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Role of third intracellular loop of the melanocortin 4 receptor in the regulation of constitutive activity

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Abstract

The melanocortin 4 receptor (MC4R) has been reported to display constitutive activity, which is probably relevant to the maintenance of a normal energy balance. Among the clinically reported mutants of MC4R in human obesity patients, we investigated the functional characteristics of seven mutants characterized by mutations in the third intracellular (i3) loop of MC4R. Via a CRE (cAMP responsive element)-mediated luciferase reporter gene assay, we show that most of these mutants displayed significantly reduced basal activity with reduced reporter gene activity, whereas the P230L mutant manifested significantly increased basal activity. When the dominant negative G_s mutant was co-expressed, the majority of the mutants, including the P230L mutant, showed reduced basal activity. These results suggest that the i3 loop of MC4R is essential not only for the functional activity but also for the regulation and maintenance of an optimal constitutive activity of MC4R in association with G protein coupling, in the control of energy homeostasis.

Keywords: Melanocortin; Melanocortin 4 receptor; Constitutive activity; G protein; Intracellular loop; Obesity; Mutation

The melanocortin 4 receptor (MC4R) is a G protein-coupled receptor (GPCR), which is involved in the regulation of both central energy homeostasis and body weight, the genetic disruption of MC4R has been shown to induce obesity in mice [1–4]. Therefore, MC4R receptor-mediated signaling is currently being widely considered as a potential target for our understanding of abnormal eating behaviors, most notably obesity and anorexia.

It has been demonstrated that heterologously expressed MC3R and MC4R are coupled to the cAMP pathway [5–7]. We have previously shown that the third intracellular (i3) loop of MC4R performs a pivotal function in G protein coupling specificity [5,6].

MC4R has been reported to display constitutive activity, increasing basal cAMP production in the absence of a ligand [8]. The physiological relevance of the constitutive activity of MC4R in the context of obesity remains poorly understood. Interestingly, it has been determined that some of the MC4R mutants found only in obese individuals evidence similar binding affinities and expression levels as wild-type receptors when they are expressed in *in vitro* systems. However, these receptor variants have lost the constitutive activity normally associated with wild-type MC4R, thereby suggesting that the constitutive activity of MC4R is probably relevant to the maintenance of a normal energy balance [9].

It has recently been suggested that the constitutive activity of MC4R is provided by its N-terminal domain, which functions as a tethered intramolecular ligand for the receptor, and that the obesity-related mutations in the N-terminal domain of MC4R attenuate its constitutive activity [10]. However, as many GPCRs display constitutive activity, the molecular mechanism which induces constitutive activity in MC4R remains incompletely understood.

In the present study, we have investigated the functional characteristics of MC4R mutants with mutations in the

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third intracellular loop of MC4R, which have been previously known to be associated with obesity (Table 1). We have observed that the third intracellular loop of MC4R is essential not only for the functional activity but also for the constitutive activity of MC4R in association with G protein coupling.

Experimental procedures

Construction of MC4R mutant receptors. The human melanocortin 4 receptor (hMC4R) sequence was obtained from the NCBI database (NM_005912). Primers were designed from the sequences in the third intracellular loop of hMC4R using the recommended protocols (Quikchange™ Site-directed Mutagenesis Kit, Stratagene), as previously described [6].

Transient transfection and luciferase reporter gene assay. HEK 293T cells were grown in Dulbecco's modified Eagle's medium (Gibco, NY) supplemented with 10% fetal bovine serum (Sigma, St. Louis, USA) and transfected with melanocortin 4 receptor or melanocortin 4 receptor mutants, CREB response element (CRE)-lucifrease, and pCH110. Thirty-six hours after transfection, the HEK 293T cells were treated for 3 h with various concentrations of NDP-MSH (Peptron, KOREA) or Agouti-related protein (AGRP) (83–132) amide (Phoenix Pharmaceuticals Inc., CA, USA). After treatment, the cells were lysed and assayed for luciferase activity using a luciferase assay system (Promega, WI, USA), as previously reported [6].

