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European Journal of Pharmacology
Molecular Pharmacology Section 269 (1994) 331–337

ejp
molecular pharmacology

Identification of antagonists for melanocortin MC_3, MC_4 and MC_5 receptors

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Received 8 June 1994; revised MS received 2 August 1994; accepted 16 August 1994

Abstract

Antagonists for the melanocortin receptor family were identified by analysis of the effects of four melanocortin analogues on α-MSH(α-melanocyte-stimulating hormone)-induced cAMP accumulation in 293 human embryonal kidney (HEK) cells that expressed either the rat melanocortin MC_3 receptor, the human melanocortin MC_4 receptor or the ovine melanocortin MC_5 receptor. Two peptides, [D-Arg^8]ACTH(adrenocorticotrope hormone)-(4–10) and [Pro^{8,10},Gly^9]ACTH-(4–10), antagonized the action of α-MSH on the melanocortin MC_4 and MC_5 receptors, but not the melanocortin MC_3 receptor. [Ala^6]ACTH-(4–10) inhibited the α-MSH activation of the melanocortin MC_3 and MC_5, but only weakly antagonized the activation of the melanocortin MC_4 receptor. [Phe-I^7]ACTH-(4–10) antagonized the melanocortin MC_3, MC_4 and MC_5 receptors equally well. These antagonists were also tested to block a behavioral response induced by α-MSH. α-MSH-induced excessive grooming behavior in rats was inhibited by [Phe-I^7]ACTH-(4–10), [D-Arg^8]ACTH-(4–10) and [Pro^{8,10},Gly^9]ACTH-(4–10), but not by [Ala^6]ACTH-(4–10). This suggests that α-MSH-induced excessive grooming behavior is mediated by melanocortin MC_4 receptors.

Keywords: Melanocortin receptor antagonist; Melanocortin receptor antagonist; Grooming behavior; ACTH (adrenocorticotrophic hormone) derivative; α-MSH (α-melanocyte-stimulating hormone)

1. Introduction

Several peptides derived from pro-opiomelanocortin share a heptapeptide core sequence. They include ACTH (adrenocorticotrope hormone), α-MSH (α-melanocyte-stimulating hormone), β-MSH, γ-LPH (γ-lipotropin hormone) and various forms of γ-MSH. These peptides and their synthetic derivatives are collectively called melanocortins. Melanocortins have a wide range of biological activities. Classically they stimulate pigmentation and corticosteroidogenesis (Eberle, 1988), but melanocortins also induce excessive grooming behavior (Wiegant et al., 1979), stimulate the retention of a conditioned active avoidance response (Greven and De Wied, 1977; De Wied and Jolles, 1982), increase blood pressure and heart rate (De Wildt et al., 1993), accelerate nerve regeneration (Bijlsma et al., 1981; Van Der Zee et al., 1991) and have been implicated in modulating the immune response (Catania and Lipton, 1993). Studies describing these effects were hampered by the lack of antagonists that could demonstrate the specificity of these melanocortin effects.

The cloning of melanocortin receptors formed a milestone in the research on the biology of melanocortins. Five melanocortin receptor subtypes have been cloned now (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992; Gantz et al., 1993a; Gantz et al., 1993b; Rehfuss-Roselli et al., 1993; Mountjoy et al., 1994; Barrett et al., 1994; Chhajlani et al., 1993; Low et al., 1994). They show different patterns of expression, as well as a difference in affinity for different melanocortins (such as α-MSH and γ-MSH). Melanocortins stimulate pigment dispersion via melanocortin MC_1 receptors in melanocytes and activate corticoidsteroidogenesis via melanocortin MC_2 receptors in the adrenal cortex (Mountjoy et al., 1992). Melanocortin MC_3 receptors are expressed mainly in

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SSDI 0922-4106(94)00132-4

the hypothalamus (Rehfuss-Roselli et al., 1993), whereas melanocortin MC_4 receptors are expressed more widespread in the brain (Mountjoy et al., 1994). The expression of melanocortin MC_5 receptors has only been described using the polymerase chain reaction on reverse transcribed RNA from various tissues, suggesting expression in brain, pituitary, muscle, gut, adrenal and thymus (Chhajlani et al., 1993; Barrett et al., 1994). These melanocortin MC_3, MC_4 and MC_5 receptors are thought to mediate several of the previously described actions of melanocortins. But it is not known which biological effect is mediated by which melanocortin receptor type(s).

In order to determine the role of melanocortin receptor subtypes in mediating the various effects of melanocortins, we took advantage of the cloning of melanocortin receptors to search for receptor antagonists. Here we present melanocortin receptor antagonists for the melanocortin MC_3, MC_4 and MC_5 receptors. Furthermore, we demonstrate that melanocortin MC_4 receptor antagonists inhibit α-MSH-induced excessive grooming behavior.

