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Short communication

Binding of cyclic and linear MSH core peptides to the melanocortin receptor subtypes

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Abstract

We report here the binding of 5-, 6- and 7-amino-acid-long linear and cyclic core peptides of MSH (melanocyte-stimulating hormone) to cells transiently expressing the human melanocortin MC_1, MC_3, MC_4 and MC_5 receptors. The results show that, in contrast to the natural peptides, the core peptides did not differentiate between the melanocortin MC_3 and MC_4 receptors. All tested cyclic peptides had much lower affinities than their corresponding linear homologues. Interestingly, the relative loss of binding due to the cyclisation did not change as the ring size decreased. Therefore, decreasing the ring size does not seem to force the peptide into a more unfavourable conformation.

Keywords: Melanocortin receptor subtype; MSH (melanocyte-stimulating hormone); Ligand binding

1. Introduction

Five different melanocortin receptor subtypes have been identified by us and others by use of molecular cloning (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a,b; Mountjoy et al., 1992). The presence of different subtypes of the melanocortin receptors may explain the variety of effects that are caused by melanocortic peptides (Eberle, 1988). The five melanocortin receptors show unique affinities for the melanocortic peptides (Low et al., 1994; Siegrist and Eberle, 1995; Schiöth et al., 1995, 1996a,b,c). The melanocortin MC_1 receptor shows high affinity for α-MSH (melanocyte-stimulating hormone), but lower affinities for β-MSH, γ-MSH and ACTH (adrenocorticotropin). The melanocortin MC_2 receptor (the ACTH receptor) binds ACTH with high affinity, but it does not bind the MSH peptides. The melanocortin MC_3 receptor shows slightly higher affinity for γ-MSH compared to β-MSH and α-MSH. The melanocortin MC_4 receptor shows slight preference for β-MSH over α-MSH and a very low affinity for γ-MSH. The melanocortin MC_5 receptor, finally, shows the same potency order for the MSH peptides as the melanocortin MC_1 receptor, although (at least for the human case) it binds the peptides with much lower affinities.

Early structure-activity studies using pigment dispersing activity of melanophores (presumed melanocortin MC_1 receptor mediated effect) identified the His-Phe-Arg-Trp as the core sequence for melanotropic activity (Eberle, 1988). This core is shared by the natural melanocortic peptides: α-MSH, β-MSH, γ-MSH and ACTH.

Currently, there are selective substances available for the MC_1 receptor subtype, like α-MSH and [Nle^4,D-Phe^7]α-MSH, but there are only few reports on specific analogues for the other subtypes. Cyclic lactam analogues (Hruby et al., 1995) and ACTH-(4–10) analogues (Adan et al., 1994b) were reported to show certain selectivity. More basic knowledge is needed about the binding of the different receptor subtypes to different regions and conformations of the MSH peptides to elucidate the subtype specific properties and to allow construction of selective compounds. Docking of long peptides into molecular models of the melanocortin receptors is simplified by adding a constraint (e.g. by formation of a ring) into the peptide ligand. A cyclic [Cys^4,D-Phe^7,Cys^{10}]α-MSH was used for

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ligand docking to a preliminary 3-dimensional model of the melanocortin MC_1 receptor (Prusis et al., 1995). Binding data for small cyclic MSH analogues should facilitate the interpretation of results from ligand docking experiments.

The aim of the present study was therefore to synthesise new cyclic and linear analogues based on the core MSH peptide and test the subtype specific binding using the human melanocortin MC_1, MC_3, MC_4 and MC_5 receptors transiently expressed in an eukaryotic cell line.

2. Materials and methods

2.1. Chemicals

[Nle^4,D-Phe^7]α-MSH (Sawyer et al., 1980) was purchased from Saxon Biochemicals, Germany. [Nle^4,D-Phe^7]α-MSH was radioiodinated by the chloramine T method and purified by HPLC (high performance liquid chromatography). His-(D-Phe)-Arg-Trp-Gly-NH_2 (HdFAWG), Asn-His-(D-Phe)-Arg-Trp-Gly-NH_2 (NHdFAWG), Met-Asn-His-(D-Phe)-Arg-Trp-Gly-NH_2 (MNHdFAWG) and their corresponding cyclic homologues, i.e., $HdFAWG_c$, $NHdFAWG_c$ and $MNHdFAWG_c$, were synthesised in our laboratories using the solid phase approach and purified by HPLC. The correct molecular weights of the peptides were confirmed by mass spectrometry. His-Phe-Arg-Trp-OH (HFAW) was purchased from Bachem, Switzerland. In the text, we use the position numbering of α-MSH: Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}.

