Metal Ion-mediated Agonism and Agonist Enhancement in Melanocortin MC1 and MC4 Receptors

An endogenous metal-ion site in the melanocortin MC1 and MC4 receptors was characterized mainly in transiently transfected COS-7 cells. ZnCl₂ alone stimulated signaling through the Gₛ pathway with a potency of 11 and 13 µM and an efficacy of 50 and 20% of that of α-melanocortin stimulating hormone (α-MSH) in the MC1 and MC4 receptors, respectively. In the presence of peptide agonist, Zn(II) acted as an enhancer on both receptors, because it shifted the dose-response curves to the left: most pronounced was a 6-fold increase in α-MSH potency on the MC1 receptor. The effect of the metal ion appeared to be additive, because the maximal cAMP response for α-MSH in the presence of Zn(II) was 60% above the maximal response for the peptide alone. The affinity of Zn(II) could be increased through binding of the metal ion in complex with small hydrophobic chelators. The binding affinities and profiles were similar for a number of the 2,2'-bipyridine and 1,10-phenanthroline analogs in complex with Zn(II) in the MC1 and MC4 receptors. However, the potencies and efficacies of the metal-ion complexes were very different in the two receptors, and close to full agonism was obtained in the MC1 receptor. Metal ion-chelator complexes having antagonistic properties were also found. An initial attempt to map the metal-ion binding site in the MC1 receptor indicated that Cys²⁷¹ in extracellular loop 3 and possibly Asp¹³⁹ at the extracellular end of TM-III, which are both conserved among all MC receptors, are parts of the site. It is concluded that the function of the MC1 and MC4 receptors can be positively modulated by metal ions acting both as partial agonists and as potentiators for other agonists, including the endogenous peptide ligand α-MSH at Zn(II) concentrations that could be physiological. Furthermore, the metal ion-chelator complexes may serve as leads in the development of novel melanocortin receptor modulators.

The melanocortins, adrenocorticotropic hormone and α-, β-, and γ-melanocyte-stimulating hormone (MSH), are all derived from the precursor protein pro-opiomelanocortin. They are peptide hormones and neuropeptides, which exert their function through the five different members of the melanocortin receptor family, MC1 to MC5. These receptors are widely distributed both in the periphery and in the central nervous system. The physiological functions of the receptors cover a correspondingly diverse spectrum ranging from regulation of pigmentation (MC1 receptor), adrenal cortical steroidogenesis (MC2/adrenocorticotropic hormone receptor), and exocrine secretion (MC5 receptor) to energy homeostasis, appetite regulation, and penile erection (MC3 and -4 receptors) (1, 2). The focus on this receptor family has greatly increased following the discovery of the involvement of initially the MC4 receptor and later the MC3 receptors in appetite regulation and metabolic control (3–7). Mice with a genetically disrupted MC4 receptor display an obese phenotype characterized by excessively increased food intake and a decreased metabolic rate (4, 8). A similar phenomenon of pathological obesity is seen in humans with inactivating mutations of the MC4 receptor (9, 10). Both observations indicate that the MC4 receptor could be a useful target in the treatment of obesity and type II diabetes and, that agonists for this receptor would be needed. Importantly, intra-cerebrospinal injection of peptide agonists for the melanocortin receptors does reduce obesity in animal models, an effect that was reversible by co-injection of the corresponding antagonists (11). However, only very recently have the first high potency non-peptide agonists been described for the melanocortin receptors (12).

Metal ions are required for the function of numerous proteins. Zn(II) is known to serve as a part of the active site in, for example metalloenzymes, and to act as a stabilizer of protein structure, for example in the Zn(II) finger binding motif of transcription factors. More recently it has been discovered that zinc ions also bind with high affinity to and modulate the function of a number of membrane proteins, particularly in neural tissues. This has been shown to be the case for ion channels such as the glutamatergic N-methyl-d-aspartate receptor and the nicotinic acetylcholine receptor (13–15); for membrane transporters such as the dopaminergic and glutamatergic transporter (16, 17); and for 7TM receptors such as the tachykinin NK3 receptor, the β₂-adrenergic receptor, and the galanin receptor (18–20). Zn(II) is present in high concentrations in several regions of the brain, where it is stored in synaptic vesicles of the nerve terminals and is co-released with neurotransmitters in micromolar concentrations to the synaptic gap upon neuronal activation (21, 22).

In 7TM receptors both antagonist and agonist binding sites have been re-engineered into metal-ion sites, where free metal ions and metal ions in complex with small organic metal-ion chelators have been demonstrated to mimic the action of the antagonist or of the agonist, respectively (23–25). Because the geometry of metal-ion binding sites in general is very well characterized in respect of distance and angles, such metal-ion...
sites have provided important information about the structure and potential activation mechanism of 7TM receptors (23, 25–27) and recently of 12TM transporter molecules (16, 28). The antagonistic metal-ion sites provide important pieces of structural information due to the distance constraints such sites impose upon the molecular models of the 7TM receptors (27), for which only a single high-resolution x-ray structure of the inactive form is otherwise available (29). The few agonistic metal-ion sites, which as yet have been described, provide the first distance constraints in the active conformation of 7TM receptors. For example, introduction of a metal-ion binding site that constrained the third and the seventh transmembrane segment in a well-defined conformation results in 25% partial agonism (24, 25).

In the present study we find that both the MC1 receptor and the MC4 receptor possess endogenous metal-ion sites through which Zn(II) can function both as a partial agonist and as an enhancer or potentiator for the peptide agonists (Fig. 1). The high affinity of the metal-ion site suggests that the metal ion-induced modulation of the endogenous melanocortin receptor activity is of physiological relevance. Moreover, not only free Zn(II) but also the metal ion in complex with small organic, hydrophobic chelators could activate the MC receptors even with higher efficacy and potency than the free metal ion, suggesting a possible route for the design of novel modulating agents for this receptor family.

