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Structural Insight into the MC4R Conformational Changes via Different Agonist-Mediated Receptor Signaling

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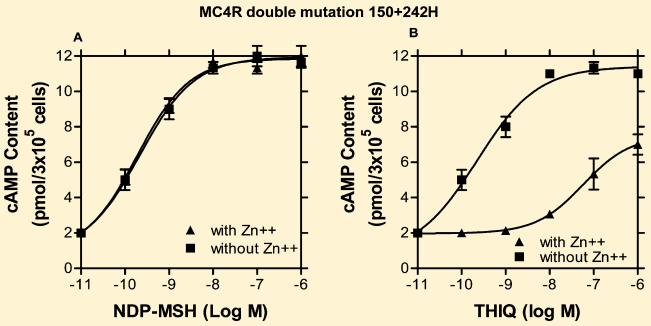
ABSTRACT: The melanocortin-4 receptor (MC4R) plays a key role in the regulation of food intake and body weight. Previous studies indicate that α-melanocyte stimulating hormone (α-MSH) binds to MC4R and activates three signal pathways (cAMP, calcium, and mitogen-activated protein kinase pathways), whereas MC4R synthetic agonist THIQ can activate only the cAMP pathway. The molecular basis of the MC4R responsible for different ligand-mediated signaling is unknown. We hypothesize that different MC4R agonists can stabilize different MC4R conformations and result in ligand-mediated signal transduction. In this study, we examined the effect of the MC4R conformational change in cAMP signaling pathways mediated by different agonists by cross-linking two transmembrane helices (TM3 and TM6). We generated and tested 11 single and 8 double mutations that are located at the end of TM3 and beginning of TM6 in MC4R. Our results indicate that (1) single or double mutations of the MC4R did not significantly alter cAMP production induced by NDP-MSH compared to that of wild-type MC4R except single mutation 243H (the mutation 243H significantly decreased cAMP production mediated by NDP-MSH or THIQ due to a low level of receptor expression at the cell surface), (2) the mutation 247H significantly decreased THIQ-mediated cAMP production but not NDP-MSH, and (3) the receptor cAMP signaling pathway activation by THIQ is blocked in the presence of Zn^{2+} with the double mutation I150/242H but activation by NDP-MSH is not, suggesting that the activated conformation of MC4R mediated by NDP-MSH and THIQ is different. This study provides insight into the molecular basis of MC4R responsible for receptor signaling mediated by different agonists.



The melanocortin-4 receptor (MC4R), a seven-transmembrane G-protein-coupled receptor (GPCR) expressed mainly in the hypothalamus, plays a key role in the regulation of food intake and body weight. MC4R is currently considered a potential therapeutic target for obesity.[1−6] MC4R inhibits food intake through activation by α-melanocyte stimulating hormone (α-MSH). Extensive studies over the last several years have determined that transmembrane (TM) helices TM3 and TM6 of the MC4R are responsible for ligand binding and signaling.[7−9] Furthermore, MC4R can couple to three major classes of G-proteins (Gs, Gi/o, and Gq), activating second-messenger cAMP, calcium, and mitogen-activated protein kinase (MAPK).[10,11] However, these signal pathways can be activated by different agonists. The molecular mechanism of MC4R responsible for receptor signaling mediated by different ligands is unclear.

Extensive studies indicate that GPCRs can be activated through binding to different agonist ligands. The discrepancy may be due to the disruption of different combinations of receptor intramolecular interactions, leading to different receptor conformations and different effects on downstream signaling proteins.[12] Several studies indicate that different transmembrane helix movement of the receptor is involved in different agonist-mediated receptor activation. For example, TM3 and TM6 of the rhodopsin and β2-adrenergic receptors moved away from each other after agonist stimulation, but the movement of TM1 and TM7 of the muscarinic 1 receptor is responsible for agonist-induced receptor activation.[13−18] As part of the GPCR family, MCRs share many structural features with other GPCRs. The computer model of MC4R shows there is a structural change that accompanies receptor activation.[19,20] MC4R is proposed to undergo TM movement during activation. The aim of this study is to examine whether a conformational change of the MC4R plays an important role in receptor activation mediated by different agonists by using metal ion binding sites. We hypothesize that MC4R can adopt multiple conformations mediated by different agonists, leading to different signaling events. These conformations can be stabilized by different ligands, causing ligand-mediated signal transduction. The experiment was designed to determine whether there is any movement between TM3 and TM6 of the MC4R when the receptor is activated. Our results provide some evidence that MC4R adopts different conformations in cAMP production mediated by NDP-MSH or THIQ.