2. Materials and methods

2.1. Peptides

All peptides used were synthesized by Drs. H.M. Greven and J. Van Nispen and kindly provided by Organon International (Oss, The Netherlands). ACTH fragments used have a free N^{α}-amino group and carboxyl terminus. The ACTH analogues used are shown in Table 1 (D- = a D-enantiomer instead of the natural L-enantiomer of the indicated amino acid; Phe-I = para-iodo-phenylalanine). Peptides were dissolved and diluted in Hanks' balanced salt solution (HBBS).

2.2. Cells and transfection

293 human embryonal kidney (HEK) cells, grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were transfected, using the Calcium-Phosphate precipitation method as described previously (Van der Eb and Graham, 1980), with 10 μg of pcDNA1/MC_4 (encoding the human melanocortin MC_4 receptor) (Mountjoy et al., 1994) or with a mixture of 9 μg pcDOR8, which had been constructed by inserting the ovine melanocortin MC_5 receptor gene into pcDNA1 (invitrogen) (Barrett et al., 1994) and 1 μg pAG-60-NEO, which contains the neomycin gene under control of the Rous sarcoma virus promoter (Colbere-Garapin et al., 1981). Stably expressing clones of cells were selected in geniticin (G418) supplemented medium for 4 weeks. The expression of melanocortin MC_4 and MC_5 receptors was confirmed by measurement of the cAMP content of cells treated 30 minutes with 1 μM α-MSH as compared to non-transfected cells. 293 HEK cells expressing the rat melanocortin MC_3 receptor were described previously (Rehfuss-Roselli et al., 1993).

2.3. cAMP assay

293 HEK cells expressing either melanocortin MC_3, MC_4 or MC_5 receptor were washed once with HBSS (supplemented with 2 mM calcium chloride and 0.1 mM isobutylmethoxyxanthine (IBMX), hereafter called HBSS +) and treated for 20 min with ACTH-peptides in HBSS + . If antagonists were applied, they were mixed with α-MSH in HBSS + . Cells were harvested in 1 ml of HBSS + supplemented with 0.02% Triton X-100, centrifuged 15 min at $12000 \times g$. 50 μl of the supernatant was succinylated and used for determination of cAMP content, exactly as described in the protocol supplied by the cAMP-RIA-kit from Sigma (Frandsen and Krishna, 1977). An analysis of variance for repeated measurements was performed on the data presented in Figs. 1 and 2, to test the hypothesis that the curves do differ from each other. In case of statistical significant difference ($P < 0.05$), an ANOVA followed by Tukey's honestly significant difference test was performed at one point (20 nM α-MSH) to investigate which curves differed significantly ($P < 0.05$).

2.4. pA2 value determination

The $-\log$ of the concentration of antagonist was plotted on the X-axis against the $\log [(A'/A) - 1]$ on the Y-axis. A represents the concentration of agonist needed to elicit a certain level of cAMP accumulation. A' represents the concentration of agonists needed in

Table 1
Primary structures of ACTH-(4–10), [Phe-I^7]ACTH-(4–10), [D-Arg^8]ACTH-(4–10), [$Pro^{8,10}$,Gly^9]ACTH-(4–10) and [Ala^6]ACTH-(4–10) are shown

	4	5	6	7	8	9	10
ACTH-(4–10)	MET -	GLU -	HIS -	PHE -	ARG -	TRP -	GLY
[Phe-I^7]ACTH-(4–10)				PHE-I			
[Ala^6]ACTH-(4–10)			ALA				
[D-Arg^8]ACTH-(4–10)					D-ARG		
[$Pro^{8,10}$,Gly^9]ACTH-(4–10)					PRO -	GLY -	PRO

The D in D-Arg indicates a D-enantiomer in stead of a L-enantiomer. Phe-I is para-iodo-phenylalanine.

presence of antagonist to reach the same amount of cAMP accumulation as when agonist was added alone at concentration A. The results of two or three different experiments in which one ACTH-(4–10) analogue was tested for antagonism were combined in this plot. The p$A2$ value is represented by the intercept on the X-axis as described previously (Arunlakshana and Schild, 1959).

2.5. Grooming assay

Male Wistar rats of approximately 150 g, bred and housed under standard laboratory conditions, received an intracerebroventricular (i.c.v.) cannula one week prior to the experimental session, according to the protocol of Brakkee and colleagues (Brakkee et al., 1979). Fifteen min after the i.c.v. injection of either saline, α-MSH or ACTH-(4–10) analogues dissolved in saline or mixtures of α-MSH and ACTH-(4–10) analogues dissolved in saline, were assayed for grooming behavior as described previously (Gispen et al., 1975).