2.2. Expression of receptor clones

The human melanocortin MC_1 and human melanocortin MC_5 receptors (Chhajlani and Wikberg, 1992; Chhajlani et al., 1993) were cloned into the expression vector pRc/CMV (InVitrogen). The human melanocortin MC_3 and human melanocortin MC_4 receptors, cloned into the expression vector pCMV/neo, were a gift 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. Eighty percent confluent cultures were transfected with the DNA mixed with liposomes in serum-free medium (for details, see Schiöth et al., 1996b). After transfection, the serum-free medium was replaced with the serum-containing medium and the cells were cultivated for about 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

2.3. 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 with 0.05 ml binding buffer in each well containing a constant concentration of [^{125}I][Nle^4,D-Phe^7]α-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 of 0.1 M NaOH. Radioactivity was counted (Wallac, Wizard automatic gamma counter) and data analysed with BindAid, a software package (Wan System, Umeå, Sweden). Data were either analysed by fitting them to formulas derived from the law of mass action by the method generally referred to as computer modelling, or by fitting to the four-parameter logistic function. K_i values were calculated by using the Cheng and Prusoff equation. The K_d values for [^{125}I][Nle^4,D-Phe^7]α-MSH for the melanocortin MC_1, melanocortin MC_3 and melanocortin MC_5 receptors were taken from Schiöth et al. (1995) and for melanocortin MC_4 from Schiöth et al. (1996b). 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][Nle^4,D-Phe^7]α-MSH.

3. Results

In order to probe the binding of the core of the MSH peptides to the melanocortin receptor we synthesised and evaluated three linear peptides, and three corresponding cyclic homologues. The smallest peptide was 5 amino-acids long that included the main MSH core: His^6-(D-Phe^7)-Arg^8-Trp^9 and Gly^{10}. The other peptides had in addition Asn^5 or Met^4-Asn^5 at the N-terminus. We used the D-isomer of Phe^7 as these peptides have higher affinity compared to L-Phe-containing compounds, as high affinity was a prerequisite to obtain reliable data. It may be pointed out here that [Nle^4,D-Phe^7]α-MSH is a peptide that shows the highest affinities of all ligands for all the melanocortin receptor subtypes, except for the melanocortin MC_2 receptors. According to earlier structure-activity data (Eberle, 1988), and reports of alanine scanning of the MSH peptide on the murine melanocortin MC_1 and the rat melanocortin MC_3 receptors (Sahm et al., 1994a,b), the Glu^5 and the Gly^{10} on each side of the MSH peptide main core are not very important for ligand binding. We included the Gly^{10} but replaced Glu^5 with Asn^5 to facilitate the synthesis of the $NHdFAWG_c$ and $MNHdFAWG_c$. The ring closure of the cyclic peptides was created by ordinary peptide bonds.

The K_i values for the linear and cyclic peptides were evaluated in competition with [^{125}I][Nle^4,D-Phe^7]α-MSH on melanocortin MC_1, MC_3, MC_4 and MC_5 receptor clones and the data obtained are given in Table 1. In Table 1 is also given the K_i value for [Nle^4,D-Phe^7]α-MSH obtained from our previous studies for comparison. The assays were performed using the same approach as we used earlier for the evaluation of several natural melanocortic peptides (Schiöth et al., 1995, 1996b). Com-

Table 1
K_i values (mean ± S.E.M) obtained from competition curves, for MSH analogues on human melanocortin MC_1, MC_3, MC_4 and MC_5 receptor transfected COS-1 cells together with relative affinity ratios

Ligand	K_i (nmol/l)				MC_3/MC_1	MC_4/MC_1	MC_5/MC_1
	MC_1	MC_3	MC_4	MC_5)			
[^{125}I]NDP-MSH [a]	0.0851 ± 0.0080 [b]	0.396 ± 0.065 [b]	3.84 ± 0.57 [c]	5.05 ± 1.00 [b]	4.6	45	59
MNHdFRWG	2.79 ± 0.08	350 ± 120	285 ± 34	962 ± 250	130	100	340
MNHdFRWG$_c$	355 ± 65	14 600 ± 2 700	8 400 ± 1 000	58 900 ± 8 200	41	24	170
NHdFRWG	399 ± 78	7 220 ± 870	6 110 ± 1 300	24 100 ± 12 000	18	15	60
NHdFRWG$_c$	2 730 ± 270	103 000 ± 17 000	73 300 ± 13 000	208 000 ± 39 000	38	27	76
HdFRWG	292 ± 53	7 760 ± 1 900	7 170 ± 980	61 200 ± 28 000	27	25	210
HdFRWG$_c$	3 210 ± 320	113 000 ± 21 000	71 400 ± 9 300	207 000 ± 45 000	35	22	64
HFRW	69 300 ± 19 000	> 300 000	> 300 000	> 300 000	–	–	–
MNHdFRWG$_c$/MNHdFRWG	130	42	30	61	0.31	0.24	0.50
NHdFRWG$_c$/NHdFRWG	6.8	14	12	8.6	2.1	1.8	1.3
HdFRWG$_c$/HdFRWG	11	15	10	3.4	1.3	0.88	0.30