**EXPERIMENTAL PROCEDURES**

**Materials**—The peptides α-MSH and NDP-α-MSH were purchased from Peninsula (St. Helens, Merseyside, UK). The metal ion chelators 2,2'-bipyridine, 1,10-phenanthroline, 4,7-diphenyl-1,10-phenanthroline (1a), 5-phenyl-1,10-phenanthroline (1b), 5-chloro-1,10-phenanthroline (1c), 2,9-dimethanol-1,10-phenanthroline (1e), and 2,9-bis(trichloromethyl)-1,10-phenanthroline (1f) were all obtained from Sigma-Aldrich (St. Louis, MO). 5-Amino-1,10-phenanthroline was obtained from Polysciences Europe GmbH (Eppelheim, Germany), whereas the three 2,2'-bipyridine analogs were kindly donated from 7TM Pharma A/S (Copenhagen, Denmark). The Zn(II)-chelator complexes were prepared by dissolving the chelator compounds either in ethanol or in Me6SO and mixing with aqueous solutions of ZnCl2 to a final molar ratio of 3:1 in the case of 1,10-phenanthroline and 2,2'-bipyridine and 2:1 for the rest of the compounds.

**Molecular Biology**—The human MC4 receptor cDNA was cloned by PCR from brain cDNA library, whereas the mouse MC1 receptor was kindly provided by Dr. Roger Cone (Vollum Institute, Portland, OR). The cDNA were cloned into the eukaryotic expression vector pcDNA1 or pcDNA3 (Invitrogen, Carlsbad, CA). Mutations were constructed by
PCR using the overlap expression method (30). The PCR products were digested with appropriate restriction endonucleases, purified, and cloned into the pcDNA3. All PCR experiments were performed using Pfu polymerase (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. All mutations were verified by restriction endonuclease mapping and subsequent DNA sequence analysis using an ABI 310 automated sequencer.

**Transfections and Tissue Culture**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. The expression plasmids containing the cDNAs encoding the wild-type or the mutated receptors were transiently expressed after transfection according to the instructions of the manufacturer. The malignant melanoma cell line COLO 829 was grown in RPMI 1640 medium supplemented with 2 mM t-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum.

**Binding Assay**—One day after transfection, the cells were transferred and seeded into multwell plates for assay. The number of cells plated per well was chosen so as to obtain 5–10% binding of the radioligand added. Two days after transfection the cells were assayed in competition binding assays using 125I-NDP-α-MSH as a tracer. Radioligand was bound in a buffer composed of 0.5 ml of 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl₂, 5 mM MgCl₂, and 0.1% bovine serum albumin, and displaced in a dose-dependent manner by unlabeled ligands. The assay was performed in duplicate for 3 h at 25°C and stopped by washing twice in the buffer. Cell-associated, receptor-bound radioligands were determined by the addition of lysis buffer (48% urea, 2% Nonidet P-40 in 3 mM acetic acid). The concentration of radioligands in the assay corresponds to a final concentration of ~25 μM. The metal ion-chelating compounds were added in a 2-fold molar excess to ensure that no free metal ion was present.

**cAMP Assay**—COS-7 cells (2.5 × 10⁵ cells per well) or the melanoma cell line (2 × 10⁵ cells per well) were incubated for 24 h with 2 μCi of [3H]adenine (Amersham Biosciences, TRK 311) in 1 ml of medium. Cells were washed twice and incubated for 15 min at 37°C in 1 ml of freshly prepared binding buffer supplemented with 1 mM isobutylmethylxanthine (Sigma, 15879), 40 μg/ml bacitracin, and various concentrations of agonists or 50 μM forskolin. After incubation, cells were placed on ice, medium was removed, and cells were lysed with 1 ml of 5% (w/v) trichloroacetic acid, supplemented with 0.1 mM cAMP and 0.1 mM ATP for 30 min. The lysis mixtures were loaded onto a Dowex 50W-X4 (Bio-Rad, 142–1351) column (Bio-Rad, poly-prep columns, 731–1550), which was washed with 2 ml of water and placed onto the top of alumina columns (Sigma, A9003) and washed again with 10 ml of water. The columns were eluted with 6 ml of 0.1 M imidazole (Sigma, I0125) into 15 ml of scintillation fluid (Highsafe III). Columns were determined by the addition of lysis buffer (48% urea, 2% Nonidet P-40 in 3 mM acetic acid). The concentration of radioligands in the assay corresponds to a final concentration of ~25 μM. The metal ion-chelating compounds were added in a 2-fold molar excess to ensure that no free metal ion was present.

**Data Analysis**—IC₅₀ and EC₅₀ values were determined by non-linear regression using the Prism 3.0 software (GraphPad Software, San Diego, CA). Values of the dissociation and inhibition constants (Kᵢ and Kᵢ) were estimated from competition binding experiments using the equation, Kᵢ = IC₅₀ − L and Kᵢ = IC₅₀/(1 + [L/Kᵢ]), where L is the concentration of the radioactive ligand. For determination of statistical significance and correlation, the Spearman test was applied.

