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Molecular identification of melanocortin-2 receptor responsible for ligand binding and signaling

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Abstract

The melanocortin-2 receptor (MC2R), also known as the adrenocorticotropic hormone (ACTH) receptor, plays an important role in regulating and maintaining adrenocortical function, specifically steroidogenesis. Mutations of the MC2R gene have also been identified in humans with familial glucocorticoid deficiency. However, the molecular basis of MC2R responsible for ligand binding and signaling remains unclear. In this study, both truncated ACTH peptides and site-directed mutagenesis studies were utilized to determine the molecular basis of MC2R responsible for ACTH binding and signaling. Our results indicate that ACTH1-16 is the minimal peptide required for MC2R binding and signaling and mutations of the conserved amino acid residues E80 in transmembrane domain 2 (TM2), D107 in TM3, F178 in TM4, F235, H238 in TM6 and F258 in TM7 significantly reduced ACTH binding affinity and signaling, which is similar to other MCRs. Furthermore, mutations of unique amino acids D104 and F108 in TM3, and F168 and F178 in TM4, significantly decreased ACTH binding and signaling. In conclusion, our results suggest that the residues in TM2, TM3 and TM6 of hMC2R share similar binding sites with other MCRs but the residues identified in TM4, TM7 of hMC2R are unique for ACTH binding. Our study suggests that hMC2R may have a broad binding pocket in which both conserved and unique amino acid residues are involved, which may be the reason why α-MSH was not able to bind hMC2R.

Keywords

stress; POMC; MC2R; agonist; GPCR

The melanocortin-2 receptor (MC2R), also known as the adrenocorticotropic hormone (ACTH) receptor, plays an important role in the regulation of adrenal cortisol secretion (9). Mutations of the hMC2R have been identified in a potentially fatal disease called familial glucocorticoid deficiency (FGD) in which affected individuals are deficient in cortisol and likely to succumb to hypoglycemia or overwhelming infection in infancy or childhood if they are not treated (8, 10, 16, 35, 39, 40, 44, 49, 50, 52, 58–60). Over expression of this receptor is also found in adrenal adenomas or hyperplasia associated with glucocorticoid overproduction (Cushing syndrome) (1, 22, 37, 38).

Extensive studies had been performed to determine the molecular basis of ACTH in receptor function in the last two decades but the results are not consistent. In early years, ACTH1-14, ACTH11-19 and ACTH11-24 were reported to increase corticosterone levels in the isolated

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adrenal cell system and ACTH11-24 plays an important role in adipocyte function (14, 24, 42, 45). However, ACTH1-14 was also reported not able to bind MC2R (48). The explanation for these variations is that different methods of adrenal cell preparation and separate receptors may be involved in these peptide activities (53). Further studies of some ACTH peptide truncations indicated that ACTH1-17 is the minimal peptide required for ligand binding and signaling (14, 24, 42, 45). However, detailed structure-function of ACTH responsible for MC2R binding and signaling is still unclear.

Cloning of melanocortin receptors (MCRs) has greatly improved understanding of the ligand receptor interactions between melanocortins and MCRs. Structure function studies and mutagenesis studies of the receptor have provided great insights into the ligand receptor interaction (26, 61, 63, 64). During the past several years, many studies have indicated that the transmembrane domains 2, 3 and 6 of melanocortin 1, 3 and 4 receptors (MCRs) are important for α-MSH binding and signaling (15, 26, 30, 46, 62, 64). However, the molecular determinants of the hMC2R responsible for agonist ACTH binding remain unknown.

MC2R is one of five known melanocortin receptors belonging to the seven transmembrane G-protein coupled receptor family (GPCR) (41). It shares a nearly 50% homology with other melanocortin receptor subtypes but it is unique among MCRs because of its ligand selectivity. ACTH is the only known endogenous agonist at MC2R, whereas ACTH as well as α–, β–, and γ–MSH bind to other MCRs (2, 4, 11–13, 48, 54, 55, 57). All melanocortins have a shared core amino acid sequence, His-Phe-Arg-Trp (HFRW), which is critical for melanocortin binding and signaling (27, 28, 47). In this study, both truncated ACTH peptides and site-directed mutagenesis studies were utilized to determine the molecular basis of MC2R responsible for ACTH binding and signaling. Our results indicate that ACTH1-16 is minimal peptide for MC2R binding and both conserved and unique residues in TMs of MC2R are involved in ACTH binding and signaling. Not only are TM2, TM3 and TM 6 of hMC2R are important for ACTH recognition but also TM4, TM5 and TM7 are required for ACTH binding and signaling.

Materials and Methods

Peptides

ACTH1-39 and the truncated ACTH peptides were purchased from Peninsula Laboratories, Inc. (Belmont, CA). ACTH1-15 was purchased from Genscript Corporation (Piscataway, NJ).

Site-directed mutagenesis

Single residue mutation was constructed using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The entire coding region of the mutated receptors was sequenced by the University of Alabama at Birmingham Sequence Core to confirm that the desired mutation sequences were present and that no sequence errors had been introduced. The mutated receptors are shown in Figure 1. The mutant receptors were subcloned into the eukaryotic expression vector pCDNA 3.1 (Invitrogen; Carlsbad, CA).

Cell culture and transfection

The OS3 adrenal cell line was cultured in DMEM medium containing 10% bovine fetal serum 2% CBS and HEPES. Cells at 80% confluence were washed twice with DMEM, and the receptor constructs were transfected into cells using lipofectamine (Life Technologies, Rockville MD). The permanently transfected clonal cell lines were selected by resistance to the neomycin analogue G418 (62).

