Life Sciences, Vol. 45, pp. 1141-1148 Pergamon Press
Printed in the U.S.A.

CURRENT CONCEPTS:

Melanin Concentrating Hormone

III.

MELANIN CONCENTRATING HORMONE (MCH): THE MESSAGE SEQUENCE

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Summary

Melanin concentrating hormone (MCH) is a heptadecapeptide synthesized by the hypothalamus and secreted by the neurohypophysis of the teleost pituitary gland. MCH stimulates melanosome aggregation within teleost melanocytes but also exhibits MSH-like (melanosome dispersing) activity on tetrapod (frog and lizard) melanocytes. We have synthesized a number of MCH analogues to determine the essential features of the primary structure necessary to stimulate either melanosome aggregation or dispersion in fish or tetrapod melanocytes, respectively. An analysis of the potencies and actions of these analogues on vertebrate melanocytes is provided and demonstrates that the two activities have different structural requirements.

Studies on biologically active peptides usually involve the following sequence of research activity: structure elucidation, total synthesis, biological and pharmacological evaluations, and structure-activity and conformational studies. Based on these results, the design, synthesis and evaluation of conformationally constrained analogues are performed to investigate the biologically active form of the peptide. These experimental results often lead to selective agonists or antagonists (inhibitors). This approach is well exemplified by the newly discovered peptide, melanin concentrating hormone (MCH). The hormone was isolated from salmon pituitary glands over 30 years ago [1], but it was not sequenced until much later [2,3]. MCH is a heptadecapeptide possessing the following primary sequence: Asp-Thr-Met-Arg-Cys-Met-Val-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Glu-Val. Soon after sequence elucidation, the synthesis of MCH was achieved in several laboratories either by the solid phase technique [4,5] or by classical solution methods [6]. At the same time, structure-activity studies were directed toward the determination of: 1) the shortest biologically active fragment, 2) the particular amino acid residues critical for activity (with the potential for radiolabelling at these positions within the molecule), and 3) the

0024-3205/89 $3.00 + .00

significance of the disulfide bridge (or the conformational space available to the molecule).

MCH stimulates melanin granule (melanosome) aggregation within teleost melanocytes leading to a lightening of the skin [7,8]. Unexpectedly, MCH was shown to stimulate melanosome dispersion within tetrapod (frog, lizard) melanocytes (4). Even though some discrepancies between laboratories exist relative to the reported of the MSH-like activity of MCH [4,5,9], the existence of the dual activity of MCH is generally accepted [10-12]. In the present report we document the structural features of MCH that relate to the contrasting actions of the hormone.

Materials and Methods

Melanotropin Synthesis. Several types of MCH analogues were synthesized. One group of peptides contained truncated sequences, while a second group contained analogues with a contracted cysteine bridged ring (also combined, in some cases, with a shortened peptide sequence). MCH as well as nineteen fragment analogues were synthesized [13] to determine the minimal sequence for equipotency and for signal transduction (Table I). In addition, nine ring-contracted analogues were synthesized [14,15] to determine the effects of ring size on potency (Table 2). In all cases, the synthetic schemes employed the Merrifield solid phase synthesis followed by cyclization and purification as described in detail elsewhere.

TABLE I
MCH and Fragment Analogues Synthesized and Bioassayed for MCH-like and MSH-like Activities

		Potency*				Potency	
		Fish	Frog			Fish	Frog
I	MCH_{1-17}	1.0[a]	1.0[b]	XI	MCH_{1-15}	1.0	0.03
II	MCH_{2-17}	1.0	0.005	XII	MCH_{2-15}	1.0	0.01
III	MCH_{3-17}	1.0	0.005	XIII	MCH_{3-15}	1.0	0.01
IV	MCH_{4-17}	1.0	0.005	XIV	MCH_{4-15}	1.0	0.004
V	MCH_{5-17}	1.0	<0.001	XV	MCH_{5-15}	1.0	0.004
VI	MCH_{1-16}	1.0	0.03	XVI	MCH_{1-14}	1.0	0.006
VII	MCH_{2-16}	1.0	0.005	XVII	MCH_{2-14}	0.07	0.05
VIII	MCH_{3-16}	1.0	0.04	XVIII	MCH_{3-14}	0.05	<0.001
IX	MCH_{4-16}	1.0	0.01	XIX	MCH_{4-14}	0.023	<0.001
X	MCH_{5-16}	1.0	0.007	XX	MCH_{5-14}	0.014	<0.001

*The potency of MCH was considered as 1.0 in each of the bioassays. The relative potencies were calculated as: analogue ED_{50}/MCH ED_{50}.
[a]Melanosome aggregation within teleost melanocytes [7,13] ED_{50} 10^{-11}M.
[b]Melanosome dispersion within frog melanocytes [17], ED_{50} 4 x 10^{-8}M.

