Functional Analysis of Transmembrane Domain 2 of the M₁ Muscarinic Acetylcholine Receptor*\textsuperscript{S}

Received for publication, May 11, 2007, and in revised form, August 10, 2007. Published, JBC Papers in Press, September 6, 2007. DOI 10.1074/jbc.M703909200

Mark S. Bee\textsuperscript{1} and Edward C. Hulme\textsuperscript{2}

From the Division of Physical Biochemistry, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

Ala substitution scanning mutagenesis has been used to probe the functional role of amino acids in transmembrane (TM) domain 2 of the M₁ muscarinic acetylcholine receptor, and of the highly conserved Asn\textsuperscript{43} in TM1. The mutation of Asn\textsuperscript{43}, Asn\textsuperscript{414}, and Leu\textsuperscript{64} caused an enhanced ACh affinity phenotype. Interpreted using a rhodopsin-based homology model, these results suggest the presence of a network of specific contacts between this group of residues and Pro\textsuperscript{415} and Tyr\textsuperscript{418} in the highly conserved NP\_XXY motif in TM7 that exhibit a similar mutagenic phenotype. These contacts may be rearranged or broken when ACh binds. D71A, like N414A, was devoid of signaling activity. We suggest that formation of a direct hydrogen bond between the highly conserved side chains of Asp\textsuperscript{71} and Asn\textsuperscript{414} may be a critical feature stabilizing the activated state of the M₁ receptor. Mutation of Leu\textsuperscript{67}, Ala\textsuperscript{70}, and Ile\textsuperscript{74} also reduced the signaling efficacy of the ACh-receptor complex. The side chains of these residues are modeled as an extended surface that may help to orient and insulate the proposed hydrogen bond in the outer half of TM2 primarily reduced the expression of functional receptor binding sites. These residues may mediate contacts with TM1 and TM7 that are preserved throughout the receptor activation cycle. Thermal inactivation measurements confirmed that a reduction in structural stability followed the mutagenic phenotype. These contacts may be rearranged or broken when ACh binds. D71A, like N414A, was devoid of signaling activity. We suggest that formation of a direct hydrogen bond between Asp\textsuperscript{71} and Asn\textsuperscript{414}. Mutation of Leu\textsuperscript{72}, Gly\textsuperscript{75}, and Met\textsuperscript{79} in the outer half of TM2 primarily reduced the expression of functional receptor binding sites. These residues may mediate contacts with TM1 and TM7 that are preserved throughout the receptor activation cycle. Thermal inactivation measurements confirmed that a reduction in structural stability followed the mutation of Met\textsuperscript{79} as well as Asp\textsuperscript{71}.

The 5 subtypes of muscarinic acetylcholine receptors (mACHRs)\textsuperscript{3} exemplify the group of G protein-coupled receptors (GPCRs) activated by the reversible binding of small ligands (1). mACHRs are known classically to be the mediators of the parasympathetic actions of acetylcholine (ACh), but recent gene knock-out studies have also emphasized their importance in modulating synaptic plasticity in the central nervous system (2). In the forebrain, M₁ mAChRs mediate most of the ACh-induced phosphoinositide (PI) turnover and extracellular signal-regulated kinase (ERK) kinase activation (3), and help to mediate the enhancement of hippocampal long-term potentiation by physiologically released ACh (4). The importance of these processes in cognition and memory motivates efforts to understand the structure and activation of M₁ mACHRs, especially for the purposes of selective drug development (5).

The crystal structure of bovine rhodopsin in the ground state (6) has provided a template for modeling the inactive state of M₁ mAChRs (7). However, a model does not in itself yield the secret of how ligand binding promotes the transition from an inactive to an active conformation that is transduced into a signal. Alanine-scanning mutagenesis with phenotypic classification of the mutants is one means of fleshing out the structural skeleton with functional data (8).

Transmembrane domain 2 (TM2) is one of the core domains of the rhodopsin-like GPCRs. It contains the highly conserved sequence motif (N/S)LA\textsuperscript{2.50} (9). (SLACAD in the M₁ mACHR) within which Asp\textsuperscript{2.50} (in standard nomenclature (10)) is one of the most highly conserved residues in the GPCR superfamily. In rhodopsin, it is directly hydrogen bonded to Asn\textsuperscript{1.50} (corresponding to Asn\textsuperscript{43} in the M₁ mACHR), and indirectly, via a water-mediated network, to Asn\textsuperscript{2.49} (Asn\textsuperscript{414}, M₁) (6, 11, 12). In the M₁ mACHR, Asn substitution first established the critical role of Asp\textsuperscript{71} in signal transduction (13), a finding subsequently confirmed in the M₃ (14) and M₄ (15) subtypes as well as a host of other receptor types (16). Reciprocal mutagenesis experiments on a number of receptors (17–22) have suggested that pairing between positions 2.50 and 7.49 is important for receptor activation.