**RESULTS**

**Effect of Zn(II) on Ligand Binding**—Competition binding studies were performed for both the MC1 and MC4 receptors in transiently transfected COS7 cells using 125I-NDP-α-MSH as radioligand. Zn(II) displaced the 125I-NDP-α-MSH with an IC₅₀ value of 13 μM in the MC1 receptor and 19 μM in the MC4 receptor, corresponding to the affinity observed for example in bis-His metal-ion sites, previously engineered in 7TM receptors (Fig. 2 and Table 1) (23, 26). However, in the dose-response experiments Zn(II) only displaced ~50% of the maximally bound 125I-NDP-α-MSH, whereas the peptide ligands α-MSH and NDP-α-MSH displaced the radioligand fully, i.e. down to ~5% unspecific binding (Fig. 2). In receptors with artificially engineered metal-ion binding sites, for example in the NK1 receptor and the kappa opioid receptor, Zn(II) has previously been shown to fully displace the employed radioligands (23, 26, 27). In the MC1 and the MC4 receptors, Zn(II) in complex with, for example, the metal-ion chelator 1c (see below) also displaced the radioligand fully (Fig. 2). Cu(II) competed for radioligand binding with an affinity at 66 μM for the MC1 receptor and 93 μM for the MC4 receptor, i.e. in both cases ~5-fold lower affinity than Zn(II).

**Effect of Zn(II) on Signal Transduction**—In a melanoma cell line expressing the human MC1 receptor, Zn(II) stimulated cAMP production with an efficacy similar to that of MSH and with a potency of 79 μM. However, to ensure that the effect of Zn(II) was in fact mediated through the MC receptor, the further functional characterization was performed in transiently transfected COS7 cells (Fig. 3). Here, Zn(II) acted as a partial agonist both in cells transfected with the MC1 receptor and the MC4 receptor. On the MC1 receptor, Zn(II) had an efficacy of ~50% as compared with α-MSH (Fig. 4A), whereas on the MC4 receptor Zn(II) showed only 20% efficacy compared with α-MSH (Fig. 4B). The potency of Zn(II) was 11 and 13 μM for the MC1 and the MC4 receptors, respectively (EC₅₀ values), which corresponds to the affinity for Zn(II) determined in the competition binding experiments. In contrast, Cu(II) had no specific stimulatory effect on either the MC1 or the MC4 receptor. In mock transfected cells Zn(II) only showed a minimal stimulatory effect and only at very high concentration (Fig. 4B). In cells transfected with two other Gα₃-coupled receptors the β₂-adrenergic and the V2 receptor Zn(II) had no stimulatory effect.

According to basic pharmacological theory (32), a partial agonist should behave as an antagonist of the full agonist and dose-dependently bring the cAMP turnover down to the level observed with maximal occupancy of the partial agonist alone, i.e. Zn(II). However, the Zn(II)-mediated inhibition of the ago-
Metal Ion-mediated Agonism and Agonist Enhancement

**Table I**

Ligand affinities in the MC1 and MC4 receptors

| Ligand   | MC1 receptor | MC4 receptor |
|----------|--------------|--------------|
|          | $K_d$ (µM) ± S.E. | $K_i$ (nM) ± S.E. |
| α-MSH    | 0.0050 ± 0.0004 (6) | 0.0072 ± 0.001 (6) |
| NDP-α-MSH| 0.0001 ± 0.0001 (7) | 0.0045 ± 0.001 (7) |
| Zn(II)   | 13 ± 3 (7) | 19 ± 4 (7) |
| Zn[Phe]  | 24 ± 4 (3) | 33 ± 5 (3) |
| Zn[1a]   | 1.4 ± 0.2 (3) | 1.2 ± 0.1 (3) |
| Zn[1b]   | 1.2 ± 0.1 (3) | 1.4 ± 0.2 (3) |
| Zn[1c]   | 7.3 ± 0.3 (3) | 5.3 ± 1.0 (3) |
| Zn[1d]   | 9.7 ± 1.7 (3) | 6.9 ± 0.4 (3) |
| Zn[1e]   | 8.9 ± 2.5 (3) | 10 ± 3 (3) |
| Zn[1f]   | 20 ± 7 (3) | 12 ± 1 (3) |
| Zn[Dip]  | 22 ± 7 (3) | 22 ± 7 (3) |
| Zn[2a]   | 14 ± 1 (3) | 18 ± 5 (3) |
| Zn[2b]   | 7.0 ± 3.1 (3) | 9.5 ± 2.5 (3) |
| Zn[2c]   | 4.5 ± 1.1 (3) | 9.9 ± 4.1 (3) |

Fig. 3. Cyclic AMP accumulation in response to Zn(II) and α-MSH in a human melanoma cell line. Dose-response curves for α-MSH (circles) and Zn(II) (squares). Data are mean ± S.E. from five independent experiments. The $E_{max}$ is 4.7 fmol/10⁵ cells, and $EC_{50}$ is 7.5 nM for α-MSH; the $E_{max}$ is 5.2 fmol/10⁵ cells and $EC_{50}$ is 79 µM for Zn(II).

**Fig. 2** Cyclic AMP accumulation in response to Zn(II) and α-MSH in a human melanoma cell line. Dose-response curves for α-MSH (circles) and Zn(II) (squares). Data are mean ± S.E. from five independent experiments. The $E_{max}$ is 4.7 fmol/10⁵ cells, and $EC_{50}$ is 7.5 nM for α-MSH; the $E_{max}$ is 5.2 fmol/10⁵ cells and $EC_{50}$ is 79 µM for Zn(II).

