A Network of Conserved Intramolecular Contacts Defines the Off-state of the Transmembrane Switch Mechanism in a Seven-transmembrane Receptor*

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Activation of the rhodopsin-like 7-transmembrane (7-TM) receptors requires switching interhelical constraints that stabilize the inactive state to a new set of contacts in the activated state, which binds the cognate G-protein. The free energy to drive this is provided by agonist binding, which has higher affinity to the active than to the inactive conformation. We have sought specific interhelical constraint contacts, using the M1 muscarinic acetylcholine receptor as a model. Histidine substitutions of particular groups of amino acids in transmembrane domains 3, 6, and 7, created high-affinity Zn2+ binding sites, demonstrating the close proximity of their side chains in the inactive state. Alanine point substitutions have shown the effect of weakening the individual intramolecular contacts. In each case, the acetylcholine affinity was increased, implying promotion of the activated state. These amino acids are highly conserved throughout the 7-TM receptor superfamily. We propose that they form an important part of a network of conserved interhelical contacts that defines the off-state of a general transmembrane switch mechanism.

Mutations which cause agonist-independent activation of 7-TM1 receptors have suggested the presence of structural constraints; for instance, a salt bridge between TM 3 and TM 7 may stabilize the ground-state conformation of rhodopsin (1) and the a1B receptor (2). Recently, we identified a patch of amino acids in TM 3 of the M1 mAChR, including the highly conserved residues Leu116 and Ser120, whose mutation increased ACh affinity and caused constitutive activation (3, 4). We proposed that this follows the deletion of specific intramolecular contacts.

Although the high-resolution structures of the 7-TM receptors are unknown, a general model of the a-carbon backbone has been published, based on sequence analysis in the context of a low-resolution electron crystallographic structure of rhodopsin (5, 6). Interactions between highly conserved amino acids within the receptor core were proposed to mediate receptor conformational changes. In reference to the model, potential contacts for Leu116 are Phe374 (TM 6), Asn414 (TM 7), and Tyr418 (TM 7), while Ser120 may contact Tyr208 (TM 5). Mutations of Phe374 cause constitutive activation of the M2 mAChR (7), whereas Asn414 and Tyr418 have proved important for signal transduction in all of the 7-TM receptors in which they have been investigated (8–12). The locations of these residues are indicated in Fig. 1, a and b. We have used polymerase chain reaction mutagenesis to make combinatorial histidine substitutions of these residues, creating Zn2+ binding sites which have allowed us to explore this network of contacts in the M1 mAChR.

EXPERIMENTAL PROCEDURES

Mutagenesis and Binding Studies—Mutants were constructed by a polymerase chain reaction method and validated by di-deoxy sequencing. Mutant receptors were subcloned into the pCD expression vector and expressed transiently in COS-7 cells by electroporation as described (4, 15–17). Binding studies were conducted in 20 mM Na-Hepes buffer plus 100 mM NaCl, pH 7.5, for 2 h at 30 °C. (–)-[3H]methylscopolamine ([3H]MS) binding curves were fitted to a one-site model to yield a total concentration of binding sites, B, (expressed relative to a wild-type control in each transfection) and an affinity constant, Kd

Expression of the wild-type M1 mAChR varied from 0.7 to 1.2 pmol/mg of protein in different transfections. The affinity of [3H]MS for the wild-type receptor was 1.0 ± 0.05 × 106 M⁻¹. Inhibition curves for ACh and ZnCl2 were fitted to the Hill equation, and the binding constants, KACH and KZn, were corrected for the Cheng-Prusoff shift, as necessary (4, 15). KACH for the wild-type M1 mAChR was 1.1 ± 0.1 × 10⁷ M⁻¹.

Functional Studies—Phosphoinositide (PI) dose-response curves to ACh were determined as described (4, 15) and fitted to a four-parameter logistic function, yielding an EC50 value and a maximum response (Emax). Emax values are expressed relative to wild-type control in each transfection.