Binding Assays

After removal of media, OS3 cells expressing hMC2R wild type or mutants were incubated with various non-radioligand in 0.5 ml MEM (Fisher Scientific., Pittsburgh, PA) containing 0.2% BSA and radioligand. Binding experiments were performed using conditions previously described (62, 65). Briefly, 2×10^5 cpm of ^{125}I-ACTH (Amersham, Arlington Heights, IL) was used in combination with non-radiolabeled ligand ACTH. Binding reactions were terminated by removing the media and washing the cells twice with MEM containing 0.2% BSA. The cells were lysed with 0.2 N NaOH, and the radioactivity in the lysate was quantified in an analytical gamma counter. Nonspecific binding was determined by measuring the amount of ^{125}I-label bound in the presence of 10^{-6} M unlabeled ligand. Specific binding was calculated by subtracting nonspecifically bound radioactivity from total bound radioactivity.

cAMP Assay

cAMP generation was measured using a competitive binding assay (TRK 432, Amersham, Arlington Heights, IL). Briefly, OS3 cells stably expressing hMC2R were used in these assays (6, 65). Cell culture media was removed, and cells were incubated with 0.5 ml Earle's Balanced Salt Solution (EBSS), containing ACTH (10^{-10}–10^{-6} M), for one hour at 37°C in the presence of 10^{-3} M isobutylmethylxanthine. The reaction was stopped by adding ice-cold 100% ethanol (500μl/well). The cells in each well were scraped, transferred to a 1.5 ml tube, and centrifuged for 10 min at 1900 × g, and the supernatant was evaporated in a 55°C water bath with pre-purified nitrogen gas. cAMP content was measured according to instructions accompanying the assay kit. Each experiment was performed a minimum of three times with duplicate wells.

Receptor Expression

For receptor protein expression studies, we utilized PCR to insert a FLAG tag onto the NH_2 terminus of hMC2R in order to characterize receptor protein cell surface expression by flow cytometry using fluorescence-activated cell sorting (FACS). The FLAG protein is an eight amino acid peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), useful for immunoaffinity purification of fusion proteins (25, 57). hMC2R or mutant receptor transfected cells were harvested using 0.2% EDTA and washed twice with phosphate buffer saline (PBS). Aliquots of 3×10^6 cells were centrifuged and fixed with 3% paraformaldehyde in PBS (pH 7.4). The cells were incubated with 50 μl of 10 μg/ml murine anti-FLAG M1 monoclonal antibody(Sigma, catalog No. 316, St. Louis, MO) in incubation buffer for 45 minutes. Under these conditions, the primary antibody binds only to receptors located at the cell surface. The cells were collected by centrifugation and washed three times with incubation buffer. The cell pellets were suspended in 100 μl of incubation buffer containing CY™3-conjugated Affinity Pure Donkey Anti-Mouse Ig G (ImmunoResearch Lab, Inc., West Grove, PA) and incubated at room temperature for 30 minutes. Flow cytometry was performed on a fluorescence-activated cell sorter (Becton Dickinson FACStar plus six parameter cytometer/sorter with a dual Argon ion laser, San Jose, California). The results were analyzed using the software CellQuest (Beckton-Dickinson Immunocytometry Systems, San Jose, California).

Statistical analysis

Each experiment was performed at three separate times with duplicated wells. Data are expressed as mean ± SEM. The mean value of the dose-response data of binding and cAMP production was fit to a sigmoid curve with a variable slope factor using non-linear squares regression analysis (Graphpad Prism, Graphpad Software, San Diego, CA). Significant differences were assessed by one-way ANOVA, with $p < 0.05$ considered to be statistically significant.

Results

Structure-function study of ACTH peptides at hMC2R wild type

To determine which region of ACTH is essential for high affinity binding and potency at hMC2R, we performed structure-activity studies using the truncated ACTH peptides. The sequences of the truncated peptides are shown in Table 1. Our results indicate that ACTH1-39, ACTH1-24, ACTH1-17 and ACTH1-16 possess full agonist activities. ACTH1-24 is the most potent and ACTH1-16 is the least potent among the ACTH peptides tested (Figure 2 and table 1). The peptides with less than 16 amino acids lost their binding affinity and biological activities. Further studies indicate that the peptides without first six amino acids also lost their biological activities, suggesting that three key regions of ACTH are required for ligand binding and signaling at hMC2R which is different from that of other MCRs (Figure 3 and table 1).

Substitutions of the conserved amino acid residues of hMC2R on ACTH binding and potency