n-stimulated cAMP accumulation was in fact biphasic. As shown in Fig. 4C, Zn(II) in concentrations from 1 to 10 µM inhibited the α-MSH-induced cAMP stimulation on the MC1 receptor down to ~60% of the α-MSH response. However, at higher concentrations of zinc ions an increase in the cAMP accumulation to ~160% of the maximal α-MSH response was observed. In the MC4 receptor a similar biphasic pattern was observed, although the inhibitory component was more pronounced, because Zn(II) in concentrations from 1 to 10 µM inhibited NPD-α-MSH (α-MSH has a potency at 350 nM on the MC4 receptor in COS-7 cells, therefore, the more potent agonist NDP-α-MSH was used on the MC4 receptor)-induced cAMP production down to ~35% (Fig. 4D). As in the MC1 receptor, at higher concentrations the inhibitory effect of the metal ion apparently disappeared, and from 10 to 100 µM Zn(II) the cAMP accumulation was increased to the level observed with NPD-α-MSH alone (Fig. 4D). These experiments indicate that Zn(II) functions not only as a partial agonist on the MC1 and MC4 receptors but apparently also as a potentiator of the agonistic function of the peptide agonists, α-MSH and NDP-α-MSH. This was directly studied by performing dose-response experiments with α-MSH/NDP-α-MSH in the presence and absence of Zn(II). Addition of a constant concentration of Zn(II) (10⁻⁴ M) shifted the dose-response curve for α-MSH and NDP-α-MSH to the left in the MC1 and the MC4 receptors, respectively, indicating that Zn(II) does act as an enhancer or potentiator of the peptide agonist (Fig. 5). The enhancing effect of Zn(II) was most pronounced in the MC1 receptor, where the α-MSH potency ($EC_{50}$ in respect of cAMP stimulation) without Zn(II) was 116 nM, and in the presence of zinc (10⁻⁴ M) it was increased to 20 nM (Fig. 5A). Zn(II) at 10⁻³ M gave ~50% of the maximal α-MSH-induced stimulation; however, as shown in Fig. 4 the combination of Zn(II) and α-MSH gave a supra-maximal, additive response, because the dose-response curve for α-MSH was basically shifted upward in the presence of Zn(II) (Fig. 5A). A similar, but smaller, enhancing and additive effect of Zn(II) on the α-MSH stimulation of cAMP accumulation was observed in the MC4 receptor; however, instead of a 6-fold shift in potency only a 2-fold shift of the dose-response curve for α-MSH from 1.1 to 0.6 nM was observed (Fig. 5B).

The Effect of Metal Ion Chelator Complexes on the Melanocortin Receptors—Previously we have found that metal ions may bind to metal-ion sites in receptors in complex with certain small organic, hydrophobic metal-ion chelators (24, 25). In the melanocortin receptors, Zn(II) in complex with 1,10-phenanthroline and with 2,2'-bipyridine analogs approximately the same affinity as the Zn(II)-1,10-phenanthroline. Only the compound 1f of the tested 1,10-phenanthroline analogs in complex with Zn(II) showed a 2- to 20-fold increase in affinity compared with the unmodified Zn(II)-1,10-phenanthroline. Only the compound 1f where the 1,10-phenanthroline was substituted with trichloromethyl groups in positions 2 and 9, i.e. in the position next to the free metal ion, i.e. with $K_d$ values of 24 and 22 µM, respectively, at the MC1 receptor (Table I). However, in contrast to the free metal ion, the complex between the chelator and the metal ions inhibited the radioligand as efficiently as the peptide agonists did, i.e. down to the unspecific level (Fig. 2).

Six 1,10-phenanthroline analogs and three 2,2'-bipyridine analogs were tested for their ability to bind to and activate both the MC1 and the MC4 receptors (Tables I and II). In general, the tested 1,10-phenanthroline analogs in complex with Zn(II) showed a 2- to 20-fold increase in affinity compared with the unmodified Zn(II)-1,10-phenanthroline. Only the compound 1f, where the 1,10-phenanthroline was substituted with trichloromethyl groups in positions 2 and 9, i.e. in the position next to the metal ion chelating nitrogen, bound the MC1 receptor with approximately the same affinity as the Zn(II)-1,10-phenanthroline or free Zn(II). Similarly, most of the 2,2'-bipyridine analogs in complex with zinc had an increased affinity for the MC1 and MC4 receptor compared with Zn(II)-2,2'-bipyridine (Table I). Interestingly, the two melanocortin receptors had a very similar pharmacological profile for the metal ion-chelator complexes when tested in competition binding analysis. That is, the affinity of the different metal ion-chelator complexes to the MC1 and the MC4 receptors correlated closely, with an $r$ value of 0.89 ($p < 0.0001$, Spearman test). This could indicate that the metal-ion site is conserved among the MC receptors.
Zn(II)-1,10-phenanthroline and Zn(II)-2,2'-bipyridine as well as the analogs were tested for their ability to activate the receptors (Table II). In contrast to the findings in the competition binding experiments, the MC1 and MC4 receptors exhibited very different pharmacological profiles for the metal ion chelator compounds when tested in the functional assay. Thus, despite the fact that the metal ion-chelator complexes bound with very similar affinity in the two receptors, seven out of the eleven complexes acted as agonists on the MC1 receptor but only two on the MC4 receptor. Furthermore, the most efficacious complex on the MC1 had no effect on the MC4 receptor. The molecular structure of the chelators was important for the degree of activation they induced on the MC1 receptor. Zn(II)-1,10-phenanthroline and Zn(II)-2,2'-dipyridine had a lower efficacy than the free zinc ion (Fig. 6A and Table II). However, when these simple chelators were chemically modified, higher efficacies could be obtained. One of the compounds, 1c (Zn-(5-chloro-1,10-phenanthroline), was not only 2-fold more potent than the free metal ion but also had a higher efficacy reaching 167% of the maximally Zn(II)-induced cAMP production (Fig. 6A). Other Zn(II)-chelator complexes, 1d (Zn-(5-amino-1,10-phenanthroline), and the three bipyridine derivatives activated the MC1 receptor with an efficacy slightly lower than zinc but better than the chelator scaffolds without substitutions.

On the MC4 receptor, where the free Zn(II) was only a 20% partial agonist, none of the metal-ion complexes had a higher efficacy than free Zn(II). However, two of the 2,2'-bipyridine compounds, 2b and 2c, stimulated the receptor with a higher potency than the free zinc ion and with appreciable efficacies of 62 and 53% that of the free metal ion (Fig. 6B).

