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The melanocortin (MC3) receptor from rat hypothalamus: photoaffinity labelling and binding of alanine-substituted α-MSH analogues

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Abstract

Membrane preparations of cells expressing the cloned rat hypothalamus melanocortin receptor, MC3, have been photoaffinity labelled using a radiolabelled photoreactive analogue of α-MSH, [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH. SDS-PAGE followed by autoradiography showed a single band at 53–56 kDa for the native receptor or 35 kDa after deglycosylated with PNGase F, consistent with the predicted cDNA sequence. Receptor binding studies with α-MSH, γ-MSH and [Nle^4,D-Phe^7]α-MSH established that α-MSH and γ-MSH had similar affinities while [Nle^4,D-Phe^7]α-MSH bound 100 times more strongly. These results suggest that the receptor recognises the conserved 'core sequence' (-Met-Glu/Gly-His-Phe-Arg-Trp-) of MSH/ACTH peptides. The binding affinities of alanine-substituted analogues of α-MSH were determined to investigate the role of individual residues in ligand–receptor interactions. While in the terminal regions only the replacement of Tyr^2 reduced the affinity of the peptide, replacement of Met^4, Phe^7, Arg^8 and Trp^9 within the peptide core led to a significant loss of affinity. Glu^5 appeared unimportant for receptor recognition.

Key words: Melanocyte-stimulating hormone; Melanocortin receptor; MC3 receptor; Photoaffinity labelling; Receptor binding; Alanine scan

1. Introduction

Recently, several receptors for melanocortin peptides have been discovered in the brain [1–6] where MSH peptides have a multitude of functions [7–11]. Screening of cDNA libraries revealed the existence of structurally related receptors in the human limbic system [3] and rat hypothalamus [6]. These receptors have subsequently been named MC3 [3,12]. Studies with endogenous MSH and ACTH ligands have led to the proposal that this receptor might provide a possible site of action for a specific physiological role for γ-MSH [6], but the use of synthetic ligands has been limited. In contrast, structure–activity relationships for the MC1 receptor found in melanocytes and melanoma cells have been widely investigated [13].

In this study we have analysed the molecular weight and extent of glycosylation of the cloned MC3 receptor from rat hypothalamus expressed in 293 cells. A potent derivative of α-MSH, [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH, which had previously been used to successfully characterise the α-MSH (MC1) receptor from B16 murine melanoma cells [14] and rat Schwann cells [15], has been employed in these photoaffinity labelling experiments. Binding of α-MSH, γ-MSH and [Nle^4,D-Phe^7]α-MSH to the receptor was studied and alanine-substituted analogues of α-MSH were also tested in this assay to investigate the influence of individual amino acids on receptor recognition. Although the binding of various MSH peptides to this receptor has been studied previously [6], this is the first attempt to systematically study the relationship between ligand structure and MC3 receptor affinity by using an alanine scan. We have previously used this method to investigate the structure-activity relationships for the MC1 receptor from murine melanoma cells [16].

2. Materials and methods

2.1. Peptide synthesis and radioiodination

α-MSH [17], [Nle^4,D-Phe^7]α-MSH, [Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH [14] and alanine analogues of α-MSH [16] were synthesised and purified as previously described. γ$_1$-MSH was obtained commercially (Sigma). [Nle^4,D-Phe^7]α-MSH and [Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH were iodinated at Tyr^2 using the method of Eberle [13]. The mono-iodinated product was used in all experiments.

2.2. Cell culture

Transformed human 293 cells expressing the rat MC3 receptor were generously provided by R.D. Cone [6] and were cultured in RPMI 1640 medium (Gibco) containing 10% heat-inactivated foetal calf serum (ICN), 2 mM L-glutamine, 1% non-essential amino acids, 50 IU/ml penicillin, 50 μg/ml streptomycin and 250 μg/ml amphotericin B in an humidified atmosphere of 95% air/5% CO_2.