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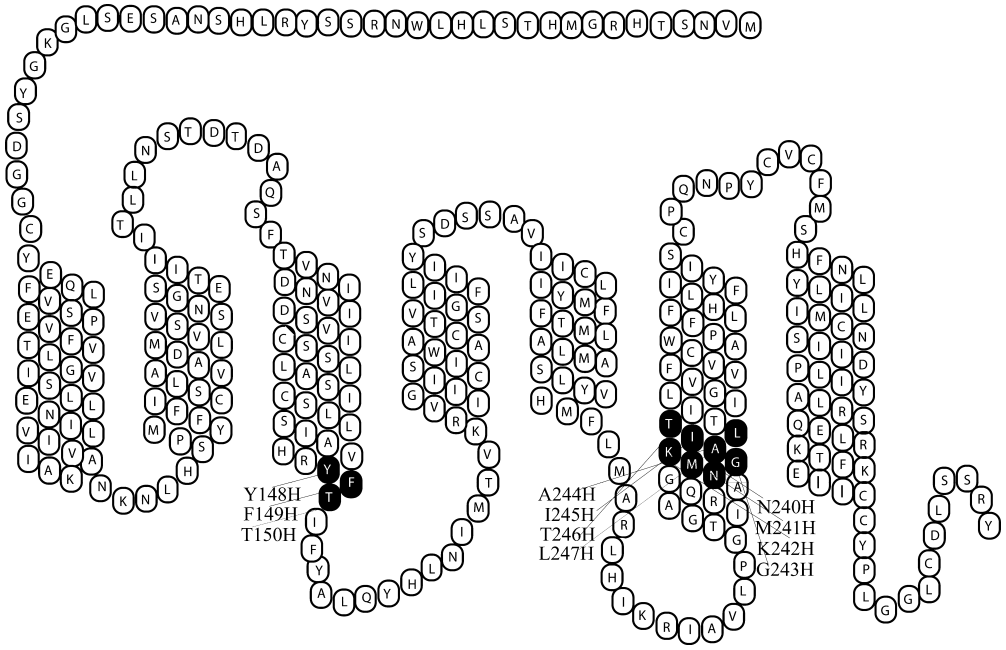

Figure 1. Two-dimensional representation of the seven-TM structure of the hMC4R. The unique amino acid residues mutated in these experiments are denoted by black highlighting.

MATERIALS AND METHODS

MC4R Agonists. (Nle[4],D-Phe[7])-α-MSH (NDP-α-MSH) was purchased from Peninsula Laboratories, Inc. (Belmont, CA). THIQ was synthesized by Hruby's group.[21]

Site-Directed Mutagenesis. Single and double MC4R mutations (Figure 1) were constructed using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the entire coding region of the MC4R mutants was sequenced at the University of Alabama at Birmingham Sequence Core to confirm that the desired mutation sequences were present.

Cell Culture and Transfection. The HEK-293 cell line was purchased from ATCC (Manassas, VA). HEK cells were plated in a six-well plate 12 h before transfection. Cells were transfected with MC4R mutation vectors using lipoD293 DNA *in vitro* (SignaGen Laboratories, Ijamsville, MD). The permanently transfected clonal cell lines were selected by resistance to the neomycin analogue G418.[7]

Binding Assays. Binding experiments were performed using conditions previously described.[22] Briefly, cells with MC4R mutations were plated 12 h before the experiment. [^{125}I]NDP-α-MSH (2×10^5 cpm) was added to the medium in combination with nonradiolabeled ligand NDP-α-MSH. Binding reactions were terminated by removing the medium, and the cells were washed twice. 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 assessed 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. Cellular cAMP generation was measured using a cAMP assay kit (Cisbio Bioassays, San Diego, CA). This cAMP kit is based on a competitive immunoassay using a cryptate-labeled anti-cAMP antibody and d2-labeled cAMP. Cells were plated in a 96-well plate overnight before the experiment. The medium was removed, and the cells were incubated with 50 μL of Earle's Balanced Salt Solution (EBSS), containing the melanocortin agonist NDP-MSH or THIQ, for 1 h at 37 °C in the presence of 10^{-3} M isobutylmethylxanthine. The reaction was stopped by adding lysis buffer (50 μL/well). The cAMP content was measured according to instructions accompanying the assay kit.

Receptor Expression. To determine the MC4R protein expression at the cell surface, the FLAG tag was inserted into the NH_2 terminus of hMC4R. The FLAG protein is an eight-amino acid peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) that can be detected with an antibody.[23] The cells with hMC4R or a mutant receptor were harvested and washed with phosphate-buffered saline (PBS). Cells were centrifuged and fixed with 3% paraformaldehyde in PBS (pH 7.4). The cells were incubated first with an anti-FLAG M1 monoclonal antibody (catalog no. 316, Sigma, St. Louis, MO). The cells were then centrifuged and washed with incubation buffer. The cell pellets were then suspended in buffer containing a second antibody, CY3-conjugated affinity pure donkey anti-mouse IgG (Immuno Research Lab, Inc., West Grove, PA), and incubated at room temperature for 30 min. Receptor expression was detected by flow cytometry (FACS) (FACStar plus six parameter cytometer/sorter with a dual argon ion laser, Becton Dickinson, San Jose, CA). The results were analyzed using the software CellQuest (Beckton-Dickinson Immunocytometry Systems, San Jose, CA).

Statistical Analysis. Each experiment was performed three separate times with duplicate wells. Data are expressed as means ± the standard error of the mean. 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 nonlinear least-squares regression analysis (Graphpad Prism, Graphpad Software, San Diego, CA). A Student's *t* test was used to determine the statistical significance of changes in affinity and potency ($p < 0.05$ was considered significant).

