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Cardiovascular effects of γ-MSH/ACTH-like peptides: structure-activity relationship

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Abstract

Intravenous administration of γ_2-melanocyte-stimulating hormone (γ_2-MSH) to conscious rats causes a dose-dependent increase in blood pressure and heart rate, while the structurally related peptide adrenocorticotropic hormone-(4–10) (ACTH-(4–10)) is 5–10 times less potent in this respect. This prompted us to investigate which amino acid sequence is determinant for the cardiovascular selectivity of peptides of the γ-MSH family. Lys-γ_2-MSH, most likely the endogenously occurring γ-MSH analog, was as potent as γ_2-MSH in inducing increases in blood pressure and heart rate. Removal of C-terminal amino acids resulted in γ-MSH-fragments which were devoid of cardiovascular activities. Removal of amino acids from the N-terminal side of γ_2-MSH resulted in fragments which were less potent, but had an intrinsic activity not different from that of γ-MSH. Surprisingly, γ-MSH-(6–12) was more potent than γ_2-MSH. The shortest fragment which displayed pressor and tachycardiac responses was the MSH 'core', His-Phe-Arg-Trp (= γ-MSH-(5–8)), which is identical to ACTH-(6–9). This was corroborated by testing fragments of ACTH-(4–10). We conclude that the message essential for cardiovascular effects resides in the γ-MSH-(5–8)/ACTH-(6–9) sequence. Proper C-terminal elongation is required for full expression of cardiovascular activity of γ_2-MSH, as the sequence of Asp^9-Arg^{10}-Phe^{11} appears to play an important role in establishing intrinsic activity. The amino acids N-terminal to the MSH 'core' sequence appear to be essential for the potency of the peptides.

Keywords: γ_2-MSH (γ_2-melanocyte-stimulating hormone); ACTH-(4–10) (adrenocorticotropic hormone-(4–10)); Blood pressure; Heart rate; Structure-activity; Melanocortin receptor; (Rat)

1. Introduction

The dodecapeptide γ_2-melanocyte-stimulating hormone (γ_2-MSH) and the heptapeptide adrenocorticotropic hormone-(4–10) (ACTH-(4–10)) have been reported to affect cardiovascular paradigms. They are peptides with partial homology: the ACTH-(4–9) sequence occurs in γ_2-MSH with one amino acid replacement, i.e. glutamic acid in glycine (see Table 1). Following intravenous (i.v.) administration to conscious rats these peptides cause pronounced increases in blood pressure and heart rate (Klein et al., 1985; Sun et al., 1992; De Wildt et al., 1993), in combination with a strong enhancement of carotid and cerebrocortical blood flow (De Wildt et al., 1995). γ_2-MSH is 5–10 times more potent than ACTH-(4–10) (Klein et al., 1985; De Wildt et al., 1993), while γ_1-MSH (= des-Gly^{12}-γ_2-MSH) is either as potent or slightly less potent than γ_2-MSH (Gruber et al., 1985; Sun et al., 1992). γ_3-MSH, which is γ_2-MSH with a C-terminal extension of 13 amino acids (see Eberle, 1988), has no cardiovascular effects (Gruber et al., 1985; Klein et al., 1985). Likewise, structurally related peptides such as α-MSH (= [$AcSer^1$]ACTH-(1–13)) (Klein et al., 1985; De Wildt et al., 1993) and the behaviorally active, stable ACTH/MSH-(4–9) analog Org 2766 (=

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[$MetO_2^4$,D-Lys^8,Phe^9]ACTH-(4–9)) (De Wildt et al., 1993) are devoid of cardiovascular effects in conscious rats. These data indicate that other structural characteristics than the Met-x-His-Phe-Arg-Trp sequence, which γ_1-MSH and γ_2-MSH share with ACTH-(4–10), are responsible for the considerable cardiovascular selectivity of the γ-MSHs.