2.3. Binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH and competitive binding assay

The binding assay was based on a previously described method [17] adapted from Siegrist et al. [18]. Cells were distributed into 96-well filtration plates (Millipore) at a density of 10^5 cells per well and incubated for 4 h at 37°C. Cells were then washed with serum-free RPMI 1640 by filtration using a vacuum manifold (Millipore). They were then incubated with 0.1 ml binding medium for 24 h at 0–4°C. The binding buffer consisted of serum-free RPMI 1640 medium containing 25 mM

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N-(2-hydroxyethyl)piperazine-*N'*-2-ethane sulphonic acid (HEPES)/NaOH (pH 7.4), 0.2% bovine serum albumin (BSA) and various concentrations of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. Non-specific binding was determined in the presence of a 1000-fold excess unlabelled [Nle^4,D-Phe^7]α-MSH. Four replicates were assayed for each concentration point. After the incubation, cells were washed twice with serum-free RPMI 1640 and the filters punched out using the Multiscreen Assay System (Millipore). The filters were then counted on a LKB Wallac 1277 gammamaster.

Competitive binding experiments were carried out as above but in the presence of 1×10^{-10} M [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH and varying concentrations of the unlabelled analogues. Significant differences were determined from one-way analysis of variance using Fisher's multiple comparison procedure at the 99% confidence interval unless otherwise stated. The coefficient of variance associated with replicate wells was less than 10%.

2.4. Photoaffinity labelling and SDS/polyacrylamide gel electrophoresis

Membrane preparations of transformed 293 cells were photoaffinity labelled with [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH and subsequent SDS-PAGE of the solubilised labelled proteins was carried out as previously described [14].

2.5. Enzymatic deglycosylation

[^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH labelled membrane preparations were suspended in 50 mM phosphate buffer pH 7 containing 20 mM EDTA, 1% octylglucoside, 0.1% SDS, 1% 2-mercaptoethanol and protease inhibitors (antipain, aprotinin, leupeptin and pepstatin at 1 mg·ml^{-1} each). PNGase F was added to a final concentration of 10 units·mg^{-1}. The samples were incubated at 37°C for 3 h, solubilised in electrophoresis sample buffer and subjected to SDS-PAGE.

3. Results and discussion

The dissociation constant (K_d) for the interaction of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH with the MC3 receptor was obtained from three replicate binding isotherms and was estimated by *MINSQ* non-linear least-squares regression analysis to be 1.66 nM. Scatchard analysis of the binding isotherm showed only one population of receptors. The number of receptors per cell was relatively high, varying between 35,000 and 50,000.

Photoaffinity labelling of the MC3 receptor using [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH followed by SDS-PAGE and autoradiography showed a protein band at 53–56 kDa (Fig. 1, lanes 2 and 4). In the presence of a 1000-fold excess unlabelled [Nle^4,D-Phe^7]α-MSH this band was completely suppressed (Fig. 1, lanes 3 and 5), suggesting that it represented the specifically labelled melanocortin receptor. Treatment with hot buffer solution, which was routinely performed with SDS-PAGE samples, resulted in the radioactivity remaining at the origin of the gel, indicating aggregation of the protein (Fig. 1, lane 6). This phenomenon has also been observed with the α-MSH receptor from B16 murine melanoma cells [14]. The intense band at the front of lanes 2, 4 and 6 represents specifically bound ligand not removed by the washing procedure employed before solubilisation which dissociates from the receptor on introduction to the SDS buffer. In lanes 3, 5 and 7 the band at the front represents the small amount of free ligand remaining after washing.

