Identification of Gβγ Binding Sites in the Third Intracellular Loop of the M₃-muscarinic Receptor and Their Role in Receptor Regulation*

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Gβγ binds directly to the third intracellular (i3) loop subdomain of the M₃-muscarinic receptor (MR). In this report, we identified the Gβγ binding motif and G-protein-coupled receptor kinase (GRK2) phosphorylation sites in the M₃-MR i3 loop via a strategy of deletional and site-directed mutagenesis. The Gβγ binding domain was localized to Cys²⁸⁹–His³³⁰ within the M₃-MR-Arg²⁵⁵–Gln⁴⁹⁰ i3 loop, and the binding properties (affinity, influence of ionic strength) of the M₃-MR-Cys²⁸⁹–His³³⁰ i3 loop subdomain were similar to those observed for the entire i3 loop. Site-directed mutagenesis of the M₃-MR-Cys²⁸⁹–His³³⁰ i3 loop subdomain indicated that Phe³¹², Phe³¹⁴, and a negatively charged region (Glu³²⁴–Asp³²⁸) were required for interaction with Gβγ. Generation of the full-length M₃-MR-Arg²⁵⁵–Gln⁴⁹⁰ i3 peptides containing the F312A mutation were also deficient in Gβγ binding and exhibited a reduced capacity for phosphorylation by GRK2. A similar, parallel strategy resulted in identification of major residues (S³³³SS³³³ and S³⁴⁴SASS³⁵¹) phosphorylated by GRK2, which were just downstream of the Gβγ binding motif. Full-length M₃-MR constructs lacking the 42-amino acid Gβγ binding domain (Cys²⁸⁹–His³³⁰) or containing the F312A mutation exhibited ligand recognition properties similar to wild type receptor and also effectively mediated agonist-induced increases in intracellular calcium following receptor expression in Chinese hamster ovary and/or COS-7 cells. However, the M₃-MRACys²⁸⁹–His³³⁰ and M₃-MR(F312A) constructs were deficient in agonist-induced sequestration, indicating a key role for the Gβγ-M₃-MR i3 loop interaction in receptor regulation and signal processing.

Signal efficiency/specificity for G-protein-coupled receptors is likely determined in part by accessory proteins found in the microenvironment of the receptor, which, together with the three core signaling entities (receptor, G-protein, and effector), contribute to the formation of a signal transduction complex at the cytoplasmic face of the receptor. The existence of such a complex is suggested by the detection of multimeric forms of G-protein subunits or receptors (1–4), the isolation of receptor or G-protein subunits together with some effectors (5–8), the existence of proteins that influence the activation state of G-proteins (9–19), and the identification of proteins interacting with G-protein subunits, receptor subdomains, or intact receptors (19–26). The dynamics of such a complex are not understood, and it is not known whether such a complex is preformed and stabilized by agonist binding to receptor or whether the agonist initiates the formation of a signal transduction complex de novo.

As an initial approach to identify components of this putative signal transduction complex, we designed two experimental systems. One strategy was based upon initial observations in our laboratory concerning the transfer of signal from R to G and focused on a functional readout involving G-protein activation (9, 10). This approach resulted in the partial purification and characterization of the NG108-15 G-protein activator and the “activators of G-protein signaling” group of proteins that activated heterotrimeric G-protein signaling pathways in the absence of a typical receptor (10–12). A second experimental approach utilized protein interaction technology to identify proteins that might exist within a putative signal transduction complex (20, 21). We initially focused on the large i3 loop of muscarinic receptors (MRs) and α₁-adrenergic receptors and used these domains as probes to screen bovine brain cytosol for interacting proteins. The first interacting protein identified by this approach was brain arrestin. The interaction of arrestins with G-protein-coupled receptors is a key component of signal termination.

The properties of arrestin binding to the i3 peptides were identical to those observed for an intact agonist-activated G-protein-coupled receptor (20). The latter observation is consistent with the hypothesis that when the large i3 loop is expressed free of the conformational constraints imposed by membrane spans, it assumes a conformation similar to that attained with receptor activation (20). The identification of arrestin as one of the cytosolic proteins interacting with the i3 peptide receptor subdomain underscores the potential utility of this experimental approach to identify additional interacting proteins that may contribute to the formation of a signal transduction complex. Subsequently, we extended this series of studies to evaluate the interaction of the i3 loop of M₂- and M₃-MR with brain G-protein. This work led to the surprising observation.

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‡The abbreviations used are: MR, muscarinic receptor; CHO, Chinese hamster ovary; [H]QNB, quinuclidinyl benzilate; [H]NMS, scopolamine methyl chloride; GST, glutathione S-transferase.
that Gβγ directly interacted with the i3 loop of the M₃r and M₅rRs (21). The binding of Gβγ to the i3 loop was inhibited by Ga and was required for effective phosphorylation of the i3 loop by the receptor kinase GRK2.

