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Discovery of novel melanocortin$_4$ receptor selective MSH analogues

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1 We synthesized a novel series of cyclic melanocyte stimulating hormone (MSH) analogues and tested their binding properties on cells transiently expressing the human melanocortin$_1$ (MC_1), MC_3, MC_4 and MC_5 receptors.

2 We discovered that compounds with 26 membered rings of [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11) displayed specific MC_4 receptor selectivity. The preference order of the different MC receptor subtypes for the novel [Cys^{4}D-Nal^{7}Cys^{11}]α-MSH(4–11) analogues are distinct from all other known MSH analogues, particularly as they bind the MC_4 receptor with high and the MC_1 receptor with low relative affinities.

3 HS964 and HS014 have 12 and 17 fold MC_4/MC_3 receptor selectivity, respectively, which is much higher than for the previously described cyclic lactam and [Cys^4,Cys^{10}]α-MSH analogues SHU9119 and HS9510.

4 HS964 is the first substance showing higher affinity for the MC_5 receptor than the MC_1 receptor.

5 HS014, which was the most potent and selective MC_4 receptor ligand (K_i 3.2 nM, which is ~300 fold higher affinity than for α-MSH), was also demonstrated to antagonize α-MSH stimulation of cyclic AMP in MC_4 receptor transfected cells.

6 We found that a compound with a 29 membered ring of [Cys^3,Nle^{10},D-Nal^7,Cys^{11}]α-MSH(3–11) (HS010) had the highest affinity for the MC_3 receptor.

7 This is the first study to describe ligands that are truly MC_4 selective and a ligand having a high affinity for the MC_3 receptor. The novel compounds may be of use in clarifying the physiological roles of the MC_3, MC_4 and MC_5 receptors.

Keywords: Melanocortin (MC) receptor subtypes; MSH (melanocyte stimulating hormone); cyclic MSH analogues; ligand binding; cyclic AMP; HS964; HS014

Introduction

The melanocortic peptides, ACTH (adrenocorticotropin) and MSH (melanocyte stimulating hormone), are primarily known for their role in the regulation of adrenal steroid production (Simpson & Waterman, 1988), and skin pigmentation (Eberle, 1988). The MSH and ACTH peptides also induce a variety of central and peripheral effects. The melanocortins have, for example, been shown to affect memory, behaviour, inflammation, pyretic control, pain perception, blood pressure, nerve growth and regeneration, and to influence events surrounding parturition (Eberle, 1988; O'Donahue & Dorsa, 1982).

Molecular cloning of five melanocortin receptor subtypes (MC_1–MC_5) (Chhajlani & Wikberg, 1992; Mountjoy *et al*., 1992; Gantz *et al*., 1993a,b; Chhajlani *et al*., 1993) has provided tools for systematic studies of the molecular mechanisms underlying the above mentioned effects. The natural hormones are not known to be subtype selective, except that α-MSH is selective for the MC_1 receptor and ACTH is selective for the MC_2 receptor. The MC_2 receptor is distinguishable from the other MC receptor subtypes in that it does not bind to the MSH peptides. The MC_3 receptor has a slight preference for the γ-MSH, and the MC_4 receptor for β-MSH. Still these natural hormones are not selective for these MC receptor subtypes as they both have highest affinity for the MC_1 receptor (Schiöth *et al*., 1995; 1996a,b; Siegrist & Eberle, 1995).

The physiological roles of the newly discovered receptors are not fully known but it has been speculated that they might participate in eliciting the various peripheral and central effects of melanocortins. The MC_3 receptor is mainly expressed in the brain, but it is also present in the periphery where it has been found in the placenta, gut tissues and the human heart (Gantz *et al*., 1993a; Chhajlani, 1996). The MC_4 receptor is found only in the central nervous system, where it is widely distributed, including in the cortex, thalamus, hypothalamus, brain stem and spinal cord (Gantz *et al*., 1993b; Mountjoy *et al*., 1994). Recent findings with knock-out techniques (Huszar *et al*., 1997) and i.c.v. injections of the cyclic MSH analogues SHU9119 and MTII (Fan *et al*., 1997) relate the MC_4 receptor to feeding behaviour and weight homeostasis. The MC_5 receptor is also found in the brain but, more importantly, it has a wide peripheral distribution, although its physiological role is still much less well defined (Labbé *et al*., 1994; Barrett *et al*., 1994).

The demand for substances that discriminate between the newly discovered MC receptors is high as such compounds would help to clarify the physiological roles of these receptors. MC_4 receptor-selective substances might also have a potential in the treatment of eating disorders (e.g. overweight, anorexia and bulimia) where good pharmacological remedies are presently not available.

In this study, we designed and synthesized a novel series of cyclic MSH analogues and tested these on cells expressing the cloned human MC_1, MC_3, MC_4 and MC_5 receptors.

