Kinetic Analysis of M₂ Muscarinic Receptor Activation of Gi in Sf9 Insect Cell Membranes*

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A steady-state kinetic mechanism describing the interaction of M₂ muscarinic acetylcholine receptors and the guanine nucleotide-binding protein Gₐ₅β₂γ₃ is presented. Data are consistent with two parallel pathways of agonist-promoted GTPase activity arising from receptor coupled to a single or multiple guanine nucleotide-binding proteins. An aspartate 103 to asparagine receptor mutation resulted in a receptor lacking the ability to catalyze the binding of guanosine-5'-O-(3-thiotriphosphate) or guanosine triphosphate hydrolysis by the G protein. An aspartate 69 to asparagine receptor mutant was able to catalyze agonist-specific guanine nucleotide exchange and GTPase activity. A threonine 187 to alanine receptor mutation resulted in a receptor that catalyzed guanine nucleotide exchange comparable with wild-type receptors but had reduced ability to stimulate GTP hydrolysis. A tyrosine 403 to phenylalanine receptor mutation resulted in an increase in agonist-promoted GTPase activity compared with wild type. The observation that the threonine 187 and tyrosine 403 mutants promote guanine nucleotide exchange similarly to wild type but alter GTPase activity compared with wild type suggests that the effects of the mutations arise downstream from guanine nucleotide exchange and may result from changes in receptor-G protein dissociation.

The activation of a G protein by an agonist-ligated receptor molecule consists of at least four steps as follows: 1) a conformational change in the receptor that transduces the ligand-binding signal from the ligand-binding site to the intracellular receptor:G protein interface; 2) a conformational change at the receptor:G protein interface that communicates the ligand-binding signal from the receptor to the G protein; 3) a conformational change in the G protein that promotes GDP/GTP exchange; and 4) a conformational change in the quaternary structure of the G protein that promotes the dissociation of the G protein α and βγ subunits and allows their interaction with effectors.

G protein coupling has been examined with mACHRs and other cationic amine-binding GPCRs. Most of these studies examine the coupling of heterologously expressed receptors through the activation of cellular effectors by endogenous G proteins. The primary limitations of the use of cellular effector assays to examine G protein coupling are that these assays do not offer insight into the individual steps in G protein activation and the fact that many heterologous expression systems are immortalized mammalian cell lines that contain a mixture of different G proteins and GPCRs.

The present study uses the baculovirus-mediated expression of M₂ mAChR and a defined Gₛ preparation in Sf9 insect cells. The Sf9 system has been used previously to express both mACHR and G proteins (1). Coupling is examined with receptor-stimulated GTPγS binding and GTPase activity. GTPγS binding has been extensively used to characterize receptor:G protein coupling for muscarinic (2), angiotensin (3), bradykinin (4), dopamine (5, 6), serotonin (7), and neurotensin receptors (8). GTPase activity has been used as a measure of GPCR-mediated signaling in a reconstituted system of M₂ mACHR and Gₛ (9) and in enriched membranes for the β₂-adrenoceptor and Gₛ (10). A kinetic mechanism is presented that describes the receptor-stimulated GTPase activity of Gₛ. Kinetic constants derived from this mechanism allow the examination of the effects of several site-directed mutants on GTPase activity. The site-directed mutants examined all involve alterations in amino acids that are conserved in the cationic amine ligand-binding GPCR.

The mutants are an asparagine 69 to aspartate (D69N) mutant in transmembrane sequence (TM) 2, an aspartate 103 to asparagine mutant in TM 3, a threonine 187 to alanine mutant in TM 5, and a tyrosine 403 to phenylalanine mutant in TM 6.

EXPERIMENTAL PROCEDURES

Materials—[35S]GTPγS, [α-32P]GDP, and [γ-32P]GTP were purchased from PerkinElmer Life Sciences. [3H]QNB, oxotremorine M, and recombinant TEV protease were purchased from Amersham Biosciences, Research Biochemicals, and Invitrogen, respectively.

Cloning—The wild-type coding region of the M₂ mAChR in the pSVE expression vector (11) was a gift from Dr. Daniel Capon (Genentech). Epitope-tagged receptors were constructed in the pSVE vector and then transferred as an EcoRI/EcoRV fragment into the EcoRI/SmaI site of the baculovirus transfer vector pVL1392 (PharMingen). Generation of the D69N, D103N, and Y403F mutants has been described (12). The Thr-187 to Ala mutant pm2.musc.short.T187A was produced by oligonucleotide-directed mutagenesis (13). The wild-type coding region of the M₂ mAChR was ligated into M13mp18 as a HinR/EcoRI fragment into the dIII/R site of [35S]GTPγS; [α-32P]GDP, and [γ-32P]GTP were purchased from PerkinElmer Life Sciences. [3H]QNB, oxotremorine M, and recombinant TEV protease were purchased from Amersham Biosciences, Research Biochemicals, and Invitrogen, respectively.

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quinoxalinyl benzilate; HA, hemagglutinin antigen 1; TEV, tobacco etch virus; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; TM, transmembrane; MOPS, 4-morpholinepropanesulfonic acid; m.o.i., multiplicity of infection.
introduced to create a unique BglII restriction site for screening. Mutant sequences were confirmed by dideoxy sequencing (14). The HA epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) was inserted at the extreme amino terminus of the \( M_2 \) mACHR receptor sequence and is separated from the receptor sequence by the consensus sequence (underlined) for the TC10 cleavage site (Glu-Leu-Thr-The-Gly-Thr-Ser). Thr and Ser were added in the cloning process.

**Polycotylan HA Epitope Antiseria**—New Zealand White rabbits were injected with 250 \( \mu \)g of keyhole limpet hemocyanin-conjugated HA epitope peptide in PBS:Freund’s complete adjuvant (1:1). A booster was performed after 1 month. Sera was aliquoted and stored at minus 80°C.

**Cell Culture**—Cells were maintained in 75 cm² tissue culture flasks in Trichoplusia ni media formulation Hink media and in suspension in SF-900 II media. Cells were grown to 1/4 to 1/3 final desired volume in SF-900 II media, and the remaining volume was \( T. \) ni media formulation Hink. One liter of cells was pelleted 1 min in a clinical centrifuge (400°G), resuspended in PBS, and pelleted in a clinical centrifuge. The pellet was resuspended in 4 volumes of 20 mM HCl, pH 8.0 (HCl), 2 mM EDTA with protease inhibitors (1 \( \mu \)g/ml benzamidine, 10 \( \mu \)g/ml bacitracin, 0.5 \( \mu \)g/ml pepstatin A, 17 \( \mu \)g/ml PMSF, 2.5 \( \mu \)g/ml leupeptin, and aprotinin, 1 \( \mu \)g/ml E-64), and stored at −80°C.

**Baculovirus Expression Constructs**—Recombinant baculovirus containing the \( M_2 \) mACHR were generated using the BaculoGold™ transfection kit (PharMingen). Viruses containing the sequences for the \( G_i \) subunits (\( G_{ia}, G_{ib}, \) and \( G_{ic} \)) were a generous gift from Dr. Jim Garrison, Department of Pharmacology, University of Virginia.

**Enriched Membranes**—Cells were thawed at room temperature (2 h), placed on ice (2 h), and lysed with 20 mM tris buffer from a Polytron homogenizer (Brinkmann Instruments) at setting 90. The homogenate was pelleted 1 min in a clinical centrifuge (400 \( \times \)g), and the supernatant was layered onto a sucrose step gradient and centrifuged (1 h, 93,500 \( \times \)g). Sucrose was dissolved in MEE (10 mM MOPS, pH 8.0, HCl), 1 mM EDTA, and 1 mM EGTA and protease inhibitors. Membranes were collected from the 20-60% sucrose interface, diluted with MEE buffer, and pelleted (20 min, 189,600 \( \times \)g) in a Beckman Ti 70 rotor. Membranes were resuspended in MEE, passed 2–3 times through a 21-gauge needle, frozen in liquid nitrogen, and stored at −80°C.

