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MELANIN CONCENTRATING HORMONE ANALOGUES: CONTRACTION OF THE CYCLIC STRUCTURE. II. ANTAGONIST ACTIVITY

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Summary

Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val, melanin concentrating hormone (MCH), is a cyclic hormone possessing both MCH-like (melanin granule aggregating effect) and melanocyte stimulating hormone (MSH)-like (melanin granule dispersing effect) activities. Nine ring-contracted analogues were synthesized and characterized for their melanotropic activity on the fish (Synbranchus marmoratus) and frog (Rana pipiens) bioassays. In most cases, these analogues were totally devoid of MCH-like agonist activity, demonstrating the essential role of the disulfide bridge between residues 5 and 14 of the hormone. [Ala[5], Cys[10]]MCH, for example, was totally devoid of MCH-like activity. This analogue, like α-MSH, however, antagonized the melanosome aggregating actions of MCH on fish melanocytes. The antagonistic activity of the analogue, like that of α-MSH, was Ca^{2+}-dependent. Evidence suggested that this antagonism of MCH activity was related to the intrinsic MSH-like activity of the analogue. These results suggest that MCH and α-MSH may be structurally and, therefore, evolutionarily related.

Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val (melanin concentrating hormone, MCH) is a heptadecapeptide synthesized by the hypothalamus and secreted by the neurohypophysis of fish [1]. MCH stimulates melanosome (melanin granule) aggregation within integumental melanocytes, and this results in a lightening of the skins as an adaptive response [1,2]. MCH has been synthesized in several laboratories [3-6]. We reported previously that MCH also possessed melanocyte stimulating hormone (MSH)-like activity when used at higher concentrations [4,7,8]. We proposed that the two molecules were probably evolutionarily related [8,9].

We have synthesized a number of MCH analogues in which the disulfide bridge between amino acid 5 and 14 was contracted to the 7-14, the 8-14, and 10-14 positions within these peptides

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[10]. Most of these ring-contracted analogues were devoid (or nearly so) of MCH-like activity as determined in a fish skin (melanosome aggregating) bioassay [10,11]. Surprisingly, some of these analogues, although devoid of MCH-like activity, still exhibited MSH-like activity. In this report we demonstrate that several of the analogues lacking MCH-like activity are inhibitory to the actions of MCH and that this antagonistic activity is most likely related to their intrinsic MSH component of activity.

Materials and Methods

Melanin concentrating hormone synthesis. MCH (Figure 1) was prepared by solid phase synthesis as reported elsewhere [3]. Nine melanin concentrating hormone (MCH) analogues were synthesized as previously reported [10]. They were purified by a combination of ion exchange chromatography on CM-cellulose, reverse phase high pressure liquid chromatography on a Vydac C_{18} column followed by gel filtration on a Bio-Gel P-4 column. Purity was assessed by HPLC and TLC in several solvent systems and by electrophoresis. The properties were as those previously reported; for example, they were 98% pure or better [10].

Melanin concentrating hormone bioassay. The teleost fish, Synbranchus marmoratus, a fresh-water eel, was used. Skins were removed and prepared as previously described for the frog [12] and lizard [13] skin bioassays, and as detailed elsewhere [11]. In this bioassay, skins become light in response to MCH due to melanosome aggregation within melanocytes. The skins can be redarkened by removal of the hormone with Ringer rinses or by the addition of MSH which causes dispersion of melanosomes within the pigment cells. These changes in reflectance are monitored by a Photovolt reflectometer and recorded as percent changes from the initial base value. When $[Ala^5, Cys^{10}]$MCH was used as a MCH inhibitor, the skins were pre-incubated for 60 minutes in the blocker and the assays performed in its presence. A 1 h preincubation in the blocker has been standardized since that is the average time for a maximal response of the system to an agonist.

Melanocyte stimulating hormone bioassay. To determine the MSH-like activity of the MCH analogues, the frog, Rana pipiens, skin bioassay was used as originally described by Shizume et al., [12]. In this bioassay, skins darken due to melanosome dispersion within melanocytes in response to melanotropin stimulation.

	1 3 5 7 9 11 13 15 17
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
MCH	Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Ala^5, Cys^{10}]MCH	Asp-Thr-Met-Arg-Ala-Met-Val-Gly-Arg-Cys-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Ala^5, Cys^{8}]MCH	Asp-Thr-Met-Arg-Ala-Met-Val-Cys-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Ala^5, Cys^{7}]MCH	Asp-Thr-Met-Arg-Ala-Met-Cys-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Ala^5, Cys^{10}]MCH_{5-17}	Ala-Met-Val-Gly-Arg-Cys-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Ala^5, Cys^{8}]MCH_{5-17}	Ala-Met-Val-Cys-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Ala^5, Cys^{7}]MCH_{5-17}	Ala-Met-Cys-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Cys^{10}]MCH_{10-17}	Cys-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Cys^{8}]MCH_{8-17}	Cys-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val
[Cys^{7}]MCH_{7-17}	Cys-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val

Fig. 1 Primary structures of α-MSH, MCH and related analogues.

