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 radiiodination was designed in which Tyr13 was replaced by Phe and Val19 by Tyr. The resulting monoiodinated [125I][Phe13,Tyr19]-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 Kd of 1.18 ± 10-10 mol/l. Receptors for MCH were also found on rat PC12 pheochromocytoma 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. RatMCH(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 NO2 group onto the tyrosine residue, located in the central region of the MCH molecule, reduced bioactivity by 500-1000-fold [3,8,9], which molecular modelling suggests is probably due to distortion of the shape of the cyclic structure [9]. N-Terminal 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 [Phe13,Tyr19]-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 spectrometric analysis of the final product [11]. Monoiodinated [125I][Phe13,Tyr19]-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 [Phe13,Tyr19]-MCH was determined with the microscopic melanophore assay using scales from the Chinese grass carp, Ctenopharyngodon idella [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 pheochromocytoma, 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 cm2 tissue culture flasks at 37°C in a humidified atmosphere of 95% air and 5% CO2. The cells were detached with 0.02% EDTA in phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 0.2 g KH2PO4, 1.44 g Na2HPO4·2H2O per liter). Cell numbers were determined in a haemocytometer or in a Coulter counter.

2.4. Receptor binding assay

The binding method 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 × 107 cells/ml) to 12 × 75 mm polypropylene tubes containing (i) 50 μl containing 0.05 pmol (240,000 cpm) of [125I][Phe13,Tyr19]-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–
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 1.013 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 Ïγ-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$_{50}$ = 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 $^{[}$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...
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 Bmax 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 PC12 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.

The cyclic peptide rANF(1-28) was a weak competitor for [125I][Phe13, Tyr19]-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 [Phe13, Tyr19]-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 [125I][Phe13, Tyr19]-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, \[^{[25]}I\]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 Sharp & Dohme for financial support to B.I.B.

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