hMC2R shares 50% conserved amino acid residues with other MCRs and 67% conserved amino acid residues in the transmembrane domains (17–19, 41). However, the pharmacological profile of MC2R is substantially different from other MCRs. The rationale for selecting amino acids of hMC2R in the mutagenesis experiments is based on our previous studies of other melanocortin receptors. Our previous results indicate that the conserved amino acid residues in TMs of MCRs are important for ligand binding and signaling (6, 62, 64). Moreover, our recent studies of chimeric receptors with hMC4R and hMC2R indicate that the conserved amino acid residues of hMC2R are important for NDP-MSH binding and signaling (7). The substitutions of the TM2, 3, 4, 5 and 6 of the hMC4R with the corresponding regions of the hMC2R did not significantly alter or abolish NDP-MSH specific activity, suggesting that conserved amino acid residue of TMs may be crucial for NDP-MSH and ACTH binding and activity (7). Moreover, ACTH is a 39 amino acid peptide and does not have access to the receptor's deep transmembrane region, the conserved ionic and aromatic amino acid residues in the upper region of the transmembrane regions of hMC2R are therefore potential candidates for ACTH binding and signaling. A sequence alignment of hMC2R with other MCRs was therefore compared and ten amino acid residues in TM2, TM3, TM4, TM5 and TM6 are identified conserved between hMC2R and other MCRs in the upper regions of the transmembrane regions of hMC2R. We hypothesized that these conserved amino acid residues of hMC2R are involved in ACTH binding and receptor activation which is similar to that of other MCRs. Besides the conserved amino acid residues, unique amino acid residues of hMC2R are also important for ACTH binding and signaling because MSH was not able to bind MC2R. Based on this theory, the conserved and unique ionic and aromatic amino acid residues in TMs of the hMC2R were selected in this study. To determine whether these amino acid residues are involved in ACTH binding and signaling, these residues were individually mutated with alanine and the ACTH binding affinity and potency were evaluated (Figure 1). Alanine is the generally accepted amino acid of choice for mutagenesis substitution in this type of analysis since its small neutral nature theoretically makes it unlikely to disturb receptor tertiary structure. Asparagine instead of alanine is utilized for some mutations when alanine resulted in complete loss of the receptor function (64). Our results indicate that all of the mutant receptors were expressed at cell surface and their expressions are shown in Table 2. The expressions of K77A, E80A, D103N, D107N, F178A, F235A, H238A and F258A are lower than that of the hMC2R-WT. Receptor function study indicates that ACTH dose dependently displaced ^{125}I ACTH binding at the mutations, K77A, E80A, D103N, D107N, F178A, F235A, H238A and F258A, but their binding affinities for ACTH were significantly reduced compared to that of the hMC2R WT (Figure 4A, 5A and table 2). Consistent with

the binding results, the mutations, K77A, E80A, D103N, D107N, F178A, F235A, H238A and F258A, significantly reduced ACTH mediated cAMP production (Figure 4B, 5B). Their Ki and EC_{50} are shown in Table 2.

It has been reported that mutations of aspartic acid residues in TM2 and TM7 of hMC3R and hMC4R resulted in not only altered ligand binding, but also altered receptor signaling (6, 64). To determine whether the corresponding residues of hMC2R are also involved in agonist mediated receptor activation, we mutated residue D70 in TM2 and D272 in TM7 of hMC2R with alanine and tested their function. Our results indicate that mutations D70A and D272A resulted in a complete loss of receptor-ligand binding and activation. To examine the possibility that these mutations may not be properly expressed at the cell surface, we utilized FACS to determine whether the alanine substitution alters receptor expression. Our results show that strong signal was detected at FLAG tagged MC2R WT but no signal was detected in FLAG-tagged mutants D70A and D272A, suggesting that these two mutants were not properly expressed at the cell surface.

Substitutions of the unique amino acids in the transmembrane domains of hMC2R on ACTH binding and activation

ACTH is the only endogenous ligand for hMC2R and three regions of ACTH are proposed for receptor ligand binding and signaling (3, 36, 48). Our results indicate that the residues 15–17 Lys15-Lys16-Arg17 are important for hMC2R binding and signaling because loss of this region (ACTH1-14) impairs receptor activation. This is a very positively charged domain and we therefore postulate that additional binding sites at hMC2R may be required for ACTH15-17 binding. Unique amino acid residues are therefore potential candidates responsible for this binding. The sequence of amino acids in TMs of hMC2R was compared with other MCRs and six amino acid residues are revealed to be unique. These residues are N81 in TM2, D104 and F108 in TM3, T164 and F168 in TM4 and F244 in TM6. To determine whether they are involved in ACTH specific binding and activity, these residues were individually mutated with alanine and evaluated. Our results indicate that these mutant receptors were expressed at cell surface but the expression levels of the mutations, N81A, D104N, F108A and F168A, are lower than that of the hMC2R-WT (Table 3). ACTH dose dependently displaced ^{125}I ACTH binding at these mutations but the binding affinity of ACTH at mutations, N81, D104, F108 and F168, was significantly reduced (Figure 6A, 7A and Table 3). Consistent with the binding results, the mutations, N81A, D104A, F108A and F168A, significantly reduced ACTH mediated cAMP production (Figure 6B, 7B and Table 3).

Discussion

The melanocortin peptides α-MSH and ACTH belong to a group of neuropeptides derived from the pro-opiomelanocortin prohormone and contain the common amino acid sequence, His-Phe-Arg-Trp (23, 27, 51). These residues in MSH and ACTH were identified as important residues for ligand binding and biological activities at hMC1R, hMC3R and hMC4R (31–34, 56). However, this tetrapeptide was not able to bind and activate MC2R. As long with the cloning MC2R, the studies with cells expressing transfected hMC2R indicated that α-MSH was not able to bind to MC2R and ACTH1-17 is the minimal peptide for receptor binding and activation (3, 36, 48). In our study, we transfected hMC2R into OS3 cells which is an adrenal cell lines lacking endogenous MC2R and determined which region of ACTH is important for MC2R binding and activation. Our results indicate that ACTH1-24 is the most potent truncated peptide among the truncated peptides tested. The peptide loses its activity at MC2R if the length of ACTH peptide is less than 16 amino acids in N terminus, which is one amino acid less than the length of peptide reported before (36). Our results also indicate that N terminus of ACTH is also important for ligand binding and

receptor activation because peptide ACTH6-24 completely loses its agonist activity which are consistent with the previous results (5, 24). Our results therefore supports the theory raised by other groups that three key regions of ACTH are important for ACTH binding and signaling at hMC2R (36). One region is located in ACTH1-5 which is important for receptor activation but not for ligand binding. The second region is located in ACTH 6-9 (HFRW) which is important for binding and activation. The third one is located between residue 14 and 16 of ACTH which is critical for hMC2R binding and activation, which is different from that of other MCRs. The results of truncated ACTH peptides also suggest that the binding pockets of MC2R may differ structurally from that of other MCRs.

