Identification of Orthosteric and Allosteric Site Mutations in M_2 Muscarinic Acetylcholine Receptors That Contribute to Ligand-selective Signaling Bias*\textsuperscript{5}

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Muscarinic acetylcholine receptors contain at least one allosteric site that is topographically distinct from the acetylcholine, orthosteric binding site. Although studies have investigated the basis of allosteric modulation at these receptors, less is known about putative allosteric ligands that activate the receptor in their own right. We generated M_2 muscarinic acetylcholine receptor mutations in either the orthosteric site in transmembrane helices 3 and 6 (TM3 and -6) or part of an allosteric site involving the top of TM2, the second extracellular (E2) loop, and the top of TM7 and investigated their effects on the binding and function of the novel selective (putative allosteric) agonists (AC-42 (4-n-butyl-1-(4-(2-methylphenyl)-4-oxo-1-butyl)piperidine HCl), 77-LH-28-1 (1-(3-(4-butyl-1-piperidinyl)propyl)-3,3-dihydro-2(1H)-quinolinone), and N-desmethylclozapine) as well as the bitopic orthosteric/allosteric ligand, McN-A-343 (4-(m-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium). Four classes of agonists were identified, depending on their response to the mutations, suggesting multiple, distinct modes of agonist-receptor interaction. Interestingly, with 77-LH-28-1, allosteric site mutations had no effect on the affinity of any of the agonists tested, but some mutations in the E2 loop influenced the efficacy of both orthosteric and novel selective agonists, highlighting a role for this region of the receptor in modulating activation status. Two point mutations (Y1043.33A (Ballesteros and Weinstein numbers in superscript) in the orthosteric and Y177A in the allosteric site) unmasked ligand-selective and signaling pathway-selective effects, providing evidence for the existence of pathway-specific receptor conformations. Molecular modeling of 77-LH-28-1 and N-desmethylclozapine yielded novel binding poses consistent with the possibility that the functional selectivity of such agents may arise from a bitopic mechanism.

The five subtypes of muscarinic acetylcholine receptors (mACHRs)\textsuperscript{4} are prototypical Family A G protein-coupled receptors (GPCRs) that are widely expressed throughout the central nervous system and periphery and mediate most of the actions of the neurotransmitter acetylcholine (ACh). These receptors are also potential therapeutic targets for a diverse range of pathologies, including schizophrenia, Alzheimer disease, bladder dysfunction, and irritable bowel syndrome (1, 2). Unfortunately, the development of highly efficacious mAChR therapeutic agents with acceptable side effect profiles has remained largely elusive. This is most likely due to the fact that all current mAChR-based therapeutics target the Ach-binding (orthosteric site), which is predominantly made up of highly conserved residues within the top third of the transmembrane (TM) domains (3–9). One way to overcome this lack of selectivity at the mAChRs is to target allosteric sites, which are topographically distinct and generally consist of regions that show greater sequence divergence among receptor subtypes relative to residues comprising the orthosteric pocket (10–19).

The mAChRs, and in particular the M_2 subtype, are among the best characterized Family A GPCRs with respect to allosteric modulation by small molecules. Some of the earliest examples of mAChR allosteric modulators are exemplified by a series of neuromuscular blockers and bis-onium compounds, which either inhibit or enhance orthosteric ligand affinity, depending on the combination of ligands present and the receptor subtype (20–24). Although there is evidence that they may act as inverse agonists under certain experimental conditions (25–27), these prototypical mAChR modulators are often inactive in the absence of orthosteric ligand and mechanistically described by a simple allosteric ternary complex model (26, 28, 29).

More recently, it has become evident that there is a second class of allosteric ligand, collectively referred to as “allosteric agonists,” that are able to engender receptor activation in their own right (30, 31); many such agonists are emerging from drug discovery programs. This emerging approach to mAChR drug development is supported by a recent study that demonstrated that the allosteric agonists, McN-A-343 and the bitopic orthosteric/allosteric ligand, McN-A-334, were able to reverse the cholinergic muscle weakness associated with the use of the cholinergic anticholinesterase drug, physostigmine, in a mouse model of myasthenia gravis (32).

\textsuperscript{5} This work was supported by National Health and Medical Research Council (NHMRC) of Australia Program Grant 519461. The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1 and 2.

\textsuperscript{4} The abbreviations and trivial names used are: mAChR, muscarinic acetylcholine receptor; GTP\gamma S, guanosine 5'-[\gamma-thio]triphosphate; Gpp(NH)p, guanosine 5'-[\beta,\gamma-imido]triphosphate; GPCR, G protein-coupled receptor; ACh, acetylcholine; NDMC, N-desmethylclozapine; FBS, fetal bovine serum; QNB, quinuclidinyl benzilate; ERK, extracellular signal-regulated kinase; BPMC, biased probability Monte Carlo; TM, transmembrane; ANOVA, analysis of variance; CCh, carbachol; AC-42, 4-n-butyl-1-(4-(2-methylphenyl)-4-oxo-1-butyl)piperidine HCl; 77-LH-28-1, 1-(3-(4-butyl-1-piperidinyl)propyl)-3,3-dihydro-2(1H)-quinolinone; McN-A-343, 4-(m-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium.
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discovery programs, suggesting that their prevalence is likely to increase in the coming years. However, the term “allosteric agonist” has been broadly used to encompass a number of different possible receptor activation mechanisms. For example, a “pure” allosteric agonist would be a ligand that mediates both receptor activation and modulation of the orthosteric site via interaction with a topographically distinct allosteric binding site; the compound LY2033298, acting at the M₂ mAChR, is one such ligand (32, 33). Another variation is if the ligand can recognize both orthosteric and allosteric sites to engender agonism via the former site and modulation via the latter (34). Furthermore, the interaction between the two sites with such ligands can conceivably occur concomitantly, as recently demonstrated for the bitopic orthosteric/allosteric ligand McN-A-343 (35) and novel ceivably occur concomitantly, as recently demonstrated for the bitopic orthosteric/allosteric ligand McN-A-343 (35) and novel

Experimental Procedures

Materials—Dubacco’s modified Eagle’s medium, hygromycin-B, penicillin/streptomycin, metafectene, and zeocin were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Thermo Electron Corp. (Melbourne, Australia). [³H]Quinuclidinyl benzilate (QNB) (520 Ci/mmol), [³⁵S]GTPγS (1191 Ci/mmol), and AlphaScreen reagents were purchased from PerkinElmer Life Sciences. The SureFire™ cellular ERK1/2 assay kits were a gift from TGR Biosciences (Adelaide, Australia). AC-42 and 77-LH-28-1 were a generous gift from GlaxoSmithKline (Harlow, UK). N-Desmethyclozapine was sourced from Tocris Biosciences (Bristol, UK). All other reagents were purchased from Sigma or BDH Merck (Victoria, Australia) and were of an analytical grade.