In this report, we identified the amino acid residues in the M₃-MR i3 loop required for Gβγ binding and phosphorylation by GRK2. Full-length M₃-MR lacking Gβγ binding motifs did not internalize in response to agonist, further indicating functional aspects of the Gβγ-M₃-MR i3 loop interaction. These observations are particularly interesting relative to a possible role for Gβγ as an adaptor protein in receptor trafficking and other aspects of signal processing.

**EXPERIMENTAL PROCEDURES**

**Materials**—HeterotrimERIC G-protein and Gβγ subunits were purified from bovine brain as described previously (21, 27). Antisera to the M3-MR construct lacking the Gβγ carboxyl-terminal 10 amino acids of Gβγ were kindly provided by Dr. Tom Bonner (Laboratory of Cell Biology, NIMH, National Institutes of Health, Bethesda, MD). Quinacridinyl benzilate (1-[benzilic acid 4, 4'-H] (H-QNB)) and scopolamine methyl chloride (H-N-methyl-H-H-QNMS) were purchased from NEN Life Science Products. Probenecid, carbachol and atropine were purchased from Molecular Probes Inc. (Eugene, OR). Superfect reagent was from Qiagen (Valencia, CA). LipoFectAMINE and Geneticin were obtained from Life Technologies, Inc. All other materials were obtained as described elsewhere (20, 21).

**Plasmid Constructions and Protein Expression**—The constructs encoding the full-length M₃-MR i3 peptide Arg252-Gln490 and the i3 loop subdomains Lys492-Thr498, Ser499-Leu500, Arg502-Glu508, Arg502-Ser505, Arg502-Ser505, and Cys508-His510 were generated by DNA amplification using the polymerase chain reaction and inserted into the BamHI and EcoRI restriction sites of the pGEX-4T-1 vector. The constructs encoding M₃-MR i3 loop amino acids Arg502-Gln409 and Val390-Gln409 were generated from the Arg525-Gln409 construct by taking advantage of a restriction site of the pGEX-4T-1 vector. The constructs encoding the full-length rat M₃-MR lacking Gβγ carboxyl-terminal 10 amino acids of Gβγ were generated as described (28). GRK2 was purified from SF9 insect cells infected with recombinant virus as described (29). Peptides corresponding to amino acids Gly308-Asp334 and Ser335-Ile356 of the M₃-MR were synthesized without amino or carboxyl modifications and purified by Biosynthesis Inc. (Leuwisville, TX). The full-length rat M₃-MR was kindly provided by Dr. Tom Bonner (Laboratory of Cell Biology, NIMH, National Institutes of Health, Bethesda, MD). GST and GST fusion proteins were eluted from the resin with 10 mM glutathione-Sepharose matrix as described previously (21). GST and GST fusion proteins were eluted from the resin with 10 mM glutathione-Sepharose matrix as described previously (21).

**Construction of M₃-MR Subdomains by GRK2**—The incubation conditions for phosphorylation reactions were essentially as described previously (21). Briefly, the reaction was carried out in a total volume of 50 μl of buffer (20 mM Tris-HCl, pH 7.2, 2 mM EDTA, 7 mM MgCl₂, 300 μM phosphatidylcholine, 140 mM NaCl, and 50 mM GRK2) containing Gβγ (120 nm) and the GST-M₃-MR subdomain fusion proteins (40–80 nm) until terminated. Under these incubation conditions, the stoichiometry of phosphorylation was 0.8–1.5 mol of phosphate/mol of peptide for the full-length M₃-MR i3 loop. Reactions were initiated by addition of 0.1 μM [3H]-ATP (500–1000 cpmm/μl), incubated at 30 °C for 30 min and terminated by the addition of 50 μl of 2% Laemmli sample buffer and subsequent electrophoresis on 10% SDS-polyacrylamide gels. The gels were dried and exposed to Kodak XAR-5 film for 1–12 h. Phosphorylated species were cut from the dried gels, and subsequent phosphorylation was quantitated by liquid scintillation spectrometry.