Extensive studies have been performed to examine the molecular basis of MC1R, MC3R and MC4R responsible for ligand binding and signaling (6, 62, 64). The results indicate that conserved residues in TM of melanocortin receptors are involved in MSH binding and signaling and electrostatic and hydrophobic forces have been proposed to be involved in MSH binding and receptor activation (29). Melanocortin receptor subtypes are believed to have the same basic molecular architecture and hMC2R has near 50% identity with other MCRs in the putative TM regions (18–20, 41). To determine the molecular basis of hMC2R responsible for ACTH binding and activity, we first examined the roles of the highly conserved residues across the MC2R, MC1R, MC3R and MC4R subtypes which have been identified to be crucial in other MCRs. We examined ten conserved amino acid residues of hMC2R that have previously been identified to be involved in ligand binding and activity in MC1R, MC3R and MC4R (6, 7, 15, 26, 30, 62, 64). Our results support this hypothesis and the conserved residues E80 in TM2, D107 in TM3, F178 in TM4, F235, H238 in TM6 are involved in ACTH binding which are similar to that of other MCRs (6, 15, 26, 62, 64). However, significant differences between MC2R and other MCRs were also identified in this study. hMC2R has three aspartic acids (D103, D104 and D107) in TM3 but other MCRs have only two (D117 and D121 in hMC1R; D154 and D158 in hMC3R, D122 and D126 in hMC4R). Residue D104 in TM3 of hMC2R is unique among the MCRs. Our results indicate that unlike MC1R, MC3R and MC4R, the residue D103 in hMC2R may be not important for ACTH binding because mutation of D103 did not dramatically alter ACTH binding affinity and potency. Instead of the residue D103, the residue D104 in TM3 is important in ACTH binding and activity because mutation of this residue significantly decreased ACTH binding affinity and potency. Our result of D107N is also consistent with the results identified in FGD in which mutation of D107N resulted in receptor malfunction (43). Our results suggest that hMC2R shares similar ionic binding sites with hMC1R, hMC3R and hMC4R. We incorporated our results into the model for hMC2R ligand receptor interaction which is shown in Figure 8. An ionic pocket formed by amino acid residues E80 in TM2 and D104, D107 in TM3 of hMC2R and a second hydrophobic binding pocket consisted of F235 and H238 in TM6 of hMC2R. In addition, two unique amino acid residues, F178 in TM5 and in TM7 of hMC2R may be involved in Lys15-Lys16 interaction.

MC2R is unique among MCRs due to the fact that ACTH is its only endogenous ligand. We speculate that the binding pockets of MC2R may be different from that of other MCRs and D104 may play an important role in differential recognition of ACTH or MSH at MC2R. We examined the effect of α-MSH at mutation D104N but this mutation did not increase MSH binding, hereby suggesting that this residue is not crucial for MSH binding (data not shown). Combined with truncated peptides and mutagenesis studies, our present data indicate that several similarities exist between the hMC2R and other hMCRs. First, all MCR subtypes are fully activated by the ACTH1-16. Secondly, the mutation of homologous residues in TM2, TM3, and TM6 of the hMC1R, hMC2R, hMC3R and hMC4R are found to affect agonist binding affinity (hMC1R residues E94, D117, D121, and H260, hMC3R residue E131, D154, D158 and H258, hMC4R residues E100, D122, D126 and H264 are homologous to hMC2R residue E80, D104, D107, H238) (6, 62, 64). However, the present

studies did identify a potentially important subtype specific difference between the hMC2R and other hMCRs. TM4, TM5 and TM7 of hMC2R are important for ACTH binding and signaling because mutation of residue F168 (Y182 in MC1R; Y219 in MC3R, Y180 in MC5R; MC4R Y187) in TM4, F178 (L192 in MC1R; L229 in MC3R, L190 in MC5R) in TM5, and F258 in TM7 affects both ACTH binding affinity and potency. This observation suggests that hMC2R may have a broad binding pocket in which conserved amino acid residues are involved in ACTH binding which are similar to that of other MCRs but some unique amino acid residues are perhaps crucial for ACTH14-16 binding and activity. That may be the reason why α-MSH was not able to bind hMC2R although α-MSH shares the first 13 amino acids with ACTH1-39.

Activation of GPCRs has been proposed to involve the rotation of TM domains with outward movement of their cytoplasmic ends (21). This theoretically would enable G proteins to interact with some of the intracellular loops as well as the C terminal tail of GPCRs. Some previous MCR studies support this theory since mutations of some TM residues of MCRs can result in inactivation of MCRs (6, 64). Our current results indicate that mutations of the homologous residues of hMC2R (D70A and D272A) abolish both ACTH binding and receptor signaling because these two mutated receptors are not fully expressed at the cell surface. Altered receptor function may reflect loss of cell surface receptor expression. Thus, this suggests that D70 and D272 in hMC2R play important roles in receptor expression on plasma membrane, and their role in hMC2R is likely similar to that of MC3R and MC4R.

