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Biphasic Effect of MCH on α-MSH Release
From the Tilapia (Oreochromis mossambicus) Pituitary

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GRÖNEVELD, D., P. H. M. BALM AND S. E. WENDELAAR BONGA. Biphasic effect of MCH on α-MSH release from the tilapia (Oreochromis mossambicus) pituitary. PEPTIDES 16(5) 945–949, 1995.—The effect of melanin-concentrating hormone (MCH) on the release of α-melanocyte stimulating hormone (α-MSH) from the tilapia pituitary gland was studied in vitro. In a superfusion set up, 10 nM to 1 μM synthetic salmon MCH caused a concentration-dependent inhibition of α-MSH release from tilapia neurointermediate lobes (NILs). Immunoneutralization of MCH in tilapia NILs further indicated that endogenous MCH has an inhibitory effect on the melanotropes. The release of monoacetylated α-MSH release was more strongly inhibited by MCH than that of des-, and diacetylated α-MSH, indicating that MCH modulates the secretory signal of the melanotropes in a quantitative and qualitative manner. A high concentration of MCH (10 μM) substantially increased the release of α-MSH. Further evidence in support of a stimulatory action of high concentrations of MCH was provided by the observation that the MCH analogue MCH(2–17) at 10 and 35 μM enhanced α-MSH release as well. Therefore, we conclude that the response of pituitary melanotropes to MCH is biphasic, as was reported previously for the effects of MCH on other targets in fish and mammals. Under physiological conditions the inhibitory action of MCH on fish melanotropes most likely dominates.

Melanin-concentrating hormone (MCH)  α-Melanocyte-stimulating hormone (α-MSH)  Teleost  Pituitary

Many lower vertebrates alter the color of their skin in response to variations in background coloration. Background adaptation is of paramount importance to these animals for survival. The regulation of background adaptation represents an attractive model for the analysis of the interactions between peptidergic neuroendocrine factors. In most species α-melanocyte-stimulating hormone (α-MSH) dominates the hormonal control of pigment migration in response to changes in background coloration. α-MSH is produced in the melanotropes of the pars intermedia of most vertebrates, and induces pigment dispersion (1). In teleosts a neurohormone, melanin-concentrating hormone (MCH), antagonizes α-MSH in this respect (5). As in other teleosts (4), in tilapia (Oreochromis mossambicus) MCH is synthesized in the hypothalamus and transported to the pituitary pars nervosa (18). Also in higher vertebrates, MCH is produced in the hypothalamus (4), and it plays a role in the stress response, in osmoregulation, and in lactation (2). In teleosts the MCH system appeared to be responsive to stress and ionic challenges as well (4,6,15–17), in addition to its function in the regulation of skin color.

There are indications that MCH may affect background adaptation at the level of the pituitary by inhibiting α-MSH release from the pars intermedia. Firstly, MCH nerve terminals are located in the vicinity of pars intermedia cells (10,29). Furthermore, it has been reported that trout injected with MCH had decreased plasma α-MSH levels (7), and immunoabsorbance of endogenous MCH in trout and eel pituitary tissue enhanced the release of α-MSH in vitro (9).

At present, little is known on the sensitivity of pituitary melanotropes to MCH, and on species differences for the effect of MCH on melanotropes. In the teleost skin, MCH induces aggregation of melanosomes in the blood (3,4), whereas administration of high concentrations of MCH to melanophores induces pigment dispersion. The latter has been explained by binding of MCH to α-MSH receptors on the melanophores (12,19). The MCH concentration–response curve of MCH on auditory gating in rats is also biphasic (27). This raises the question whether the concentration–response relationship of MCH on α-MSH release is biphasic as well.

Three forms of α-MSH are known: des-, mono-, and diacetylated α-MSH. In tilapia, all three α-MSH forms are released from the neurointermediate lobe (NIL) (25). Acetylation of α-MSH is of functional significance, because the isoforms of α-MSH have different melanotropic and corticotropic potencies in fish (22,24). Monoacetylated and diacetylated α-MSH are most effective on pigment dispersion in trout (22), whereas in particular diacetylated α-MSH has corticotropic activity in tilapia (8,24).

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The purpose of the present study was to gain more insight into the action of MCH at the pituitary level. We therefore quantitatively and qualitatively analyzed the effects of MCH and MCH(2–17), a MCH analogue lacking the N-terminal amino acid, which was helpful in elucidating the mechanisms of action of MCH on the teleost skin (11,26), on the release of α-MSH in vitro from the tilapia pituitary gland.