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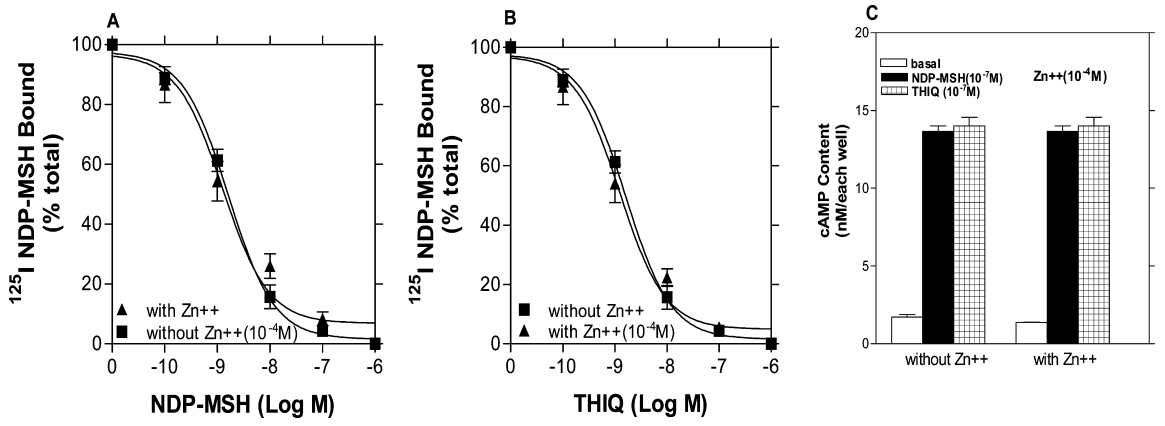

Figure 2. Effect of Zn^{2+} on ligand binding affinity and potency at the cells stably transfected with the wild-type hMC4R. (A) Effect of different doses of unlabeled NDP-MSH on [^{125}I]NDP-MSH binding in the presence of Zn^{2+} (10^{-4} M). (B) Effect of different doses of unlabeled THIQ on [^{125}I]NDP-MSH binding in the presence of Zn^{2+} (10^{-4} M). (C) Effect of NDP-MSH or THIQ on cAMP production in the presence of Zn^{2+} (10^{-4} M).