In summary, our results indicate that ACTH1-16 is the minimal peptide required for MC2R binding and signaling. Both conserved and unique residues of MC2R are required to form a broad binding pocket for ACTH binding and signaling which includes TM2, TM3, TM4, TM6 and TM7 of hMC2R. These results may provide important information about the molecular determinants of hMC2R responsible for ACTH binding and receptor signaling.

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Abbreviations

MCR	Melanocortin receptor
hMC2R	human melanocortin-2 receptor
GPCR	G-protein coupled receptor
ACTH	Adrenocorticotropic Hormone
α–MSH	α-melanocyte stimulating hormone
ASIP	Agouti-signaling protein
TM	transmembrane domains
IBMX	3-Isobutyl-methylxanthine
PCR	Polymerase chain reaction
FACs	Flow cytometry

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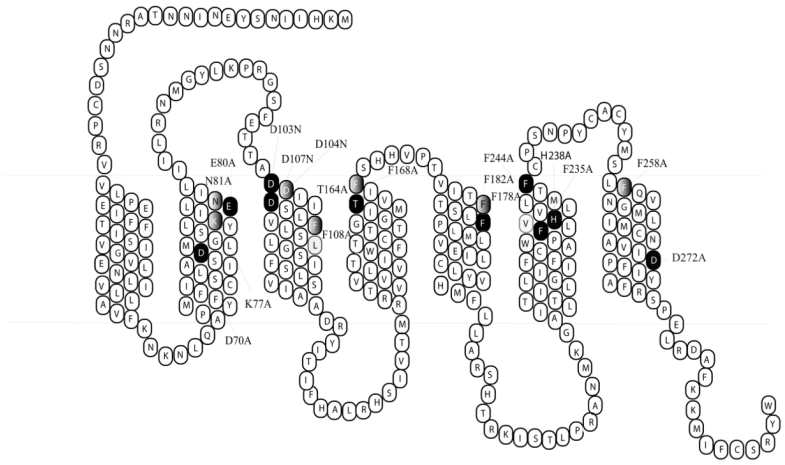

Figure 1.

Two-dimensional representation of the seven TM structure of the hMC2R. The conserved TM residues mutated in these experiments are denoted by gray highlighting. The unique TM residues mutated in these experiments are denoted by black highlighting.

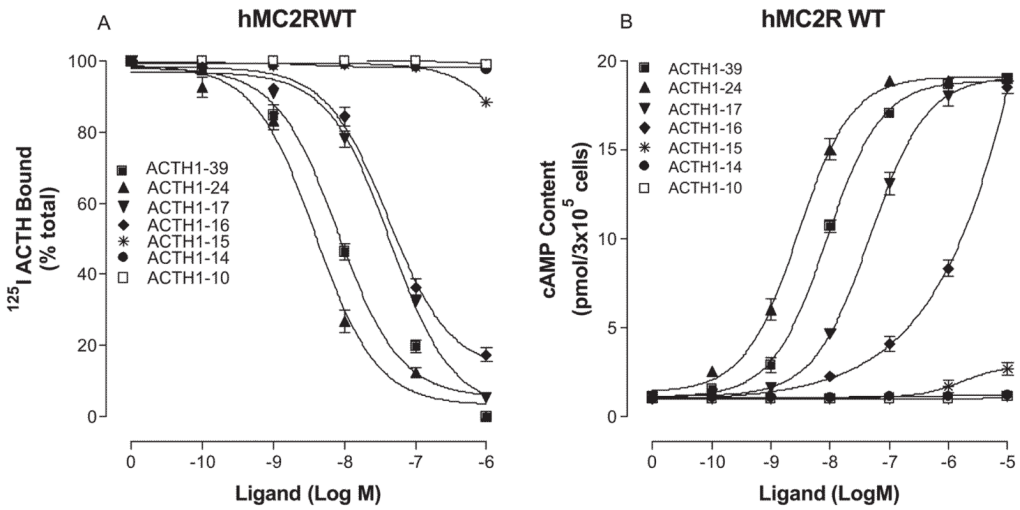

Figure 2.

Binding affinity and potency of truncated ACTH peptides in OS3 cells stably transfected with the wild-type hMC2R. Panel A shows that OS3 cells transfected with hMC2R were incubated with ^{125}I-ACTH in the presence of the indicated amounts of unlabeled ligands, and total ^{125}I-ACTH binding was determined. Panel B shows that the cells were incubated with the indicated amounts of peptides and total cAMP accumulation was determined. (n = 3; see Table 1 for K_i and EC_{50} values).

