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Differential Effects of Melanocortin Peptides on Neural Melanocortin Receptors

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SUMMARY

Melanocortins (MCs) have various physiological actions on the brain. The recent cloning of neural MC receptors opened new avenues to study the effects of these neuropeptides on the nervous system. Here we investigated the structure-activity relationships (SARs) of peptides derived from adrenocorticotropic hormone (ACTH) with cloned MC3 and MC4 receptors *in vitro* and correlated these with central effects of MCs *in vivo*. Analysis of the effects of various MC peptides on cAMP accumulation in and binding to cells that expressed either the rat MC3 receptor or the human MC4 receptor demonstrated that ACTH-4–9-NH_2 was the core sequence of ACTH able to activate these receptors. Furthermore, γ-melanocyte-stimulating hormone (MSH) displayed selectivity for the MC3 receptor, whereas [D-Phe^7]ACTH-4–10 more efficiently activated the MC4 receptor than the MC3 receptor. The activities of MC fragments that lacked the three carboxyl-terminal amino acids (residues 11–13) of ACTH-1–13 were much lower than that of α-MSH, for both receptors. Furthermore, the three amino-terminal amino acids (residues 1–3) of α-MSH were more important for full activation of the MC4 receptor, compared with the MC3 receptor. The SAR for the MC4 receptor resembled that for the induction of excessive grooming behavior by MC peptides. Therefore, we suggest that this behavioral response is mediated by MC4 receptors. The SAR for the MC3 receptor did not overlap with that for *in vivo* effects of MCs. ORG2766, an ACTH-4–9 analog that is very potent in an active avoidance task, did not activate, antagonize, or bind to the MC3 and MC4 receptors. This suggests the presence of still other MC receptors, in addition to the MC3 and MC4 receptors, in the brain. These data identify peptides with selectivity for either the MC3 receptor or the MC4 receptor, which may be used for development of novel MC receptor-specific ligands. Furthermore, this is the first report that discusses behavioral effects of MCs in light of data on cloned MC receptors.

The recent cloning of MC receptors that are expressed in brain has opened new avenues for the study of behavioral, neurochemical, and neurotrophic effects of MC peptides (1–9). More than 30 years ago it was recognized that ACTH and fragments of ACTH had effects on behavior that were independent of the endocrine effects of ACTH (10, 11). Even ACTH fragments like ACTH-4–10 and analogs of ACTH-4–10 and ACTH-4–9 were shown to have potent effects on the facilitation of active avoidance behavior, whereas these peptides had no effect on the adrenal gland, for which ACTH is the most potent peptide (12, 13). This suggested that MC receptors in brain differed from those in the adrenal cortex. This was also indicated by the difference in processing of the pro-opiomelanocortin peptide precursor in the brain, where the majority of ACTH is cleaved to form α-MSH, compared with processing in the anterior lobe of the pituitary gland, where ACTH is formed (14).

Not only could brain MC receptors be distinguished from the adrenal MC (ACTH) receptor, but pharmacological studies further indicated the existence of multiple receptor subtypes in the brain (12, 15). The structural requirements for ACTH fragments and ACTH analogs to influence different behavioral responses were markedly different (12, 15). For instance, ACTH-4–10 stimulated the facilitation of retention of active avoidance behavior, whereas it was inactive in inducing excessive grooming behavior (16). Surprisingly, substitution of phenylalanine at position 7 in ACTH-4–10 by its D-enantiomer generated a peptide that could induce excessive grooming behavior (16), whereas this peptide had an effect opposite to that of ACTH-4–10 on active avoidance behavior (12, 17). Furthermore, effects of ACTH fragments and ACTH peptides in these behavioral assays could be discriminated pharmacologically from effects of ACTH-4–10 on blood pressure and heart rate (18) and from effects of α-MSH on nerve regeneration (19, 20), as well as from stimulatory effects of intracerebroventricularly administered α-MSH on plasma corticosterone levels (16).

To date, five MC receptors have been cloned, i.e., the melanocyte MSH receptor (MC1 receptor), the adrenal ACTH recep-

ABBREVIATIONS: MC, melanocortin; ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone; NDP-MSH, [Nle^4,D-Phe^7]-α-melanocyte-stimulating hormone; HBSS+, Hanks' balanced salt solution supplemented with 2 mM $CaCl_2$ and 0.1 mM isobutylmethylxanthine; SAR, structure-activity relationship; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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tor (MC2 receptor), the brain MC3 and MC4 receptors, and the MC5 receptor (1–8). These MC receptors are highly homologous to each other and form a subclass within the G protein-coupled receptor superfamily. They all couple to adenylate cyclase. *In situ* hybridization analysis with antisense RNA probes demonstrated that the MC3 receptor is predominantly expressed in hypothalamus, whereas the MC4 receptor has a more widespread expression in brain (3–6). The expression pattern of the MC5 receptor has not yet been mapped extensively. The distribution of MC3 and MC4 receptor transcripts correlates well with the localization of binding sites for iodinated NDP-MSH in brain that has been determined previously (21, 22).

SARs for central effects of ACTH, ACTH fragments, and ACTH analogs in various bioassays can now be compared with the SARs for the cloned receptors. Here we describe the effect of these peptides on cAMP formation and displacement of ACTH binding in cell lines that stably express either the MC3 receptor or the MC4 receptor, and we discuss the correlates between SARs of central effects and cloned receptors present in the brain.

Materials and Methods

Peptides. All peptides used were synthesized and kindly provided by Organon International (Oss, The Netherlands), except for γ-MSH (Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly), which was purchased from Bachem (Feinchemikalien, Bubendorf, Switzerland). α-MSH is *N*-acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val. The ACTH fragments used do not have the acetylated amino terminus and amidated carboxyl terminus as in α-MSH, except for ACTH-4–9-NH_2, which has an amidated carboxyl terminus. ORG2766 is Met$(O)_2$-Glu-His-Phe-D-Lys-Phe. Peptides were dissolved and diluted in Hanks' balanced salt solution.