**Ligand Binding**—\( M_2 \) mACHR-binding sites in whole cells were determined in \( T. \) ni media formulation Hink media as described previously (17). Methods for determining binding sites in membranes and dissociation constants for ligands have been described (17). Briefly, receptor expression in whole cells and in enriched membranes was determined using the tritiated muscarinic antagonist \( \tau \)-quinuclidinyl benzilate (\( ^3 \)HQNB). Dissociation constants for different classes of agonist binding were determined by competition binding experiments with \( ^3 \)HONB. Data from experiments performed in binding buffer (10 mM Hepes, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, pH 7.4 (NaOH)) were identical to those performed in GTPase buffer (10 mM MOPS, pH 7.4 (HCl), 1 mM EDTA, 1 mM EGTA, 120 mM NaCl, 20 mM MgCl₂).

**GTP-S Binding**—Membranes with or without ligands were equilibrated for 20 min at 25°C before the initiation of the reaction by the addition of \( [35S] \)GTP-S to 50 mM (1 \( \times \)10⁻⁸ Ci/μmol). Aliquots were added to the reservoir of a filtration manifold containing 2 ml of ice-cold wash buffer (reaction buffer minus membranes and ligands) and immediately filtered through a Schleicher & Schuell 32605 glass fiber filter and rinsed with an additional 2 ml of buffer. Filters were placed inside glass vials and counted for \( ^{38} \)P (Cerenkov).

**GTPase Assay**—For each data point 40 \( \mu \)g of membranes (1 mg/ml) plus 0.1 mM ouabain and 1 mM dithiothreitol in GTPase buffer were incubated with or without ligands at 25°C for 30 min prior to the addition of 10 \( \mu \)l of \([γ-32\text{P}]\)GTP, 0.5 mM ATP. Assays without ligands gave a measure of the nonmuscarinic plus constitutive (agonist-independent) muscarinic-stimulated GTPase activity, whereas reactions in the presence of saturating concentrations (10 \( \mu \)M) of hyoscymamine allowed determination of the nonmuscarinic GTPase activity. To terminate the reaction, 250 \( \mu \)l of 5% (v/v) activated charcoal in 50 mM sodium phosphate, pH 2.2 (4°C), was added and the tube and briefly vortexed. After centrifugation for 1 min on ice the be was vortexed again and spun in a micro centrifuge at full speed for 5 s. 200 \( \mu \)l of the supernatant was removed and counted (Cerenkov).

**Electrophoresis and Western Blotting**—Samples were subjected to SDS-PAGE on 7–15% gradient gels using standard protocols and transferred to 0.2-μm nitrocellulose membrane. Nitrocellulose was blocked in TNT (10 mM Tris, pH 8 (HCl), 150 mM NaCl, 0.05% Tween 20 plus 2% polyvinylpyrrolidone and 4% nonfat dry milk. Membranes were incubated with primary antibody (polyclonal rabbit oH), diluted in TNT (10 mM Tris, pH 8 (HCl), 150 mM NaCl), and horseradish peroxidase secondary antibody in TNT. Membranes were rinsed with TNT and developed with SuperSignal™ ECL (Pierce).

**Data Analysis**—GTPase data were fit to Equations 1 or 2, where \( v_i \) is the initial velocity. In Equation 1, \( V_1 \) and \( V_2 \) are the maximum velocity of agonist-promoted and agonist-independent GTPase activity; \( A \) is agonist concentration; GTP is GTP concentration; and \( K_a \) and \( K_{GTP} \) are the Michaelis constants for agonist and GTP. Equation 2 assumes two pathways of agonist-promoted GTPase activity arising from different pools of receptor. \( V_1 \) and \( V_2 \) represent the maximum velocities for the two pathways and the respective Michaelis constants for agonist are given as \( K_{GTP} \) and \( K_a \). The single Michaelis constant for GTP (\( K_{GTP} \)) is independent of the pathway and is the same for both agonist-promoted and constitutive GTPase activity. Parameter values are from global analysis of 8–12 data sets, each representing an agonist titration at a given [GTP]. Data were fit by global analysis where \( V_{max} \) and \( K_a \) values were shared between data sets, and [GTP] was fixed for each data set. The best fit (Equation 1 or 2) was determined using a \( F \) test applied at the 95% confidence interval.

\[
V_1 \left[ \frac{A}{[GTP]} \right] + V_2 \left( K_a ([GTP]) \right)
\]

\[
\frac{v_i}{[GTP]} = \frac{K_a ([GTP]) + K_{GTP}([A]+[A])}{K_a ([GTP]) + [GTP]} \quad \text{(Eq. 1)}
\]

\[
V_{11}[A][GTP] + V_2 \left( K_a ([GTP]) \right)
\]

\[
\frac{v_i}{[GTP]} = \frac{K_a ([K_{GTP}]+[GTP]) + [A] \left( [GTP] \right)}{[GTP]} + \frac{V_2 \left( [A][GTP] \right) + V_2 \left( [A][GTP] \right)}{[GTP]} \quad \text{(Eq. 2)}
\]

Association or dissociation reactions were fit to Equation 3 or 4 where \( y(t) \) is

\[
y(t) = A_0 + \sum_{i=1}^{n} A_i \left( 1 - e^{-k_i t} \right) \quad \text{(Eq. 3)}
\]

\[
y(t) = A_0 + \sum_{i=1}^{n} A_i e^{-k_i t} \quad \text{(Eq. 4)}
\]

counts/min bound at time \( t \); \( A_0 \) and \( A_i \) are the counts/min bound at time 0 or equilibrium, and \( A_0 \) and \( k_i \) are the amplitudes and observed rate constants of the kinetic phases. Data for determination of the initial rate of GTP-S binding were fit to Equation 3. For a single exponential, the first derivative of Equation 3 evaluated at \( t = 0 \) equals the value of the initial velocity of GTPyS binding.

\[
\frac{dy}{dt} \left( t = 0 \right) = A_k k_i
\]

The dissociation constant for GTP binding to \( G_i \) (\( K_i \)) was determined from the inhibition of the initial rate of GTPyS binding (Equation 6), where \( I \) is GTP, \( K_i \) is the Michaelis constant for GTPyS, and \( V \) is the maximum velocity.

\[
v_i = \frac{V[S]}{1 + \frac{I}{K_i} + [S]} \quad \text{(Eq. 6)}
\]

All data were fit by nonlinear least-squares procedures using Marquardt’s algorithm (19) in Microcal Origin, and parameter values are reported as the mean and associated S.E. from the nonlinear curve fitting routine.
RESULTS

Expression of M₂ mAChR and G:—Fig. 1 is a Western blot of HA-tagged M₂ mAChR from baculovirus-infected Sf9 cells. Epitope tags have been used with a number of GPCR and have shown to have little effect on ligand binding or effector coupling (20–23). Fig. 1 shows a low and high molecular weight species in both the membrane-bound (lane 2) and solubilized receptor (lane 5). The molecular weights of the two species determined from Ferguson plots (24) are consistent with a receptor monomer (69 ± 13 kDa) and dimer (126 ± 20 kDa). Receptor expression (measured by specific [3H]QNB binding) increased with increasing multiplicity of infection (m.o.i.) reaching a plateau at an m.o.i. of 10 (data not shown). G₂ protein expression (measured by the appearance of [35S]GTPγS-binding sites) increased with increasing m.o.i. and reached a plateau when an m.o.i. of 3–5 was used for each G₂ protein subunit (data not shown).