[a] K_d values. Data taken from [b] Schiöth et al. (1995) and [c] Schiöth et al. (1996b).

petition curves for MNHdFRWG, MNHdFRWG$_c$, NHdFRWG, NHdFRWG$_c$, HdFRWG and HdFRWG$_c$ are shown in Fig. 1.

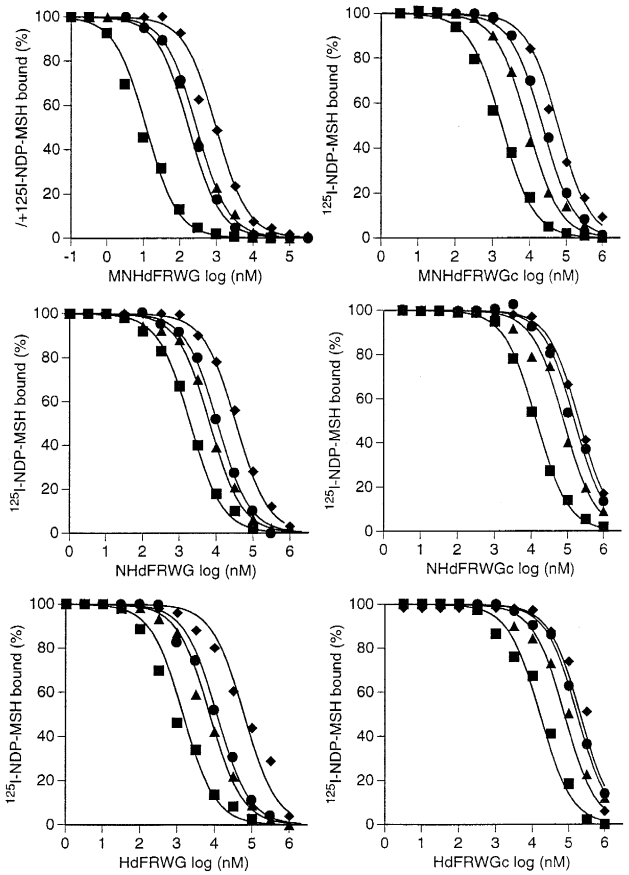

Fig. 1. Competition curves of MNHdFRWG, MNHdFRWG$_c$, NHdFRWG, NHdFRWG$_c$, HdFRWG, HdFRWG$_c$ on COS-1 cells transfected with the MC_1 (■), MC_3 (●), MC_4 (▼), or MC_5 (◆) receptor obtained by using a fixed concentration of ~2 nM [^{125}I][Nle^4,D-Phe^7]α-MSH and varying concentrations of the non-labelled competing peptide. Competing peptides used are indicated on the abscissa for each panel.

As can be seen from Table 1 the melanocortin MC_1 receptor shows the highest affinity for all the tested ligands. The melanocortin MC_3 receptor shows 18–130-fold lower affinity for the ligands compared to the melanocortin MC_1 receptor. The melanocortin MC_4 receptor shows similar or slightly higher affinities for the ligands, compared to the melanocortin MC_3 receptor. The melanocortin MC_5 receptor shows overall lower affinities for all the tested peptides.

Thus, the melanocortin MC_3 and the melanocortin MC_4 receptors show very similar affinities to the core peptides. This contrasts quite strongly to our recent results that show that the melanocortin MC_3 receptor has a 10-fold higher affinity for [Nle^4,D-Phe^7]α-MSH, a 30-fold higher affinity for α-MSH and an about 6000-fold higher affinity for γ-MSH, compared with the melanocortin MC_4 receptor.