**Receptor Expression and Characterization**—COS 7 cells were grown on Falcon Primaria plates at 37 °C (5% CO₂) in Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter), supplemented with 10% fetal bovine serum plus penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). CHO cells were grown on Falcon tissue culture dishes at 37 °C (5% CO₂) in Ham's F-12 medium supplemented with 10% fetal bovine serum plus penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). For transient receptor expression, COS 7 or CHO cells at 70–80% confluency (100-mm dish) were transfected with 10 μg of M₃-MR, M₃-MRAcys289–His310, or M₃-MR(F312A) in pcDNAs by the DEAE-dextran method (30) or using Superfect reagent (Qiagen) in accordance with the manufacturer's instructions. Cells were harvested for membrane preparation 72 h after transfection for analysis in competition and saturation binding studies as described previously (30). To generate cell lines stably expressing the receptor constructs, CHO cells in six-well plates (70% confluent) were transfected with 5 μg of M₃-MR, M₃-MRAcys289–His310, or M₃-MR(F312A) in pcDNAs by the DEAE-dextran method (30) or using Superfect reagent (Qiagen) in accordance with the manufacturer's instructions. The entire system was controlled by a Windows NT-based software interface. The 488 nm argon line at 100 mW was used as an excitation source. Stable CHO transfectants or transiently transfected CHO cells (24 h following transfection) were seeded in 96-well clear-bottomed black microplates (Corning Costar Corp., Cambridge, MA) 18 h before the experiments. On the day of assay, 25 μl aliquots (one for each microplate) of 2 mM Fluo-3 AM ester (Molecular Probes Inc.) containing 20 mM Hepes, pH 7.4, 2.5 mM probenecid, and 1% fetal bovine serum. The final Fluo-3 concentration in the dye-loading buffer was washed three times with 0.5 ml of Buffer A, and the retained proteins were solubilized in Laemmli sample buffer for denaturing gel electrophoresis (10% polyacrylamide). Polyvinylidene difluoride membrane filters were evaluated by immunoblotting with anti-Gβγ antibody. In each experiment, the membrane filters used for immunoblotting where blocked for 1 h with 3% bovine serum albumin and incubated with GST fusion proteins or either GST fusion proteins by either immunoblotting with anti-GST antibody or amido black staining of proteins to control for sample processing in individual experiments. Unless stated otherwise, the concentration of Gβγ used in the protein interaction assays was 30 nm.

**Phosphorylation of M₃-MR Subdomains by GRK2**—The incubation conditions for phosphorylation reactions were essentially as described previously (21). Briefly, the reaction was carried out in a total volume of 50 μl of buffer (20 mM Tris-HCl, pH 7.2, 2 mM EDTA, 7 mM MgCl₂, 300 μM phosphatidylcholine, 140 mM NaCl, and 50 mM GRK2) containing Gβγ (120 nm) and the GST-M₃-MR subdomain fusion proteins (40–80 nm) until terminated. Under these incubation conditions, the stoichiometry of phosphorylation was 0.8–1.5 mol of phosphate/mol of peptide for the full-length M₃-MR i3 loop. Reactions were initiated by addition of 0.1 μM [3H]-ATP (500–1000 cpmm/μl), incubated at 30 °C for 30 min and terminated by the addition of 50 μl of 2% Laemmli sample buffer and subsequent electrophoresis on 10% SDS-polyacrylamide gels. The gels were dried and exposed to Kodak XAR-5 film for 1–12 h. Phosphorylated species were cut from the dried gels, and subsequent phosphorylation was quantitated by liquid scintillation spectrometry.

To evaluate receptor-effector coupling, we determined the ability of agonist to increase intracellular calcium following transient and stable expression of M₃-MR, M₃-MRAcys289–His310, or M₃-MR(F312A) in CHO cells using the fluorometric imaging plate reader system (Molecular Devices Corp., Sunnyvale, CA) (31, 32). The instrument included a temperature controller, and a 488 nm argon laser (Coherent Inc., Santa Clara, CA), a scanner with a proprietary optical system, an integrated 96-tip pipettor (to add test agents from two separate microplates), a temperature controller, and a CCD camera. The entire system was controlled by a Windows NT-based software interface. The 488 nm argon line at ~300 mW laser power was used as an excitation source. Stable CHO transfectants or transiently transfected CHO cells (24 h following transfection) were seeded in 96-well clear-bottomed black microplates (Corning Costar Corp., Cambridge, MA) 18 h before the experiments. On the day of assay, 25 μl aliquots (one for each microplate) of 2 mM Fluo-3 AM ester (Molecular Probes Inc.) in dimethyl sulfoxide were mixed with an equal volume of 20% plumeric acid and then diluted with Hanks’ balanced salt solution (without phenol red; Life Technologies, Inc.) containing 20 mM Hepes, pH 7.4, 2.5 mM probenecid, and 1% fetal bovine serum. The final Fluo-3 concentration in the dye-loading buffer.
was 4 μM. Cells were dye-loaded for 1 h at 37 °C in a 5% CO₂ incubator by replacing the growth medium with 100 μl of the dye-loading buffer. Cells were then washed four times with loading buffer lacking dye on a Danley plate washer and transferred to the fluorometric imaging plate reader system. During a data run, cells in different wells were exposed to different concentrations of carbachol, and the system recorded fluorescent signals for all 96 wells simultaneously in subsecond range intervals. Increases in intracellular calcium were observed as sharp fluorescent signals for all 96 wells simultaneously in subsecond range intervals. Increases in intracellular calcium were observed as sharp fluorescent signals for all 96 wells simultaneously in subsecond range intervals.

