃ and final purification on a QHR15 column. Gi1 α was expressed in Sf9 insect cells and purified as described (Bae *et al.*, 1999). Gi1Q6N and Gi1Q6N35C were expressed and purified from Sf9 cells using identical procedures as for Gi1 α . All G protein α subunits except Gi1Q35C and Gqi5 contained only native protein sequences.

Intrinsic tryptophan fluorescence assay. An assay was performed to verify that G α subunits could bind GDP and adopt the active conformation in the presence of AlF₄⁻ (Skiba *et al.*, 1996). Intrinsic fluorescence was measured with a QM-2000-4 spectrofluorimeter (PTI fluorescence system hardware and Felix software) at room temperature in a buffer containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM MgCl₂ and 1 μ M GDP. The AlF₄⁻-dependent conformational changes of activated G α subunits were monitored by intrinsic tryptophan fluorescence changes with excitation at 280 nm and emission at 340 nm. The relative increase in fluorescence of 400 nM G α subunits was determined from absorbance readings before and after addition of 10 mM NaF and 20 μ M AlCl₃ in a total volume of 100 μ l. The increase in fluorescence was calculated as $\Delta F\% = (F - F_0)/F_0 \times 100\%$. The relative change in intrinsic fluorescence for individual G α subunits was consistent with the literature (Skiba *et al.*, 1996) and as follows: Gi1 48.3 $\pm$ 3.2 (n=11), q6N 45 $\pm$ 12.2 (n=8), q35C 56.7 $\pm$ 5.2 (n=3), q6N35C 54.5 $\pm$ 15.6 (n=4), qi5C 47.7 $\pm$ 8.4 (n=3), Gq 50 $\pm$ 22.9 (n=4). Protein concentrations were determined using the BCA protein assay with BSA as a standard.

Preparation of Sf9 cell membranes containing recombinant receptors. Baculoviruses expressing M1 and M2 muscarinic receptors were the kind gift of Dr. Elliott Ross (University of Texas Southwestern Medical Center, Dallas, Texas). Sf9 cell membranes containing recombinant

muscarinic receptors were produced as described (Bae *et al.*, 1999). Briefly, Sf9 cells were infected at a density of 3.0×10^6 cells/ml with a recombinant baculovirus expressing M1 or M2 muscarinic receptors at a 2 fold multiplicity of infection. Infected cells were harvested 60-72 hrs post-infection for M1 receptors and 48-56 hrs post-infection for M2 receptors. To prepare membranes, cell pellets were thawed in 15x their wet weight of ice-cold SGHB1 buffer (10 mM Tris/HCl, 25 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, pH 8.0) with protease inhibitors (2 mg/ml aprotinin, 20 mg/ml benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin and 0.1 mM PMSF) and burst by nitrogen cavitation (20 minutes at 600 PSI). Cavitated cells were centrifuged at 4°C for 10 minutes at 500xg. The supernatant was centrifuged at 4°C for 30 min at 28,000xg. The pellets were resuspended in 35 ml of HE buffer (5 mM Hepes, 1 mM EDTA, pH 7.5) with protease inhibitors. The membranes were washed twice, resuspended in HE buffer at 1-3 mg protein/ml, snap frozen in liquid nitrogen and stored at -70°C. M1 receptor coupling required removal of endogenous proteins with 6M Urea essentially as described by Northup (Hartman and Northup, 1996). For urea stripping, membranes were thawed, resuspended at about 0.15 mg/ml in binding buffer (50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA) with 120 µM Oxotremorine-M and 6 µM GTPγS, and incubated for 30 min at 25°C. Following centrifugation at 288,000xg for 30 min at 4°C, pellets were resuspended at approximately 0.6 mg/ml in stripping buffer (HE+6M Urea) and extracted on ice for 30 minutes. The urea extract was centrifuged at 142,000xg for 30 min at 4°C and pellets were washed twice in HE buffer. Urea stripped membranes were resuspended in HE buffer at a concentration of about 2 mg/ml and snap frozen in liquid nitrogen. Receptor number in membrane preparations was determined using the muscarinic receptor antagonist [³H]-N-methyl Scopolamine (NMS) at concentrations from 0.03-8 nM of free [³H]-

NMS under the same conditions as used in the affinity shift assay (see below). Membrane protein concentrations were determined using the BCA protein assay with BSA as a standard.

Reconstitution of receptors with exogenous G proteins. Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge at 10,000xg for 10 minutes and resuspended at 5-10 mg/ml in the reconstitution buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.08% CHAPS). G protein α and $\beta\gamma$ subunits were mixed at a ratio of 1:1.3 and diluted in reconstitution buffer to keep the reconstitution volume the same for each sample. Typically, 0.8 pmol of receptor was reconstituted with 80 pmol of G protein heterotrimers (based on G α). The mixture was incubated at 25°C for 20 minutes and held on ice briefly until the start of the binding assay.

Affinity Shift Assay. Just prior to the binding assay the reconstitution mixture was diluted 10-12 fold with binding buffer (50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA). Agonist binding was determined in the presence of 5 nM [³H]-Oxotremorine-M. Non-specific binding was determined in the presence of 10 μ M atropine sulfate. Binding to equilibrium was for 1 hour at 25°C in a temperature controlled shaker and was terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed three times with 4 ml of ice cold wash buffer (50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% NaAzide), placed in 4.5 ml CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter. The final concentrations of receptors and G protein heterotrimers in the binding assay were 1.5-1.7 nM and 150-500 nM respectively, in a final volume of 150 μ l. Specific binding in the absence of exogenous G-proteins averaged 50 DPM for M1 receptors and 326 DPM for M2 receptors. Because muscarinic receptors exhibit ratios as high as 30,000-fold for the high and low affinity states for agonists such as oxotremorine M

(Oxo-M) and acetylcholine (Berrie *et al.*, 1984; Florio and Sternweis, 1989) specific binding for M1 receptors reconstituted with Gq averaged 1614 DPM while that for M2 receptors reconstituted with Gi1 averaged 7682 DPM. The actual concentrations of agonist varied slightly among experiments which resulted in different magnitudes of absolute binding. In order to compare the results among experiments it was necessary to express the data as affinity shift activities. Affinity shift is defined as the fold-enhancement above buffer controls of high affinity agonist binding in membranes expressing recombinant receptors reconstituted with G protein heterotrimers. Subunits that can't stabilize the high affinity state of receptors for agonist (as well as buffer controls) have affinity shift activities of 1, while active subunits have affinity shift activities significantly greater than 1. Control experiments determined the concentrations of G proteins required to saturate the affinity shift activities of all receptor G-protein combinations used in this study.

Gα saturation binding experiments. To determine the concentration of Gα needed to saturate the affinity shift, increasing amounts of G protein heterotrimers were added to the receptors in the reconstitution and agonist binding was detected in the binding assay as described above. Typically, 7 different G protein concentrations in triplicates in the range 10-500 nM were used. EC₅₀ values were generated from nonlinear regression analysis of data fit to a one-site interaction model between receptor and G protein.

ADP-Ribosylation of Gα by Pertussis Toxin. Pertussis Toxin (1.1 µg/µl) was activated by the addition of an equal volume of activation buffer (5 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 0.08% CHAPS, 40 mM DTT, 2µM GDP) and incubation for 40 min at 32°C. G protein heterotrimers (3 µg α plus 3.6 µg βγ) were ribosylated for 1 hr at 32°C in a total volume of 20 µl containing 10 µl of activated PTX and 400 µM NAD⁺. Ribosylation

reactions were reconstituted with membranes under the same conditions described above. At the end of the reconstitution incubation the mixture was diluted 9 fold with binding assay buffer and used in the affinity shift assay.

Muscarinic receptor-stimulated [^{35} S]-GTP γ S Binding Assay. A GTP γ S binding assay was used to analyze receptor-catalyzed GDP/GTP exchange on G protein α subunits. Typically 1.3 pmol of receptors was reconstituted with 80 pmol of G protein heterotrimers (1.3 fold excess of α over $\beta\gamma$) for 15 min at 30°C in reconstitution buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 40 μ M GDP, 0.08% CHAPS). After reconstitution the mixture was diluted 10 fold with GTP γ S Binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 25 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA). Reactions were initiated by the addition of 25 μ l of membrane mixture to 25 μ l of GTP γ S with or without Oxotremorine-M. Final assay conditions were 50 nM G α , 0.8 nM receptor, 2 μ M GDP, 100 nM GTP γ S as a mixture of 1nM [^{35} S]-GTP γ S (~125,000 cpm/well) and cold GTP γ S, with or without 2 μ M Oxotremorine-M in a final volume of 50 μ l. Use of MultiScreen 96 well plates (Millipore) allowed collection of samples at 30 second intervals for 4-6 minutes for M2 receptors or 3 minute intervals for 18 minutes for M1 receptors. Filters were rinsed three times with 100 μ l of ice-cold wash buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM MgCl₂). Radioactivity retained on the filters was counted to constant error in a liquid scintillation counter. Greater than 1500 cpm were observed at the shortest time points and ligand depletion was less than 15% at the longest time-points. Data were analyzed by linear regression, and agonist driven GTP γ S binding was reported as the ratio of the slopes in the presence and absence of agonist provided the slopes were significantly different ($p < 0.05$). If the slopes did not differ significantly a value of 1.0 was used for the slope ratio. GTP γ S binding in

the absence of agonist (moles of GTP γ S per mole of receptor) are reported in the legend to Figure 5.

Analysis of the data. Data analysis was done using the GraphPad Prism software package (GraphPad Software, San Diego, CA). For affinity shift assays, triplicate determinations were used within each experiment and experiments were repeated 2-5 times as indicated. Rates of GTP γ S binding were determined from linear regression of eight individual determinations at 30 second or 3 minute intervals within each experiment and experiments were repeated 3-5 times as indicated. Data represent the mean $\pm$ SEM from multiple experiments unless otherwise indicated.

RESULTS AND DISCUSSION

Molecular mechanisms of selectivity in G protein-receptor coupling are still not completely understood (Wess, 1998). Intact organisms employ various mechanisms including regulation of receptor and G protein expression levels, compartmentalization of signaling complexes, and the activity of accessory proteins, to regulate the selectivity of signaling along G protein mediated pathways. However, at the level of the G protein-receptor interface, the primary recognition is governed by the amino acid sequences of both G protein and receptor. G protein α subunits are considered the major determinant of coupling selectivity and several domains of $G\alpha$ have been shown to be important for receptor coupling. In this study we investigated the roles of the N- and C- termini of $Gq\alpha$ and $Gi1\alpha$ in directing the selectivity of M1 and M2 muscarinic receptor coupling in a reconstituted system. To do so we tested a series of chimeric $G\alpha$ subunits composed from different portions of $Gq\alpha$ and $Gi1\alpha$ for their ability to stabilize the high affinity agonist binding state of receptors and for their ability to undergo agonist stimulated guanine nucleotide exchange.

Figure 1A shows the secondary structure of the chimeric $G\alpha$ subunits used in this study. The q6N chimera has been constructed by addition of the unique six amino acid N-terminal extension of $Gq\alpha$ (MTLESI) to the N- terminus of $Gi1\alpha$. Q35C has been constructed by the replacement of thirty-five C-terminal amino acids of $Gi1\alpha$ with the corresponding amino acids of $Gq\alpha$. There are fourteen amino acids that are different between $Gi1\alpha$ and $Gq\alpha$ in this region. Q6N35C has been constructed by addition of the six N- terminal amino acids of $Gq\alpha$ (MTLESI) to the q35C construct. The qi5C chimera (Conklin *et al.*, 1993) has five C-terminal amino acids of $Gq\alpha$ (EYNLV) replaced with the corresponding amino acids of $Gi1\alpha$ (DCGLF). All constructs used for protein expression were verified by DNA sequencing. All proteins except

q35C were purified after expression in Sf9 cells. Q35C was purified after expression in bacteria and is the only G α subunit in this study with a HIS₆ tag at the extreme N-terminus. Previous work, with bacterially expressed subunits containing a HIS₆ tag at the extreme N-terminus, demonstrated that their EC₅₀ values for receptor interactions are indistinguishable from those of native or baculovirus expressed subunits (Bae *et al.*, 1997). Furthermore, all of the α subunits used in this study bound GDP and adopted the active conformation in the presence of AlF₄⁻ as shown by an enhancement of intrinsic tryptophan fluorescence (see Materials and Methods) comparable to that reported for other G protein preparations (Skiba *et al.*, 1996). In order to verify that the purified proteins had the expected domain switching between G α subunits, chimeric proteins were separated on 12% SDS-polyacrylamide gel and immunoblotted with antibodies selective for unique sequences within the G α subunits. Immunoblots of purified chimeric G α subunits are shown in Figure 1B. As expected, Gi1, q6N, q6N35C and q35C, but not qi5C or Gq, were recognized by an antibody against internal G α i1 sequence. Although the antibody directed against G α i1/2 C-terminal sequence recognized Gi1 and q6N, it did not recognize q6N35C and q35C verifying that the C-terminal domains of q6N35C and q35C are different from the C-terminal domain of Gi1. In addition, the antibody directed against G α i1/2 C-terminal sequence recognized the qi5C chimera demonstrating that C-terminal amino acids of Gi1 α are present in qi5C. Q6N35C and q35C were also recognized by the antibody against G α q/11 C-terminal sequences, as was Gq α , further supporting the domain switching. The antibody against Gq α internal sequence recognized Gq only. This antibody did not recognize qi5C because an internal HA tag in the qi5C construct (amino acid positions 125-130) disrupts the recognition epitope of the antibody against Gq α internal sequence (amino acids 115-133). The presence of the qi5C chimera was proven by immunoblotting with anti-HA tag antibodies

(Fig.1B). Therefore, immunoblotting with different antibodies verified the domain switching between wild type α subunits in the α subunit chimeras used in this study. Figure 1C shows Coomassie Blue stained G α subunits resolved on a 12% SDS-polyacrylamide gel. All of the α subunits used in this study were estimated to be at least 80% pure. The presence of doublets and triplets on the gel is likely to be due to the presence of α subunits in different states of lipid modification or guanine nucleotide binding. This interpretation is supported by the fact that these bands are recognized by the antibodies specific to the protein of interest (Figure 1B).

In order to study the role of N- and C- termini of Gq α and Gi1 α in M1 and M2 muscarinic receptor coupling, G protein heterotrimers containing chimeric α subunits were reconstituted with Sf9 cell membranes containing expressed M1 or M2 muscarinic receptors and their ability to form a high affinity ternary complex with the expressed receptors was determined by affinity shift assay. Figure 2 shows the affinity shift activities of wild type and chimeric α subunits with M1 and M2 muscarinic receptors which were determined with saturating concentrations of G proteins. The affinity shift assay has been completely described for several receptors expressed in Sf9 cells (Figler *et al.*, 1996; Clawges *et al.*, 1997) and is based on earlier work with native receptors (Asano *et al.*, 1985; Pobiner *et al.*, 1991). Briefly, the affinity shift assay detects the formation of the high affinity state of the receptor as an enhanced level of agonist binding upon ternary complex formation. Control experiments, shown in Figure 3, in which G proteins were titrated at fixed agonist concentration (5 nM [^{3} H]-OXO-M), were used to determine the concentration of G protein required to saturate affinity shift activity. The EC₅₀ values for reconstitution of high affinity agonist binding for individual G α subunits shown in the inset tables of Figure 3 are not significantly different, which is in agreement with earlier work suggesting that differences in G protein-receptor coupling manifest themselves as differences in

agonist affinities rather than differences in EC₅₀ values (Bae *et al.*, 1997). The G α subunits for which inset data are lacking displayed either weak saturable activity (as shown in experiments where doubling G protein concentrations did not increase affinity shift activity) or no activity even at high concentrations (300-500 nM) of G proteins (data not shown). By saturating affinity shift activities we eliminated the possibility that the differences in the affinity shifts shown in Figure 2 were caused by differences in specific activities of the G α subunit preparations.