Receptor Mutagenesis and Generation of Stable Cell Lines—Mutations were introduced into the wild type human M₂ mAChR in pENTR/D-TOPO using site-directed mutagenesis prior to transfer to pEFS/FRT/V5-DEST as described previously (19, 46), which resulted in the insertion of the V5 epitope tag at the C terminus of the receptor. This receptor sequence, referred to as “wild type” throughout this study, has previously been shown to possess pharmacological properties equivalent to those of the non-tagged human M₂ mAChRs (47). The primers for the E172Q/D173N/E175Q/Y177A/T423 7.36A constructs have been reported previously (19, 35). For additional mutations, the following primers were used: Y802.61A, forward (5'- GTTTTCTCATGAACCTTGCCACCCCTCTACACTGTG-3') and reverse (5'-CACAGTGTAAGGTTGCGCAAGTTCACTGAGAAAAAC-3'); W993.28A, forward (5'-GGTGTTGACCTTGGGCTAGCCCTGCTCTTG-3') and reverse (5'-CCACGAGGTCTACACACCCAGCCTGCTCTTG-3') and reverse (5'-CACAGTGTAAGGTTGCGCAAGTTCACTGAGAAAAAC-3'); L1003.29A, forward (5'-GGTGTTGACCTTGGGCTAGCCCTGCTCTTG-3') and reverse (5'-CCACGAGGTCTACACACCCAGCCTGCTCTTG-3'). In all instances, these compounds displayed the highest functional efficacy at the M₁ mAChR subtype, but they are able to bind to all mAChR subtypes with similar affinities. This suggests that their activation mechanisms vary between subtypes. To date, however, structure-function analyses of the mode of action of such novel selective agonists have focused almost exclusively on the M₁ mAChR (39–41, 43, 44). Furthermore, these studies have examined regions of the receptor predominantly associated with the orthosteric binding pocket. Thus, the aim of the current study was to introduce selected mutations into either the orthosteric site or the prototypical allosteric binding site on the M₂ mAChR (Fig. 1) and determine the consequences of these mutations on the binding and signaling properties of a number of novel selective agonists in comparison with the effects of the mutations on orthosteric ligands. By applying an operational model of agonism to the analysis of the data (45), we have also been able to differentiate mutational effects on ligand affinity from effects on ligand signaling efficacy and have identified mutations in both orthosteric and allosteric pockets that selectively affect coupling to the ERK1/2 pathway. Collectively, we have identified modes of interaction that are probably shared by more than one mAChR subtype as well as differences that may contribute to the observed functional selectivity of these novel agonists.

5 Ballesteros and Weinstein numbers are provided (in superscript) to indicate relative position of residues within the transmembrane domains (48). Residues located within the second extracellular loop are denoted by their position within the M₁ mAChR sequence only.
FIGURE 1. Agonist structures, and snake diagram of M₂ mAChR highlighting mutated residues. Residues previously reported to be involved in prototypical allosteric modulator binding are shown in squares, whereas residues implicated in orthosteric ligand binding are shown in heavy circles. Trp-99₁.₂₈ is highlighted within a diamond, because this residue has been implicated in both orthosteric and allosteric ligand binding.
TABLE 1
Radioligand binding parameter estimates for ligands at M2 mAChR constructs

| M2 receptor | Bmax | pKd | ACh | Arecoline | CCh | Pilocarpine | AC-42 | 77-LH-28-1 | Mcn-A-343 | NDMC |
|-------------|------|-----|-----|----------|-----|-------------|-------|------------|------------|------|
| Wild type   | 4.12 | 3.65| 4.53| 4.48     | 4.84| 4.88        | 4.84  | 4.64       | 4.16       | 4.16|
| W99A-A     | 3.65| 3.65| 4.53| 4.48     | 4.84| 4.88        | 4.84  | 4.64       | 4.16       | 4.16|
| L1003.33A  | 3.65| 3.65| 4.53| 4.48     | 4.84| 4.88        | 4.84  | 4.64       | 4.16       | 4.16|
| Y1043.33A  | 3.65| 3.65| 4.53| 4.48     | 4.84| 4.88        | 4.84  | 4.64       | 4.16       | 4.16|
| S1073.36A  | 3.65| 3.65| 4.53| 4.48     | 4.84| 4.88        | 4.84  | 4.64       | 4.16       | 4.16|
| Y4036.51A  | 3.65| 3.65| 4.53| 4.48     | 4.84| 4.88        | 4.84  | 4.64       | 4.16       | 4.16|
| E172Q/D173N/E175Q | 3.65| 3.65| 4.53| 4.48     | 4.84| 4.88        | 4.84  | 4.64       | 4.16       | 4.16|

Hygromycin-B at 37 °C in a humidified incubator containing 5% CO2, 95% O2. Non-transfected CHO-FlpIn cells were maintained in similar medium except that zeocin (50 μg/ml) was used instead of hygromycin-B as the selection agent.

Preparation of Cell Membranes for Binding—Membrane preparations were prepared from CHO-FlpIn cells stably expressing the wild type M2 mAChR and mutants thereof as described previously (35).

Radioligand Binding Assays—For saturation experiments, membrane preparations were diluted to 5–25 μg/tube using GTPγS Assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl2) containing 1 μM guanosine-3’,5’-diphosphate. [35S]GTPγS (0.1 nm) was then added and incubated for a further 30 min at 30 °C. The assay was terminated by rapid filtration through GF/B filters (prewet with 0.9% NaCl), followed by three washes with 3 ml of ice-cold 0.9% NaCl. Filters were then dried under a heat lamp before being transferred to scintillation vials, and 4 ml of Ultima Gold scintillation mixture was added. After 2 h, radioactivity was counted using a Packard 1600 TR liquid scintillation counter.

Radioligand Binding Assays—For saturation experiments, membrane preparations were diluted to 5–25 μg/tube using GTPγS Assay buffer and incubated with 0.003–3 nM [3H]QNB for 90 min at 30 °C in a shaking water bath. For [3H]QNB inhibition binding experiments, membrane preparations were diluted to 5–25 μg/tube using GTPγS assay buffer with 100 μM Gpp(NH)p and incubated with 0.1–0.3 nM [3H]QNB for 90 min in the presence of a range of concentrations of agonist. 10 μM atropine was used to determine nonspecific binding for all receptor constructs, except Y1043.33A and Y4036.51A, where 100 μM methoctramine was used. The assay was terminated, and radioactivity was determined as described above.

