Melanin-concentrating hormone binding to mouse melanoma cells in vitro

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Received 10 December 1994

Abstract An analogue of human melanin-concentrating hormone (MCH) suitable for radioiodination was designed in which Tyr[13] was replaced by Phe and Val[19] by Tyr. The resulting monoiodinated [^{125}I][Phe[13],Tyr[19]]-MCH radioligand was biologically active and led to the discovery of high-affinity binding sites on mouse B16-F1, G4F and G4F-7 melanoma cells. Saturation binding analysis with G4F-7 cells revealed 1090 MCH receptors per cell and a K_D of 1.18×10^{-10} mol/l. Receptors for MCH were also found on rat PC12 phaeochromocytoma cells, human RE melanoma cells and COS-7 cells. Competition binding analyses with other peptides such as α-MSH, NPY and PACAP demonstrated that MCH receptor binding is specific. rANF(1–28) was found to be a weak competitor of MCH, indicating topological similarities between MCH and rANF(1–28) when interacting with MCH receptors.

Key words: B16-F1, B16-G4F mouse melanoma cell; Melanin-concentrating hormone (MCH); Melanocyte- stimulating hormone (MSH); Atrial natriuretic factor (ANF); Receptor binding

1. Introduction

The neuropeptide melanin-concentrating hormone (MCH) is a colour-regulating hormone in fish, binding to skin pigment cells to induce concentration of the intracellular pigment granules and hence skin pallor [1]. In tetrapods, MCH is inactive in this respect, although high concentrations of salmonid MCH will induce melanin dispersion rather than concentration in isolated amphibian or reptile skin [2,3], and stimulate tyrosinase activity in mouse melanoma cells [3]. This effect has been attributed to pharmacological interaction of MCH with the receptors for α-melanocyte-stimulating hormone (α-MSH) [4]. In higher vertebrates, MCH is abundant in the brain where its role is probably to serve as a widespread neurotransmitter/neuromodulator [5]. Human MCH, the structure of which is identical with that of rat MCH [6], is mainly located in hypothalamic neurons projecting to various other brain areas [7]. Evidence suggests that it also occurs and exerts actions in peripheral organs of the body.

Little is known about the central effects of MCH, and attempts to examine MCH binding sites in the brain have so far proved unsuccessful. A major problem has been the development of a satisfactory radioligand. The introduction of an iodine atom or NO_2 group onto the tyrosine residue, located in the central region of the MCH molecule, reduced bioactivity by 500- to 1000-fold [3,8,9], which molecular modelling suggests is probably due to distortion of the shape of the cyclic structure [9]. N-Terminally iodinated MCH analogues, e.g. labelled with iodinated Bolton–Hunter reagent, bind to brain membranes but are rapidly degraded (unpublished observations).

The present paper describes a very potent analogue of human MCH which is C-terminally iodinated and which exhibits specific binding to mouse melanoma cells, whether or not they possess MSH receptors. The results indicate the existence of specific MCH receptors on these cells.

2. Materials and methods

2.1. Peptides and chemicals

Human (rat) MCH was obtained from Bachem (Bubendorf, Switzerland) or synthesized in our own laboratory. The analogue [Phe[13],Tyr[19]]-MCH was prepared by the continuous-flow solid-phase method [10] using an automated Milligen 9050 peptide synthesizer. Cyclization of the linear peptide was performed by iodine oxidation followed by RP-HPLC purification and FAB mass spectrometic analysis of the final product [11]. Monoiodinated [^{125}I][Phe[13],Tyr[19]]-MCH was obtained through enzymatic iodination using solid-phase bound glucose oxidase/lactoperoxidase, as described for α-MSH [12], followed by a first purification step on Spherisorb ODS minicolumns [13] and a second, preceding each experiment, by RP-HPLC. Rat ANF(1–28), α-MSH, pituitary adenylate cyclase activating polypeptide (PACAP) and neuropeptide Y (NPY) were purchased from Bachem (Bubendorf, Switzerland). All chemicals and solvents were of analytical grade.

2.2. Biossay

The biological activity of [Phe[13],Tyr[19]]-MCH was determined with the microscopic melanophore assay using scales from the Chinese grass carp, *Ctenopharyngodon idellus* [3]. Human (rat) MCH and salmon MCH served as standards.

