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$ACTH_{1-4}$ Potentiates α-MSH-Induced Melanophore Dispersion and Excessive Grooming

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DE GRAAN, P. N. E., B. M. SPRUIJT, A. N. EBERLE, J. GIRARD AND W. H. GISPEN. *$ACTH_{1-4}$ potentiates α-MSH-induced melanophore dispersion and excessive grooming*. PEPTIDES 7(1) 1–4, 1986.—The biological activity and a possible modulatory role of the N-terminal tetrapeptide Ser-Tyr-Ser-Met from α-MSH/ACTH was tested in the *Anolis* melanophore assay, the *Xenopus* melanophore assay, tyrosinase stimulation in mouse melanoma cells and in excessive grooming in the rat. $ACTH_{1-4}$ did not exhibit biological activity in any of these four assays nor did it have modulatory properties in the *Xenopus* and the melanoma cell assay. However, in the *Anolis* assay $ACTH_{1-4}$ potentiated pigment dispersion induced by α-MSH, α-MSH_{5-13} and $ACTH_{1-24}$ by a factor of about 2. In the grooming assay $ACTH_{1-4}$ potentiated the effects of α-MSH, α-MSH_{5-13}, $ACTH_{1-16}$ and $ACTH_{5-16}$, but not those of $ACTH_{1-24}$. Oxidized $ACTH_{1-4}$ was without biological activity and potentiating properties in all four assays. This study shows that small fragments of the pro-opiomelanocortin precursor, which are devoid of biological activity, can modulate peripheral and central actions of α-MSH/ACTH.

Melanotropins Structure-activity $ACTH_{1-4}$ Potentiation Grooming behavior Pigment dispersion

α-MSH is the most potent pro-opiomelanocortin-derived peptide hormone regarding pigment dispersion [12] and ACTH has the highest activity in inducing excessive grooming [13]. Extensive structure-activity studies using fragments and derivatives of these hormones have revealed that the hormonal information within the common tridecapeptide sequence 1–13 is arranged in more or less distinct regions of the molecule. The sequences 4–9 and 10–13 are regarded as message sequences, since they possess melanotropic activity in a number of melanophore systems [6,10]. Both sequences are also important for the induction of excessive grooming in the rat [13]. The sequence 4–10 contains all the information required for activity in learning tasks (for a review see [1]).

The N-terminal sequence Ser-Tyr-Ser-Met of α-MSH/ACTH is regarded as a potentiating element within the molecule when covalently attached to the 2 message sequences [8]. The free tetrapeptide was shown to have no melanotropic activity in the *Rana* and *Anolis* melanophore assays [7]. However, so far its effects have not been studied in combination with α-MSH/ACTH. Several examples are known where fragments of the pro-opiomelanocortin precursor modulate the effect of other pro-opiomelanocortin-derived peptides. MSH-induced pigment dispersion is potentiated by β-endorphin [4]. ACTH-induced corticosterone production is increased by the amino-terminal region of the pro-opiomelanocortin precursor [18]. ACTH-induced excessive grooming is suppressed by [des-Tyr¹]-γ-endorphin [14].

The aim of this study was to investigate possible modulatory effects of $ACTH_{1-4}$ as a free peptide, simultaneously present with α-MSH, α-MSH_{5-13} and $ACTH_{1-24}$. Modulatory effects were studied on pigment dispersion in the *Anolis* and the *Xenopus* melanophore assay, on tyrosinase stimulation in mouse melanoma cells and on excessive grooming in the rat.

METHOD

Anolis *Melanophore Assay*

The *in vitro* bioassay using skin pieces of *Anolis carolinensis* was performed according to Tilders *et al.* [22] as modified by Eberle and Girard [11]. Briefly, dorsal skins of male *Anolis carolinensis* were cut into 50–60 pieces of approximately 2.5×2.5 mm and floated on Krebs-Ringer solution. Serial dilutions of the peptides were tested with three skin pieces for each concentration and the melanophore response was assessed after a 15 min incubation period by visual comparison with a standard series and expressed as percentage of the maximal response (for details see [11]). $ACTH_{1-4}$ (1 or 10 μg/ml) was added concomitantly with the melanotropic peptide.