Western blot analysis. HEK 293T cells were transfected with 1 μg of pCRE-Luc, 0.5 μg of pCH110, 1 μg of hMC4R plasmid, and Gs mutant plasmid DNA ($G\alpha_s$-$A^{366}S/G^{226}A/E^{268}A$). Thirty-six hours after transfection, the HEK 293T cells were washed with ice-cold PBS and lysed. Protein (30 μg) was separated on 10% SDS–PAGE, and then blotted onto pre-wetted polyvinylidene difluoride nitrocellulose membranes (PVDF) (Millipore, MA). Mouse monoclonal anti-HA (hemaglutinin) (1:1000; Santa Cruz) was used as a primary antibody. HRP-linked mouse IgG (Amersham, England) was utilized as a secondary antibody. Specific bands were detected via enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and analyzed using an LAS3000 image analysis system (Fuji, Tokyo, Japan).

Competition ligand binding assay. [^{125}I]-[Nle4, D-Phe7]-α-melanocytes stimulating hormone (^{125}I-NDP-MSH) was purchased from Perkin-Elmer Life Sciences (Boston, MA). The assay system contained 20 mM HEPEs, 5 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml BSA, and 0.5 mM Na acetate (pH 7.4) in a final volume of 0.125 ml. Eighty micrograms of membrane protein were used for the ligand binding assay with ^{125}I-NDP-MSH (specific activity 81.4 TBq/mmol). For the competition experiments, unlabeled-NDP-MSH (Peptron, Korea) was used in a range of 10^{-12} to 10^{-6} M with 0.3 nM of ^{125}I-NDP-MSH. Non-specific binding was evaluated in the presence of 0.1 μM Agouti-related protein (AGRP) (83–132) amide (Phoenix Pharmaceuticals Inc., CA, USA). All binding data were analyzed with GraphPad Prism software, using a one-site binding model for a one-site competition model for the competition experiments.

Statistical analysis. Cellular responses to the various peptides utilized in this study were compared via one-way analysis of variance and Dunnett's *t*-test with Instat software (GraphPad Prism4).

Results and discussion

Binding and CRE-mediated reporter gene activity of mutant receptors

In order to assess the role of the third intracellular (i3) loop and to determine whether mutations in the i3 loop of MC4R elicit alterations in receptor function, we constructed seven mutant MC4R receptors, according to the reported human MC4R mutations in the i3 loop of MC4R, including A219V, I226T, P230L, G231S, G238D, N240S, and A244E [11–19] (Fig. 1, Table 1). Among these mutants, except G238D, most of mutants were previously examined for their cAMP production [11–19], however, our present study is a first systematic investigation by comparing these mutants and examining in terms of the role of the i3 loop of MC4R in the constitutive activity of MC4R involving G protein coupling. These mutant receptors were evaluated with regard to their abilities to bind melanocortin receptor-specific ligands, in addition to their abilities to transduce signals at the cAMP level.

All mutant receptors were determined to bind to [^{125}I]-NDP-MSH, thereby showing that they were all expressed on the plasma membrane. Table 2 summarizes the ligand affinities for MC4R and the seven mutant MC4 receptors. In general, no significant differences were observed with regard to the binding of [^{125}I]-NDP-MSH to the mutant receptors. These data show that the substitution of the third intracellular loop with these mutant sequences exerted no significant conformational influence on ligand binding domains.

Table 1
Identified variants in the third cytoplasmic loop of the melanocortin receptor 4 gene in obese and control subjects

Effect on amino acid sequence	Nucleotide substitution	Study group (BMI, kg/m^2)	Interpretation (references)
A219V	656C→T	Obese (33.3 ± 2.4)	Significant reduction in cAMP signal [11]
I226T	677T→C	Obese (40)	I226T did not differ from the wild-type MC4R in response to a, β, γ-MSH and AGRP [12]
P230L	689C→T	Obese children and adolescents (32.5 ± 6.3)	Elevated basal cAMP level, [13]
G231s	691G→A	Obese (44)	Decreased basal activity [14,15]
G238D	713G→A	Obese group	Not examined [16]
N240S	719A→G	Control (⩽30)	Partial decrease in maximal binding and had decreased basal activities [17,18]
A244E	731C→A	Obese children and adolescents	Decreased basal activity and decreased cAMP production [13,19]

The MC4R variants are listed according to their amino acid number relative to the N-terminal of the receptor. Nucleotide position is given relative to the A of the start codon.