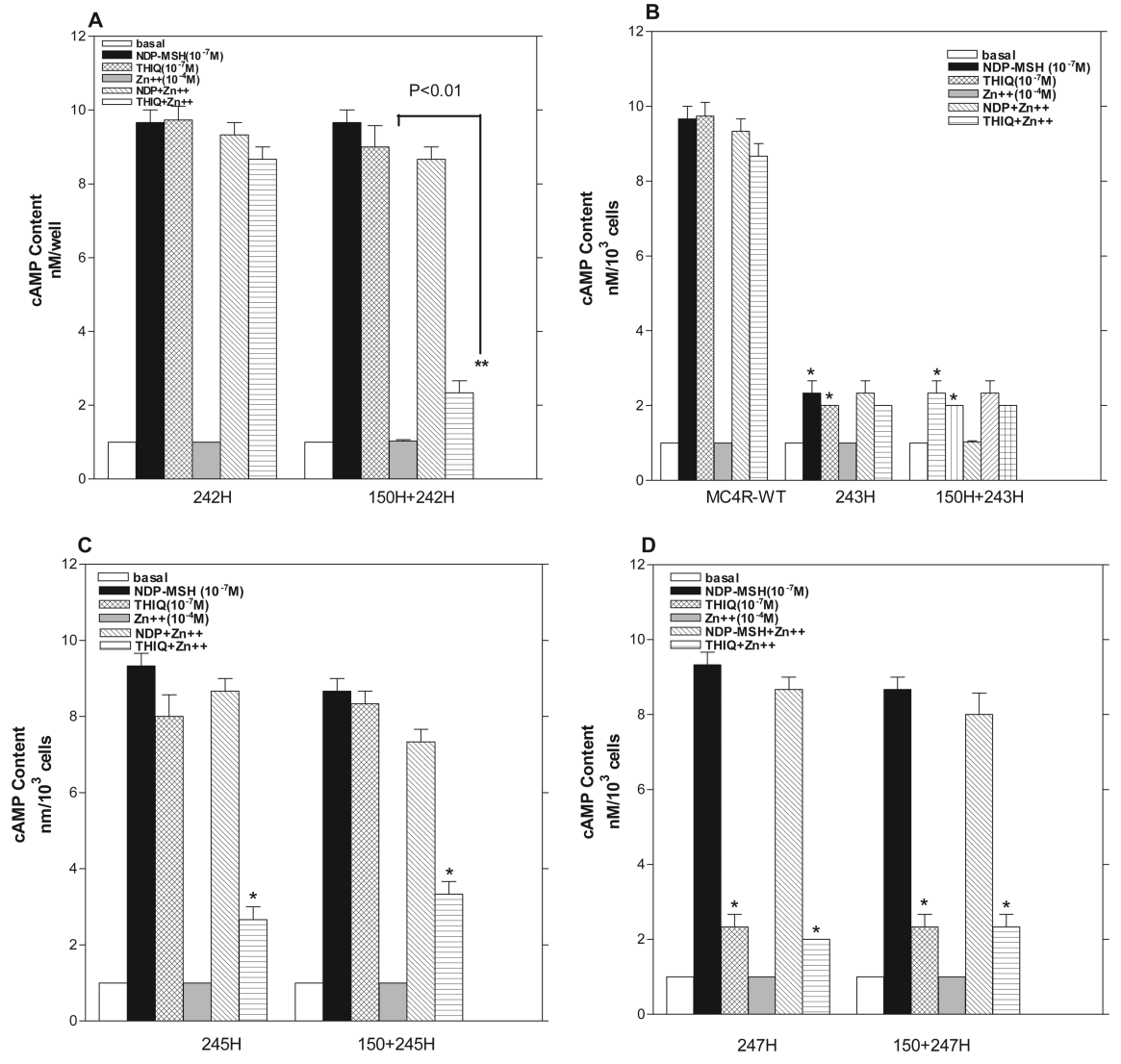

Figure 3. Effect of NDP-MSH or THIQ on cAMP production at the mutation in the presence of Zn^{2+} (10^{-4} M). (A) Profile of the single mutation 242H and double mutation 150 + 242H. (B) Profile of the single mutation 243H and double mutation 150 + 243H. (C) Profile of the single mutation 245H and double mutation 150 + 245H. (D) Profile of the single mutation 247H and double mutation 150 + 247H. The cells with mutations were incubated with NDP-MSH (10^{-7} M) or THIQ (10^{-4} M) in the presence of Zn^{2+} (10^{-4} M). Total cAMP accumulation was determined ($n \geq 3$; see Table 1 for total cAMP values). *$p < 0.05$ compared to wild-type MC4R; **$p < 0.05$ compared to that without Zn^{2+}.

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Table 1. Effects of Metal Ion Zn^{2+} on Agonist-Mediated cAMP Production of MC4R Mutants

		cAMP level (without Zn^{2+}) (nM)		cAMP level (with Zn^{2+}) (nM)	
	receptor expression (% of WT)	NDP-MSH (10^{-7} M)	THIQ (10^{-7} M)	NDP-MSH (10^{-7} M)	THIQ (10^{-7} M)
wild-type hMC4R	100	9.6 ± 1.0	9.7 ± 1.0	9.1 ± 1.0	9.2 ± 0.2
Y148H	96 ± 3.5	9.4 ± 1.5	9.2 ± 0.5	9.3 ± 1.0	9.0 ± 0.9
F149H	97 ± 2.6	9.7 ± 1.0	9.2 ± 0.7	9.6 ± 1.0	9.1 ± 0.4
I150H	95 ± 6.1	9.5 ± 1.1	9.1 ± 1.4	9.4 ± 0.9	9.1 ± 0.3
240H	93 ± 6.7	9.3 ± 1.0	9.0 ± 1.4	9.2 ± 1.0	9.2 ± 0.3
241H	94 ± 6.1	9.2 ± 1.0	9.7 ± 1.0	9.6 ± 1.0	9.1 ± 1.0
242H	95 ± 4.7	9.1 ± 1.0	9.2 ± 1.3	9.1 ± 0.5	8.2 ± 0.4
243H	38 ± 7.2[a]	2.4 ± 0.4[a]	2.1 ± 0.4[a]	2.3 ± 0.5[a]	1.9 ± 0.3[a]
244H	96 ± 2.2	9.2 ± 1.0	9.1 ± 1.4	9.6 ± 1.0	9.1 ± 0.3
245H	94 ± 5.7	9.5 ± 1.0	8.0 ± 1.5	8.3 ± 1.0	2.5 ± 0.5[b]
246H	98 ± 12.7	9.4 ± 1.0	9.4 ± 1.4	9.6 ± 1.0	9.1 ± 0.5
247H	95 ± 4.9	9.3 ± 1.0	2.3 ± 1.0[a]	8.6 ± 1.0	2.0 ± 0.2[a]

[a] $p < 0.05$ compared to the value of wild-type MC4R. [b] $p < 0.05$ compared to the value without Zn^{2+}.

Table 2. Effects of Metal Ion Zn^{2+} on Agonist-Mediated cAMP Production with MC4R Double Mutants