The objective of the present investigations was to study in detail the structural features of the amino acid sequence of the γ-MSHs which are determinant for cardiovascular selectivity. To this end we measured the effects of a considerable number of γ-MSH/ACTH-related peptides and fragments on blood pressure and heart rate of the conscious, freely moving rat. Three series of peptides were used: (1) Lys-γ_2-MSH, which is one of the endogenously occurring γ-MSHs (see Eberle, 1988), and C-terminally shortened fragments, (2) γ_2-MSH and N-terminally shortened fragments, and (3) ACTH-(4–10) and various fragments thereof (for amino acid sequences, see Table 1). The results, together with previously published data (Klein et al., 1985; De Wildt et al., 1993), indicate that the tripeptide C-terminal of the His-Phe-Arg-Trp MSH/ACTH 'core' structure is also the core sequence for cardiovascular selectivity. The C-terminal part of γ_1-MSH and γ_2-MSH appears to be crucial for intrinsic activity, and the N-terminal part for potency.

2. Materials and methods

2.1. Animals

The experiments were carried out with male Wistar rats (U:WU cpb), weighing between 200–345 g. Prior to the operations the rats were housed three to a cage. Food pellets and tap water were provided to the rats ad libitum. The room temperature was kept at 20 ± 1°C

Table 1
Amino acid sequences of various γ-MSH/ACTH fragments

Lys-γ_2-MSH	Lys-Tyr^1-Val^2-Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}
Lys-γ-MSH-(1-10)	Lys-Tyr^1-Val^2-Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}
Lys-γ-MSH-(1-8)	Lys-Tyr^1-Val^2-Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**
Lys-γ-MSH-(1-8)-NH_2	Lys-Tyr^1-Val^2-Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**-NH_2
γ_2-MSH	Tyr^1-Val^2-Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}
γ-MSH-(2-12)	Val^2-Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}
γ-MSH-(3-12)	Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}
γ-MSH-(4-12)	Gly^4-**His^5-Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}
γ-MSH-(5-12)	**His^5-Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}
γ-MSH-(6-12)	**Phe^6-Arg^7-Trp^8**-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}
γ-MSH-(3-8)	Met^3-Gly^4-**His^5-Phe^6-Arg^7-Trp^8**
γ-MSH-(5-8) = ACTH-(6-9)	**His^5-Phe^6-Arg^7-Trp^8**
ACTH-(4-10)	Met^4-Glu^5-**His^6-Phe^7-Arg^8-Trp^9**-Gly^{10}
ACTH-(5-10)	Glu^5-**His^6-Phe^7-Arg^8-Trp^9**-Gly^{10}
ACTH-(6-10)	**His^6-Phe^7-Arg^8-Trp^9**-Gly^{10}
ACTH-(4-9)	Met^4-Glu^5-**His^6-Phe^7-Arg^8-Trp^9**
ACTH-(4-7)	Met^4-Glu^5-**His^6-Phe^7**

The MSH 'core' sequence His-Phe-Arg-Trp is highlighted.

with lights on from 7:00 a.m. to 7:00 p.m. and a relative humidity of 50–60%. Following operations the rats were housed individually under the same conditions.

The design of the experiments was approved by the Experimental Animal Boards of the Medical Faculty of the Utrecht University and the National Institute for Public Health and Environmental Protection.