Materials—(–)-N-[3H]methylscopolamine (85 Ci/mmol), (–)-[3H]quinuclidinyl benzilate (50 Ci/mmol), and myo-[3H]inositol (80 Ci/mmol) were obtained from Amersham Pharmacia Biotech. ZnCl2 was from Merck.

RESULTS

Ligand Binding and Receptor Expression—With one exception (N414H-Y418H), the single, double, and triple His mutants were expressed at between 30 and 170% of the wild-type level in COS-7 cells, assayed by the specific binding of a high-affinity antagonist [3H]MS (Table I). Most of the mutations caused less than a 3-fold change in [3H]MS affinity. However, the F374H mutation caused a 7-fold reduction, which was partly reversed by the addition of a second His at position 116 or 414. The N414H-Y418H mutant gave 1% of wild-type expression, whereas its affinity was reduced by more than 10-fold. Both of these effects were substantially reversed by the incorporation of a third His at position 116 or 374. ACh-stimulated PI Signaling—Signaling efficacies for the

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1 The abbreviations used are: TM, transmembrane domain; mAChR, muscarinic acetylcholine receptor; ACh, acetylcholine; NMS, (–)-N-methylscopolamine; PI, phosphoinositide.
L116H and F374H mutants, calculated as described (3, 4), were close to wild-type. Decreases were found for Y208H (4-fold), S120H (25-fold), and Y418H (35-fold), whereas the N414H mutant had, essentially, zero efficacy. These reductions mirror the decreased maximum PI responses for these mutants. The L116H-F374H, L116H-Y418H, F374H-Y418H, and L116H-F374H-Y418H mutants gave detectable PI responses to ACh, but multiple mutants in which Asn414 was altered were inactive (Table I). None of the His mutants showed raised basal PI signaling.

The binding and functional data (Table I) suggested that, with the exception of N414H-Y418H, the His mutations caused only minor perturbations of the ground-state structure of the M1 mAChR. Thus, probing the substituted structures with Zn$^{2+}$ ions, and determination of the chelate effect, should give information about the spatial proximities of the parent residues.

Zn$^{2+}$ Inhibition of Antagonist Binding—Zn$^{2+}$ binding was assayed by inhibition of the binding of $[^3$H]NMS (2 $K_{d}$ concentration). The apparent $K_{d}$ of Zn$^{2+}$ for the wild-type receptor was 0.72 mM. The results are exemplified in Fig. 2a and summarized in Fig. 3a, which are expressed relative to the wild-type receptor.

Single histidine substitutions of Ser$^{120}$, Tyr$^{208}$, Phe$^{374}$, Asn$^{414}$, and Tyr$^{418}$ gave less than 6-fold increases in the Zn$^{2+}$ affinity. However, the mutation of Leu$^{116}$ increased the affinity by 14-fold.

The double histidine mutant L116H-S120H, with a favorable $i$ and $i + 4$ helical spacing, displayed a 43-fold increase in Zn$^{2+}$ affinity (Fig. 2a). The contribution to the free energy arising from cooperativity of the two histidines, calculated from the chelate effect ($\Delta G_{chelate}^o$), was 2.6 kJ/mol (legend to Fig. 3). L116H-F374H and L116H-N414H yielded 71- and 32-fold increases in affinity, respectively, also giving positive chelate effects. However, L116H-Y208H, L116H-Y418H, and Y418H did not show increased Zn$^{2+}$ affinity relative to the single mutants and gave no chelate effect. The double mutations F374H-N414H and F374H-Y418H increased the affinity of Zn$^{2+}$ by 8–14-fold (Fig. 3c).