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Methods

Peptide synthesis

The peptides tested in this study (except NDP-MSH) were synthesized by use of the solid phase approach and purified by high performance liquid chromatography (h.p.l.c). The correct molecular weights of the peptides were confirmed by mass spectrometry. The peptide sequences were assembled with a Pioneer peptide synthesis system (PerSeptive Biosystems). Fmoc (9-fluorenylmethoxycarbonyl)-amino acid derivatives were used in coupling steps. When OPfp (pentafluorophenyl) esters were used the synthesis cycle was as follows: (a) the Fmoc group was removed by 20% piperidine in DMF (N,N-dimethylformamide) (5 min); (b) to form a new peptide bound side chain protected Fmoc-amino acid OPfp ester (4 eq.) and HOAt (1-hydroxy-7-azabenzotriazole) (4 eq.) were dissolved in DMF and circulated through the reaction column for 30–60 min; (c) to cap residual amino groups the support was treated with 0.3 M Ac_2O (acetic anhydride) in DMF for 5 min. If free acids were used only step (b) was different: side chain protected Fmoc-amino acid (4 eq.), HATU (O-[7-azabenzotriazol-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate) (4 eq.) and DIEA (N,N-diisopropylethylamine) (4 eq.) were applied. For the deprotection a reagent mixture (TFA (trifluoroacetic acid)-phenol-anisole-1,2-ethanedithiol-water, 92 : 2 : 2 : 2 : 2) was used for 2.5 h. The S-S bond was formed by dissolving the product in a minimal amount of DMSO (dimethylsulphoxide) and heating at 65°C under argon for 36 h. The raw peptides were purified by h.p.l.c. (10×250 mm Vydac RP C18, 90A 201HS1010 column eluted with 20–30% MeCN (acetonitrile) in water + 0.1% TFA and detection at 240 nm).

Expression of receptor clones

The human MC_1 and human MC_5 receptor (Chhajlani & Wikberg, 1992; Chhajlani *et al*., 1993) were cloned into the expression vector pRc/CMV (InVitrogen). The human MC_3 and human MC_4 receptor DNAs, cloned into the expression vector pCMV/neo, were gifts from Dr Ira Gantz (Gantz *et al*., 1993a,b). For receptor expression, COS-1 (*C*V-1 *O*rigin, *S*V40) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eight percent confluent cultures were transfected on 100 mm cell culture dishes with the DNA (approximately 1 μg DNA for every 1×10^6 cells) mixed with liposomes in serum free medium (for details see Schiöth *et al*., 1996b). After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged and used for radioligand binding.

Binding studies

The transfected cells were washed with binding buffer (see Schiöth *et al*., 1995) and distributed into 96 well plates. The cells were then incubated for 2 h at 37°C (Siegrist *et al*., 1989) with 0.05 ml binding buffer in each well, containing a constant concentration of [^{125}I]-NDP-MSH and appropriate concentrations of an unlabelled ligand. After incubation the cells were washed with 0.2 ml of ice-cold binding buffer and detached from the plates with 0.2 ml 0.1 N NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with a software package for radio ligand binding analyses (Wan System, Umeå, Sweden). Data were analysed by fitting it to formulae derived from the law of mass-action by the method generally referred to as computer modelling. The binding assays were performed in duplicate wells and repeated three times. Untransfected COS-1 cells did not show any specific binding to [^{125}I]-NDP-MSH. The non specific binding was less than 5% of the total binding at 2 nM [^{125}I]-NDP-MSH when determined in presence of 3 μM unlabelled NDP-MSH.

Cyclic AMP assay

The transfected cells and the melanoma B16 cells (cultivated in the same media as the COS cells (see above)) were harvested and incubated for 30 min at 37°C with 0.05 ml serum free Dulbecco's modified Eagle's medium in each tube, containing 0.5 mM IBMX (isobutylmethylxantine) and appropriate concentrations of α-MSH or HS014. After incubation with the indicated drugs, cyclic AMP (adenosine 3′ : 5′-cyclic monophosphate) was extracted with perchloric acid at final concentration 0.4 M. After centrifugation, the protein free supernatants were neutralized with 5 M KOH/1 M Tris (tris-(hydroxymethyl)aminomethane). Then 0.05 ml of the neutralized cyclic AMP extract or a cyclic AMP standard (dissolved in distilled water) was added to a 96 well microtitre plate. The content of cyclic AMP was then estimated essentially according to Nordstedt & Fredholm (1990), by adding to each well [^{3}H]-cyclic AMP (0.14 pmol, approximately 11,000 c.p.m., specific activity 54 Ci mmol^{-1}, Amersham) and bovine adrenal binding protein and incubating at 4°C for 150 min. Standards containing unlabelled cyclic AMP were also assayed concomitantly with the samples. The incubates were thereafter harvested by filtration on Whatman GF/B filters by a semiautomatic Brandel cell harvester. Each filter was rinsed with 3 ml 50 mM Tris/HCl pH 7.4. The filters were punched out and put into scintillation vials with scintillation fluid and counted. The cyclic AMP assays were performed in duplicate wells and repeated three times.