Ligand Binding—Table I summarizes ligand binding experiments in enriched membranes from Sf9 cells expressing either wild-type or mutant M₂ mAChR as well as the three subunits of G₂. The fraction of agonist binding in the high affinity (G-i-coupled) state is summarized in Table II. The wild-type data presented are for the HA-tagged receptor. In most cases, the presence of the HA tag had only a small (<4-fold) effect on binding constants. The exceptions were acetylcholine where the high and low affinity dissociation constants were increased by 18- and 9-fold, respectively, and for oxotremorine M where the high affinity dissociation constant was decreased by 6-fold. These effects do not interfere with interpretation of the values of kₗcat from the GTPase data because determination of kₗcat involves extrapolation of both GTP and agonist concentration of infinitely high values. Antagonist binding to the D69N (TM2) mutant was similar to wild type with the largest deviation being less than 3-fold. D69N displayed both high and low affinity agonist binding for the full agonists carbachol, acetylcholine, and oxotremorine M but no high affinity binding with the partial agonist pilocarpine. The dissociation constants for agonists deviate less than 3-fold from wild-type values suggesting that Asp-69 is not directly involved in ligand binding. Although high affinity (G protein-coupled receptor) agonist binding was not sensitive to the addition of guanine nucleo-

![Fig. 1. Western blot of membrane-bound and solubilized M₂ mAChR.](image)

### Table I

Dissociation constants for muscarinic ligands determined in enriched membranes from baculovirus-infected Sf9 cells

| Ligand   | WT (nM) | D69N (nM) | D103N (nM) | T187A (nM) | Y403F (nM) |
|----------|---------|-----------|------------|------------|------------|
| NMS      | 3.7 ± 0.7 | 6.0 ± 0.7 | 6.4 ± 0.7 | 6.7 ± 0.7 | 6.9 ± 0.7 |
| Oxo M    | 2.5 ± 0.5 | 2.7 ± 0.5 | 2.8 ± 0.5 | 2.9 ± 0.5 | 3.0 ± 0.5 |
| Pilo     | 2.0 ± 0.1 | 2.2 ± 0.1 | 2.3 ± 0.1 | 2.5 ± 0.1 | 2.6 ± 0.1 |

### Table II

Receptor-coupled G₂ and G-i-coupled receptor in enriched membranes from baculovirus-infected Sf9 cells

| Ligand   | WT (nM) | D69N (nM) | D103N (nM) | T187A (nM) | Y403F (nM) |
|----------|---------|-----------|------------|------------|------------|
| ACh      | 5.8 ± 0.7 | 6.0 ± 0.7 | 6.2 ± 0.7 | 6.4 ± 0.7 | 6.6 ± 0.7 |
| Carb     | 5.9 ± 0.7 | 6.0 ± 0.7 | 6.1 ± 0.7 | 6.2 ± 0.7 | 6.3 ± 0.7 |
| Oxo M    | 5.4 ± 0.7 | 5.5 ± 0.7 | 5.6 ± 0.7 | 5.7 ± 0.7 | 5.8 ± 0.7 |
| Pilo     | 5.8 ± 0.7 | 5.9 ± 0.7 | 6.0 ± 0.7 | 6.1 ± 0.7 | 6.2 ± 0.7 |
| Constitutive | 1.6 ± 0.1 | 1.7 ± 0.1 | 1.8 ± 0.1 | 1.9 ± 0.1 | 2.0 ± 0.1 |

a QNB binding with high affinity for agonist/mg protein.

b Values in parentheses indicates percent of total G₂ protein or receptor.
Fig. 2. $M_2$ mACHR-stimulated GTP-$S$ binding. Membranes were pretreated with the muscarinic agonist carbachol (●), or were untreated (▲). Data were fit to Equation 3 and gave the following amplitudes: hyoscyamine, $A_1 = 0.4, A_2 = 41 \pm 1$; untreated, $A_1 = 7.8 \pm 2.0, A_2 = 43 \pm 1$; and carbachol, $A_1 = 22 \pm 2, A_2 = 27 \pm 1$ over a value at time 0 (shared for all data sets) of 4.0 ± 1.7 pmol/mg and observed rate constants (shared for all three data sets) of $k_1 = 2.1 \pm 0.3$ and $k_2 = 0.11 \pm 0.05$ min$^{-1}$.

In order to test whether GTP promotes GTPase activity we first investigated the antagonist and agonist-promoted stimulation of GTPase activity. The partial agonist pilocarpine shows no statistically significant stimulation of GTPase activity compared with the wild-type mACHR. 

The Y403F mutant (TM 6) decreased antagonist affinity by 2.1-fold with wild-type receptor and showed only low affinity agonist binding for both full and partial agonists. The antagonist binding was not affected by the presence of guanine nucleotides suggesting that the low affinity site represents the free receptor. Agonist affinities decreased 28–86-fold for full agonists and 15-fold for pilocarpine compared with wild type.

Antagonist affinities for T187A (TM 5) showed small deviations from wild-type (3–5-fold increase). T187A displayed both high- and low-affinity agonist binding for the full agonist but only a single affinity for pilocarpine. The dissociation constants for agonists show only small deviations from wild type.

The Y403F mutant (TM 6) decreased antagonist affinity by 4–7-fold compared with wild-type receptor. Y403F displayed high and low affinity agonist binding for both full and partial agonists. The high affinity site was sensitive to the addition of guanine nucleotides. The dissociation constants for the high affinity site increased over 10-fold for carbachol and oxotremorine M and 15-fold for pilocarpine. Y403F showed similarly large deviations in low affinity binding.

GTP-$S$ Binding—Fig. 2 shows the results of a GTP-$S$ binding experiment in membranes from cells expressing the HA-tagged wild-type $M_2$ mACHR and $G_i$. The data indicated a small burst phase followed by the presence of a fast and slow phase of $[^{35}S]$GTP-$S$ binding. Compared with membranes preincubated with the antagonist hyoscyamine, untreated membranes or membranes preincubated with carbachol showed a larger portion of the total GTP-$S$ binding occurring in the fast phase (Fig. 1A) indicating that this phase represents the receptor-stimulated GTP-$S$ binding. Subtraction of the value of A1 for the antagonist-treated from agonist-treated membranes yields the amount of agonist-promoted GTP-$S$ binding. Subtraction of the value of A1 for antagonist-treated from untreated membranes gives the amount of constitutive GTP-$S$ binding.

The D103N mutant did not show any agonist-promoted GTP-$S$ binding. Oxotremorine M was the only agonist tested that promoted GTP-$S$ binding (17% of total $G_i$) over constitutive levels for the D69N mutant. GTP-$S$ binding in T187A and Y403F is similar to the wild-type $M_2$ mACHR for full agonists. The partial agonist pilocarpine shows no statistically significant promotion of GTP-$S$ binding for T187A and stimulated less GTP-$S$ binding (11%) in Y403F compared with wild type (20–25%). Constitutive (agonist-independent) GTP-$S$ binding was similar to wild type for all the mutants examined. The GTP-$S$ binding data are summarized in Table II. Table II also summarizes the amount of receptor in the high affinity (G protein-coupled) state determined by agonist competition with $[^{3}H]$QNB.