ERK1/2 Phosphorylation Assay—Receptor-mediated ERK1/2 phosphorylation was determined using the AlphaScreen-based ERK Surefire kit as described in detail previously (19, 46).

Ca2+ Mobilization Assay—The cell-permeant Ca2+ indicator dye Fluo-4 was used to assay receptor-mediated Ca2+ mobilization as described previously (49). A five-point smoothing function was applied to the raw Ca2+ traces, and the magnitude of the peak response was determined over the first 20 s and then corrected for the basal fluorescence of the individual well. Data were then normalized to the peak response elicited by 2 μM ionomycin.

Molecular Modeling—A homology model of the M2 mAChR was constructed based on the high resolution structure of the β2 adrenergic receptor (Protein Data Bank code 2RH1) (50) using the Molsoft ICM program (51). An alignment between the M2 mAChR and β2 adrenergic receptor sequences based on conserved TM residues of biogenic amine receptors was used in the creation of the model. To enable docking of the agonists, 77-LH-28-1 or NDMC, the E2 loop was not included in the preliminary homology model. The protocol involved tethering the M2 mAChR model backbone to the β2 adrenergic receptor template, utilizing residues where the sequences were aligned (as well as side chains where coordinates could be obtained from the template). To generate the lowest energy model, the system then underwent multiple rounds of biased probability Monte Carlo (BPMC) simulations. The resultant model was analyzed using the ICM Protein Health algorithm to ensure there were no stereochemical anomalies.

The docking of the agonists to the resultant homology model was then performed in ICM using the same BPMC algorithm. Initially, a low energy agonist starting conformation was generated with the six-membered ring of either compound in the boat conformation, and the functional groups were oriented in the equatorial positions with the nitrogen protonated, as is consistent with a physiological pH environment. The compounds were then posed manually in an approximate starting position for docking, such that Asp-1033.32 and the protonated nitrogen of either agonist could interact, based on the critical role of this Asp residue in the binding of prototypical biogenic amine receptor ligands (5, 52–54). This position was used to define a 16-Å simulation sphere around either ligand. The ligand and side chains within 11 Å of the ligand were subsequently allowed to move freely in the BPMC simulation. A distance restraint of 4.5 Å between an oxygen of Asp-1033.32 and the protonated
nitrogen of either agonist was imposed to ensure that the salt bridge was maintained. Multiple rounds of extensive BPMC simulations were performed (128 million energy evaluations), and the best energy pose was taken as the preferred docking position/binding mode.

Sixteen new homology models were then built around the agonist, utilizing the previously calculated pose. These latter models included the intact E2 loop and had Glu-172–Cys-176 aligned with the equivalent Glu-186–Cys-191 of the H9252 adrenergic receptor, whereas the remaining loop residues were unaligned and able to move freely in the modeling protocol. The highly conserved disulfide bond between Cys-963.25 and Cys-176 (E2 loop) of the M2 mAChR was maintained in the model.

Data Analysis—All computerized nonlinear regression was performed using Prism 5.01 (GraphPad Software, San Diego, CA). Total and nonspecific binding data sets from [3H]QNB saturation binding assays were globally fitted to a one-site saturation binding model to derive estimates of the radioligand dissociation constant (KD) and maximal density of binding sites (Bmax). Inhibition binding data sets were fitted to a one-site inhibition binding model to obtain estimates of inhibitor potency, which were then converted to inhibitor dissociation constants (KI) (55). Agonist concentration-response curves were fitted to either a three- or four-parameter logistic equation to derive estimates of agonist potency (EC50) and maximal agonist effect (Emax). Subsequently, to compare agonist profiles between the M2 wild type and mutant receptors in terms of separating effects on affinity from signaling efficacy, agonist concentration-response curves were also fitted to the following form of an operational model of agonism (45, 56),

\[
Y = \text{Basal} + \frac{E_m - \text{basal}}{1 + \left(\frac{10^{0} + 10^{0}}{10^{0} \times 10^{0}}\right)^n}
\]  

(1)

where \(E_m\) is the maximal possible response of the system (not the agonist), basal is the basal level of response in the absence of agonist, \(K_A\) denotes the functional equilibrium dissociation constant of the agonist (A), \(\tau\) is an index of the coupling efficiency (or efficacy) of the agonist and is defined as \(R_T/K_E\) (where \(R_T\) is the total concentration of receptors and \(K_E\) is the concentration of agonist-receptor complex that yields half the maximum system response (\(E_{m}\))), and \(n\) is the slope of the transducer function that links occupancy to response. To define the \(E_m\) and \(\tau\) for each mutant and assay, the \(K_A\) for all high efficacy agonists was constrained to equal the \(K_I\) value derived from radioligand binding assays (see “Results”) in the nonlinear regression procedure. For all other agonists that did not produce the maximal system response, \(K_A\) was estimated directly from the functional data via nonlinear regression.

**FIGURE 2. Novel selective agonists display low efficacies at the wild type M2 mAChR.** A and B, peak levels of phosphorylated ERK1/2 (pERK1/2) were assessed as described under “Experimental Procedures,” corrected for basal level, and normalized to the response elicited by 10% FBS. Data represent the mean ± S.E. of 5–16 experiments performed in duplicate. C and D, agonists were pre-equilibrated with membranes from FlpInCHO cells stably expressing the wild type M2 mAChR prior to exposure to [35S]GTPγS, as indicated under “Experimental Procedures.” Data were corrected for basal level and normalized to the maximal response elicited by ACh. Data represent the mean ± S.E. of 3–5 experiments performed in duplicate. E, ordinate response data for equivalent concentrations of each agonist at each pathway in A–D were plotted against one another. In this manner, the bias for a given agonist toward one pathway relative to the other can be visualized. The error bars not shown lie within the dimensions of the symbol.

The highly conserved disulfide bond between Cys-964 and Cys-176 (E2 loop) of the M2 mAChR was maintained in the model.
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All affinity, potency, and efficacy parameters were estimated as logarithms (57). All results are expressed as the mean ± S.E. Statistical analyses were performed where appropriate using one-way ANOVA with Dunnett’s post-test, and statistical significance was taken as p < 0.05.