Functional analysis of other positions in TM2 remains patchy and incomplete. The most systematic studies have been a substituted cysteine scan from residues 2.47 to 2.68 in the D2 receptor (23), and random mutagenesis from residues 2.43 to 2.63 of the C5a receptor (24). In the former case, the accessibility of the mutant side chains to sulphydryl reagents was the issue, whereas in the latter the criterion was preservation of receptor signaling, which defined important positions but did not directly address the function of the side chains. The recent use of a yeast genetic screen to isolate inactivating mutations in the M₁ mACHR (25) identified a number of non-conservative mutations in TM2. Of a total of 20 mutants, none were Ala substitutions, and only 9 were amenable to characterization at the level of binding or function. This study therefore confirmed the functional importance of the highly conserved amino acids

---

* This work was supported in part by the Medical Research Council, United Kingdom. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{S} The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Fig. S1.

1 Supported by a Medical Research Council postgraduate studentship.

2 To whom correspondence should be addressed. Tel.: 44-208-816-2057; Fax: 44-208-906-4477; E-mail: ehulme@nimr.mrc.ac.uk.

3 The abbreviations used are: mACHR, muscarinic acetylcholine receptor; ACh, acetylcholine; TM, transmembrane domain; NMS, (−)-N-methylscopolamine; PI, phosphoinositide.
of TM2, but again did not probe the role of the side chains within the structure.

Several other studies have been guided by naturally occurring allelic variants, some of which show constitutive activity, e.g. M2.53V in the thyroid-stimulating hormone receptor (26); interestingly, the adjacent position 2.52 in the angiotensin 2 receptor has been reported to exhibit an altered orientation in a constitutively active mutant (27). Thus, sequences surrounding position 2.50 are important for receptor activation.

The aim of this study was to make a systematic analysis of the functional roles of all of the amino acid side chains from positions 2.40 (Asn\(^{61}\)) to 2.60 (Leu\(^{81}\)) of the M1 mAChR by alanine substitution mutagenesis (Ala itself was mutated to Gly). Additionally, we have substituted Asn\(^{43}\} (1.50). The replacement of an amino acid by alanine deletes the side chain beyond the \(\beta\)-carbon atom, ablating any interactions that it makes, leaving a small hole in the three-dimensional structure without introducing new contacts to confuse the functional effect (28). By measuring the changes in expression of receptors that are appropriately folded to bind ligands, and the effects on antagonist and agonist affinity and signal transduction we have been able to assign the interactions made by the amino acid side chains to different functional categories. We have used this classification to group them with residues from other parts of the receptor structure. In this way, we have been able to suggest functional roles for most of the important residues in TM2.

**EXPERIMENTAL PROCEDURES**

The experimental procedures were as described in detail previously (29–31).

**Materials**—(–)-N-[\(^3\)H]Methylscopolamine (84 Ci/mmole) and myo-D-[\(^3\)H]inositol (80 Ci/mmole) were purchased from Amersham Biosciences. Unlabeled ligands were from Sigma. The QuikChange\textsuperscript{TM} kit was from Stratagene.

**Mutagenesis and Expression**—Briefly, residues of the rat M1 mAChR were mutated to Ala using the QuikChange method. Ala itself was mutated to Gly. Mutant receptors, cloned into the pCD expression vector, and validated by dideoxy sequencing, are expressed in COS-7 cells by electroporation in a pCD expression vector, and validated by dideoxy sequencing, were transiently expressed in COS-7 cells by electroporation in a pCD expression vector, and validated by dideoxy sequencing, or PI turnover assays, as described above.

**Data Analysis**—Saturation binding curves for [\(^3\)H]NMS were fitted to a one-site model of binding using SigmaPlot 8.0 to yield a rate constant for the formation of [\(^3\)H]NMS binding sites per cell based on high levels (>75%) of transfection assessed by immunocytochemistry with an antibody directed against the C-terminal 13 amino acids of the M1 mAChR that were similar to those reported previously (29).

**Receptor Thermal Stability Assays**—Membranes were resuspended in binding buffer and aliquots preincubated at a predetermined 53 °C for time periods between 2 and 240 min. Control samples and membranes not being incubated at the time were kept on ice. After preincubation, the samples were diluted into binding buffer to give a concentration of 5–20 \(\mu\)g of protein/ml and re-homogenized. A single concentration of \(10^{-9}\) M (final concentration) of [\(^3\)H]NMS was mixed with membrane preparations, vehicle (binding buffer), or vehicle containing \(10^{-6}\) M atropine to determine total and nonspecific binding as described above.