2.3. Cell lines and cell culture

The following cell lines were used: mouse B16-F1 melanoma, G4F melanoma (originating from B16-F1 and not expressing MSH receptors [14]), G4F-7 melanoma (G4F cells with transfected human MSH receptor; cells constructed in our laboratory) as well as human RE melanoma (isolated in our laboratory from a metastasis); rat PC12 phaeochromocytoma, COS-7, CHO (Chinese hamster ovary) and human fibroblasts. The cells were grown in modified Eagle's medium (MEM) with Earle's salt (Gibco, Paisley, UK), supplemented with 10% heat-inactivated foetal calf serum (Amimed, Basel, Switzerland), 2 mM L-glutamine, 1% MEM non-essential amino acids (100×; Gibco), penicillin (50 units/ml) and streptomycin (50 μg/ml), using Falcon 75 and 175 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells were detached with 0.02% EDTA in phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.44 g $Na_2HPO_4 \cdot 2H_2O$ per liter). Cell numbers were determined in a haemocytometer or in a Coulter counter.

2.4. Receptor binding assay

The binding medium consisted of modified Eagle's medium (MEM) with Earle's salts (Gibco) containing 25 mM HEPES, 0.2% BSA, 0.3 mM 1,10-phenanthroline (Merck, Darmstadt) and 0.16 mM PMSF. The binding reaction was started by adding 0.5 ml of a cell suspension ($0.5–2 \times 10^7$ cells/ml) to 12×75 mm polypropylene tubes containing (i) 50 μl containing 0.05 pmol (240,000 cpm) of [^{125}I][Phe[13],Tyr[19]]-MCH and 50 μl of unlabelled peptide in a 1:3 dilution series (→competition binding experiments) or (ii) 50 μl containing 0.008–0.2 pmol (40,000–

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SSDI 0014-5793(95)00043-7

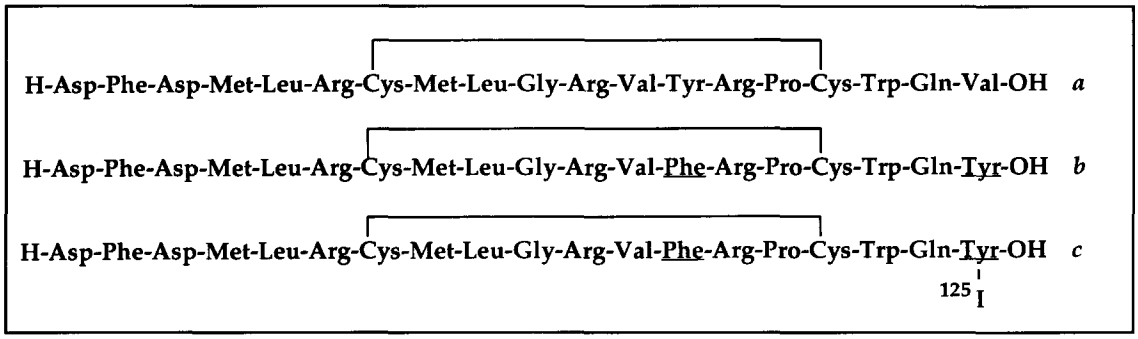

Fig. 1. Primary sequence of human (rat) MCH (a), [Phe[13],Tyr[19]]-MCH (b), and [[125]I][Phe[13],Tyr[19]]-MCH (c). Altered residues are underlined.

1,000,000 cpm) of [[125]I][Phe[13],Tyr[19]]-MCH and 50 μl of 0.6 μM unlabelled MCH or buffer (→saturation experiments). The cells were incubated for 90 min at 10°C. Unbound radioactivity was removed by centrifugation of triplicate aliquots (150 μl) through a layer of 150 μl silicon oil in 0.4 ml polyethylene microtubes [15]. The oil was made up to a density of 1013 kg/cm^3 by mixing equal volumes of AR-20 and AR-200 silicon oil (Wacker Chemie, Munich, Germany). The radioactivity was counted in an Packard Riastar γ-counter and the binding data were analyzed with Ligand [16], an iterative non-linear regression program established for Mac personal computers.