Fig. 3. $M_2$ mACHR-stimulated GDP dissociation. Membranes were pretreated with the muscarinic antagonist hyoscyamine (●), or were untreated (▲). Data were fit to Equation 4 and showed 3 kinetic phases with the following amplitudes: hyoscyamine, $A_1 = 12.3 \pm 1.9$, $A_2 = 16.2 \pm 5.3$, $A_3 = 16.6 \pm 8.8$, $A_4 = 55.0 \pm 7.9$; untreated, $A_1 = 7.3 \pm 1.5$, $A_2 = 34.9 \pm 6.1$, $A_3 = 46.7 \pm 6.6$, $A_4 = 11.1 \pm 5.3$; and carbachol, $A_1 = 8.3 \pm 1.7$, $A_2 = 50.5 \pm 6.5$, $A_3 = 31.7 \pm 5.7$, and $A_4 = 9.5 \pm 4.2$. Observed rate constants were shared for the three data sets and resulted in values of 3.4 ± 0.7 × 10$^{-1}$, 3.2 ± 0.7 × 10$^{-2}$, and 4.8 ± 0.7 × 10$^{-3}$ s$^{-1}$.

GDP Dissociation—Fig. 3 shows a GDP dissociation experiment in membranes from cells expressing the wild-type $M_2$ mACHR and $G_i$. GDP dissociation showed three kinetic phases, and data were globally fit to Equation 4. The three rate constants were shared, and the amplitudes in each phase were determined as fitted parameters. The fastest phase (3.4 ± 0.7e-1 s$^{-1}$) of GDP dissociation results from $M_2$ mACHR-stimulated GDP dissociation from $G_i$, and $G_m$. The intermediate phase (3.2 ± 0.7e-2 s$^{-1}$) represents $M_2$ mACHR-independent GDP dissociation from $G_i$. The slow phase (4.8 ± 0.7e-3 s$^{-1}$) represents GDP dissociation from $G_m$ and other GDP-binding proteins. Membranes from S9 cells expressing $G_i$ only (data not shown) had only two kinetic phases corresponding to the intermediate and slow phase of GDP dissociation (4.0 ± 0.5e-2 and 2.4 ± 0.4e-3 s$^{-1}$).

Dissociation Constant ($K_f$) of GTP—The $K_f$ for GTP was determined as the $K_f$ for GTP as an inhibitor of the initial velocity of GTP-$S$ binding (Fig. 4 and 5), in membranes from cells expressing the HA-tagged receptor and $G_i$. The initial velocity of agonist-promoted $M_2$ mACHR-stimulated GTP-$S$ binding was determined by calculating the difference in initial velocity between agonist-treated and antagonist-treated membranes. Fig. 4 shows the results of an experiment to determine the initial velocity of agonist-promoted $M_2$ mACHR-stimulated GTP-$S$ binding. The initial velocity was determined at several concentrations of GTP, and the inverse of the initial velocity was plotted versus the inverse of the GTP-$S$ concentration (Fig. 5). A replot of the slope from Fig. 5 versus concentration of GTP according to Equation 6 gave a dissociation constant for GTP binding equal to 30 ± 10 nm (Fig. 5, inset).

GTPase Activity—The results of a typical agonist titration of $M_2$ mACHR-stimulated GTPase activity are shown in Fig. 6. Table III presents the fitting parameters from fits of GTPase data. The two values of $k_{cat}$ are the agonist-promoted and constitutive $M_2$ mACHR-stimulated GTPase, and $K_{GTP}$ and $K_A$ are the Michaelis constants for the GTP agonist. For data fit to Equation 2, $K_{A1}$ and $K_{A2}$ are the Michaelis constants for the two agonist-promoted pathways of GTPase activity. The ratio of these two constants ($K_{A2}/K_{A1}$) was 17, 48, and 75 for acetylcholine, carbachol, and oxotremorine M, respectively, and indicates the tendency to activate one pathway relative to the other. Data were best fit to Equation 1 or 2 depending on the agonist and/or G/R ratio (Table IV).

The D103N mutant $M_2$ mACHR did not demonstrate agonist-promoted stimulation of GTPase activity.

Oxotremorine M was the only muscarinic agonist tested that promoted GTPase activity with the D69N mutant, and data were best fit to Equation 2. The $K_{GTP}$ for D69N showed a very
M were the same as wild type. The changes in \( k_{cat} \) values for T187A compared with wild-type receptor were highly agonist-dependent.

GTase data for Y403F were best fit to Equation 2 for all agonists tested. This was a surprising result because data for the partial agonist pilocarpine were best fit to Equation 1 for the wild-type receptor (see “Discussion”). \( K_{GT} \) for Y403F does not deviate more than 2-fold from wild-type values except with carbachol where \( K_{GT} \) increased 4-fold. The values of \( K_A \) (and \( K_{AX}K_A \)) show the most significant deviations from wild type with the Y403F mutant. A 6-fold decrease and 19-fold increase in \( K_A \) and \( K_{AX} \) lead to an overall 100-fold increase in \( K_{AX}K_A \) for acetylcholine. Increases in both \( K_A \) and \( K_{AX} \) with carbachol (100- and 500-fold) result in a smaller (5-fold) increase in \( K_{AX} \) with this agonist. \( K_A \) values for oxotremorine M show only small deviations from wild type, and there is no significant change in \( K_{AX} \). The changes in the values of \( k_{cat} \) for Y403F compared with wild-type receptor were also highly agonist-dependent.

**DISCUSSION**

The goal of this investigation was to examine the effects of different receptor mutations on the coupling of the \( M_2 \) mACHR and \( G_i \) Coupling was analyzed in terms of activated G protein, G protein-coupled receptor (receptor having high affinity guanine nucleotide-sensitive agonist sites), and the kinetic properties of receptor-stimulated GTase activity of \( G_i \).

**GTase Mechanism**—Initially, GTase data were fit to a logistic equation that resulted in poor fits (large residuals) due to the fact that agonist-promoted activity spanned several orders of magnitude. The observation that receptor-stimulated GTase activity of \( G_i \) shows both constitutive and agonist-promoted activity led to the examination of several mechanisms that included both pathways of GTase activity. Equations derived from the mechanism shown in Fig. 7 gave the best fit and were used to analyze experimental data (see “Appendix”). Agonist and G protein binding to the receptor are in rapid equilibrium (Fig. 7, box). GDP dissociation from agonist-receptor-\( G_i \) (or receptor-\( G_i \)) gives two GDP free receptor-G protein complexes ARG (C1) and RG (C3). The steady-state binding of GTP then forms a second set of complexes, ARG-GTP (C2) or RG-GTP (C4), followed by the dissociation of the agonist-receptor-\( G_i \) or receptor-\( G_i \) to yield the activated G protein (G*). G* is returned to G-GDP by the intrinsic GTase activity of the Ga subunit (\( k_{cat} \)). Initially GTP binding was assumed to be in rapid equilibrium; however, the kinetic equations derived from this model required that \( K_{GT} \leq K_G \) GTP. Experimental data were inconsistent with this model. The \( K_d \) for GTP of 30 \( nM \) determined by the inhibition of the initial rate of GTP\( \gamma \)S binding by GTP was lower than the \( K_{GT} \) of 150 \( nM \). A second mechanism with GTP binding in steady state yielded a kinetic equation (Equation 1) with no constraint on the relationship of \( K_{GT} \) and \( K_G \). In some Sf9 membrane preparations (and CHO cell membranes, not shown), however, the data were not adequately fit by Equation 1. The mechanism was expanded to contain two paths of agonist-promoted GTase activity (Equation 2). The two paths were assumed to represent different populations, possibly oligomers, of receptor and/or G proteins. Equation 1 contains two velocity terms (\( V_1 \) and \( V_2 \)) that represent the maximum velocity of agonist-promoted and constitutive \( M_2 \) mACHR-stimulated GTase activity. Equation 2 contains three velocity terms as follows: \( V_1 \) and \( V_2 \) represent the maximum velocity of the agonist-promoted GTase activity for the two receptor pools, and \( V_2 \) represents the maximum velocity of the constitutive GTase activity. Because the velocity was a simple hyperbolic function of GTP concentration at both 0 and saturating agonist concentration, the maximum
velocity at saturating GTP and agonist derived from Equation 2 must be of the form $V_{max} = V_1 + V_2$. Thus, the agonist-promoted $k_{cat}$ value ($k_{cat1}$) equals $(V11 + V12)/(G_{total})$. The maximum velocity at 0 agonist (saturating GTP) is of the form $V_{max} = 2V2$ and the constitutive $k_{cat}$ value ($k_{cat2}$) equals $2V2/G_{total}$.