2.2. Experimental procedure

Cannulations were carried out 3–4 days prior to the experiment. The rats were anesthetized with Nembutal in a dose of 0.11 ml/100 g body weight, intraperitoneally. Prior to the operation the rats received a single injection of antibiotics (0.05 ml s.c.). For the measurement of blood pressure and heart rate cannulation of the aorta was performed as described by Weeks and Jones (1960) as adapted by Nijkamp (1975), with slight modifications. The cannula, consisting of a 9 cm polythene tubing (PP25, i.d. 0.40 mm, o.d. 0.80 mm) with a 540° loop in the middle and melted into a polyethylene tubing (PE100, i.d. 0.86 mm, o.d. 1.52 mm). The cannula was inserted into the abdominal aorta, approximately 1 cm above the bifurcation to the lower extremities. Histoacryl blue was used to close the wound and to fix the cannula in place. The tubing was guided underneath the skin towards the skull. The cannula was filled with a PVP (50% polyvinylpyrrolidone (M = 25 000); 500 IU/ml heparin) solution. For i.v. administration of peptides the jugular vein was cannulated as described by Steffens (1969), with slight modifications. The jugular vein cannula consisted either of a Silastic cannula (i.d. 0.63 mm, o.d. 1.19 mm) or a polyethylene tubing (PE50, i.d. 0.58 mm, o.d. 0.965 mm). The cannula was guided underneath the skin towards the skull and filled with saline. Both cannulas were connected to a stainless steel connector, stoppered and fixed on the skull with dental cement. The rats were kept warm in either a temperature-controlled box or under a lamp until they regained consciousness.

2.3. Measurement of blood pressure and heart rate

Arterial blood pressure and heart rate were measured by connecting the aortic cannula to a pressure transducer (Viggo-Spectramed, disposable DTX/plus). A PE100 tubing of a length sufficient to enable the rat to move around in its cage relatively undisturbed was connected to the stainless-steel loop of the aorta cannula on the head of the rat. The pressure transducer was connected to a pressure signal pre-amplifier/biotachometer (Instrument Service, Utrecht University) coupled to a Wekagraph WK-812 AR recording system. Blood pressure and heart rate were recorded continuously at a chart speed of 10 mm/min.

2.4. Experimental protocol

For i.v. administration the peptides were freshly dissolved in saline (0.9% NaCl) or diluted from frozen stock solution. After a 45 min stabilization period the peptides were infused into the jugular vein in the doses indicated in the Results section in a volume of 0.1 ml by means of a Braun infusion pump (Braun Perfusor) set at a rate of 500 μl/min during 12 s. Each infusion of a peptide was followed by 0.1 ml saline in order to flush the cannula. Saline served as vehicle control. Only those rats were used which had a significant increase in mean arterial pressure (Δ mean arterial pressure = 40–50 mm Hg) after i.v. administration of either phenylephrine (5 μg/kg body weight) or γ_2-MSH (50 nmol/kg body weight). Measurements started between 10:00 and 11:00 a.m. Per rat only one peptide was tested. The various doses were given with an interval of 10–15 min.

2.5. Statistics

Mean arterial pressure was calculated according to the formula:

$$\frac{(2 \times P_d + P_s)}{3}$$

in which P_d is diastolic pressure and P_s systolic pressure. For dose-response relationships the change in mean arterial pressure and heart rate was calculated at the time of maximal effect as compared to pre-injection values. In order to calculate ED_{50} value the data of the dose-response curves were transformed by means of the Hill equation:

$$\frac{E_D}{E_{max}} = \log \frac{D}{D + ED_{50}}$$

where E_D is the effect at dose D, E_{max} the (estimated) maximal effect at plateau, D dose, and ED_{50} effective dose required to produce half-maximal effect. These data were fitted using the non-linear steepest gradient method (Levenberg, 1944; Marquardt, 1963). From this fit the ED_{50} and E_{max} were calculated which reflect a measure of potency and intrinsic activity (α), respectively. For those peptides which did not show a complete dose-response curve of mean arterial pressure or heart rate after i.v. administration to rats, the E_{max} was visually estimated if possible.

Basal values of mean arterial pressure, heart rate, and ED_{50} and E_{max} were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Data of the dose-response curves were analyzed by repeated-measures ANOVA followed by Student t-test as post-hoc test. The level of significance

was set at the 95% confidence limit. All data are expressed as the mean ± S.E.M.