3. Results and discussion

The replacement of Tyr[13] by Phe and of Val[19] by Tyr of the human (rat) MCH sequence (Fig. 1) did not alter the biological activity of the peptide when tested in the fish scale melanophore assay, the only bioassay for MCH peptides known to date: the analogue [Phe[13],Tyr[19]]-MCH showed exactly the same potency as the parent human MCH ($EC_{50} \approx 10^{-11}$ M). This demonstrates that the C-terminal valine of mammalian MCH and the hydroxy group of the Tyr[13] residue are not crucial for the stimulation of fish MCH receptors, which confirms similar findings with analogues of fish MCH [8,9]. Thus, radioiodination of MCH at its C-terminus appears to be much more advantageous than when the radiolabel is introduced at the N-terminus or within the ring structure.

Binding analyses with [[125]I][Phe[13],Tyr[19]]-MCH and three mouse melanoma cell lines (B16-F1, G4F, G4F-7) and with human RE melanoma cells revealed that (i) all these melanoma cells showed specific MCH binding and (ii) binding was optimal between 10–15°C using a 60–90 min incubation period (Fig. 2). These conditions were chosen because a number of preliminary experiments showed that non-specific binding increases considerably at even lower temperatures, and ligand degradation and internalization into the cells rises markedly at higher temperatures and after prolonged incubation (i.e. >120 min). Highest specific binding was seen on the B16-F1 and G4F-7 cells. Using these latter cells for a detailed saturation binding analysis, a dissociation constant (K_D) of 0.118 nmol/l was found for the iodinated form of [Phe[13],Tyr[19]]-MCH and a B_{max} of 1090 binding sites/cell (Fig. 3). Only a single class of binding sites was apparent from these data. Studies with other cell lines showed that MCH receptors are also present on COS-7 cells and rat PC12 phaeochromocytoma cells, whereas they are absent on human skin fibroblasts and CHO cells (not shown).

Competition binding analyses with G4F-7 cells comparing

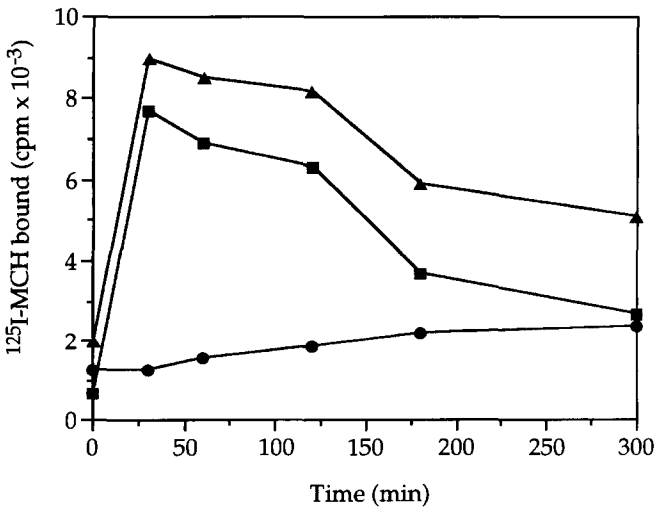

Fig. 2. Time–course of specific binding of [[125]I][Phe[13],Tyr[19]]-MCH to G4F-7 cells at 10°C. The cells were incubated with 0.3 nM radioligand in the absence (total binding; ▲) or presence of 0.7 mM MCH (non-specific binding; ●) for various time periods. Specific binding (■) was calculated as the difference between total and non-specific binding.