As expected, in the affinity shift assay M1 receptors coupled with Gq and M2 receptors coupled with Gi1 (Figure 2). We used both loss of function and gain of function approaches to investigate the role of the C- terminus of Gq α in M1 muscarinic receptor coupling. As shown in Figure 2, the qi5C chimera has very little ability to couple M1 receptors compared with native Gq α , verifying the important role of the Gq α C- terminus. Because Conklin, Bourne and Wess have demonstrated in transfection studies that the five C- terminal amino acids of Gi1 α , when placed in the Gq α context, were sufficient to couple M2 muscarinic receptors (Conklin *et al.*, 1993; Liu *et al.*, 1995), we used a gain of function approach as a criteria to test if the C- terminus of Gq α placed in the Gi1 α context was sufficient to couple M1 muscarinic receptors. Interestingly, even thirty-five C-terminal amino acids of Gq α in the Gi1 α context were not sufficient to couple M1 receptors (Figure 2). We have also tested chimeras with three, five and eleven C- terminal amino acids of Gq α in place of those from Gi1 α and found they were not sufficient for M1 receptor coupling (data not shown). We conclude, therefore, that the C- terminus of Gq α , although required, is not sufficient for proper Gq-M1 receptor coupling and that other domains of Gq α must be present. Intriguingly, Natochin et al. reported that eleven C-terminal residues of Gt α in the Gs α context were sufficient for rhodopsin coupling in a reconstituted system (Natochin *et al.*, 2000). Such differences seem to indicate that molecular

determinants of G protein-receptor coupling selectivity differ among individual receptors and their cognate G proteins.

In order to investigate the role of the unique six amino acid N- terminal extension of Gq α (MTLESI) in M1 receptor coupling, we added these amino acids of Gq α to the N- terminus of Gi1 α . However, this chimera (q6N), also did not gain the ability to couple M1 receptors (Figure 2, compared with Gi1 and Gq). To investigate the possibility that both N- and C- termini of Gq α are required for proper M1 receptor coupling, we created the double chimera, q6N35C, which has the six N- terminal amino acids and thirty-five C- terminal amino acids of Gq α in the Gi1 α context. Interestingly, q6N35C had a significantly enhanced ability ($p < 0.01$ - 0.001 , Tukey's Multiple Comparison Test) to shift the agonist affinity of M1 muscarinic receptors, indicating that the appropriate context of N- and C- termini is required for proper receptor coupling (Figure 2). However, the activity of q6N35C chimera in the affinity shift assay was significantly lower ($p < 0.05$, Tukey's Multiple Comparison Test) than the activity of Gq (Figure 2). At least two explanations can be suggested for this difference in activity. First, other domains of Gq α or, possibly, a longer N- terminal portion of Gq α , are needed for full M1 receptor coupling. Second, since Cys9 and Cys10 of Gq α have been shown to be important but not critical for receptor coupling (Hepler *et al.*, 1996), the lack of Cys10 in the q6N35C chimera may explain the lower activity. Kostenis and others have suggested that the unique six amino acid N- terminal extension of Gq α prevents Gi/o-coupled receptors (including M2 muscarinic) and Gs-coupled receptors from coupling to Gq (Kostenis *et al.*, 1997a). We tested if these six N- terminal amino acids of Gq α could restrict M2 receptor coupling in the affinity shift assay. As shown in Figure 2, the six N- terminal amino acids of Gq α added to the N- terminus of Gi1 α did not prevent M2 receptor coupling in a reconstitution system.

The important role of the C- terminus of Gi1 α in receptor coupling was verified by the loss of function of the q35C chimera in the affinity shift assay with M2 receptors (Figure 2). In similar experiments, q5C and q11C chimeras also failed to couple with M2 receptors (data not shown). The q6N35C chimera also had no coupling activity with the M2 receptor (Figure 2). As discussed below, the low level affinity shift activity of both Gq and qi5C in coupling the M2 receptor is due to a small amount of contaminating Sf9 cell Gi/o-like protein in these preparations (see Figure 4). Therefore, the data indicate that the M2 receptor has an absolute requirement for the proper context of the C-terminus of the coupling G protein. Interestingly, gain of function experiments indicated that the five C- terminal amino acids of Gi1 α placed in the context of Gq α were not sufficient for optimal M2 receptor coupling as measured by the affinity shift assay (qi5C chimera, Figure 2). Because qi5C only minimally couples M2 receptors, it is tempting to speculate that cooperation between appropriate N- and C- termini are also critical for full Gi1-M2 muscarinic receptor coupling. Previous reconstitution studies demonstrated a role for a poorly defined region within the first 210 amino acids of Gi1 α that is important for 5-HT_{1B} receptor coupling (Bae *et al.*, 1997). However, since only the six amino acid N-terminal extension that is unique to Gq α subunits was tested in this study such speculation may be premature and the structural determinants of coupling may differ significantly between M1 and M2 receptors. The failure of qi5C to couple appreciably in our reconstitution assays is in contrast with the results of Liu, Conklin and others, who demonstrated in transfection assays that the qi5C construct conferred coupling to several Gi/o coupled receptors, including the M2 receptor (Liu *et al.*, 1995). While the precise reasons for such different observations in transfection versus reconstitution assays are unknown, several factors are likely to contribute. In transfection assays proteins of interest are overexpressed in a limited

percentage of cells and coupling is inferred from effector activities which are substantially amplified at several points in the signal transduction cascade. On the other hand, all cellular components are present and the integration and assembly of membrane structures occurs in a cellular context. Reconstitution experiments directly examine the G protein-receptor interface and allow controlled stoichiometries of components, however they require the G protein to associate with the receptor after the receptor has been integrated into cellular membranes and may not allow the participation of all components responsible for receptor-G protein coupling. Thus, reconstitution experiments are likely to more precisely identify the G protein domains directly involved in the receptor-G protein interface, while transfection experiments are likely to identify the domains that permit coupling in a cellular context without revealing the precise mechanism or required stoichiometries. Ultimately both contribute to our understanding of coupling although neither may completely reflect the actual situation in living tissues.

In addition to the expected Gq-M1 and Gi1-M2 receptor coupling, we observed a low level of Gi1-M1 and Gq-M2 receptor coupling in the affinity shift assay (Figure 2). The basis for this coupling was explored with the use of pertussis toxin (PTX). PTX is known to uncouple Gi/o proteins from their cognate receptors by ADP-ribosylating a C-terminal cysteine in Gi/o α subunits (West, Jr. *et al.*, 1985). We ADP-ribosylated G protein heterotrimers using PTX and reconstituted them with M1 or M2 muscarinic receptors. The results shown in Figure 4 indicate that PTX treatment completely uncoupled Gi1 from M2 receptors as expected. Surprisingly, PTX treatment also completely uncoupled Gq from M2 receptors, but not Gq from M1 receptors. As Gq α is not a substrate for ADP-ribosylation by PTX we attribute the Gq-M2 receptor coupling to the presence of small amounts of endogenous Sf9 Gi/o family proteins in our Gq preparation which was undetectable by immunoblotting (Figure 1A). A similar contamination of Gq purified

after expression in Sf9 cells by endogenous Gi/o proteins was reported by Kozasa (Kozasa and Gilman, 1995). This contaminating activity is not present in the q35C and q6N35C preparations (see Figure 2) as they are purified from the soluble fraction of expressing cells which contains few, if any, endogenous G proteins, while Gq and qi5C are purified from a detergent extract of the particulate fraction from expressing cells which would be expected to contain endogenous G proteins (see Materials and Methods). In addition, PTX treatment prevented Gi1-M1 receptor coupling (Figure 4). Therefore, our data indicate that M1 muscarinic receptors weakly couple with Gi1, while M2 muscarinic receptors probably don't couple with Gq. Our data agree well with the data of Burford et al. who demonstrated that M1 receptors, especially when expressed at high levels, can couple to both pertussis toxin sensitive and insensitive G proteins, while M2 receptors couple only with pertussis toxin sensitive G proteins (Burford *et al.*, 1995). DeLapp et al. also demonstrated that M1 receptors were able to couple to inhibitory G proteins while M2 receptors coupled to Gq/11 only weakly (DeLapp *et al.*, 1999).

In order to compare the ability of G α subunits to be activated by receptors, muscarinic receptor-catalyzed GDP/GTP exchange was measured as agonist-stimulated GTP γ S binding. Figure 5 shows the fold differences in slopes between agonist-driven and basal nucleotide exchange on various G α subunits. The inset graph shows moles of GTP γ S bound in membranes expressing M2 muscarinic receptors with or without reconstitution with Gi1 in the presence or absence of agonist. All chimeras were analyzed in the same fashion and the rates of GTP γ S binding were determined from the slopes of the lines. Data for M2 receptor-catalyzed GTP γ S binding demonstrated that q6N exchanged guanine nucleotides at a rate comparable to Gi1, while the rest of the G α subunits were inactive (Figure 5). With the M1 receptor, both Gq and q6N35C exchanged guanine nucleotides upon agonist stimulation, while the rest of the G α subunits were

inactive (Figure 5). These data agree well with the affinity shift data (Figure 2) and support our main conclusion that both the N- and C- termini of Gq α are required for effective M1 receptor coupling.

Although the affinity shift data indicate that the M1 receptor couples weakly with Gi1 (Figure 2), and that this coupling is abolished by PTX treatment (Figure 4), we did not see agonist stimulated M1 receptor mediated exchange on Gi1. Thus while the high affinity agonist binding state of G protein coupled receptors is thought to be a ternary complex containing nucleotide free G protein, and GDP release is accepted as the rate-limiting step in nucleotide exchange (Gilman, 1987), our data indicate that it is possible to stabilize the high affinity state of the receptor without stimulating guanine nucleotide exchange. Such discrepancies have been noted in a previous study (Bae *et al.*, 1999) and are likely to be related to the fact that the affinity shift assay measures an equilibrium condition among agonist, receptor and G protein in the absence of guanine nucleotides after an hour or more of incubation, while the agonist driven GTP γ S binding assay measures the kinetics of GTP γ S binding in the first few minutes after mixing these components.

The N- terminus of G α subunits, together with amino acids from switch I and switch II are binding sites for G $\beta\gamma$ subunits in the G protein heterotrimer (Wall *et al.*, 1995). Mutations at the N- terminus of G α may affect its interactions with $\beta\gamma$, which acts as a guanine nucleotide dissociation inhibitor for G α . Kostenis *et al.* have suggested that the six N- terminal amino acids of Gq α (MTLESI) form a tightly folded protein subdomain that prevents Gi/o- and Gs-coupled receptors from accessing Gq α , thereby regulating coupling selectivity (Kostenis *et al.*, 1998). Our results demonstrate that the addition of the six N- terminal amino acids of Gq α to the N-terminus of Gi1 α does not restrict M2 receptor coupling. Therefore we can say that the q6N

chimera interacted with $\beta\gamma$ properly. Our data suggest that N- and C- termini of Gq α cooperate to couple M1 receptors. While the crystal structure of Gq is not available, and the crystal structure of the Gi1 heterotrimer (with GDP bound) lacks four amino acids from the N- terminus and six amino acids are from the C- terminus (Wall *et al.*, 1995), it appears that the G α C- terminus in the heterotrimer is accessible and not buried within a compact microdomain. The N-terminus and α N helix are turned away from the body of G α to interact with $\beta\gamma$. The structure of the Gi1 heterotrimer indicates that Val34 and Arg32 are the N- terminal amino acids of Gi1 α that come close to Leu348, the most C- terminal G α residue in the structure. However, because the structural orientation of the extreme N- and C- termini in Gq is not known, and also because the crystal structure of a nucleotide-free G protein heterotrimer is not available, the structural basis for cooperativity between the extreme N- and C- termini of Gq α is not yet understood. One possibility is that the N- and C- termini of G α might interact to form a single receptor binding site. Alternatively, there might be two separate sites on the receptor for the N- and C- termini which have to be recognized for the receptor to activate G proteins. Muradov and Artemyev suggested that coupling between N- and C- terminal domains of Gt α is responsible for low basal GDP/GTP exchange in Gt α (Muradov and Artemyev, 2000). Since Gq α is also known to have low basal GDP/GTP exchange this concept may in principal apply to Gq α .

Taken together, our data indicate that the context of the C- terminus of G α , although critical, is not sufficient for M1 or M2 muscarinic receptor coupling. In addition, the unique six amino acid N- terminal extension of Gq α is neither sufficient to allow M1 muscarinic receptor coupling, nor sufficient to prevent M2 muscarinic receptor coupling. Rather, the appropriate context of both N- and C- termini of G α is required for functional coupling to M1, and possibly, M2, muscarinic receptors. Q6N35C represents an easily purified, relatively stable, and soluble

G α subunit with the ability to functionally couple with M1 muscarinic receptors. As a reagent it therefore offers significant advantages to native Gq α , which is difficult to purify, requires detergents for solubility and is easily inactivated by denaturation.

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FIGURE 1. A. Secondary structure of chimeric G α subunits. Numbers on the right of the wild type forms of G α i1 and G α q indicate their total amino acid residues. Numbers within the designations for the chimeras indicate the number of amino acids switched between G α i1 and G α q. The diagram on the bottom depicts the secondary structural domains common to G α subunits. **B. Immunoblot analysis of purified chimeric α subunits.** Proteins were purified as described in Methods, electrophoresed on SDS-polyacrylamide gels and immunoblotted with antibodies indicated at the right of each panel. Each lane contained 75 ng of the indicated purified α subunit. **C. Coomassie stain of purified G α subunits.** 500 ng of purified alpha subunits were resolved on a 12% SDS-polyacrylamide gel and visualized with coomassie blue stain. The order of proteins on coomassie gel is the same as indicated above the immunoblot. Lane 1 is Gi1, lane 2 is q6N, lane 3 is q35C, lane 4 is q6N35C, lane 5 is qi5C, lane 6 is Gq.

FIGURE 2. Affinity shift activity of G α subunits with M1 and M2 muscarinic receptors.

Affinity shift activities represent the fold enhancement above buffer controls of high affinity [^{3} H]-Oxo-M binding in membranes expressing the indicated muscarinic receptor (1-2 nM) reconstituted with a saturating excess (100-200 nM) of G protein heterotrimers containing the indicated G α subunits. Data are the mean $\pm$ SEM from 3-5 experiments where the concentration of Oxo-M was $\sim$ 5 nM. The dotted line represents an affinity shift activity of 1, the level of activity observed in the absence of exogenous G proteins or in the presence of inactive G protein heterotrimers.

FIGURE 3. Concentration dependence of G α subunits in agonist binding to M1 and M2 muscarinic receptors. Sf9 cell membranes expressing either M1 (left panel) or M2 (right panel) muscarinic receptors were reconstituted with increasing concentrations of G protein heterotrimers containing the indicated G α subunit. Receptor concentrations were 0.95 nM for M1 and 0.82 nM for M2. High affinity agonist binding was determined in the presence of 5 nM [^{3} H]-OXO-M. Data were fit to a single-site interaction between receptor and G protein and are the mean $\pm$ SD of triplicate determinations from a representative experiment. The inset tables show the EC₅₀ values (mean $\pm$ SEM) from the indicated number of experiments for individual G α subunits. For each receptor, the individual EC₅₀ values did not differ significantly from one another (One-way ANOVA or t-test).