Chemicals

[Nle^4,D-Phe^7]α-MSH (NDP-MSH) (Sawyer *et al*., 1980) (Bachem, Switzerland) was radio iodinated by the chloramine T method and purified by h.p.l.c. β-Cyclohexyl-D-alanine (D-Cha), p-benzoyl-D-phenylalanine (D-Bpa) and β-(2-naphthyl)-D-alanine (D-Nal) were purchased from Bachem, Switzerland. All other amino acid derivatives and chemicals for peptide synthesis (unless specified otherwise) were purchased from PerSeptive Biosystems (Kebo, Sweden). All medium and serum for cell cultivation were purchased from Gibco-BRL (Life Technologies, Sweden). All other chemicals were purchased from Sigma-Aldrich (Sweden), unless specified otherwise.

Results

We designed and synthesized a new series of cyclic MSH analogues which have a disulphide bridge between Cys residues in position 4 and 11. This ring includes an extra Gly in position 10 as compared to the earlier known cyclic [Cys^4,Cys^{10}]α-MSH analogues. This ring includes 8 amino acids or totally 26 atom members when the slide chains of Cys are accounted. The structure of the novel substances are aligned with α-MSH, NDP-MSH, [Cys^4,Cys^{10}]α-MSH(1–13), HS9510, MTII and SHU9119 in Table 1. The human DNAs for the MC_1, MC_3, MC_4 and MC_5 receptors were transiently and independently expressed in COS-1 cells for competitive receptor binding with [^{125}I]-NDP-MSH as radioligand. The expression levels of the different receptor subtypes were similar

(data not shown). The K_i values for the different peptides resulting from calculations of the competition curves of binding with [^{125}I]-NDP-MSH are summarized in Table 2. For comparison, we also included in Table 2 the K_i values for α-MSH, NDP-MSH, [Cys^4,D-Phe^7,Cys^{10}]α-MSH(1–13), HS9510, MTII and SHU9119, the values of which we recently demonstrated (Schiöth *et al*., 1997a,b) by use of the same method as in the present study. Competition curves for HS963, HS964, HS007, HS010, HS012 and HS014 obtained on cells expressing the human MC_1, MC_3, MC_4 and MC_5 receptors are shown in Figure 2.

HS963, HS964, HS005 and HS006 are all [Cys^4,X^7,Cys^{11}]α-MSH(4–11) analogues and differ only in position 7. HS964, which contains D-Nal^7 (β-(2-naphthyl)-D-alanine) (Figure 1), exhibited the highest affinity for the MC_4 receptor and showed 12, 63 and 7 fold selectivity compared to the MC_1, MC_3 and MC_5 receptors, respectively. Moreover, HS964 showed a preferential order $MC_4 > MC_5 > MC_3 > MC_1$, which is a preference order not shared by any other known melanocortic peptide. HS963, which contains D-Phe^7, proved not to be MC_4 receptor-selective. It showed a 44 fold lower affinity for the MC_4 receptor than HS964 and also had much lower affinities for the MC_3 and the MC_5 receptors, but higher affinity for the MC_1 receptor. HS005, which contains

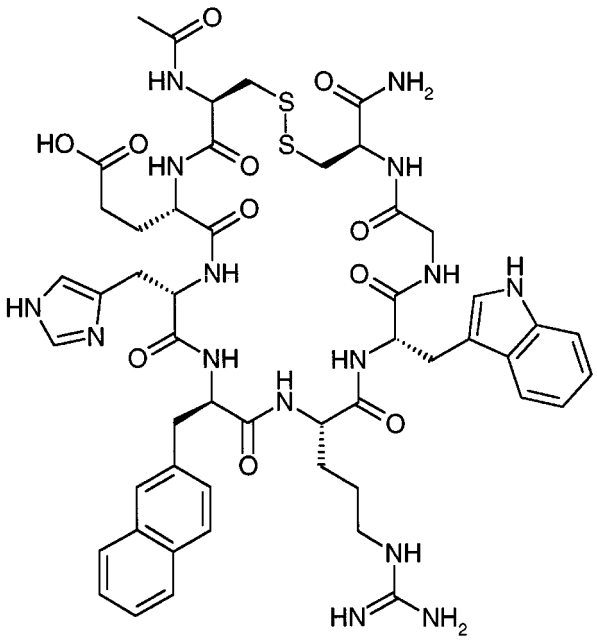

Figure 1 Structure of the MC_4 receptor-selective analogue; HS694 (cyclic [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11)).