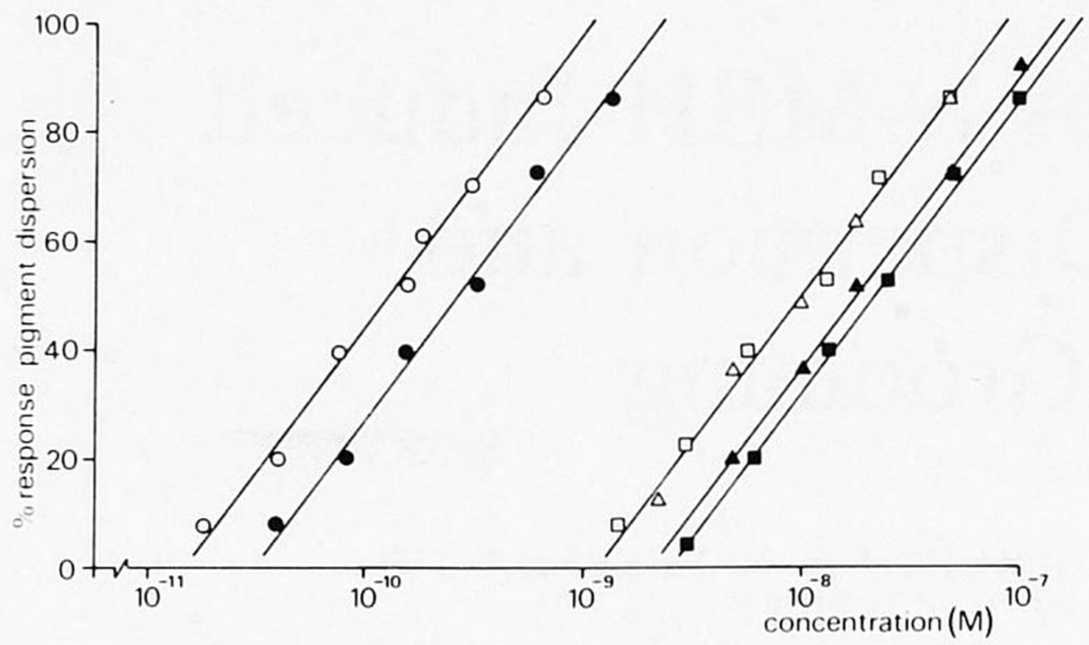

FIG. 1. Pigment dispersion in Anolis melanophores induced by α-MSH (●), $ACTH_{1-24}$ (■) and α-MSH_{5-13} (▲) in the absence (closed symbols) or presence (open symbols) of 10 μg/ml $ACTH_{1-4}$. Each point represents the mean of 8–10 measurements. SEM values are about 10% (see [11]).