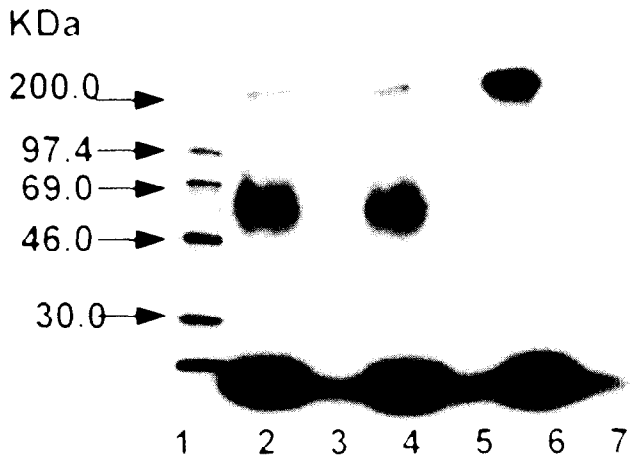

Fig. 1. Autoradiography of gel of solubilized membrane proteins labelled with [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH. Lane 1, ^{14}C-labelled molecular mass standards; lanes 2 and 4, labelled membrane proteins, lanes 3 and 5, membrane proteins in the presence of radioligand and excess non-labelled [Nle^4,D-Phe^7]α-MSH; lane 6, labelled membrane proteins after SDS solubilisation in hot buffer solution; lane 7, same treatment as in lane 6 in the presence of excess non-labelled [Nle^4,D-Phe^7]α-MSH.

PNGase F treatment of the photoaffinity labelled membranes demonstrated the presence of *N*-linked carbohydrate side-chains on the receptor protein. PNGase F is known to cleave asparagine-bound *N*-glycans to give proteins free of *N*-linked carbohydrate chains [19,20]. After 3 h of treatment, the labelled receptor complex was reduced to an apparent molecular weight of approximately 35 kDa (Fig. 2, lane 6). This molecular weight is in agreement with the amino acid sequence of the receptor [6] and suggests that the MC3 receptor, like the MC1

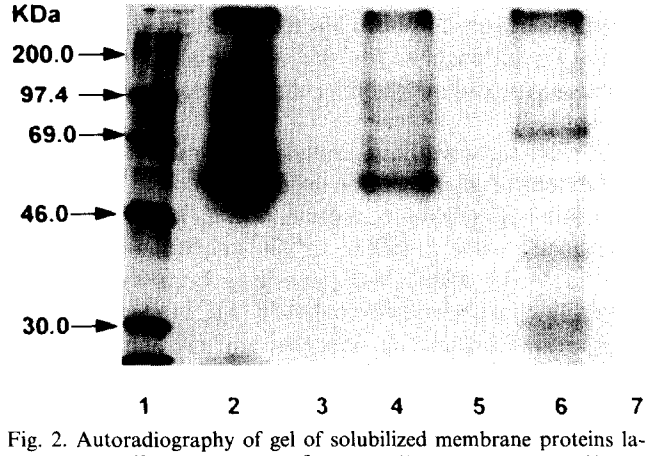

Fig. 2. Autoradiography of gel of solubilized membrane proteins labelled with [^{125}I-Tyr^2,Nle^4,D-Phe^7,ATB-Lys^{11}]α-MSH. Lane 1, ^{14}C-labelled molecular mass standards; lane 2, labelled membrane proteins; lane 3, membrane proteins in the presence of radioligand and excess non-labelled [Nle^4,D-Phe^7]α-MSH; lanes 4 and 5, as lanes 2 and 3 with lower loading; lane 6, labelled membrane proteins after treatment with PNGase F; lane 7, labelled and PNGase F treated membrane proteins in the presence of excess non-labelled [Nle^4,D-Phe^7]α-MSH.

Table 1
Dissociation constants (± S.D.) and relative binding affinities of MSH peptides

	Binding (K_d)	Relative binding
[Nle^4,D-Phe^7]α-MSH	$2.15 \times 10^{-8} \pm 1.73 \times 10^{-8}$ ($n = 14$)	96.28
α-MSH	$2.07 \times 10^{-6} \pm 1.18 \times 10^{-6}$ ($n = 4$)	1
γ-MSH	$3.24 \times 10^{-6} \pm 2.15 \times 10^{-6}$ ($n = 4$)	0.64

receptor [21], does not contain additional *O*-linked carbohydrates. Two other bands appear at 70 and 28 kDa and are believed to represent a dimer of the receptor and a degradation product respectively. All three bands could be inhibited in the presence of excess unlabelled ligand (Fig. 2, lane 7). Incubations with PNGase F for longer periods of time did not result in any further change in the apparent molecular weight, therefore it was assumed that the deglycosylation was complete. Broadening of the specifically labelled bands in lanes 2, 4 and 6 of Fig. 2 and the routinely observed specific ^{125}I activity at higher apparent molecular weights was attributed to poor solubilisation and aggregation of the receptor proteins.