2.6. Drugs

Nembutal (sodium pentobarbital, 60 mg/ml, benzylalcohol 9 mg/ml) was purchased from Sanofi, Maassluis, Netherlands; heparin from either Organon Technika, Boxtel, Netherlands (Tromboliquin, heparin sodium, 5000 IU/ml) or Leo Pharmaceutical Products, Weesp, Netherlands (heparin sodium 5000 IU/ml); Histoacryl blue (enbucrilate 1 g/ml) from B. Braun Melsungen, Melsungen, Germany; Combi-kel 20/20 (procaine benzylpenicillin 200000 IU/ml, dihydrostreptomycin sulphate 200 mg/ml) from Kombivet, Etten-Leur, Netherlands; Strepto-pen 20/20 (procaine penicillin G 200000 IU/ml, streptomycin sulphate 200 mg/ml) from Alfasan, Woerden, Netherlands; and PVP (polyvinylpyrrolidone) from Sigma Chemical Co., St. Louis, MO, USA.

ACTH-(4–7) and ACTH-(4–9) were donated by Organon International, Oss, Netherlands. Lys-γ_2-MSH, Lys-γ-MSH-(1–10), Lys-γ-MSH-(1–8), Lys-γ-MSH-(1–8)-NH_2, ACTH-(5–10), ACTH-(6–10), γ-MSH-(2–12), γ-MSH-(3–12), γ-MSH-(4–12), γ-MSH-(5–12), and γ-MSH-(6–12) were synthesized at and kindly provided by the National Institute for Public Health and Environmental Protection (RIVM), Bilthoven, Netherlands. ACTH-(4–10) was either donated by Organon or synthesized at the RIVM. γ_2-MSH was either purchased from Bachem, Bubendorf, Switzerland, or synthesized at the RIVM. The MSH 'core' sequence (ACTH-(6–9) = γ-MSH-(5–8)) and γ-MSH-(3–8) were obtained from Bachem.

The peptides were analyzed by fast atom bombardment mass spectrometry in the positive ion mode. In all cases, the protonated molecular ion (MH^+) was detected at the expected values of m/z (i.e. ± 0.3 amu, $z = 1$).

3. Results

3.1. Baseline mean arterial pressure and heart rate values

Baseline mean arterial pressure and heart rate values were not significantly different for the various groups. Pre-administration values of mean arterial pressure varied from 100 ± 3 mm Hg in the experiment with γ-MSH-(3–8) ($n = 6$) to 113 ± 7 mm Hg in the experiment with ACTH-(4–9) ($n = 6$). Basal heart rate varied from 330 ± 12 bpm in the experiment with ACTH-(4–10) ($n = 11$) to 375 ± 13 bpm in the experiment with ACTH-(4–7) ($n = 6$). Pre-administration values for mean arterial pressure and heart rate did not significantly differ before the administration of the various doses of a peptide in a given experiment. Saline had no significant effect on mean arterial pressure and heart rate (data not shown).

3.2. Effect of γ_2-MSH and Lys-γ_2-MSH; result of removal of C-terminal amino acids

In a first series of experiments dose-response curves were made for the effects of γ_2-MSH and Lys-γ_2-MSH

Table 2
Potency (ED_{50}) and intrinsic activity (E_{max}) of various γ-MSH/ACTH peptides with respect to mean arterial pressure and heart rate