		cAMP level (without Zn^{2+}) (nM)		cAMP level (with Zn^{2+}) (nM)	
	receptor expression (% of WT)	NDP-MSH (10^{-7} M)	THIQ (10^{-7} M)	NDP-MSH (10^{-7} M)	THIQ (10^{-7} M)
wild-type hMC4R	100	9.6 ± 1.0	9.7 ± 1.0	9.6 ± 1.0	9.2 ± 0.2
150H+240H	93 ± 6.7	9.3 ± 1.3	9.0 ± 1.4	9.2 ± 1.4	9.2 ± 0.3
150H+241H	94 ± 6.1	9.2 ± 1.4	9.7 ± 1.0	9.6 ± 1.0	9.1 ± 1.0
150H+242H	**95 ± 4.7**	**9.1 ± 1.2**	**8.4 ± 1.3**	**8.1 ± 2.5**	**3.5 ± 0.4[b]**
150H+243H	38 ± 7.2[a]	2.2 ± 1.0[a]	2.1 ± 0.4[a]	2.6 ± 1.0[a]	2.1 ± 0.3[a]
150H+244H	94 ± 5.7	9.2 ± 1.0	9.1 ± 0.4	9.6 ± 1.0	9.1 ± 0.3
150H+245H	94 ± 5.7	8.5 ± 1.0	8.2 ± 0.5	7.3 ± 2.0	3.3 ± 0.5[a]
150H+246H	98 ± 12.7	9.4 ± 1.0	9.4 ± 0.4	9.6 ± 1.0	9.1 ± 0.5
150H+247H	95 ± 4.9	8.8 ± 2.0	2.5 ± 0.4[a]	8.0 ± 2.0	2.1 ± 0.2[a]

[a] $p < 0.05$ compared to that of the wild type. [b] $p < 0.05$ compared to that without Zn^{2+}.

RESULTS

Effect of Metal Ion Zn^{2+} on Ligand-Mediated Signaling at Wild-Type MC4R. Histidine residues can complex Zn(II) and thus form a bridge between different receptor residues, thereby allowing the study of helix−helix interactions. Substitution of histidine in the different receptor helices will form a bridge in the presence of Zn^{2+} if they are close enough when the receptor is activated. To examine the role of the MC4R conformational change in ligand-mediated receptor signaling, we introduced histidine residues into the end of MC4R TM3 and the beginning of the TM6 helices. Human MC4R (hMC4R) was transfected into HEK cells that lacked endogenous MC4R, and receptor function was examined. Cells expressing hMC4R were incubated with [^{125}I]NDP-MSH and different concentrations of unlabeled NDP-MSH. Ligand binding affinity was determined. Our results indicate that NDP-MSH dose-dependently displaced [^{125}I]NDP-MSH binding at hMC4RWT. Consistent with the binding results, NDP-MSH was able to stimulate cAMP production at hMC4RWT in a dose-dependent manner (Figure 2). To examine the effect of metal ion Zn^{2+} on ligand-mediated receptor signaling, cells expressing MC4R WT were treated with NDP-MSH or THIQ in the presence of Zn^{2+} (10^{-4} M) . Our results indicate that cAMP production mediated by NDP-MSH or THIQ was not altered by Zn^{2}+ at the dose of 10^{-4} M (Figure 2), which is consistent with a previous report.[24] The maximal response of the cAMP level stimulated by NDP-MSH or THIQ was not altered by the presence of Zn^{2+} at a dose of 10^{-4} M, suggesting that metal ion Zn^{2+} does not inhibit ligand-mediated receptor signaling at wild-type MC4R at a dose of 10^{-4} M.

Effect of Metal Ion Zn^{2+} on Ligand-Mediated Receptor Signaling at the Single Mutation at the End of the MC4R TM Regions. The MC4R single mutation was transfected into HEK cells, and the receptor function was examined. Three amino acid residues at the end of MC4R TM3 (Y148, F149, and T150) were substituted with histidine. Eight amino acid residues at the end of TM6 (N240, M241, K242, G243, A244, I245, T246, and L247) were mutated. Cells expressing these hMC4R mutations were incubated with [^{125}I]NDP-MSH and different concentrations of unlabeled NDP-MSH. Ligand binding affinity was determined. Our results indicate that NDP-MSH dose-dependently displaced [^{125}I]NDP-MSH binding at these single mutations, and their binding affinities were not significantly altered compared to that of wild-type MC4R except that of the G243H mutant. Substitution of G243 with histidine at MC4R significantly decreased ligand binding affinity and potency (Figure 3 and Table 1). A further experiment shows that mutation 243H is poorly expressed at the cell surface (Table 1), suggesting that a low level of receptor expression at the cell surface weakened receptor function.

Cells expressing hMC4R mutants were then treated with THIQ, and cAMP production was examined (Table 2). THIQ was able to stimulate cAMP production of these MC4R mutants that are similar to wild-type MC4R except 247H. The THIQ-mediated cAMP production ability of this mutant was significantly weakened (Figure 3).

To test whether Zn^{2+} alters receptor function at these single mutations, cAMP production mediated by different agonists

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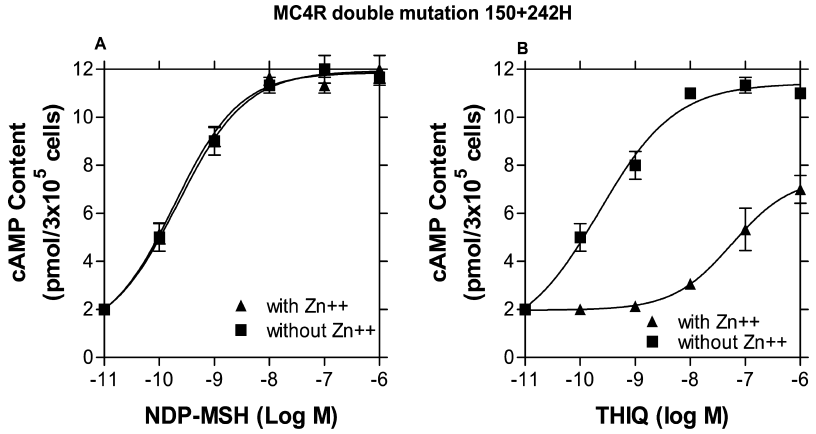