**PI dose-response curves were fitted to a four-parameter logistic function, yielding a \(pEC_{50}\) value and a slope factor \((R_{S})\). The \(pIC_{50}\) values were corrected for the Cheng-Prusoff shift, as necessary. Thermal inactivation curves were fitted to single (or where appropriate double) exponential functions yielding a rate constant for thermal decay.**

**Plasticity and Functional Assays**—Membrane binding and PI assays were performed as described previously with small variations (29, 31). Briefly, binding of (–)-N-[\(^3\)H] methylscopolamine ([\(^3\)H]NMS) to membrane preparations (5–20 \(\mu\)g of membrane protein/ml) was measured at 30 °C in a buffer containing 20 mM Na-Hepes, 100 mM NaCl, and 1 mM MgCl\(_2\), pH 7.5, using radioligand interactions ranging from 1 \(\times\) \(10^{-11}\) to 3 \(\times\) \(10^{-9}\) M with 2 concentrations per decade in a final volume of 1 ml using an incubation time of 2 h. Nonspecific binding was defined with \(10^{-6}\) M atropine. Assays were performed in triplicate. The binding reaction was terminated by rapid filtration on a Brandel or TomTec cell harvester. The mean expression level of wild-type receptor binding sites for [\(^3\)H]NMS was 1.3 ± 0.4 pmol/mg protein (mean ± S.D.). The binding of ACh was measured by inhibition of the binding of [\(^3\)H]NMS (usually 3 \(\times\) \(10^{-10}\) M) using ACh concentrations ranging from \(10^{-8}\) to \(10^{-2}\) M with 2 concentrations per decade.

ACh-stimulated PI breakdown was assayed for 30 min at 37 °C in Krebs-bicarbonate solution supplemented with 10 mM LiCl to inhibit inositol monophosphatases following pre-labeling of the transfected COS-7 cells in 12-well plates with [\(^3\)H]inositol (1 \(\mu\)Ci/ml) for 48 h (31). ACh concentrations were \(10^{-10}\)–\(10^{-3}\) M.

Whole cell binding capacity of [\(^3\)H]NMS was measured by incubating transfected cells in 12-well plates with 0.5 ml of \(10^{-9}\) M [\(^3\)H]NMS in phosphate-buffered saline at 4 °C for 4 h. Nonspecific binding was defined with \(10^{-6}\) M atropine. The cells were washed three times in ice-cold phosphate-buffered saline, and harvested in 0.5 ml of 1% Triton-X-100. The lysate was counted in 4.5 ml of scintillation fluid. The mean expression level of the wild-type receptor binding sites for [\(^3\)H]NMS was 1.5 ± 0.3 pmol/mg of protein (mean ± S.D.). This corresponds to \(\sim\)\(10^{-9}\) [\(^3\)H]NMS binding sites per cell based on high levels (>75%) of transfection assessed by immunocytochemistry with an antibody directed against the C-terminal 13 amino acids of the M1 mAChR that were similar to those reported previously (29).
We have shown previously, by blockade with an irreversible antagonist, that the apparent ratio of functional wild-type M1 mAChR to G protein is about 20 after expression in COS-7 cells (29). The levels of expression of functional receptors are affected by mutations, and this can affect the basal activity, ACh potency, and maximum response obtained in PI assays. These variations were taken into account in calculating values of the signaling efficacy of the ACh-receptor complex (eA) using equations described previously (30, 33, 34) that are based on the free association of receptors and G-proteins in the cell membrane (35) as shown,

\[ e_A = \left( \frac{(IC_{50}/EC_{50}(1 - B_{\text{asat}})) - 1}{RT} \right) \tag{Eq. 1} \]

when the \( E_{\text{max}} \) value of the mutant (as a fraction of that of the wild-type receptor) is greater than 0.9 or

\[ e_A = \left( \frac{E_{\text{max}}/(1 - E_{\text{max}})}{RT} \right) \tag{Eq. 2} \]

when the \( E_{\text{max}} \) of the mutant is less than 0.9. The two equations yield equivalent results, but when the potency ratio IC(50)/EC(50) is large, \( E_{\text{max}} \) is close to 1; conversely, when \( E_{\text{max}} \) is substantially less than 0.9, \( IC_{50} \approx EC_{50} \).

In the above equations, \( B_{\text{asat}} \) represents the receptor-dependent agonist-independent signal calculated relative to the wild-type ACh-stimulated maximum signal. In COS-7 cells, the agonist-independent constitutive signaling of the wild-type receptor represents not more than 25% of the total basal signal, assessed by treatment with the inverse agonist atropine (30, 34). Thus the \( B_{\text{asat}} \) parameter had a value less than 0.1 for the wild-type mAChR, and its variations had a negligible effect on the estimation of \( e_A \) in the present experiments. \( e_A \) values computed for the mutants were expressed relative to the wild-type receptor.

**Statistical Analysis**—Experiments were repeated at least 3 times. Values are tabulated as mean ± S.E. Statistical comparisons of affinity and rate constants for mutants and wild-type controls were carried out by one-way analysis of variance followed by Dunnett’s post-hoc test. Where values, such as expression levels, had been normalized to the wild-type control, two-tailed \( t \)-tests were used to ascertain the level of significance of differences from the wild-type.

**Molecular Modeling**—Results were analyzed in the context of a rhodopsin-based homology model of the M1 mAChR, as described previously (7).