Equation 2 contains two Michaelis constants, $K_{A1}$ and $K_{A2}$, for agonist-promoted GTPase activity of the two pathways.

Heitz et al. (1) determined that there was no endogenous Gi1, Gi2, Gi3, or Go detected in Sf9 cells. It is therefore reasonable to assume that the mAChR-stimulated GTPase activity in our membrane preparations arose solely from the exogenously expressed Gi. The rate of dissociation of GDP from Gi was determined to confirm that GDP release was not the rate-limiting step in the mechanism. In the presence of muscarinic agonists, the receptor promotes GDP dissociation with a rate constant slower than the rate of GDP dissociation.

**FIG. 7.** Kinetic scheme for $M_2$ mAChR-stimulated GTPase activity.

| Agonist          | $k_{cat}^{a,c}$ | $K_A^{b}$ | $K_{GTP}$ | Fit |
|------------------|----------------|-----------|-----------|-----|
| Wild type        |                |           |           |     |
| Acetylcholine    | 2.7 ± 0.5      | 0.13 ± 0.04 | 228 ± 13 | 2   |
| Carbachol        | 1.4 ± 0.4      | 2.2 ± 1.4 |          |     |
| Oxotremorine M   | 3.1 ± 0.5      | 0.048 ± 0.03 | 147 ± 12 | 2   |
| Pilocarpine      | 2.2 ± 0.4      | 2.2 ± 0.5 |          |     |
| D69N             |                |           |           |     |
| Acetylcholine    | NR$^{d}$       | NR        | NR        |     |
| Carbachol        | NR$^{d}$       | NR        | NR        |     |
| Oxotremorine M   | 1.25 ± 0.16    | 0.085 ± 0.054 | 340 ± 29 | 2   |
| T187A            | 0.32 ± 0.29    | 72 ± 5    |          |     |
| Y403F            |                |           |           |     |
| Acetylcholine    | 1.4 ± 0.4      | 0.029 ± 0.025 | 118 ± 10 | 2   |
| Carbachol        | 0.27 ± 0.28    | 5.3 ± 0.8 |          |     |
| Oxotremorine M   | 0.28 ± 0.4     | 0.023 ± 0.016 | 130 ± 10 | 2   |
| Pilocarpine      | 0.97 ± 0.98    | 3.14 ± 1.23 |        |     |
| D103N            |                |           |           |     |
| Acetylcholine    | NR$^{d}$       | NR        | NR        |     |
| Carbachol        | NR$^{d}$       | NR        | NR        |     |
| Oxotremorine M   | 2.1 ± 0.6      | 0.035 ± 0.019 | 351 ± 53 | 2   |
| Pilocarpine      | 0.097 ± 0.98   | 3.14 ± 1.23 |        |     |

**TABLE IV**
Summary of best fit to GTPase equations

| Acetylcholine | Carbachol | Oxotremorine M | Pilocarpine |
|--------------|-----------|----------------|-------------|
| M2:G5$^a$    | 2$^b$ (1.7) | 2 (2.5) | 2 (1.8) (3.6) |
| M1:G5        | 2 (1.1) | 2 | 1 |
| M1:G1        | 2 (0.9) | 2 (1.1) | 1 |
| M10:G1       | 1 (0.3) | 1 (0.7) | (0.7) |

$^a$ Multiplicity of infection of $M_2$ mAChR (M) and G protein (G).
$^b$ Equation that gave best fit.

The analysis required two equations for the description of receptor-stimulated GTPase activity. Both equations assume an agonist-promoted and constitutive pathway of GTPase activity, but Equation 1 describes an agonist-stimulated pathway of GTPase activity arising from a single pool of receptor and/or G protein, where Equation 2 describes activity from two pools.

One explanation for the existence of two pathways of agonist-stimulated GTPase activity is the existence of functional $M_2$ mAChR dimers. Immunoblot analysis of the epitope-tagged mAChR show the presence of a high molecular weight species at a molecular weight consistent with a receptor dimer. If receptor dimers were responsible for the second pathway of GTPase activation, one would expect the second pathway to be present at the highest expression levels of receptor. The data are inconsistent with this explanation as the membranes with the highest concentrations of receptor (lowest G:R ratio) show only a single pathway of GTPase activation (Table IV). In addition the amount

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of dimer relative to monomer observed on Western blots was not dependent on receptor concentration (data not shown).

Another explanation is that the second pathway of GTPase activity represents receptor coupled to G protein oligomers and that the receptor activates G proteins in an oligomeric array differently than monomeric G proteins. The existence and functional significance of G protein oligomers has been extensively studied for transducin (G<sub>T</sub>) with light scattering and direct binding studies with labeled nucleotides (25). G<sub>T</sub> oligomers were demonstrated in cross-linking experiments (26), and cooperative binding of G<sub>T</sub> to rhodopsin has been demonstrated with [35S]labeled G<sub>T</sub> (27). The existence of G protein oligomers has also been examined in solubilized G<sub>S</sub> (28–30). The conclusion of these experiments and observations of the behavior of other G proteins was that G proteins exist as multimers, and receptor activation results in the release of heterotrimer monomers that mediate signal transduction (31).

The mechanism presented here that gives rise to Equation 2 assumes two paths of agonist-promoted GTPase activity. If G protein oligomers exist, then the two pathways would represent receptors with either a single or multiple associated G proteins. Different preparations of membranes were best fit to either Equation 1 or 2, depending on the expression levels of receptor and G protein (Table IV). Wild-type data for full agonists were best fit to Equation 2 except at very high ratio of receptor to G protein suggesting that the second pathway arises at higher concentrations of G protein relative to receptor. The poor fits of GTPase data from preparations with a lower G:<R ratio to Equation 2 suggest that G protein oligomers were not present in these preparations. Pilocarpine data were fit to Equation 1, suggesting that the partial agonist pilocarpine was only able to stimulate one of the pools of receptor, or the V<sub>max</sub> terms for the two pools were indistinguishable.

The two values of K<sub>A</sub> represent the Michaelis constant for agonist for the two pathways of agonist-promoted GTPase activity. The lower value (K<sub>A1</sub>) may represent the pathway with receptor coupled to a G protein oligomer and may result from the absence of receptor dissociation from the G protein that has been thought to precede GTP hydrolysis on Go. The higher value (K<sub>A2</sub>) is for the pool of agonist-promoted GTPase activity of receptor coupled to a single G protein. The ratio of K<sub>A2</sub>:K<sub>A1</sub> is 17, 48, and 73 for acetylcholine, carbachol, and oxotremorine M, respectively. This increasing tendency to activate one pathway over the other results from changes in K<sub>A1</sub>, because K<sub>A2</sub> is independent of agonist structure and is unchanged for all agonists at 2–2.5 μM. Because K<sub>A1</sub> and K<sub>A2</sub> are dependent on a number of rate and equilibrium constants as well as the free receptor concentration interacting with each G protein pool (Equation A9), it is not possible to assign changes in K<sub>A1</sub> to a specific step in the mechanism.