3. Results

Approximately 100 analogues of ACTH-(4–10) and ACTH-(4–9) were screened for inhibition of α-MSH-induced cAMP accumulation in 293 HEK cells express-

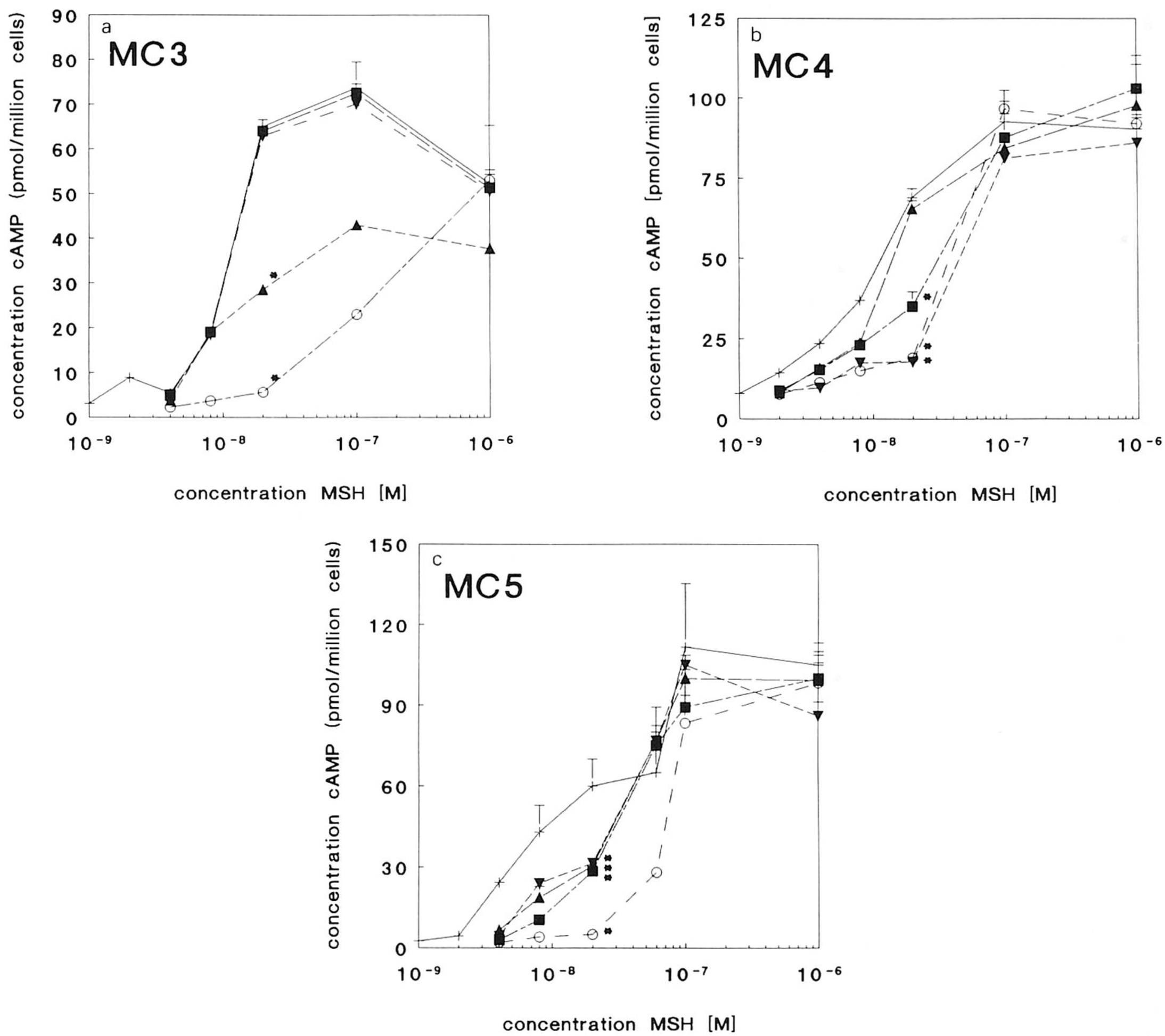

Fig. 1. The effects of ACTH analogues on α-MSH-induced cAMP accumulation in 293 HEK cells, stably expressing either the rat melanocortin MC_3 (a), human melanocortin MC_4 receptor (b) or ovine melanocortin MC_5 receptor (c). Cells were treated with α-MSH (+), or mixtures of α-MSH at the indicated concentrations with either 1 μM [Phe-1^7]ACTH-(4–10) (○), 1 μM [Ala^6]ACTH-(4–10) (▲), 1 μM [D-Arg^8]ACTH-(4–10) (▼) or 1 μM [Pro^{8,10},Gly^9]ACTH-(4–10) (■). Each value represents the mean of three separate treatments with standard deviation. Similar results were obtained repeatedly. Statistical significant differences ($P < 0.05$) as compared to α-MSH are indicated with asterisks (*).