Figure 4. Effect of Zn^{2+} on ligand potency at double MC4R mutation 150H + 242H. (A) Effect of Zn^{2+} on NDP-MSH-mediated cAMP production. (B) Effect of Zn on THIQ-mediated cAMP production. The cells with double mutation 150H + 242H were incubated with different doses of NDP-MSH or THIQ in the presence of Zn^{2+} (10^{-4} M). Total cAMP accumulation was determined ($n \geq 3$; see Table 2 for total cAMP values).

was examined at these mutations in the presence of Zn^{2+} (10^{-4} M). Cells expressing mutant receptors were incubated with NDP-MSH or THIQ in the presence of Zn^{2+}, and cAMP production was examined. Our results indicate that Zn^{2+} does not significantly alter NDP-MSH or THIQ potency at these single mutations except 245H. In the presence of Zn^{2+}, cAMP production mediated by THIQ with mutation 245H was significantly decreased. In summary, our results indicate that single amino acid mutations 150H, 242H, and 245H did not alter NDP-MSH binding or signaling in the presence of Zn^{2+} while single mutation 245H decreased the level of THIQ binding and signaling in the presence of Zn^{2+}.

Effect of Metal Ion Zn^{2+} on Ligand-Mediated Receptor Signaling with the Double Mutations at the End of the MC4R TM Regions. We then tested whether double mutations of the MC4R alter NDP-MSH- or THIQ-mediated receptor activity. Cells expressing hMC4R double mutations were treated with NDP-MSH (10^{-7} M) or THIQ (10^{-4} M). Our results indicate that double histidine mutations do not significantly affect NDP-MSH or THIQ activities except that of 150H + 247H. NDP-MSH is able to stimulate cAMP production at the mutated receptor 150H + 247H, but THIQ-mediated cAMP production was significantly decreased, which is similar to that of the same single mutation 247H.

To test whether movement of MC4R TM3 and TM6 is involved in ligand-mediated receptor activation, we examined NDP-MSH- or THIQ-mediated receptor activation in the presence of Zn^{2+} with these double mutations. Cells expressing mutant receptors were incubated with NDP-MSH (10^{-7} M) or THIQ (10^{-4} M) in the presence of Zn^{2+} (10^{-4} M), and cAMP production was examined. Our results indicate that Zn^{2+} does not significantly alter NDP-MSH or THIQ potency at these double mutations except the double mutation 150H + 242H. THIQ-mediated cAMP production was significantly decreased with the double mutation 150H + 242H in the presence of Zn^{2+}. A dose response experiment indicates that Zn^{2+} significantly inhibits THIQ-mediated cAMP production (Figure 4). A further experiment shows that the binding affinity of THIQ at the double mutation was decreased in the presence of Zn^{2+}.

DISCUSSION

Our study indicates that THIQ-mediated but not NDP-MSH-mediated cAMP production at double mutation 150H + 242H was inhibited by Zn^{2+}, suggesting that different agonists may induce distinct MC4R conformational changes.

Many studies show that the movements of GPCR helices during activation are accompanied by a common set of local movements in the intracellular parts of GPCRs.[12] A common activation-related feature is that receptor helix VI moves away from helix III.[25] The magnitude of this motion may vary among different GPCRs and different activated states. The variety of conformational and functional responses of GPCR may depend on the structurally distinct ligands. Extensive studies indicate that the conformational alteration of the GPCR may play an important role in the specific signaling pathway of the GPCR activated by different agonists. Different agonists can induce multiple conformations and stabilize different active states. The distinct receptor conformations induced by ligands result in distinct receptor−effector complexes, which produce different levels of activation or inhibition of subsequent signaling cascades. MC4R has been proposed to have two major different conformations associated with the inactive or active G-protein-coupled state. Furthermore, MC4R agonists, NDP-MSH and THIQ, activate different MC4R intracellular effectors. NDP-α-MSH binds to MC4R and activates cAMP, calcium, and MAPK pathways, whereas MC4R synthetic agonist THIQ can activate only the cAMP pathway. All these results suggest that different MC4R agonists may result in different MC4R conformational changes. The ability of these ligands to induce and/or stabilize a specific MC4R conformation that couples the receptor may be a key molecular mechanism underlying this functional selectivity.