Melanotropin Bioassays. The MCH-like activity of MCH was studied in the teleost fish, Synbranchus marmoratus, as described elsewhere [7]. In this in vitro bioassay, centripetal migration

of melanosomes within integumental melanocytes results in a lightening of the skin which can be monitored by a Photovolt reflectometer. Conversely, in the frog (Rana pipiens) and lizard (Anolis carolinensis) skin bioassays, melanosome dispersion within melanocytes in response to a melanotropin leads to a darkening of the skins which similarly can be measured [16].

Results

Structure-Activity Studies. Tables I, II, and III list the MCH analogues synthesized by our laboratory as well as by others and the potencies of these peptides as determined in several bioassays.

MCH-like Activity. Shortening of the peptide chain of MCH showed (in our experiments) that for the MCH-like component of activity, tryptophan at position-15 of the carboxy-terminal part of the molecule was important (compare XV to X and XX) whereas the amino-terminal tetrapeptide sequence could be eliminated (I-V) without a loss of potency. We determined that the shortest sequence for equipotency to MCH was MCH_{5-15}, while the central sequence, MCH_{5-14}, possessed about $1/70^{th}$ the potency of MCH [13].

TABLE II
Relative MCH- and MSH-Like Activities of Ring-Contracted MCH Analogues

	Analogue	MCH ACTIVITY[a] Fish Bioassay	MSH ACTIVITY[b] Frog & Lizard Bioassays	
	MCH[c]	1.0	1.0	1.0
XXI	[Ala^5,Cys^{10}]MCH	Inactive[d]	1.0	ND[+]
XXII	[Ala^5,Cys^8]MCH	Inactive[d]	0.06	0.8
XXIII	[Ala^5,Cys^7]MCH	Inactive[d]	0.04	0.9
XXIV	[Ala^5,Cys^{10}]MCH_{5-17}	Inactive[d]	0.025	7.0
XXV	[Ala^5,Cys^8]MCH_{5-17}	0.00001	Inactive[e]	
XXVI	[Ala^5,Cys^7]MCH_{5-17}	Inactive[d]	0.01	0.5
XXVII	[Cys^{10}]MCH_{10-17}	0.0001	Inactive[e]	
XXVIII	[Cys^8]MCH_{8-17}	0.000001	Inactive[e]	
XXIX	[Cys^7]MCH_{7-17}	Inactive[d]	0.006	7.0

[a]Melanosome aggregation within fish skin melanocytes.
[b]Melanosome dispersion within frog and lizard skin melanocytes.
[c]The potency of MCH was considered as 1.0 in each of the bioassays. The relative potencies were calculated as: analogue ED_{50}/MCH ED_{50}.
[d]Inactive at 10^{-6}M, or lower.
[e]Inactive at 10^{-5}M or lower.
[+]ND, not determined

To address the question of ring size and potency, it was necessary to shift the position of the disulfide bridge [14,15].

This may lead to a conformational change of the molecule, but since the original ring size allows for great flexibility, the conformations of a smaller disulfide ring may still encompass one of the conformations in the parent hormone important for receptor binding. Thus, we synthesized a group of analogues having either a shift of the cystine bridge and/or a shortened peptide chain from the amino terminus (Table II). The analogues in this series, which otherwise still contained the full sequence of MCH, did not show any MCH-like activity (XXI - XXIII), yet rather surprisingly, several ring-contracted analogues with shortened peptide chains possessed some MCH-like activity (XXV, XXVII, XXVIII). The highest activity (1/1000th that of MCH) was expressed by the shortest analogue [Cys^{10}]MCH$_{10-17}$ (XXVII), which was a full agonist in the fish skin bioassay.

MSH-like Activity. In the frog skin bioassay for the MSH-like activity of MCH, the importance of the amino-terminal sequence of the peptide was demonstrated [17]. Removal of the N-terminal amino acid, aspartic acid, resulted in a near total loss of the MSH-like activity of MCH. This analogue, MCH$_{2-17}$, as well as MCH$_{3-17}$, MCH$_{4-17}$ and MCH$_{5-17}$ (Table I, II-V) were nearly devoid of MSH-like activity. We also documented that some component of the C-terminal side chain of MCH was important to the MSH-like activity of MCH, since MCH$_{1-14}$ (XVI) was less potent than MCH in stimulating melanosome dispersion (Table I, I, XVI). We then determined that Trp^{15} and Glu^{16} also contributed to this activity since MCH$_{1-15}$ and MCH$_{1-16}$ possessed only about 1/100th the MSH-like activity of MCH (Table I, VI, XI).