ing either the rat melanocortin MC_3, human melanocortin MC_4 or ovine melanocortin MC_5 receptor (data not shown). Four of these analogues, displaying antagonistic activity, were examined in more detail. Table 1 shows the primary structure of these ACTH-(4–10) analogues. These ACTH-(4–10) analogues, when incubated with the melanocortin receptor-expressing cell lines at 1 μM, did not affect the basal cAMP level in these cells. The effect of these analogues on α-MSH-induced cAMP accumulation in 293 HEK cells expressing the melanocortin MC_3, MC_4 or MC_5 receptor is shown in Fig. 1. Typically the maximal cAMP accumulation in these three cell lines was reached at a concentration of 100 nM α-MSH, whereas the half maximal cAMP accumulation was reached at approximately 10 nM α-MSH. The effect of α-MSH on the melanocortin MC_3 receptor (Fig. 1a) was antagonized by [Phe-I^7]ACTH-(4–10) and to a lesser extend by [Ala^6]ACTH-(4–10). The p*A2* values of these compounds were 8.7 and 6.5 respectively (Table 2). [D-Arg^8]ACTH-(4–10) and [$Pro^{8,10}$,Gly^9]ACTH-(4–10) did not antagonize the melanocortin MC_3 receptor. The melanocortin MC_4 receptor (Fig. 1b) was antagonized by [$Pro^{8,10}$,Gly^9]ACTH-(4–10), [D-Arg^8]ACTH-(4–10) and [Phe-I^7]ACTH-(4–10) (p*A2* values were 8.6, 8.2 and 8.4 respectively). In contrast to the