FIGURE 4. Affinity shift activity of G α subunits following ADP-ribosylation by Pertussis Toxin. Affinity shift activities represent the fold enhancement above buffer controls of high affinity [^{3} H]-Oxo-M binding in membranes expressing the indicated muscarinic receptor (1-2 nM) reconstituted with a saturating excess (100-200 nM) of G protein heterotrimers containing the indicated G α subunits with or without ADP-ribosylation by Pertussis Toxin. Data are the mean $\pm$ SD of triplicate determinations from a representative experiment repeated twice with similar results. G α subunits without pertussis toxin treatment contained all of the reagents (except Pertussis Toxin) and underwent the same treatment as those treated with toxin. The dotted line represents an affinity shift activity of 1, the level of activity observed in the absence of exogenous G proteins or in the presence of inactive G protein heterotrimers.

FIGURE 5. Muscarinic receptor-catalyzed GDP/GTP exchange on G α subunits. GDP/GTP exchange was measured as GTP γ S binding in membranes expressing the indicated muscarinic receptor reconstituted with G protein heterotrimers containing the indicated G α subunits. Bars represent the fold-enhancement by agonist above basal nucleotide exchange in the absence of agonist on G α subunits and are the mean $\pm$ SEM from 3-8 experiments. Final conditions in the binding assay were 50 nM G α , 0.8 nM receptor, 2 μ M GDP, 100 nM GTP γ S (~125,000 cpm/well), with or without 2 μ M Oxotremorine-M in a final volume of 50 μ l. The asterisks above the bars indicate values significantly different than 1.0 ($p < 0.05$, one-sample t-test) and the absence of error bars indicates that there was no significant difference between basal and agonist driven rates in any experiment. The inset graph depicts moles of GTP γ S bound per mole of receptor in membranes expressing M2 muscarinic receptors with or without reconstitution with Gi1 in the presence and absence of 2 μ M Oxo-M as indicated. The lines are least squares regression lines and the slopes represent the rate of GTP γ S binding. Rates of GTP γ S binding to all G α subunits were determined as shown in the inset graph and the mean basal rates of GTP γ S binding (mol GTP γ S/mol receptor/min) were as follows- Gi1, 0.23; Q6N, 0.25; Q35C, 0.10; Q6N35C, 0.12; Gqi5C, 0.04; Gq, 0.08. The basal rate for Gi1 was significantly greater than all other subunits except Q6N ($p < 0.05$, Tukey's Multiple Comparison Test).

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CHAPTER III. RESULTS

PART II

Closely related G protein coupled receptors use multiple and distinct domains on G protein α subunits for selective coupling

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Abbreviations and Textual Footnotes—¹The abbreviations used are: GPCRs, G protein coupled receptors; GTP γ S, guanosine 5'-3-*O*-(thio)triphosphate; OXO-M, Oxotremorine-M; 5-HT, Hydroxytryptamine; CCPA, chloro-N⁶-cyclopentyladenosine, R-PIA, R-phenylisopropyl adenosine.

ABSTRACT

The molecular basis of selectivity in receptor-G protein coupling has been explored by comparing the abilities of G protein heterotrimers containing chimeric G α subunits, comprised of various regions of Gi1 α , Gt α and Gq α , to stabilize the high affinity state of serotonin, adenosine and muscarinic acetylcholine receptors. The data indicate that multiple and distinct determinants of selectivity exist for individual receptors. While the A1 adenosine receptor does not distinguish between Gi1 α and Gt α sequences, the 5-HT_{1A} and 5-HT_{1B} serotonin and M2 muscarinic receptors can couple with Gi1 but not Gt. It is possible to distinguish domains that eliminate coupling and hence are defined as “critical”, from those that impair coupling and hence are defined as “important”. Domains within the N terminus, α 4 helix, and α 4 helix- α 4/ β 6 loop of Gi1 α are involved in 5-HT and M2 receptor interactions. Chimeric Gi1 α /Gq α subunits verify the critical role of the C terminus in receptor coupling, however, the individual receptors differ in the G α amino acids that are required for coupling. Furthermore, the EC₅₀ for interactions with Gi1 differ among the individual receptors. These results suggest that coupling selectivity ultimately involves subtle and cooperative interactions among various domains on both the G protein and the associated receptor as well as the G protein concentration.

INTRODUCTION

A large number of diverse seven transmembrane spanning cell surface receptors mediate signaling to a variety of intracellular effectors by coupling to the heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) (Pierce *et al.*, 2002). The mechanisms responsible for selectivity in G protein mediated signaling pathways are not fully understood (Wess, 1998; Albert and Robillard, 2002). Although it is known that at the molecular level the selectivity in G protein-receptor coupling is determined by amino acid sequences of both receptor and G protein, the individual amino acids involved in this selective recognition have not been completely identified. Different receptor systems and different methodologies indicate that the in the G α subunit C terminus and $\alpha 5$ helix (Hamm *et al.*, 1988; Conklin *et al.*, 1993; Gilchrist *et al.*, 1998; Natochin *et al.*, 2000), N terminus and αN helix (Hamm *et al.*, 1988; Hepler *et al.*, 1996; Kostenis *et al.*, 1997; Swift *et al.*, 2000), $\alpha 4$ helix and $\alpha 4/\beta 6$ loop (Bae *et al.*, 1997; Natochin *et al.*, 1999; Blahos *et al.*, 2001), $\alpha 2$ helix and $\alpha 2/\beta 4$ loop (Lee *et al.*, 1995), $\alpha 3/\beta 5$ loop (Grishina and Berlot, 2000), $\alpha N/\beta 1$ loop (Blahos *et al.*, 2001) and amino acids 110-119 from the α helical domain (Krieger-Brauer *et al.*, 1999) are involved in receptor coupling selectivity. Some of these domains contact the receptor directly, while others regulate receptor coupling selectivity indirectly by playing a role in nucleotide exchange. Despite the fact that many of the receptor interacting domains have been identified, the relationship between receptor subtypes and G α domains involved in receptor coupling has not been clearly established. Thus, it is difficult to predict which G α domains will be utilized by a specific receptor. Here we propose that individual receptors recognize specific patterns formed by amino acids of G α thus making G protein interface look different for different receptors. The C terminus of G α is a well accepted receptor recognition domain, which contacts receptors directly (Conklin and Bourne,

1993). Although individual C terminal amino acids important for receptor coupling have been identified in several G α subunits, the specific G α amino acids participating in receptor recognition may differ among receptors. The α 4 helix- α 4/ β 6 loop domain, first described as an effector domain, has been shown to be important for 5-HT_{1B} receptor coupling to Gi1 (Bae *et al.*, 1997). Later it was demonstrated that Gln304 and Glu308 in the α 4 helix of Gi1 α are important for 5-HT_{1B} receptor coupling (Bae *et al.*, 1999). However the generality of the role for the α 4 helix- α 4/ β 6 loop domain in receptor coupling selectivity has not been determined.

Gi1 α and Gt α are closely related G α subunits, which belong to the Gi/o class of G protein α subunits, share 68% homology, and have nearly identical overall structures. Although the 5-HT_{1B} receptor discriminates between Gi1 and Gt (Skiba *et al.*, 1996; Bae *et al.*, 1997) the fact that their C termini are identical render Gi1 α /Gt α chimeras useless for exploring the role of this domain in receptor coupling. However, the extreme C terminus of Gq α differs from that of Gi1 α by four amino acids, while their α 5-helices differ by additional nine amino acids. Thus Gi1 α /Gq α chimeras are ideal for studying the role of this domain in coupling. Since several different GPCRs¹ can couple to the same G protein, we wanted to test the hypothesis that individual receptors utilize slightly different domains on G α subunits to achieve coupling. G protein-receptor coupling selectivity may also be regulated at the level of G protein concentration. In fact, Clawges *et al.* demonstrated that 5-HT_{1A} and 5-HT_{1B} receptors distinguish themselves by the affinity with which they interact with G proteins (Clawges *et al.*, 1997). Therefore we also wanted to test the generality of this mechanism with different receptors. Here we compare the coupling behavior of four Gi/o-coupled receptors (5-HT_{1A} and 5-HT_{1B} serotonin, A1 adenosine and M2 muscarinic) by reconstituting them with G protein heterotrimers containing native or chimeric G α 's composed of G α i1, G α t and G α q. Our data

demonstrate that selective coupling between Gi1 and the members of Gi/o-coupled receptor family is directed by multiple and distinct G α domains and is regulated at the level of G protein concentration.

EXPERIMENTAL PROCEDURES

Materials. [^3H]-Oxotremorine-M Acetate ([^3H]-OXO-M) (85.8 Ci/mmol), [^3H]-Hydroxytryptamine Binoxalate ([^3H]-5-HT) (25.5 Ci/mmol) and [^3H]-Chloro-N⁶-cyclopentyladenosine ([^3H]-CCPA) (30 Ci/mmol) were from New England Nuclear Life Science Products, Inc. (Boston, MA). Atropine Sulfate, 5-Hydroxytryptamine (5-HT) and R-phenylisopropyl adenosine (R-PIA) were from Sigma-Aldrich Corporation (St. Louis, MO). Adenosine deaminase was from Roche Molecular Biochemicals (Indianapolis, IN). The BCA Protein Assay reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO) or EMD Biosciences (formerly Calbiochem-Novabiochem Corporation; San Diego, CA).

Expression and purification of proteins. The expression and purification of the G α_{i1} and G $\beta\gamma$ subunits was as previously described (Graber *et al.*, 1992a; Graber *et al.*, 1992b). The chimeric G α_{i1} /Gat subunits were constructed, expressed in *E. coli* and purified as described (Skiba *et al.*, 1996). The Gi1/Q3C, Gi1/Q5C and Gi1/Q11C chimeras were made from pHis₆G α_{i1} using the silent BamHI site introduced at amino acid position 212 (Skiba *et al.*, 1996). The pHis₆G α_{i1} cDNA was amplified by PCR reaction with primer oligonucleotides containing the desired mutations. The PCR products were digested with BamHI and HindIII, and the BamHI-HindIII fragment was used to replace the corresponding fragment from pHis₆G α_{i1} . To construct Gi1/Q35C, the C-terminal portion of a Gq α cDNA was amplified by PCR reaction, followed by digestion with BglII and HindIII. The digested PCR fragment was inserted into the BglII and HindIII sites of the Chi13 plasmid (Bae *et al.*, 1997). Functional characterization of all bacterial subunits included GTP γ S binding, AlF₄⁻-dependent conformational change (measured as an

increase in intrinsic tryptophan fluorescence) or binding to the cGMP phosphodiesterase γ subunit (Skiba *et al.*, 1996; Bae *et al.*, 1997; Bae *et al.*, 1999).

Preparation of Sf9 membranes containing expressed receptors. Sf9 cells were infected with a recombinant baculovirus expressing the desired receptor, cultured and harvested as previously described (Graber *et al.*, 1992b). To prepare membranes, harvested cells were thawed in 15x their wet weight of ice cold homogenization buffer (10 mM Tris-Cl, pH 8.0 at 4 °C, 25 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 μ g/ml of benzamidine and 2 μ g/ml of each of aprotinin, leupeptin and pepstatin A) and burst by nitrogen cavitation (600 psi, 20 minutes). Cavitated cells were centrifuged at 4 °C for 10 minutes at 500 x g to remove the unbroken nuclei and cell debris. The supernatant from the low speed spin was centrifuged at 4 °C for 30 min at 28,000 x g. The supernatant was discarded and the pellets were resuspended and pooled in 35 ml of HE buffer (5 mM NaHEPES, 1 mM EDTA, pH 7.5) containing the same protease inhibitors as used in the homogenization buffer. Adenosine receptor HE buffer included 100 mM NaCl in addition to the above components. The membranes were washed twice in HE, resuspended in the same buffer at a concentration of 1-3 mg protein/ml, aliquoted, snap frozen in liquid nitrogen, and stored at -70 °C.

Reconstitution of receptors with exogenous G-proteins. Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge (10 min, 12,000 rpm) and resuspended at about 10 mg/ml in a reconstitution buffer consisting of 5 mM NaHEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.04% CHAPS (0.08% CHAPS for M2 receptor), pH 7.5. G protein subunits were diluted in the same buffer such that the desired amount of subunit was contained in 1-5 μ l. Typically, 1-2 μ l of G protein subunits were added to 40 μ l of membrane suspension, the mixture was incubated at 25 °C for 15 minutes and held on ice until the start of the binding assay.

Radioligand binding. Just prior to the start of the binding assay the reconstitution mixture was diluted 10-12 fold with binding assay buffer appropriate to the receptor of interest such that the desired amount of membranes (5-25 $\mu\text{g}/\text{assay tube}$) were contained in 10-50 μl . Binding buffer for 5-HT and M2 receptors was 50 mM Tris, 5 mM MgCl_2 , 0.5 mM EDTA, pH 7.5. Binding buffer for A1 adenosine receptor was 10 mM HEPES, 5 mM MgCl_2 , 1 mM EDTA, pH 7.4. Radioligand binding in the affinity shift assay was determined in the presence of the [^3H]-OXO-M for M2 muscarinic receptor, [^3H]-5-HT for 5-HT serotonin receptors and [^3H]-CCPA for A1 adenosine receptor. Adenosine deaminase was added to the [^3H]-CCPA solution at 12 $\mu\text{g}/\text{ml}$ in binding buffer. Non-specific binding was determined by addition of 1000-fold excess of unlabeled ligand– 5-HT for 5-HT receptors, atropine sulfate for M2 receptor and R-PIA for A1 receptor. Incubations were for times sufficient to achieve equilibrium in a temperature controlled shaker (1 hr for M2 receptor, 1.5 hrs for 5-HT receptors, 2 hrs for A1 receptor) and were terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed thrice with 4 ml ice cold 50 mM Tris-Cl, 5 mM MgCl_2 , 0.5 mM EDTA, 0.01% NaAzide, pH 7.5 at 4 $^{\circ}\text{C}$, placed in 4.5 ml CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter. For reconstitution of high affinity agonist binding in affinity shift assays, a single concentration of radioligand near the high affinity K_D of the receptor of interest was used in a final volume of 150 μl . [^3H]-5-HT radioligand purity was monitored by HPLC or TLC using an appropriate mobile phase. Radioligands were repurified or replaced when the radiochemical purity fell below 85%.

Affinity shift activity assay. The Sf9 cell membranes expressing individual receptors were reconstituted with saturating amounts of native or chimeric Gi1 protein heterotrimers (≥ 25 nM or 40-400 fold molar excess over receptors) to achieve the maximal specific binding during the

binding assays. Because the magnitude of the affinity shifts observed with native Gi1 protein heterotrimers varied significantly among the individual receptors affinity shift activity was normalized to Gi1 activity and expressed as % affinity shift activity, which is $(\text{Chimera Reconstituted Binding} - \text{Control Binding}) / (\text{Gi1 Reconstituted Binding} - \text{Control Binding}) \times 100$.