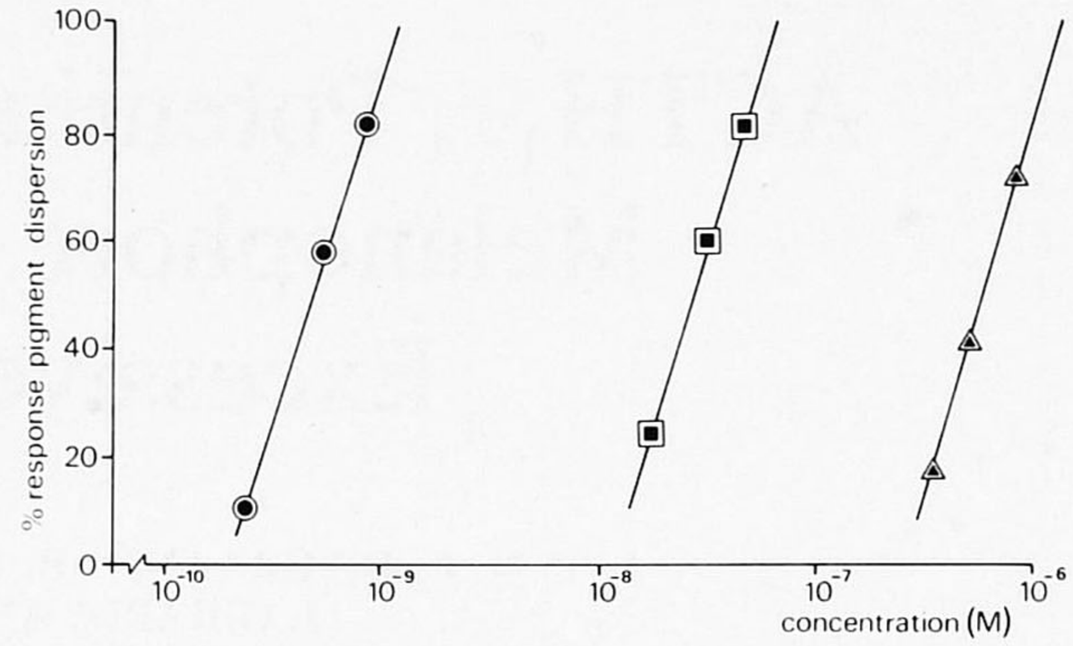

FIG. 2. Pigment dispersion in *Xenopus* melanophores induced by α-MSH (●), $ACTH_{1-24}$ (■) and α-MSH_{5-13} (▲) in the absence (closed symbols) or presence (open symbols) of 10 μg/ml $ACTH_{1-4}$. Each point represents the mean of 8–12 measurements. SEM values are about 10% (see [5]).

Xenopus *Melanophore Assay*

The *in vitro* melanophore bioassay, using tail-fins of *Xenopus* tadpoles, was performed as described in detail by De Graan *et al.* [5]. Tadpoles of *Xenopus laevis* were used at stage 51–53 according to Nieuwkoop and Faber [17] and adapted to a white background. Pieces of 2×2 mm (containing about 100 melanophores) were excised from the ventral tail-fins, equilibrated and subsequently treated with the peptides for 60 min. The average melanophore response was quantified microscopically using the melanophore index of Hogben and Slome [15]. $ACTH_{1-4}$ was added 15 min prior to the melanotropic peptide.

Tyrosinase Stimulation in Mouse Melanoma Cells

Tyrosinase activity in Cloudman (S-91) mouse melanoma cells in monolayer culture was assayed according to Pomerantz [19] as modified by Eberle and Schwyzer [9]. In brief, Cloudman S-91 Clone M-3 (CCL 53.1) mouse melanoma cells were maintained in a rapidly growing monolayer culture (doubling time: 24 hours) in 25 cm^2 Falcon tissue culture flasks (inoculation: 10^6 cells per flask in 10 ml of NCTC-135 medium with 10% horse serum). Sterile solutions with 4–9 different concentrations of each peptide were added together with 10 μCi of ^{3}H-tyrosine to the flasks when they contained 2×10^6 cells. The activity of tyrosinase was determined by measuring the release of ^3H_2O after 48 hours (elimination of ^{3}H-tyrosine from the samples by treatment with charcoal). A maximal response of about 200% above the basal level of ^3H_2O of non-stimulated cells was obtained with a 10^{-7} M α-MSH solution. This value was arbitrarily set as 100% of hormonal tyrosinase-stimulating potency. $ACTH_{1-4}$ was added concomitantly with the melanotropic peptide. The cells were incubated with the peptides for 48 hr.

Excessive Grooming in the Rat

Male rats of an inbred Wistar strain were used (TNO, Zeist, NL). They were bred at our laboratory and weighed about 145 g at the commencement of the experiments. The animal rooms were kept at a 12 hr light/12 hr dark schedule (8.00 hr a.m./20.00 hr p.m.). Peptides were administered in 3.0 μl saline by means of an intracerebroventricular injection [2] and the grooming behavior was recorded essentially as described by Gispen *et al.* [13]. During a 55 min session a maximum score of 220 was possible. $ACTH_{1-4}$ was always injected 10 min prior to the grooming-inducing peptide. The dose of the grooming-inducing peptide was chosen to produce a grooming score between 60 and 120, in order to allow detection of modulatory effects of $ACTH_{1-4}$. Several doses of $ACTH_{1-4}$ were tested; the lowest dose producing modulatory effects—if any—is presented (see Fig. 4). Controls received two 3.0 μl saline injections with an interval of 10 min.