METHOD

Animals

Male and female tilapia (Oreochromis mossambicus), average weight 80 g, were bred in the aquarium facility of the Dept. of Animal Physiology of the University of Nijmegen. They were kept in fresh water at 28°C and were fed a commercial dried fish food (Tetramin). The fish were kept in glass aquaria, illuminated by overhead TL tubes, with a day–night rhythm of 12 h light and 12 h darkness. Immediately after removal from the tank, the animals were sacrificed by spinal transection and pituitary glands were dissected from the brain.

Superfusion of Neurointermediate Lobe Tissue

One or two freshly dissected pituitaries or NILs were placed on a nylon gauze in a 10-µl superfusion chamber. A multichannel peristaltic pump (Whatson Marlow) was used to pump carbogen-aerated incubation medium (IM) (142 mM NaCl, 2 mM KCl, 2 mM CaCl2, 15 mM HEPES (pH 7.38), 0.3 mg/ml bovine serum albumin (Sigma), 2.5 mg/ml glucose) through each chamber at a rate of 30 µl/min. The superfusion chambers as well as the IM were thermostat controlled at 28°C. The effluent from each chamber was collected in fractions over varying periods with an Isco (Retriever II model) fraction collector. During at least 3 h superfusion fractions were collected before switching to pulse medium containing MCH or MCH(2–17) (both synthesized by Dr. T.O. Matsunaga at the lab of Dr. V. J. Hruby and kindly provided by Dr. M. E. Hadley, Tucson, AZ) (32), for a 25–30-min period. After the pulse the superfusion was continued with IM. The collected fractions were stored immediately at −20°C until α-MSH radioimmunoassay.

Immunoneutralization of MCH From Tilapia NILs

NILs from tilapia kept in dark glass aquaria were dissected and individually preincubated in 100 µl IM, containing 5 mg/ml (w/v) glucose, during 1 h at 28°C. Subsequently, the NILs were transferred into 30 µl IM containing either 0.1% (v/v) MCH antiserum (a kind gift of Dr. B.I. Baker and Dr. H. Kawauchi) (28) or 0.1% (v/v) normal rabbit serum. The vials were aerated with carbogen, sealed, and shaken gently at 28°C for a 15-h incubation period. After this static incubation, media were collected and stored at −20°C until α-MSH radioimmunoassay; subsequently, the NILs were superfused as described above.

Reversed-Phase HPLC Analysis

To determine the effect of MCH on the profile of the secretory signal, superfusate was submitted to HPLC analysis. Different forms of α-MSH were separated on a Spherisorb 10 ODS column (Bischoff) as described previously (25). In short, the primary solvent was buffer B (0.5 M formic acid, 0.14 M pyridine, pH 3.0) and elution was accomplished with a gradient of 1-propanol at a flow rate of 2 ml/min. Fractions of 0.6 ml were collected. The fractions were dried in a Speedvac concentrator (Savant), diluted into 0.05 N HCl, 50% methanol, and submitted to α-MSH radioimmunoassay.

α-MSH Radioimmunoassay

The α-MSH radioimmunoassay (RIA) with L9 α-MSH antiserum has been described previously (24). The antiserum is equally sensitive to des-, mono- and diacetyl α-MSH. Cross-reactivity of the α-MSH antiserum with MCH [preliminary results have been reported by De Koning et al. (13)] and MCH(2–17) was assessed (see the Results section).

Processing of Data and Statistics

The results of the superfusions are either expressed as pg α-MSH per min per NIL, or as a percentage of the basal release (=100%). Basal release was defined as the average a-MSH release in 20–30 min prior to addition of secretagogue, and basal release values ranged from 30 to 200 pg/min/NIL. For the concentration–response relationships of MCH- or MCH(2–17)-induced effects, values for maximum inhibition or stimulation of α-MSH release during the secretagogue pulse were expressed as a percentage of the prepulse values. Correction of α-MSH values for cross-reactivity after a 10-µM MCH pulse was performed as follows: proceeding from 0.0032% cross-reactivity (see the Results section).

![FIG. 1. Inhibitory (A) and stimulatory (B) effects of MCH on α-MSH release from tilapia NILs during in vitro superfusion expressed as percentage of basal release (=100%). (A) 250 nM MCH, n = 12; (B) 10 µM MCH, n = 9. The time of addition of secretagogue at least 220 min of superfusion is designated t0. (C) Concentration–response relationship of MCH induced effects on α-MSH release. Basal release in this presentation is designated 0%. Numbers of incubations are given in parentheses for each concentration tested. *p < 0.05, **p < 0.001.](image)
FIG. 2. Cross-reactivity of MCH with α-MSH antiserum. Concentration series of MCH (●) and MCH(2−17) (▲) in 50 μl incubation medium were tested for cross-reactivity in α-MSH radioimmunoassay. B/B₀ is compared with the α-MSH standard curve (●). Sample size 3 or 4. SEMs not shown fall within the area of the symbol. EC₅₀ α-MSH = 1.8 nM, ED₅₀ MCH = 57 μM. MCH(2−17) did not cross-react.