RESULTS

Rationale for the Choices of Orthosteric and Allosteric Site Mutations—Fig. 1 shows a snake diagram of the secondary structure of the M₂ mAChR, indicating residues mutated in the current study. The orthosteric site on the mAChRs is composed of a series of conserved amino acid residues that line the upper third of the receptor’s extracellular facing transmembrane regions (58). In the current study, we have focused on key residues in TM3 and TM6, specifically Asp-103,28, Leu-104,29, Tyr-104,33, Ser-107,36, and Tyr-403,51 (6, 7, 40, 59). It should be noted that the conserved Trp,28 residue has also been reported to affect the properties of allosteric ligands, such as gallamine, at the M₁ mAChR (60). In general, however, the allosteric site recognized by prototypical modulators is thought to comprise residues that are located more extracellularly relative to the orthosteric pocket and usually involve amino acids that are not as highly conserved across subtypes (31). At the M₂ mAChR, these include Glu-172, Asp-173, Glu-175, and Tyr-177 in the E2 loop and a cluster of residues bordering the extracellular face of TM7 (Arg-419,32, Thr-420,33, Trp-422,35, and Thr-423,36) (10–19). More recently, mutation of Tyr,261 at the top of TM2 in the M₁ mAChR has been shown to alter the allosteric properties of the prototypical modulator, gallamine, as well as the putative allosteric agonists, 77-LH-28-1 and AC-42 (44, 61). Thus, the equivalent residue of the M₂ mAChR, Tyr-80,61, was substituted for alanine and included in the present study.

Orthosteric and Allosteric Agonists Bind with Similar Affinities to the Wild Type M₂ mAChR—Agonist affinities for the ground state of the wild type M₂ mAChR were initially determined from inhibition binding assays against the orthosteric antagonist, [³H]QNB, in the presence of 100 μM Gpp(NH)₇; the latter was included to promote the uncoupling of the receptor from its cognate G protein(s) and thus maintain an inactive state. The pKᵢ values determined from these experiments are listed in Table 1, where it can be seen that the differences in affinities for the M₂ mAChR between the orthosteric compounds, ACh, CCh, arecoline, and pilocarpine, and the novel selective agonists, AC-42, 77-LH-28-1, and NDMC, were not large. The bitopic ligand, McN-A-343, displayed the lowest affinity for the receptor, but overall all agonists bound with affinities in the low (~1–50) micromolar range.

Evidence for Functional Selectivity at the Wild Type M₂ mAChR—To assess the potential for functional selectivity of the various agonists at the M₂ mAChR, two pathways linked to receptor activation were chosen: ERK1/2 phosphorylation and [³⁵S]GTPγS binding. The former assay was chosen specifically because it is downstream of receptor activation and represents a convergence point for multiple cellular inputs, some of them potentially G protein-independent (62). The latter assay was chosen specifically as a proximal measure of receptor activation at the level of the G protein. For each agonist, ERK1/2 phosphorylation was determined to peak between 5 and 10 min (data not shown), and subsequent concentration-response curves were constructed at the peak time points. The orthosteric ligands ACh, arecoline, and CCh were all high efficacy agonists for M₂ mAChR-mediated ERK1/2 phosphorylation, whereas pilocarpine displayed partial agonism. In comparison, the novel selective agonists, 77-LH-28-1 and NDMC, exhibited weak partial agonism, as did the bitopic ligand, McN-A-343; AC-42 did not display any discernible agonism at the wild type M₂ mAChR (Fig. 2 and Table 2). The rank order of potencies was ACh > CCh ≥ arecoline > NDMC > pilocarpine > McN-A-343 = 77-LH-28-1. In agreement with the ERK1/2 phosphorylation data, ACh, arecoline, and CCh also showed high efficacy for promoting [³⁵S]GTPγS binding (Fig. 2 and Table 2). In contrast, pilocarpine, NDMC, McN-A-343, and 77-LH-28-1 were clear partial agonists for [³⁵S]GTPγS binding. AC-42 did not elicit a detectable response. Interestingly, when compared with the ERK1/2 phosphorylation data, a different rank order of potencies was evident: ACh = NDMC > arecoline ≥ 77-LH-28-1 > CCh ≈ pilocarpine > McN-A-343.

Fig. 2E illustrates a novel means of graphing the potential signaling bias of the various agonists between the two pathways. The normalized responses for each agonist signaling to ERK1/2 and [³⁵S]GTPγS binding were replotted against each other. In this manner, the bias of each agonist for one pathway relative to the other is immediately apparent. It can be seen from this plot that ACh, CCh, and arecoline have a bias for signaling to ERK1/2 relative to promoting [³⁵S]GTPγS binding, whereas 77-LH-28-1, AC-42, McN-A-343, and NDMC show the reverse preference. Interestingly, pilocarpine appears to have equal preference for signaling to both pathways in this cellular background. If it is assumed that the signaling bias demonstrated by the endogenous agonist for a given GPCR represents the “natural” (non-selective) cellular state, then any bias toward different pathways by other agonists is evidence of functional selectivity by those agonists.

Subsequently, we applied an operational model of agonism (45) to the data, which allowed for the separate determination of functional agonist affinity (pKᵢ; Table 3) and relative signal-

### TABLE 2

Potency (pEC⁰) and maximal agonist effect (E_max) parameters for muscarinic ligands at wild type M₂ mAChR in signaling assays

| Ligand       | pEC⁰  | E_max  |
|--------------|-------|--------|
| ACh          | 8.18  | 95.1   |
| Arecoline    | 7.23  | 85.2   |
| CCh          | 7.30  | 84.1   |
| Pilocarpine  | 5.69  | 72.0   |
| 77-LH-28-1   | 5.22  | 34.1   |
| AC-42        | NR    | NR     |
| McN-A-343    | 5.27  | 16.2   |
| NDMC         | 6.58  | 35.8   |

Values represent the mean ± S.E. from 5–16 experiments performed in duplicate. NR, no response detected.

*Negative logarithm of the agonist concentration required to elicit half the maximal response.

Maximal response elicited by the ligand expressed as a percentage of the response to 10% FBS.

Maximal response elicited by the ligand expressed as a percentage of the maximal response to ACh.

Significantly different from the ACh value, as determined by one-way ANOVA, p < 0.05.
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### TABLE 3

| M$_2$ receptor | pERK1/2/pK$_A$ | pERK1/2/pK$_A$ |
|---------------|----------------|----------------|
| 77-LH-28-1    | AC-42         | Arocine        |
| 4.99$^a$      | 4.85$^b$      | 5.19 ± 0.06    |
| 7465          | 7465          | 7465           |

Values represent the mean ± S.E. from 3–16 experiments performed in duplicate. The spacing within each signaling pathway section divides mutations into predominantly orthosteric (top) versus allosteric site (bottom). NR, no response detected; ND, not determined.

### TABLE 4

| M$_2$ receptor | pERK1/2/log$\tau$ | pERK1/2/log$\tau$ |
|---------------|-------------------|-------------------|
| 77-LH-28-1    | AC-42             | Arocine           |
| 2.51 ± 0.25   | 2.58 ± 0.30       | 2.67 ± 0.30       |

Values represent the mean ± S.E. from 3–16 experiments performed in duplicate. The spacing within each signalling pathway section divides mutations into predominantly orthosteric (top) versus allosteric site (bottom). NR, no response detected; ND, not determined.