**RESULTS**

**N-[3H]Methylscopolamine Affinity and Functional Receptor Expression Levels**—The levels of functional expression of the Ala substitution mutants expressed in COS-7 cell membranes were derived from analysis of [3H]NMS saturation curves with a one-site model of binding (“Experimental Procedures”) and compared with contemporaneously transfected wild-type controls. This provides a measure of the proportion of receptors that are appropriately folded to bind the antagonist ligand. A graphical summary is given in Fig. 1a. Full details are given in supplementary Table S1. Most of the mutants were expressed at levels from 22 to 144% of the wild-type (1.3 ± 0.4 pmol/mg of protein). Some were expressed at lower levels, particularly

function of M1 mAChR Transmembrane Domain 2

**FIGURE 1.** The effects of Ala substitution of residues in TM1 and TM2 on the level of expression and affinity of [3H]NMS binding sites. Mutant M1 mAChRs were expressed in COS-7 cells. \( a \), effects on functional receptor expression level, calculated from saturation curves with [3H]NMS. Bars show the change in expression level relative to contemporaneously transfected wild-type controls, and represent the weighted mean ± S.E. of three to five independent measurements, displayed on a log scale. *, \( p < 0.05; **, p < 0.01; ***, p < 0.001 \) with respect to wild-type. The [3H]NMS binding sites on transfected whole cells assayed on 12-well plates for selected mutants were as follows: [3H]NMS binding WT, 100% (100 fmol/well); A70G, 75%; L72A, 16%; and G75A, 10%. \( b \), effects on affinity for NMS. Changes in affinity relative to wild-type are displayed on a log scale, and represent the mean ± S.E. of three to five independent measurements. Values for D71A, G75A, and M79A were obtained after atropine rescue of expression. *, \( p < 0.05; **, p < 0.01; ***, p < 0.001 \) with respect to corresponding wild-type (\( n = 21 \)) or atropine-rescued wild-type (\( n = 7 \)) controls. Full details are given in supplementary Table S1. N61A (13 ± 3%), D71A (2 ± 1%), L72A (12 ± 4%), G75A (17 ± 2%), and M79A (5 ± 1%). 8 of 10 of the positions that showed significant reductions were concentrated in the outer C-terminal part of the transmembrane sequence. The N43A (TM1) mutant also showed reduced expression, to 8.7 ± 2% of the wild-type value. Interestingly, L65A showed a 50% enhanced level of expression relative to wild-type. To examine the location of these binding sites, we measured cell-surface binding using the hydrophilic antagonist [3H]NMS on intact cells transfected with selected mutants. This gave results that were consistent with those obtained using membrane preparations (Fig. 1, legend), confirming findings in other TM domains (7). We have found that certain mutants that yield very low levels of [3H]NMS binding sites also give reduced or undetectable PI signaling, but that signaling can be rescued by atropine treatment of the transfected cells in culture, in parallel with the
Function of M1 mAChR Transmembrane Domain 2

restoration of [3H]NMS binding (7, 30). This suggests that atropine treatment promotes the formation of fully folded receptors, and that only these can bind [3H]NMS and signal. Following the procedure described previously (30), culture of the cells transfected with the D71A, G75A, and M79A mutants with 10⁻⁶ M atropine prior to harvesting rescued their expression levels, raising them to 65 ± 6, 111 ± 10, and 88 ± 13% of wild-type, respectively (supplementary Table S1), thus enabling their binding and functional properties to be characterized; expression of the wild-type receptor increased by only 43% as a result of this procedure.

None of the mutants showed much change in affinity for [3H]NMS relative to the wild-type value (pKᵦ = 10.08 ± 0.03), the largest effect being a reduction of 3-fold (defined as Kᵦ,mutant/Kᵦ, wild-type) for G75A (Fig. 1b; supplementary Table S1).

Acetylcholine Binding—Ach binding affinities were measured by competition with [3H]NMS and analyzed using the Hill equation. For the wild-type receptor, the p(IC50) was 5.00 ± 0.03 and the slope factor (nH) was 0.88 ± 0.02. Under the assay conditions used here, there is no reproducible GTP effect on Ach affinity (36), and a more complex form of data analysis did not seem to be justified. The effects of the mutations on Ach affinity are summarized in Fig. 2a. Full details are given in supplementary Table S2.

Reductions in Ach affinity of about 5-fold were seen for A70G and I74A, with 2-fold reductions for S66A and L67A. In contrast, two of the mutations, L64A and N61A, yielded 5–7-fold increases in Ach affinity, whereas N80A gave a 3-fold increase. A 10-fold increase was also seen for N43A (pLC₅₀ = 6.01 ± 0.09). The mutations did not significantly affect the slope factor for Ach binding.

Phosphoinositide Functional Response—The effects of the mutations on receptor function were assessed by measurement of the Ach-induced total PI response following labeling of the cells with [3H]inositol. Representative PI dose-response curves for a selection of mutants are shown in supplementary Fig. S1. Values for basal (ACh-independent) and maximal signaling were computed as a fraction of the values measured for contemporaneously transfected wild-type controls. The wild-type M₁ mAChR typically gave a maximum Ach-stimulated signal equivalent to 3–5 times the basal activity (300–600 dpm) with a mean pEC₅₀ value of 6.91 ± 0.03 (n = 34). In the case of D71A, G75A, and M79A, which gave low levels of expression of [3H]NMS binding sites, additional measurements were carried out after enhancement of the expression levels by atropine rescue. The atropine rescue protocol, applied to the wild-type receptor, decreased basal activity by 23% and Ach potency by about 3-fold with little effect on the Eₘₐₓ possibly indicating the persistence of some residual atropine. Values for atropine-rescued mutants were related to the corresponding atropine-treated controls.