Table 1 Alignment of α-MSH, NDP-MSH, cyclic [Cys^4,Cys^{10}]α-MSH(1–13), HS9510, MTII and SHU9119 to the new analogues evaluated in this study

	Position														
Peptide	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
α-MSH	Ser	Tyr	Ser	Met	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val		
NDP-MSH	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val		
HS9510				*Cys*	Glu	His	D-Nal	Arg	Trp	*Cys*					
MTII				Nel	*Asp*	His	D-Phe	Arg	Trp	*Lys*					
SHU9119				Nle	*Asp*	His	D-Nal	Arg	Trp	*Lys*					
HS964				*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS963				*Cys*	Glu	His	D-Phe	Arg	Trp	Gly	*Cys*				
HS005				*Cys*	Glu	His	D-Cha	Arg	Trp	Gly	*Cys*				
HS006				*Cys*	Glu	His	D-Bpa	Arg	Trp	Gly	*Cys*				
HS007				*Cys*	Arg	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS009				*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS011				*Cys*	Glu	Ala	D-Nal	Arg	Trp	Asp	*Cys*				
HS010			*Cys*	Nle	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS012			Nle	*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*				
HS014				*Cys*	Glu	His	D-Nal	Arg	Trp	Gly	*Cys*	Pro	Pro	Lys	Asp

All peptides have an acetyl-group on the N-terminus and an amide group on the C-terminus. The amino acid residues which make up the ring closure in the cyclic compounds are shown underlined in italics.

Table 2 K_i values of MSH analogues obtained from computer analysis of competition curves on human MC_1, MC_3, MC_4 and MC_5 receptor transfected COS cells

Ligand	*MC_1*	*MC_3*	*MC_4*	*MC_5*
α-MSH*	0.230±0.089	31.5±3.9	900±97	7160±860
NDP-MSH*	0.109±0.010	0.469±0.038	2.93±0.34	5.50±0.11
MTII*	0.686±0.109	34.1±4.4	6.60±0.82	46.1±7.9
SHU9119*	0.714±0.161	1.20±0.30	0.360±0.059	1.12±0.31
HS9510*	148±48	216±47	37.0±5.2	5150±530
HS964	1460±440	281±40	23.2±4.4	164±27
HS963	468±109	3120±610	1780±220	6040±700
HS005	10000±4700	5880±1280	1000±230	7770±1240
HS006	6490±1940	6440±1110	2760±480	3440±1210
HS007	200±72	8.14±3.55	10.1±4.1	42.7±9.4
HS009	69200±36000	68800±13000	5170±920	91400±24600
HS011	2030±1060	17.1±2.9	4.01±1.48	405±35
HS010	170±38	48.5±15.5	179±58	253±91
HS012	396±170	140±22	33.2±6.3	120±25
HS014	108±62	54.4±37.6	3.16±1.16	694±237

Data are presented as means±s.e.mean ($n=3$). *Data from Schiöth *et al*. (1997), by courtesy of the publisher.

D-Cha[7] (β-cyclohexyl-D-alanine), had much lower affinity than HS964 for all the MC receptors. Still, HS005 is MC_4 receptor-selective, as it had about 6 fold higher affinity for the MC_4 receptor than the MC_3 receptor. HS006, which contains D-Bpa[7] (p-benzoyl-D-phenylalanine), also had the highest relative affinity for the MC_4 receptor, although it showed much lower all over affinities than HS964 for all the MC receptors. HS006 showed about 2 fold selectivity over the MC_3 receptor, although it had 120 fold lower affinity for the MC_4 receptor compared to HS964.

Three of the peptides (HS007, HS009 and HS011) are based on HS964, but differ in positions 5, 6 and 10. HS007,