**D69N and D103N mACHR**—The transmembrane domains of muscarinic receptors contain several conserved aspartic acid residues. Specific interaction of these residues with muscarinic ligands is indicated by alkylation of a conserved aspartate (99 in M<sub>1</sub> and 103 in M<sub>2</sub>) in TM 3 by the muscarinic antagonist analogue [3H]propyl benzilyl choline mustard (32). Mutation of aspartate 71 (69 in M<sub>3</sub>mACHR) in TM 2 of the rat M<sub>1</sub> mACHR resulted in a receptor that showed carbachol binding but drastically decreased efficiency and potency in agonist-induced activation of phospholipase C (33). Mutation of Asp-105 to Asn in the M<sub>1</sub> mACHR greatly decreases both antagonist affinity and agonist-mediated activation of phospholipase C in Chinese hamster ovary (CHO) cells (32). Mutation of aspartate 99 or 105 to asparagine in the rat M<sub>1</sub> mACHR decreases ligand binding and/or covalent incorporation of [3H]propyl benzilyl choline mustard suggesting that these residues are involved in ligand binding (33). The ligand binding and effector coupling characteristics of the M<sub>2</sub> mACHR mutant D69N expressed in CHO cells have been described (12). D69N showed no high affinity (G protein-coupled) agonist binding but did demonstrate agonist-specific (oxotremorine M) activation of cellular effectors. This result suggests not only the presence of the G protein-coupled state (possibly at levels too low to detect in ligand binding experiments) but also the ability of this mutant to adopt different agonist-stabilized active states. Sf9 cell membrane preparations showed high affinity binding for all three full agonists tested and low affinity binding for both full and partial agonists. The observation of high affinity binding for D69N in Sf9 cell membrane preparations that was undetectable in CHO cells was probably due to the higher levels of G<sub>S</sub> present in the Sf9 cell membrane preparations. The fact that ligand binding constants for D69N did not deviate significantly from wild-type values agrees with previous data indicating that Asp-69 is not directly involved in ligand binding (12). As seen in physiological assays in CHO cells, the only agonist that was able to stimulate GTP·γ·S binding and GTPase activity with D69N was oxotremorine M. Furthermore, the GTPase activity was fit to Equation 2, which suggests that the D69N mutant is capable of supporting both pathways of GTPase activity. The fact that high affinity oxotremorine M binding was not sensitive to guanine nucleotides may result from impaired ability of the D69N to dissociate from the activated G protein. This hypothesis is consistent with the decreased values of k<sub>cat</sub> for D69N.

The ligand binding and effector coupling characteristics of the D103N mutant expressed in CHO cells have been described (12). D103N was very poorly expressed in CHO cells, and only the comparison of other single and D103N containing double mutant constructs allowed the characterization of the effects of the D103N mutation. The large shifts in ligand affinities in D103N containing mutants suggests that aspartate 103 serves as the cationic amine ligand counterion. Changes in the effector coupling properties of the D103N containing double mutants compared with the single mutants suggests that aspartate 103 also has a role in transduction of the ligand binding signal to the receptor-G protein interface. The high expression levels of the D103N mutant (1 × 10<sup>6</sup>/ml receptors/cell) in Sf9 cells allowed the direct characterization of the ligand binding and G protein coupling characteristics of this mutant. D103N showed increases in antagonist binding constants compared with wild type and showed only low affinity agonist binding that was significantly weaker than with the wild-type receptor. Consistent with the lack of high affinity binding, D103N was not able to support agonist-promoted GTP·γ·S binding or GTPase activity.

**T187A and Y403F mACHR**—Conserved tyrosines and threonines of mACHRs have been implicated in ligand binding in studies using receptor chimeras, site-directed mutagenesis, and photoaffinity labeling (32). Wess et al. (34) demonstrated that a tyrosine 506 to phenylalanine or threonine 234 to alanine mutation in the M<sub>3</sub> mACHR substantially decreased acetylcholine binding and severely impaired the ability of the receptor to stimulate agonist-dependent activation of phospholipase C. They proposed that tyrosine and threonine hydroxyls on the inner face of the ligand binding pocket create hydrophilic environments for acetylcholine ester side chain binding in the M<sub>3</sub> mACHR.

The T187A mutant shows decreased agonist affinity in enriched membranes from CHO cells (data not shown) but differential changes in effector coupling cannot be explained by the change in agonist affinity alone. Changes in the EC<sub>50</sub> for the activation of phospholipase C are similar to changes in agonist binding affinities. The EC<sub>50</sub> values for the inhibition of aden-
lyl cyclase, however, is shifted 4–5000-fold compared with wild type with changes in agonist affinity of only 1000-fold. The data suggest that the T187A mutant results in a receptor conformation that differentially activates different classes of G proteins involved in coupling to different pathways. In Sf9 cell membrane preparations the T187A mutant had relatively small effects (less than 10-fold) on ligand binding. Despite the fact that GTPγS binding was similar to wild type, there were agonist-specific effects on GTPase activity. Acetylcholine and oxotremorine M were both able to promote GTPase activity that involved both receptor pools. Carbachol data were best fit to nist-specific effects on GTPase activity. Acetylcholine and oxotremorine M were both able to promote GTPase activity that involved both receptor pools. Carbachol data were best fit to Equation 1, and the single $K_A$ value correlated with the pool of receptor associated with a single G protein. Pilocarpine was unable to promote GTPase activity. The lack of two receptor pools for carbachol suggests that the T187A mutant is not able to stimulate GTPase activity in one of the pools with carbachol but can do so with other full agonists. Increases in $K_A$ for acetylcholine were accompanied by a 1.9-fold decrease in $k_{cat1}/k_{cat2}$ (constitutive) showed a larger (5.2-fold) decrease in T187A compared with wild type. This differential effect of the mutation on the agonist-promoted and constitutive $k_{cat}$ values can be explained by examination of the equations that give rise to the kinetic constants. Assuming that $K_A/[R] < 1$ in Equation A9, we can express the ratio of the $k_{cat}$ values in the two pathways as Equation 7,

$$\frac{k_{cat1}}{k_{cat2}} = \left(\frac{1}{k_{11}} + \frac{1}{k_{12}}\right) \left(\frac{1}{k_{21}} + \frac{1}{k_{22}}\right)$$

(Eq. 7)

This equation predicts that if GTP hydrolysis ($k_{17}$) is rate-determining for both pathways ($k_{17} < \frac{k_{15}}{k_{15}}$, $k_{15}$, $k_{13}$, $k_{12}$), then the two $k_{cat}$ values will be equal, and the ratio will equal 1. Because $k_{cat1}/k_{cat2}$ is always greater than 1, we know that GTP hydrolysis is not the rate-limiting step for both pathways. GDP dissociation experiments with the wild-type receptor showed that the rate constant for muscarinic stimulated GDP dissociation is 0.34 s⁻¹ (18 min⁻¹). Because the rate of GDP dissociation is 10-fold greater than the values of $k_{cat}$, GDP dissociation cannot be the rate-limiting step for wild-type. For a mutation to make GDP dissociation the rate-limiting step, it would have to decrease the dissociation rate of GDP such that it would be in the range that we found for muscarinic independent GDP dissociation 0.036 s⁻¹ (2.16 min⁻¹). Thus, for a mutant to reduce GDP dissociation ($k_g$ and $k_{13}$) to the extent that it becomes the rate-limiting step, the GDP dissociation has to be essentially the same as that seen for muscarinic independent GDP dissociation. This is not the case because the mutants were still able to promote GTPγS binding and GTPase activity (therefore GDP dissociation). This leaves G protein dissociation from the receptor as the rate-limiting step in this kinetic model. Mutations could have differential effects on the two values of $k_{cat}$ because they have differential effects on G protein dissociation from the agonist-bound versus the free receptor.