	Mean arterial pressure		Heart rate	
	ED_{50} (nmol/kg)	Δ E_{max} (mm Hg)	ED_{50} (nmol/kg)	Δ E_{max} (bpm)
Lys-γ_2-MSH	29 ± 8	52 ± 4	45 ± 16	105 ± 8
Lys-γ-MSH-(1–10)	NA	NA	NA	NA
Lys-γ-MSH-(1–8)	NA	NA	NA	NA
Lys-γ-MSH-(1–8)-NH_2	NA	NA	NA	NA
γ_2-MSH	30 ± 5	53 ± 4	41 ± 9	104 ± 5
γ-MSH-(2–12)	60 ± 12 [a]	65 ± 5	37 ± 9	91 ± 5
γ-MSH-(3–12)	42 ± 9	57 ± 3	43 ± 9	83 ± 4 [a]
γ-MSH-(4–12)	43 ± 10	60 ± 4	42 ± 12	103 ± 13
γ-MSH-(5–12)	172 ± 28 [a]	59 ± 6	151 ± 28 [a]	101 ± 16
γ-MSH-(6–12)	18 ± 1 [a]	56 ± 5	22 ± 3	95 ± 7
γ-MSH-(3–8)	NA	NA	NA	NA
γ-MSH-(5–8) = ACTH-(6–9)	NA	NA	NA	NA
ACTH-(4–10)	287 ± 37	51 ± 4	313 ± 57	104 ± 13
ACTH-(5–10)	312 ± 38	48 ± 8	243 ± 36	105 ± 29
ACTH-(6–10)	464 ± 32 [b]	57 ± 2	423 ± 103	107 ± 9
ACTH-(4–9)	NA	NA	NA	NA
ACTH-(4–7)	NA	NA	NA	NA

Data are expressed as mean ± S.E.M; [a] $P < 0.05$ as compared to γ_2-MSH; [b] $P < 0.05$ as compared to ACTH-(4–10); NA = not available/incorrect fit.

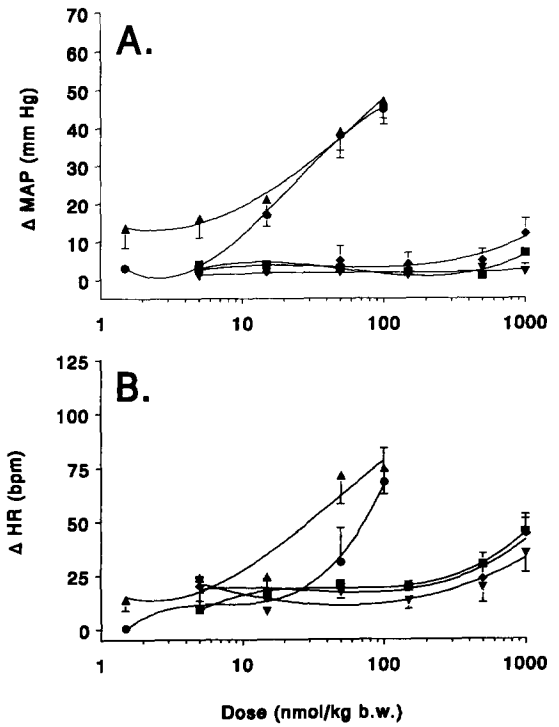

Fig. 1. Dose-response relationship for various N-terminal γ-MSH fragments with respect to their effects on mean arterial blood pressure (MAP) (A) and heart rate (HR) (B) after i.v. administration to conscious, freely moving rats. The results are expressed as absolute change from pre-administration values and as mean ± S.E.M. (n = 6–8). (●) γ_2-MSH, (▲) Lys-γ_2-MSH, (■) Lys-γ-MSH-(1–10), (▼) Lys-γ-MSH-(1–8), and (◆) Lys-γ-MSH-(1–8)-NH_2.

and for three fragments of this latter peptide with a shortened C-terminus. A dose-dependent and acute pressor response of short duration was observed following i.v. administration of Lys-γ_2-MSH to conscious rats (Fig. 1A). There was no significant difference between the dose-response curves of Lys-γ_2-MSH and γ_2-MSH (dose × treatment interaction $F(5,75) = 1.48$; $P > 0.05$); at the ED_{50} level Lys-γ_2-MSH was equipotent to γ_2-MSH and had a similar intrinsic activity (Table 2). Removal of the C-terminal dipeptide Phe^{11}-Gly^{12} and of the C-terminal tetrapeptide Asp^9-Arg^{10}-Phe^{11}-Gly^{12} yielded peptides which were devoid of cardiovascular effects (Fig. 1A). Only in the highest dose Lys-γ-MSH-(1–8)-NH_2 induced a small, but significant increase in mean arterial pressure (12 ± 4 mm Hg).