Cells and transfection. HEK 293 cells, grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were transfected with 10 μg of pcDNA1/MC4 (6), using the calcium phosphate precipitation method described previously (23). Stably expressing clones of cells were selected in G418-supplemented medium for 4 weeks. The expression of the human MC4 receptor was confirmed by measurement of the cAMP content of cells treated for 30 min with 1 μM α-MSH, compared with nontransfected cells. HEK 293 cells expressing the rat MC3 receptor were described previously (5).

cAMP determination. HEK 293 cells expressing either the MC3 receptor or the MC4 receptor were washed once with HBSS+ and incubated with ACTH peptides in HBSS+ for 20 min, except when indicated otherwise. Cells were harvested in 1 ml of HBSS+ supplemented with 0.02% Triton X-100 and were centrifuged at 12,000 × *g* for 15 min. Fifty microliters of the supernatant were succinylated and used for determination of the cAMP content, exactly as described in the protocol supplied with the cAMP radioimmunoassay kit from Sigma (24).

Binding assay. Binding assays were performed as described previously by Rainey *et al.* (25). HEK 293 cells expressing either the MC3 or MC4 receptor were plated on gelatin-coated 24-well Costar plates. Approximately 20,000 cells were incubated with 100,000 cpm of ^{125}I-Tyr^{23}-ACTH-1–39 (specific activity, 2000 Ci/mmol; Amersham, Buckinghamshire, UK) and different concentrations of ACTH peptides in binding buffer (Dulbecco's modified Eagle's medium/F-12 medium with 0.5% bovine serum albumin, 0.1% bacitracin, and 15 mM HEPES, pH 7.4), at 25° for 90 min. After the cells were washed twice in ice-cold binding buffer, they were lysed with 0.4% deoxycholate. Radioactivity of the lysates was determined in a γ counter. Typically, the total binding was ~1800 cpm/well for the MC3-expressing cells and ~1300 cpm/well for the MC4-expressing cells, whereas nonspecific binding was ~400 cpm/well. HEK 293 cells without stably transfected MC3 or MC4 receptors did not display specific binding of ^{125}I-Tyr^{23}-ACTH-1–39.

Grooming assay. Male Wistar rats of approximately 150 g, bred and housed under standard laboratory conditions, were implanted with an intracerebroventricular cannula 1 week before the experimental session, according to the protocol of Brakkee *et al.* (26). Fifteen minutes after the intracerebroventricular injection of either saline solution or 1.5 or 3 μg of ACTH-4–13 in saline solution, animals were scored individually for grooming behavior (forepaw vibration, face washing, body grooming, anogenital grooming, tail sniffing, scratching, and body shaking) every 15 sec for a period of 55 min, as described previously (27).

Results

The incubation time for maximal cAMP formation in MC3 receptor-expressing HEK 293 cells in response to peptide was determined by treating cells with 10 nM α-MSH or 1 μM ACTH-4–10 for various time periods. Because the highest cAMP level was reached after incubation of cells with α-MSH for 20 min, this incubation time was used in all other assays (Fig. 1). The basal cAMP content in the HEK 293 cells, after removal of medium and incubation in HBBS+ for 20 min, was estimated to be 2 ± 1 pmol/10^6 cells. Treatments of these cells with <1 nM α-MSH did not stimulate cAMP levels significantly above basal levels (data not shown).

The effects of α-MSH, NDP-MSH, γ-MSH, and ACTH-4–10 on cAMP accumulation in HEK 293 cells expressing either the MC3 or MC4 receptor were determined. Fig. 2A shows that dose-response curves for α-MSH, NDP-MSH, and γ-MSH with the MC3 receptor were similar, as reported previously (3, 5). ACTH-4–10, however, had lower activity; at 1 μM ACTH-4–10 only 30% of the maximal cAMP amount was formed. Even at 10 μM, ACTH-4–10 did not stimulate cAMP levels to >30% of the maximal response (data not shown). We also investigated

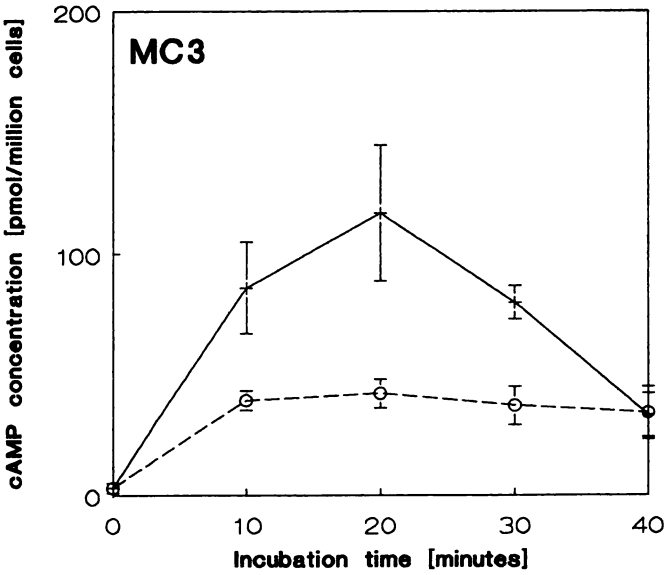

Fig. 1. HEK 293 cells stably expressing the MC3 receptor were incubated with either 10 nM α-MSH (+) or 1 μM ACTH-4–10 (○) for the periods indicated. cAMP levels were measured as described in Materials and Methods. Each value represents the mean ± standard deviation of two separate experiments.