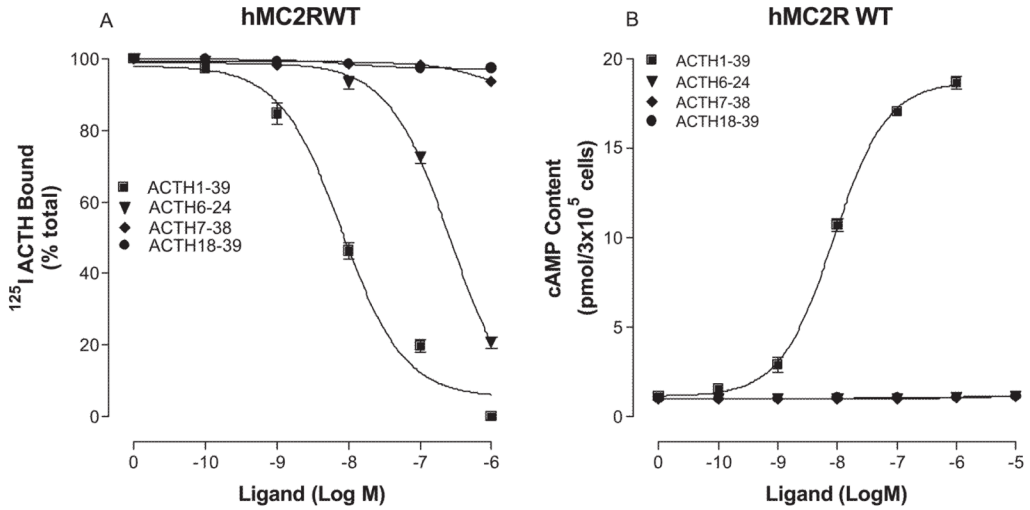

Figure 3.

Binding affinity and potency of truncated ACTH peptides in OS3 cells stably transfected with the wild-type hMC2R. Panel A shows that OS3 cells transfected with hMC2R were incubated with ^{125}I-ACTH in the presence of the indicated amounts of unlabeled ligands, and total ^{125}I-ACTH binding was determined. Panel B shows that the cells were incubated with the indicated amounts of peptides and total cAMP accumulation was determined. (n = 3; see Table 1 for K_i and EC_{50} values).

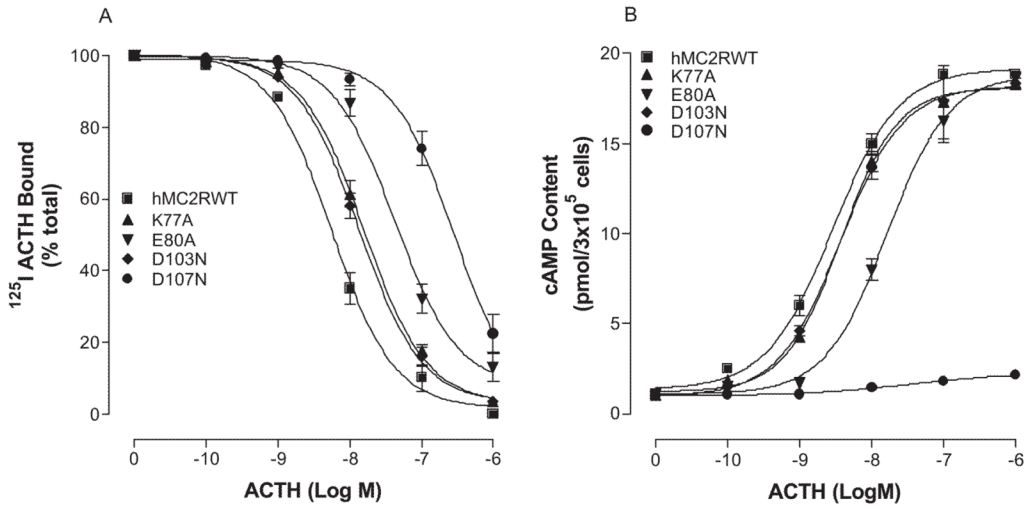

Figure 4.

Effects of the mutations of the conserved charged amino acid hMC2R TM residues with alanine on ACTH binding affinity and receptor activity. Panel A shows the ACTH binding affinity of these mutants. Panel B shows the ability of ACTH stimulated cAMP production at these mutants. (n = 3; see Tables 2 for actual K_i and EC_{50} values).

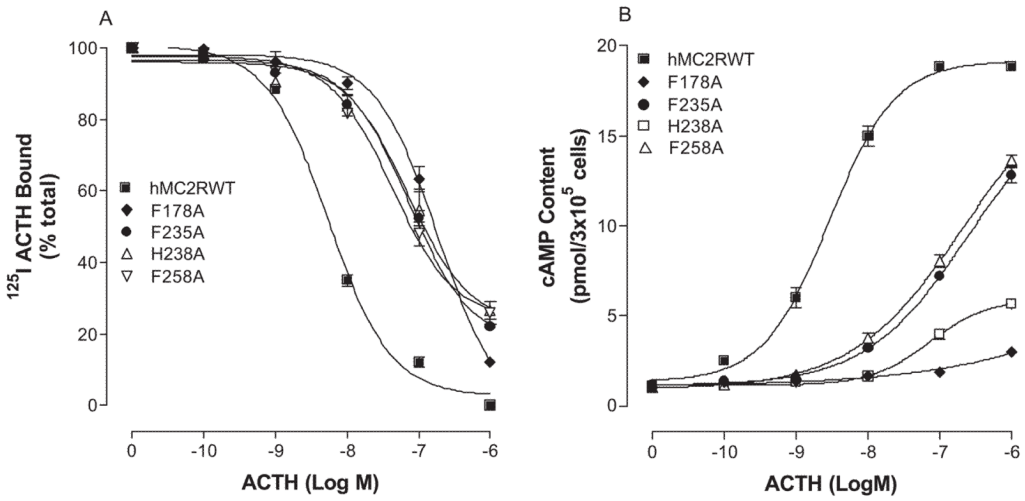

Figure 5.