Analysis of the data. Data analysis was done using the GraphPad Prism software package (GraphPad Software, San Diego, CA). For affinity shift assays, triplicate determinations were used within each experiment and experiments were repeated 3 or more times. Data represent the mean $\pm$ SEM from multiple experiments. One-way ANOVA with Tukey's multiple comparison post test was used to compare the activities of chimeras.

RESULTS

Previously we have shown that amino acids 299-318 and 1-219 of Gi1 α are molecular determinants of 5-HT_{1B} receptor coupling (Bae *et al.*, 1997) and that two amino acids in the α 4 helix of Gi1 α (Gln304 and Glu308) are especially important for 5-HT_{1B} receptor coupling (Bae *et al.*, 1999). The goal of the present study was to examine the generality of these findings among closely related members of the Gi/o-coupled receptor family. Our general strategy involves reconstitution of purified G proteins containing chimeric α subunits with receptors expressed in Sf9 insect cell membranes and comparison of the abilities of these chimeric G proteins to stabilize the high affinity agonist binding state of the receptors in an affinity shift activity assay. In the present study we compared the coupling behavior of four different Gi/o-coupled receptors: 5-HT_{1A} and 5-HT_{1B} serotonin receptors, M2 muscarinic receptors and A1 adenosine receptors.

Affinities of individual receptors for G proteins- First we determined the concentration of G proteins in the binding assay that produced the maximum affinity shift for each receptor. Increasing amounts of G protein heterotrimers were reconstituted with individual receptors and EC₅₀ values for reconstitution of high affinity agonist binding were determined. The data indicate that A1, 5-HT_{1A}, 5-HT_{1B} and M2 receptors have different EC₅₀ values for Gi1 (Figure 1). A1 receptors have the highest apparent affinity (0.4 nM) and M2 receptors have the lowest apparent affinity (47 nM) for the Gi1 heterotrimer. 5-HT receptors have intermediate EC₅₀ values of 3.7 nM and 16.2 nM for the 5-HT_{1A} and 5-HT_{1B} receptors respectively. Titration experiments similar to those shown in Figure 1 were used to determine the concentration of chimeric G proteins needed to saturate affinity shift activities with individual receptors. For individual receptors, the EC₅₀ values of the active chimeras were not significantly different and

even high concentrations (>600 nM) of inactive chimeras did not have affinity shift activity (data not shown). All affinity shift activities were determined with saturating concentrations of G proteins.

Affinity shift activity of chimeric G α subunits- Figure 2 depicts the secondary structures of the Gi1 α /Gt α chimeras used in this study. All of these chimeras have been previously described and were used to study Gi1 α domains involved in 5-HT_{1B} receptor coupling (Skiba *et al.*, 1996; Bae *et al.*, 1997; Bae *et al.*, 1999). Figure 3, in which 100% activity corresponds to the affinity shift activity of Gi1, shows the per cent affinity shift activity of Chi2, Chi3, Chi6, Chi13 and Chi21. Chi6 was constructed as a soluble analog of Gt α and has the same functional properties as Gt α (Skiba *et al.*, 1996). Chi6 is primarily Gt α in character as it includes N- terminal amino acids 1-215 and C- terminal amino acids 295-350 of Gt α with the amino acids corresponding to 216-294 from Gi1 α to maintain solubility. In this region there are just 26 amino acids that differ between Chi6 and Gt α . As shown in Figure 3, Chi6 was inactive with 5-HT_{1A}, 5-HT_{1B} and M2 muscarinic receptors. Earlier experiments with native transducin demonstrated it also failed to couple with the 5-HT receptors (Clawges *et al.*, 1997). In contrast, the data in Figure 3 demonstrate Chi6 was 74% active with the A1 adenosine receptor, indicating that A1 adenosine receptor doesn't discriminate well between Gt and Gi1 sequences. Although the activity of Chi6 with the A1 adenosine receptor was significantly lower ($p < 0.001$) than the activity of Gi1, the magnitude of the difference was too small to be of use in identifying the precise domains responsible for the reduced activity. However, the inability of the 5-HT_{1A}, 5-HT_{1B} and M2 muscarinic receptors to couple with Chi6 allowed us to use additional chimeras containing less Gt α sequence to more precisely identify the domains required for coupling.

We first examined whether the N- terminal or C- terminal portion of Gi1 α was critical for receptor coupling. Chi21 has N- terminal amino acids 1-215 of Gt α with the rest of the molecule Gi1 α sequence (Figure 2). Chi21 was fully active with the A1 adenosine receptor, indicating that the A1 receptor does not distinguish between N- terminal amino acid sequences of Gi1 α and Gt α (Figure 3). The activity of Chi21 with 5-HT_{1A}, 5-HT_{1B} and M2 receptors was significantly ($p < 0.001$) reduced (44%, 57% and 42% respectively, Figure 3) demonstrating that amino acids 1-219 of Gi1 α contain an important determinant of Gi coupling with these receptors. Chi2 has the C- terminal amino acids 295-350 of Gt α with the rest of the chimera Gi1 α sequence (Figure 2). Figure 3 demonstrates that amino acids 299-354 of Gi1 α contain residues critical for 5-HT_{1A}, 5-HT_{1B} and M2 receptor coupling because the affinity shift activity of Chi2 with these receptors (2%, 9% and 23% respectively) was not significantly different from Chi6 activity. In contrast, Chi2 was fully active with A1 adenosine receptors supporting our conclusion that A1 adenosine receptor does not distinguish well between Gi1 α and Gt α sequences. To further evaluate the role of amino acids 299-354 of Gi1 α in 5-HT and M2 receptor coupling we tested two additional chimeras, Chi3 and Chi13 (Figure 2). Chi3 has amino acids 299-319 of Gi1 α replaced with the corresponding amino acids of Gt α (amino acids 295-315) while Chi13 has the 35 C-terminal amino acids of Gi1 α replaced with the corresponding amino acids of Gt α . As shown in Figure 3, the affinity shift activities of Chi3 show that amino acids 299-319 of Gi1 α (α 4-helix and α 4/ β 6-loop) are critical for 5-HT_{1A}, 5-HT_{1B} and M2 receptor coupling, but not for A1 adenosine receptor coupling. In contrast, Chi13, with six amino acids variant from Gi1 α , was active with all four receptors indicating that the 35 C-terminal amino acids of Gi1 α and Gt α are functionally interchangeable in coupling these receptors. Nevertheless, the significantly ($p < 0.01$) reduced activity of Chi13 (85.9%) with the

M2 receptor and the significantly ($p<0.01$) increased activity with both 5-HT_{1A} (128%) and 5-HT_{1B} (124.5%) receptors suggest subtle differences in the coupling mechanism of these receptors. The role of the extreme C-terminus of Gi1 α cannot be evaluated with these chimeras because the eight C-terminal amino acids of Gi1 α and Gt α are identical.

Role of the $\alpha 4$ -helix and $\alpha 4/\beta 6$ -loop of Gi1 α in receptor coupling- In order to investigate $\alpha 4$ - $\alpha 4/\beta 6$ region of Gi1 α in more detail we used several additional chimeras to subdivide this region (Figure 4). Chi22 has the $\alpha 4$ helix of Gi1 α replaced with that from Gt α while Chi25 has the $\alpha 4/\beta 6$ loop of Gi1 α replaced with that from Gt α . Chi23 has the $\alpha 4/\beta 6$ loop of Gi1 α replaced with that from Gt α and also switches the Glu in Gi1 α at the end of the $\alpha 4$ helix for the Leu found in Gt α . Chi24 has the central part of the $\alpha 4/\beta 6$ loop with two variant amino acids switched between Gi1 α and Gt α . These chimeras were fully active with the A1 adenosine receptor (data not shown), supporting our conclusion that the A1 receptor does not use the $\alpha 4$ - $\alpha 4/\beta 6$ region to distinguish between Gt and Gi1 (see Figure 3). Figure 5 shows the affinity shift activity of these chimeras with 5-HT_{1A}, 5-HT_{1B} and M2 receptors. Chi22 had low affinity shift activity with all three receptors indicating that a critical determinant of coupling selectivity for these receptors is located in the $\alpha 4$ helix of Gi1 α (Figure 5). For the 5-HT_{1B} receptor, the activity of Chi22 was significantly higher than the activity of Chi3 ($p<0.01$), indicating that the $\alpha 4/\beta 6$ loop may also play a role in 5-HT_{1B} receptor coupling. This conclusion is supported by the Chi25 activity with the 5-HT_{1B} receptor (73%), which was significantly ($p<0.001$) lower than the activity of Gi1 (100%). However, Chi25 was 91% as active with M2 muscarinic receptor) which was not significantly different ($p>0.05$) from Gi1 activity) and was 121% as active with the 5-HT_{1A} receptor (which was significantly ($p<0.001$) higher than Gi1). Clearly the $\alpha 4/\beta 6$ loop is utilized differently by these receptors. Chi24 was fully active with all three

receptors (Figure 5) which suggests that the reduced activity of Chi25 with the 5-HT_{1B} receptor is due to the replacement of Asp309 by Glu at the beginning of the $\alpha 4/\beta 6$ loop (Figure 4). Figure 5 also shows the affinity shift activity of Chi23 was significantly reduced ($p < 0.001$) compared with the activity of both Gi1 and Chi25 for all three receptors. Chi23 differs from Chi25 by just one amino acid (replacement of Glu308 from Gi1 α for Leu from Gt α) indicating that Glu308 is important for coupling to 5-HT_{1A}, 5-HT_{1B} and M2 receptors. Taken together, the data indicate that the $\alpha 4$ helix (Glu308 in particular) is important for all three receptors, and that the $\alpha 4/\beta 6$ loop (probably Asp309) is also important for 5-HT_{1B} receptors.

Defining individual amino acids in the $\alpha 4$ - $\alpha 4/\beta 6$ region of Gi1 α – To prove the role of Glu308 in receptor coupling and also to study the role of other amino acids in the $\alpha 4$ - $\alpha 4/\beta 6$ region of Gi1 α we used chimeras in which amino acids Ala301, Gln304, Cys305, Glu308, Lys312 and Thr316 of Gi1 α were replaced individually or in combinations with the corresponding amino acids of Gt α . All of the mutants used here have been previously described (Bae *et al.*, 1999). First we studied the role of these amino acids with a loss of function assay. Mutants in which amino acids of Gi1 α were replaced individually or in combinations with the corresponding amino acids of Gt α would be expected to exhibit reduced affinity shift activities if these amino acids were important for coupling. Replacement of Ala301 with Asn did not reduce activity (Gi1A301N, Figure 6) demonstrating that Ala301 is not important for coupling any of the receptors tested. When Gln304 was changed to Lys (Gi1Q304K, Figure 6) activity with 5-HT_{1A} and M2 receptors was significantly ($p < 0.001$) reduced, but as reported previously (Bae *et al.*, 1999), this single amino acid replacement did not significantly reduce affinity shift activity with 5-HT_{1B} receptors (Figure 6). The activity of Gi1C305V shows that Cys305 is important for M2 muscarinic receptors (67% activity, $p < 0.001$) but not important for either 5-

HT receptor (Figure 6). Glu308 is an important amino acid for all three receptors as the Gi1E308L mutant displays 62%, 73% and 61% of activity with 5-HT_{1A}, 5-HT_{1B} and M2 receptors respectively ($p < 0.001$) (Figure 6). Lys312 and Thr316 are not important for coupling these receptors and the increased activity of Gi1K312M and Gi1T316V with the 5-HT_{1A} receptor ($p < 0.001$) is consistent with the increased activity of Chi25 with this receptor.

Data obtained with three double mutants (Gi1Q304K/C305V, Gi1Q304K/E308L, Gi1C305V/E308L) and a triple mutant (Gi1Q304K/C305V/E308V) support the conclusions drawn from the point mutants (Figure 6). The activity of the Gi1Q304K/E308L mutant was lower than the activity of either Gi1Q304K or Gi1E308L for all receptors supporting the importance of both Gln304 and Glu308 in receptor coupling. The role of Cys305 in M2 receptor coupling is supported by the observation that the activity of Gi1Q304K/C305V mutant was significantly lower than the activity of the Gi1Q304K mutant ($p < 0.05$). Furthermore, the activity of the triple mutant (Gi1Q304K/C305V/E308V) was the lowest of all with the M2 receptor, supporting the idea that Gln304, Cys305 and Glu308 are all important for M2 receptor coupling. On the other hand, the conclusion that Cys305 is not important for coupling the 5-HT_{1A} and 5-HT_{1B} receptors is supported by the observations that the 304/305 and 305/308 double mutants have similar activities with these receptors as the Q304K and E308L single mutants and the 304/305/308 triple mutant is similar in activity to the 304/308 double mutant with these receptors.

Gain of function assays, in which amino acids from Gi1 α replaced those from Gt α in Chi22 were used to confirm the role of the amino acids identified in the loss of function assay. The data in Figure 7 demonstrate that substituting back Ala301 does not lead to gain of function with any of the receptors tested, supporting the conclusion that Ala301 of Gi1 α is not important

for receptor coupling. Substituting back Gln304 (Chi22K300Q) resulted in significant ($p<0.001$) gain of activity with 5-HT_{1B} receptors which is in contrast to the absence of a loss of activity with 5-HT_{1B} receptors when Gln304 was mutated to Lys in Gi1 α . Similarly, substituting back Cys305 in the Chi22V301C mutant also resulted in significant ($p<0.05$) gain of activity with 5-HT_{1B} receptors. The precise reasons for these anomalies are unknown but may be related to the actual role of these amino acids in the context of their neighbors. Substituting back Glu308 alone (Chi22L304E) resulted in a gain of affinity shift activity of 48% with 5-HT_{1A} receptors ($p<0.001$), 38% with 5-HT_{1B} receptor ($p<0.001$) but only 17% ($p>0.05$) with M2 receptors. However, when both Gln304 and Glu308 were substituted back into Chi22 sequence (Chi22K300Q/L304E), a full gain of activity was observed with 5-HT_{1A} and 5-HT_{1B} receptors, as Chi22K300Q/L304E activity was not significantly different from activity of Gi1 (100%). The gain of function with M2 receptors was significant (45% gain of activity, $p<0.001$), though still less than the activity of Gi1. Taken together, the data indicate that Gln304 and Glu308 of Gi1 α are important for 5-HT_{1A}, 5-HT_{1B} and M2 receptor coupling, and that Cys305 of Gi1 α is important for M2 receptor coupling in addition to Gln304 and Glu308.

Role of C terminus of Gi1 α in receptor coupling- Alignment of the C-terminal sequences of Gi1 α and Gt α indicates that their extreme eight C-terminal amino acids are identical (Figure 8). Because numerous studies have indicated the C terminus of G α plays a significant role in receptor coupling, we decided to investigate the role of C terminus of Gi1 α in 5-HT_{1A}, 5-HT_{1B}, A1 and M2 receptor coupling using Gi1 α /Gq α C-terminal chimeras. As shown in the sequence alignments in Figure 8, the extreme C-terminus of Gq α differs from that of Gi1 α in just four amino acids. Loss of function experiments may demonstrate partial or complete loss of activity. As shown in Figure 9, replacement of just two of these amino acids

with those from Gq α in the Q3C mutant significantly lowers the affinity shift activity with all four receptors. The nearly complete loss of affinity shift activity (0.3% and 11.2%, respectively) with 5-HT_{1B} serotonin and A1 adenosine receptors suggests that these amino acids are critical for coupling, while the more modest decrease in activity (65% and 68% activity, respectively) with the 5-HT_{1A} and M2 receptors suggest these amino acids are important, but not critical, for coupling. Substitution of the five C-terminal amino acids of Gi1 α with those from Gq α eliminates coupling with the A1 adenosine receptor while substitution of 11 C-terminal amino acids are required for complete loss of 5-HT_{1A} receptor coupling (Figure 9). These data indicate that the 5-HT_{1A}, 5-HT_{1B}, A1 adenosine and M2 muscarinic receptors differ in their utilization of the C-terminal amino acids of Gi1 α for coupling.