The triple histidine mutant generated by the addition of a third His at position 374 to L116H-S120H gave a further in-
increase in Zn\textsuperscript{2+} affinity of 5.7-fold, to 245-fold the wild-type value (\(\Delta G_{\text{chelate}}^{o} = 6.3 \text{ kJ/mol}\)) (Fig. 2a). In contrast, adding a third His at positions 208, 414, or 418 had little or no further effect. Combining L116H-F374H with N414H gave a 7-fold additional increase, to 504-fold the wild-type value (\(\Delta G_{\text{chelate}}^{o} = 7.9 \text{ kJ/mol}\)). Although it was impossible to measure the Zn\textsuperscript{2+} affinity of the poorly expressed double mutant N414H-Y418H, the triple mutant F374H-N414H-Y418H showed a 75-fold increase relative to the wild-type receptor. This was 6- and 16-fold greater than the F374H-N414H or F374H-Y418H mutants, corresponding to a \(\Delta G_{\text{chelate}}^{o}\) of 5.0 kJ/mol (Fig. 3, a and b). An interaction between these residues is also supported by the rescue of receptor expression and affinity in the triple mutant relative to the N414H-Y418H mutant (Table I).

Zn\textsuperscript{2+} inhibition of binding of the tertiary antagonist \(^{3}H\)quinuclinidyl benzilate was also tested for selected mutants, giving results similar to those measured by \(^{3}H\)NMS.\textsuperscript{2} Increasing the concentration of \(^{3}H\)NMS from 2 \(K_{d}\) to 6 \(K_{d}\) or 18 \(K_{d}\) shifted the inhibition curves to higher concentrations of Zn\textsuperscript{2+} (Fig. 2b) but did not reduce the maximum inhibition, thus showing high negative cooperativity of inhibition. None of these mutants increased the affinity of Ni\textsuperscript{2+} more than 8-fold.\textsuperscript{2} The inhibition of \(^{3}H\)NMS binding to the histidine mutants by Zn\textsuperscript{2+} at the \(K_{d}\) concentration was reversed by subsequent addition of 1 mM excess of EDTA to chelate the Zn\textsuperscript{2+} ions. This had less effect on the nonspecific binding of Zn\textsuperscript{2+} to the wild-type receptor (Fig. 2c). These results indicated that relatively specific Zn\textsuperscript{2+} binding sites were created and that the Zn\textsuperscript{2+} binding to the specific sites was reversible.

Zn\textsuperscript{2+} Effects on the PI Response—Zn\textsuperscript{2+} (100–300 \(\mu\text{M}\)) inhibited the PI response evoked by ACh at the L116H-F374H mutant (70\% inhibition of the effect of 10\(^{-4}\) M ACh at 300 \(\mu\text{M}\) Zn\textsuperscript{2+}) while having little effect on the wild-type receptor (20\% inhibition of the effect of 10\(^{-5}\) M ACh at 300 \(\mu\text{M}\) Zn\textsuperscript{2+}) or the F374H and L116H mutants (less than 20\% inhibition of the effect of 10\(^{-4}\) M ACh at 300 \(\mu\text{M}\) Zn\textsuperscript{2+}). However, Zn\textsuperscript{2+} (10–100 \(\mu\text{M}\)) also doubled the basal PI signal in untransfected COS-7 cells, indistinguishably from cells transfected with the inactive L116H-N414H, F374H-N414H, and L116H-F374H-N414H mutants. The occurrence of this background of nonspecific stimulation made it impossible to quantitate the effects of Zn\textsuperscript{2+} on the ACh-induced PI response, and these experiments were not pursued further.

**DISCUSSION**

Three of the triple His mutants showed strong positive cooperativity of Zn\textsuperscript{2+} binding by histidine (Fig. 3b), corresponding to a \(\Delta G_{\text{chelate}}^{o}\) in the range of 5.0–7.9 kJ/mol. The \(K_{d}\) values for Zn\textsuperscript{2+} of these triads (L116H-S120H-F374H, 2.9 \(\mu\text{M}\); L116H-F374H-N414H, 1.4 \(\mu\text{M}\); F374H-N414H-Y418H, 9.5 \(\mu\text{M}\)) are comparable with those reported for triple-His mutants in the NK-1 (18) and \(\kappa\)-opioid receptors (19) and suggest the successful creation of high-affinity Zn\textsuperscript{2+} binding sites. The Hill coefficients of Zn\textsuperscript{2+} inhibition of \(^{3}H\)NMS binding to these triple mutants were close to 1.0, consistent with the ligation of a single Zn\textsuperscript{2+} ion. The inhibition mechanism appeared near competitive, over the concentration ranges studied. It is likely that the introduction of a positively charged Zn\textsuperscript{2+} ion into the central cleft of the receptor strongly favors the binding of the positively charged radiolabeled antagonist, even though the altered residues do not overlap with the primary ligand binding residues (Fig. 1a).

