The Functional Topography of Transmembrane Domain 3 of the M1
Muscarinic Acetylcholine Receptor, Revealed by Scanning
Mutagenesis*

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Alanine-scanning mutagenesis has been applied to residues 100–121 in transmembrane domain 3 of the M1
muscarinic acetylcholine receptor. This study comple-
ments a previous investigation of the triad Asp122,
Arg120-Tyr124 (Lu, Z.-L., Curtis, C. A., Jones, P. G., Pavia,
The results demonstrate the α-helical secondary struc-
ture of the domain and suggest its orientation with re-
spect to the other transmembrane domains. The C-ter-
minus part of the helix appears to be largely buried
within the receptor structure. On its surface, there is
a patch of three residues, Val113, Leu116, and Ser120, which
may form intramolecular contacts that help to stabilize
the inactive ground state of the receptor. Mutagenic
disruption of these increased agonist affinity and signal-
ing efficacy. In two cases (L116A and S120A), this led to
constitutive activation of the receptor. Parallel to the
helix axis and spanning the whole transmembrane re-
gion, a distinct strip of residues on one face of trans-
membrane domain 3 forms intermolecular (acyethyl-
choline-receptor, receptor-G protein) or intrareceptor
bonds that contribute to the activated state. The binding
of acetylcholine may destabilize the first set of contacts
while favoring the formation of the second.

EXPERIMENTAL PROCEDURES

Experimental procedures were performed as described previously
(10, 12, 13).

Mutagenesis and Expression—Briefly, residues 100–121 of the rat
M1 mACHr were individually mutated to Ala (or, if they were Ala in the
parent sequence, to Gly), using a polymerase chain reaction method.
After verification by dideoxy sequencing, mutant receptors were sub-
cloned into the pCD expression vector and expressed transiently
in COS-7 cells by electroporation using a Bio-Rad Gene Pulser at 180 V
and 960 microfarads with 10 μg of DNA/0.4-cm cuvette (4 × 107 cells,
0.8 ml). Membrane preparations were made after 72 h as described
previously (13).

Binding and Functional Assays—Binding of (-)-N-[3H]methylscopol-
amine ([3H]NMS), and (−)-[3H]quinuclidinyl benzilate ([3H]QNB) to
membrane preparations was measured at 30 °C in a buffer containing
20 mM Na-HEPES, 100 mM NaCl and 1 mM MgCl2 using an incubation
time of 60 min to 3 h, as described previously (10). The binding of ACh
and other unlabeled ligands was measured by inhibition of the binding
of the tritiated antagonist. For the study of guanine nucleotide shifts,
MgCl2 was replaced by 2 mM MnCl2 (12).

Phosphoinositide (PI) turnover was assayed as described previously
(12, 13). Briefly, transfected COS-7 cells were prelabeled with [3H]
inositol for 48 h, followed by washing and preincubation with Krebs
bicarbonate solution containing 10 mM LiCl for 30 min, and then ACh or
other agonists were added for 30 min. When the effects of antagonists
on the basal phosphoinositide response were to be studied, the anti-
agonist was added at different times up to 48 h before the performance
of the PI assay. In some instances, the antagonist was washed out (three
changes of Krebs bicarbonate solution without Li+ over a period of
about 15 min) before adding Li+ for 30 min to initiate the PI assay.

Immunocytochemistry—Expression of receptor protein was assessed
72 h after transfection by incubation of fixed, permeabilized cells with
a 1:100 dilution of an immunoaffinity-purified antiserum raised against

* This work was supported by the Medical Research Council, United
Kingdom, and by a Travelling Fellowship from the Wellcome Trust (to
Z.-L. L.). The costs of publication of this article were defrayed in part by
the payment of page charges. This article must therefore be hereby
solely to indicate this fact.
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Printed in U.S.A.
the C-terminal 13 amino acids of the M₁ mACHR sequence (13). This was followed by incubation with a 1:5000 dilution of an alkaline phosphatase-labeled second antibody and visualization as described (13).

Materials—(-)-(1H)methylisocyanate (85 Ci/mmol), (-)-1H-quinuclidinyl benzilate (50 Ci/mmol), and myo-1H[3Hinositol (80 Ci/mmol) were obtained from Amersham Pharmacia Biotech. Guanosine 5′-O-(3-thiotriphosphate) tetrathiolum salt, was from Boehringer Mannheim.