Intracellular domains of the M3-MR (Fig. 1B, right panel) were generated as GST fusion proteins and evaluated in GRK2 phosphorylation and/or Gβγ binding assays as described under “Experimental Procedures.” B, left panel, purified GST-M3-MR subdomain fusion proteins (2–4 pmol) were incubated with GRK2 (50 nM) and phosphatidylinositol (300 μM), and samples were processed for SDS-PAGE/autoradiography. Right panel, GRK2-mediated phosphorylation of the GST M3-MR-Arg252–Gln490 fusion protein was determined in the presence of heterotrimeric brain G-protein (120 nM) with added nucleotide (20 μM) and compared with the results of similar experiments conducted with Gβγ with no added nucleotide. G-protein was preincubated with nucleotide for 10 min at 24 °C. GST itself was not phosphorylated by GRK2 under any incubation conditions. C, the full-length M3-MR and two subdomains were evaluated for GRK2-mediated phosphorylation (autoradiograph) as described in B and for their ability to bind Gβγ (immunoblot) as described previously (21). Std, 100 ng Gβγ. Each series of experiments was repeated 2–4 times with similar results. V and VI in the left panel of C indicate the putative fifth and sixth membrane spans of the receptor, respectively.

Fig. 1. Phosphorylation of M3-MR subdomains by GRK2. Intracellular domains of the M3-MR (A) were generated as GST fusion proteins and evaluated in GRK2 phosphorylation and/or Gβγ binding assays as described under “Experimental Procedures.” B, left panel, purified GST-M3-MR subdomain fusion proteins (2–4 pmol) were incubated with GRK2 (50 nM) and 0.1 mM γ-ATP in the presence of Gβγ (120 nM) and phosphatidylinositol (300 μM), and samples were processed for SDS-PAGE/autoradiography. Right panel, GRK2-mediated phosphorylation of the GST M3-MR-Arg252–Gln490 fusion protein was determined in the presence of heterotrimeric brain G-protein (120 nM) with added nucleotide (20 μM) and compared with the results of similar experiments conducted with Gβγ with no added nucleotide. G-protein was preincubated with nucleotide for 10 min at 24 °C. GST itself was not phosphorylated by GRK2 under any incubation conditions. C, the full-length M3-MR and two subdomains were evaluated for GRK2-mediated phosphorylation (autoradiograph) as described in B and for their ability to bind Gβγ (immunoblot) as described previously (21). Std, 100 ng Gβγ. Each series of experiments was repeated 2–4 times with similar results. V and VI in the left panel of C indicate the putative fifth and sixth membrane spans of the receptor, respectively.

RESULTS

Phosphorylation of the M3-MR i3 Loop by GRK2—We reported previously the direct interaction of Gβγ with the i3 loop of the M3-MR and M5-MR, the requirement of this interaction for phosphorylation of the i3 peptide by the receptor kinase GRK2, and the interaction of these three proteins within a ternary complex (21). We initiated a series of experiments to identify the Gβγ binding site in the i3 peptide, its spatial relationship to residues phosphorylated by GRK2, and the role of this domain in signal processing. We first asked whether GRK2 phosphorylated intracellular domains of the M3-MR other than the i3 loop. Only the i3 loop of the receptor was phosphorylated by GRK2 (Fig. 1A and B, left panel), and this observation parallels the protein interaction data previously published (21) indicating that Gβγ also did not interact with the i1, i2, or carboxyl-terminal region of the M3-MR. Both the interaction of Gβγ with the M3-MR i3 loop and GRK2-mediated phosphorylation of the i3 loop preferred a Gβγ subunit free of Go (Fig. 1B, right panel), consistent with the concept that the interaction of Gβγ with the M3-MR i3 peptide and its putative role in positioning GRK2 on its substrate is dependent upon receptor-G-protein activation.