Characterization of the agonist binding and effector coupling characteristics of the Y403F mutant expressed in CHO cells has been described (17). This mutation decreased the affinity of muscarinic ligands and changed the amount of the nonhydrolyzable guanine nucleotide analogue GDPβS needed to dissociate the receptor-G protein complex but did not alter the coupling of the receptor to physiological pathways. In Sf9 cell membrane preparations the Y403F mutant showed differential effects on ligand binding. The largest effects were on antagonlist binding affinities that were decreased 13–17-fold. The largest effect on agonist binding was seen with acetylcholine where there was a 10-fold decrease in affinity for both the high and low affinity binding. The decreases in affinity for the other agonists were less pronounced (2–5-fold). GTPγS binding in Y403F was the same as wild type except with the partial agonist pilocarpine (11 versus 25% for wild type). Despite the fact that pilocarpine supported less GTPγS binding with the Y403F mutant, this was the only construct examined where pilocarpine was able to promote GTPase activity in both pools of receptor.

The other agonists had differential effects on GTPase activity. Carbachol showed a 3–4-fold increase in $k_{cat}$ values and very large increases (100- and 500-fold) in $K_A$ and $K_{cat}$ for Y403F. The data suggest that this mutation affected the GTPase activity for both agonist-promoted and constitutive GTPase activity to the same extent but had differential effects on the $K_A$ for the two pools of receptor. Acetylcholine-stimulated GTPase activity shows differential effects on $K_A$ for the two pools of receptor (6-fold increase in $K_A$ and 19-fold decrease in $K_{cat}$). $k_{cat}$ values were unchanged from wild type with acetylcholine. GTPase activity with oxotremorine showed a 1.7-fold increase in $K_A$ and 2.4-fold increase in $K_{cat}$ increased 1.9-fold, and $k_{cat}$ was the same as wild-type.

**Summary and Conclusions**—The GTPase assay is useful for the examination of the effect of mutations on receptor:G protein coupling. Wild-type receptor appeared to support two pathways of agonist-promoted GTPase activity with different Michaelis constants for agonist. The data are consistent with a model where the two pathways represent receptors coupled to a single or multiple G proteins. The lower $K_A$ of the pathway with G protein oligomers may arise from the absence of receptor dissociation from the G protein thought to precede GTP hydrolysis on Gα. The ability of the receptor to act catalytically and activate multiple G proteins is supported by a recent paper by Janetopoulos et al. (35). Their observations of the activation of G proteins by the cAMP receptor suggest that cAMP receptors act catalytically and undergo multiple rounds of G protein activation upon agonist stimulation.

These wild-type data are useful for the examination of the effects of site-directed mutants of the M₂ mACHR on the activation of G proteins. Three of the four mutant M₂ mACHR examined in this study have been partially or fully characterized with respect to ligand binding and effector activation in CHO cells. The goal of this study was to examine the utility of the GTPγS binding assay and GTPase assay to assess the coupling characteristics of these mutants at the mechanistic level. Assays for the individual steps in the activation of G proteins by mutant M₂ mACHR provide a more detailed understanding of the effects of these mutations on the activation of a defined G, preparation and provide insight into the mechanism of wild-type activation of G proteins. The data from this study support and extend the conclusions of the previous work and confirm that Asp-69 is involved in both the creation of different agonist-stabilized states of the receptor and in events downstream from guanine nucleotide exchange (possibly G protein dissociation from receptor) in the activation of Gα. The data confirm the importance of Asp-103 in ligand binding and also suggest that this residue is also involved in stabilization of the receptor-G protein complex. It is interesting to note that the low levels (D69N) or absence (D103N) of the receptor-G protein complex in ligand binding assays do not interfere with the levels of constitutive GTPγS binding that were similar to wild type. This observation suggests that low steady-state levels of the receptor-G protein complex need not interfere with the ability of the receptor to promote GTPγS binding to the same extent seen with the wild-type receptor. This result points out the potential inaccuracy in extending ligand binding data to functional interpretations of receptor activity. The differential
effects of mutations on the \( k_{cat} \) values for the agonist-promoted and constitutive pathways of GTPase activity seen with the T187A and Y403F mutants may arise from the ability of the mutant receptor to differentially affect dissociation of the G protein from the agonist bound versus free receptor.

**APPENDIX**

The mechanism for the hydrolysis of GTP by \( G \) coupled to the \( \text{M}_2 \) mACHR in Sf9 membranes shown in Fig. 6, where \( A \) is agonist, \( R \) is \( \text{M}_2 \) mACHR, \( G \) is \( G_i \), C1 to C4 indicate intermediate complexes, and \( X = G + ARG + RG \) can be rewritten as Equation A1.

This form of the equation treats \( X \) as a rapid equilibrium segment and allows the application of the method of Cha (36) to determine the rate equation for the mechanism. The value of \( f_i \) represents the fractional concentration factor of the enzyme form in the rapid equilibrium segment that reacts with rate constant \( k_i \) (see Equations A2, A4, and A5),

\[
f_i = \frac{[ARG]}{[A]} \quad \text{and} \quad f_{11} = \frac{[RG]}{[G]} \quad \text{(Eq. A2)}
\]

by defining the following values (Equations A3 and A4),

\[
K_i = \frac{[R][G]}{[RG]}, \quad K_0 = \frac{[A][RG]}{[ARG]}, \quad K_3 = \frac{[AR][G]}{[ARG]} \quad \text{and} \quad K_4 = \frac{[AR]}{[AR]} \quad \text{(Eq. A3)}
\]

\[
f_5 = \frac{[R][A]}{[K_0][K_2]} \quad \text{and} \quad f_{11} = \frac{[R][A]}{[K_0][K_2]} \quad \text{(Eq. A4)}
\]

the King and Altman method (37) can then be used to derive distribution equations describing the amount of G protein present in each enzyme form as a fraction of the total G protein (Equation A5),

\[
(D) \quad \frac{[X]}{[G_0]} = k_{h}k_{12}k_{13}k_{[GTP]}^2
\]

\[
(D) \quad \frac{[C_1]}{[G_0]} = k_{h}k_{12}k_{13}k_{[GTP]}^2 k_{h} + k_3
\]

\[
(D) \quad \frac{[C_2]}{[G_0]} = k_{h}k_{12}k_{13}k_{[GTP]}^2
\]

\[
(D) \quad \frac{[C_4]}{[G_0]} = k_{h}k_{12}k_{13}k_{[GTP]}^2 k_{h} + k_5
\]

\[
(D) \quad \frac{[C_3]}{[G_0]} = k_{h}k_{12}k_{13}k_{[GTP]}^2 k_{h} + k_4
\]

\[
(D) \quad \frac{[G]}{[G_0]} = k_{h}k_{12}k_{13}k_{[GTP]}^2 k_{h} + k_5
\]

where \([G_0]\) is total \([G]\) and \([C_1] = [X] + [C_2] + [C_3] + [C_4] + [G']\) and \((D)\) is the sum of all the values on the right side of Equation A5. The initial velocity equation for the simplified mechanism is \( v_i = k_{17} [G']. \) Substituting for \([G']\) in Equation A6,

\[
\frac{V_i}{G_0} = \frac{k_{h}k_{12}k_{13}k_{[GTP]}^2}{k_{17} + k_{17} + k_{3} + k_{5}} \quad \text{(Eq. A6)}
\]

Rearranging Equation A6 gives Equation A7.

\[
\frac{V_i}{G_0} = \frac{k_{h}k_{12}k_{13}k_{[GTP]}^2}{k_{17} + k_{17} + k_{3} + k_{5}} \quad \text{(Eq. A7)}
\]

The values of steady-state kinetic constants are defined (Equation A8) as follows.

\[
k_{cat1} = \frac{V_1}{[G_0]} = \frac{k_{h}k_{12}k_{13}k_{[GTP]}^2}{k_{17} + k_{17} + k_{3} + k_{5}} \quad \text{(Eq. A8)}
\]

\[
k_{cat2} = \frac{V_2}{[G_0]} = \frac{k_{h}k_{12}k_{13}k_{[GTP]}^2}{k_{17} + k_{17} + k_{3} + k_{5}} \quad \text{(Eq. A8)}
\]