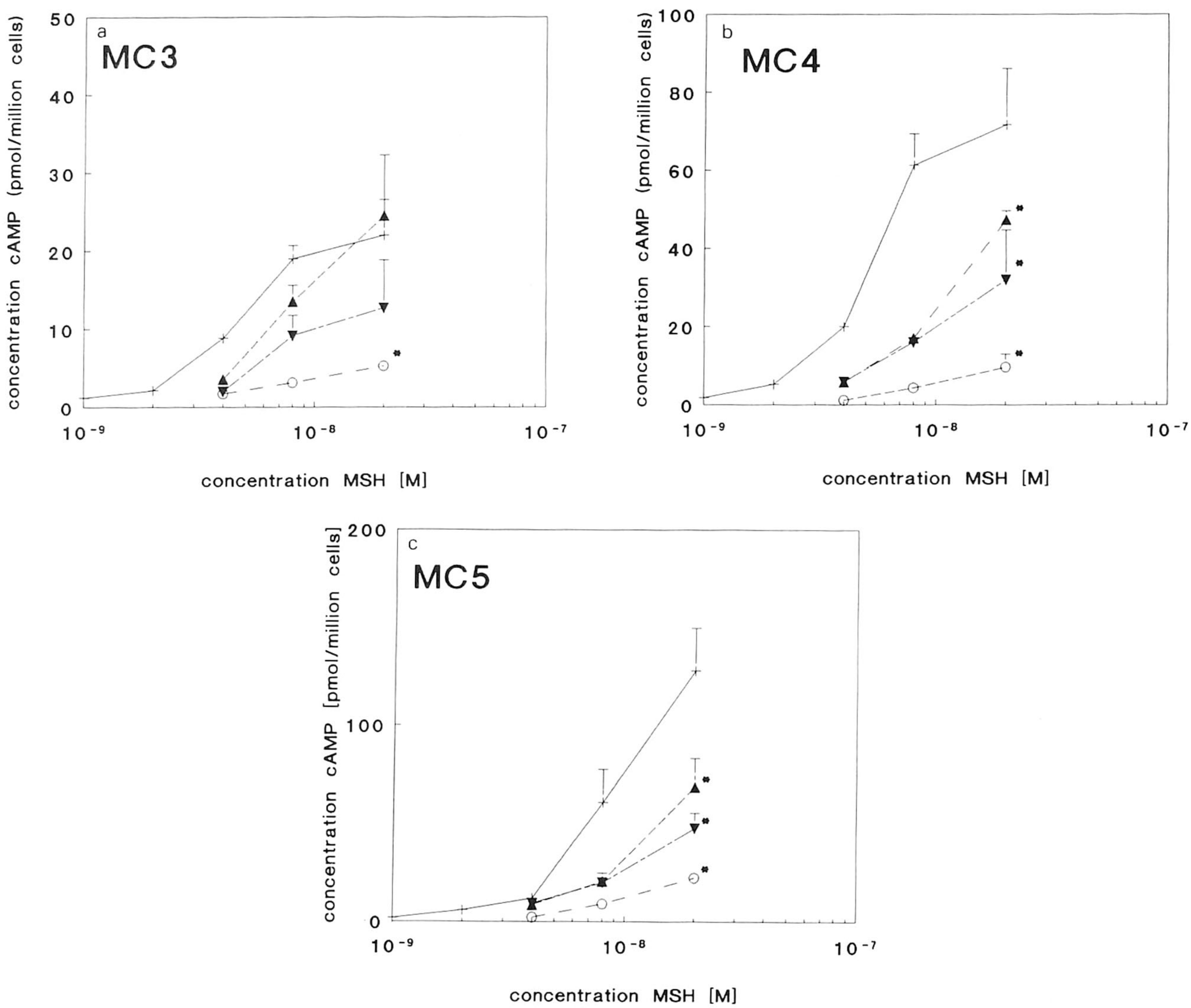

Fig. 2. The effects of different concentrations of [Phe-I^7]ACTH-(4–10) on α-MSH-induced cAMP accumulation in 293 HEK cells, stably expressing either the rat melanocortin MC_3 (a), human melanocortin MC_4 receptor (b) or ovine melanocortin MC_5 receptor (c). Cells were treated with α-MSH (+) or mixtures of α-MSH at the indicated concentrations, and either 10 nM (▲), 100 nM (▼) or 1 μM [Phe-I^7]ACTH-(4–10) (○) at concentrations of α-MSH indicated in the figure. Each value represents the mean of three separate treatments with standard deviation. Similar results were obtained repeatedly. Statistical significant differences ($P < 0.05$) as compared to α-MSH are indicated with asterisks (*).

Table 2
pA_2 values of 4 ACTH-(4–10) analogues on the melanocortin MC_3, MC_4 and MC_5 receptors

	MC_3-R	MC_4-R	MC_5-R
PEPTIDE			
[Phe-I^7]ACTH-(4–10)	7.4	8.4	7.9
[Ala^6]ACTH-(4–10)	6.5	< 6	8.7
[Pro^{8,10},Gly^9]ACTH-(4–10)	–	8.6	6.5
[D-Arg^8]ACTH-(4–10)	–	8.2	8.1

melanocortin MC_3 receptor, the melanocortin MC_4 receptor was only weakly antagonized by [Ala^6]ACTH-(4–10) (p*A2* value was below 6). The melanocortin MC_5 receptor was antagonized by all of these four ACTH-(4–10) analogues (Fig. 1c). The p*A2* values were between 6.5 and 8.7 for the 4 ACTH-(4–10) analogues (Table 2). 1 μM of the ACTH-(4–10) analogues did not inhibit the effect of 1 μM α-MSH on the melanocortin receptors (Fig. 1a, 1b and 1c).

For each of the three melanocortin receptors, the activity of the antagonists was investigated at lower concentrations. Fig. 2 shows the effect of [Phe-I^7]ACTH-(4–10) at 1 μM, 100 nM and 10 nM on the dose response curve of α-MSH on 293 HEK cells expressing the melanocortin MC_3, MC_4 or MC_5 receptors. From these data the p*A2* values were calculated (Table 2).

The effect of these ACTH analogues on grooming behavior in rats was also investigated. None of these peptides when injected i.c.v. at a dose of 1.5 μg or 15 μg per animal induced excessive grooming behavior (data not shown). Next these analogues were tested for their ability to antagonize the induction of excessive grooming behavior by α-MSH. Therefore, the dose response relation of α-MSH on eliciting excessive grooming behavior was investigated (Fig. 3). A submaximal dose of α-MSH (1.5 μg) was chosen to study the effect of antagonists on α-MSH-induced excessive grooming behavior. Rats received i.c.v. injections of mixtures of 1.5 μg α-MSH and 15 μg of the ACTH-(4–10) analogues. Fig. 4 shows that [D-Phe-I^7]ACTH-(4–10), [D-Arg^8]ACTH-(4–10) and [Pro^{8,10},Gly^9]ACTH-(4–10) inhibited the induction of excessive grooming behavior when co-administered with α-MSH. [Ala^6]ACTH-(4–10) did not inhibit α-MSH-induced grooming behavior. When the dose of α-MSH was raised from 1.5 to 5 μg, [D-Phe-I^7]ACTH-(4–10) at a dose of 15 μg was not able to inhibit the grooming response (Fig. 5). At 7.5 μg, [D-Phe-I^7]ACTH-(4–10) inhibited the effect of 1.5 μg α-MSH similarly as at 15 μg (data not shown).