The compounds that bound to the melanocortin receptors but did not activate the receptors would be expected to function as antagonists. The dose-response curve on the MC1 receptor for one of these, compound 1b, is shown in Fig. 7. As expected the metal ion-chelator complex inhibited the α-MSH-stimulated cAMP production with a potency of 1.1 μM, which is similar to the affinity of the compound determined in the binding assays. For comparison, the biphasic inhibition stimulation curve for the free Zn(II) is also shown.

**Attempt to Locate the Metal-ion Binding Site in the MC1 Receptor**—Fourteen potential metal binding residues (cysteines, histidines, and acidic residues), located in the extracellular part of the transmembrane segments, in the extracellular loops, or in the membrane-close part of the N-terminal extension of the MC1 receptor, were substituted with a residue without metal ion-coordinating properties, i.e. either with an alanine or with another, more structurally conservative substitution (Fig. 1). Dose-response curves with respect to stimulation of cAMP production were performed for both Zn(II) and α-MSH in each of the mutant receptors, and the EC50 values for the two agonists are listed in Table III.

Most of the mutations affected neither the zinc-induced nor the α-MSH-induced stimulation of cAMP production. In two cases, Glu92 and Glu100, the Ala substitutions were not tolerated, but substitution with the structurally more similar but non-metal binding Gln residue revealed no or only minor effect on the potency of the two agonists (Table III). Substitution of His258 located on the close interface between TM-VI and TM-VII eliminated both α-MSH and Zn(II) stimulation, conceivably due to misfolding of the receptor.

Cys residues located in the extracellular part of 7TM receptors are frequently but not always involved in the forma-
tion of structurally stabilizing disulfide bridges (33). In the MC receptors, one Cys residue is located in the N-terminal segment close to the start of TM-I, and surprisingly even three Cys residues are found in the extracellular loop 3. Ala substitution of Cys38 in the N-terminal segment affected neither α-MSH nor Zn(II) stimulation of the receptor, indicating that this Cys residue is not involved in disulfide bridge formation (Fig. 8 and Table III) or that such a bridge is not important for agonist stimulation of the receptor. In contrast, substitution of Cys265 and Cys273 located in extracellular loop 3, in both cases eliminated both α-MSH and Zn(II) stimulation of the mutant receptors, indicating that these Cys residues are important for the structure and function of the receptor, for example by forming an intra-loop disulfide bridge (Table III). Interestingly, Ala substitution of the last cysteine residue in extracellular loop 3, Cys271, gave a molecular phenotype, where the α-MSH stimulation of cAMP production was almost identical to that observed in the wild-type MC1 receptor, whereas Zn(II) stimulation was eliminated (Fig. 8 and Table III). This indicates that Cys271 is part of the agonistic metal-ion site in the MC1 receptor but not directly involved in peptide agonist binding.

The inner face of TM-III is an important interaction area for agonists in rhodopsin-like 7TM receptors in general. In the MC receptors, three potential metal-binding residues are found in this location: Asp115, Asp119, and Cys123. Substitution of Asp115 and Cys123 had only a minor effect on α-MSH and Zn(II) stimulation of the receptor (Table III). In contrast, both Ala and Asn substitution of Asp119 eliminated not only α-MSH stimulation, which is in agreement with reports in the literature (34, 35), but also Zn(II) stimulation (Table III). Although it cannot be directly demonstrated here, it is possible that Asp119 could be important not only for α-MSH binding and action but also for the binding and action of Zn(II).

DISCUSSION

In the present study activation of both the MC1 and the MC4 melanocortin receptors was achieved by zinc ions both alone and in complex with small hydrophobic metal ion-chelating compounds. The free metal ion was able to induce 20 and 50% partial agonism as compared with α-MSH in the MC4 and the MC1 receptors, respectively. Furthermore, it was found that Zn(II) modulates the α-MSH function to increase both its potency and efficacy at concentrations, which indicates a possible physiological importance especially in the CNS. The observation that Zn(II) binds and stimulates two different melanocortin receptors suggests that this could be a common feature among all members of this subfamily of rhodopsin-like 7TM receptors. This notion is supported by the similar pharmacological profile observed in the MC1 and MC4 receptors with respect to Zn(II) being both a partial agonist and a potentiator of α-MSH action and by the similar affinity profiles for a series of different metal ion-chelator complexes in the two different receptors.

Attempt to Map the Activating Metal-ion Site—Our mutational analysis of fourteen potential metal-ion binding residues in the MC1 receptor identified only a single Cys residue in the loop between TM-VI and TM-VII as being part of the activating metal-ion site. That is, the mutant receptor responded normally to α-MSH but not at all to Zn(II) (Fig. 8). The potency of

![Figure 5. Zn(II) modulation of agonist dose-response curves in the MC1 and MC4 receptors.](image)

**Table II**

| Efficacy | MC1 receptor | | | MC4 receptor | | |
|---|---|---|---|---|---|---|
| | % of max Zn(II) ± SEM | EC50 (μM) | (n) | % of max Zn(II) ± SEM | EC50 (μM) | (n) |
| Zn | 100 ± 20 | 11 ± 4 | (7) | 100 ± 30 | 13 ± 4 | (8) |
| Zn[Phe] | 43 ± 15 | 12 ± 3 | (3) | NS | NS | (3) |
| Zn[Asp] | NS | 2 | (2) | NS | NS | (2) |
| Zn[Cys] | 167 ± 40 | 8.1 ± 1.5 | (4) | NS | NS | (3) |
| Zn[Cys] | 60 ± 15 | NS | (3) | NS | NS | (2) |
| Zn[Cys] | NS | (3) | NS | NS | (2) |
| Zn[Cys] | 30 ± 10 | 82 ± 13 | (3) | NS | NS | (2) |
| Zn[Cys] | 65 ± 12 | NS | (3) | NS | NS | (2) |
| Zn[Cys] | 78 ± 11 | 62 ± 15 | (3) | NS | 2.7 ± 0.8 | (3) |
| Zn[Cys] | 87 ± 19 | 53 ± 12 | (3) | NS | NS | (2) |

* The chelator compounds without zinc did not stimulate the receptor.