The initial velocity for mACHR-stimulated GTPase activity was a hyperbolic function of [GTP] under conditions where agonist was saturating and in the absence of agonist (e.g. constitutive). The fitted \( K_m \) values for GTP were the same under both conditions. This allowed the simplifying assumption that \( K_{GTP1} = K_{GTP2} = K_{GTP} \). Equation A8 can then be rewritten in terms of steady-state kinetic constants (see Equation A9),

\[
V_i = \frac{V_1[A][GTP] + V_2[K_0][GTP]}{[A][GTP] + [K_0][GTP] + K_{GTP}[A] + [K_0]} \quad \text{GTase1} \quad \text{(Eq. A9)}
\]

where \( V_1 \) and \( V_2 \) are the maximum velocities for agonist-stimulated and agonist-independent (constitutive) \( \text{M}_2 \) mACHR-
promoted GTPase activity, respectively; [A] equals the concentration of agonist; $K_a$ is the Michaelis constant for agonist; and $K_{GTP}$ is the Michaelis constant for GTP. If more than one class of G proteins is present and both behave as described in the above derivation but have different kinetic constants, then there are essentially two parallel reaction pathways, and Equation A9 becomes Equation A10.

$$V_{11} = \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} \quad \text{(Eq. A10)}$$

Because the $K_m$ values for GTP were the same (e.g., $K_{GTP}$, $K_{GTP}$, and $K_{GTP}$ equal $K_{GTP}$, Equation A10 was simplified to give Equation A11.

$$V_{11} = \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} \quad \text{(Eq. A11)}$$

Equation A11 predicts that as the agonist concentration goes to 0 the constitutive (agonist independent) $M_2$ mACHR-promoted GTPase activity at the two classes of receptor sites will equal $(V_1 + V_2) (K_{GTP} + [GTP])$ which yields the form of the equation used for data analysis where $V_2 = (V_1 + V_2)/2$ because the constitutive activity has a simple hyperbolic dependence on [GTP].

$$V_{11} = \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} + \frac{[A][GTP]}{[K_{GTP}][GTP]} \quad \text{(Eq. A12)}$$

In Equation A12 $V_{11}$ and $V_{12}$ are the maximum velocities of agonist-stimulated $M_2$ mACHR-promoted GTPase activity at the two classes of receptor sites, and $V_2$ is agonist-independent (constitutive) $M_2$ mACHR-promoted GTPase activity. $[A]$ is concentration of agonist; $K_{A,2}$ are Michaelis constants for agonist in pathway one or two; and $K_{GTP}$ is Michaelis constant for GTP (same for both agonist promoted pathways and constitutive activity).

REFERENCES

1. Heitz, F., McClue, S. J., Harris, B. A., and Guenet, C. (1995) J. Recept. Signal. Transduct. Res. 15, 55–70
2. Rinken, A., Kameyama, K., Haga, T., and Engstrom, L. (1994) Biochem. Pharmacol. 48, 1245–1251
3. Sane, T., Ohyama, K., Yamano, Y., Nakagomi, Y., Nakazawa, S., Kikyo, M., Shirai, H., Blank, J. S., Exton, J. H., and Inagami, T. (1997) J. Biol. Chem. 272, 23831–23836
4. Miyamoto, M., Laufl, U., Pardo, C., and Liao, J. K. (1997) J. Biol. Chem. 272, 19661–19669
5. Grunewald, S., Reiland, H., and Michel, H. (1996) Biochemistry 35, 15162–15173
6. Grunewald, S., Haase, W., Reiland, H., and Michel, H. (1996) Biochemistry 35, 15149–15161
7. Barr, A. J., Brass, L. F., and Manning, D. R. (1997) J. Biol. Chem. 272, 2293–2299
8. Hermans, E., Geurts, M., and Maloteaux, J. M. (1997) Br. J. Pharmacol. 121, 1817–1823
9. Tota, M. R., Kahler, K. R., and Schimerlik, M. I. (1987) Biochemistry 26, 8175–8182
10. Seifert, R., Wenzel-Seifert, K., Lee, T. W., Gether, U., Sanders-Bush, E., and Kublika, B. K. (1996) J. Biol. Chem. 271, 5109–5116
11. Perrot, E., Winslow, J. W., Peterson, G. L., Smith, D. H., Ashkenazi, A., Ramachandran, J., Schimerlik, M. I., and Capon, D. J. (1987) Science 236, 660–665
12. Vogel, W. K., Peterson, G. L., Broderick, D. J., Mosher, V. A., and Schimerlik, M. I. (1999) Arch. Biochem. Biophys. 361, 283–294
13. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 156, 367–382
14. Sangar, P., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
15. Peterson, G. L. (1983) Methods Enzymol. 91, 95–119
16. Peterson, G. L., Tomnade, A., Johnson, W. C., Jr., and Schimerlik, M. I. (1995) J. Biol. Chem. 270, 17808–17814
17. Vogel, W. K., Sheehan, D. M., and Schimerlik, M. I. (1997) Mol. Pharmacol. 52, 1087–1094
18. Passonneau, J. V., and Lowery, O. H. (1962) Biochem. Biophys. Res. Commun. 7, 10–15
19. Marquardt, D. W. (1963) J. Soc. Indust. Appl. Math. 11, 431–441
20. Hebert, T. E., Moffett, S., Morello, J. P., Lisere, T. P., Bichet, D. G., Barret, C., and Bovier, M. (1996) J. Biol. Chem. 271, 16384–16392
21. January, B., Seibold, A., Whaley, B., Hipkin, R. W., Lin, D., Schonbrunn, A., Barber, R., and Clark, R. B. (1997) J. Biol. Chem. 272, 23871–23879
22. Keller, K. J., Whitehorn, E. A., Tate, E., Ries, T., Aguilar, B., Chervonu-Rogin, T., Davis, A. M., Dobbs, A., Yen, M., and Barrett, R. W. (1997) Anal. Biochem. 250, 51–60
23. Kamenis, E., Gomez, J., Lerche, C., and Wess, J. (1997) J. Biol. Chem. 272, 23675–23681
24. van Holde, K. E., Johnson, W. C., Jr., and Ho, P. S. (1998) Principles of Physical Biochemistry, 1st Ed, pp. 217–218, Prentice-Hall Inc., Upper Saddle River, NJ
25. Bennett, N., and Dupont, Y. (1985) J. Biol. Chem. 260, 4156–4168
26. Vanlanceurt, R. R., Dhanasekaran, N., Johnson, G. L., and Ruoho, A. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3645–3649
27. Willardson, B. M., Poo, B., Yoshida, T., and Bitemny, M. W. (1993) J. Biol. Chem. 268, 6371–6382
28. Nakamura, S., and Rodbell, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6413–6417
29. Nakamura, S., and Rodbell, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7150–7154
30. Coulter, S., and Rodbell, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5842–5846
31. Jahangeer, S., and Rodbell, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8792–8796
32. Hulme, E. C., Birdsall, N. J., and Buckley, N. J. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 633–673
33. Fraser, C. M., Wang, C. D., Robinson, D. A., Gocayne, J. D., and Venter, J. C. (1989) Mol. Pharmacol. 36, 840–847
34. Wess, J., Liu, J., Blin, N., Yun, J., Lerche, C., and Kostenis, E. (1997) Life Sci. 60, 1007–1014
35. Janetopulos, C., Jin, T., and Devreest, P. (2001) Science 291, 2408–2411
36. Cha, S. (1968) J. Biol. Chem. 243, 820–825
37. King, E. L., and Altman, C. (1966) J. Phys. Chem. 60, 1375–1381
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