Effect of the mutations of various conserved aromatic amino acids in the hMC2R on ACTH binding affinity and receptor activity. Panel A shows the ACTH binding affinity to these mutant receptors. Panel B shows the ability of ACTH to stimulate cAMP production upon binding to the mutant receptors. (Panels A and B) (n = 3; see Tables 2 for actual K_i and EC_{50} values).

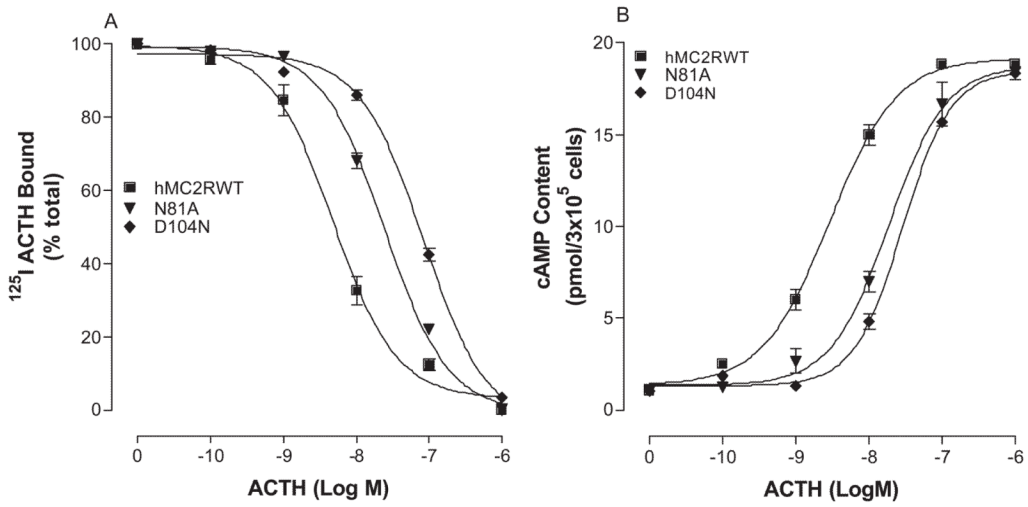

Figure 6.

Effects of the mutations of unique charged amino acid hMC2R TM residues on ACTH binding affinity and receptor activity. Panel A shows the ACTH binding affinity of these mutants. Panel B shows the ability of ACTH stimulated cAMP production at these mutants. (n = 3; see Tables 3 for actual K_i and EC_{50} values).

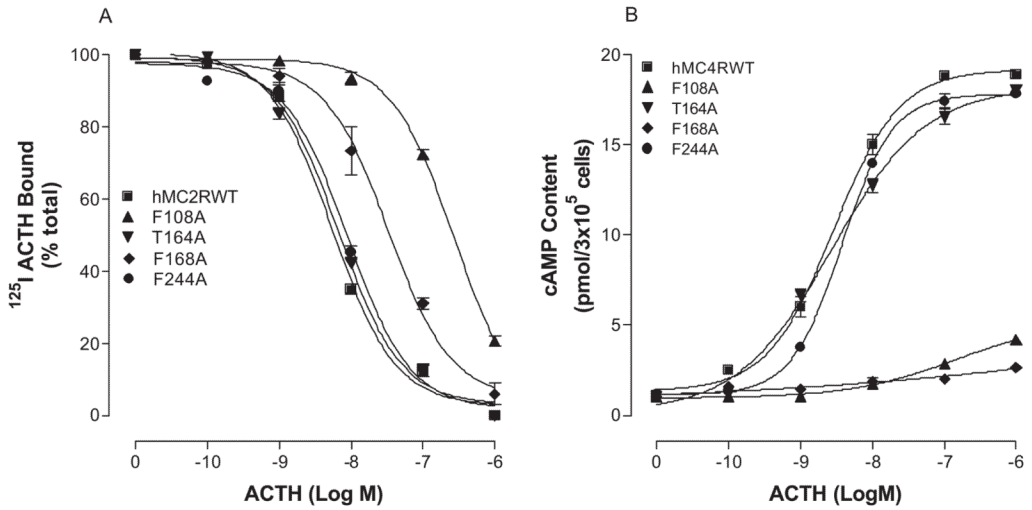

Figure 7.

Effect of the mutations of various unique aromatic amino acids in the hMC2R on ACTH binding affinity and receptor activity. Panel A shows the ACTH binding affinity to these mutant receptors. Panel B shows the ability of ACTH to stimulate cAMP production upon binding to the mutant receptors. (Panels A and B) (n = 3; see Tables 3 for actual K_i and EC_{50} values).