$^a$ Negative logarithm of the equilibrium dissociation constant for each ligand, determined utilizing an operational model of agonism.

$^b$ In all cases, ACh was assumed to be a full agonist, and as such, the pK$_A$ was constrained to equal the pK$_A$ derived from [3H]QNB inhibition binding assays (Table 1); for other ligands, if the maximal agonist response was not different from ACh, this same constraint was introduced.

$^c$ Significantly different ($p < 0.05$) from the wild type receptor as determined by one-way ANOVA.

The coupling efficiencies of ligands at M$_2$ mAChR constructs determined from signaling assays (Values represent the mean ± S.E. from 3–16 experiments performed in duplicate. The spacing within each signaling pathway section divides mutations into predominantly orthosteric (top) versus allosteric site (bottom). NR, no response detected; ND, not determined.

$^d$ Relative coupling efficiency parameter, $\tau$, was determined via nonlinear regression of the data to an operational model of agonism and corrected for receptor expression levels to yield a corrected $\tau$ parameter.

$^e$ Significantly different ($p < 0.05$) from the wild type receptor as determined by one-way ANOVA.

Significantly different ($p < 0.05$) from orthosteric (top) versus allosteric site (bottom) according to the pK$_A$, corrected for the orthosteric values derived from radioligand binding studies (Table 1), with the exception of McN-A-343, where the Km values were ~10-fold lower (i.e. significantly higher affinity). This latter finding suggests that the signaling assays are capturing a higher affinity conformation of the receptor unique to the bitopic ligand, McN-A-343, that is not detected in the radioligand binding assays. For the remaining agonists, it is likely that the stimulus bias they exhibit for one pathway relative to the other arises from differences in relative signaling efficacies.
Mutations within the Predicted Orthosteric or Allosteric Binding Pockets Have Differential Effects on Agonist Binding—Mutations were introduced into regions of the M₂ mAChR predicted to contribute to either the prototypical allosteric site or the orthosteric site (Fig. 1). Mutation of D1033.32A yielded no discernible binding (or functional response) to any of the agonists tested, consistent with previous studies (5, 54), and was not evaluated any further. Saturation binding assays performed on the orthosteric site mutations revealed a decreased affinity for the orthosteric antagonist, [³H]QNB, of between 2- and 40-fold, depending on the residue mutated (Table 1). In contrast, allosteric site mutations had little or no effect on the affinity of the orthosteric radioligand.

With respect to agonist affinity estimates, four different types of effects were revealed by the mutational analysis. First, alanine substitutions of the orthosteric site residues, Leu-1003.29, Tyr-1043.33, Ser-1073.36, or Tyr-4036.51, resulted in significantly reduced affinity for the orthosteric agonists, ACh, arecoline, CCh, and pilocarpine (Table 1), compared with the wild type M₂ mAChR. Similarly, the pKᵦ values for ACh, CCh, and pilocarpine at W993.28A were also reduced, although only the reduction in pKᵦ for ACh reached statistical significance; allosteric site mutations had little effect on the affinity of the orthosteric agonists tested. In contrast, AC-42 showed a 70-fold increase in affinity for the W993.28A mutant, with no change observed at all other mutants (Table 1). Even more strikingly, 77-LH-28-1 displayed a >1000-fold increase in binding affinity for the W993.28A mutant M₂ mAChR. In addition, 77-LH-28-1 showed affinity increases (3–30 fold) at Y403.51A, Y802.61A, E172Q/D173N/E175Q and the combined E172Q/D173N/E175Q/Y177A/T4237.36A mutant. Third, the affinity of NDMC was similar at all mutants, with the exception of L1003.29A, where a significant 10-fold reduction was observed. Fourth, none of the mutants altered the affinity of the bitopic ligand, McN-A-343.

Mutations within the Orthosteric or Allosteric Sites Lead to Distinct Effects on M₂ mAChR-mediated ERK1/2 Phosphorylation Depending on the Agonist—None of the mutations introduced into the M₂ mAChR had any discernible effect on the basal level of ERK1/2 phosphorylation or on the receptor-independent ERK1/2 response to 10% FBS (data not shown). Agonist concentration-response curves were generated as described above for the wild type receptor and analyzed according to the operational model of agonism to delineate effects on functional affinity (Table 3) and efficacy (Table 4). To account for the potential confounding effect of different receptor expression levels for each of the mutants on estimates of agonist signaling efficacy, the τ values from the operational model analyses were normalized to what they would be if the mutant were expressed at the same level as the wild type receptor, using the B₅₀ values obtained from [³H]QNB saturation binding studies (Table 1). These corrected τ values are referred to as τₑ values herein (Table 4). This allowed for a direct comparison of agonist functional affinities (Kₑ) and signaling efficiencies (τₑ) across different receptor mutants (Figs. 3 and 4).

In general, the functional estimates of agonist affinities (pKₐ values) followed a similar trend as those determined via radio-ligand binding (pKᵦ values) for the various mutants studied, with the exception of L1003.29A, where significant reductions were noted in pKᵦ values for arecoline, pilocarpine, and NDMC but...
As with the binding data, the functional assays also revealed distinct modes of agonism at the mutant M2 mAChRs that depended on the nature of the agonist. For the orthosteric site mutations W993.28A, L1003.29A, S1073.36A, and Y4036.51A, variable reductions were noted in the efficacy of ACh (2–30-fold). Arecoline, CCh, and pilocarpine followed a similar trend, although not all reductions were statistically significant (Table 4 and Fig. 4). In contrast, a different profile of effects was noted for 77-LH-28-1 and AC-42, which displayed gains in signaling efficiency at the W993.28A M2 mAChR (Table 4 and Fig. 5) and either modest (77-LH-28-1) or no apparent (AC-42) sensitivity to mutation of L1003.29A, S1073.36A, or Y4036.51A. The last mutation, however, had a marked effect on NDMC, increasing its efficacy such that the ligand elicited the maximal system response for ERK1/2 phosphorylation (Fig. 5), whereas the L1003.29A and S1073.36A mutations affected the signaling efficacy of the bitopic ligand, McN-A-343, completely abolishing its response (Fig. 4). Another interesting finding from the operational model analysis was that the transducer slope function, which is a measure of the sensitivity by which agonist occupancy is linked to final response, was dramatically steepened at the W993.28A mutation for 77-LH-28-1 and AC-42 in the analysis of the ERK1/2 data (supplemental Table 1); this is suggestive of a selective change in the sensitivity of coupling of these two agonists to the ERK1/2 response upon mutation of the Trp-993.28 residue.