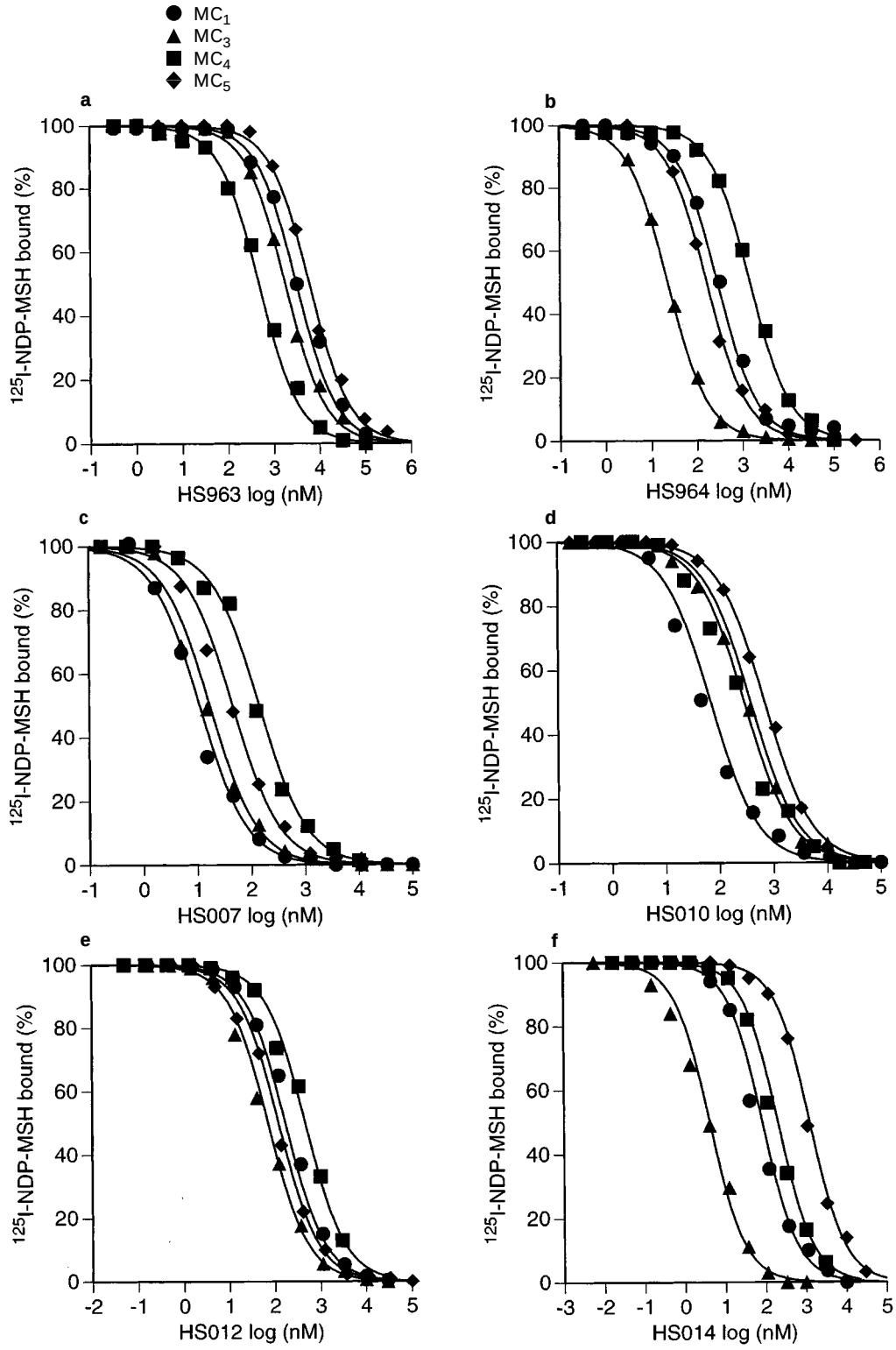

Figure 2 Competition curves for (a) HS963, (b) HS964, (c) HS007, (d) HS010, (e) HS012 and (f) HS014 obtained on COS-1 cells transfected with the MC_1, MC_3, MC_4 or MC_5 receptor clones, obtained by using a fixed concentration of [^{125}I]-NDP-MSH and varying concentrations of the unlabelled competing peptide. Competing peptides used are indicated on abscissa scale. Each experiment was performed in duplicate and repeated three times.

in which the acidic hydrophilic Glu^5 is replaced by the basic hydrophilic Arg^5, had about 2 fold higher affinity for the MC_4 receptor than HS964. Interestingly, HS007 had a 35 fold higher affinity for the MC_3 receptor than HS964. Thus, HS007 had similar or slightly higher affinity for the MC_3 receptor than the MC_4 receptor. HS009, where the non polar Gly^{10} is replaced with the acidic hydrophilic Asp^{10}, had 13 fold selectivity for the MC_4 receptor when compared to the MC_3 receptor, but it showed 220 fold lower affinity for the MC_4 receptor than HS964, as well as much lower affinity for the other MC receptor subtypes. In HS011, the basic hydrophilic His^6 is replaced by less polar and slightly hydrophobic Ala^6. HS011 had a similar affinity profile to that of HS964, although it had 16 and 6 fold higher affinity for the MC_3 and MC_4 receptors, respectively, and slightly lower affinities for the MC_1 and MC_5 receptors compared to HS964.

Further expansion of the ring size was attempted in HS010 ([Cys^3,Nle^4,D-Nal^7,Cys^{11}]α-MSH(3–11)) where Nle^4 is added into the ring. This lowered the affinity for the MC_4 receptor but increased it for the MC_3 receptor. HS010 had indeed highest affinity for the MC_3 receptor when compared to its affinity for the other evaluated MC receptors; HS010 showed 4 fold MC_3/MC_1, 4 fold MC_3/MC_4 and 5 fold MC_3/MC_5 selectivity, respectively.