The highest MSH-like activity (equal to the potency of MCH) of the ring-contracted analogues was found in the analogue with the most contracted disulfide bridge (Table II, XXI). The MSH-like activity of the analogues generally decreased in relation to the length of the N-terminal sequence of the molecule (compare XXI - XXII with XXIV - XXV) and also in relation to the length of the peptide past the amino-terminal cysteine residue. Unexpectedly, the fragment analogues [Cys^{7}]MCH$_{7-17}$ (XXIX), [Ala^{5},Cys^{10}]MCH$_{5-17}$ (XXIV), and [Ala^{5},Cys^{7}]MCH$_{5-17}$ (XXVI) possessed some MSH-like activity. One analogue, [Cys^{7}]MCH$_{7-17}$, was quite potent in the frog skin bioassay whereas two related analogues, [Cys^{8}]MCH$_{8-17}$ and [Cys^{10}]MCH$_{10-17}$, lacked such activity (Table II).

In the lizard skin bioassay, the MSH-like activity of the analogues generally followed the results obtained in the frog skin bioassay (XXI - XXIX). The analogues exhibited more MSH-like activity relative to MCH in the lizard skin bioassay than in the frog skin bioassay [14,15]. Again, one analogue, [Cys^{7}]MCH$_{7-17}$ (XXIX) was very potent whereas two closely related peptides, [Cys^{8}]MCH$_{8-17}$ (XXVIII) and [Cys^{10}]MCH$_{10-17}$ (XXVII), lacked significant melanotropic activity (Table II). In fact, [Cys^{7}]MCH$_{7-17}$ exhibited full MSH-like activity at a potency about seven-fold greater than MCH (Table II). In contrast, lizard melanocytes were less sensitive to MSH than frog pigment cells.

Discussion

Structure-Activity Studies. In Tables I-III, the MCH-like and MSH-like potencies of MCH analogues synthesized by several laboratories are summarized. Probably, the first synthetically

produced analogue was [Gln^{16}]MCH, which was synthesized in parallel with the first synthesis of MCH for the reasons of structural clarification [18]. The replacement of the methionine residues by norvaline led to only a slight decrease of the potency (XXXV). This observation led to the labeling of MCH through the tritiation of the unsaturated propargylglycine side chains within these residues [9]. In addition, oxidation of the methionines did not completely eliminate biological activity (XXXVII) of MCH [9,19]. In contrast, reduction of the disulfide bridge of MCH followed by alkylation of the resulting SH groups (peptides XXXI and XXXII in Table III) led to a dramatic decrease in the potency of MCH [19,20]. However, this modification was combined with alkylation of cysteine with a rather bulky S-acetamidomethyl or carboxamidomethyl group, neither of which are good models for attaining pseudoisosteric conversion of a cyclic peptide to a linear one. Because of large steric bulk and the larger differences in stereoelectronic properties of these groups, one still has difficulty in assessing the importance of the intact disulfide bridge.

Kawazoe et al. [19] studied the effect of modification of various functional groups in MCH to determine their influence on the biological activity of the peptide. Modification of tryptophan was compatible with high biological potency; the resulting analogue, [Trp(Nps)^{15}] MCH, (XXXVIII) was fully active

TABLE III.
Biological Potencies of MCH Analogues Synthesized in Other Laboratories

	Peptide	Bioassay		Reference
		Fish	Frog	
	MCH	1[a]	1[b]	
XXX	[Gln^{16}]MCH			[18]
XXXI	[Cys(Acm)^{5,14}]MCH[c]	0.003		[9]
XXXII	[Cys(Cam)^{5,14}]MCH[d]	0		[19]
XXXIII	MCH-alkali treated	0	30[e]	[9]
XXXIV	MCH-iodinated	0.002		[9]
XXXV	[Nva^{3,6}]MCH	0.37		[9]
XXXVI	[Pra^{3,6}]MCH[f]	0.15		[9]
XXXVII	[Met(O)^{3,6}]MCH	0.88		[9]
XXXVIII	[Trp(Nps)^{15}]MCH	1.0		[19]
XXXIX	[Arg(DHCH)^{4,9,12}]MCH[d]	0.001		[19]
XL	[Tyr(NO$_2$)^{11}]MCH	0.001		[19]
XLI	MCH$_{1-14}$	1.0[e]		[19]
XLII	MCH$_{5-14}$	1.0[e]		[19]

[a]Melanosome aggregation within melanocytes, ED_{50} 6 x 10^{-11}M
[b]Melanosome dispersion within melanocytes.
[c]Acm, acetamidomethyl
[d]Cam, carboxamidomethyl; DHCH, dihydroxycylohex-1,2-ylene.
[e]Pra, propargylglycine
[f]see new data in this report (Table 1)

on fish melanocytes in vitro. However, the tyrosine in position-11 (XL) and at least one of the arginines (at positions -4, -9 or -12) appeared to be critical for biological activity since analogues with modifications of these residues only had about 0.1% of the potency of MCH (XXXIX).