We then prepared and evaluated receptor subdomain constructs from the i3 loop in Gβγ binding and phosphorylation
assays (Fig. 1C). The M₃-MR i3 loop contains 53 Ser/Thr residues, and there are 6 Ser/Thr-rich regions (285SSRSCSS291, 315TTKS318, 331SSS333, 348SASS351, 439TSDTNSS445, and 449TTAT452) (Fig. 2A). Both the Gbg binding domain and the primary GRK2 phosphorylation sites are found in the amino-terminal half of the M₃-MR i3 loop (Fig. 1C). Phosphorylation of the amino-terminal half of the i3 loop (Arg 252–Gln389) was comparable to that of the entire i3 loop. There are four Ser/Thr-rich motifs in the Arg252–Gln490 segment of the i3 loop (Ser285–Ser291, Thr315–Ser318, 331SSS333, and 348SASS351) (Fig. 2A). Two of these serine-rich sequences (331SSS333 and 348SASS351) are inserted between acidic residues. Acidic residues at the amino terminus of a serine-rich region are important for phosphorylation by GRK2 (35–37). Acidic residues at the carboxyl terminus of the serine-rich regions are important for the action of rhodopsin kinase, although such a positioning of these amino acids may actually inhibit phosphorylation by GRK2 (35). To determine whether these serine motifs are phosphorylated by GRK2, we mutated the serine-rich motifs in the context of the full-length M₃-MR i3 loop. Mutation of either serine-rich motif in M₃-MR-Arg252–Gln490 resulted in reduced GRK2-mediated phosphorylation (331SSS333 to 331AAA333, 45% reduction, and 348SASS351 to 348AAAA351, 34% reduction), and mutation of both serine-rich motifs reduced phosphorylation by ~75% (Fig. 2B). Synthetic peptides encompassing either serine cluster were also evaluated as substrates for GRK2. The Gly308–Asp334 peptide was phosphorylated by GRK2, and it inhibited GRK2-mediated phosphorylation of the full-length M₃-MR i3 loop (IC₅₀ ~ 40 μM) (Fig. 2C).² In contrast, the Ser335–Ile356 peptide was not phosphorylated by GRK2 and did not inhibit GRK2 phosphorylation of the full-length M₃-MR i3 loop.

Localization of Gbg Binding Motifs in the M₃-MR i3 Loop—The M₃i3-II domain containing the Gbg binding motif was further truncated at the amino and/or carboxyl terminus to generate constructs M₃-MR-IV–VIII (Fig. 3A). Each construct was evaluated for Gbg binding. This strategy localized the Gbg binding motif to Cys289–His330 within the M₃-MR i3 loop (Fig. 3B). The binding affinity and influence of ionic strength were identical for the Cys289–His330 subdomain and the full-length i3 loop. Gbg binding to the full-length i3 loop and the Cys289–His330 subdomain increased at lower concentrations of NaCl (0–70 mM) and subsequently decreased at higher concentrations of NaCl (140–800 mM) (Fig. 3C). Of particular note is that the GRK2 phosphorylation sites (331SSS333 and 348SASS351) in the M₃-MR i3 loop are adjacent to the Gbg binding motif (Fig. 3D), consistent with the hypothesis concerning the role of Gbg in positioning GRK2 on its substrate (21).

Amino Acid Residues Required for Gbg Binding—The Gbg binding domain Cys289–His330 in the i3 loop of M₃-MR possesses a grouping of glutamate residues (296QQQ297), a positively charged motif (300KRSSRRR306), a hydrophobic cluster (312FWF314), and a negatively charged cluster (324EQM-

² G. Wu and S. M. Lanier, unpublished observations.
DQD^{329}) of amino acids (Fig. 4A). To define the amino acid residues in this domain required for Gβγ binding, we used modified alanine scanning mutagenesis to generate 15 mutants (6 multiple residue mutations (Y292A, Y293A, L294A, Y307A, R309A, H311A, K317A, W319A, and K320A)) in the Gβγ binding domain Cys^{289}–His^{330}. GST fusion proteins encoding wild type or mutated Cys^{289}–His^{330} peptide were purified, and their abilities to interact with Gβγ were assessed. Five of the 15 mutants altered Gβγ binding (Fig. 4B). The Y292A and Y307A mutations reduced Gβγ binding. Mutation of the hydrophobic (E324WF^{314} to AAA) or acidic (E324EQDQD^{329} to AQMAQA) site and GRK2 phosphorylation sites on the Cys^{289}–His^{330} i3 loop subdomain were compared with the M3-MR Arg^{252}–Gln^{490} i3 loop peptide. Similar data were obtained in two experiments. Data for the M3-MR Arg^{252}–Gln^{490} in the left panel were taken from Ref. 21. D, location of Gβγ binding sites and GRK2 phosphorylation sites on the M3-MR Arg^{252}–Gln^{490} i3 loop peptide.