Two peptides were made, which have either C- or N-terminal extensions to the core cyclic structure of HS964. HS012 has Nle^3 on the N-terminal end and HS014 has a 4 amino acid C-terminus which is identical to that of β-MSH. HS012 had very similar affinity profile to HS964 but slightly lower MC_4 receptor selectivity. HS014 had about 7 fold higher affinity for the MC_4 receptor, 14 fold higher affinity for the MC_1 receptor and 5 fold higher affinity for the MC_3 receptor compared to HS964, but similar affinity for the MC_5 receptor. It is also the most MC_4 receptor-selective of all the evaluated compounds, its selectivity for the MC_4 receptor being 34, 17 and 220 fold higher than that for the MC_1, MC_3 and MC_5 receptors, respectively.

We tested the cyclic AMP response of α-MSH and HS014 in COS-1 cells expressing the human MC_1, MC_3, MC_4 and MC_5 receptors (see Figure 3) and in murine B16 melanoma cells (see Figure 4). As can be seen from the figures α-MSH stimulated accumulation of cyclic AMP in all the cell types. For the MC_3 and MC_4 receptor expressing cells HS014, in concentrations up to 100 μM, did not affect the cyclic AMP levels. Instead 1 μM HS014 was found to block completely the cyclic AMP increasing action of α-MSH (Figure 3). In the murine B16 melanoma cells (presumed MC_1 receptor effect) (Figure 4), and in the COS cells transiently expressing MC_1 and MC_5 receptors (Figure 3) the addition of HS014 caused a dose-dependent

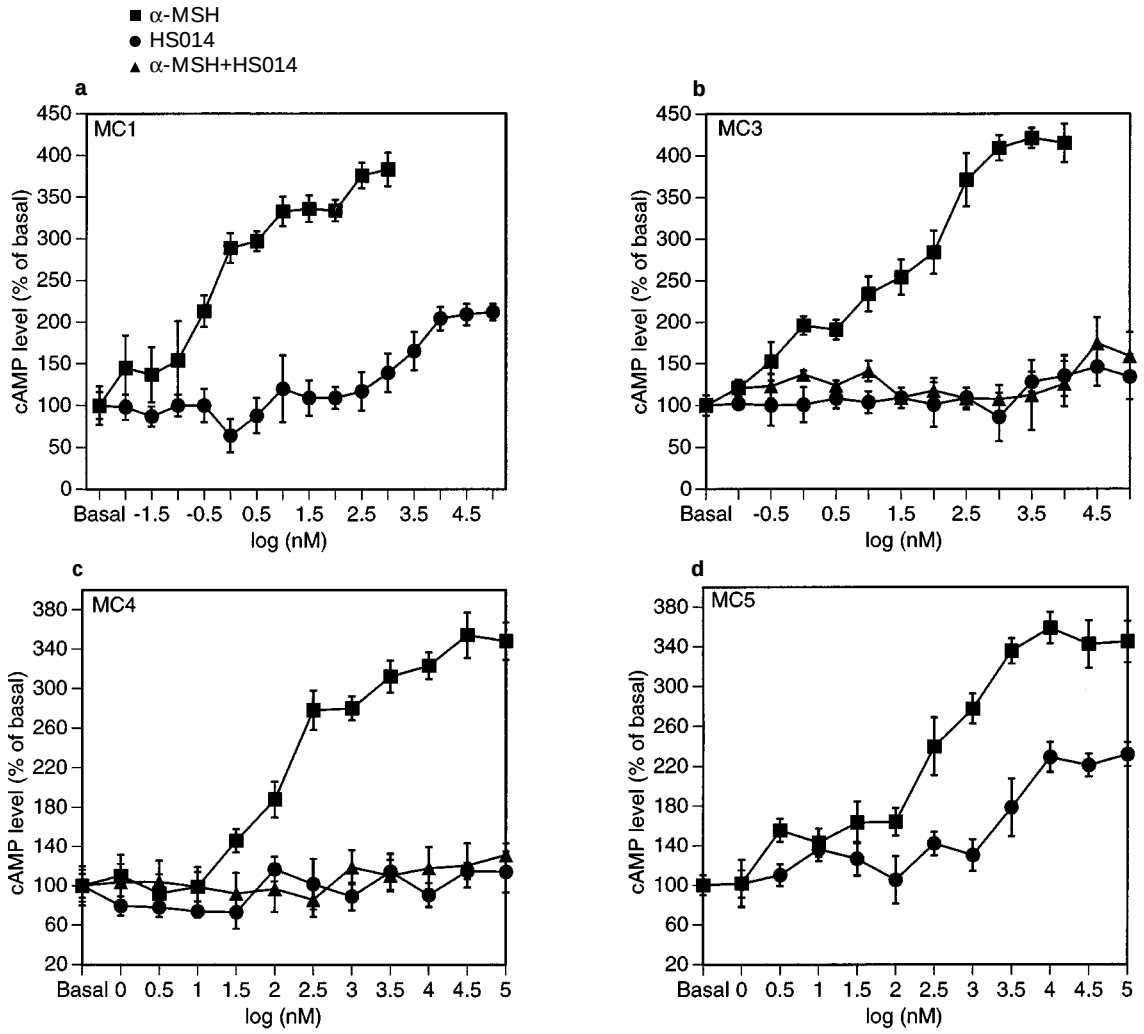

Figure 3 Generation of cyclic AMP in response to α-MSH, HS014 or α-MSH + 1 μM HS014 for the (a) MC_1, (b) MC_3, (c) MC_4 and (d) MC_5 receptors in transfected COS-1 cells. Each point represents the mean and vertical lines show s.e.mean ($n = 6$).