DISCUSSION

G protein-receptor coupling can be regulated by a variety of mechanisms (Wess, 1998; Albert and Robillard, 2002). At the G protein-receptor interface, the selectivity of coupling is regulated by the amino acid sequences of both receptor and G protein. By comparing the coupling mechanism of four closely related receptors to the same G proteins, we found that receptors use multiple domains on G α to achieve selective coupling. Coupling selectivity is also regulated by the G protein concentration as demonstrated by the significant differences among the EC₅₀ values for Gi1-receptor interactions. This suggests that in living cells the expression levels of specific G protein subunits may regulate receptor coupling preferences.

At the level of G α domains, the major difference we found is that the A1 adenosine receptor does not discriminate well between Gi1 α and Gt α sequences. In contrast, the 5-HT and M2 receptors couple with Gi1 but fail to couple with Gt. This selectivity allowed us to use Gi1 α /Gt α chimeras to define domains on Gi1 α important for coupling with these receptors. Our findings indicate that amino acids especially important for receptor coupling are located in the α 4 helix. In addition, the 5-HT_{1B} receptor may require Asp309 at the beginning of α 4/ β 6 loop for optimal coupling. The corresponding amino acid in Gt α is Glu305, and while both are negatively charged, glutamate is one -CH₂ group bigger than aspartate. Thus replacement of aspartate with glutamate may decrease 5-HT_{1B} receptor coupling because of the change in the size of the receptor interacting surface on G α . In addition, we demonstrated that within the α 4 helix- α 4/ β 6 loop region of Gi1 α the amino acids that are involved in receptor coupling differ slightly among the receptors. While all three receptors utilize Gln304 and Glu308, the M2 receptor also uses Cys305 and the 5-HT_{1B} receptor may use Asp309. Interestingly, interaction of the 5-HT_{1A} receptor with the K312M mutant actually leads to an increased affinity shift. This

increase in affinity shift activity may represent tighter coupling of the receptor with the chimera. Other investigators have also demonstrated the importance of this region of $G\alpha$ in receptor coupling. Natochin *et al.* demonstrated the role of Arg310 and Asp311 in interaction of $Gt\alpha$ with rhodopsin (Natochin *et al.*, 1999). Blahos *et al.* demonstrated that $\alpha 4$ - $\alpha 4/\beta 6$ - $\beta 6$ - $\alpha 5$ region of $G\alpha 16$ is important but not critical for interaction with metabotropic glutamate receptor 8 (Blahos *et al.*, 2001). In contrast, the work of Grishina and Berlot shows that $\alpha 4/\beta 6$ loop of $G\alpha s$ is not important for interactions with $\beta 2$ adrenergic receptor (Grishina and Berlot, 2000). Using gain of function experiments, Ho and Wong demonstrated that incorporation of $\alpha 4/\beta 6$ loop of $G\alpha z$ into a $G\alpha t$ backbone was not sufficient for δ -opioid receptor coupling (Ho and Wong, 2000). Taken together, these results support the idea that even if different receptors recognize the same general domain on $G\alpha$ subunits, the specific amino acids involved in receptor interactions may be different.

Another region of $Gi1\alpha$ important for 5-HT and M2 receptor coupling is the N terminus, as affinity shift activity with Chi21 was lower than with $Gi1$ for these receptors. According to the literature, the amino acids that bind to the receptor map to approximately positions 1-30 of the α subunits (Hamm *et al.*, 1988). This region, which includes the N terminus and the αN helix, contains the most differences between $Gi1\alpha$ and $Gt\alpha$ with 15 variant amino acids compared with just 9 variants from amino acids 31 to 219. Another significant difference between $Gi1\alpha$ and $Gt\alpha$ is that the αN helix of $Gt\alpha$ is 4 amino acids shorter than the αN helix of $Gi1\alpha$. Thus it is possible that amino acids 1-30 are important but not critical for 5-HT and M2 receptor coupling.

Although the C terminus of $G\alpha$ subunits is postulated to directly contact the receptor and mediate receptor coupling selectivity, our data show that the specific amino acids involved in

this recognition differ among the receptors studied. Cys351 (position -4), Gly352 (position -3) and Phe354 (position -1) in Gi family members have been shown to be important for mediating selectivity of receptor coupling (reviewed in) (Wess, 1998). Gain of function studies with Gq/i chimeras (Conklin *et al.*, 1993; Conklin *et al.*, 1996) indicate that five C terminal amino acids of Gi are sufficient for coupling to A1 and M2 receptors while three C terminal amino acids of Gi are not enough for A1 receptor coupling (Conklin *et al.*, 1993). Although so far it has not been possible to successfully solve the structure of the G α C terminus in the context of the whole molecule (the C terminus is disordered in the crystal), the structure of the C- terminal undecapeptide of Gt α bound to activated rhodopsin has been resolved by NMR spectroscopy (Koenig *et al.*, 2002). In this C- terminal decapeptide, the first eight residues form an α helix which is terminated by an α_L type C-cap (Aurora *et al.*, 1994) with C terminal glycine (Gly348 in Gt α , Gly352 in Gi1 α) in the center of the reverse turn (Kisselev *et al.*, 1998). Thus the observation that in case of A1 receptor three C terminal amino acids of Gi1 α are critical in the loss of function experiments but five C terminal amino acids are required to gain coupling may be explained by the fact that this α_L C-cap, which is disrupted in Gi1/Q3C chimera, is required for A1 receptor coupling. This is probably also true for the 5-HT_{1B} receptor. Our M2 receptor data indicate that although this α_L C-cap structure is important, it is not critical for receptor coupling. For the 5-HT_{1A} receptor three C terminal amino acids of Gi1 α are important while amino acids at the positions -4 and -5 (Asp350 and Cys351) are not important (activities of Gi1/Q3C and Gi1/Q5C are the same). Gi/Q5C and Gi/Q11C are different in three amino acids, which are probably involved in 5-HT_{1A} receptor coupling. Some additional amino acids involved in 5-HT_{1A} receptor coupling are located in the α_5 helix (see Figure 8) as evident from the activity of Gi1/Q35C chimera. Taken together, our results support the idea that different

receptors may recognize a specific pattern of amino acids which form receptor recognition surfaces.

Figure 10 depicts the structure of the $G\alpha_{i1}\beta_1\gamma_2$ G protein heterotrimer. Six amino acids from the C terminus and four amino acids from the N terminus are missing in this structure (Wall *et al.*, 1995). The domains of $Gi1\alpha$ discussed herein are surface exposed and located on the G protein surface that is presumed to face the receptor. They are therefore available for receptor coupling. However, while some amino acids may be involved in coupling by making direct contact with receptors, others may be involved indirectly by playing a role in guanine nucleotide exchange. Amino acids Glu304, Glu308 and Asp309 are surface exposed and so are also available for receptor coupling. Molecular modeling indicates that $Gi1Q304K$, $Gi1E308L$ and $Gi1304/308$ mutations alter the surface potential (Bae *et al.*, 1999), while $Gi1D309E$ mutation alters steric interactions because Glu is one CH_2 group larger than Asp (water-accessible surfaces of native $Gi1$ and $Gi1D309E$ were constructed and superimposed in Insight II; not shown). Therefore, structural considerations are consistent with our conclusions. In summary, here we demonstrated that four closely related Gi/o coupled receptors distinguish themselves by the affinity with which they interact with $Gi1$ and by their use of multiple domains of $Gi1\alpha$ for selective coupling.

FIGURE 1. Concentration dependence of Gi1 in affinity shift assays for individual Gi1-coupled receptors. Sf9 cell membranes expressing the indicated Gi1-coupled receptors were reconstituted with increasing concentrations of Gi1 heterotrimer. The affinity shift activities, percent of Gi1 for each receptor were fit to a single-site interaction between receptor and G protein. Saturation was achieved for each receptor, however for visual purposes the curves have been extended to a common endpoint. Shown are the data from representative experiments. EC50 data are the mean $\pm$ SEM from 3 or more independent experiments.

FIGURE 2. Secondary structure of Gα subunits. Numbers above the chimeric structures indicate the junction points of G α t and G α i1 sequences and refer to the amino acid positions in G α . Numbers for the wild type forms of G α t and G α i1 represent their total amino acid residues. The bottom diagram depicts the secondary structural domains common to Gα subunits.

FIGURE 3. Functional coupling of receptors to the indicated Gi1/Gt chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the affinity shift activities, percent of Gi1 as mean ± SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

FIGURE 4. Primary sequence alignment of the $\alpha 4$ - $\alpha 4/\beta 6$ loop region of Gi1 α and Gt α . The boxes indicate the regions of Gi1 α that were substituted with the corresponding sequences from Gt α to generate the indicated Gi1 α /Gt α chimeras.

FIGURE 5. Functional coupling of receptors to the indicated Gi1/Gt chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric G α and $\beta\gamma$ subunits. Data represent the affinity shift activities, percent of Gi1 as mean $\pm$ SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

FIGURE 6. Functional coupling of receptors to the indicated Gi1α point mutants. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the affinity shift activities, percent of Gi1 as mean ± SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

FIGURE 7. Functional coupling of receptors to the indicated Chi22 point mutants. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric G α and $\beta\gamma$ subunits. Data represent the affinity shift activities, percent of Gi1 as mean $\pm$ SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

FIGURE 8. Sequence alignment of 35 C terminal amino acids of Gtα, Gi1α and Gqα. The sequences of Gtα and Gqα are compared to Gi1α sequence. Depicted in bold are amino acids of Gtα and Gqα that are different from corresponding amino acids of Gi1α.

FIGURE 9. Functional coupling of receptors to the indicated Gi1/Gq chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric G α and $\beta\gamma$ subunits. Data represent the affinity shift activities, percent of Gi1 as mean $\pm$ SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-400 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

FIGURE 10. Spacefilling representation of the $G\alpha\beta1\gamma2$ heterotrimer. The image was generated using GRASP (developed by A. Nicholls and B. Honig, Columbia University) with coordinates from Wall et al. (Wall *et al.*, 1995). The α subunit is shown in metallic blue and the $\beta\gamma$ dimer is in yellow. The regions involved in receptor coupling as discussed herein are the $\alpha4$ -helix and $\alpha4/\beta6$ -loop in *pink*, Gln304 in *red*, Glu308 in *green*, Asp309 in *blue*, the C-terminus in *cyan*, and the N-terminus and αN -helix in *gray*.

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CHAPTER III. RESULTS

PART III

Agonist and G Protein May Stabilize Multiple and Distinct Active Receptor Conformations

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ABSTRACT

Molecular mechanisms of G protein coupling to M2 muscarinic and A1 adenosine receptors were investigated in a Sf9 cell membrane reconstitution system. Functional G protein-receptor interactions were assessed with affinity shift, agonist competition and agonist-driven nucleotide exchange assays. Chi3, a chimeric G α with the $\alpha 4$ - $\alpha 4/\beta 6$ domain of Gt α in a Gi1 α context, required significantly higher concentrations of agonist for functional interactions with both receptors. Intriguingly, at high concentrations of agonist, Chi3 had a greater nucleotide exchange rate than Gi1 with both receptors, indicating that the $\alpha 4$ helix- $\alpha 4/\beta 6$ loop domain may play a fundamental role in nucleotide exchange. A second chimera, Q6N35C, which contains the $\alpha 4$ - $\alpha 4/\beta 6$ domain of Gi1 α , also required significantly higher concentrations of agonist for functional interactions with M2 muscarinic receptors. However, nucleotide exchange rates on Q6N35C were significantly lower than on Gi1 at all concentrations of agonist. Our results suggest that agonist and G protein may stabilize multiple and distinct active conformations of receptors.

INTRODUCTION

G protein coupled receptors (GPCRs) can be activated by a variety of extracellular signals which they transduce through heterotrimeric G proteins to different intracellular effectors (Pierce *et al.*, 2002). The molecular mechanisms of GPCR action are not completely understood. The two-state modified ternary complex model is currently the most widely accepted receptor model (Samama *et al.*, 1993). According to this model, GPCRs exist in equilibrium between inactive R and active R* conformations. The R* conformation is stabilized by guanine nucleotide free G proteins and binds agonist with higher affinity than the R conformation. Accumulating data suggest that multiple R* conformations of the same receptor subtype for different agonists may exist (Perez *et al.*, 1996; Krumins and Barber, 1997; Watson *et al.*, 2000; Waelbroeck, 2001; Ghanouni *et al.*, 2001; Kenakin, 2001). Also it has been demonstrated that G protein type can have an effect on agonist efficacy (Yang and Lanier, 1999). Presumably, ligand-specific R* states have different affinities for different G proteins resulting in different response (Cordeaux *et al.*, 2001). In addition to the ligand-specific receptor states the existence of receptor-specific G protein conformations has also been suggested (Wenzel-Seifert and Seifert, 2000). This may indicate that G proteins play an active role in receptor coupling by either stabilizing or inducing a specific R* conformation already produced by agonist. This hypothesis is partially supported by the suggestion of Seifert *et al.* that receptor state that promotes GDP release and the state that stabilizes the ternary complex can be different (Seifert *et al.*, 2001).

Here we used a Sf9 cell membrane reconstitution system to investigate how G protein type and agonist concentration can regulate G protein-receptor coupling. In this study we used two Gi-coupled receptors - A1 adenosine and M2 muscarinic receptors. Using chimeric Gi1 α /Gt α and Gi1 α /Gq α subunits we demonstrated previously that the C terminus of Gi1 α is

critical for M2 muscarinic and A1 adenosine receptor coupling and that the $\alpha 4$ - $\alpha 4/\beta 6$ region of $G\alpha$ is critical for M2 muscarinic receptor coupling but is not important for A1 adenosine receptor coupling (Ma *et al.*, 2000). Here we used two chimeric $G\alpha$ subunits. Chi3 is a $Gi1\alpha$ based chimera in which the $\alpha 4$ - $\alpha 4/\beta 6$ region of $Gi1\alpha$ has been replaced with the corresponding region of $Gt\alpha$ (Bae *et al.*, 1997). Q6N35C is also a $Gi1\alpha$ based chimera which has 35 C-terminal amino acids of $Gi1\alpha$ replaced with the corresponding amino acids from $Gq\alpha$ and 6 N-terminal amino acids of $Gq\alpha$ added to the N terminus of $Gi1\alpha$ (Slessareva JE, 2001). Using affinity shift, agonist competition and agonist-stimulated GDP/GTP exchange assays we demonstrate that the agonist affinity of the R^* conformation depends on the identity of the coupling G protein. Our results indicate that there may be multiple active receptor conformations depending on both the nature of G protein with which receptor interacts and the agonist concentration. The two-state model does not adequately explain the existence of multiple activated states of receptors and therefore our results are relevant with respect to receptor theory.