Statistical differences were assessed by an analysis of variance followed by a supplemental *t*-test [3]; significance was assigned to the $p<0.05$ level (two-tailed).

Peptides

α-MSH and α-MSH_{5-13} were synthesized by a classical solution approach as described by Eberle *et al.* [7]. $ACTH_{1-24}$ used in the *Xenopus* bioassay, the *Anolis* bioassay and the tyrosinase assay were a generous gift of CIBA-Geigy (Basel, CH); $ACTH_{1-24}$ and the ACTH fragments used in the grooming studies were kindly provided by Organon Int. BV (Oss, NL). $ACTH_{1-4}$ was synthesized from BOC-Ser-Tyr-Ser-Met, obtained from CIBA-Geigy (Basel, CH). Oxidation of Met^4 was performed with standard techniques using H_2O_2 and acetic acid.

RESULTS

Pigment Cell Systems

The free tetrapeptide $ACTH_{1-4}$, tested in concentrations up to 1 mg/ml, did not show any melanotropic activity in the *Anolis* or the *Xenopus* melanophore assay. In the *Anolis* assay 10 μg/ml $ACTH_{1-4}$ significantly ($p<0.01$) potentiated the dispersion induced by α-MSH, α-MSH_{5-13} and by $ACTH_{1-24}$ (Fig. 1). The factor of potentiation as calculated from the EC_{50} values was 2.5 for α-MSH, 2.0 for α-MSH_{5-13} and 2.5 for $ACTH_{1-24}$. At a dose of 1 μg/ml $ACTH_{1-4}$ potentiated the effect of α-MSH only slightly and not over the full

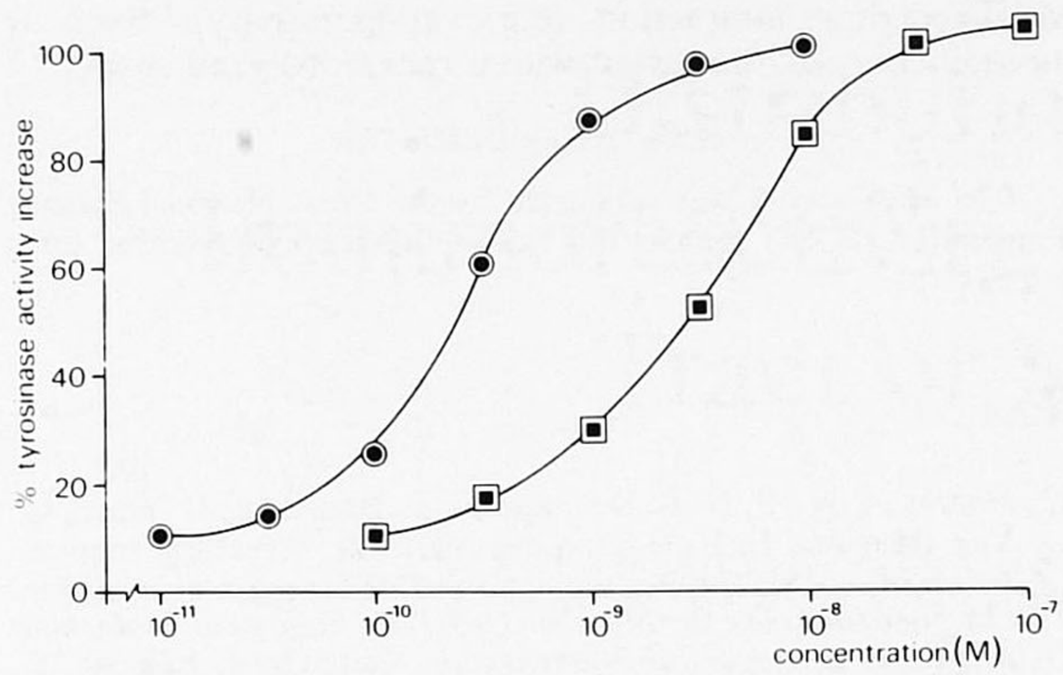

FIG. 3. Tyrosinase activity in Cloudman S-91 mouse melanoma cells induced by α-MSH (●) and ACTH$_{1-24}$ (■) in the absence (closed symbols) or presence (open symbols) of 10 μg/ml ACTH$_{1-4}$. Each point represents the mean of 8 measurements. SEM values are about 6% (see [13]).