Data Analysis—Binding curves were analyzed (10, 12, 13) using SigmaPlot (SPSS Software Inc.). Ligand saturation curves were fitted to a one-site model of binding to yield an affinity constant, \(K_{\text{in}}\), and a total concentration \(C_{\text{max}}\). Competitive curves were fitted to the Hill equation, or to one- or two-site models of binding. Binding constants were corrected for the Cheng-Prusoff shift, as necessary (13). Unless otherwise stated, the agonist \(K_{\text{in}}\) is taken to be the reciprocal of the corrected IC₅₀ value. PI dose-response curves were fitted to a four-parameter logistic function, yielding a basal activity (PIbas,wild), a maximum response (PImax), an EC₅₀ value, and a slope factor. The slopes of the PI dose-response curves were, on average, not significantly different from 1.0. The \(K_{\text{act}}\) value is defined as 1/EC₅₀, \(E_{\text{max}}\) is the maximum receptor-induced signal, defined as \((\text{PI}_{\text{max}} - \text{PI}_{\text{bas,wild}})/(\text{PI}_{\text{max}} - \text{PI}_{\text{bas,wild}})\). For two constitutively active mutants, the basal activity parameter, denoted \(\text{Basal}\), was calculated relative to the wild-type receptor as \((\text{PI}_{\text{bas,wild}} - \text{PI}_{\text{bas,wild}})/(\text{PI}_{\text{max}} - \text{PI}_{\text{bas,wild}})\). With these two exceptions, \(\text{Basal} = 0\).

Agonist signaling efficacy values were calculated by fitting the ratio \([\text{RG}]\) to the ternary complex model (17) to the dose-response data, where [RG] is the concentration of the receptor-G protein binary complex, [ARG] is the concentration of the agonist-receptor-G protein ternary complex, and \([G_2]\) is the receptor-accessible concentration of G protein. Unless the basal activity is raised, the contribution of [RG] can be ignored. The calculation uses the agonist binding constant, \(K_{\text{in}}\), and an estimate of the effective ratio of total receptor concentration to receptor-accessible G protein, \([\text{RG}]/[G_2]\), denoted \(R_T\). In performing these calculations, we have used the value of 20 for \(R_T\) for the wild-type receptor estimated in a previous study in which the effect of an irreversible blocking agent on the ACh dose-response curve was studied (13). Values of \(R_T\) for the different mutants were calculated from the expression of [3H]NMS binding sites. For the L116A mutant, the calculated \(R_T\) value of 0.4 is consistent with the experimentally determined \(E_{\text{max}}\) of 40% of wild type (legend to Fig. 3).

The agonist signaling efficacy parameter is \(K_G/[G_2]\) (denoted \(K_G\)), where \(K_G\) is the apparent affinity constant of the G protein for the ensemble of agonist-receptor complexes. \(K_G\) was computed to reproduce the \(pIC_{\text{50}}\) of the PI dose-response curve.

For values of \(R_T > 1\), the efficacy parameter, \(K_G\), was calculated as follows.

\[
K_G = \left(\frac{K_{\text{act}}}{K_{\text{in}}(1 - \text{Basal})}\right) - 1/R_T \quad \text{(when } E_{\text{max}} > 1) \quad \text{(Eq. 1)}
\]

or

\[
K_G = \left(\frac{E_{\text{max}}(1 - E_{\text{max}})}{R_T} \right) \quad \text{(when } E_{\text{max}} < 1) \quad \text{(Eq. 2)}
\]

These equations are extensions of those derived by Whaley et al. (18). Other details are given by Hulme and Lu (16). When \(R_T\) was <1, the efficacy was calculated from a fit of the ternary complex model to the dose-response data, as described previously (13).

RESULTS

**3H-Labeled Antagonist Binding and Receptor Expression—** Several Ala substitution mutations in the N-terminal half of TMD 3 strongly reduced the affinity of the quaternary antagonist NMS (Fig. 1a). D105A and Y106A gave 100-fold reductions, measured by direct radioligand saturation or by competition binding assays (10). 10-fold reductions followed mutations of Trp₁₀¹, Leu₁₀², Asn₁₁⁰, and Val₁₁³. Large decreases were also seen in the affinity of the tertiary antagonist QNB (legend to Fig. 1), 6000-fold for D105A (measured by competition with [3H]NMS), and 31-fold for Y106A. In contrast, mutations in the C-terminal part of TMD 3 had little effect on NMS affinity. The largest change was a 2-fold reduction, from I₁₁⁹A (Fig. 1a).