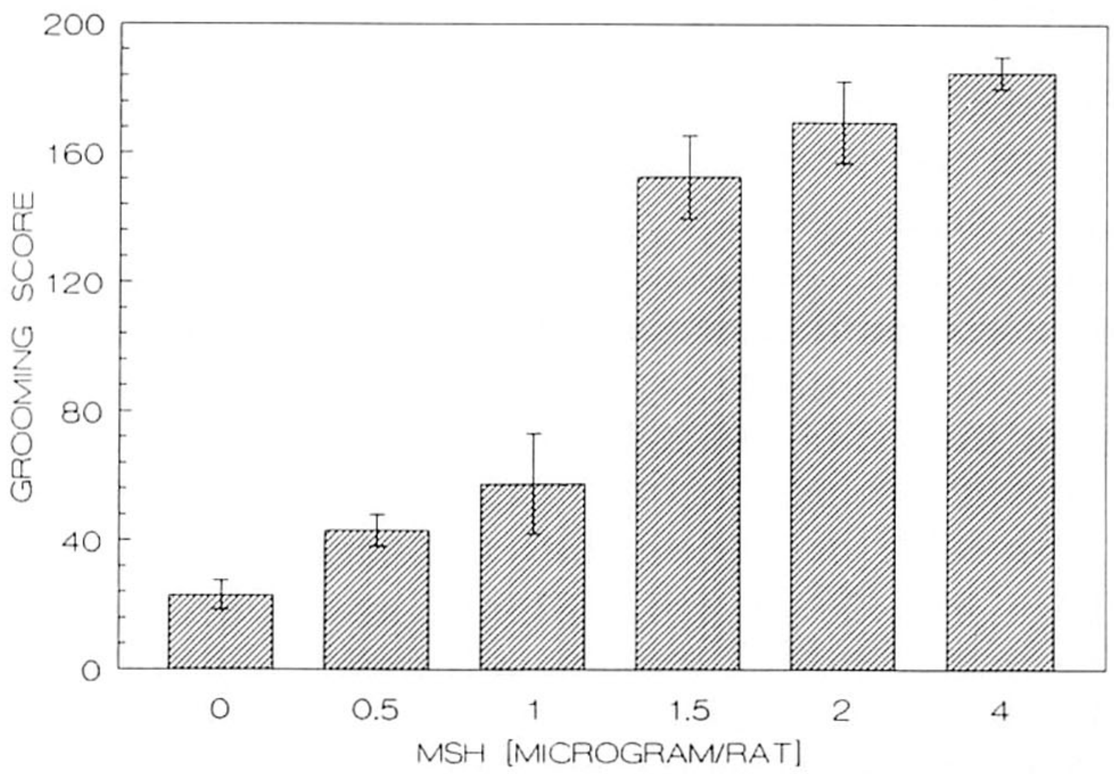

Fig. 3. Dose dependent effect of α-MSH to induce grooming behavior in rats. Rats received i.c.v. injection of either saline or different doses of α-MSH. The data are expressed as the mean with standard error of the mean.