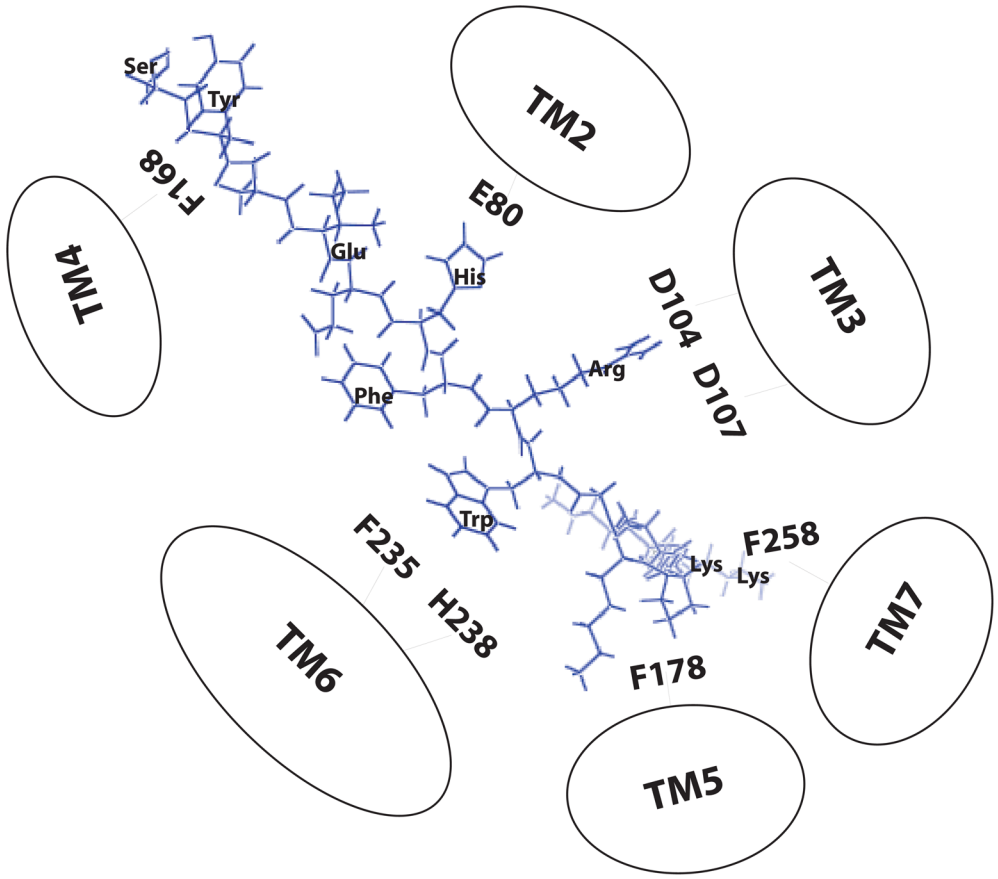

Figure 8.

Two-dimensional representation of a proposed three dimensional model illustrating ACTH1-16 docked inside the hMC2R. Based on our results, three main receptor binding pockets are proposed. The first is a predominantly ionic pocket formed by E80, D104 and D107. The second binding pocket is formed by aromatic residues F235 and H238 in TM6. The third binding pocket is formed by F178 in TM5 and F258 in TM7.

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Table 1

Effect of ACTH truncations on ^{125}I ACTH binding and cAMP generation

		Ki (nM)	EC_{50} (nM)
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-AA14-39	(ACTH1-39)	9.8 ± 2.1	8.59 ± 0.65
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-AA14-24	(ACTH1-24)	5.4 ± 1.5*	2.8 ± 0.5*
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg	(ACTH1-17)	78 ± 3.7*	46.8 ± 8.5*
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys	(ACTH1-16)	92 ± 12.3*	567 ± 34*
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys	(ACTH1-15)	No	NR
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly	(ACTH1-14)	No	NR
Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly	(ACTH1-10)	No	NR
His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro	(ACTH6-24)	839 ± 13.5*	NR
Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu	(ACTH7-38)	No	NR
Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe	(ACTH18-30)	No	NR

No: No binding; NR - No response n>3, mean ± S.E.M.

*P < 0.01 compared with ACTH1-39.

Table 2

Effect of the substitutions of the conserved amino acid residues of hMC2R on ^{125}I ACTH binding and cAMP production

	Receptor expression (% of WT)	^{125}I- ACTH binding Ki (nM)	cAMP production EC_{50} (nM)
hMC2RWT	100	5.6 ± 1.0	0.8 ± 0.2
D70A	No	NB	NR
K77A	73 ± 9.5	25.2 ± 4.1*	6.2 ± 0.9*
E80A	75 ± 12.3	65.2 ± 7.1*	7.8 ± 1.1*
D103N	68 ± 14	15.7 ± 0.7*	5.8 ± 1.2*
D107N	66 ± 11	>10^3*	>10^3*
F178A	67 ± 8.7	>10^3*	>10^3*
F235A	84 ± 6.8	98 ± 6.9*	216 ± 18*
H238A	69 ± 11.7	>10^3*	>10^3*
F258A	78 ± 12.7	78 ± 4.4*	187 ± 10*
D272A	No	NB	NR

*P<0.05 compared with WT receptor.

Table 3

Effect of the substitutions of unique amino acid residues of hMC2R on ^{125}I ACTH binding and cAMP production

	Receptor expression (% of WT)	^{125}I- ACTH binding Ki (nM)	cAMP production EC_{50} (nM)
hMC2RWT	100	8.6 ± 1.0	4.8 ± 0.1
N81A	89 ± 12.4	15.9 ± 0.6*	11.6 ± 1.2*
D104N	78 ± 13.4	87.3 ± 9.8*	87.7 ± 9.3*
F108A	65 ± 10.2	>10^3*	>10^3*
T164A	93 ± 14.2	6.4 ± 0.7	3.9 ± 0.1
F168A	56 ± 9.5	217.8 ± 13*	327.6 ± 30.3*
F244A	95 ± 11.2	9.9 ± 0.9	4.9 ± 0.9

*P<0.05 compared with WT receptor