The dissociation constants for α-MSH, γ_1-MSH, [Nle^4,D-Phe^7]α-MSH and alanine-substituted analogues of α-MSH were obtained from three or more replicate experiments using *MINSQ* non-linear least square regression (Tables 1 and 2). All peptides studied were able to fully inhibit binding of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH to the receptor at sufficiently high concentrations. In this study, α-MSH and γ_1-MSH showed a similar affinity as shown previously by Roselli-Rehfuss et al. [6], whereas [Nle^4,D-Phe^7]α-MSH bound 100 times more strongly

Table 2
Dissociation constants (± S.D.) of alanine analogues of α-MSH and binding relative to α-MSH

	Binding (K_d)	Relative binding
α-MSH	$2.07 \times 10^{-6} \pm 1.18 \times 10^{-6}$ ($n = 4$)	1
[Ala^1]α-MSH	$3.08 \times 10^{-6} \pm 2.28 \times 10^{-6}$ ($n = 3$)	0.67
[Ala^2]α-MSH	$1.50 \times 10^{-5} \pm 1.42 \times 10^{-5}$ ($n = 3$)	0.14
[Ala^3]α-MSH	$3.41 \times 10^{-6} \pm 4.18 \times 10^{-6}$ ($n = 3$)	0.61
[Ala^4]α-MSH	$2.95 \times 10^{-5} \pm 2.20 \times 10^{-5}$ ($n = 5$)	0.070
[Ala^5]α-MSH	$2.39 \times 10^{-6} \pm 2.63 \times 10^{-6}$ ($n = 4$)	0.87
[Ala^6]α-MSH	$1.25 \times 10^{-5} \pm 1.13 \times 10^{-5}$ ($n = 3$)	0.17
[Ala^7]α-MSH	$1.94 \times 10^{-4} \pm 7.36 \times 10^{-5}$ ($n = 3$)	0.011
[Ala^8]α-MSH	$4.21 \times 10^{-4} \pm 2.62 \times 10^{-4}$ ($n = 3$)	0.0049
[Ala^9]α-MSH	$1.72 \times 10^{-4} \pm 8.42 \times 10^{-5}$ ($n = 3$)	0.012
[Ala^{10}]α-MSH	$2.72 \times 10^{-6} \pm 9.80 \times 10^{-7}$ ($n = 4$)	0.76
[Ala^{11}]α-MSH	$7.49 \times 10^{-6} \pm 4.27 \times 10^{-6}$ ($n = 3$)	0.28
[Ala^{12}]α-MSH	$7.25 \times 10^{-7} \pm 1.74 \times 10^{-7}$ ($n = 3$)	2.86
[Ala^{13}]α-MSH	$1.57 \times 10^{-6} \pm 1.08 \times 10^{-6}$ ($n = 3$)	1.32

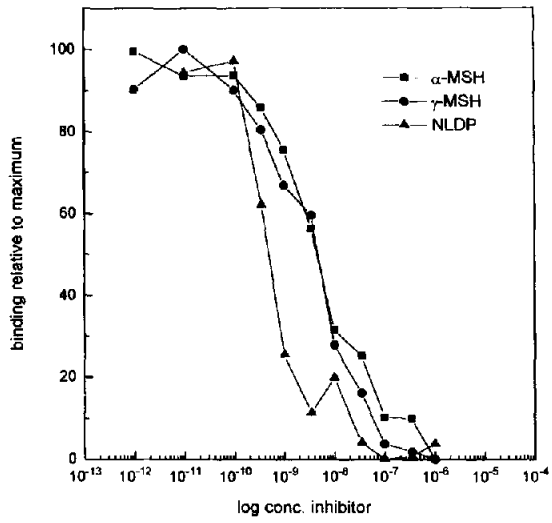

Fig. 3. Competitive binding isotherms of [Nle^4,D-Phe^7]α-MSH, α-MSH and γ_1-MSH.