Introduction of histidine side chains into complex metal ions has made a great contribution to the understanding of the receptor conformational changes that occur during receptor activation; these techniques have been widely utilized.[26] The basic principle of this technique is to introduce histidine residues that can form a complex with Zn, thus forming a bridge between residues in different helices of the GPCR and thereby allowing the study of different helix movements. This allows a more detailed investigation of the surroundings of the attachment site within the helical bundle. The metal ion chelator approach was applied in an inhibitory mode by cross-linking two helices and thus inhibiting receptor activation. Those studies were performed using a variety of receptors, including the κ-opioid receptor, rhodopsin,[14] the β_2-adrenoceptor,[27] and the M_1-ACh receptor.[18] The results from these

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studies demonstrated that different receptor conformations would be required for G-protein activation.[28] Previous studies show that the activity of metal ion Zn at MCRs is different. Zn can increase cAMP production at MC1R without an agonist. Zn has no agonist activity at MC4R but can block the binding of the inverse agonist AGRP to the MC4R. Zn can increase cAMP production at the mutation of MC4R TM3 residues.[24,29] To study the molecular mechanisms responsible for ligand efficacy and functional selectivity at hMC4R, we utilized histidine replacement to directly monitor conformational changes between TM3 and TM6 induced by different ligands within MC4R. To determine whether Zn^{2+} has an effect on agonist-mediated receptor signaling because both NDP-MSH and THIQ have functional groups that may readily coordinate Zn(II), we examined the effect of different doses of Zn^{2+} on agonist-mediated MC4R activation. Our results indicated that Zn^{2+} at a dose of 10^{-4} M has no effect on agonist-mediated receptor signaling. Our results also indicate that Zn^{2+} did not alter the single-mutation pharmacological profile that is we expected except that of 245H. THIQ-mediated cAMP production was significantly decreased in the presence of Zn^{2+} with this mutation. In this case, only one amino acid residue substitution with histidine results in the inhibition of receptor activation, suggesting that a histidine at position 245 may interact with other histidine residues in the receptor and inhibit the change in the receptor conformation. A further experiment is required to investigate this phenomenon. Our results indicate that metal Zn^{2+} has no inhibitory effect on NDP-MSH- or THIQ-mediated cAMP production with any double mutation except mutation 150H + 242H. Mutation 150H + 242H significantly decreased the level of THIQ-mediated receptor activation but not NDP-MSH in the presence of Zn^{2+}, suggesting that the movement of these two residues occurs when MC4R is activated by THIQ. The computer model of MC4R developed by the Mosberg group[19] shows that MC4R undergoes TM movement during activation. TM6 shifts outward during activation, moving its intracellular end away from TM3.[19] Our result provides evidence that the movement of residue 150 in TM3 and residue 242 in TM6 is crucial for THIQ-mediated cAMP production but not for NDP-MSH. NDP-MSH and THIQ that result in different receptor conformational changes also suggest that different ligands can stabilize different receptor conformations and result in receptor signaling. The conformational flexibility of MC4R allows it to be stabilized in a specific conformation or a subset of conformations depending on ligand characteristics that can then trigger a very specific intracellular response or a set of responses. An individual ligand induces or stabilizes a unique conformational state that can be distinguished by the activity of that state toward different signaling pathways. Our finding provides indirect evidence that a distinct ligand can induce different MC4R conformations, thereby translating specific receptor-active conformations into selective activation of individual functional responses.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Huszar, D., Lynch, C. A., Fairchild-Huntress, V., Dunmore, J. H., Fang, Q., Berkemeier, L. R., Gu, W., Kesterson, R. A., Boston, B. A., Cone, R. D., Smith, F. J., Campfield, L. A., Burn, P., and Lee, F. (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell 88*, 131−141.

(2) Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature 385*, 165−168.

(3) Giraudo, S. Q., Billington, C. J., and Levine, A. S. (1998) Feeding effects of hypothalamic injection of melanocortin 4 receptor ligands. *Brain Res. 809*, 302−306.

(4) Cone, R. D. (1999) The Central Melanocortin System and Energy Homeostasis. *Trends Endocrinol. Metab. 10*, 211−216.

(5) Fisher, S. L., Yagaloff, K. A., and Burn, P. (1999) Melanocortin-4 receptor: A novel signalling pathway involved in body weight regulation. *Int. J. Obes. 23* (Suppl. 1), 54−58.

(6) Fani, L., Bak, S., Delhanty, P., van Rossum, E. F., and van den Akker, E. L. (2014) The melanocortin-4 receptor as target for obesity treatment: A systematic review of emerging pharmacological therapeutic options. *Int. J. Obes. 38*, 163−169.

(7) Yang, Y., Dickinson, C., Haskell-Luevano, C., and Gantz, I. (1997) Molecular basis for the interaction of [Nle4,D-Phe7]-melanocyte stimulating hormone with the human melanocortin-1 receptor. *J. Biol. Chem. 272*, 23000−23010.

(8) Yang, Y., Chen, M., Loux, T. J., Georgeson, K. E., and Harmon, C. M. (2005) Molecular mechanism of the intracellular segments of the melanocortin-4 receptor for NDP-MSH signaling. *Biochemistry 44*, 6971−6979.

(9) Yang, Y., Chen, M., Lai, Y., Gantz, I., Georgeson, K. E., and Harmon, C. M. (2002) Molecular determinants of human melanocortin-4 receptor responsible for antagonist SHU9119 selective activity. *J. Biol. Chem. 277*, 20328−20335.

(10) Mountjoy, K. G., Kong, P. L., Taylor, J. A., Willard, D. H., and Wilkison, W. O. (2001) Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells. *Physiol. Genomics 5*, 11−19.