MATERIALS AND METHODS

Materials. [^3H]-Oxotremorine-M Acetate ([^3H]-OXO-M) (85.8 Ci/mmol), [^3H]-N-Methyl Scopolamine (70 Ci/mmol), [^3H]-Cyclopentyl-1,3-dipropylxanthine ([^3H]-DCPX) and [^{35}S]-GTP γ S (1250 Ci/mmol) were from New England Nuclear Life Science Products, Inc. (Boston, MA). [^{125}I]-Aminobenzyladenosine ([^{125}I]-ABA) was a kind gift of Joel Linden (University of Virginia). Oxotremorine-M, Atropine Sulfate, Chloro-N ^{6} -cyclopentyladenosine (CCPA) and R-phenylisopropyl adenosine (R-PIA) were from Sigma-Aldrich Corporation (St. Louis, MO). Adenosine deaminase was from Roche Molecular Biochemicals (Indianapolis, IN). The BCA Protein Assay reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO) or Calbiochem-Novabiochem Corporation (San Diego, CA).

Preparation of Sf9 cell membranes containing recombinant receptors. To produce Sf9 cell membranes containing recombinant receptors Sf9 insect cells were infected at a density of 3.0×10^6 cells/ml with recombinant baculoviruses expressing receptors of interest at a 2-fold multiplicity of infection. Cells were harvested by centrifugation (170xg for 7.5 minutes at 4°C) 48-60 hrs postinfection depending on cell density and viability. Cell pellets were washed three times in ice-cold IPBS (7.3 mM NaH $_2$ PO $_4$, 60 mM KCl, 47 mM NaCl, 2.0 mM CaCl $_2$, pH 6.2), resuspended in SGHB1 buffer (10 mM Tris/HCl, 25 mM NaCl, 10 mM MgCl $_2$, 1 mM EGTA, pH 8.0) with protease inhibitors (2 mg/ml aprotinin, 20 mg/ml benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin and 0.1 mM PMSF) at 0.25 - 0.5 g/ml and snap-frozen in liquid nitrogen. To prepare membranes cell pellets were thawed in 15x their wet weight of ice-cold SGHB1 buffer with protease inhibitors and burst by nitrogen cavitation (600 psi, 20 minutes). Cavitated cells were centrifuged at 4°C for 10 minutes at 500xg to remove nuclei and cell debris. The

supernatant from the low speed spin was centrifuged at 4°C for 30 min at 28,000xg. The supernatant was discarded and the pellets were resuspended in 35 ml of HE buffer (5 mM Hepes, 1 mM EDTA, pH 7.5) with protease inhibitors at the concentrations indicated above. For the adenosine receptor HE buffer was supplemented with 100 mM NaCl. Membranes were washed twice, resuspended in the HE buffer at 1-3 mg protein/ml, snap frozen in liquid nitrogen and stored at -70°C.

Reconstitution of receptors with G proteins. Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge at 12,000 rpm for 10 minutes and resuspended at 5-10 mg/ml in reconstitution buffer (5 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.08% CHAPS for muscarinic receptors and 0.04% CHAPS for adenosine receptors). G protein subunits were purified as previously described (Bae *et al.*, 1997). To produce G protein heterotrimers α and $\beta\gamma$ subunits were mixed at the ratio of 1:1.3 and diluted in reconstitution buffer to keep the reconstitution volume the same for each sample. G protein heterotrimers were added to the membranes at the 60–150 fold molar excess over the recombinant receptors which was sufficient to saturate the affinity shift activity as shown in Figure 1. The mixture was incubated at 25°C for 20 minutes and hold on ice until the start of the binding assay.

Radioligand binding assays. Just prior to the start of the binding assay the reconstitution mixture was diluted 10-12 fold with binding assay buffer appropriate to the receptor of interest such that the desired amount of membranes (5-25 μ g/assay tube) were contained in 10-50 μ l. Binding buffer for M2 receptors was 50 mM Tris, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.5. Binding buffer for A1 adenosine receptor was 10 mM HEPES, 5 mM MgCl₂, 1 mM EDTA, pH 7.4. Radioligand binding in the affinity shift assay was determined in the presence of 5 nM [³H]-Oxotremorine M for M2 muscarinic receptor and 0.75 nM [³H]-Cyclopentyl-1,3-

dipropylxanthine for A1 adenosine receptor in a final volume of 150 μ l. Adenosine deaminase was added to the [^{3} H]-CCPA solution at 12 μ g/ml in binding buffer. Non-specific binding was determined by addition of 1000-fold excess of unlabeled ligand – atropine sulfate for M2 receptor and R-phenylisopropyl adenosine for A1 receptor. Incubations were for times sufficient to achieve equilibrium in a temperature controlled shaker (1 hr for M2 receptor and 2 hrs for A1 receptor) and were terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed thrice with 4 ml ice cold 50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% NaAzide, pH 7.5 at 4 °C, placed in 4.5 ml CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter. M2 receptor number was determined in the presence of [^{3} H]-Scopolamine methyl chloride at concentrations 0.031-7.41 nM of free [^{3} H]-NMS. A1 receptor number was determined in the presence of saturating amounts of Gi1 and [^{3} H]-CCPA at concentrations 0.2–40 nM.

Competition binding experiments. The extent of receptor coupling with individual G proteins and the K_D for the high and low agonist affinity states were determined in competition binding experiments between a single concentration of radiolabeled antagonist and increasing concentrations of non-labeled agonist. [^{3} H]-Scopolamine methyl chloride was used for M2 receptors and [^{3} H]-Cyclopentyl-1,3-dipropylxanthine for A1 receptors. The agonists used were Oxotremorine M for M2 receptors and Chloro-N⁶-cyclopentyl adenosine for A1 receptors. Receptors were reconstituted with saturating amounts of G proteins as described above. Agonist concentrations were equally spaced on the log scale in the range of 10⁻¹²-3X10⁻² M for M2 receptors and 10⁻¹⁴-3X10⁻⁵ M for A1 receptors. Data were analyzed by nonlinear regression and the F test was used to compare one-site vs two site fits. The Cheng-Prusoff correction was used to calculate K_D values.

Receptor stimulated [^{35} S]-GTP γ S binding assay. A GTP γ S binding assay was used to analyze receptor-catalyzed GDP/GTP exchange on G protein α subunits. Typically 1.3 pmol of receptors were reconstituted with 80 pmol of G protein heterotrimers (1.3 fold excess of α over $\beta\gamma$) for 15 min at 30°C in reconstitution buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 200 nM GDP, 0.08% CHAPS for M2 receptor and 0.04% CHAPS for A1 receptor). After reconstitution the mixture was diluted 10 fold with GTP γ S Binding buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 25 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA). Reactions were initiated by the addition of 25 μ l of membrane mixture to 25 μ l of GTP γ S with or without agonist. Final assay conditions were 50 nM G α , 0.8 nM receptor, 10 nM GDP, 10 nM GTP γ S as a mixture of 1nM [^{35} S]-GTP γ and cold GTP γ S, with or without agonist in a final volume of 50 μ l. Use of MultiScreen 96 well plates (Millipore) allowed collection of samples at 30 second intervals for 4-6 minutes. Filters were rinsed three times with 100 μ l of ice-cold wash buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM MgCl₂) Radioactivity retained on the filters was counted to constant error in a liquid scintillation counter. Greater than 1500 cpm were observed at the shortest time points and ligand depletion was less than 15% at the longest time-points. The rate of GTP γ S binding for each G α at each agonist concentration was taken as the slope determined by linear regression. If the slope was significantly different from basal (zero agonist), the fold-enhancement over basal GTP γ S binding was calculated as the ratio of the slopes. If the slopes were not significantly different the ratio was assigned a value of 1. The ratios were plotted against log agonist concentration and the data fit to a sigmoidal dose-response relationship.

Analysis of the data. The data were analyzed using GraphPad Prism software package (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

The goal of this paper was to understand how the G protein-receptor coupling is regulated by agonist concentration and also to investigate how identity of G α can regulate the affinity of agonist for receptor. A1 adenosine receptors and M2 muscarinic receptors are both capable to couple to Gi1. The important difference between these receptors however is that A1 receptors can couple Gt but M2 receptors can not (Ma *et al.*, 2000). As reported previously, the $\alpha 4$ - $\alpha 4/\beta 6$ region of Gi1 α is critical for M2 receptor coupling and the C terminus of Gi1 α is critical for both A1 and M2 receptor coupling (Ma *et al.*, 2000). Figure 1 shows G α proteins used in this study. Chi3, which has the $\alpha 4$ - $\alpha 4/\beta 6$ region of Gi1 α replaced with the corresponding region from Gt α , can couple A1 adenosine receptors but can not couple M2 muscarinic receptors very well, while Q6N35C was inactive with both receptors as shown in the insets in Figure 2. To ensure that all experiments were done at saturating G protein concentration, G α saturation experiments were performed as shown in Figure 2. Our results demonstrate that the increase in high affinity agonist binding following reconstitution with exogenous G proteins is a saturable phenomenon. Chi3 enhances agonist binding with adenosine receptors almost as well as Gi1 but has very little activity with muscarinic receptors while q6N35C is unable to couple either receptor. The ability of individual G proteins to enhance high affinity agonist binding to different extents suggests that the actual agonist affinity of a receptor depends on the identity of the coupling G protein.

To investigate how the G protein-receptor coupling is regulated by agonist concentration, agonist competition experiments against a fixed concentration of radiolabeled antagonist and increasing concentrations of agonist were performed. These experiments can detect the formation of the ternary complex of agonist, receptor and nucleotide-free G protein and determine the actual affinity of the high affinity state of the receptor. Figure 3 shows the agonist competition

experiments with A1 and M2 receptors reconstituted with Gi1, Chi3 and q6N35C. Insets in Figure 3 show the percentages of receptors coupled with G proteins and the K_D for the high affinity state of receptors. The percentages of the coupled receptors were not significantly different between different G proteins, indicating that the differences between G proteins manifest themselves as the differences in the K_D for the formation of the high affinity state of the receptor. Gi1, used as a positive control, had highest affinity for high affinity receptor state with both A1 adenosine and M2 muscarinic receptors (0.01 nM and 2.6 nM respectively). With A1 adenosine receptor Chi3 had the same activity as Gi1 and the K_D for the formation of high affinity state was not much different for Gi1 and Chi3 (Figure 3A). Q6N35C was inactive in affinity shift assay with A1 receptors but could form the ternary complex as indicated by the agonist competition assay (Figure 3A). However as seen in inset in Figure 3, K_D for the formation of the high affinity state was 155 times higher for q6N35C than for Gi1 (1.71 nM vs. 0.011 nM). With M2 muscarinic receptor, Chi3 was only weakly active and q6N35C was completely inactive in the affinity shift assay (Figure 2B). In the agonist competition assay (Figure 3B) Chi3 formed the ternary complex with high affinity K_D of 26.9 nM, which is 10 times higher than Gi1. Q6N35C, which was completely inactive in the affinity shift assay with M2 muscarinic receptor, formed the ternary complex with the high affinity K_D of 239.8 nM (92 times higher than Gi1) (Figure 3B). Data in Figure 3 demonstrate that G proteins unable to couple receptors in the affinity shift assay gained the ability to couple the receptors at higher agonist concentration. Our results indicate that the actual affinity of the high-affinity agonist binding state of a receptor depends on the identity of the coupling G protein and may vary over several orders of magnitude. This suggests that there are multiple active receptor conformations and that agonist and G protein may differentially stabilize such states.

Agonist competition experiments look at the G protein binding to the receptor as indicated by the formation of the high affinity receptor state. However they don't provide information about G protein activation step. To investigate if G protein binding at higher agonist concentration seen in agonist competition assay leads to G protein activation we performed agonist dose-response of GDP/GTP exchange assay. Figure 4 shows the agonist concentration dependence of GDP/GTP exchange on Gi1, Chi3 and q6N35C for A1 adenosine (Figure 4A) and M2 muscarinic (Figure 4B) receptors. As seen in inset table in Figure 4, the functional coupling of individual G proteins to receptors at significantly different concentrations of agonist is also seen in GDP/GTP exchange assays. With the exception of q6N35C and the A1 adenosine receptor, there is a strong correlation between the high affinity agonist K_D (Figure 3) and the agonist EC_{50} values for GDP/GTP exchange (Figure 4) which supports the conclusion that there are multiple active receptor states that are differentially stabilized by agonist and G proteins. The inability of the A1 adenosine receptor to catalyze GTP γ S binding to q6N35C at any concentration of agonist is not understood. However the fact that q6N35C can bind to A1 adenosine receptors but can not be activated supports the idea of multiple active receptor conformations. Intriguingly, with both receptors, at high concentrations of agonist Chi3 has a significantly greater nucleotide exchange rate than Gi1. Transducin is known to have very low basal GTP binding and to undergo the greatest receptor stimulation of GTP binding when coupled to rhodopsin (Fawzi and Northup, 1990) suggesting that the $\alpha 4$ - $\alpha 4/\beta 6$ domain (which is from transducin in Chi3) may play a fundamental role in nucleotide exchange. Figure 5 shows different views of Gi1 $\alpha\beta_1\gamma_2$ heterotrimer in order to illustrate the location of $\alpha 4$ - $\alpha 4/\beta 6$ domain relative to the guanine nucleotide binding site. The $\alpha 4$ - $\alpha 4/\beta 6$ domain is not a part of the guanine

nucleotide binding site (Sprang, 1997). However it can play a role by affecting conformation of the regions directly involved in guanine nucleotide exchange.

GPCRs are proteins in nature, so they can exist in multiple conformations. However not all conformations have a physiological role. The idea that different agonists stabilize different active receptor conformations has been actively discussed and experimentally tested (Tucek, 1997; Kenakin, 1997; Kenakin, 2001). The important characteristic of receptor active states is their ability to interact with G proteins or other membrane proteins and initiate a response. Supposedly different active receptor conformations expose different sequences in intracellular loops which may account for the ability of one receptor subtype to couple to several G proteins. Thus the nature of agonist dictates which active receptor conformation will prevail and signal through a certain pathway. However in this scheme G proteins play rather a passive role. Earlier work of Clawges *et al.* demonstrated that different $G\alpha$ subunits ($G\alpha$, $Gi1\alpha$, $Gi2\alpha$, $Gi3\alpha$) enhanced the high affinity [^3H]-HT binding to 5-HT receptors to different extent, suggesting that actual affinity of high affinity state can be different depending on the nature of G protein (Clawges *et al.*, 1997). Yang and Lanier demonstrated that G protein type have an effect on agonist efficacy by showing that the expression of $G\alpha1$ together with $\alpha2$ adrenergic receptor increased the relative efficacy of clonidine but $Gi1\alpha$ did not, without changing the ability of clonidine to interact with and stabilize the receptor (Yang and Lanier, 1999). Cordeaux *et al.* showed that D2 Dopamine receptors have higher affinity for G_o than $Gi2$ and that this preference depends on agonist used, indicating that different active receptor conformations have different abilities to couple with G proteins (Cordeaux *et al.*, 2001). Using fusion protein approach Wenzel-Seifert and Seifert demonstrated that $\beta2$ adrenergic receptor couples better with G_s than Gi and G_q , and is able to discriminate between different members of Gi and G_q families, as

judged by the ternary complex formation (Wenzel-Seifert and Seifert, 2000). Using full and partial agonists they also demonstrated that G protein type have an effect on pharmacological properties of the receptor. Here we used one agonist for each receptor and different G proteins to investigate how G proteins affect agonist affinity for the receptor in a reconstitution system. We demonstrated that the G proteins unable to couple to receptors in the affinity shift assay were able to couple to receptors at higher agonist concentrations. Importantly, the ability of G proteins to bind to receptor at higher agonist concentrations correlated with G protein activation at higher agonist concentrations, with the exception of q6N35C and A1 adenosine receptors. Our results indicate that agonist and G protein may stabilize multiple and different active receptor conformations. We propose the following mechanism by which G proteins can regulate receptor coupling. Receptor activation by agonist produces at least one activated R^* state capable to couple with G proteins. G proteins in turn can bind to receptor and change its conformation so that GDP release could occur and ternary complex with high affinity for agonist could form. If G protein is unable to change receptor conformation, ternary complex will not form. If the receptor conformation in the formed ternary complex is not able to activate the G protein, G protein binding but not activation will be seen, as in case of q6N35C and A1 adenosine receptors. Thus different G proteins will stabilize different R^* conformations. Importantly, the formation of these different R^* is regulated by agonist concentration. The physiological relevance of this however is not understood.