* NS, no stimulation.

---

**Fig. 5.** Zn(II) modulation of agonist dose-response curves in the MC1 and MC4 receptors. A. α-MSH-induced cAMP accumulation for the MC1 receptor in the presence (circles) and absence (squares) of Zn(II) at a concentration of 10⁻⁴ M. B. NDP-α-MSH-induced cAMP accumulation in the presence and absence of Zn(II) at a concentration of 10⁻⁴ M. The experiments in each panel were performed in parallel in transiently transfected COS-7 cells. Data are mean ± S.E. from four independent experiments made in duplicate.
Zn(II) in the wild-type MC receptors and our general experience with metal-ion site engineering in 7TM receptors would suggest that the metal ion binds in a bidentate or perhaps even a tridentate metal-ion site (23, 26). Thus, it is most likely that there is at least one more residue involved in the coordination of the zinc ion. Substitution of four other possible metal ion-coordinating residues impaired not only the zinc-induced activation but also the \(^{\text{H9251}}\)-MSH-induced receptor activation. One or more of these residues could potentially be part of the activating metal-ion site, besides being important for the \(^{\text{H9251}}\)-MSH action. Among these residues, we find Asp119 in TM-III (AspIII:05) to be the most attractive candidate for having such a dual role. The inner face of the extracellular end of TM-III is a classic location for agonist interactions in rhodopsin-like 7TM receptors, with the important amine-binding AspIII:08 of, for example, the monoamine and opioid receptors being perhaps the most well characterized case (36, 37). In the melanocortin receptors, AspIII:05 (Asp119 in the MC1 receptor) has previously been described as a crucial interaction point for the binding and function of peptide agonists (34, 35). The carboxylic group of the aspartic acid has been suggested to form a salt bridge with the functionally important Arg6 of the melanocortin peptide ligands. In the present study we can confirm that AspIII:05 is important for peptide agonist function, in this case in the MC1 receptor.

In a scenario where Zn(II) would bind between Asp119 at the extracellular end of TM-III and Cys271 in extracellular loop 3 and thereby activate the receptor, it would be envisioned that part of the loop would bend down toward Asp119 at position III:05. In this connection it is important to note that especially in the MC receptors this is a likely possibility. The reason being that extracellular loop 2, which in nearly all other 7TM receptors is rather long and occupies the space between TM-III, -V, and VI, because it is connected to the top of TM-III by a disulfide bridge (to CysIII:01), is in fact not present in the melanocortin receptors, because TM-IV basically continues directly into TM-V with a "mini-loop" consisting of only one or two residues (Fig. 1). Thus, the inner face of the extracellular end of TM-III, including AspIII:05, which in the x-ray structure of rhodopsin is covered by layers of \(^{\text{H9252}}\)-strands from extracellular loop 2, is freely exposed in the MC receptors to interact with extracellular ligands and, for example, with residues such as Cys271 in extracellular loop 3 through an intercalated metal ion. Extracellular loop 3 obviously connects TM-VI and -VII, and much evidence, including EPR spectroscopy in combination with site-directed spin labeling, fluorescence spectroscopy, and metal-ion site engineering, suggests that interchanges in the movement between TM-III, -VI, and -VII are the most crucial conformational changes involved in activation of 7TM receptors (25, 38–41). The fact that mutational substitution of the two other cysteines in extracellular loop 3 leads to impairment of both \(^{\text{H9251}}\)-MSH and Zn(II) stimulation is here interpreted as an indication that these residues probably are involved in a structurally important disulfide bridge. This is in agreement with the occurrence of natural loss of function mutations of these two Cys residues in the MC4 receptor leading to obesity (42). However, we cannot rule out the possibility that these

---

**FIG. 6.** cAMP accumulation induced by metal ion chelators in complex with Zn(II) in the MC1 and MC4 receptors. A, cAMP accumulation induced by ZnII (squares) and ZnII in complex with the metal-ion chelators, 1,10-phenanthroline (triangles), and the 1,10-phenanthroline analog 1c (circles) in the MC1 receptor. B, cAMP accumulation induced by ZnII (squares) and ZnII in complex with the 2b analog of 2,2'-bipyridine (circles) in the MC4 receptor. The experiments were performed in transiently transfected COS-7 cells. Data are mean ± S.E. for three to seven independent experiments made in duplicate. C, structures of a number of metal ion chelators for which their ability to bind to and stimulate the MC1 and/or the MC4 receptor is shown in A and B or in Tables I and II.

**FIG. 7.** Antagonistic properties of a metal-ion chelator in complex with Zn(II) in the MC1 receptor. Inhibition of the \(^{\text{H9251}}\)-MSH-induced cAMP accumulation by compound 1b in complex with ZnII in transiently transfected COS-7 cells. Data are mean ± S.E. from three to seven independent experiments made in duplicate. The complex, biphasic inhibition/enhancement curve for the free metal ion, ZnII, is shown for comparison.
residues instead are involved more directly in both peptide and metal-ion binding. However, it would be most unusual to have three freely exposed thiol-containing side chains in close proximity in an extracellular loop.