Ala substitutions in the C-terminal part of the domain had the largest effects on the expression of [3H]NMS binding sites. The L116A mutant was expressed at 2% of the wild-type level.

Mutations of Val₁¹³, Asn₁¹⁵, and Leu₁¹⁷ also caused greater than 3-fold reductions (Fig. 1b). In the N-terminal region, only the mutation of Asp₁⁰⁵ decreased receptor expression, to 10% of the wild-type level. Unusually, mutation of Asn₁¹⁰ somewhat enhanced receptor expression.

Alterations in the expression of the receptor protein were also visualized by immunocytochemical staining of transfected cells using an antibody directed against the C terminus of the receptor (Fig. 2). As noted previously, the apparent variation in staining intensity was less than might be expected from the variation in expression of binding sites, suggesting that, particularly in the case of poorly expressing mutants such as D105A (Fig. 2b), part of the receptor protein was misfolded or was inappropriately localized (13).

**Acetylcholine Binding—** Acetylcholine binding was assayed by inhibition of the binding of [3H]NMS. ACh binding to the wild-type receptor expressed in COS-7 cells gave a slightly flattened isotherm (\(n_H = 0.9\)) with a \(K_{\text{Bin}}\) of 1.26 × 10⁵ M⁻¹ (10).
Mutations that diminished NMS affinity caused reductions in the affinity of ACh (Fig. 3a). Mutation of Ser110, which was without effect on NMS binding, gave a 10-fold reduction in the affinity of ACh.

Three mutations located between the center and the C terminus of TMD 3 increased the affinity of ACh by between 4- and 30-fold. They were V113A, L116A, and S120A.

ACh-stimulated Phosphoinositide Response—In COS-7 cells transfected with the wild-type M1 mAChR, ACh elicits a robust phosphoinositide response, with an EC50 of 70 nM and a maximum stimulation of 3 times the basal activity (13). Qualitatively, the effects of the Ala substitution mutations on the PI response were parallel to their effects on ACh binding. Mutations that decreased ACh affinity decreased the KAct (defined as 1/EC50), while mutations that increased ACh affinity increased the KAct (Fig. 3b). However, S112A decreased ACh potency without affecting its affinity.

The quantitative effects of the D105A, Y106A, and I119A mutations on ACh potency in the PI response were much greater than their effects on ACh binding. In particular, the D105A mutant completely failed to give a PI response to ACh stimulation, and ACh potency was decreased by more than 1000-fold by both the Y106A and I119A mutations. The basal PI activities of most of the mutant receptors were between 92% (I119A) and 112% of the wild-type basal, but that of the S120A mutant was significantly raised (122% ± 6%; p < 0.01; Basal = 0.07).

Computation of Agonist Signaling Efficacy—Receptor-transducer models (19), such as the ternary complex model (17), can be used to quantify the effects of mutations on signaling efficacy. Provided that signal generation is directly proportional to the sum of the concentrations of the RG binary and ARG ternary complexes, there exists a quantitative relationship between the maximum response, Emax (as a fraction of the maximum obtainable signal); the KAct, the basal activity parameter, Basal; and the agonist binding constant, Kbas (parameter definitions are given under “Experimental Procedures”). When the ratio of receptor concentration to accessible G protein concentration is greater than 1, the relationship is as follows.

\[
E_{\text{max}} = 1 - (1 - \text{Basal}) \cdot \frac{K_{\text{bas}}}{K_{\text{Act}}} \quad (\text{Eq. 3})
\]

For the present set of mutants, as well as R123A, R123K (12), and D105E (10), this relationship is reasonably well obeyed (Fig. 4a). In only one case, N115A, was the deviation of the measured from the predicted value greater than 20% of the total signal, suggesting a reduction in the signaling activity of the ternary complex in this case.