In all cases, the cyclic peptides have lower affinities than the corresponding linear homologues. The difference is largest for the 7-amino-acid-long cyclic and linear peptides, especially in the case of the melanocortin MC_1 receptor. The affinity differences between the 6- and 5-amino-acid-long cyclic and linear peptides are in the same order of magnitude for all the receptors. Moreover, the 5- and the 6-amino-acid-long peptides show very similar affinities. Thus, the addition of the Asn^5 does not affect the affinities of the peptide for the different receptors.

In addition to the tests described above we also evaluated the binding of the α-MSH main core HFRW, which has an L-Phe^7, instead of the D-Phe^7 that was used for all the other core peptides evaluated in this study. As expected, HFRW showed much lower affinities for all the melanocortin receptors compared to the D-Phe^7 peptides.

4. Discussion

Each of the melanocortin receptor subtypes binds the natural melanocortic peptides with specific affinity. It is

not known how different these receptors bind the core of the MSH peptide (i.e. His^6-Phe^7-Arg^8-Trp^9). There is evidence that the C- and/or the N-terminal side chains may play an important role in determining the subtype specific binding, as the γ-MSH has much higher affinity for the MC_3 than for the MC_4 receptor (Adan et al., 1994a; Miwa et al., 1995; Schiöth et al., 1995, 1996b). Elaboration of the MSH core residues resulted in specific antagonists for the different subtypes (Adan et al., 1994b; Hruby et al., 1995). Cyclic MSH analogues were synthesised by Hruby and co-workers more than 15 years ago. A classical example of these compounds is the disulfide bridged cyclic [Cys^4,Cys^{10}]α-MSH which shows high potency and prolonged biological activity (Sawyer et al., 1982). It was proposed that a β turn or other reversed chain structure of the main core could contribute to the bioactivity of the MSH peptide.

Our present data show that the melanocortin MC_1 receptor shows the highest affinities, the melanocortin MC_3 and MC_4 receptors intermediate affinities, and the melanocortin MC_5 receptor low affinities for the core MSH peptides. These data thus clearly indicate that differences in core peptide binding may also have a role for the high, intermediate and low affinities of the melanocortin receptor subtypes for the natural MSH peptides. However, it was a very interesting observation that the melanocortin MC_3 and MC_4 receptors show very similar affinities to all the evaluated core peptides, and these data may thus indicate that the C- and/or N-terminal parts of the natural MSH peptides are the sites responsible for the selectivities of α-, β- and γ-MSH for the melanocortin MC_3 and MC_4 receptors.

That Met^4 along with the His^6, Phe^7, Arg^8 and Trp^9 are the most important amino acids for receptor binding in the MSH peptide is supported by our present data which show that the addition of Met^4 to the linear NHdFRWG leads to a more than 100-fold higher affinity for the melanocortin MC_1 receptor, and about 20-fold higher affinity for the other subtypes. The importance of the Met^4 was shown in early structure-activity studies (Eberle, 1988), as well as by alanine scanning of the α-MSH peptide (Sahm et al., 1994a,b).

The cyclic analogues of the present study show lower affinities for the melanocortin receptor subtypes compared to their linear homologues. This may be due to constraint in the cyclic peptides or that free N- and/or C-termini are important for the binding. It is notable that the differences in affinities between the cyclic and linear peptides (displayed as relative ratios in Table 1), are not decreased as the rings become larger. One might have anticipated that an increase in the size of the ring would result in greater flexibility and allow the peptide to take a more optimal binding conformation. However, cyclisation of the 7-amino-acid-long core peptide led to a relatively larger loss of binding affinities than was observed for the 6- and 5-amino-acid-long peptides (Table 1). The ring closure in the $MNHdFRWG_c$ is based on a peptide bond which connects the Met^4 to Gly^{10}. As can be observed in Table 1 the addition of a Met^4 leads in the linear case to a drastic increase in affinities. One plausible explanation for the low affinity of the cyclic $MNHdFRWG_c$ might therefore be that cyclisation forces the peptide to an unfavourable conformation for Met^4 interactions with the receptors. Another very interesting observation was that the cyclic 6- and 5-amino-acid-long peptides show virtually identical affinities for the respective melanocortin receptor subtype. Besides indicating that Asn^5 does not have any important role in the binding of the ligand (cf., also the results for 5- and 6-amino-acid-long linear peptides), this shows that the decreasing ring size does not force the peptide into unfavourable conformations for binding to the receptors. Nevertheless, still further synthesis of MSH peptides, molecular modelling of receptor structure and mutagenesis studies will be necessary to achieve full insight into the mechanisms for the binding of peptides to the different melanocortin receptor subtypes.

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