**A Possible Physiological Relevance of Zn(II) Modulation on Melanocortin Receptors**—The melanocortin system is unique among T7M receptors in the sense that MC receptor activity is regulated by two endogenous peptides with opposing activities: the pro-opiomelanocortin-derived melanocortins, and, on the other hand, the Agouti protein and Agouti-related peptide, which act as antagonists or inverse agonists on MC receptors (43, 44). The present data suggest that endogenous zinc ions could be a third player in the regulation of the melanocortin receptor activity. On its own, Zn(II) acts as a partial agonist of receptors and overall difficulty in working with these cells prevented us from further characterization of the metal-ion effect in this system. At higher concentrations Zn(II) modulates the α-MSH-induced receptor stimulation as an enhancer or potentiator, which is evident both in the lack of total displacement of the peptide ligands by the free metal ions (Fig. 2) and in the functional potentiating effect (Fig. 5). In this connection it should be noted that in the brain Zn(II) is found stored in synaptic vesicles and can be co-released with the neurotransmitter to reach concentrations of up to 300 μM in the synaptic cleft (45). Vesicular zinc is found also in the hypothalamus, and its staining pattern suggests an overlap with the MC4 receptors (46–50).

It may be relevant that mice lacking metallothioneins I and II exhibit a phenotype characterized by obesity, high food intake, and increased levels of leptin (48). A similar complex of symptoms is observed in the MC4 knock-out mice. In this case, however, the symptoms are much more pronounced. Metallothioneins are proteins serving to protect the body from toxic effect of heavy metals by sequestering an excess level of the metal ions (49). Whether there is a direct connection between metallothionein and MC4 receptor, apart from the fact that they are both zinc binding and involved in obesity, is yet unclear.

It is suggested in the literature that zinc is able to modify the immune system (50). Likewise, it is well described that α-MSH may affect the immune system through the MC1 receptors (51); however, it is not possible to conclude that zinc under physiological conditions modulates the MC1 receptor function.

**Metal Ion-Chelator Complexes as Leads for Novel Melanocortin Receptor Modulators**—Several of the MC receptors are highly interesting as potential drug targets, especially in the control of food intake but also, for example, for the treatment of erectile dysfunction (6, 52). More or less selective, high affinity peptide ligands have been developed for all of the MC receptors (53). Importantly, however, based on the cyclic, non-selective oligopeptide melanotin II, Patchett and coworkers (12) have recently been able to develop selective, nanomolar affinity non-peptide compounds, which are full agonists on the human MC4 receptor. It is likely that these compounds will lead to the discovery of non-peptide ligands for other MC receptors as well.

The activating metal-ion site of the melanocortin receptors described in the present study could possibly be used as an anchor site for the development of a novel class of compounds, i.e. metal ion chelators, that can modulate the function of these receptors. Although the metal-ion affinity and the binding profiles of the site in the MC1 and MC4 receptors are very similar, it was possible to obtain not only selectivity but also close to full agonism through only minor chemical modifications of the chelator. Most interestingly, binding of the metal ion potentiates the binding and function of the endogenous peptide agonist on the MC receptors. Such an effect can be achieved through a

---

**Table III**

| Construct Position | EC50 (nM) | α-MSH (nM) |
|--------------------|-----------|------------|
| mMC1-wt N-terminal | 11 ± 2 (7) | 90 ± 7 (7) |
| Glu90Ala N-terminal | 16 ± 4 (5) | 87 ± 21 (5) |
| Cys273Ala N-terminal | 31 ± 9 (3) | 110 ± 11 (3) |
| Glu92Ala II:20 | NS | NS |
| Glu92Gln II:20 | 37 ± 15 (3) | 450 ± 90 (3) |
| Glu100Ala ec1 | NS | NS |
| Glu100Gln ec1 | 32 ± 7 (3) | 320 ± 80 (3) |
| Asp115Ala III:01 | 18 ± 4 (3) | 400 ± 21 (3) |
| Asp115Asn III:01 | 19 ± 5 (3) | 540 ± 30 (3) |
| Asp119Ala III:05 | NS | NS |
| Asp119Asn III:05 | NS | NS |
| Cys123Ser III:09 | 12 ± 1 (3) | 330 ± 70 (3) |
| His183Ala V:02 | 14 ± 6 (3) | 58 ± 20 (3) |
| Cys189Ala V:08 | 15 ± 4 (4) | 88 ± 21 (4) |
| His258Ala VI:19 | NS | NS |
| Cys265Ala ec1 | NS | NS |
| His268Ala ec1 | 23 ± 4 (3) | 110 ± 20 (3) |
| Cys271Ala ec1 | NS ± 4 (5) | 356 ± 90 (5) |
| Cys273Ala ec1 | NS | NS |

* NS, no stimulation.
direct modification of the state of the receptor by the potentiating compound, for example, through stabilization of a conformation of the receptor with an increased tendency to be bound to the G protein, which is essential for high affinity agonist binding (54, 55). In the case of the melanocortin receptors, it is even possible that the enhancement is obtained through binding of the metal ion in between the receptor and the peptide, because the ligands all have a metal-ion binding imidazole side chain, which is part of the core His-Phe-Arg-Trp sequence. Whatever the molecular mechanism may be, compounds that can increase the potency of the endogenous ligand and that even have additive effects leading to supra-maximal stimulation could possibly be developed into interesting drug candidates.

Acknowledgments—We thank Susanne Hummelgaard, Mette Simons, and Heidi Pedersen for expert technical assistance.