A measure of agonist signaling efficacy, adjusted for differences in receptor expression level, is provided by the affinity of the G protein for the ensemble of agonist-receptor complexes,
Mn$^{2+}$ complex model. Predicted G protein, \[ G \] mutations of Leu 116 and Ser 120 caused 2.2- and 1.4-fold increases in the ACh signaling efficacy, respectively. The D105A mutant had zero efficacy. There were also tendencies in the calculated signaling efficacy of ACh of 100-fold or greater. The D105A mutant had zero efficacy. Calculation of the efficacy of pilocarpine gave a value equal to 1.4% of the efficacy of ACh at the wild-type receptor. This was increased to 5% (3.6-fold) for the S120A and V113A mutants and to 30% (21-fold) for the L116A mutant. Tests for Constitutive Activity—The mutants that showed enhanced ACh and pilocarpine efficacies were examined for inverse agonist effects. Preincubation of transfected cells with NMS for 24–48 h before the addition of LiCl and the isolation of phosphoinositides reduced the basal activity of the S120A mutant (Basal % of the wild-type receptor, V113A and L116A gave approximately 10-fold increases in affinity for pilocarpine, while S120A gave less than a 2-fold enhancement (Fig. 5). The potencies of pilocarpine in the PI response were increased in all three mutations. At the V113A and S120A mutants, pilocarpine became a full agonist. At the L116A mutant, its maximum effect was maintained with respect to that of ACh, despite the very low expression of the mutant receptor.

GTP Shifts—The effects of the nonhydrolyzable GTP analogue GTP$\gamma$S (10$^{-5}$ M) were investigated on the mutants that showed enhanced ACh affinity and efficacy. Binding studies were performed in the presence of 2 mM Mn$^{2+}$ ions to promote the stability of the agonist-receptor G protein complex (12). Mn$^{2+}$ increased the affinity of ACh for the wild-type receptor by 1.5-fold relative to the standard Mg$^{2+}$-containing buffer, but no significant GTP$\gamma$S shift resulted. The V113A, and L116A mutants also showed no GTP shift. Mn$^{2+}$ enhanced the affinity of ACh for the S120A mutant by 3-fold; in this case, the binding data could be described by a two-site model of binding (pK$_{dH}$ = 5.74 ± 0.01 (60% of total sites); pK$_{dH}$ = 7.33 ± 0.06 (40% of total sites)). GTP$\gamma$S gave a significant decrease in the ACh affinity for this mutant. Analysis showed a 3.5-fold decrease in the high affinity binding constant (pK$_{dH}$ = 6.79 ± 0.11; p < 0.05) without an effect on the low affinity binding constant. The partial nature of this effect indicates that only a fraction of the high affinity sites were affected.

Pilocarpine Activation—The mutants that showed increased ACh affinity and efficacy were probed with the partial agonist pilocarpine. Pilocarpine gave 78% of the maximum PI response elicited by ACh at the wild-type receptor (Fig. 5). With respect to the wild-type receptor, V113A and L116A gave approximately 10-fold increases in affinity for pilocarpine, while S120A gave less than a 2-fold enhancement (Fig. 5). The potencies of pilocarpine in the PI response were increased in all three mutations. At the V113A and S120A mutants, pilocarpine became a full agonist. At the L116A mutant, its maximum effect was maintained with respect to that of ACh, despite the very low expression of the mutant receptor.

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wild-type receptors. Atropine pretreatment of the transfected cells, followed by washout, before measurement of the PI response increased the basal activity of the L116A mutant to a level equivalent to 70–120% of the maximum signal obtained in cells transfected with the wild-type receptor. The enhanced basal activity was partially inhibited by the addition of atropine, with an IC50 of $10^{-6}$ M (Fig. 6c). The PI signal was stimulated by 40% by the addition of carbachol (Fig. 6c) or ACh (not shown). The Basal parameter was equivalent to 70% of the maximum signal in these cells. In contrast, pretreatment with atropine had no effect on the PI response of the wild-type receptor.

**DISCUSSION**

One category of residues is those whose mutation had minimal effect, defined as less than a 3-fold change in the level of receptor expression, in NMS and ACh affinities and less than a 6-fold change in ACh functional potency. Five of these (Leu105, Ala103, Leu104, Val107, and Ala108) occur in the N-terminal half of TMD 3, and three (Met114, Leu118, and Phe121) occur in the C-terminal half. In a helical wheel plot, they are clustered in one sector (Fig. 7a), strongly supporting an α-helical conformation for TMD 3. Cys substitution mutants of the equivalent positions in the D2 receptor failed to react with a polar sulfhydryl reagent (6). In both receptor types, these residues probably face toward the lipid bilayer. A helical net representation (Fig. 7b) shows that the sector of TMD 3 in which mutations can be made without major effect narrows toward the cytoplasmic end of the helix, in agreement with models based on the structure of rhodopsin, which suggest that this part of the helix is mostly surrounded by the other TMDs (1).