Subsequently, we assessed mutations that were expected to influence predominantly the prototypical allosteric site of the M2 mAChR with respect to their impact on agonism of novel selective/bitopic ligands in parallel with two representative orthosteric ligands, ACh and pilocarpine. Intriguingly, Ala substitution of Tyr-802.61 in TM2 actually increased the efficacy of the orthosteric ligand pilocarpine and abolished the response to 77-LH-28-1 but had no effect on NDMC and McN-A-343 (Fig. 4). Allosteric site mutations in TM7 and the E2 loop had no effect on the functional affinity or efficacy of pilocarpine (Figs. 3

FIGURE 4. Signaling efficacy estimates from pERK1/2 assays of novel selective agonists display a different pattern of responses to receptor mutations relative to orthosteric agonists. The bars represent the difference in log $\tau_c$ estimates (coupling efficiency corrected for receptor expression level) of each agonist derived from an operational model of agonism (see “Experimental Procedures”), relative to the wild type receptor value for that agonist. Data are not shown for AC-42, because this compound did not elicit any response at the wild type receptor (but see Table 4). All other details are as for Fig. 3.
and 4 and Tables 3 and 4) but caused small reductions in the efficacy of ACh (Fig. 4 and Table 4). The functional affinity of 77-LH-28-1 was increased (7–25-fold) at Y177A, T4237.36A, and the combined E172Q/D173N/E175Q/Y177A/T4237.36A mutant (Fig. 3 and Table 3). Small reductions (~3-fold) in 77-LH-28-1 efficacy were seen for the N4197.32A, T4237.36A, and E172Q/D173N/E175Q mutants. Only T4237.36A significantly reduced the efficacy of NDMC (~3-fold; Fig. 4 and Table 3). The efficacy of McN-A-343 was significantly increased at Y177A and E172Q/D173N/E175Q/Y177A/T4237.36A (7- and 17-fold, respectively; Table 4), such that McN-A-343 elicited a similar maximal response to ACh. Collectively, these results highlight that residues previously suggested to affect the potency of prototypical allosteric modulators have more complex effects on agonists, irrespective of whether the latter are orthosteric or potentially allosteric.

**Effects of Selected Mutations on M2 mAChR-mediated \[^{35}S\]GTP\(\gamma\)S Binding Provide Evidence for Pathway-specific Changes in Coupling Efficiency**—From the preceding studies, the most striking effects on the signaling properties of the novel selective/bitopic agonists were noted at the W993.28A, Y177A, and Y4036.51A mutations. To establish whether the differential effects on agonist function of these mutations were signal pathway-dependent, \[^{35}S\]GTP\(\gamma\)S binding was utilized as a second measure of receptor activation (Fig. 6). The orthosteric site mutants, W993.28A and Y4036.51A, decreased the efficacy and functional affinity of ACh, arecoline, CCh, and pilocarpine, although these reductions did not all reach significance (Tables 3 and 4). Consistent with the ERK1/2 data, the functional affinity of AC-42 and 77-LH-28-1 derived using the operational model fit of the \[^{35}S\]GTP\(\gamma\)S data were dramatically increased at the W993.28A mutant (Fig. 7 and Table 3), although the compounds were not able to elicit the maximal system response with respect to G protein activation (Fig. 6). At the Y4036.51A mutant, NDMC achieved a similar response level to ACh (Fig. 6), due to an increase in its efficacy (4-fold; Fig. 8 and Table 4), again consistent with the ERK1/2 results. Similarly, the efficacy of pilocarpine was increased at Y802.61A with respect to \[^{35}S\]GTP\(\gamma\)S binding, whereas the efficacy of 77-LH-28-1 was decreased (Fig. 8 and Table 4). Interestingly, however, for \[^{35}S\]GTP\(\gamma\)S binding at the Y177A mutant (Fig. 6), the efficacy of McN-A-343 was not increased (Fig. 8), which is in contrast to what was seen in the ERK1/2 experiments. Thus, it is possible that this E2 loop residue in the prototypical allosteric modulator pocket plays a previously unappreciated role in signal pathway selectivity. Further evidence for a role of Tyr-177 in receptor activation was provided by the significant decreases in efficacy that were evident for ACh, arecoline, CCh, pilocarpine, 77-LH-28-1, and NDMC at this mutation (Fig. 8 and Table 4).

**Identification of Tyr-1043.33 as a Key Residue That Selectively Couples Receptor Activation to the ERK1/2 Pathway Irrespective of the Agonist**—Another novel finding in the current study was noted with mutation of Tyr-1043.33 in TM3. Specifically, we found that alanine substitution of this residue resulted in almost complete abrogation of agonist signaling to the ERK1/2 pathway; ACh could only maximally elicit ~10% of the wild type M2 mACHr response, whereas no response could be detected for any of the other orthosteric or novel selective agonists (Fig. 9A). Nonetheless, the receptor was still able to couple to G protein activation, as evidenced in the \[^{35}S\]GTP\(\gamma\)S binding assays (Fig. 9, B and C). Although the efficacy and/or affinities for the orthosteric ligands, ACh, arecoline, CCh, and pilocarpine, at the Y1043.33A mutant were decreased relative to the M2 wild type mAChR (Fig. 8), NDMC and 77-LH-28-1 elicited similar maximal responses to ACh, indicating significantly increased efficacy (4 and 17-fold, respectively) at this mutant with respect to G protein activation. The efficacy of McN-A-343 in promoting \[^{35}S\]GTP\(\gamma\)S binding was unaffected by the mutation, whereas AC-42 gained efficacy to a similar degree as observed at the Y4036.51A mutation in this same assay (Table 4).

To ensure that the selective lack of activation of ERK1/2 phosphorylation by the Y1043.33A mutant (in contrast to its effects in the membrane-based \[^{35}S\]GTP\(\gamma\)S binding assays)
was not simply due to lack of cell surface expression of the construct, we monitored whole cell intracellular Ca\textsuperscript{2+} mobilization as a third functional measure of receptor activity. Although the M\textsubscript{2} mAChR is not as efficiently coupled to Ca\textsuperscript{2+} mobilization as the M\textsubscript{1}, M\textsubscript{3}, and M\textsubscript{5} mAChR subtypes (63, 64), responses were clearly noted at both wild type and Y1043.33A mAChRs for the most efficacious agonists, ACh, CCh, and arecoline (Fig. 9, D and E). Moreover, the Ca\textsuperscript{2+} mobilization response mediated by ACh at the Y1043.33A M\textsubscript{2} mAChR was sensitive to inhibition by the hydrophilic antagonist, NMS (supplemental Fig. 1), providing further evidence that activation of the receptor was occurring at the cell surface.