(11) Nickolls, S. A., Fleck, B., Hoare, S. R., and Maki, R. A. (2005) Functional selectivity of melanocortin 4 receptor peptide and nonpeptide agonists: Evidence for ligand-specific conformational states. *J. Pharmacol. Exp. Ther. 313*, 1281−1288.

(12) Taddese, B., Simpson, L. M., Wall, I. D., Blaney, F. E., and Reynolds, C. A. (2013) Modeling active GPCR conformations. *Methods Enzymol. 522*, 21−35.

(13) Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gether, U., and Javitch, J. A. (2001) Activation of the β2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J. Biol. Chem. 276*, 29171−29177.

(14) Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. *Nature 383*, 347−350.

(15) Meng, E. C., and Bourne, H. R. (2001) Receptor activation: What does the rhodopsin structure tell us? *Trends Pharmacol. Sci. 22*, 587−593.

(16) Deupi, X., and Kobilka, B. (2007) Activation of G protein-coupled receptors. *Adv. Protein Chem. 74*, 137−166.

(17) Kobilka, B. K., and Deupi, X. (2007) Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol. Sci. 28*, 397−406.

(18) Lu, Z. L., and Hulme, E. C. (2000) A network of conserved intramolecular contacts defines the off-state of the transmembrane switch mechanism in a seven-transmembrane receptor. *J. Biol. Chem. 275*, 5682−5686.

(19) Pogozheva, I. D., Chai, B. X., Lomize, A. L., Fong, T. M., Weinberg, D. H., Nargund, R. P., Mulholland, M. W., Gantz, I., and Mosberg, H. I. (2005) Interactions of human melanocortin 4 receptor with nonpeptide and peptide agonists. *Biochemistry 44*, 11329−11341.

(20) Xiang, Z., Pogozheva, I. D., Sorenson, N. B., Wilczynski, A. M., Holder, J. R., Litherland, S. A., Millard, W. J., Mosberg, H. I., and Haskell-Luevano, C. (2007) Peptide and small molecules rescue the functional activity and agonist potency of dysfunctional human melanocortin-4 receptor polymorphisms. *Biochemistry 46*, 8273−8287.
(21) Yang, Y., Cai, M., Chen, M., Qu, H., McPherson, D., Hruby, V., and Harmon, C. M. (2009) Key amino acid residues in the melanocortin-4 receptor for nonpeptide THIQ specific binding and signaling. *Regul. Pept. 155*, 46−54.
(22) Chen, M., Cai, M., Aprahamian, C. J., Georgeson, K. E., Hruby, V., Harmon, C. M., and Yang, Y. (2007) Contribution of the conserved amino acids of the melanocortin-4 receptor in [corrected] [Nle4,D-Phe7]-α-melanocyte-stimulating [corrected] hormone binding and signaling. *J. Biol. Chem. 282*, 21712−21719.
(23) Guan, X. M., Kobilka, T. S., and Kobilka, B. K. (1992) Enhancement of membrane insertion and function in a type IIIb membrane protein following introduction of a cleavable signal peptide. *J. Biol. Chem. 267*, 21995−21998.
(24) Lagerstrom, M. C., Klovins, J., Fredriksson, R., Fridmanis, D., Haitina, T., Ling, M. K., Berglund, M. M., and Schioth, H. B. (2003) High affinity agonistic metal ion binding sites within the melanocortin 4 receptor illustrate conformational change of transmembrane region 3. *J. Biol. Chem. 278*, 51521−51526.
(25) Schwartz, T. W., Frimurer, T. M., Holst, B., Rosenkilde, M. M., and Elling, C. E. (2006) Molecular mechanism of 7TM receptor activation: A global toggle switch model. *Annu. Rev. Pharmacol. Toxicol. 46*, 481−519.
(26) Elling, C. E., Frimurer, T. M., Gerlach, L. O., Jorgensen, R., Holst, B., and Schwartz, T. W. (2006) Metal ion site engineering indicates a global toggle switch model for seven-transmembrane receptor activation. *J. Biol. Chem. 281*, 17337−17346.
(27) Sheikh, S. P., Vilardarga, J. P., Baranski, T. J., Lichtarge, O., Iiri, T., Meng, E. C., Nissenson, R. A., and Bourne, H. R. (1999) Similar structures and shared switch mechanisms of the β2-adrenoceptor and the parathyroid hormone receptor. Zn(II) bridges between helices III and VI block activation. *J. Biol. Chem. 274*, 17033−17041.
(28) Vilardaga, J. P., Frank, M., Krasel, C., Dees, C., Nissenson, R. A., and Lohse, M. J. (2001) Differential conformational requirements for activation of G proteins and the regulatory proteins arrestin and G protein-coupled receptor kinase in the G protein-coupled receptor for parathyroid hormone (PTH)/PTH-related protein. *J. Biol. Chem. 276*, 33435−33443.
(29) Holst, B., and Schwartz, T. W. (2003) Molecular mechanism of agonism and inverse agonism in the melanocortin receptors: Zn^{2+} as a structural and functional probe. *Ann. N.Y. Acad. Sci. 994*, 1−11.