The mutant D71A showed no Ach-induced PI signal, either before or after atropine rescue of expression. In contrast, all of the other TM2 mutants, as well as N43A, gave Eₘₐₓ values between 76 and 150% of the wild-type value (see supplementary Table S3 for full details). None of the reductions were statistically significant, but A68G and C69A gave statistically significant increases in Eₘₐₓ of up to 50% (n = 6; p < 0.05, p < 0.01, respectively). Some of the mutants also showed statistically significant reductions in Ach-independent signaling relative to wild-type, namely N61A (83 ± 3%), S66A (75 ± 2%), A68G (83 ± 2%), L72A (76 ± 3%), and I74A (71 ± 8%); in the case of N61A, S66A, and L72A this might reflect reduced expression levels. C69A showed a slightly increased level of basal signaling (115 ± 4%; p < 0.05). Because the receptor-dependent component of the basal signal is about 25%, these effects were hard to quantitate more precisely.

Ala substitution of particular residues in the N-terminal (cytoplasmic) two thirds of TM2 caused reductions (defined as EC₅₀,mutant/EC₅₀, wild-type) in the signaling potency of Ach of up to 70-fold (Fig. 2b). The largest (>20-fold) effects were due to mutation of Ser⁶⁶, Leu⁶⁷, Ala⁷⁰, and Ile⁷⁴, whereas Tyr⁶² gave a 9-fold effect. We noted a periodic distribution of the mutational effect on PI signaling peaking at Asp⁷¹, whose muta-
tion completely abolished signaling. The N43A mutant showed a 4-fold reduction in signaling potency ($pEC_{50} = 6.27 \pm 0.07$).

Basal and maximal PI signaling are dependent on the expression level of functional mutant receptors relative to the cognate G protein. The potency of ACh is directly proportional to its binding affinity as well as dependent on the expression of fully folded receptors that can bind ligand and signal (29). To compensate for these dependencies we have computed a parameter designed to estimate the efficacy of the ACh-receptor complex in signaling to the PI pathway (30, 33, 34) (see “Experimental Procedures”). The values for the atropine-rescued mutants G75A and M79A were calculated relative to the atropine-treated wild-type controls. The results are plotted in Fig. 3.

This representation emphasizes the primary importance of the integrity of Asp71 for the mediation of PI signaling. Mutation of this residue led to zero signaling efficacy. Ten-fold reductions in efficacy also resulted from mutation of its neighboring residues Ala70, Leu67, and Ile69. Smaller reductions (3–5-fold) followed mutation of Tyr62, Leu64, Leu65, and Ser66. The increased ACh affinity observed for the L64A mutation (Fig. 2a) was not translated into increased PI potency (Fig. 2b), whereas the reduction in PI potency seen for S66A may be partly attributable to decreased expression (Fig. 1a) and decreased affinity for ACh (Fig. 2a). The N43A mutant showed an ~10-fold reduction in its calculated signaling efficacy, also reflecting the failure of the increased binding affinity to be reflected in enhanced signaling potency.

Investigations of Mutant Receptor Stability—To further understand the origin of the reduced expression of functional binding sites found for the M79A and D71A mutants and the apparently slightly enhanced expression found for the L65A mutant, we performed studies of receptor thermal stability following a protocol modeled on that used by Rasmussen and coworkers (37).

Preliminary experiments showed that all of the receptors were stable at temperatures up to 40 °C, but that incubation at 60 °C led to 60% loss of binding of the wild-type receptor in 30 min. After further investigation, a temperature of 53 °C was chosen as suitable for simultaneous measurements on atropine-rescued D71A and M79A (with time points of 0, 2, 4, 8, 16, and 32 min) and wild-type and L65A (with time points of 0, 15, 30, 60, 120, and 240 min). Following the incubations, residual binding capacity was measured by incubation of the membranes with $10^{-5} \text{M} ^{3} \text{H} \text{NMS}$ for 60 min at 30 °C in the absence or presence of $10^{-6} \text{M}$ atropine to determine nonspecific binding. The results were fitted to single or double exponentials.

Most of the thermal inactivation curves were adequately fitted by single exponentials. An example is shown in Fig. 4. This shows that, at 53 °C, the D71A mutant decayed at a rate of 0.22 min$^{-1}$, 55-fold faster than the wild-type receptor with a rate of 0.004 min$^{-1}$, whereas the M79A mutant had an intermediate rate of 0.04 min$^{-1}$. The inactivation rate of the L65A mutant was similar to that of the wild-type receptor. In the case of L65A and wild-type receptor from atropine-pretreated cells, a small proportion (about 30%) of an initial faster inactivation phase was seen in some experiments (data not shown). The full set of results is presented in Table 1.