The fact that responses could be observed for a traditionally poorly coupled M\textsubscript{2} mAChR pathway, such as Ca\textsuperscript{2+} mobilization, but not for the pERK1/2 pathway in intact cells suggests that the effect of the Y1043.33A mutation was indeed to functionally and selectively uncouple the M\textsubscript{2} mAChR from the pERK1/2 response, irrespective of the nature of the activating agonist.

Docking of 77-LH-28-1 or NDMC to a Homology Model of the M\textsubscript{2} mAChR Reveals Novel Binding Poses—In a previous molecular modeling study, we identified a binding pose for the bitopic ligand McN-A-343 that indicated a lack of prominent interactions with a number of residues involved in binding prototypical orthosteric ligands (35). This model is also consistent with findings reported herein for McN-A-343. Thus, for the purposes of the current study, we attempted to rationalize the findings obtained with the agonists 77-LH-28-1 and NDMC. Computerized BPMC-based docking of each agonist using ICM yielded the docking poses depicted in Fig. 10. Interestingly, despite modeled interactions with Asp-1033.32, both compounds are predicted to sit higher up near the junction of the transmembrane regions and extracellular loops compared with the classic orthosteric pocket that is located further within the TM bundle. This is consistent with the lack of effect on the affinity of the agonists by mutation of Y1043.33A or S1073.36A. The significant effects that mutation of W993.28A, Y802.61A, or Y4036.51A have on the binding affinity of 77-LH-28-1 may be due to the removal of steric constraints imposed on the binding of the dihydroquinolinone ring or other parts of the ligand by the side chains of these amino acids; mutation of these residues clearly results in receptor conformations more favorable for 77-LH-28-1 binding. With NDMC, the only mutation directly found to reduce its binding affinity was L1003.29A, which may be due to the removal of a direct hydrophobic interaction between the residue and NDMC.

If the binding mode of the two agonists was indeed bitopic, one would expect that they should functionally compete with other purely orthosteric agonists. We have previously found this to be the case for the interaction between 77-LH-28-1 and...
ACh (19) (see also supplemental Fig. 2) and have now identified a similar interaction between NDMC and ACh as well (supplemental Fig. 2).

**DISCUSSION**

This is the first study to simultaneously probe the effects of key mutations in the conserved orthosteric site and extracellular allosteric binding site on the binding and signaling of both orthosteric and novel selective (putative allosteric) agonists at the M2 mAChR. We have identified four broad classes of agonist, which indicates multiple modes of agonist-receptor engagement. Moreover, we have identified two amino acid residues likely to contribute to ERK1/2-specific functional selectivity: Y1043.33A in the orthosteric site, which selectively abolished signaling to the ERK1/2 pathway while retaining the ability to activate G proteins and mediate intracellular Ca\(^{2+}\) mobilization, and Y177A in the allosteric site, which selectively enhanced signaling of the bitopic ligand, McN-A-343 to the ERK1/2 pathway.

Distinct Patterns of Agonist Response to Orthosteric Site Mutations—With one recent exception (19), detailed structure-function studies of novel selective mAChR agonists have centered on the M\(_1\) mAChR and predominantly focused on residues within the orthosteric pocket (39, 40, 44). The mutational data in our current study now reveal four agonist classes at the M\(_2\) mAChR with respect to their patterns of response to mutation, the first of which is exemplified by prototypical orthosteric agonists, such as ACh, arecoline, CCh, and pilocarpine, and responds predictably with a reduction in affinity and/or signaling efficacy upon mutation of key orthosteric site residues, particularly Ala substitution of Tyr-1043.33 and Tyr-4036.51. The second ligand class is exemplified by AC-42 and 77-LH-28-1, which demonstrated a dramatic increase in binding affinity and signaling efficacy at the W993.28A mutant M2 mAChR and also gained efficacy for activating G proteins at the Y1043.33A and Y4036.51A mutants. NDMC and McN-A-343 probably represent a third and fourth class, respectively, with the former agent selectively displaying a sensitivity to alanine substitution of Leu-1003.29 and a gain in efficacy at Y4036.51A, whereas the latter ligand shows essentially unaltered affinity at all mutant receptors (with the exception of the key D1033.32A substitution, where neither binding nor function was quantifiable).

Our results at the M\(_2\) mAChR are in general agreement with recent findings made for some of the novel selective agonists at the M\(_1\) mAChR (40, 44), which is not surprising given that the studies all focused on orthosteric site residues conserved across subtypes. However, our study has also identified differences in ligand behavior at certain equivalent mutations between subtypes. For instance, mutation of Y2.61A at the M\(_1\) mAChR did not affect 77-LH-28-1 affinity (44), whereas we noted a significant enhancement in affinity at the W993.28A mutant M2 mAChR and also gained efficacy for activating G proteins at the Y1043.33A and Y4036.51A mutants. NDMC and McN-A-343 probably represent a third and fourth class, respectively, with the former agent selectively displaying a sensitivity to alanine substitution of Leu-1003.29 and a gain in efficacy at Y4036.51A, whereas the latter ligand shows essentially unaltered affinity at all mutant receptors (with the exception of the key D1033.32A substitution, where neither binding nor function was quantifiable).
eling (Fig. 10) suggests a different binding pose for 77-LH-28-1 at the M₂ mAChR compared with that presented by Lebon et al. (44) for the M₁ mAChR.

Novel Selective Agonists Are Unlikely to be Interacting Predominantly with the Prototypical M₂ mAChR Allosteric Site—Mutation of residues within the allosteric site previously shown to decrease the affinity of prototypical allosteric modulators, such as W84, C₁₇₃-phth, and gallamine (11–14, 16, 17, 19, 66), had no effect on the affinity of any of the agonists examined in the current study, with the intriguing exception of 77-LH-28-1, which showed an increased affinity when the charged Glu-172/Asp-173/Gly-174/Glu-175 (EDGE) sequence in the E₂ loop was mutated to Gln/Asn/Gly/Gln. This finding was also noteworthy given that the structurally related AC-42 did not display a change in affinity when tested at the same mutation. The implications of these findings are 2-fold. First, it is likely that there are no substantial direct interactions between any of the novel selective agonists and the prototypical allosteric site residues mutated in the current study; the selective effect on 77-LH-28-1 of neutralizing the charged E₂ loop EDGE sequence probably reflects a complex indirect conformational effect that cannot be modeled due to the current lack of high resolution data on the extracellular loop regions of the mAChRs. Second, despite high structural similarity, the differences noted for 77-LH-28-1 and AC-42 suggest that the presence or absence of an aliphatic ring region can have a profound effect on the engagement of this class of agonist with the receptor.