Lys-γ_2-MSH increased the heart rate dose-dependently, which was not significantly different from γ_2-MSH (dose × treatment interaction $F(5,75) = 1.58$; $P > 0.05$) (Fig. 1B). The various C-terminally shortened peptides had no significant effect on heart rate (Fig. 1B).

3.3. Result of removal of N-terminal amino acids

In a second series of experiments the consequences of sequential removal of N-terminal amino acids from the γ_2-MSH sequence were examined. Removal of Tyr^1, Val^2 and Met^3, yielding the fragments γ-MSH-(2–12), γ-MSH-(3–12) and γ-MSH-(4–12), did not result in a significant difference in pressor response as compared to γ_2-MSH (dose × treatment interactions $F(9,69) = 1.12$; $P > 0.05$) (Fig. 2A). However, a small, but significant loss of potency was observed for γ-MSH-(2–12) (Table 2). Intrinsic activity of these fragments was not significantly affected (Table 2).

Removal of the N-terminal tetrapeptide Tyr^1-Val^2-Met^3-Gly^4 (γ-MSH-(5–12)) or Tyr^1-Val^2-Met^3-Gly^4-His^5 (γ-MSH-(6–12)) resulted in significant shifts of the dose-response curves (dose × treatment interactions $F(6,57) = 6.84$; $P < 0.01$) (Fig. 3A). A significant loss of potency, demonstrated by a shift to the right of the dose-response curve, was observed with γ-MSH-(5–12), whereas γ-MSH-(6–12) was significantly more potent than γ_2-MSH in inducing a pressor response (Fig. 3A and Table 2). Both γ-MSH-(5–12) and γ-MSH-(6–12) had similar intrinsic activities as γ_2-MSH (Table 2).

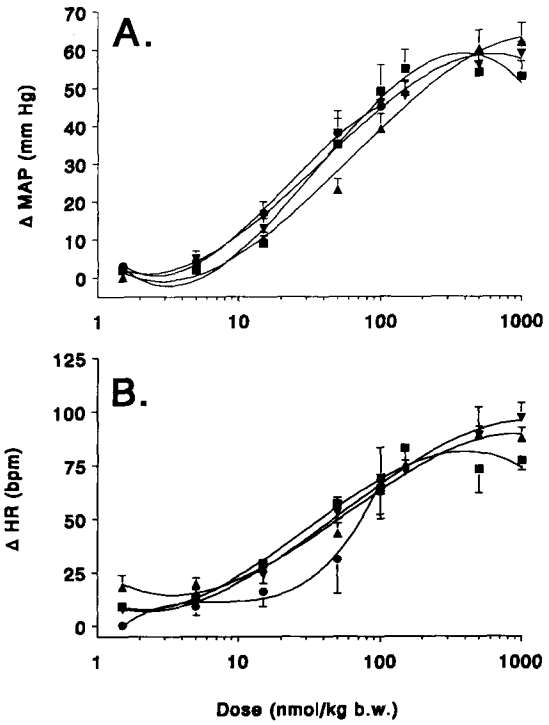

Fig. 2. Dose-response relationship for various C-terminal γ-MSH fragments with respect to their effects on mean arterial blood pressure (MAP) (A) and heart rate (HR) (B) after i.v. administration to conscious, freely moving rats. The results are expressed as absolute change from pre-administration values and as mean ± S.E.M. (n = 6–8). (●) γ_2-MSH, (▲) γ-MSH-(2–12), (■) γ-MSH-(3–12), and (▼) γ-MSH-(4–12).

γ_2-MSH dose-dependently and significantly increased heart rate (Fig. 2B). All γ-MSH fragments were equipotent to γ_2-MSH, except for γ-MSH-(5–12) (Fig. 2B and Fig. 3B; Table 2).