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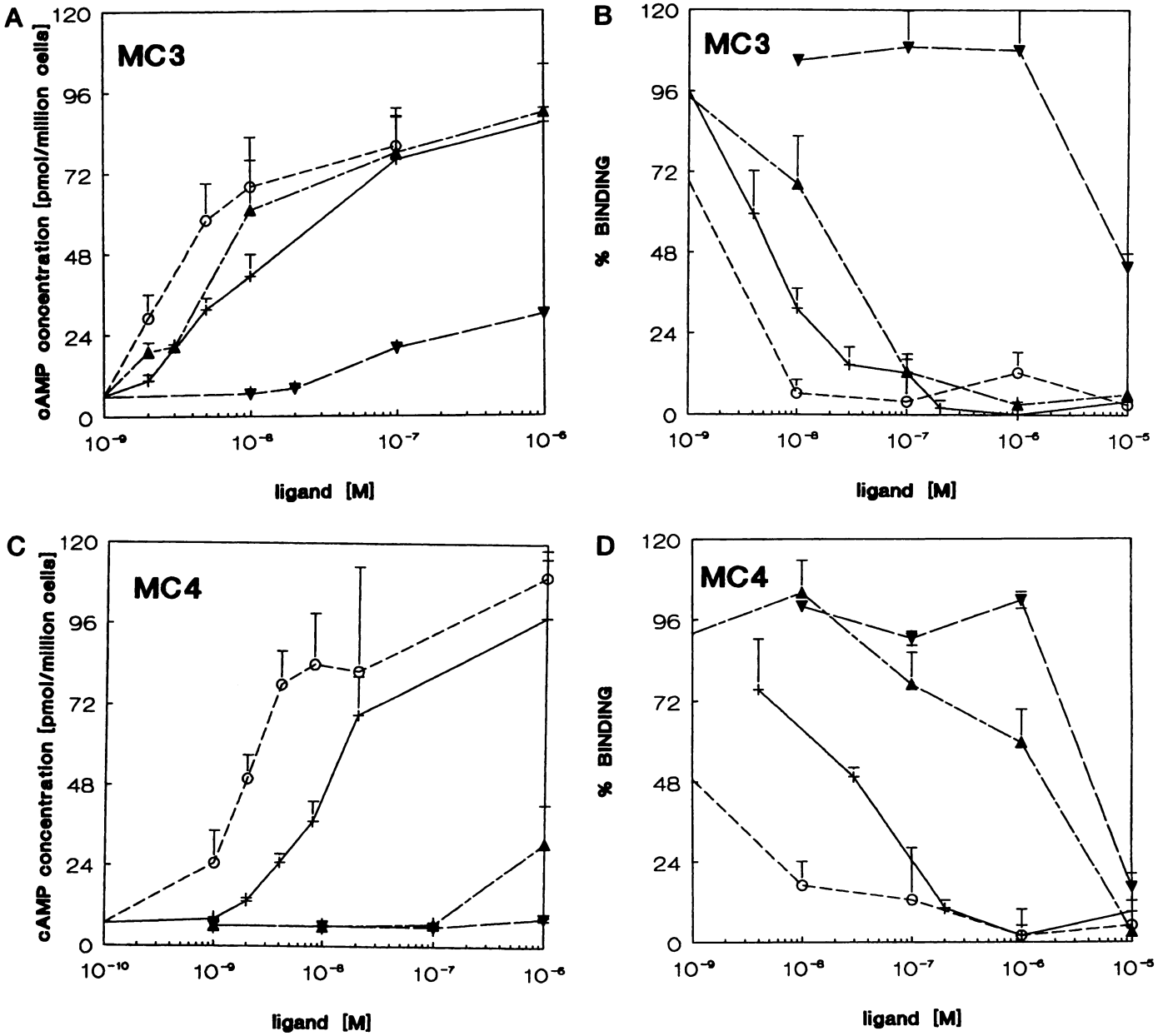

Fig. 2. A and C, HEK 293 cells stably expressing either the MC3 (A) or MC4 (C) receptor were treated with α-MSH (+), NDP-MSH (○), γ-MSH (▲), or ACTH-4–10 (▼) at the concentrations indicated. Each value represents the mean ± standard deviation of three separate treatments. All data were obtained from one experiment in which MC receptor stimulation by four different peptides was determined. Similar results were obtained in several other experiments. B and D, Binding of ^{125}I-Tyr^{23}-ACTH-1–39 to HEK 293 cells stably expressing either the MC3 (B) or MC4 receptor (D) was displaced with different concentrations of α-MSH (+), NDP-MSH (○), γ-MSH (▲), or ACTH-4–10 (▼). Total specific binding of ^{125}I-Tyr^{23}-ACTH-1–39 was set at 100%. The *y*-axis indicates the percentage of total specific binding in the presence of different concentrations of ACTH peptides.

the displacement by these peptides of ^{125}I-Tyr^{23}-ACTH-1–39 binding to these MC3 receptor-expressing cells. As shown in Fig. 2B, α-MSH and γ-MSH displaced this radiolabel equally well, whereas NDP-MSH displaced it more efficiently. ACTH-4–10 only poorly displaced ^{125}I-Tyr^{23}-ACTH-1–39 from MC3 receptor-expressing cells. The displacement of ^{125}I-Tyr^{23}-ACTH-1–39 by 10 μM α-MSH was taken as the maximum.

Fig. 2C shows that NDP-MSH was more potent than α-MSH in stimulating the MC4 receptor, although the maximal effects of these peptides were not different. γ-MSH and ACTH-4–10 activated the MC4 receptor only very poorly; 1 μM concentrations of these peptides raised cAMP levels to approximately 31% and 9%, respectively, of the maximal response reached with α-MSH. Both of these findings were also consistent with previous reports (4, 6). NDP-MSH displaced ^{125}I-Tyr^{23}-ACTH-1–39 binding to these cells more efficiently than did α-MSH (Fig. 2D) (the displacement of ^{125}I-Tyr^{23}-ACTH-1–39 by 10 μM α-MSH was taken as maximum). γ-MSH and ACTH-4–10 displaced this radiolabel from MC4 receptor-expressing cells only at high doses. These data indicate that γ-MSH is selectively recognized by the MC3 receptor, whereas α-MSH and its analog NDP-MSH act on the MC3 and MC4 receptors equally well.