RESULTS
Quantitative Effects of MCH on α-MSH Release

At concentrations below 1 μM, MCH caused a concentration-dependent inhibition of α-MSH release during in vitro superfusion of tilapia NILs [Fig. 1(A,C)], and at 10 μM α-MSH release was stimulated [Fig. 1(B)]. The effect of MCH on the α-MSH release lasted for the duration of the pulse; after the pulse α-MSH release did not restore to basal levels. The MCH concentration inducing 50% of maximum inhibition of α-MSH release (EC₅₀), using the data points up to and including 250 nM, was estimated to be 19 nM with a maximum inhibition of 42%. At 10 μM MCH the α-MSH release was stimulated with a maximum stimulation of 96 ± 17% (p < 0.001) [Fig. 1(C)]. Taking into account the slight cross-reactivity of MCH with the α-MSH antiserum (0.0032%) (Fig. 2), this percentage decreased marginally to 87 ± 22% (p < 0.001 compared with basal release). However, because the dilution curve for MCH only approximately paralleled the α-MSH dilution curve in the α-MSH radioimmunoassay (Fig. 2; the slope of log transformed curves was significantly different, p < 0.01) an additional control was performed. To mimic pulse conditions, MCH was added to prepulse superfusates to a final concentration of 10 μM. This yielded a nonsignificant increase (p = 0.88) of immunoreactive α-MSH of 2 ± 10 pg (and 16 ± 16%; n = 6) compared with untreated prepulse fractions. During the 10 μM MCH pulse the increase of immunoreactive α-MSH was 108 ± 33 pg (95 ± 28%; n = 5), which was significantly higher (p < 0.01) than the increase measured when MCH was added afterwards to superfusates. MCH(2−17), a MCH analogue that, in contrast to MCH, did not cross-react with the α-MSH antiserum (Fig. 2), induced a significant stimulation of the α-MSH release at concentrations of 10 and 35 μM (Fig. 3). The stimulation of α-MSH release by 10 μM MCH(2−17) was comparable with that evoked by 10 μM MCH.

Effect of Endogenous MCH on α-MSH Secretion

After incubation of tilapia NILs with 0.1% MCH antiserum, to immunoneutralize endogenous MCH, α-MSH release significantly increased compared with NILs incubated in medium containing normal rabbit serum [Fig. 4(A)]. After 15 h of static incubation, the antiserum was washed out by superfusion of the NILs with fresh incubation medium. During this superfusion the α-MSH release of the NILs incubated with the MCH antiserum returned to control levels within 2 h [Fig. 4(B)].

Effects of MCH on the Release of α-MSH Isoforms

To study the qualitative effect of MCH on α-MSH release, the isoforms of α-MSH released before and during pulses with 100 and 250 nM MCH were analyzed by HPLC. Des-, mono-, and diacetylated α-MSH were released during MCH pulses as well as under control conditions (Fig. 5). During the 100 and 250 nM MCH pulses the amount of monoacetylated α-MSH decreased relatively more than the amounts of des- and diacetylated α-MSH, when compared to control. Taking the area under the curve of diacetylated α-MSH (di), monoacetylated α-MSH (mono), and desacetylated α-MSH (des), we calculated di/mono and des/mono ratios of 0.45 and 0.38 for control, 0.73 and 0.50 during a 100 nM MCH pulse, and 0.95 and 0.61 during a 250 nM MCH pulse, respectively. The highest increase of the ratios was found at the concentration (250 nM) where the overall inhibitory effect was the strongest (Figs. 1 and 5).