3.4. Effects of shorter γ-MSH/ACTH fragments on mean arterial pressure and heart rate

Subsequently, the effects of two short γ-MSH fragments were examined. We tested γ-MSH-(3–8) and the ACTH/MSH 'core', His-Phe-Arg-Trp (γ-MSH-(5–8) = ACTH-(6–9)), in doses up to 1000 nmol/kg body weight. Both peptides caused a small, but significant dose-dependent increase in mean arterial pressure (Fig. 4A) and heart rate (Fig. 4B) (effect of treatment $F(5,55) = 14.49$, $P < 0.01$; $F(5,55) = 29.24$, $P < 0.01$, respectively).

Finally, the potency of a number of ACTH-(4–10) fragments in inducing increases in mean arterial pressure and heart rate were compared with that of ACTH-(4–10) (Fig. 5). A significant dose × treatment interaction was present for the effects on mean arterial pressure ($F(25,180) = 7.03$, $P < 0.01$). ACTH-(4–10), 15–1000 nmol/kg body weight, caused a dose-dependent increase in mean arterial pressure. This peptide was about 10 times less potent than γ_2-MSH (Table 2).

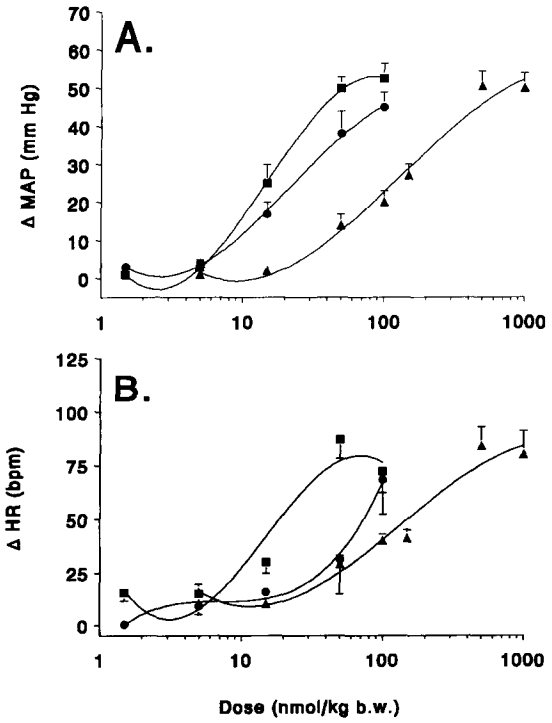

Fig. 3. Dose-response relationship for various C-terminal γ-MSH fragments with respect to their effects on mean arterial blood pressure (MAP) (A) and heart rate (HR) (B) after i.v. administration to conscious, freely moving rats. The results are expressed as absolute change from pre-administration values and as mean ± S.E.M. (n = 6–8). (●) γ_2-MSH, (▲) γ-MSH-(5–12), and (■) γ-MSH-(6–12).