(Fig. 3). This is in disagreement with the earlier findings [6], which showed only a 5-fold difference in binding between α-MSH and [Nle^4,D-Phe^7]α-MSH. These discrepancies may be explained by the experimental conditions used: equilibrium studies at 4°C were used in the work reported here whereas Roselli-Rehfuss et al. employed 10 min incubations at 37°C [6]. The human equivalent of the MC3 receptor was also reported to respond similarly to a range of MSH peptides including α-MSH and [Nle^4,D-Phe^7]α-MSH [3] in the cAMP assay, although no binding data was reported.

The K_d values of the alanine substituted analogues were higher than that of α-MSH with two exceptions, the Ala^{12} and Ala^{13} compounds. The latter two analogues had affinities of 2.9 and 1.3 relative to α-MSH which were not statistically different to α-MSH. The relatively low affinity of all the peptides except [Nle^4,D-Phe^7]α-MSH was surprising but consistent, suggesting that another endogenous ligand for the MC3 receptor may exist. Except for the Tyr^2, alanine replacements can be made in the terminal regions of the α-MSH molecule (amino acids 1–3 and 10–13) without significant loss of affinity. [Ala^2]α-MSH showed only 14% of the affinity of α-MSH. Within the 'core sequence', substitution of Glu^5 can be made without altering the binding properties of the peptide, but replacement of all other residues leads to a significant loss of affinity. [Ala^4]α-MSH and [Ala^6]α-MSH retained 7% and 17% of the affinity of α-MSH, respectively. The most important residues for receptor binding occurred in the -Phe^7-Arg^8-Trp^9-triplet, with [Ala^8]α-MSH, [Ala^8]α-MSH and [Ala^9]α-MSH having affinities of 0.011, 0.0049 and 0.012 times that of α-MSH (Fig. 4). Similar results have also been obtained for a mouse MC1 receptor [16].

The results presented in this study are generally in

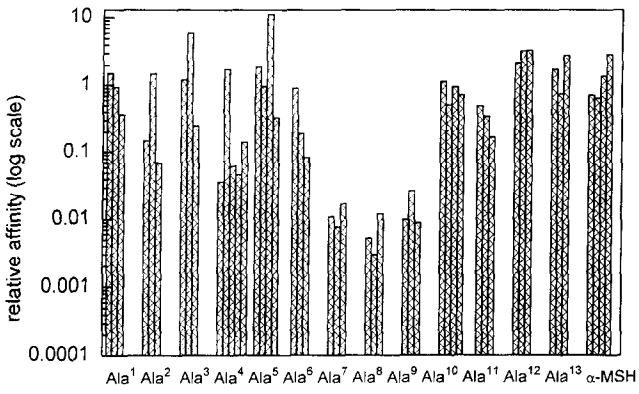

Fig. 4. Relative binding affinities of alanine-substituted analogues of α-MSH to the MC1 and MC3 receptor. Values are relative to the affinity of α-MSH on the respective receptor.

agreement with the view that this receptor might recognise the α-MSH$_{(4-10)}$ 'core sequence' [3], although we have shown that the residue in position 10 is not crucial for the affinity. There is evidence that a tyrosine residue in position 2 is favourable for receptor binding. As yet, there is no evidence that γ-MSH is the endogenous ligand for the MC3 receptor but it is significant that α-MSH and γ-MSH had similar affinities, a situation which is not the case for the MC1 receptor found on melanoma cells [22].

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