Allosteric, Orthosteric, or Bitopic Agonism?—Throughout this study, we have described AC-42, 77-LH-28-1, and NDMC as novel selective agonists and/or “putative” allosteric agonists. The former category is descriptive, whereas the latter indicates the current uncertainty whether these ligands exert their agonism purely via an allosteric site or whether they activate the receptor predominantly via orthosteric site residues while also being able to recognize an allosteric site with a different affinity. To date, the evidence that these agents can interact allosterically...
with the mAChRs is based largely on their ability to allosterically retard the dissociation rate of the orthosteric antagonist, [3H]N-methylscopolamine, from M₁ or M₂ mAChRs (19, 40, 42) or on their different patterns of response to mutations of key orthosteric site residues that are deleterious to the binding and/or function of ACh-like agonists (39–41). A limitation of radioligand dissociation rate studies is that they monitor interactions on a receptor that has been pre-equilibrated with orthosteric ligand; these experiments can reveal that a ligand may adopt an allosteric binding mode if the orthosteric site is already occupied, but they cannot be used alone to conclude that an agonist binds allosterically to activate the unoccupied receptor. With respect to the interpretation of mutagenesis studies, a differential sensitivity to specific mutations can certainly be indicative of a different mode of binding, as also described herein, but may not necessarily be proof of interaction with an entirely topographically distinct region from the orthosteric site.

As outlined above, it is unlikely that the novel selective agonists are interacting solely with key residues of the prototypical extracellular allosteric site on the M₂ mAChR. However, an alternative possibility is that the ligands may be bitopic, in that they recognize epitopes within both the orthosteric site and part of the allosteric site. We have recently proposed this as the mechanism of action for McN-A-343 at the M₂ mAChR (35). The molecular models shown in Fig. 10 for 77-LH-28-1 and NDMC indicate that these agonists can sit higher up in the binding cavity of the M₂ mAChR than prototypical orthosteric agonists, thus having the potential to interact with either the orthosteric or an allosteric site.

**New Insights into mAChR Functional Selectivity**—In contrast to their effects on binding affinity, allosteric site mutations in the E₂ loop and the top of TM7 differentially affected the efficacy of orthosteric agonists as well as that of the novel selective agonists, implicating these regions of the receptor in the adoption of active states. Interestingly, we have found that the single point mutation of Y₁₀₄₃.₃₃A in the E₂ loop generated a pathway-selective (ERK1/2) efficacy increase for McN-A-343. These findings contribute to a growing body of evidence of the importance of the extracellular loops in GPCR activation (46, 67–71).

![FIGURE 9. Mutation of Y₁₀₄₃.₃₃A selectively abrogates agonist coupling to the ERK1/2 pathway. A, agonist concentration-response curves for ERK1/2 phosphorylation determined in FlpInCHO cells stably expressing the Y₁₀₄₃.₃₃A M₂ mAChR. Data represent the mean ± S.E. of 4–5 experiments performed in duplicate. All other details are as for Fig. 2. B and C, agonist concentration-response curves for [35S]GTPγS binding at the same mutant. Data represent the mean ± S.E. of 4–5 experiments performed in duplicate. All other details are as for Fig. 2. D and E, agonist concentration-response curves for receptor-mediated Ca²⁺ mobilization at the wild type M₂ mAChR (D) or the Y₁₀₄₃.₃₃A M₂ mAChR (E). The peak Ca²⁺ response, determined as described under “Experimental Procedures,” was corrected for the background level of fluorescence and normalized to the response elicited by 2 μM ionomycin. Data represent the mean ± S.E. of 4–5 experiments performed in duplicate. Data represent the mean ± S.E. of 4–5 experiments performed in duplicate. The error bars not shown lie within the dimensions of the symbol.](image-url)
Y104<sup>3,33</sup>A selectively impairs M<sub>2</sub> mAChR-mediated phosphorylation of ERK1/2. Although Tyr<sup>3,33</sup> has previously been shown to be a critical residue for mAChR activation (6, 40), a pathway-selective impact of alanine substitution of this residue has not previously been reported. This is probably due to the fact that many structure-function studies rely on a limited number of functional end points when assessing the impact of receptor mutations. To fully appreciate the potential of ligand-directed signaling and functional selectivity, however, it is necessary to utilize a broader range of functional assays. In this regard, the ERK1/2 pathway is particularly relevant, because it is known that the coupling of various GPCRs to this pathway can involve both G protein-dependent and -independent (e.g. β-arrestin-mediated) events. Indeed, our discovery of single-point mutations both in the orthosteric (Y104<sup>3,33</sup>A) and allosteric (Y177A) sites that selectively affect the activation of the ERK1/2 pathway points to the existence of multiple activation regions of GPCRs that may be exploited to achieve functional selectivity for non-canonical signaling pathways.

Numerous elegant biochemical and biophysical studies of GPCRs have described global conformational changes that result from ligand binding biasing different states (72–80). A direct example from our current study was noted with the Y104<sup>3,33</sup>A mutation. Given that this mutant can drastically reduce the affinity for one class of compounds (orthosteric full agonists) and enhance the efficacy of another class (orthosteric AC-42, 77-LH-28-1, and NDMC) for coupling to [35S]GTP-SγS turnover, while abolishing signaling of all classes of agonists to the ERK1/2 pathway, we can infer that these different classes of agonists must recognize (or stabilize) different active conformations of the receptor that are pathway-specific.

In conclusion, this study has found that orthosteric site mutations had differential effects on the affinity and/or efficacy of novel selective/bitopic agonists, suggesting that these compounds utilize alternate binding modes to engender receptor activation that also differ across mAChR subtypes. Only the affinity of 77-LH-28-1 was affected by allosteric site mutations, although some mutations in the E2 loop and the top of TM7 altered the efficacy of both orthosteric and allosteric ligands. The differential effects on ligand efficacy, in addition to evidence of pathway-selective effects, highlight the propensity for ligand- and pathway-selective GPCR activation states/conformations, likely to be responsible for the phenomenon of ligand-directed signaling of GPCRs.

Through exploitation of allosteric/bitopic ligands, there is significant scope for the development of not only receptor subtype-selective ligands but also pathway selective modulation.

Acknowledgments—We are grateful to Dr. Greg Stewart for performing the intracellular Ca<sup>2+</sup> mobilization experiments shown in supplemental Fig. 1 and to GlaxoSmithKline (Harlow, UK) for the generous gifts of 77-LH-28-1 and AC-42.