Next, the activities of ACTH-1–13 fragments with shortened amino and carboxyl termini were determined. These experiments were performed to determine what part of the peptide

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was responsible for the loss of activity of ACTH-4–10 at the MC3 receptor, compared with the parent peptide α-MSH. Fig. 3A shows the activities of α-MSH, ACTH-1–10, ACTH-4–13, and ACTH-4-9-NH_2 at the MC3 receptor. ACTH-4–13 elicited the same response, compared with α-MSH, at the MC3 receptor. Removal of the residue 11–13 sequence of α-MSH, as in ACTH-1–10, led to a loss of activity at the MC3 receptor, to a level comparable to that of ACTH-4–10. The order of potency of these peptides for cAMP accumulation in MC3 receptor-expressing cells was similar to the order of potency for displacement by these peptides of ^{125}I-Tyr^{23}-ACTH-1–39 binding (Fig. 3B). Thus, amino acids 11–13 of α-MSH are more important than amino acids 1–3 of α-MSH for eliciting a full response with and binding to the MC3 receptor.

The activities of these peptides at the MC4 receptor were also investigated (Fig. 3C). ACTH-4–13 at 100 nM significantly stimulated the MC4 receptor, and at 1 μM peptide an almost full response was observed. ACTH-4-9-NH_2 and ACTH-1–10 stimulated the MC4 receptor only at 1 μM, to approximately 18% and 11%, respectively, of the maximal response reached with α-MSH. The order of potency of these peptides to increase cAMP levels in MC4 receptor-expressing cells was the same as the order of potency of these peptides to displace binding of ^{125}I-Tyr^{23}-ACTH-1–39 from these cells (Fig. 3D). Thus, amino

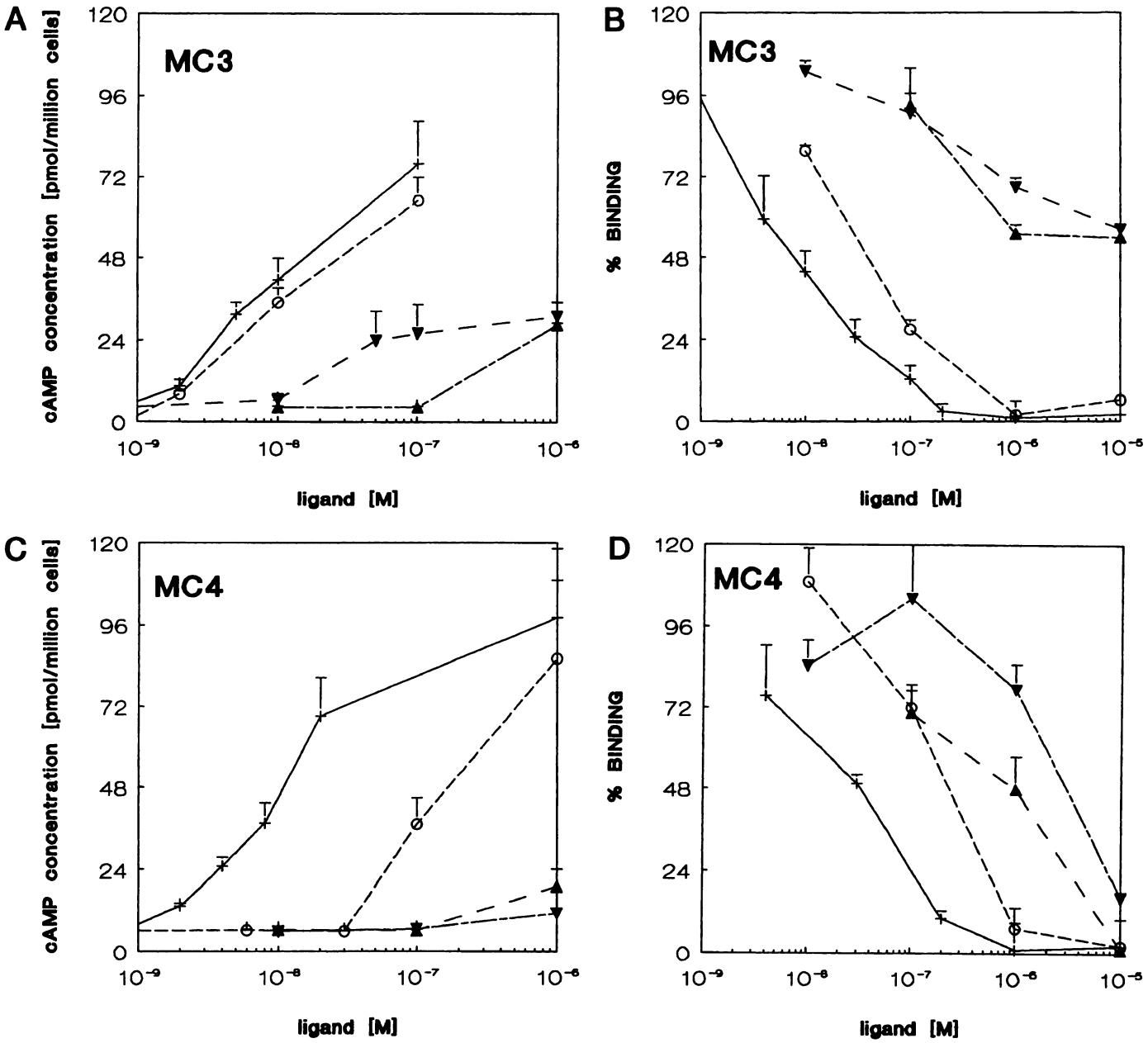

Fig. 3. A and C, HEK 293 cells stably expressing either the MC3 (A) or MC4 (C) receptor were treated with α-MSH (+), ACTH-4–13 (○), ACTH-4–9-NH_2 (▲), or ACTH-1–10 (▼) at different concentrations. Each value represents the mean ± standard deviation of three separate treatments. All data were obtained from one experiment in which MC receptor stimulation by four different peptides was determined. Similar results were obtained in several other experiments. B and D, Binding of ^{125}I-Tyr^{23}-ACTH-1–39 to HEK 293 cells stably expressing either the MC3 (B) or MC4 (D) receptor was displaced with different concentrations of α-MSH (+), ACTH-4–13 (○), ACTH-4–9-NH_2 (▲), or ACTH-1–10 (▼). Total specific binding of ^{125}I-Tyr^{23}-ACTH-1–39 was set at 100%. The *y*-axis indicates the percentage of total specific binding in the presence of different concentrations of ACTH peptides.