DISCUSSION

Systematic alanine-scanning mutagenesis has proved valuable for the detection of hot spots in receptor-ligand interfaces...
Interpreted in the context of multistate models of GPCR activation, Ala substitution studies have also provided insight into intramolecular interactions, and their role in transducing binding energy into the activated state (8, 39, 40).

Ala substitution of a particular amino acid deletes the side chain beyond the β-carbon atom. The consequent effect on receptor function depends on interactions made by the deleted side chain. The extended ternary complex model of GPCR activation (41) provides a suitable predictive framework. Here the receptor exists in a conformational equilibrium between a ground state that does not productively bind the cognate G-protein, and an activated state that catalyzes GTP-GDP exchange. Ligands that bind to the receptor act by perturbing this equilibrium.

Four canonical classes of mutant are predicted. First, a null phenotype when the target side chain does not make energetically significant interactions in either the ground or the activated state. Second, a stability phenotype when the side chain interactions are equally important in the ground state and the activated state. Their deletion reduces either the stability of the protein fold if they are solely intramolecular potentially leading to a reduction in functional expression, or the affinity of the ligand if they are intermolecular and involved in ligand anchoring. Third, an enhanced agonist affinity phenotype when the side chain makes intramolecular interactions in the ground state that are broken when the agonist-activated state is formed. A group of related effects ensues, not only a reduction in the stability of the ground state, but also an enhancement of agonist affinity, because binding energy no longer has to be used to rupture the intramolecular contacts. Ligand-independent basal signaling may also be promoted. Fourth, a reduced signaling phenotype when the side chain forms interactions in the activated state that are absent in the ground state. Their deletion increases the free energy difference between the ground and activated states thus reducing the signaling efficacy of the agonist-receptor complex. No major effects on ground state stability are expected, but there may be reductions in agonist affinity and basal signaling, depending on whether the interactions are intramolecular or intermolecular, with the ligand or G-protein. In more complex cases, a superposition of these fundamental phenotypes may occur.

In TM2 of the M1 mAChR, F63A, L65A, A68G, C69A, I73A, T76A, F77A, S78A, N80A, and L81A showed little functional effect, and can be classified as null positions. L72A (2.51), G75A (2.54), and M79A (2.58) showed pure functional expression phenotypes. N61A (2.40) and L64A (2.43) showed enhanced ACh affinity phenotypes; this characteristic was shared by N43A (2.50). L67A (2.46), A70G (2.49), and I74A (2.53) showed pure reduced signaling efficacy phenotypes. Finally D71A (2.50) showed major effects on signaling efficacy and expression level, consistent with a dual role for this key residue. The mutations caused a maximum 3-fold reduction in NMS and ACh affinity, probably ruling out a primary role in anchoring these ligands for any of the target residues.

To interpret the functional effects of the Ala substitutions, we have used a rhodopsin-based homology model of the M1 mAChR (7). Like the crystal structure of rhodopsin (42), this emphasizes the close spatial relationships that exist between TM1, TM2, and TM7.

Interestingly, Ala substitution of Pro415 and Tyr418 in the highly conserved NPXYY sequence of TM7 evoked an increased ACh affinity phenotype very similar to that caused by the mutations of Asn43, Asn61, and Leu64 (7). As summarized in Fig. 5a, this occurred without major changes in NMS affinity, and was accompanied by variable reductions in expression and signaling efficacy. As shown in Fig. 6b, the clustering of these residues by phenotype is reflected in the context of the model by the existence of hydrogen bond between the amide moiety of Asn61 and the hydroxyxyl group of Tyr418 (also proposed in the M3 mAChR (25)), reinforced by a van der Waals contact between Leu64 and the aromatic ring of Tyr418; homologous contacts exist in the high-resolution crystal structure of rhodopsin (6, 12, 42). Again guided by the rhodopsin crystal structure, Asn43 is modeled as forming a hydrogen bond with the backbone carbonyl of Ser411 (TM7), and may be in van
der Waals contact with the pyrrolidine ring of Pro415, as well as H-bonded to Asp71 (Fig. 6b). These proposed ground state contacts may explain the important destabilizing effect of mutating Asn43 on receptor expression. Some of them (e.g. with Asp71) may be maintained in the activated state to account for the accompanying reduction in signaling efficacy, whereas others (e.g. with TM7) may be destabilized. These observations suggest that there is a cluster of intra-molecular contacts between the inner sections of TM1, TM2, and TM7 that is broken or modified when ACh binds. Conformational linkage between this inner membrane domain and the ACh binding site may account for the increased ACh affinity observed after Ala substitution of the participating residues. This interpretation is consistent with a Cys substitution and cross-linking study suggesting that a rotation and translation of the inner part of TM7 occurs after agonist binding to the M1 mACHR (43).