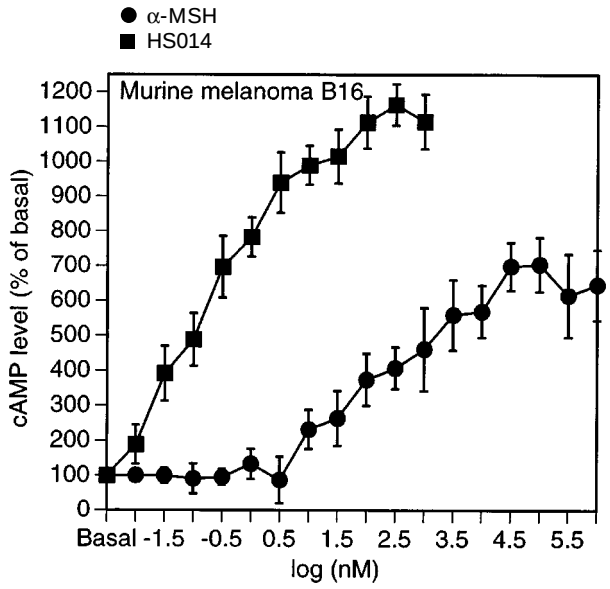

Figure 4 Generation of cyclic AMP in response to α-MSH and HS014 for murine B16 melanoma cells. Each point represents the mean and vertical lines show s.e.mean ($n=6$).

increase in the cyclic AMP levels. However, the increase induced by HS014 did not, in these cases, reach the same maximum levels as did α-MSH. Moreover, HS014 appeared also to be less potent than α-MSH in increasing cyclic AMP via the MC_1 and MC_5 receptors.

Discussion

A large amount of data have for a long time been accumulated about the structure-activity relationships of different MSH analogues from tests on the biological responses of frog and lizard melanophores, and on melanoma cells, systems which are presumed to involve the MC_1 receptor. The most potent linear MSH analogue obtained from these earlier studies was the α-MSH analogue NDP-MSH (Sawyer *et al*., 1980). Later, it was shown that cyclic [Cys^4,Cys^{10}]α-MSH analogues are also very potent and may be more long acting and more stable against enzymatic degradation than the linear MSH analogues (Sawyer *et al*., 1982; Knittel *et al*., 1983). Further development led to the invention of cyclic lactam analogues, like MTII and later SHU9119 (Al-Obeidi *et al*., 1989; Hruby *et al*., 1995). The lactam analogues differ from [Cys^4,Cys^{10}]α-MSH as their ring structure is made of a lactam bridge between the Asp and Lys side chains instead of a disulphide bridge between two Cys residues. The ring of both the [Cys^4,Cys^{10}]α-MSH and the lactams have 23 members. NDP-MSH, cyclic [Cys^4,Cys^{10}]α-MSH analogues and MTII, which are all very potent in melanophore assays, were developed before the cloning and identification of the MC receptor subtypes. In a more recent study, we found that cyclic [Cys^4,Cys^{10}]α-MSH(4–10) analogues favour binding to the MC_4 receptor (Schiöth *et al*., 1997a). Moreover, the replacement of D-Phe^7 by D-Nal^7 in [Cys^4,Cys^{10}]α-MSH(4–10), resulted in a novel compound, HS9510, with increased selectivity for the MC_4 receptor. In an earlier study the cyclic lactam analogue SHU9119, which also contains D-Nal^7, was found to show some selectivity for the MC_4 receptor (Hruby *et al*., 1995), but in our assays its selectivity proved to be only 2 fold over the MC_3 receptor (Schiöth *et al*., 1997a).

In the present study, we discovered that increasing the ring size of [Cys^4,D-Nal^7,Cys^{10}]α-MSH(4–10) by adding one amino acid (Gly^{10}) greatly favoured selectivity for the MC_4 receptor. Gly^{10} is not expected to play an important role in the binding of MSH peptides, at least not for the MC_1 and MC_3 receptors (Sahm *et al*., 1994a,b). The 26 membered cyclic [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11) analogue (HS964) had more than 12 fold MC_4/MC_3 selectivity, and more than 60 fold MC_4/MC_1 selectivity. Our results show that both the ring size and the D-Nal^7 is crucial to obtain these selective properties. Replacement of the bulky hydrophobic and aromatic D-Nal^7 with D-Phe^7 or with the bulky hydrophobic D-Cha^7 or with the bulky aromatic and hydrophobic D-Bpa^7 abolished the selective binding properties and lowered the affinities, in particular for the MC_4 receptor.