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acids 11–13 are important for activation of and binding to the MC4 receptor, whereas amino acids 1–3 are needed to achieve maximal activation of the MC4 receptor at low peptide concentrations.

In additional experiments, the core sequence for activation of and binding to the MC3 and MC4 receptors was determined. ACTH-4–9 and ACTH-5–10 as well as all shorter peptides tested, at 10 μM peptide, did not stimulate cAMP levels in HEK 293 cells that stably expressed the MC3 receptor or the MC4 receptor, nor did these peptides displace binding of ^{125}I-Tyr^{23}-ACTH-1–39 from these cells. The presence of a carboxyl-terminal carboxamide instead of the carboxylic acid in ACTH-4–9 had a significant effect on the activity of the peptide. Although ACTH-4–9 was virtually inactive, ACTH-4–9-NH_2 displayed activity at and binding to MC3 and MC4 receptors (Fig. 3). Extension of the ACTH-5–10 sequence to ACTH-5–13 poorly restored the activity at MC3 and MC4 receptors to the level reached with ACTH-4–10. At 1 μM ACTH-5–13, the receptors were activated to the same extent as with ACTH-4–10 at 1 μM. Thus, ACTH-4–9-NH_2 is the core sequence for activation of the MC3 and MC4 receptors.

The activity of ORG2766, an ACTH-4–9 analog that has modifications at three positions of the ACTH-4–9 peptide, was also studied for its ability to activate and bind to the MC3 and MC4 receptors. ORG2766 at 10 μM only very poorly activated the MC3 receptor; the cAMP level was increased <2-fold above basal levels, which was <3% of the full response to α-MSH. ORG2766 had no effect at the MC4 receptor. ORG2766 lacks the amidated carboxyl terminus, and two other modifications in ORG2766 are the sulfonated methionine at position 4 and the substitution of tryptophan by phenylalanine at position 9. Each of these modifications, when introduced separately into ACTH-4–10, resulted in loss of activity at the MC3 or MC4 receptors when tested at 1 μM peptide (Table 1). No antagonistic activity could be observed for 1 μM ORG2766 at the MC3 or MC4 receptors when they were stimulated with 10 nM α-MSH (Table 1). Furthermore, ORG2766 (at 10 μM) was not able to displace binding of ^{125}I-Tyr^{23}-ACTH-1–39 from MC3 and MC4 receptor-expressing cells.

The substitution of phenylalanine at position 7 of ACTH-4–10 by its D-enantiomer was shown to alter its activity in several behavioral assays; in active avoidance behavior assays introduction of D-Phe^7 into ACTH-4–10 reversed its activity (the retention time of this behavior was decreased), whereas in grooming behavior assays the introduction of D-Phe^7 induced excessive grooming behavior and ACTH-4–10 itself was inactive (12, 16, 17). Therefore, we determined the activity and binding of [D-Phe^7]ACTH-4–10, in comparison with α-MSH and ACTH-4–10, at MC3 and MC4 receptors. Although [D-Phe^7]ACTH-4–10 stimulated the MC3 receptor more efficiently than did ACTH-4–10, 1 μM [D-Phe^7]ACTH-4–10 only half-maximally stimulated the MC3 receptor. The MC4 receptor, however, could be fully stimulated by [D-Phe^7]ACTH-4–10. At 30 nM [D-Phe^7]ACTH-4–10 significantly stimulated the MC4 receptor, whereas at this concentration the MC3 receptor was not activated (Fig. 4, A and B). The displacement by [D-Phe^7]-ACTH-4–10 of ^{125}I-Tyr^{23}-ACTH-1–39 binding to MC4 receptor-expressing cells was much more efficient than displacement from MC3 receptor-expressing cells (Fig. 4, C and D).

Because ACTH-4–13 was the shortest MC peptide that activated the MC3 receptor maximally at low doses and the MC4 receptor submaximally at higher doses, the activity of ACTH-4–13 to induce excessive grooming behavior was tested. Fig. 5 shows that ACTH-4–13 was indeed able to induce the excessive grooming response. However, the maximal grooming score obtained with α-MSH was not reached, even at a higher dose of ACTH-4–13.

Discussion

We took advantage of the cloning of neural MC receptors by using these cloned receptors to study their pharmacological properties *in vitro*. This allowed us to compare the reported effects of ACTH peptides and analogs on behavior, nerve regeneration, blood pressure, and heart rate with pharmacological properties of the cloned receptors. However, one should keep in mind that the SARs of cloned MC3 and MC4 receptors described here were obtained in an *in vitro* system. The behavioral assays that are discussed here were performed *in vivo* and the peptides were applied either systemically or intracerebroventricularly. The peptide fragments on which the SARs of these behavioral assays were based may have different pharmacokinetic properties, which may influence the *in vivo* activity of these fragments in these assays. In the *in vitro* assays described here, no decrease in activity (stimulation of cAMP accumulation) of the MC peptides was observed when they were tested after the incubation period, with fresh cells. Therefore, the SAR for bioassays and the SAR for receptors *in vitro*, as described here, may not overlap completely although the same receptor mediates the effect of the peptides *in vivo*. In the future, local applications of selective agonists may help to

TABLE 1

ACTH peptide structures and activities

HEK 293 cells lines, stably expressing either the MC3 or MC4 receptor, were treated with ACTH peptides at 1 μM (10 μM in the case of ORG2766) (four experiments). To investigate the antagonistic properties of ORG2766 at MC3 and MC4 receptors, 1 μM ORG2766 was mixed with 10 nM α-MSH and the activation was compared with the activation of MC3 and MC4 receptors by treatment of cells with 10 nM α-MSH alone (three experiments).