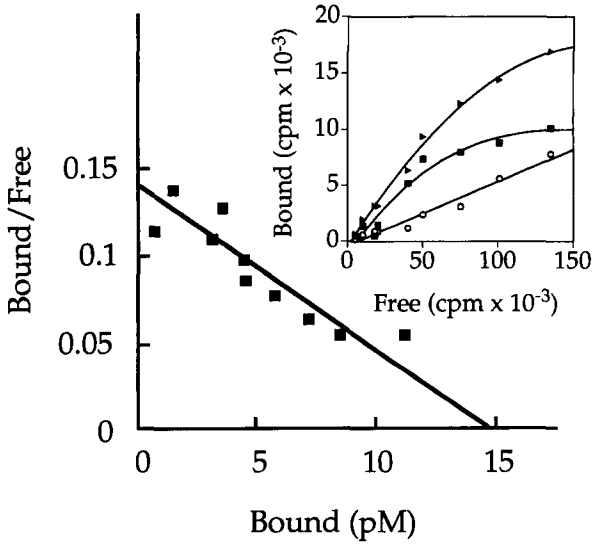

Fig. 3. Scatchard analysis of a typical saturation binding experiment using G4F-7 cells and 0.014–0.35 nM [[125]I][Phe[13],Tyr[19]]-MCH in the presence and absence of 60 nM MCH (main graph). In this particular experiment the K_D was 0.1 nmol/l and B_{max} was 1090 sites/cell. Total (▲), non-specific (○) and specific binding (■).

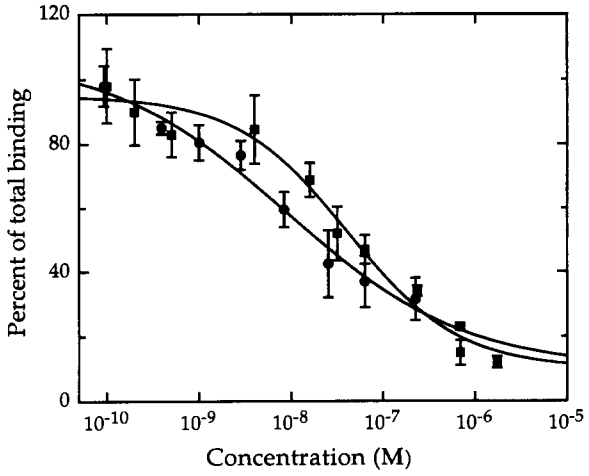

Fig. 4. Log dose–response curve of human MCH (■) and its analogue [Phe[13],Tyr[19]]-MCH (●) in a competition binding assay with G4F-7 cells and [[125]I][Phe[13],Tyr[19]]-MCH as radioligand. The results are the mean ± S.E.M. of three independent experiments.

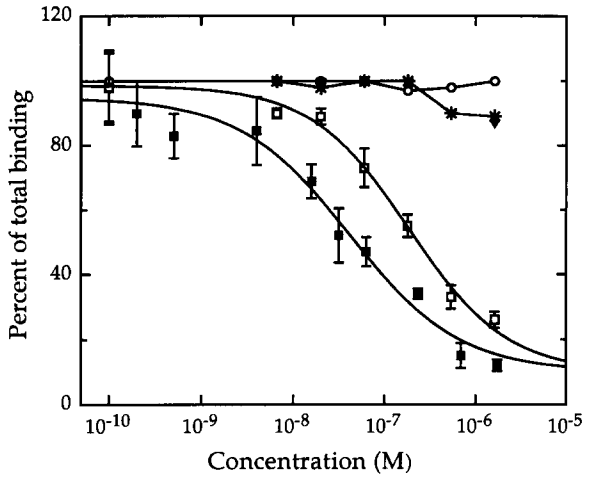

Fig. 5. Log dose–response curve of human MCH (■) and rANF(1–28) (□) in a competition binding assay with G4F-7 cells and [[125]I][Phe[13],Tyr[19]]-MCH as radioligand. PACAP (*), NPY (▼) and α-MSH (○) were also tested as competitors. The results are the mean ± S.E.M. of three independent experiments.

human MCH with the analogue [Phe[13],Tyr[19]]-MCH demonstrated that the latter is about twice as potent as the naturally occurring peptide (Fig. 4). Using PC12 cells, the analogue was still 50% more potent than human MCH (Table 1). This suggests that the binding characteristics of [Phe[13],Tyr[19]]-MCH to mammalian MCH receptors hardly differ from those to fish MCH receptors. The different K_D values for [Phe[13],Tyr[19]]-MCH obtained in saturation and competition binding experiments may be explained by a possibly higher affinity of the iodinated form of [Phe[13],Tyr[19]]-MCH as compared to non-iodinated MCH peptides and/or by the fact that MCH peptides are extremely hydrophobic, resulting in high non-specific absorption to plastic and tissue surfaces, which may significantly reduce the actual peptide concentration in the vicinity of the receptor, thus lowering the amount of tracer available for receptor binding. Nevertheless, competition binding experiments with MCH radioligands are useful for comparisons between different peptides and cell types.