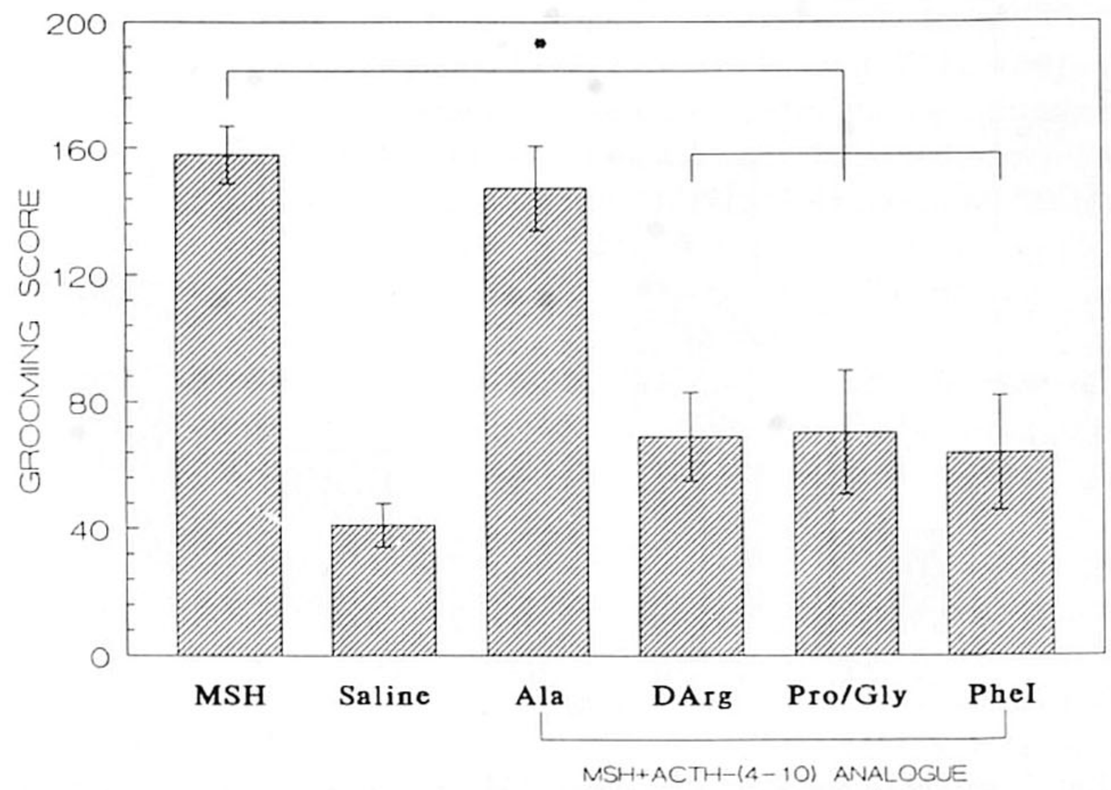

Fig. 4. The effect of ACTH-(4–10) analogues on α-MSH-induced excessive grooming behavior. Rats received i.c.v. saline, 1.5 μg α-MSH or mixtures of 1.5 μg α-MSH and 15 μg of one of the ACTH-(4–10) analogues indicated. The data are expressed as the mean with standard error of the mean. * Statistical significant difference as compared to α-MSH treatment (Student *t*-test; $P < 0.05$). PheI = [Phe-I^7]ACTH-(4–10); Ala = [Ala^6]ACTH-(4–10); DArg = [D-Arg^8]ACTH-(4–10); Pro/Gly = [Pro^{8,10},Gly^9]ACTH-(4–10).

4. Discussion

Antagonists are essential tools to understand the function of receptor subtypes in biological systems. Furthermore, many antagonists are used in the clinic to block the action of endogenous ligands. Thus, the characterization of receptor antagonists is essential. Although melanocortins have many actions, thus far no antagonists were available. This hampered the understanding of the mechanism of action of melanocortins. The cloning of melanocortin receptors opened new avenues, since the site of action of the different melanocortin receptor subtypes could be mapped and research on the development of selective agonists and antagonists could be accelerated.

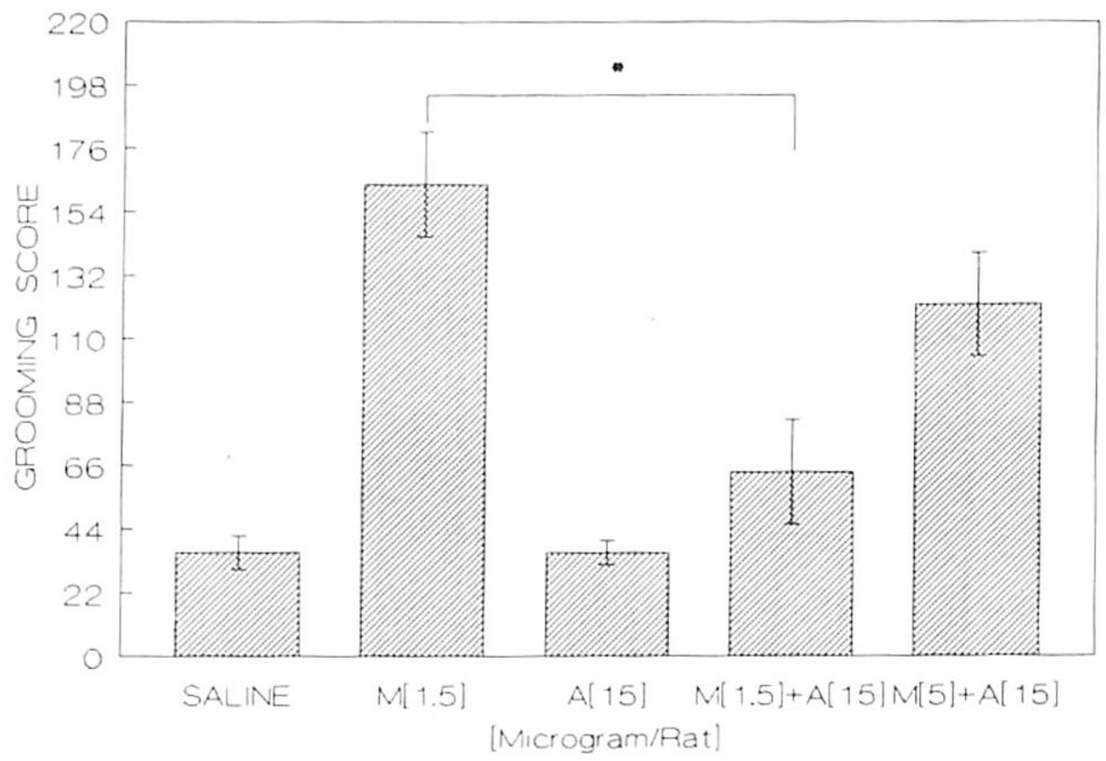

Fig. 5. Effect of the dose of α-MSH (M) on the inhibitory potency of [Phe-I^7]ACTH-(4–10) (A) in α-MSH-induced grooming behavior. Rats were scored for grooming activity following i.c.v. injections of either saline, [Phe-I^7]ACTH-(4–10), α-MSH or mixtures of [Phe-I^7]ACTH-(4–10) and α-MSH. The data are expressed as the mean with standard error of the mean. An asterisk *t*atistical significant difference as compared to α-MSH treatment (Student *t*-test; $P < 0.05$).