Peptide	Amino acid							Activity	
	4	5	6	7	8	9	10	MC3	MC4
								pmol of cAMP/10^6 cells	
ACTH-4–10	Met	Glu	His	Phe	Arg	Trp	Gly	22	8
4-Met-O_2[a]	Met(O_2)							2	2
9-Phe						Phe		2	2
ORG2766	Met(O_2)				D-Lys	Phe	None	4	2
10 nM α-MSH								54	60
10 nM α-MSH + 1 μM ORG2766								54	74

[a] 4-Met-O_2, [Met·$(O)_2^4$]ACTH-4–10; 9-Phe, [Phe^9]ACTH-4–10.

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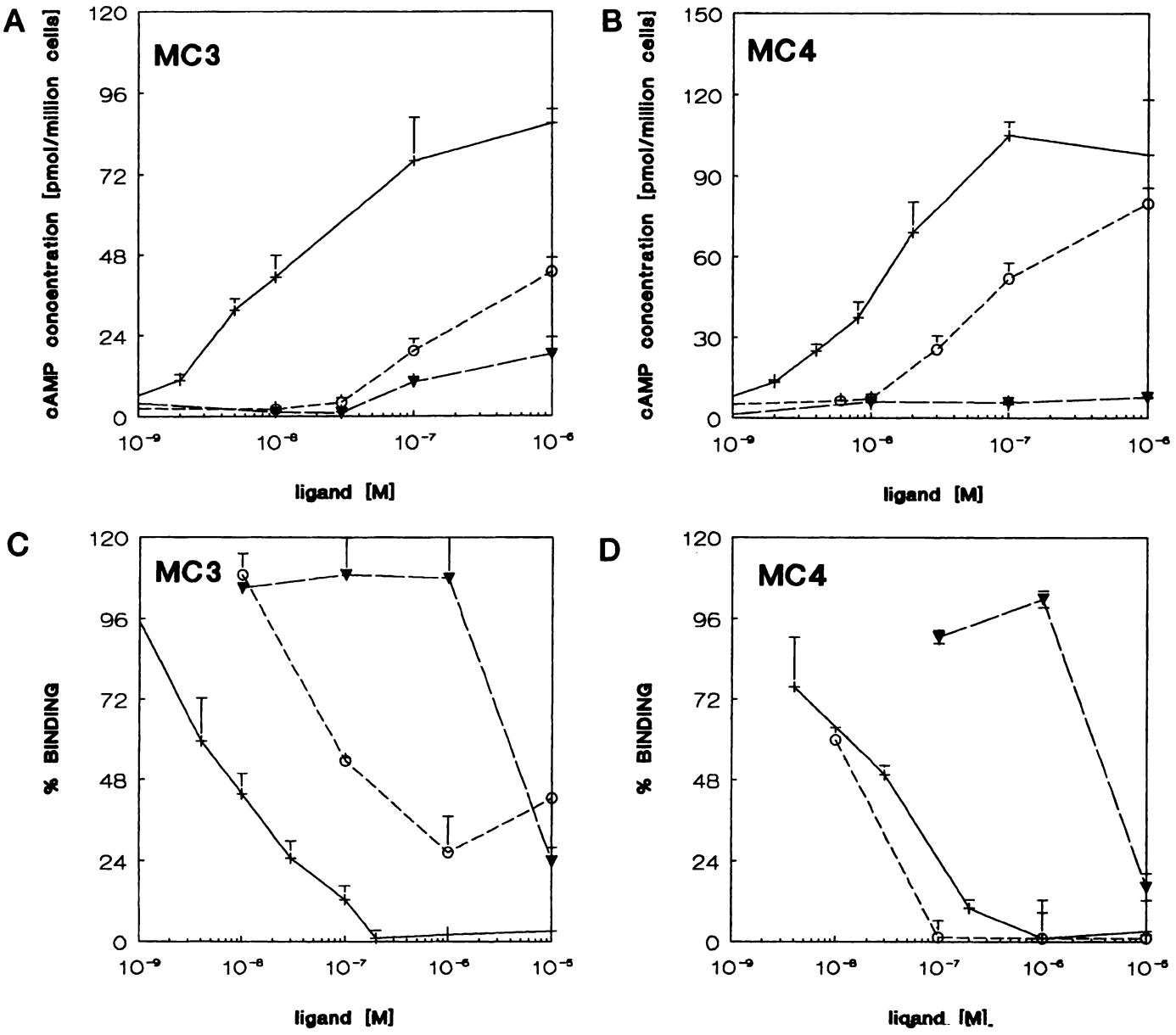

Fig. 4. A and B, HEK 293 cells stably expressing either the MC3 (A) or MC4 (B) receptor were treated with α-MSH (+), [D-Phe^7]ACTH-4–10 (O), or ACTH-4–10 (▼) at the indicated concentrations, as in Fig. 2. A and C, Binding of ^{125}I-Tyr^{23}-ACTH-1–39 to HEK 293 cells stably expressing either the MC3 (C) or MC4 (D) receptor was displaced with different concentrations of α-MSH (+), [D-Phe^7]ACTH-4–10 (O), or ACTH-4–10 (▼). Total specific binding of ^{125}I-Tyr^{23}-ACTH-1–39 was set at 100%. The *y*-axis indicates the percentage of total specific binding in the presence of different concentrations of ACTH peptides.

establish which effect is mediated by activation of particular receptors.

α-MSH, γ-MSH, and NDP-MSH activated and bound to the MC3 receptor equally well, whereas the activity of ACTH-4–10 at the MC3 receptor was lower. These results agree with earlier reports (3, 5). At 1 μM ACTH-4–10, the (sub)maximal activation of the MC3 receptor described by Roselli-Rehfuss *et al.* (5) and Gantz *et al.* (3) was not observed. In the latter studies cAMP levels were measured after 45 and 30 min, respectively, whereas here we determined cAMP content after 20-min treatment with peptide. When HEK 293 cells expressing the MC3 receptor were treated with 10 nM α-MSH for 40 min, the cAMP level reached was the same as that reached with treatment with 1 μM ACTH-4–10 for 40 min (Fig. 1). However, after 20 min the cAMP level reached with α-MSH treatment was 3 times higher than the level reached with ACTH-4–10. This explains the difference between the effects of ACTH-4–10 and α-MSH described previously (3, 5) and in this study. These data suggest that, because α-MSH activated the MC3 receptor more efficiently than did ACTH-4–10, it also caused more rapid desensitization. This explains the lower activity of α-MSH after 40-min treatment, compared with 20-min treatment.