REFERENCES

1. Wikberg, J. E. (1999) Eur. J. Pharmacol. 375, 295–310
2. Marks, D. L., and Cone, R. D. (2001) Recent Prog. Horm. Res. 56, 359–375
3. Robinson, S. W., Dinulescu, D. M., and Cone, R. D. (2000) Annu. Rev. Genet. 34, 687–745
4. Huszar, D., Brenchley, L. E., Wu, G., Kesterson, R. A., Boston, B. A., Covey, R. D., Smith, F. J., Campfield, I. A., Burn, P., and Lee, F. (1997) Cell 88, 131–141
5. Chen, A. S., Marsh, D. J., Brantjes, H. E., Frazier, E. G., Guan, X. M., Yu, H., Rosenblum, C. I., Vangs, A. P., Cao, L., Metzger, J. M., Strack, A. M., Camacho, R. E., Melkin, T. N., Nunez, C. N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D. E., Chen, H. Y., and Van Der Ploeg, L. H. (2000) Nat. Genet. 26, 97–102
6. Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J., and Baskin, D. G. (2000) Nat. Rev. Neurosci. 1, 673–685
7. Marsh, D. J., Hollister, J., Huszar, D., Lauf, R., Yagaloff, K. A., Fisher, S. L., Burn, P., and Palmer, R. D. (1999) Nat. Genet. 21, 119–122
8. Ste, M. L., Minna, G. I., Marsh, D. J., Yagaloff, K., and Palmer, R. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12339–12344
9. Farooqi, I. S., Ye, G. S., Reigh, J. M., Amimian, S., Jebb, S. A., Butler, G., Cheetham, T., and O’Rahilly, S. (2000) J. Clin. Invest. 106, 271–279
10. Vaisse, C., Clement, K., Durand, E., Herberg, S., Guy-Grand, B., and Frouk, P. (2000) J. Clin. Invest. 106, 253–262
11. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997) Nature 385, 165–168
12. Van der Ploeg, L. H., Martin, W. J., Howard, A. D., Nargund, R. P., Austin, C. P., Guan, X., Drisko, J., Cashen, D., Sebhat, I., Petchett, A. A., Figueroa, D. J., DiLella, A. G., Connolly, B. M., Weinberg, D. H., Tan, C. P., Palyha, C. P., Guan, X., Drisko, J., Cashen, D., Sebhat, I., Patchett, A. A., and Birdsall, N. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7893–7898
13. Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Cell 86, 943–954
14. Yang, Y. K., Kong, T. M., Dickinson, C. J., Mao, C., Li, J. Y., Tota, M. R., Musley, R., Van Der Ploeg, L. H., and Gantz, I. (2000) Biochemistry 39, 14900–14911
15. Yang, Y., Dickinson, C., and O’Rahilly, S. (1997) J. Biol. Chem. 272, 23000–23010
16. Suratt, C. F., Johnson, P. S., Morikawa, A., Seidell, B. K., Blaschak, C. J., Wang, J. B., and Uhl, G. R. (1998) J. Biol. Chem. 273, 26548–26553
17. Strader, C. D., Gaffney, T., Sugg, E. E., Candelore, M. R., Keys, R., Patchett, A. A., and Dixon, R. A. F. (1991) J. Biol. Chem. 266, 5–8
18. Farrar, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Rhorana, H. G. (1996) Science 274, 768–770
19. Gether, U., Lin, S., Ghannouni, P., Ballesteros, J. A., Weinstein, H., and Kohlb, B. K. (1997) EMBO J. 16, 6737–6747
20. Sheik, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Cell 86, 943–954
21. Barsh, G. S., Paroogi, I. S., and O’Rahilly, S. (2000) Nat. Rev. 444–645
22. Dinulescu, D. M., and Cone, R. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6695–6698
23. He, L., Gunn, T. M., Bouley, D. M., Lo, X. Y., Watson, S. J., Schlossman, S. F., and Duke-Cohan, S. J., and Barsh, G. S. (2000) Nat. Genet. 27, 40–47
24. Huang, E. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13386–13387
25. Perez-Castejon, C., Vera-Gil, A., Barral, M. J., Perez-Castejon, M. J., and Lahoz, M. (1994) Histol. Histopathol. 9, 259–262
26. Cowley, M. A., Panchou, X., Fan, W., Dinulescu, D. M., Colmers, W. P., and Cone, R. D. (1999) Neuron 29, 155–163
27. Beattie, J. H., Wood, A. M., Newmann, A. M., Brenner, I., Chou, K. H., Michalska, A. E., Duncan, J. S., and Trayhurn, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 358–363
28. Palmiter, R. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8428–8430
29. Rink, L. and Gabriel, P. (2001) Biometals 14, 367–383
30. Luger, T. A., Brusa, T., Schoken, T. E., Kelden, D. H., Sunderkotter, C., Armstrong, C., and Ansel, J. (2000) Ann. N. Y. Acad. Sci. 917, 232–238
31. Wessells, H., Fuciarelli, K., Hansen, J., Hadley, M. E., Hruby, V. J., Dorr, R., and Levine, N. (1998) J. Urol. 160, 399–393
32. Hadley, M. E., and Haskell-Luevano, C. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 689–694
33. Schwartz, T. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13232–13237
34. Thirstrup, R., Elling, C. E., Hjorth, S. A., and Schwartz, T. W. (1996) J. Biol. Chem. 271, 7875–7878
35. Schwartz, M. W., Woods, S. C., Porte, D., Jr., Seeley, R. J., and Baskin, D. G. (2000) Nature 404, 661–671
36. Marsh, D. J., Hollister, J., Huszar, D., Lauf, R., Yagaloff, K. A., Fisher, S. L., Burn, P., and Palmer, R. D. (1999) Nat. Genet. 21, 119–122
37. Schwartz, T. W. (1994) Biochemistry 33, 661–675
38. Lazareno, S., Popham, A., and Birdsall, N. J. (2000) Mol. Pharmacol. 57, 436–445
39. Schwartz, T. W. (1994) Curr. Opin. Biotech. 5, 434–444