In summary, we provided new evidence that agonist and G protein may differently stabilize multiple active receptor conformations and that the existence of these active states is regulated by agonist concentration. These findings are relevant with respect to receptor theory. In

addition, we propose that $\alpha 4$ - $\alpha 4/\beta 6$ region of $Gt\alpha$ may play a role in guanine nucleotide exchange by affecting the conformation of the guanine nucleotide binding site.

FIGURE 1. Secondary structure of chimeric Gα subunits. Numbers on the right of the wild type forms of Gtα, Gi1α and Gqα indicate their total amino acid residues. Numbers within the designations for the chimeras indicate the number of amino acids switched between wild type Gα subunits. The diagram on the bottom depicts the secondary structural domains common to Gα subunits.

FIGURE 2. Concentration dependence of Gi1 in affinity shift assays for A1 adenosine (A) and M2 muscarinic (B) receptors. Sf9 cell membranes expressing the indicated Gi-coupled receptors were reconstituted with increasing concentrations of Gi1 heterotrimer. High affinity agonist binding was determined in the presence of 0.4 nM [^{125} I]-ABA for A1 adenosine receptors and 5 nM [^{3} H]-OXO-M for M2 muscarinic receptors. Typically, each reconstitution used 0.8 pmol of M2 receptors and 0.1 pmol of A1 receptors. The highest concentration of G proteins used is an estimated 380-fold excess over expressed receptors. Data were fit to a single-site interaction between receptor and G protein and are the mean $\pm$ SD of triplicate determinations from a representative experiment. The inset graphs show normalized affinity shift activities obtained with saturating concentrations of heterotrimers containing chimeric G α subunits expressed as the % of that obtained with a saturating concentration of Gi1. The final concentrations of receptors and G protein heterotrimers in the binding assay were 1.5 nM and 150 nM for M2 receptors respectively and 0.7 nM and 28 nM for A1 receptors respectively in a final volume of 150 μ l. For each receptor the EC₅₀ values did not differ significantly between Gi1 and Chi3. No significant affinity shift activity was observed with q6N35C with either receptor.

A**A1 Adenosine Receptor** **B****M2 Muscarinic Receptor**

FIGURE 3. Agonist competition binding against a fixed concentration of radiolabeled antagonist for A1 adenosine (A) and M2 muscarinic (B) receptors. Receptors were reconstituted with saturating amounts of the indicated G proteins (at least 200-fold excess over receptors) as shown in Figure 1 and binding was determined in a final volume of 500 μ l. Antagonist binding was determined in the presence of 0.8 nM [^{3} H]-DCPX for A1 adenosine receptors and 1.5 nM [^{3} H]-NMS for M2 muscarinic receptors and increasing concentrations of unlabeled agonists as indicated. Data were analyzed by nonlinear regression and an F-test was used to compare one-site vs two site fits. The Cheng-Prusoff correction was used to calculate K_D values. Agonist competition experiments with control membranes (no exogenous G proteins) were best fit to a one-site interaction model indicating that the small amount of receptor coupling with endogenous G proteins does not contribute significantly to the results (data not shown). The K_D values in control membranes were 48.9 nM (n=1) for the A1 adenosine receptor and 8.6 ± 2.2 μ M (n=2) for the M2 muscarinic receptor. These values did not differ significantly from the low affinity K_D obtained from the 2-site fits with the reconstituted membranes.

A

A1 Adenosine Receptor

B

M2 Muscarinic Receptor

FIGURE 4. Dose-response of receptor-catalyzed GDP/GTP exchange on G α subunits for A1 adenosine (A) and M2 muscarinic (B) receptors. Receptors were reconstituted with saturating amounts of the indicated G proteins. The fold-enhancement by agonist in the rate of GTP γ S binding (determined by linear regression) to each G α subunit at the indicated concentrations was determined as described in Methods and the data fit to a sigmoidal dose-response relationship. Final conditions were 50 nM G α , 0.8 nM receptor, 10 nM GDP, 10 nM GTP γ S (as a mixture of 1nM [^{35} S]-GTP γ S and cold GTP γ S), with or without agonist in a final volume of 50 μ l. *There was no significant agonist stimulation of GTP γ S binding to q6N35C by the A1 adenosine receptor and the data shown are adequately fit by linear regression with zero slope.

A

A1 Adenosine Receptor

B

M2 Muscarinic Receptor

FIGURE 5. Structural representation of different views of Gi1 $\alpha\beta_1\gamma_2$ heterotrimer. Different views of Gi1 heterotrimer are shown to illustrate the spatial proximity of the α_4 helix and GDP. Pictures were generated using RASMOL. Coordinates are from Wall et al. G β subunit is shown in red/orange and G γ subunit is shown in blue/green. The rest of the molecule is G α subunit. C terminus and α_5 helix are shown in yellow, β_6/α_5 loop ("TCAT" motif) is in green, α_4 - α_4/β_6 region (amino acids 299-319) is in red. Other guanine nucleotide binding regions are shown in white as indicated by Sprang (Sprang, 1997).

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CHAPTER IV. DISCUSSION

In recent years much progress has been made in understanding the mechanisms of G protein-coupled receptor (GPCR) action. However the molecular mechanisms controlling G protein-receptor coupling are still not completely understood. Based on the human genome sequence, the total number of human GPCRs is close to 750. This number includes 376 GPCRs for endogenous ligands (Vassilatis *et al.*, 2003), about 350 olfactory receptors and 30 other chemosensory receptors (Glusman *et al.*, 2001; Zozulya *et al.*, 2001). These GPCRs presumably couple with heterotrimeric G proteins composed from α , β and γ subunits. Many different heterotrimers can be produced from 20 α subunits, 6 β subunits and 12 γ subunits. Therefore, the question of selectivity in G protein-receptor coupling arises (Gudermann *et al.*, 1996; Wess, 1998). Intact organisms employ various mechanisms to regulate the signal flow through G protein-mediated pathways, including regulation of G protein and receptor expression levels (Vassilatis *et al.*, 2003), compartmentalization of signaling complexes, and the activity of accessory proteins (Neubig, 1994; Gudermann *et al.*, 1996; Wess, 1998; Albert and Robillard, 2002). The major goal of this project was to understand how the selectivity of G protein-receptor coupling is achieved at the level of the G protein-receptor interface, where the primary recognition is governed by the amino acid sequences of both G protein and receptor. This project was specifically aimed at determining the selectivity of G protein-muscarinic receptor coupling with additional goals to assess the generality of molecular mechanisms underlying G protein-receptor coupling and to understand how the selectivity of G protein-receptor coupling is regulated by agonist concentration. Dysfunctions of G proteins and receptors are implicated in a growing number of diseases (Iiri *et al.*, 1998) and uncovering the molecular mechanisms of G protein and receptor action will ultimately lead to better disease treatments (Farfel *et al.*,

1999; Levine, 1999; Spiegel, 1999). GPCRs are encoded by 1-3 % of the genes in human genome and are the targets of more than 30% of therapeutically used drugs (Wise *et al.*, 2002). However only 10% of all GPCRs are used as drug targets at present. Therefore, studying the mechanisms of G protein-receptor coupling may suggest new approaches in treating disease states and may help to develop new drugs that act at the G protein-receptor interface.

This study utilized the Sf9 cell membrane-based reconstitution system, which is well established and described in the literature (Figler *et al.*, 1996; Clawges *et al.*, 1997; Bae *et al.*, 1997; Bae *et al.*, 1999). Using this system, the coupling behavior of receptors of interest with exogenously supplied, purified G proteins can be studied. In contrast to cell transfection experiments, reconstitution experiments directly examine the G protein-receptor interface and allow controlled stoichiometries of components. Importantly, this system allows the analysis of both the ability of G proteins to stabilize the high affinity state of receptors for agonist and the ability of the receptors to catalyze guanine nucleotide exchange on G α . An important step toward completion of this project was the establishment of an M1-Gq reconstitution system which had not been accomplished before. Compared with Gi1, Gq is harder to purify in significant amounts because Gq is not soluble and needs to be purified as a detergent extract from Sf9 membranes (Kozasa and Gilman, 1995), while the soluble Gi1 can be purified from the Sf9 cytosol (Graber *et al.*, 1992). Additionally, in contrast to the M2 reconstitution system, M1 receptors require treatment with 6 M Urea in order to couple with exogenous G proteins. The successful use of urea treatment to achieve coupling has been reported before for different receptors (Hartman and Northup, 1996; Hellmich *et al.*, 1997; Lindorfer *et al.*, 1998). The mechanism of urea action is not clear. For Gi-coupled receptors Lim and Neubig demonstrated that membrane extraction with urea uncouples receptors from endogenous G proteins by

inactivating them (Lim and Neubig, 2001). However, endogenous Sf9 G proteins alone can not account for the inability of M1 receptors to couple to exogenous Gq without urea treatment because, as revealed by the careful examination, only a small per cent (~0.3 %) of receptors is precoupled to endogenous G proteins. Possibly, in addition to endogenous G proteins urea also removes some structural constraints which interfere with receptor coupling. Since the urea treatment was not required to establish M2-Gi1 reconstitution, inactivation of endogenous G proteins is not the only mechanism of urea action.

As discussed in Chapter III Part I, in addition to the expected Gq-M1 and Gi1-M2 receptor coupling, we also saw a low level of Gi1-M1 and Gq-M2 receptor coupling in the affinity shift assay (Figure 2, page 98). The basis for this coupling was explored with the use of pertussis toxin (Figure 4, page 100). Pertussis toxin is known to uncouple Gi/o proteins from their cognate receptors by ADP-ribosylating a C- terminal cysteine in Gi/o α subunits (West, Jr. *et al.*, 1985). My data demonstrated that M1 receptors have limited ability to couple to Gi1, while M2 receptors can not couple to Gq. The low level of M2-Gq coupling seen in Figure 2 (page 98) was attributed to the contamination of the Gq preparations with low levels of endogenous Gi. A similar contamination of Gq purified after expression in Sf9 cells by endogenous Gi/o proteins was reported by Kozasa (Kozasa and Gilman, 1995). The fact that endogenous Gi in the Gq preparations could not be detected by immunoblotting suggested that contamination levels were low. Contamination with endogenous Gi was only present in Gq and Gqi5C preparations, which were purified as detergent extracts from membranes as opposed to the Gi1 and other chimeras, which was purified as soluble protein from the cytosol. At present not much is known about Sf9 endogenous Gi. Fall armyworm *Spodoptera frugiperda* and tobacco hornworm *Manduca sexta* belong to the order *Obtectomera*. A sequence of the Go

protein from the *Manduca sexta* has been published (Horgan *et al.*, 1995) and contains the C terminal cysteine which is required for the PTX ribosylation. The Sf9 endogenous Gi is a substrate for the PTX ribosylation, as demonstrated in the Chapter III Part I. Based on the closeness of the species, we may speculate that the Sf9 endogenous Gi may have similar amino acid composition. As an interesting sideline observation, my data suggest that endogenous Gi from Sf9 cell membranes has different properties than recombinant Gi1. Data in Chapter II Table II (page 67) show that the high affinity state of M2 receptor stabilized by endogenous Gi1, which is present as contamination in the Gq preparation, has higher affinity for agonist (0.04 nM) than does the high affinity state stabilized by recombinant Gi1 (2.6 nM). The value for the high affinity state stabilized by recombinant Gi1 for Oxotremorine is in good agreement with the data of Sharif *et al.* for the high affinity state of a mixture of M1-M4 muscarinic receptors from animal tissues for Oxotremorine (2.5 nM) (Sharif *et al.*, 1995). Furthermore, Dong *et al.* indicate that affinities of muscarinic receptor subtypes for Oxotremorine are not significantly different from each other (Dong *et al.*, 1995).

Data presented in Chapter III Part I deal with the molecular mechanisms of M1 and M2 muscarinic receptor coupling with Gq and Gi1 proteins. As described in the Introduction, different G α domains have been shown to be important for receptor coupling. However, Chapter III Part I focuses on the role of the N- and C- termini of Gq α and Gi1 α in muscarinic receptor coupling. The C terminus of G α has been shown to interact with receptors using different experimental approaches including transfection experiments (Conklin *et al.*, 1993), synthetic peptide competition experiments (Hamm *et al.*, 1988), ADP-ribosylation of G α by pertussis toxin (West, Jr. *et al.*, 1985) and direct reconstitution studies (Natochin *et al.*, 2000). The C terminus of G α is critical for coupling to many receptors. However, as we show in Chapter III

Part I, the C terminus although critical (in the loss of function experiments), is not sufficient (in the gain of function experiments) for muscarinic receptor coupling (Figure 2, page 98). Conklin *et al.* have demonstrated in the cell transfection experiments that substitution of the last five C terminal residues of Gq α with corresponding sequences of Gi α , Go α or Gz α allowed PLC β stimulation by receptors normally coupled to Gi/o such as A1 adenosine and D2 dopamine receptors (Conklin *et al.*, 1993). The same was demonstrated later for M2 muscarinic receptor (Liu *et al.*, 1995). My data, that the C terminus of Gi1 α in the Gq α context is not sufficient for M2 receptor coupling, are in contrast with results of Liu, Conklin and others. While the precise reasons for such different observations in transfection versus reconstitution assays are unknown, several factors are likely to contribute. In transfection assays proteins of interest are overexpressed in a limited percentage of cells and coupling is inferred from effector activities which are substantially amplified at several points in the signal transduction cascade. On the other hand, all cellular components are present and the integration and assembly of membrane structures occurs in a cellular context. Reconstitution experiments directly examine the G protein-receptor interface and allow controlled stoichiometries of components, however they require the G protein to associate with the receptor after the receptor has been integrated into cellular membranes and may not allow the participation of all components responsible for receptor-G protein coupling. Thus, reconstitution experiments are likely to more precisely identify the G protein domains directly involved in the receptor - G protein interface, while transfection experiments are likely to identify the domains that permit coupling in a cellular context without revealing the precise mechanism or required stoichiometries. Ultimately both contribute to our understanding of coupling although neither may completely reflect the actual situation in living tissues. The fact that the C termini of Gq α and Gi1 α were not sufficient for

M1 and M2 receptor coupling indicated that other domains of G α are required for proper receptor coupling. However this conclusion is not universal, because as others and we have demonstrated, molecular determinants of G protein-receptor coupling differ among individual receptors and their cognate G proteins. For example, Natochin *et al.* reported that eleven C-terminal residues of Gt α in the Gs α context were sufficient for rhodopsin coupling in a reconstitution system (Natochin *et al.*, 2000).