Characterization of Full-length M3-MR Lacking Gβγ Binding Motifs—As an initial approach to determining the functional consequences of Gβγ interaction with the M3-MR i3 loop, we generated full-length receptor constructs containing two structurally distinct modifications (deletion of Cys^{289}–His^{330} and the F312A muta) that resulted in a loss of Gβγ binding to the i3 loop subdomain. We then used transient and/or stable transfection strategies with each receptor construct to determine their ability to couple to signaling pathways and to undergo internalization in response to agonist exposure. Radioligand binding studies in transiently transfected COS7 cell membranes and stably transfected CHO cell membranes indicated that the M3-MRΔCys^{289}–His^{330} and the M3-MR(F312A) were identical to wild type M3-MR with respect to receptor binding to the peptide Cys^{289}–His^{330} were also important in the context of the entire i3 loop, we generated the M3-MR Arg^{252}–Gln^{490}(F312A) construct and evaluated the protein in Gβγ binding assays as a substrate for GRK2 (Fig. 5). Compared with wild type M3-MR i3 loop peptide, the M3-MR Arg^{252}–Gln^{490}(F312A) peptide was dramatically impaired in its ability to interact with Gβγ. The M3-MR Arg^{252}–Gln^{490}(F312A) peptide deficient in Gβγ binding was also a poorer substrate for phosphorylation mediated by GRK2 in the presence of Gβγ (Fig. 5B). Both the initial rate and the stoichiometry of GRK2-mediated phosphorylation of the F312A mutant were reduced compared with wild type. The K_{m} for GRK2-mediated phosphorylation of the i3 loop increased from 72 to 167 nM upon mutation of the Gβγ binding site, whereas the V_{max} was unaltered. These data suggested that Gβγ binding to the M3-MR i3 loop enhanced phosphorylation by lowering the K_{m} of GRK2 for the substrate, as described previously for GRK2-mediated phosphorylation of the agonist-activated, purified β_2-adrenergic receptor (38).
expression levels as well as antagonist (3H-QNB) and agonist (carbachol) affinity (Fig. 6).3 As one index of receptor-effector coupling, we determined the ability of the M3-MR Cys289–His330 i3 loop subdomain. A, site-directed mutagenesis identifies key residues required for interaction of Gβγ with the M3-MR Cys289–His330 i3 loop subdomain. A, sequence of Gβγ binding domain in M3-MR i3 loop. Site directed mutants were generated using the Stratagene Quick Change Kit and mutations confirmed by sequence analysis. Each mutant was expressed/purified as GST fusion proteins and used in Gβγ binding assays as described (21). Gβγ retained on the affinity matrices was determined by immunoblotting with Gβ selective antisera (B and C). Aliquots of purified fusion proteins corresponding to relative amounts used in the interaction assay were also evaluated by Coomassie Blue staining, indicating that each of the mutant proteins was successfully made. Experiments were repeated 3–5 times with similar results. Lane std in C corresponds to 30% of total Gβγ incubated with the i3 peptides. wt, wild type.

3 G. Bogatkevich and S. M. Lanier, unpublished observations.

\[ \text{Expression levels as well as antagonist (} ^ {3} \text{H-QNB}) \text{ and agonist (carbachol) affinity (Fig. 6).} \]

\[ \text{As one index of receptor-effector coupling, we determined the ability of the } M_{3}\text{-MR} \text{ Cys289–His330 } i_{3}\text{ loop subdomain. } A, \text{ site-directed mutagenesis identifies key residues required for interaction of } G\beta\gamma \text{ with the } M_{3}\text{-MR } i_{3}\text{ loop. Site directed mutants were generated using the Stratagene Quick Change Kit and mutations confirmed by sequence analysis. Each mutant was expressed/purified as GST fusion proteins and used in } G\beta\gamma \text{ binding assays as described (21). } G\beta\gamma \text{ retained on the affinity matrices was determined by immunoblotting with } G\beta \text{ selective antisera (B and C). Aliquots of purified fusion proteins corresponding to relative amounts used in the interaction assay were also evaluated by Coomassie Blue staining, indicating that each of the mutant proteins was successfully made. Experiments were repeated 3–5 times with similar results. Lane std in C corresponds to 30% of total } G\beta\gamma \text{ incubated with the } i_{3}\text{ peptides. wt, wild type.} \]
receptors (8, 39–41). Gβγ similarly interacts with multiple signaling proteins, including calcium channels, GRK2, selected proteins containing pleckstrin homology domains, G-proteingated inwardly rectifying potassium channels, Bruton’s tyrosine kinase, adenylyl cyclase type II, and activator of G-protein signaling 2. Activator of G-protein signaling 2 is a mammalian protein recently identified as a receptor-independent activator of the pheromone response pathway in Saccharomyces cerevisiae (11). Gβγ binding motifs were identified in several of these proteins, and none of these motifs are found in the Cys289–His330 region of the M3-MR i3 loop that binds Gβγ. The ability of Gβγ to interact with multiple proteins involved in signal processing indicates that Gβγ is capable of anchoring the formation of a signal transduction complex. Indeed, the interaction of Gβγ with GRK2 and the M3-MR i3 loop results in the formation of a ternary complex (21), as suggested several years ago based upon work with the purified β2-adrenergic receptor, G-protein, and receptor kinase (38). These data and other observations cited earlier (1–26) all support the concept of multicomponent signal transduction complexes for G-protein-coupled receptors.