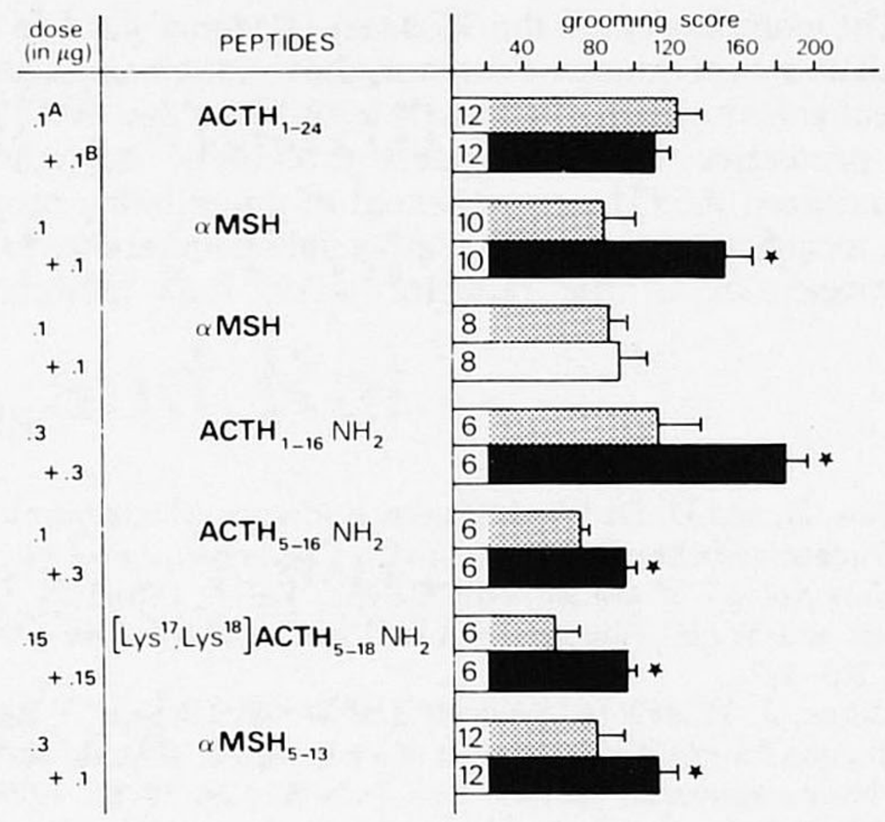

FIG. 4. Peptide-induced excessive grooming in the rat in the absence (dotted bars) or presence of ACTH$_{1-4}$ (black bars). The open bar denotes α-MSH-induced grooming in the presence of oxidized ACTH$_{1-4}$. (A) dose of the grooming-inducing peptide; (B) dose of ACTH$_{1-4}$ injected 10 min prior to the grooming-inducing peptide; *significantly different from control ($p<0.05$). Numbers in the bars indicate numbers of animals tested. Error bars indicate SEM. For experimental details see the Method section.

range of α-MSH concentrations (results not shown). Oxidized ACTH$_{1-4}$ did not potentiate the effect of any of these peptides (not shown).

In the *Xenopus* assay, which is less sensitive than the *Anolis* assay, ACTH$_{1-4}$ was devoid of potentiating properties even at concentrations up to 1 mg/ml. The dose-response curves for α-MSH, α-MSH$_{5-13}$ and ACTH$_{1-24}$ in the presence or absence of ACTH$_{1-24}$ coincide (Fig. 2).

Incubation of cultured Cloudman S-91 mouse melanoma cells with ACTH$_{1-4}$ (10 μg/ml) during 48 hr tended to decrease basal tyrosinase activity in these cells by about 20%. The presence of ACTH$_{1-4}$ did not affect the dose-response curves of α-MSH and ACTH$_{1-24}$ (Fig. 3), or ACTH$_{5-13}$ (results not shown).