The residues whose mutation lowered receptor expression levels have the opposite distribution. They are concentrated in the C-terminal half of TMD3 (Fig. 7b) and are spread over 220° of arc. We have argued that the side chains of such residues make intramolecular contacts that contribute to the stability of the transmembrane structure of the ground state of the receptor (13). Their removal reduces the free energy of the folded state with respect to inactive intermediates on the folding pathway. Besides Asp122 and Tyr124 (13), we have now found a third position, Leu116, at which a side chain deletion strongly reduced the level of expression. Three other mutations in the C-terminal part of the helix also had significant effects. Only one such sensitive residue occurs in the N-terminal half of the helix. Interestingly, it is the binding site aspartate itself. The free energy difference between the folded and unfolded states was restored by incubation of the expressing cells with a high affinity antagonist. This counteracted the effect of the L116A mutation on expression and doubled the expression of the D105A mutant.

At three of the six positions in the C-terminal half of the domain (Val113, Leu116, and, as reported in Ref. 13, Asp122), mutations that decreased expression levels increased ACh affinity; in two cases (Val113 and Leu116), signaling efficacy and/or basal activity was also increased. Mutations of acidic residues homologous to Asp122 in rhodopsin (20, 21), the a1 receptor (22), and the β2 receptor (23) increase basal activity, but we have not observed this for mutations of Asp122 in the M1 mACHR, even after antagonist preincubation to increase the expression levels.2 In contrast, S120A behaved like a classical constitutively active mutant, showing raised basal activity,

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2 Z-L. Lu, unpublished observations.
increased agonist affinity, enhanced efficacy of a partial agonist, and an increased GTP shift (24, 25). Together, Val113, Leu116, and Ser120 form a compact patch of residues located on the inward facing surface of the cytoplasmic half of TMD 3 (Fig. 7b).

In the extended ternary complex model of agonist-receptor-G protein interaction, a conformational equilibrium is postulated to exist between preexisting ground and active states of the receptor, governed by an isomerization constant, K (24). Agonists bind to the ground state conformation with low affinity, K_g, but to the activated state with a higher affinity, αK_a. In the simplest case, the G-protein binds selectively to the activated conformation. The extended ternary complex model offers three routes to mutational effects on efficacy: a change in the isomerization constant, K; a change in the agonist cooperativity, α; or a change in the affinity of the G protein for the activated receptor, K_G.

A selective increase in K provides the most economical explanation for the simultaneously increased basal activity, agonist affinity, and signaling efficacy of the group of constitutively active mutants (24), although it is not possible entirely to exclude an increase in K_g, combined with a simultaneous increase in K_o or in the cooperativity factor α. A priori, an increase in K_g seems unlikely to result from a side chain deletion.

Detailed calculations based on the assumption of a changed isomerization constant (described in Ref. 16) have suggested a value of about 2 × 10^{-4} for K for the wild-type M1 receptor and increases of about 2- and 40-fold in this parameter for the S120A and L116A mutants. Particularly in the case of L116A, this may reflect a selective destabilization of the ground state of the receptor, because the expression of the mutant was decreased by approximately the same ratio. These changes are enough to account for the observed increases in the ACh binding affinity, as well as in the ACh signaling efficacy, without an increase in the ground state binding constant, K_g (16). However, in the case of the L116A mutant, K_g for pilocarpine may be increased. The pharmacology of this mutant would repay further investigation, particularly as it retains some signaling activity even in the presence of NMS and atropine (Fig. 6, a and c). This may imply a partial uncoupling of the ligand binding site from the G protein binding site.

For V113A, there is a 30% increase in the signaling efficacy of ACh (16), and a 3-fold increase for pilocarpine. However, the 10-fold increases observed in ACh and pilocarpine binding affinity and a significant decrease in antagonist affinity imply an additional perturbation of the ground state conformation of the receptor by this mutation.

The residues whose mutation decreased the signaling efficacy of ACh form a strip extending the full length of TMD 3 (Fig. 7b). In the outer, N-terminal, part of the helix, they lie on the opposite face to the sector of null residues. In the inner, C-terminal, part, they are displaced by one residue (100° of arc) from the group of residues whose mutations enhanced signaling.