The specificity of MCH receptor binding was investigated using α-MSH, rANF(1–28), PACAP and NPY as displacing peptides (Fig. 5; Table 1). The latter two peptides were included because PC12 cells have been reported to contain receptors for both PACAP [17] and NPY [18]. Neither PACAP, NPY nor α-MSH showed any displacement activity in the different cell lines. In particular the latter finding is important because it has been suggested that, on melanophores, MCH will bind to MSH receptors [19], and conversely it could be expected that α-MSH binds to MCH receptors. This can now be ruled out for MCH receptors on melanoma cells. In order to answer the question of whether MCH may bind to MSH receptors on melanoma cells, we compared B16-F1 cells (expressing normal mouse MSH receptors) with G4F cells (lacking MSH receptors) and G4F-7 cells (containing human MSH receptors). MCH binding was observed in all three cell lines whether or not they express MSH receptors (Table 1). However, the binding affinity was about 10-fold lower in the G4F cells; a significant change in B_{max} could not be measured (not shown). Similar results were obtained with PC12 cells which also lack the MSH receptor [20]. From these studies, it is not yet clear whether MCH receptor binding is influenced by the presence or absence of the MSH receptor or whether the lower affinity observed in G4F and P12 cells is merely an unrelated coincidence. The fact that α-MSH did not displace the MCH radioligand in the different cell lines proves that the site for MCH-receptor binding is different from that for MSH.

Table 1
Binding characteristics of different peptides to MCH receptors on mouse B16-F1, G4F and G4F-7 cells and rat PC12 phaechromocytoma cells

Peptide	Dissociation constants, K_D (nmol/l ± S.E.M.)			
	B16-F1	G4F	G4F-7	PC12
h/rMCH	14 ± 5.3	120 ± 12	12 ± 4.5	120 ± 7.8
rANF (1-28)	180 ± 20	n.d.	116 ± 11.3	280 ± 35.6
α-MSH	≥ 10,000	≥ 10,000	≥ 10,000	≥ 10,000
PACAP,NPY	n.d.	n.d.	n.d.	≥ 10,000
[Phe[13],Tyr[19]]-MCH	n.d.	n.d.	6.3 ± 1.1	80.0 ± 2.1

Dissociation constants were calculated from competition binding assays using [125]I-labeled [Phe[13],Tyr[19]]-MCH; n.d., not determined.

The cyclic peptide rANF(1–28) was a weak competitor for [[125]I][Phe[13],Tyr[19]]-MCH when studied on mouse melanoma and rat phaeochromocytoma cells. Although there is a significant sequence similarity between the N-terminal regions of the prohormones for the two peptides, the primary sequence of rANF(1–28) differs from that of [Phe[13],Tyr[19]]-MCH [21]. However, it cannot be ruled out that there are topological similarities within parts of the three-dimensional structure of the two molecules. On the other hand, it is interesting to note that salmonid MCH is a very weak competitor for [[125]I][Phe[13],Tyr[19]]-MCH when tested with G4F-7 cells, although the two molecules share 80% sequence identity (unpublished observation). The physiological relevance of rANF(1–28) binding to the MCH receptor is not yet clear but may reside in a similar function of ANF and MCH with respect to water homeostasis [22,23].

In conclusion, the preparation and application of the new

MCH radioligand, [^{125}I][Phe^{13},Tyr^{19}]-MCH, has made it possible to demonstrate the existence of MCH receptors on melanoma and other cell lines and to analyze some of their binding characteristics. These studies will form the basis for a more detailed analysis of MCH receptor structure and function.

Acknowledgements: This work is part of the doctoral thesis of R.D. The authors thank the Swiss National Science Foundation and the British Council for grants to A.N.E. and B.I.B., and Merck Sharpe & Dohme for financial support to B.I.B.

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