The D71A mutation completely abolished the ability of the M1 mACHR to signal. Only two other mutations have been reported to have a similarly drastic effect, namely D105A (3.32) and N414A (7.49) (7, 30). Interestingly, all three mutations also reduced the level of expression of functional binding sites. Asp105 is part of the ACh binding site (44), so the closest phenotypic match to D71A is N414A (Fig. 5b). In the ground state of the M1 mACHR, Asp71 is modeled as being H-bonded to Asn43; in rhodopsin, the homologous Asp83 also forms indirect water-mediated H-bonds to TM3 and TM7 (positions 7.45 and 7.49) (11, 12). These important interactions may account for the large decrease in stability that follows Ala substitution of Asp71. By the same criterion, Asn414 also forms important ground state interactions. Again following the rhodopsin crystal structure, these may be with TM6 at the level of Ile370 (6.40), a residue whose mutation caused constitutive activation of the M5 mAChR (32) (Fig. 7a; the equivalent residue is Met257 in rhodopsin). This proposed interaction may help to restrain the side chain of Asn414 in the ground state, and be broken during the activating conformational change, consistent with the increased ACh affinity seen for the N414A mutant. Re-orientation of the side chain of Asn414 could allow it to form a direct H-bond to Asp71 across the central core of the receptor (Fig. 7b). The total abolition of signaling that follows Ala substitution of either of these residues implies that their interaction may be critical for the stabilization of the activated state. This would be consistent with experiments on a number of different receptor types in which signaling activity abolished by a D2.50N mutation has been restored, but usually at a lower level, by making the complementary N7.49D mutant (17–22). In the homologous group of Gq-coupled mAChRs, including the M1 and M3, the reciprocal mutant may actually have low activity, because it was not among those recovered in a comprehensive genetic screen for mutations of the M3 mAChR that restored the signaling activity of the D2.50N mutant (15). Instead, the
latter study supports the suggestion (20) that the formation of specific contacts between Asp2.50 and Asn7.49 proposed here may be energetically linked to rearrangements of the contacts made by the highly conserved Arg at the cytoplasmic end of TM3. This may require a precise three-dimensional arrangement of the participating residues.

L67A, A70G, and I74A showed a reduced signaling efficacy phenotype, accompanied by modest decreases in ACh affinity, but little effect on functional receptor expression level or NMS affinity (Figs. 1–3). In the context of the molecular model, these residues form a continuous surface that may help to orient and support a hydrogen bond between Asp71 (TM2) and Asn414 (TM7) in the active state of the receptor. Leu72, Gly75, and Met79 may mediate contacts between TM2, TM1, and TM7.

In conclusion, the rhodopsin-based homology model of the M1 mAChR gives a good account of phenotypes from Ala substitution mutagenesis of TM2. We propose that ACh binding allosterically destabilizes contacts between the inner parts of TM1, TM2, and TM7. This is likely part of a cooperative rearrangement of inter-helical contacts that accompanies receptor activation. Formation of a direct H-bond between Asp71 and Asn414 is a key to the formation of the activated state of the receptor. These residues are among the most highly conserved in the rhodopsin-like GPCRs. Bulky hydrophobic residues above and below Asp71 may help to direct and stabilize the formation of this bond. Contacts at the top of TM2 may help to stabilize the receptor fold. These appear not to be modified by receptor activation.

Acknowledgments—We acknowledge Carol Curtis and Nigel Birdsall for advice and helpful discussions.
REFERENCES