The \(\alpha\)-carbons of the residues composing the high-affinity triads must be separated by less than 13 \(\AA\) to allow the corresponding imidazole side chains to coordinate a metal ion (20). The results suggest that, in the inactive state of the M\textsubscript{1}
mAChR, a network of interactions exists between amino acid side chains, centered on Leu 116 (TM 3)-Phe374 (TM 6)-Asn414 (TM 7) and supported by Ser 120 (TM 3) and Tyr 418 (TM 7), which turn away from Leu 116 and Asn414 by 40 degrees of arc (Fig. 4). In contrast, it seems unlikely that Tyr 208 (TM 5) is close enough to Ser 120 (TM 3) to form a hydrogen bond; in the TSH receptor, the homologous tyrosine has been proposed to make a hydrogen bond with a carbonyl oxygen in the peptide backbone of TM6 (21).

Ala substitution mutagenesis has suggested functions for some of the side chains of the amino acids in this network in activation of the M1 mAChR. Ala-substitutions of Leu 116 and Ser120 simultaneously increased ACh affinity, raised basal activity, and enhanced signaling efficacy, suggesting that the intramolecular contacts made by these highly conserved residues help to stabilize the inactive ground state of the M1 mAChR (3, 4). A similar role has been proposed for Phe374 in TM 6 (7, 22). Mutation of Ser120 to histidine caused little change in agonist and antagonist binding. In contrast with mutation of S120A and S120C,2 S120H strongly reduced signaling efficacy and maximum PI response. Double mutation of L116H-S120H and S120H-Y418H was inactive, but S120H-Y208H and S120H-F374H gave detectable PI response (Table I). Substitution of the small side chain of Ser120 by histidine may affect transition from the inactive to the active state, in which relative rotation and translation of helices are necessary, but without perturbation of the ground-state structure.

His substitution of the TM 7 residues Asn414 and Tyr418 (Table I) increased ACh affinity but essentially abolished Gq-mediated phosphoinositide signaling. These findings were confirmed by Ala-substitution. In the case of N414A, as reported for L116A, there was also a large reduction in receptor expression.3 Asn414 and Tyr418 may resemble Leu116, Ser120, and Phe374 in making intramolecular contacts that stabilize the inactive ground state of the receptor. However, the loss of signal implies that they are also important for the formation of the agonist-receptor-Gq protein signaling complex. Thus, in contrast to residues which act as pure constraints, they may have an additional role.

3 Z.-L. Lu and E. C. Hulme, manuscript in preparation.
In summary, several of the specific, conserved, interhelical contacts between TM 3, 6, and 7 of the rhodopsin-like 7-TM receptors which have been proposed from mutagenesis and modeling studies have been directly supported by the engineering of Zn$^{2+}$ binding sites in the M1 mAChR. These contacts may be important in stabilizing the off-state of the receptor switch mechanism and be broken or rearranged during receptor activation.

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Fig. 4. Conserved interhelical constraint network demonstrated by Zn$^{2+}$ Sites. The white triangle shows the interhelical triad whose histidine substitution gave the highest Zn$^{2+}$ affinity, indicating close proximity of the side chains of these amino acids. The green triangles show the triads whose histidine substitution also produced high-affinity binding sites for Zn$^{2+}$. In each case, deletion of the side chain by Ala-substitution causes increases in agonist binding affinity.
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