The minimal sequence required to activate the MC3 and MC4 receptors was ACTH-4–9-NH_2, although the activity was low, compared with α-MSH (Fig. 3). The presence of the carboxamide is essential for activity and may mimic the peptide bond between residues 9 and 10. ACTH-4–9-NH_2 had the same effect as ACTH-4–10. Extension of ACTH-4–10 to ACTH-4–

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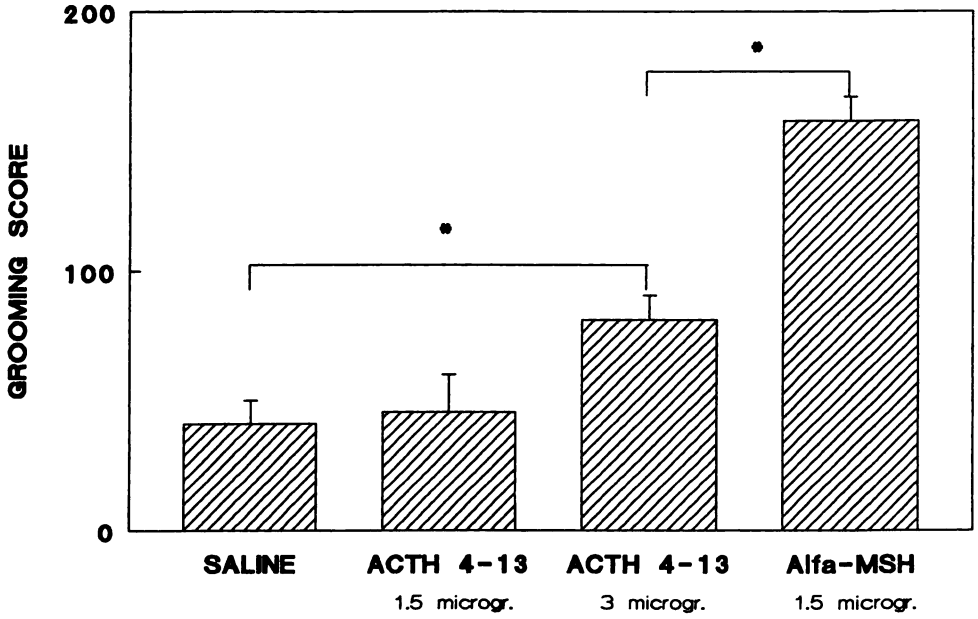

Fig. 5. The ability of ACTH-4–13 to induce grooming behavior was tested in rats. Rats received either saline, 1.5 μg or 3 μg of ACTH-4–13, or 1.5 μg of α-MSH, intracerebroventricularly. *, Statistically significant difference, compared with saline treatment (Student t test, $p < 0.05$).

13 increased the activity with the MC3 receptor to levels comparable to that of α-MSH (Fig. 3A), demonstrating that ACTH-11–13 is important for full activation of the MC3 receptor at low peptide concentrations. With the MC4 receptor, ACTH-4–13 was the shortest peptide that activated the receptor submaximally at 1 μM (Fig. 3C). Thus, in contrast to the MC3 receptor, the MC4 receptor requires amino acids 1–3 of α-MSH for full activation at low peptide concentrations. The low activity of ACTH-1–10 at the MC4 receptor demonstrated the importance of amino acids 11–13 in the activation of the MC4 receptor.

Introduction of the D-enantiomer of phenylalanine at position 7 of ACTH influenced the activity of the ACTH peptide in several bioassays (12, 16, 17, 28). The activity of ACTH peptides on corticoid production in the adrenal cortex was lost when Phe^7 was substituted by its D-enantiomer (13). In contrast, the effect on pigment dispersion was enhanced by D-Phe^7 substitutions (13).

With the MC4 receptor, ACTH-4–10 had very low activity, whereas the activity of [D-Phe^7]ACTH-4–10 was much higher (Fig. 4B). At the MC3 receptor, the activity of ACTH-4–10 was low, whereas [D-Phe^7]ACTH-4–10 activated the MC3 receptor only half-maximally at high concentrations. The fact that [D-Phe^7]ACTH-4–10 recognized the MC4 receptor better than the MC3 receptor was demonstrated by the finding that [D-Phe^7]-ACTH-4–10 displaced binding of ^{125}I-Tyr^{23}-ACTH-1–39 from the MC4 receptor as efficiently as did α-MSH (Fig. 4D), whereas it displaced this radiolabel from the MC3 receptor less efficiently (Fig. 4C). Therefore, [D-Phe^7]ACTH-4–10 is a peptide with a degree of specificity for the MC4 receptor. As shown in Fig. 2, γ-MSH is a potent agonist selective for the MC3 receptor. Using γ-MSH and [D-Phe^7]ACTH-4–10, the MC3 and MC4 receptors can be discriminated. These properties (summarized in Table 2) were used to correlate MC receptor types with *in vivo* effects of MCs.

The fact that γ-MSH fully activated the MC3 receptor with an activity comparable to that of α-MSH rules out the MC3 receptor as a mediator of MC-induced excessive grooming behavior, because γ-MSH does not induce excessive grooming

TABLE 2

Summary table presenting the estimated $-\log_{10}$ EC_{50} and IC_{50} values of the MC peptides for MC3 and MC4 receptors

EC_{50} values were calculated from dose-response curves that were based upon 15–18 separate measurements. IC_{50} values were calculated from displacement curves that were based upon 12–15 separate measurements. In cases where the EC_{50} values could not be estimated because 1 μM peptide did not elicit a maximal response, the percentage of the maximal cAMP accumulation reached with 1 μM peptide is shown.