The mutation of Trp101, Asp105, Tyr106, and Ser109 strongly reduced ACh signaling efficacy. Mutation of these residues also reduced the ground state binding of ACh and, with the exception of Ser109, decreased the affinities of NMS and QNB. Mutation of the two more peripheral residues, Leu116 and Asn110, inhibited ground state binding of ACh but had little effect on receptor activation. Our studies suggest that Tyr106 contributes almost as much free energy as Asp105 to the binding of NMS. This may indicate an amino-aromatic interaction (26), since mutation of the equivalent Tyr to Phe in the M3 aChR had little effect on the binding of NMS, although it reduced the
affinity of ACh (27). In contrast, Asp\textsuperscript{105} plays a much larger part in the binding of the tertiary amine QNB, consistent with the formation of a charge-reinforced hydrogen bond. In the case of Trp\textsuperscript{101}, the aromaticity of the side chain has been reported to be important for both NMS and ACh binding (28). In general, the data support the conjecture of Hibert et al. that aromatic as well as polar interactions are important for the binding of muscarinic ligands (29).

These findings underline the primary role of Asp\textsuperscript{105}, supported by Tyr\textsuperscript{106} in ACh binding and signal transduction (9, 10, 27). Notably, Asp\textsuperscript{105} is the only residue in this set whose mutation completely abolished signaling. However, Asp\textsuperscript{105} appears to have a complex function. It may contribute to the stability of the receptor’s ground state structure, as well as being vital for ligand contact. This may imply that its side chain also forms an intramolecular interaction, as appears to be the case for Glu\textsuperscript{113} in rhodopsin (30).

The tightly delimited strip of residues whose mutation strongly diminished ACh signaling efficacy extends to the cytoplasmic end of the helix. At this point, it becomes continuous with residues homologous to the functionally conserved residues recently identified by random saturation mutagenesis in the second intracellular loop of the M\textsubscript{3} mAChR (31), which provides a cytoplasmic extension of TMD 3.

Mutation of Ser\textsuperscript{112} and particularly Ile\textsuperscript{119} as well as Arg\textsuperscript{123} (12) had large effects on ACh signaling efficacy. However, these mutations did not strongly affect receptor expression levels, suggesting that these residues make limited contributions to the stability of the ground state.

The mutation S112A caused a small but significant enhancement of NMS affinity, while I119A decreased NMS as well as ACh affinity. We suggest that the side chains of these two residues make weak intramolecular contacts in the ground state and that these become stronger in the active conformation of the receptor. An interdomain contact has also been postulated for the residue homologous to Ile\textsuperscript{119} in the GnRH receptor (32). If this is true, the main effect of the deletion of these side chains should be to decrease the isomerization constant, K. However, it is not possible to exclude the possibility of a more direct role for them in the formation of the G protein binding pocket, leading to effects on K\textsubscript{G}; the basal activity of the I119A mutant is decreased,\textsuperscript{2} showing that there are reductions in the product K\textsubscript{G}, which governs basal activity. Arg\textsuperscript{123} also has the potential for direct binding to the G protein as well as for intrareceptor contacts (12). With the possible exception of Asn\textsuperscript{115}, there is no evidence that these residues influence the catalytic steps within the ARG ternary complex that occur subsequent to its formation.

In summary, scanning mutagenesis has revealed strong functional differentiation of the surface of TMD 3 of the M\textsubscript{1} mAChR. It has demonstrated the \( \alpha \)-helical secondary structure of the domain and suggested its orientation with respect to the other transmembrane elements and the lipid bilayer. The results suggest that the C-terminal half of the helix is deeply buried within the receptor structure. On the buried surface, we propose that a distinct group of amino acid side-chains make intramolecular bonds that selectively stabilize the ground state of the receptor. Mutagenic disruption of these characteristically decreases receptor expression and increases agonist affinity and signal transduction. Running parallel to the helix axis and spanning the whole transmembrane region, a topologically distinct strip of residues on one face of TMD 3 forms intramolecular (ACh-receptor, receptor-G protein) or intramolecular bonds that are necessary for the stability or activity of the ARG ternary complex. Mutation of these decreases signal transduction. The binding of ACh may disrupt the first set of contacts while promoting the second. This may trigger the rotational movement of TMD 3 deduced from spectroscopic studies of rhodopsin (4) and the \( \beta \)2 receptor (23), and could mobilize the cytoplasmic ends of the surrounding TMDs. We propose that TMD 3 and its cytoplasmic extension (31) may act as an intramolecular rotary switch in receptor activation. Several of the key amino acids, particularly Ser\textsuperscript{112}, Leu\textsuperscript{116}, Ile\textsuperscript{119}, Ser\textsuperscript{120}, Asp\textsuperscript{122}, Arg\textsuperscript{123}, and Tyr\textsuperscript{124}, are very highly conserved in the G protein-coupled receptor superfamily (1), suggesting the potential generality of such a mechanism.

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doi: 10.1074/jbc.274.11.7309

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