We took advantage of cloned melanocortin receptors expressed in cell lines in order to find antagonists for melanocortin receptor subtypes amongst a large collection of ACTH derivatives. Four analogues were able to antagonize melanocortin receptors with different selectivities for melanocortin MC_3, MC_4 and MC_5 receptors. [Phe-I^7]ACTH-(4–10) antagonized the activation of all three receptors by α-MSH. This suggests that modification of Phe^7 in melanocortin peptides to Phe-I changed the activity of ACTH-(4–10) from an agonist (Rehfuss-Roselli et al., 1993; Gantz et al., 1993a,b; Mountjoy et al., 1994) to an antagonist. The introduction of an iodine may lead to steric hindrance of the interaction of Phe with the receptor that usually is needed to activate the receptor. Replacement of His^6 by Ala in ACTH-(4–10), resulted in a peptide that antagonized the melanocortin MC_3 and MC_5 receptors far better than the melanocortin MC_4 receptor. [D-Arg^8]ACTH-(4–10) and [Pro^{8,10},Gly^9]ACTH-(4–10) antagonized the MC_5 receptor equally well, whereas the melanocortin MC_4 receptor was more potently antagonized by [D-Arg^8]ACTH-(4–10) than by [Pro^{8,10},Gly^9]ACTH-(4–10). The melanocortin MC_3 receptor was not antagonized by these two ACTH-(4–10) analogues. Thus, the latter ACTH analogues did not antagonize the melanocortin MC_3 receptor, but antagonized both the melanocortin MC_4 and MC_5 receptors. These two melanocortin receptor antagonists can therefore be used to investigate the involvement of the melanocortin MC_4 and MC_5 receptors in α-MSH-induced responses. Since the potency of [Ala^6]ACTH-(4–10) on antagonizing the melanocortin MC_4 receptor is low as compared to its antagonistic effect on the melanocortin MC_3 MC_5 receptors, this property provides a way to discriminate between activation of the melanocortin MC_4 and MC_5 receptors versus the melanocortin MC_3 receptor.

In vitro, the presented ACTH-(4–10) analogues at a dose of 1 μM peptide, could not antagonize the activation of the melanocortin receptors by 1 μM α-MSH. This, together with the fact that these melanocortin receptor antagonists lead to a shift to the right of the dose response curve of α-MSH on the cloned melanocortin receptors, demonstrates that these ACTH-(4–10) analogues are competitive antagonists. The induction of excessive grooming behavior at a higher dose of α-MSH (5 μg) was not antagonized by [Phe-I^7]ACTH-(4–10). This illustrates that also in vivo this ACTH-(4–10) analogue acts as a competitive antagonist.

The induction of excessive grooming behavior has been used extensively to study structure activity relationship of melanocortins on behavior (Spruijt et al., 1985; Gispen et al., 1975). It was observed that the structure activity relationship of melanocortin peptides to induce excessive grooming behavior in rats had a similarity to that of the stimulation of pigment dispersion in amphibian melanophores: [Nle^4,D-Phe^7]-α-MSH was the most potent peptide whereas γ-MSH had no or very low activity in these assays (Spruijt et al., 1985, 1992; Gispen et al., 1975). Indeed within the melanocortin receptor family, the structure activity relationship of the melanocortin MC_4 receptor resembles that of the MC_1 receptor (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992; Gantz et al., 1993a; Gantz et al., 1993b; Rehfuss-Roselli et al., 1993; Mountjoy et al., 1994). Furthermore, the structure activity relationship of melanocortin peptides to stimulate cAMP accumulation in rat brain slices resembled that of the structure activity relationship of these peptides on inducing excessive grooming behavior (Florijn et al., 1993). The melanocortin MC_4 receptor is expressed in the brain regions (Mountjoy et al., 1994) that were used by Florijn et al. (1993) to demonstrate the melanocortin effect on cAMP accumulation. Taken together these data suggest that the melanocortin MC_4 receptor mediates α-MSH-induced excessive grooming behavior. Our present data, showing that the melanocortin MC_4 receptor antagonists [Phe-I^7]ACTH-(4–10), [D-Arg^8]ACTH-(4–10) and [Pro^{8,10},Gly^9]ACTH-(4–10) inhibited the induction of excessive grooming behavior by α-MSH in rats supports this suggestion (Fig. 4). [Ala^6]ACTH-(4–10), which antagonized the melanocortin MC_4 receptor only weakly, did not block the grooming response induced by α-MSH. Furthermore, the melanocortin MC_4 receptor is expressed in the periaquaductal grey (Mountjoy et al., 1994), a site where local injections of ACTH-(1–24) induced excessive grooming behavior (Spruijt et al., 1986).

We here describe a first generation of melanocortin receptor antagonists for the melanocortin MC_3, MC_4 and MC_5 receptors. Although the activity of these antagonists is relatively low in vitro, these peptides displayed selectivity and sufficient potency to block a behavioral response induced by α-MSH. These peptides can now serve as lead compounds to design more potent and more selective antagonists. This will pave the way to understand better the function of specific melanocortin receptors in the (patho)physiology of the brain.

Acknowledgement

We thank Cor Snel for his technical assistance in performing the grooming assay.

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