The analogues we synthesized demonstrated clearly that MCH is able to activate two opposing biological responses and that modification of its structure can produce selective agonists possessing either MSH-like or MCH-like agonist activity only.

MCH Activity: The Message Sequence. Biological assay of all possible fragment analogues of MCH still retaining an intact disulfide bridge revealed that the minimal sequence for equipotency to MCH was MCH_{5-15} [14]. Since the central cyclic sequence, MCH_{5-14} (XX), was almost 100 times less active than MCH_{5-15} (XV), the importance of Trp at position-15 becomes evident. However, this is in contrast with the results obtained with MCH_{1-14} (XLI), MCH_{5-14} (XLII) and the analogue containing modified Trp (XXXVIII, Table III) which were reported to be equipotent to MCH [19]. The experimental differences might be due to the fact that our bioassay employs a different species, and that the objective reflectance technique [7] is about one order of magnitude more sensitive than the classical melanophore index employed by other laboratories [9,19,20]. Hence, an analogue that is found to be equipotent to MCH at 10^{-9}M, using the melanophore index, may indeed be less potent in our eel skin assay. In addition, our truncated peptides were prepared by direct synthesis, whereas the peptides prepared by Kawazoe and coworkers were produced by proteolytic cleavage of the parent peptide, MCH [19]. It is now clear that substitutions (e.g., D-Trp) at this position are necessary to address the importance of Trp^{15}, and in addition, to determine whether superagonists or antagonists can be produced.

Contraction of the 5-14 cyclic structure of MCH to the 7-14, 8-14 and 10-14 ring structure revealed the importance of the original ring size to biological activity (Table II). This supports, to some degree, the suggestion that an intact disulfide bridge is required for full potency of MCH [20].

Structural Requirements for the MSH-Like Activity of MCH. We have previously demonstrated that MCH and MCH_{1-14} (XVI) possessed full MSH-like activity, whereas MCH_{5-17} (V) and MCH_{5-14} (XX) did not [10,11]. This revealed the importance of the N-terminal 1-4 amino acid sequence for MSH activity. These results were generally born out again in the present studies since MCH_{2-17} (II) lacked the N-terminal amino acid and was devoid of MSH-like activity.

The C-terminal of MCH also contributes to the MSH-like activity of MCH. Removal of the C-terminal amino acid, Val (VI), led to about 100-fold decrease in MSH-like potency (Table I). These results indicate that the entire heptadecapeptide sequence of MCH contributes to the MSH component of potency of MCH.

In contrast to the total loss of MCH-like activity of MCH by contraction of the 5-14 disulfide bridge, the heptadecapeptide analogues generally possessed considerable MSH-like activity as did MCH in the frog skin bioassay. More surprising, the truncated

analogue of MCH, [Cys^{7}]MCH$_{7-17}$, was actually about 7-fold more potent than MCH in stimulating melanosome dispersion within lizard melanocytes [14].

The analogues possessing MSH-like activity were also inhibitors of the MCH-like activity of MCH on fish melanocytes [15,21], but this inhibitory activity could be observed only in the presence of extracellular Ca^{2+}. Since it is well known that Ca^{2+} ions are needed for expression of MSH-like activity on melanocytes [22,23], but not for the MCH-like activity [21], we concluded that the antagonism was noncompetitive in nature and that it involved modulation of the action on the MSH receptor. Thus, MCH exhibits auto inhibition when used at unphysiologically high concentrations [21].

Analogues with modified bridge structures were also conformationally studied by means of CD spectroscopy [24]. The results suggest that the analogue with the full sequence and the smallest ring structure [Ala^{5},Cys^{10}]MCH has a rather high tendency to form an α-helical structure. It has been suggested that this helical structure may be responsible for the selective activity, allowing the proper conformation for MSH-like activity, but not allowing the proper conformation needed for favorable interaction with the MCH receptor.

In summary, the combination of studies that we and others have made strongly support the idea that MCH and MSH receptors have different but related structural and conformational requirements for peptide-receptor interaction. More constrained analogues [25] will be needed to obtain further insight into these specific requirements.

Acknowledgements

These studies were partially supported by the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo, grants 87-0851-4, CNPq, grants 407196/87 and 408683/88 (Brazil), U.S. Public Health Service grant AM-17420, and National Science Foundation grant DCB-8615706 (USA). Terry O. Matsunaga wishes to thank the National Institute on Drug Abuse (NIDA) for a post-doctoral fellowship grant 1F32 DA05371 BI-2. The technical assistance of Mr. Michael Shea, Mr. Sean Sullivan, and Ms. Rachel Stover is gratefully acknowledged. The clerical assistance of Ms. Danielle DuBois is also gratefully appreciated.

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