Glu^5 as well as Gly^{10} is believed not to be of major importance for the binding of MSH peptides to the MC receptors (Sahm *et al*., 1994a,b). These residues might serve as coupling sites for tails which may increase the selectivity or the affinity of synthetic peptides. Our results indicate that the acidic and polar Asp^{10} does not favour the binding of the 26 membered cyclic peptide for all the receptors, without affecting the selectivity between the subtypes. On the other hand, the basic hydrophilic Arg^5 did not greatly affect the affinities except that it favoured the binding to the MC_3 receptor. This observation could be useful in the design of truly MC_3 receptor-selective substances.

His^6 together with Phe^7-Arg^8-Trp^9 make up the central core binding sequence of the MSH peptides. However, His^6 is probably less important than Phe^7, Arg^8 and Trp^9 for the binding of α-MSH. Interestingly, we have here observed that a replacement of His^6 by Ala^6 in a 26-membered ring resulted in higher affinity for the MC_3 and MC_4 receptors, but not for the MC_1 and MC_5 receptors. It is also interesting to note that we have previously shown that His^6/Ala^6 exchange in the linear ACTH(4–10) peptide resulted in great loss of affinity for the MC_1 receptor, but not the other subtypes (Schiöth *et al*., 1997b), possibly indicating that the present cyclic peptide binds differently to the MC_1 receptor compared to the linear peptides. One of the major characteristics of the cyclic [Cys^4,D-Nal^7,Cys^{11}]α-MSH(4–11) peptides is the relatively low affinity for the MC_1 receptor compared to the natural linear MSH peptides. Taken together, this might indicate that the binding of His^6 to the MC_1 receptor is already disrupted in the 26 membered cyclic peptides.

Interestingly, a further increase of the cycle to the 29 member ring in HS010 resulted in MC_3 receptor-selective properties. It is tempting to speculate that the 23 member ring is optimal for the MC_1 receptor, where the MC_4 and the MC_3 receptors may favour 26 and 29 membered rings, respectively.

An N-terminal addition of Nle^3 (HS012) did not influence the binding affinities much, which may indicate that the Nle^3 position of the present cyclic series cannot mimic the binding interaction of the relatively important $Met^4(Nle^4)$ in the linear α-MSH. However, C-terminal addition of the *β*-MSH sequence Pro^{12}-Pro^{13}-Lys^{14}-Asp^{15} did increase the affinity for all the receptors. The affinity of HS014 for the MC_4 receptor is close to that of NDP-MSH and 300 fold higher than that of α-MSH.

The most selective and potent compound for the MC_4 receptor found in the present study, HS014, was also shown to antagonize α-MSH stimulation in MC_3 and MC_4 receptor-transfected cells. It is conceivable therefore that HS014 is an MC_3 and MC_4 receptor antagonist. However, for the MC_1 and MC_5 receptors, HS014 was found to increase intracellular levels of cyclic AMP, but without reaching maximum levels.

Thus, these results seem to indicate that HS014 is a partial agonist at the MC_1 and MC_5 receptors.

The MC_3 and the MC_4 receptors are probably those MC receptors which are most abundantly expressed in the brain (Roselli-Rehfuss *et al*., 1993; Chhajlani, 1996; Alvaro *et al*., 1996). We and others have previously shown that γ-MSH has a much higher affinity for the MC_3 receptor than for the MC_4 receptor. The MC_4 receptor has in general low affinity for the natural MSH peptides and none of the peptides have a higher affinity for the MC_4 receptor than for the MC_3 receptor. Our new peptides add a new dimension to the possibilities to discriminate between the MC_3 and MC_4 receptors; the most useful substance probably being HS014 as it was the most selective and potent compound for the MC_4 receptor. The MC_5 receptor mRNA has been identified in a number of peripheral tissues and more recently also in adipocytes (Boston & Cone, 1996). The MC_1 receptor is also considered to have primarily peripheral distribution, despite evidence of some minor expression in both human and rodent brain (Xia *et al*., 1995; Rajora *et al*., 1997). The natural MSH peptides have the same potency order for the MC_5 receptor as for the MC_1 receptor, but in general much lower affinities (about or more than 100 times lower) for the MC_5 receptor. HS964 and HS007 are the first substances which have a higher affinity for the MC_5 receptor than for the MC_1 receptor. The unique MC_5/MC_1 selectivity of HS964 makes it an interesting candidate for discrimination of the peripheral effects mediated by the MC_5 and MC_1 receptors.

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