Excessive Grooming in the Rat

Intracerebroventricular injection of ACTH$_{1-4}$ (tested in doses up to 3.0 μg) did not induce excessive grooming. The grooming induced by α-MSH, α-MSH$_{5-13}$ and a number of ACTH-fragments was clearly potentiated by ACTH$_{1-4}$ (Fig. 4). However, ACTH$_{1-4}$, tested in doses from 0.1 μg–3.0 μg, did not potentiate ACTH$_{1-24}$ (doses from 0.1 μg–3.0 μg). Because of the design of the assay, the degree of potentiation of different peptides cannot directly be calculated. It is striking, however, that the grooming score induced by 0.3 μg α-MSH$_{5-13}$ in the presence of ACTH$_{1-4}$ (0.1 μg) does not differ significantly from that induced by 0.3 μg α-MSH (115±7 and 122±8, respectively). Similarly, the activity of 0.3 μg ACTH$_{5-16}$ in the presence of ACTH$_{1-4}$ (1 μg) was almost identical to that of 0.3 μg ACTH$_{1-16}$ itself (118±8 and 112±6, respectively). Oxidized ACTH$_{1-4}$ (0.1 μg–3.0 μg) was devoid of potentiating properties.

DISCUSSION

Our data confirm and extend earlier findings that ACTH$_{1-4}$ has no melanotropic activity. Furthermore, we showed that the tetrapeptide is also inactive in inducing excessive grooming in the rat. Although the peptide itself was inactive, it showed potentiating properties in the *Anolis* and the grooming assay. In the latter assay the biological activity of α-MSH$_{5-13}$ in the presence of ACTH$_{1-4}$ was almost identical to that of α-MSH itself. Similarly, ACTH$_{1-4}$ plus ACTH$_{5-16}$ produced the same effect as ACTH$_{1-16}$ alone. These results suggest that the 1–4 fragment does not necessarily have to be covalently linked to the rest of the molecule to produce the full biological response. Hence, ACTH$_{1-4}$ may interact with the fragments in a complementary way. In contrast, in the *Anolis* assay the activity of ACTH$_{1-4}$ plus α-MSH$_{5-13}$ does not reach that of α-MSH. Moreover, the concentration of ACTH$_{1-4}$ required for potentiation is high relative to the α-MSH concentration. This points to a difference in the mechanism of potentiation between covalently attached and free tetrapeptide. The difference between the grooming and the *Anolis* assay may reside in the different relative biological activities of the 5–13 fragment and des-acetyl-α-MSH compared to α-MSH.

Interestingly, also the full α-MSH sequence 1–13 is potentiated by ACTH$_{1-4}$ in the *Anolis* and the grooming assay. This unexpected finding makes it rather unlikely that ACTH$_{1-4}$ simply acts in a complementary way. The effect of ACTH$_{1-24}$ is potentiated in the *Anolis* assay, but not in the grooming assay. In the latter assay ACTH$_{1-24}$ is the most potent agonist [13]. Our results indicate that in the grooming assay highly potent agonists cannot be further potentiated by ACTH$_{1-4}$, not even at low ACTH$_{1-24}$ concentrations. Indeed, preliminary experiments with the "superagonist" [Nle^4,D-Phe^7]-α-MSH [20,21] show that grooming induced by this agonist is not potentiated by ACTH$_{1-4}$ (De Graan and Spruijt, unpublished).

At present it is difficult to explain why ACTH$_{1-4}$ potentiates only in 2 of the 4 systems tested. The lack of potentiation in the *Xenopus* and the melanoma cell assay may be due to the specific characteristics of the systems or the assay procedures. Just recently it was discovered that *Xenopus* α-MSH contains alanine in position 1 instead of serine [16]. We have no explanation for the fact that ACTH$_{1-4}$ tends to decrease basal tyrosinase activity in the melanoma cell as-

say. The complexity of the bioassay systems used in our potentiation experiments does not allow conclusions about the mechanism of potentiation. It is unlikely that $ACTH_{1-4}$ has a protective function against proteolytic degradation since oxidized $ACTH_{1-4}$ was devoid of potentiating properties. A more probable mechanism would be an interaction of the tetrapeptide at the receptor level, thus modulating MSH-receptor interaction (e.g., cooperativity). Such a mechanism could be tested with a receptor-ligand assay.

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