Results

In a previous paper we reported on the synthesis and characterization of the melanotropic actions of a number of cyclic contracted structural analogues of MCH [10]. We have demonstrated that contraction of the ring of MCH resulted in a near total loss of the MCH-like activity of the analogues. Although the MCH-like activity of most analogues was lost, several of these analogues exhibited MSH-like activity as does MCH at high concentrations. For example, [Ala^5, Cys^{10}]MCH, although totally devoid of MCH-like activity in the fish skin bioassay was equipotent to MCH relative to its MSH-like activity in the frog skin bioassay [10]. Two other heptadecapeptide ring-contracted analogues, [Ala^5, Cys^8]- and [Ala^5, Cys^7]MCH, were full MSH-like agonists but were about 1/50th less potent than the [Ala^5, Cys^{10}]-contracted analogue.

The heptadecapeptide analogues not only possessed considerable MSH-like activity in the frog skin bioassay, but they also were antagonistic to the melanosome aggregating actions of MCH in the fish skin bioassay. The agonistic actions of MCH (10^{-10} M) were diminished in a dose-dependent manner by increasing concentrations of [Ala^5,Cys^{10}]MCH (Fig. 2). The dose-response curve to MCH was shifted to the right about 50 and 500 times in the presence of [Ala^5,Cys^{10}]MCH 10^{-7} and 3 x 10^{-7}M, respectively (data not shown). However, the MCH analogues that lacked MSH-like activity were unable to antagonize the actions of MCH (data not shown).

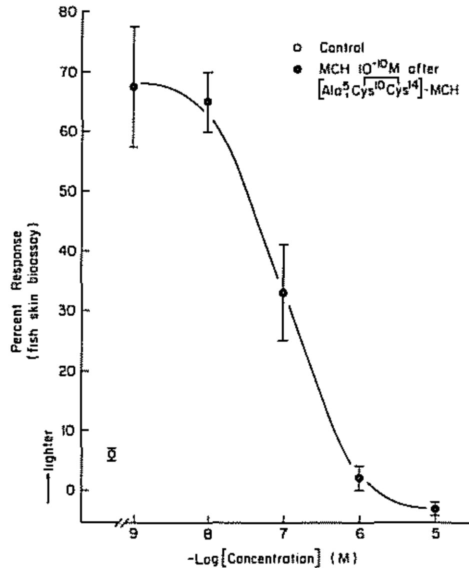

Fig. 2 Dose-related inhibition of the lightening response of fish skins to MCH (10^{-10}M) following one hour incubation of the skins in the presence of [Ala^5, Cys^{10}]MCH at increasing concentrations as noted. Each value is the mean (N=6), ± S.E., response of the skins to MCH in the presence of the antagonist.

Blockage of the MCH activity of MCH could take place at the MCH receptor (competitive antagonism) or at some other receptor (noncompetitive antagonism), most likely MSH receptors. It was clear from earlier experiments that individual fish melanocytes possess both MCH and MSH receptors and that MSH could reverse the actions of MCH [7,8]. MCH itself exhibited self antagonism when used at high concentrations [8,14]. In addition, MCH at those concentrations could also inhibit the MCH-like activity of other MCH analogues (Fig. 3). We have suggested that this inhibitory action of MCH might be due to the intrinsic MSH component of activity of the hormone.

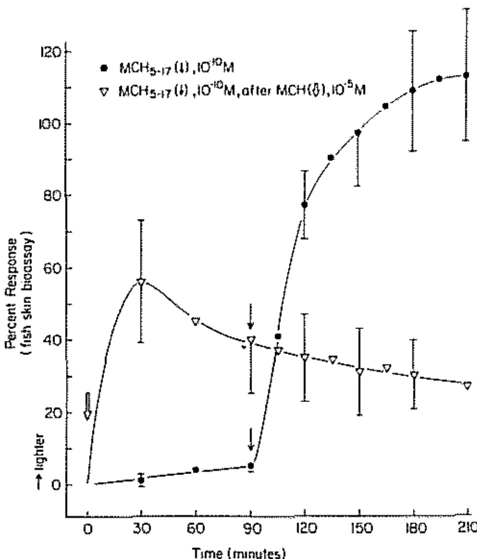

Fig. 3 <u>In vitro</u> demonstration that MCH at a high concentration (10^{-5}M) abolishes the lightening response of fish skins (N=6) to MCH_{5-17}. Each value is the mean, ± S.E.