DISCUSSION

The present results demonstrate that the MCH concentration–response curve for α-MSH release from the tilapia NIL is biphasic. At concentrations below 1 μM, synthetic MCH caused a concentration-dependent inhibition of α-MSH release. Concentration.
The different mode of action of these two inhibitors might enhance the flexibility of the fish to respond to environmental changes not only quantitatively but also qualitatively by selectively decreasing the release of mono- and diacetylated α-MSH from tilapia NILs (25). In this respect MCH, a potent inhibitor of α-MSH release in tilapia [EC$_{50}$ 10 nM], is comparable with the sensitivity to dopamine, another classical sense endocrine for the skin. Pituitary melanotropes are in close contact with the MCH-containing neurons of the neurohypophysis of tilapia (personal observation) and other teleosts (10,29), and MCH is endogenously released ([9], this study), whereas the effect of MCH on pigmentation migration was 1 nM (20). We reason that the lower sensitivity of melanotropes to MCH reflects the mode of MCH targeting, which is neuroendocrine in the pituitary and in the classical sense endocrine for the skin. Pituitary melanotropes are in close contact with the MCH-containing neurons of the neurohypophysis of tilapia (personal observation) and other teleosts (10,29), and MCH is endogenously released ([9], this study), whereas the effect of MCH on melanophores is exerted by relatively low circulating MCH levels (14,21). The difference in sensitivity of the tissues cannot be attributed to differences in the peptide sequences of synthetic salmon MCH and tilapia MCH, because we previously demonstrated that the sequences of both peptides are identical (18). The sensitivity of tilapia melanotropes to MCH is comparable with the sensitivity to dopamine, another potent inhibitor of α-MSH release in tilapia [EC$_{50}$ 10 nM; Dr. A. E. Lamers personal communication; see also (25)]. However, MCH modulates the secretory output of the melanotropes not only quantitatively but also qualitatively by selectively decreasing the release of monoacetylated α-MSH. In this respect MCH differs from dopamine, which indiscriminately inhibited the release of mono- and diacetylated α-MSH from tilapia NILs (25). The differential mode of action of these two inhibitors might enhance the flexibility of the fish to respond to environmental challenges. Tilapia melanotropes are more sensitive to MCH than to thyrotropin-releasing hormone (TRH), a stimulator of α-MSH release (EC$_{50}$ 200 nM) (23). However, with respect to the effect on differential α-MSH release MCH resembles TRH, which also enhances the di/mono ratio of released α-MSH (25).

Immunoneutralization of MCH in NILs in vitro showed that in tilapia endogenous MCH inhibits α-MSH release from the melanotropes and that this inhibitory effect persists in vitro as long as 18 h, because after washout of the MCH antisem α-MSH release restored to the low control levels. The inhibitory effect of MCH on tilapia melanotropes is in line with observations in trout and eel, that immunosorbance of endogenously released MCH enhanced the ratio of released over stored amounts of α-MSH (9). These findings indicate that under physiological conditions the effect of MCH on α-MSH is inhibitory.

High concentrations of MCH (10 µM) and MCH(2-17) (10 and 35 µM) significantly increased α-MSH release. The physiological importance of this stimulation of α-MSH release is as yet unclear. It is doubtful whether concentrations up to 10 µM MCH, which are very high compared with MCH plasma values of trout (10 to 300 pM) (14-16,21), occur in the teleost pituitary. However, it cannot be excluded that at the MCH nerve terminals in the vicinity of the melanotropes MCH occasionally reach micromolar concentrations. We demonstrated that the stimulation of α-MSH release by MCH could not be attributed to the slight cross-reactivity of MCH with the antibody used in the α-MSH RIA. The occurrence of this cross-reactivity supports the notion that the tertiary structures of α-MSH and MCH are related (11). Apparently, this structural similarity is lost when the N-terminal amino acid (Asp) of MCH is removed, because MCH(2-17) appeared not to cross-react with the α-MSH antibody (this study) and MCH(2-17) exhibited almost no α-MSH-like activity on skin melanophores (11,26). The stimulatory effect of MCH on the α-MSH release most probably is transduced by a mechanism different from that of the skin melanophores, because the effect of MCH(2-17) on α-MSH release from tilapia NILs was similar to that of MCH. Possibly, two MCH receptor subtypes, an inhibitory and a stimulatory one, occur in the teleost pituitary. However, we cannot exclude that MCH, at a high concentration, binds to receptors for other α-MSH release stimulating agents, such as TRH or CRH (25,31), although no structural similarity with these peptides has been described.

**FIG. 4.** Effect of in vitro immunoneutralization of endogenously released MCH on α-MSH release from tilapia NILs. (A) α-MSH release of NILs incubated during 15 h with 0.1% MCH antiserum (anti-MCH; n = 4) vs. NILs incubated with normal rabbit serum as controls (C; n = 4). (B) α-MSH release during subsequent superfusion with normal incubation medium of MCH antiserum-treated NILs (•) and controls (○). *p < 0.05.

**FIG. 5.** HPLC immunogram of α-MSH in pooled NIL superfusion fractions with or without MCH pulse. (A) Control, (B) 100 nM MCH, maximum inhibition of total α-MSH release in superfusion was 35 ± 7% (n = 8); (C) 250 nM MCH, maximum inhibition of total α-MSH was 47 ± 6% (n = 4). Peak identification was based on Lamers et al. (25); des = desacetylated α-MSH, mono = monoacetylated α-MSH, di = diacetylated α-MSH.
The finding that MCH interacts with the α-MSH cell not only has implications for our understanding of the roles of both cell types in background adaptation, but probably also for their functions in the response to stressors. In tilapia, both cell types are responsive to acidification as a stressor (17,24), and in trout MCH secretion and biosynthesis are affected during injection and disturbance stress (6,15).

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