A direct interaction of Gβγ with a G-protein-coupled receptor was also observed in the visual transduction system involving the photon receptor rhodopsin, where it was suggested to play an important role in signal amplification and/or receptor regulation (42–44). Additional data indicate that even within the context of G-protein heterotrimers, a receptor may make contact with all three G-protein subunits (45–49). If so, then it is unclear whether the Gβγ binding site identified in the i3 loop of the M3-MR in the present study is also involved in the interaction between receptor and Gβγ in G-protein heterotrimers. Based upon the observation that the interaction of the M3-MR with Gβγ requires dissociation of Gα and Gβγ, it is likely that there are actually two sites on the receptor for interaction with Gβγ: one Gβγ binding site that is operative within the context of the heterotrimer and another for free Gβγ.

The M3-MR is phosphorylated by GRK2 and/or casein kinase 1α in an agonist-dependent manner (50–52) and GRK2-mediated phosphorylation of the membrane-bound M3-MR expressed in the S99 cell line is enhanced by Gβγ (50). Although a number of G protein-coupled receptors are phosphorylated by GRK2 in a βγ-dependent fashion, the molecular basis for the involvement of Gβγ is unclear. The role of Gβγ as a “stimulator” of agonist-dependent receptor kinases for the muscarinic receptor was initially observed by Haga and Haga (53). The Vmax for phosphorylation of activated rhodopsin by highly enriched kinase preparation was increased 12-fold in the presence of Gβγ (53). Subsequent kinetic studies with purified β2-adrenergic receptor kinase (GRK2) and β2-adrenergic receptor indicated that the Gβγ enhancement of receptor phosphorylation was likely due to the ability of Gβγ to decrease the Kcat of GRK2 for receptor (38). The following points suggest that the interaction of Gβγ with the M3-MR i3 loop provides a mecha-

**Fig. 6.** Characterization of M3-MR lacking Gβγ interaction motifs. Wild-type M3-MR and M3-MR constructs lacking the 42-amino acid Gβγ binding domain (M3-MRΔCys289–His330) or containing the F312A mutation (M3-MR(F312A)) were expressed in COS-7 cells and evaluated in radioligand binding assays. Left panel, membranes (25 μg) from transfected COS7 cells were incubated with increasing concentrations of the muscarinic agonist [3H]-QNB (0.05–32 nM). (M3-MR Bmax = 4.24, M3-MR(F312A) Bmax = 4.24, M3-MRΔCys289–His330 Bmax = 4.49 pmol/mg of membrane protein) ([3H]-QNB affinity: M3-MR Kd = 1.24 nM, M3-MR(F312A) Kd = 1.36 nM, M3-MRΔCys289–His330 Kd = 1.30 nM). Right panel, membranes (25 μg) from transfected COS7 cells were incubated with [3H]-QNB (2 nM) and increasing concentrations of the muscarinic agonist carbachol (carbachol IC50 = 251 μM; M3-MR(F312A) IC50 = 281 μM; M3-MRΔCys289–His330 IC50 = 312 μM). Data are representative of two experiments using different transfected cell preparations.