Since we knew that MSH action on melanocytes (in all species that have been studied) is calcium-dependent [15,16], we determined whether the antagonistic action of the most active of the antagonists, [Ala^5, Cys^{10}]MCH, was also Ca^{2+} dependent. The MCH-like activity of MCH does not require Ca^{2+} [14], so its melanosome aggregating activity was not compromised in the absence of the divalent cation in the incubating medium. Under these conditions, however, the antagonistic action of [Ala^5, Cys^{10}]MCH was abolished Similar results were obtained if the skins were previously treated with α-MSH instead of [Ala^5, Cys^{10}]MCH. α-MSH (10^{-6}M) blocked the lightening actions of MCH, but only when extracellular calcium was present (Fig. 4). These results suggested that the antagonistic actions of the analogue were noncompetitive in nature and probably exerted through MSH receptors. The observation that the analogue did not now exhibit MCH-like activity in the absence of Ca^{2+} (whereas MCH did) demonstrated that contraction of the disulfide bridge abolished the MCH activity of the hormone whether in the presence or absence of Ca^{2+}.

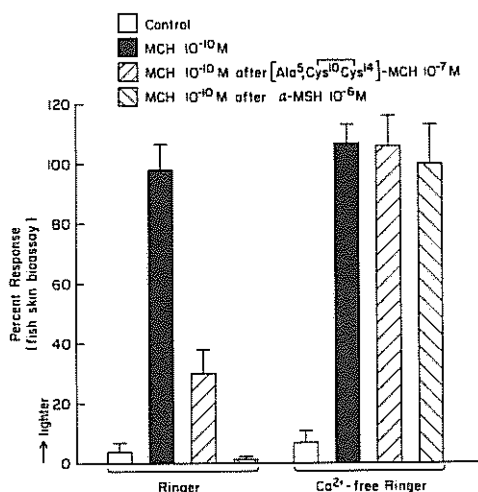

Fig. 4 In vitro demonstration that the antagonism of MCH (10^{-10}M) by [Ala^5, Cys^{10}]MCH (10^{-7}M) in normal Ringer solution is abolished in a Ca^{2+}-free Ringer solution. Each value is the mean, ± S.E., response (lightening) of the skins (N=10) to the peptides under the conditions noted.

Discussion

These results again demonstrate, as shown before [7,8], that MCH possesses both MCH as well as MSH components of biological activity. Heptadecapeptide disulfide bridge contracted analogues lose all of their MCH-like activity but often retain all or a considerable amount of the MSH-like activity exhibited by the parent hormone, MCH. Most interesting was the demonstration that the analogues possessing MSH-like activity were antagonists to the actions of MCH.

The melanosome aggregating actions of MCH have been shown to be independent of calcium in the bathing medium, but the antagonistic actions of MCH could be abolished in a Ca^{2+}-free medium. In the present experiments we could also demonstrate that the antagonistic actions of the analogues, such as [Ala^5, Cys^{10}]MCH and the other ring-contracted analogues could also be abolished in the absence of the divalent cation. We therefore conclude that the antagonistic action of the analogues, as for MCH (at high concentrations), can be accounted for by the noncompetitive actions of the peptides at the MSH receptors of melanocytes.

We previously demonstrated that the N-terminal tetrapeptide sequence of MCH was essential for the MSH component of activity in MCH fragment analogues [7]. However, some of the ring-contracted analogues, that lacked the N-terminal tetrapeptide, still possessed some MSH-like activity [10]. We also observed that MSH-like activity and antagonism of MCH are related phenomena. Conformational studies by CD spectroscopy of the MCH analogues with shortened bridge structures revealed that [Ala^5, Cys^{10}]MCH, like MCH, shows a high tendency to form an α-helical structure [17]. It might be that this helical structure allows the proper conformation for MSH-like activity, but not for a favorable interaction to the MCH receptor.

These results further strengthen our view that both MCH and MSH are evolutionarily related. This view is supported by the demonstration that immunoreactive MCH and α-MSH may coexist in the same neurosecretory granules within the fish and the rat hypothalamus [18,19]. Most fascinating was our observation that the MCH-like component of activity can be abolished leaving intact in certain analogues the MSH-like component of activity. These observations have important implications for the elucidation of the structural features of MCH and MSH that account for their overlapping activities.

Acknowledgments

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