The N terminus of G α has also been shown in variety of experiments to be a determinant of G protein-receptor coupling selectivity (Hamm *et al.*, 1988; Higashijima and Ross, 1991; Hepler *et al.*, 1996; Bae *et al.*, 1997; Swift *et al.*, 2000). Kostenis *et al.* demonstrated the importance of the N terminus of Gq α in constraining receptor coupling selectivity. They showed that deletion of six N- terminal amino acids of Gq α , which are unique for Gq family, led to promiscuous coupling so that several Gi/o and Gs coupled receptors gained the ability to productively couple with this Gq α mutant (Kostenis *et al.*, 1997). Kostenis *et al.* have suggested that the six N- terminal amino acids of Gq α (MTLESI) form a tightly folded protein subdomain that prevents Gi/o- and Gs-coupled receptors from accessing Gq α , thereby regulating coupling selectivity (Kostenis *et al.*, 1998). We demonstrated that the addition of the unique six amino acid N- terminal extension of Gq α to the N- terminus of Gi1 α neither permitted M1 receptor coupling, nor prevented M2 receptor coupling (Figure 2, page 98). Interestingly, a Gi1 α based chimera, q6N35C, containing both N- and C- terminal regions of Gq α , gained the ability to productively couple M1 receptors. This suggests that the proper context of both N- and C- termini is required for M1 muscarinic receptor coupling (Figure 2, page 98). Importantly, my data demonstrated that q6N35C chimera can couple to M1 muscarinic receptors in the affinity shift assay and be activated in the GDP/GTP exchange assay (Figure 5, page 102). Q6N35C

represents an easily purified, relatively stable, and soluble G α subunit with the ability to functionally couple with M1 muscarinic receptors. As a reagent it therefore offers significant advantages to native Gq α , which is difficult to purify, requires detergents for solubility and is easily inactivated by denaturation. Our data suggest that N- and C- termini of Gq α cooperate to couple M1 receptors. However, because the structural orientation of the extreme N- and C- termini in Gq is not known, and also because the crystal structure of a nucleotide-free G protein heterotrimer is not available, the mechanism of cooperativity between the extreme N- and C- termini of Gq α is not yet understood. It is also tempting to speculate that cooperation between appropriate N- and C- termini are also critical for Gi1-M2 muscarinic and, possible, other receptor coupling. Reconstitution studies in Chapter III Part II demonstrated a role for a poorly defined region within the first 210 amino acids of Gi1 α that is important for 5-HT_{1A} and 5-HT_{1B} serotonergic and M2 muscarinic receptor coupling (Chi21 in Figure 3, page 131). However, since only the six amino acid N-terminal extension that is unique to Gq α subunits was tested in this study such speculation may be premature and the structural determinants of coupling may differ significantly between receptors. In fact, as shown in Figure 3 (page 131) A1 adenosine receptor is different from the receptors mentioned above is that it does not distinguish between Gi1 α and Gt α sequences.

Chapter III Part II investigates the molecular determinants of Gi1-receptor coupling more precisely, making the emphasis on the role of the α 4 helix- α 4/ β 6 loop of Gi1 α in receptor coupling. The α 4 helix- α 4/ β 6 loop region of Gi1 α has been shown to be absolutely essential for 5-HT_{1B} serotonergic receptor coupling (Bae *et al.*, 1997). Our results demonstrated that the α 4 helix- α 4/ β 6 loop of Gi1 α is also critical for M2 receptor coupling (Chi3 in Figure 3, page 131) and that amino acids Gln304, Cys305 and Glu308 in the α 4 helix are especially critical for

coupling. The work of Natochin *et al.* points to the $\alpha 4/\beta 6$ loop as a receptor interacting domain and indicates that Arg310 and Asp311 are involved in the interaction with transducin (Natochin *et al.*, 1999). Mody *et al.* demonstrated that incorporation of $\alpha 4/\beta 6$ and $\alpha 5$ regions of $Gz\alpha$ into $G16\alpha$ improved recognition of resulting chimera by Gi/o-coupled receptors (Mody *et al.*, 2000). Our data in Part II demonstrated that $\alpha 4$ helix- $\alpha 4/\beta 6$ loop of $Gi1\alpha$ was also critical for 5-HT_{1A} receptor coupling (Chi3 in Figure 3, page 131). Interestingly, 5-HT_{1B} receptor in addition to the amino acids in the $\alpha 4$ helix uses Asp309 at the beginning of the $\alpha 4/\beta 6$ loop. The most intriguing finding in the Part II is that A1 adenosine receptor does not distinguish between the $\alpha 4$ helix- $\alpha 4/\beta 6$ loop region of $Gi1\alpha$ and $Gt\alpha$ (Figure 3, page 131). There are two possible explanations for this phenomenon. First, A1 adenosine receptor may not use the $\alpha 4$ helix- $\alpha 4/\beta 6$ loop region of $Gi1\alpha$ for coupling. In this case the coupling properties of A1 adenosine receptor are radically different from these of 5-HT serotonergic and M2 muscarinic receptors. Second, A1 adenosine receptor may use the $\alpha 4$ helix- $\alpha 4/\beta 6$ loop region for coupling but does not have preference for the amino acid sequence of this region. In fact, 9 from 21 amino acid difference in the $\alpha 4$ helix- $\alpha 4/\beta 6$ loop region between $Gi1\alpha$ and $Gt\alpha$ is sufficient to eliminate the coupling with 5-HT serotonergic and M2 muscarinic receptors but not with A1 adenosine receptors. To distinguish between these possibilities, other chimeric $G\alpha$ subunits need to be created and tested for the ability to couple with A1 adenosine receptor. From data in Part I we may conclude that because M1 muscarinic receptor gained a strong coupling with just the N- and C- termini of $Gq\alpha$, it is also not sensitive to the subtype specificity of the $\alpha 4$ helix- $\alpha 4/\beta 6$ loop region. However activity of q6N35C chimera in affinity shift assay with M1 receptor was lower than activity of Gq , indicating that other domains of $Gq\alpha$ may be important for M1 receptor coupling. Taken

together, these data lead to the discussion of the generality of molecular mechanisms underlying G protein-receptor coupling.

Other domains of $G\alpha$, in addition to those discussed above have been shown to be important for different receptors. Lee et al demonstrated that the $\alpha 2$ helix- $\alpha 2/\beta 4$ loop region in addition to the C terminus, which was not sufficient by itself, is important for C5a receptor activation by $G16\alpha$ subunits (Lee *et al.*, 1995). Krirgel-Brauer et al. demonstrated that amino acids 100-119 in the α helical domain of $Gs\alpha$ are a contact site for β -adrenergic receptor (Krieger-Brauer *et al.*, 1999). The $\alpha 3/\beta 5$ loop of $Gs\alpha$ has also been shown to interact with $\beta 2$ -adrenergic receptors (Grishina and Berlot, 2000). A recent report by Blahos *et al.* identified the $\alpha N/\beta 1$ loop as a novel site on $G\alpha_{16}$ which recognize metabotropic glutamate receptor 8 (Blahos *et al.*, 2001). Taken together, our data and data from other laboratories, indicate that multiple and distinct determinants of selectivity exist for individual receptor families. In Chapter III Part II we made an interesting observation regarding the critical role of the C terminus of $G\alpha$ for receptor coupling. We demonstrated that different amino acids within the C terminus of $Gi1\alpha$ are critical for different receptors (Figure 9, page 137). While 3 C terminal amino acids of $Gi1\alpha$ were important but not critical for 5-HT_{1A} and M2 receptor coupling, they were critical for 5-HT_{1B} and A1 adenosine receptor coupling. The 5 C terminal amino acids of $Gi1\alpha$ were critical for M2 receptor coupling, while more than 11 C terminal amino acids of $Gi1\alpha$ were critical for the 5-HT_{1A} receptor coupling. These differences may relate to the structure of the $Gi1\alpha$ C terminus. Although so far it has not been possible to successfully solve the structure of the $G\alpha$ C terminus in the context of the whole molecule (the C terminus is disordered in the crystal), the structure of the C- terminal decapeptide of the $Gt\alpha$ bound to rhodopsin has been resolved (Koenig *et al.*, 2002). In this C- terminal decapeptide, the first eight residues form an α helix which is

terminated by an α_L type C-cap (Aurora *et al.*, 1994) with C terminal glycine (Gly348 in Gt α , Gly352 in Gi1 α) in the center of the reverse turn (Kisselev *et al.*, 1998). This α_L C-cap structure might be critical for A1 adenosine and 5-HT_{1B} serotonin receptor coupling and important but not critical for M2 muscarinic and 5-HT_{1A} receptor coupling. Taken together, our results support the idea that different receptors may recognize a specific pattern of amino acids which form receptor recognition surfaces. Importantly, the domains of Gi1 α discussed herein are surface exposed and located on the G protein surface that is presumed to face the receptor. They are therefore available for receptor coupling. The spacefilling representation of the G $\alpha_i1\beta_1\gamma_2$ heterotrimer is shown in Figure 10 (page 138).

Another important observation that we have made regarding the generality of the mechanisms of G protein-receptor coupling deals with the apparent affinity of individual receptors for the same G protein. We showed that individual receptors distinguish themselves by the EC₅₀ with which they interact with Gi1 (Figure 1, page 129). A1 receptors have the highest apparent affinity (0.4 nM) and M2 receptors have the lowest apparent affinity (47 nM) for Gi1 heterotrimer. 5-HT receptors have intermediate EC₅₀ values (3.7 nM and 16.2 nM respectively). These results extend the earlier findings of Clawges *et al* with 5-HT serotonergic receptors (Clawges *et al.*, 1997). These data suggest that in living cells the expression levels of specific G protein subunits may regulate receptor coupling preferences. However we have to consider the existence of different mechanisms which regulate G protein-receptor coupling in the cell environment. Thus more studies are needed to test this hypothesis *in vivo*. Taken together, data indicate that coupling selectivity ultimately involves subtle and cooperative interactions among various domains on both the G protein and the associated receptor as well as the G protein concentration.

The initiating event in the activation of G protein mediated signaling is agonist binding to receptor. Therefore we also wanted to investigate how the agonist concentration might regulate G protein-receptor coupling. In Chapter III Part III we demonstrated that G proteins unable to couple receptors in the affinity shift assay, which uses a single low concentration of agonist, in fact formed a ternary complex at higher agonist concentration in competition binding assay (Figure 3, page 160). The functional coupling of individual G proteins to receptors at significantly different concentrations of agonist was also seen in GDP/GTP exchange assays (Figure 4, page 162). Interestingly, the A1 adenosine receptor was not able to catalyze the nucleotide exchange on the q6N35C chimera at any agonist concentration but was able to form a high affinity agonist binding state in agonist competition assay, indicating that G protein could bind to the receptor but could not be activated. In Part I we describe the case where M1 muscarinic receptor can couple Gi1 in the affinity shift assay but can not activate Gi1 the agonist-stimulated nucleotide exchange assay (see Figure 2, page 98 and Figure 5, page 102). These results indicate that it is possible to stabilize the high affinity state of receptor without stimulating guanine nucleotide exchange. Such discrepancies have been noted in a previous study with the 5-HT_{1B} receptor and a set of chimeric G α subunits (Bae *et al.*, 1999). Taken together these results indicate that there may be multiple active receptor conformations depending on both the nature of G protein with which receptor interacts and the agonist concentration.

The two-state modified ternary complex model is currently the most widely accepted receptor model (Samama *et al.*, 1993). According to this model, GPCRs exist in equilibrium between inactive R and active R* conformations. The R* conformation is stabilized by guanine nucleotide free G proteins and binds agonist with higher affinity than the R conformation.

Rapidly accumulating data suggest that multiple R* conformations of the same receptor subtype for different agonists may exist (Perez *et al.*, 1996; Krumins and Barber, 1997; Watson *et al.*, 2000; Waelbroeck, 2001; Ghanouni *et al.*, 2001; Kenakin, 2001; Gazi *et al.*, 2003). The idea that different agonists stabilize different active receptor conformations has been actively discussed and experimentally tested (Tucek, 1997; Kenakin, 1997; Kenakin, 2001). However in this scheme G proteins play rather a passive role. Earlier work of Clawges *et al.* demonstrated that different G α subunits (G α , Gi1 α , Gi2 α , Gi3 α) enhanced the high affinity [³H]-HT binding to 5-HT receptors to different extent, suggesting that actual affinity of the high affinity state can be different depending on the nature of the coupling G protein (Clawges *et al.*, 1997). Yang and Lanier demonstrated that G protein type have an effect on agonist efficacy by showing that the expression of G α 1 together with α 2 adrenergic receptor increased the relative efficacy of clonidine while Gi1 α did not (Yang and Lanier, 1999). Using full and partial agonists Wenzel-Seifert and Seifert demonstrated that G protein type have an effect on pharmacological properties of the receptor (Wenzel-Seifert and Seifert, 2000). They showed that efficacies of different β 2 adrenergic receptor ligands differ from each other depending on the G protein available for coupling. Furthermore, they found differences in the pharmacology of β 2 adrenergic receptor between its Gs, Gi and Gq coupled states. We propose the following mechanism by which G proteins can regulate receptor coupling. Receptor activation by agonist produces at least one activated R* state capable of coupling with G proteins. G proteins in turn can bind to receptor and produce an active receptor conformation that leads to GDP release and formation of a ternary complex with high affinity for agonist. If the G protein is unable to stabilize such a receptor conformation, the ternary complex will not form. If the receptor conformation in the ternary complex is not able to promote GTP binding to the G protein, G protein binding but not

activation will be seen, as in case of q6N35C and A1 adenosine receptors (Figure 3, page 160 and Figure 4, page 162) or in case of Gi1 and M1 muscarinic receptor. Thus different G proteins will stabilize different R* conformations. Importantly, the formation of these different R* may be regulated by agonist concentration in addition to the agonist structure. Using transferred nuclear Overhauser effect NMR spectroscopy, Dratz et al. detected the low-affinity interaction between inactive rhodopsin and C terminus of Gt α (Dratz *et al.*, 1993), supporting the idea that G proteins can bind to the receptor in the absence of agonist. Subsequently, agonist activation of receptor leads to high affinity interactions between G protein and receptor. Although the two-state model of receptor action is very useful as a model, it does not adequately explain the existence of multiple activated states of receptors. There are debates in the literature regarding the existence of the multiple R* species (Tucek, 1997; Kenakin, 1997; Strange, 1999; Kenakin, 2001). Our results support the idea of multiple active receptor conformations as opposed to the one active R* state and suggest that the two state receptor activation model needs to be changed.

In summary, this project presents interesting findings regarding the molecular mechanisms that regulate G protein-receptor coupling and helps us to understand better how the GPCRs function. Future studies should be aimed at assessing the generality of these findings and their applicability to the *in vivo* situation. Furthermore, studies investigating how the differences in the G protein-receptor coupling affect downstream signaling may suggest new ways in which signal transduction along the G protein-mediated pathways could be modulated.

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MEMBERSHIPS

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PUBLICATIONS

Slessareva JE and Graber SG: "Reconstitution reveals additional roles for N- and C- terminal domains of G α in muscarinic receptor coupling" *In revision for Biochemistry*

Slessareva JE, Depree KM, Ma H, Cabrera-Vera TM, Flood LA, Hamm HE and Graber SG: "Closely related G protein coupled receptors use multiple and distinct domains on G protein α subunits for selective coupling" *Submitted to J. Biol. Chem.*

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ABSTRACTS

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