**Fig. 7.** Agonist-induced increases in intracellular calcium mediated by M3-MR lacking Gβγ interaction motifs. The ability of agonist to increase intracellular calcium was determined following transient (A) and stable (B) expression of M3-MR, M3-MRΔCys289–His330 or M3-MR(F312A) in CHO cells. Stable CHO transfectants or transiently transfected CHO cells (24 h following transfection) were seeded (~50,000 cell/well) in 96-well microplates and loaded with Fluo-3 AM ester as described under “Experimental Procedures.” Intracellular calcium levels were determined with the fluorometric imaging plate reader. The data in A are presented as the average of three experiments with receptors densities of 1–2 pmol/mg of membrane protein for each receptor construct (carbachol EC50: M3-MR EC50 = 180 nM; M3-MR(F312A) EC50 = 200 nM; M3-MRΔCys289–His330 EC50 = 270 nM). For stable transfectants, we evaluated several individual cell lines (receptor densities, 5–8 pmol/mg membrane protein) and the results from two independent cell lines (indicated by #) for M3-MR, M3-MRΔCys289–His330, or the M3-MR(F312A) transfectants are presented in B (carbachol EC50: M3-MR 6 EC50 = 27 nM; M3-MR 7 EC50 = 25 nM; M3-MR(F312A) 6 EC50 = 12 nM; M3-MR(F312A) 11 EC50 = 18 nM; M3-MRΔCys289–His330 15 EC50 = 23 nM; M3-MRΔCys289–His330 23 EC50 = 18 nM). The experiments for B were repeated three times for each transfected cell line with similar results.
mism for translocation of GRK2 positioning the enzyme upon its substrate, the activated receptor: 1) G\(\beta\gamma\) binds to discrete regions of the M3-MR i3 loop that are in close proximity to major phosphorylation sites in the M3-MR; 2) G\(\beta\gamma\) is required for GRK2-mediated phosphorylation of the i3 peptide; 3) G\(\beta\gamma\) mediates the formation of a ternary complex consisting of the M3-MR i3 loop, G\(\beta\gamma\), and GRK2; and 4) disruption of the G\(\beta\gamma\) binding site in the M3-MR i3 loop peptide compromises GRK2-mediated phosphorylation of the i3 loop. These data are all consistent with the idea that G\(\beta\gamma\)-i3 loop interactions play an important role in GRK2-mediated phosphorylation. However, it is difficult to completely eliminate the possibility that the binding of G\(\beta\gamma\) to GRK2 induces a conformational change in GRK2 revealing a “receptor binding” domain on the enzyme that also contributes to positioning of the enzyme upon its substrate.

The marked reduction in agonist-induced receptor sequestration observed in the M3-MR constructs lacking G\(\beta\gamma\) binding motifs may be due to impaired phosphorylation of the receptor by GRK2. Disruption of the G\(\beta\gamma\) binding motif did not alter the ability of the receptor to mediate agonist-induced increases in intracellular calcium, and thus the observed changes in receptor internalization in the receptor constructs lacking the G\(\beta\gamma\) binding motif are not due to any gross alteration in receptor-G-protein coupling. One postulate is that the interaction of G\(\beta\gamma\) with the receptor i3 loop positions GRK2 upon its substrate, allowing phosphorylation to occur and the internalization process to begin. Alternatively, the interaction of G\(\beta\gamma\) with the M3-MR i3 loop may be part of a sequestration pathway that does not involve GRK2 phosphorylation (54). It is also not known whether the putative interaction of G\(\beta\gamma\) with the M3-MR i3 loop is transient or whether the G-protein subunit actually travels with the receptor through a recycling process. The precise pathway and efficiency of receptor internalization are likely cell type-specific and involve important stoichiometric considerations (55–61). Mutation of the serine cluster (548SASS\(^{551}\)) in the M3-MR i3 loop (548SASS\(^{552}\) in the human M3-MR) just downstream of the G\(\beta\gamma\) binding motif also results in a loss of receptor internalization in response to agonist exposure (33, 52). Thus, disruption of either a GRK2 phosphorylation site (548SASS\(^ {551}\)), or the G\(\beta\gamma\) binding motif in the i3 loop peptide lead to impaired receptor regulation. However, larger deletions of the i3 loop (Ala\(^{274}\)-Lys\(^{396}\)) are without effect on agonist-induced internalization of the receptor (3, 62). Thus, minimal disruption of the G\(\beta\gamma\) binding motif results in modifications in receptor regulation that are not observed when the same region is removed, together with a larger portion of the i3 loop. These data suggest that the interaction of G\(\beta\gamma\) with the i3 loop and/or receptor phosphorylation by GRK2 counteract an “internalization inhibitory factor” that involves amino acids Ser\(^{331}\)-Lys\(^{396}\). Based upon this hypothesis, the interaction of G\(\beta\gamma\) with the i3 loop and/or receptor phosphorylation by GRK2 would not be required for agonist-induced receptor internalization when the Ser\(^{331}\)-Lys\(^{396}\) region of the i3 loop is absent. Such an internalization inhibitory factor may reflect conformational issues within the i3 loop itself or the association of the i3 loop with accessory proteins in the receptor’s microenvironment. The interaction of G\(\beta\gamma\) with the i3 loop of the M3-MR and other G-protein-coupled receptors may also play a role in signal processing distinct from receptor sequestration. Several G-protein-coupled receptors are connected to mitogenic signaling pathways and/or control mechanisms for cellular architecture. The molecular interactions that allow these receptors to interface with these pathways is unresolved. Perhaps G\(\beta\gamma\) acts as a docking protein within a larger signal transduction complex and allows the interface of selected G-protein-coupled receptors to such signaling pathways that involve soluble tyrosine kinases and/or low molecular weight G-proteins.

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