1. Hulme, E. C., Birdsall, N. J. M., and Buckley, N. J. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 633–673
2. Wess, J. (2004) Annu. Rev. Pharmacol. Toxicol. 44, 423–450
3. Hamilton, S. E., and Nathanson, N. M. (2001) J. Biol. Chem. 276, 15850–15853
4. Shinoe, T., Matsui, M., Taketo, M. M., and Manabe, T. (2005) J. Neurosci. 25, 11194–11200
5. Spalding, T. A., Ma, J. N., Ott, T. R., Friberg, M., Bajpai, A., Bradley, S. R., Davis, R. E., Brann, M. R., and Burstein, E. S. (2006) Mol. Pharmacol. 70, 1974–1983
6. Palczewski, K., Kumasaka, T., Hori, T., Bahnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
7. Lu, Z.-L., Saldanha, J., and Hulme, E. C. (2001) J. Biol. Chem. 276, 34098–34104
8. Hulme, E. C., Lu, Z. L., and Bee, M. S. (2003) Receptors Channels 9, 215–228
9. Baldwin, J. M., Schertler, G. F. X., and Unger, V. M. (1997) J. Mol. Biol. 272, 144–164
10. Ballesteros, J. A., and Weinstein, H. (1995) Methods Neurosci. 25, 366–428
11. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Silow, M., Navarro, J., Landau, E. M., and Miyano, M. (2000) Science 289, 739–745
12. Li, J., Edwards, P. C., Burghammer, M., Villa, C., and Schertler, G. F. (2004) J. Mol. Biol. 343, 1409–1438
13. Fraser, C. M., Wang, C.-D., Robinson, D. A., Gocayne, J. D., and Venter, J. C. (1989) Mol. Pharmacol. 36, 840–847
14. Vogel, W. K., Peterson, G. L., Broderick, D. J., Mosser, V. A., and Schlemid, M. I. (1999) Arch. Biochem. Biophys. 361, 283–294
15. Li, B., Nowak, N. M., Kim, S. K., Jacobson, K. A., Bagheri, A., Schmidt, C., and Wess, J. (2005) J. Biol. Chem. 280, 5664–5675
16. Horn, F., Weare, J., Beukers, M. W., Horsh, S., Bairoch, A., Chen, W., Edvardsen, O., Campagne, F., and Vriend, G. (1998) Nucleic Acids Res. 26, 275–279
17. Perlman, J. H., and Gershengorn, M. C. (1997) J. Biol. Chem. 272, 11937–11942
18. Xu, W., Ozdener, F., Li, J. G., Chen, C., de Riel, J. K., Weinstein, H., and Liu-Chen, L. Y. (1999) FEBS Lett. 447, 318–324
19. Donnelly, D., Maudsley, S., Gent, J. P., Moser, R. N., Hurrell, C. R., and Findlay, J. B. (1999) Biochem. J. 339, 55–61
20. Urizar, E., Claeysen, S., Deupi, X., Govaerts, C., Costagliola, S., Vassart, G., and Pardo, L. (2005) J. Biol. Chem. 280, 17135–17141
21. Sealson, S. C., Chi, L., Ebersole, B. J., Rodic, V., Zhang, D., Ballesteros, J. A., and Weinstein, H. (1995) J. Biol. Chem. 270, 16683–16688
22. Flanagan, C. A., Zhou, W., Chi, L., Yuan, T., Rodic, V., Robertson, D., Johnson, M., Millar, R. P., Weinstein, H., Mitchell, R., and Sealson, S. C. (1999) J. Biol. Chem. 274, 28880–28886
23. Javitch, J. A., Ballasteros, J. A., Chen, J., Chiappa, V., and Simpson, S. M. (1999) Biochemistry 38, 7961–7968
24. Geva, A., Lassere, T. B., Lichtarge, O., Pollitt, S. K., and Baranski, T. J. (2000) J. Biol. Chem. 275, 35393–35401
25. Li, B., Scarselli, M., Knudsen, C. D., Kim, S. K., Jacobson, K. A., McMillin, S. M., and Wess, J. (2007) Nat. Methods 4, 169–174
26. Lee, Y. S., Poh, L., and Loke, K. Y. (2002) J. Pediatr. Endocrinol. Metab. 15, 211–215
27. Miura, S., and Karnik, S. S. (2002) J. Biol. Chem. 277, 24299–24305
28. Ward, W. H., Timms, D., and Ferdish, A. R. (1990) Trends Pharmacol. Sci. 11, 280–284
29. Lu, Z.-L., Curtis, C. A., Jones, P. G., Pavia, J., and Hulme, E. C. (1997) Mol. Pharmacol. 51, 234–241
30. Lu, Z.-L., and Hulme, E. C. (1999) J. Biol. Chem. 274, 7309–7315
31. Jones, P. G., Curtis, C. A., and Hulme, E. C. (1995) Eur. J. Pharmacol. 288, 251–257
32. Spalding, T. A., Burstein, E. S., Henderson, S. C., Ducote, K. R., and Brann, M. R. (1998) J. Biol. Chem. 273, 21563–21568
33. Hulme, E. C., and Lu, Z.-L. (1998) J. Physiol. (Paris) 92, 269–274
34. Hulme, E. C. (2006) in G Protein-coupled Receptors (Haga, T., and Takeda, S., eds) pp. 137–178, CRC Press, Boca Raton, FL
35. Arpiazu, I., and Gautam, N. (2004) J. Biol. Chem. 279, 27709–27718
36. Allman, K., Page, K. M., Curtis, C. A. M., and Hulme, E. C. (2000) Mol. Pharmacol. 58, 175–184
37. Rasmussen, S. G., Jensen, A. D., Liapakos, G., Gianouni, P., Javitch, J. A., and Gether, U. (1999) Mol. Pharmacol. 56, 175–184
38. DeLano, W. L. (2002) Curr. Opin. Struct. Biol. 12, 14–20
39. Hulme, E. C., Lu, Z.-L., Ward, S. D. C., Allman, K., and Curtis, C. A. M. (1999) Eur. J. Pharmacol. 375, 247–260
40. Lu, Z.-L., Saldanha, J. W., and Hulme, E. C. (2002) Trends Pharmacol. Sci. 23, 140–146
41. Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 4625–4636
42. Okada, T., Sugihara, M., Bondar, A. N., Elstner, M., Entel, P., and Buss, V. (2004) J. Mol. Biol. 342, 571–583
43. Han, S. J., Hamdan, F. F., Kim, S. K., Jacobson, K. A., Brichta, L., Bloodworth, L. M., Li, J. H., and Wess, J. (2005) J. Biol. Chem. 280, 24870–24879
44. Spalding, T. A., Birdsall, N. J., Curtis, C. A. M., and Hulme, E. C. (1994) J. Biol. Chem. 269, 4092–4097