Peptide	MC3		MC4	
	$-\log EC_{50}$	$-\log IC_{50}$	$-\log EC_{50}$	$-\log IC_{50}$
α-MSH	8.2	8	8.1	8.7
NDP-MSH	8.5	9	8.7	9
γ-MSH	8.3	8	30%	<6
ACTH-4–13	8	7.4	7	6.7
ACTH-1–10	34%	<6	11%	<6
ACTH-4–10	30%	<6	8%	<6
ACTH-4–9-NH_2	31%	<6	18%	<6
[D-Phe^7]ACTH-4–10	48%	6	7	8

behavior (29). NDP-MSH is the most potent peptide, both at the MC4 receptor *in vitro* and for the grooming response, followed by α-MSH (30). Removal of the three carboxyl-terminal amino acids (as in ACTH-1–10) (30) reduced excessive grooming behavior much more dramatically than did removal of the three amino-terminal amino acids (as in ACTH-4–13) (Fig. 5). ACTH-1–10 and ACTH-4–10 did not elicit excessive grooming behavior (29) and only very poorly activated the MC4 receptor *in vitro*. [D-Phe^7]ACTH-4–10 (28, 29) and ACTH-4–13 (Fig. 5) induced excessive grooming behavior, although the response was less than the response to α-MSH. This correlates with the activity of these peptides at the MC4 receptor *in vitro*. The order of potency of NDP-MSH, α-MSH, and ACTH-4–13 for eliciting excessive grooming behavior correlated with that for activating the MC4 receptor *in vitro*. Both in the grooming response and with the MC4 receptor *in vitro*, [D-Phe^7]ACTH-4–10 was active, whereas ACTH-4–10 had no or little activity, respectively. Furthermore, the MC4 receptor is expressed in the periaquaductal gray area, a site that has been demonstrated to be a neural substrate for MC-induced excessive grooming behavior (31, 32). These data suggest that the MC4 receptor mediates MC-induced excessive grooming behavior.

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Another bioassay in which the SAR of ACTH peptides has been studied extensively is the pole jump test (33). This is an active avoidance task in which a rat is taught to avoid an electric footshock. ACTH increases the avoidance retention time of this behavior. In this assay, ACTH-4–10 had a potency equal to that of ACTH-1–24 (12, 33). ACTH-4–9 was also active in this assay, which eventually led to the development of ORG2766, which is very potent in this test (33). Typically, [D-Phe^7]ACTH-4–10, NDP-MSH, and γ-MSH had effects opposite to that of ACTH-4–10 in active avoidance behavior (15). Taking into consideration the finding that ACTH-4–9 and ORG2766 were active in delaying the extinction of active avoidance behavior, whereas [D-Phe^7]ACTH-4–10, NDP-MSH, and γ-MSH enhanced the extinction of active avoidance behavior, the involvement of MC3 and MC4 receptors in mediating this behavior should be ruled out. ORG2766 and ACTH-4–9 did not activate these receptors *in vitro*, nor did they antagonize the effect of α-MSH on these receptors (Table 1). NDP-MSH and γ-MSH fully activated the MC3 receptor, whereas [D-Phe^7]-ACTH-4–10 had an activity comparable to that of ACTH-4–10 at the MC3 receptor. The MC4 receptor was activated by NDP-MSH and, at higher concentrations, [D-Phe^7]ACTH-4–10 activated the MC4 receptor. Therefore, we suggest that the effects of MCs on active avoidance behavior are mediated by another receptor.

ORG2766, whose development was guided by the activity of ACTH peptides in active avoidance behavior assays (33), has lost the ability of the native ACTH molecule to activate the members of the MC receptor family that have been cloned thus far (3–6). The substitutions in ORG2766, compared with ACTH-4–9, when introduced separately into ACTH-4–10 resulted in loss of activity at the MC3 or MC4 receptors (Table 1). Thus, considering the SARs of the MC receptors cloned thus far, ORG2766 may be acting via a receptor outside of the MC receptor family.

SARs of MC peptides have also been determined for the acceleration of recovery of sensibility seen upon treatment of rats with MCs after a sciatic nerve crush. α-MSH, NDP-MSH, ACTH-4–10, and ORG2766 are active in this test, whereas γ-MSH and [D-Phe^7]ACTH-4–10 are not (20, 34). Because γ-MSH activated the MC3 receptor *in vitro* at lower concentrations than did ACTH-4–10, the MC3 receptor is probably not involved in mediating this effect of MCs. Similarly, involvement of the MC4 receptor is unlikely, because [D-Phe^7]ACTH-4–10 is more active at the MC4 receptor than is ACTH-4–10. The fact that ORG2766 did not activate MC3 or MC4 receptors is in line with this concept. Recently, however, the effects of ORG2766 and α-MSH on primary cultures of rat spinal cord cells were shown not to overlap completely (35), which suggests that ORG2766 and α-MSH mediate their effects via different receptors, one of which may be a receptor belonging to the MC receptor family.

MCs, in particular γ-MSH, are active in increasing heart rate and blood pressure upon intravenous injections of γ-MSH (18, 36). ACTH-4–10 had a similar effect, although its potency was 5 times lower (18). Surprisingly, α-MSH was not active (18). Again, this is in contrast to the activation of all cloned MC receptors, including the MC3 and MC4 receptors, at which α-MSH is very potent. Therefore, the MC3 and MC4 receptors are probably not involved in mediating the cardiovascular responses of γ-MSH, which suggests the existence of still other MC receptors.

Here, we investigated the *in vitro* activity of MC fragments and analogs in the activation of two of the cloned MC receptors that are expressed in the brain. These data may be used for development of novel MC receptor-specific ligands. The SARs for the MC4 receptor correlated to a high degree with the SAR for the induction of excessive grooming behavior, whereas the SAR for the MC3 receptor did not fit with those for other central effects of MC peptides. We suggest that there must be more MC-activated receptors in the brain than the MC3 and MC4 receptors, because the SAR for the cardiovascular effects of γ-MSH and the SAR for the effects of ACTH peptides on active avoidance behavior do not fit with the SARs for the cloned MC receptors.

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