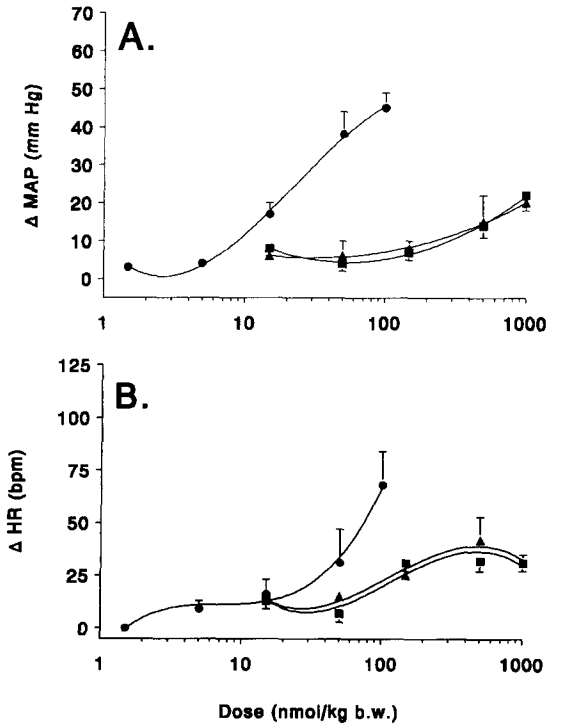

Fig. 4. Dose-response relationship for the MSH 'core' (γ-MSH-(5–8) = ACTH-(6–9) (■)) and γ-MSH-(3–8) (▲) with respect to their effects on mean arterial pressure (MAP) (A) and heart rate (HR) (B) after i.v. administration to conscious, freely moving rats. The results are expressed as absolute change from pre-administration values and as mean ± S.E.M. (n = 6–8). (●) γ_2-MSH.

Removal of Met[4] (ACTH-(5–10)) and Met[4]-Glu[5] (ACTH-(6–10)) resulted in a shift of the dose-response curves to the right (Fig. 5A). Removal of Gly[10] (ACTH-(4–9)) resulted in a drastic decrease in potency and intrinsic activity. The additional removal of Arg[8]-Trp[9] yielded a peptide, ACTH-(4–7), which had no significant effect on mean arterial pressure (Fig. 5A).

In addition to a pressor response, the ACTH fragments also had a significant effect on heart rate (dose × treatment interactions $F(25,170) = 4.07$, $P < 0.01$). ACTH-(4–10) induced a strong and dose-dependent tachycardia (Fig. 5B). The peptide was 8 times less potent than γ_2-MSH (Fig. 5B). The ACTH fragments, ACTH-(5–10), ACTH-(6–10) and ACTH-(6–9), also dose-dependently and significantly increased heart rate (Fig. 5B). ACTH-(4–9) and ACTH-(4–7) were much less potent.

4. Discussion

The family of γ-MSH-peptides consists of three members: γ_1-, γ_2-, and γ_3-MSH (see Introduction). The majority of the naturally occurring γ-MSH-peptides contains a lysine residue in the N-terminal

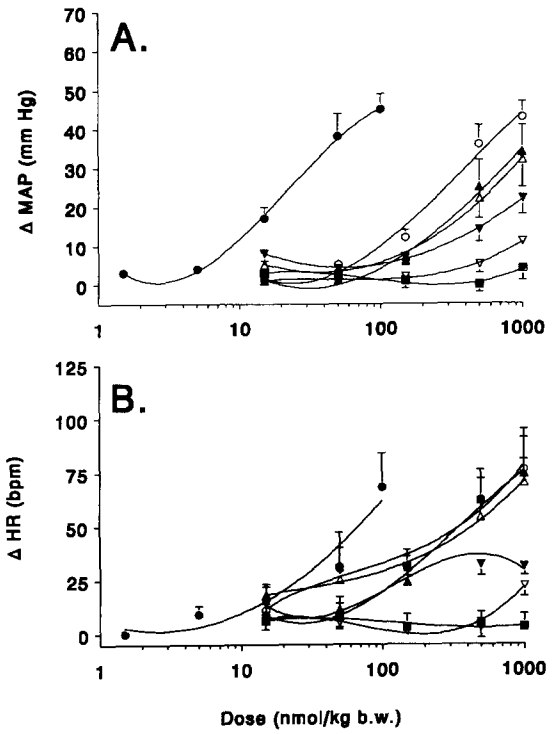

Fig. 5. Dose-response relationship for various ACTH fragments with respect to their effects on mean arterial blood pressure (MAP) (A) and heart rate (HR) (B) after i.v. administration to conscious, freely moving rats. The results are expressed as absolute change from pre-administration values and as mean ± S.E.M. ($n = 6$–11). (●) γ_2-MSH, (○) ACTH-(4–10), (▲) ACTH-(5–10), (△) ACTH-(6–10), (▼) ACTH-(6–9), (▽) ACTH-(4–9), and (■) ACTH-(4–7).

region (Eberle, 1988; Estivariz et al., 1989; Bunel et al., 1992). Since most of the cardiovascular research concerning these melanotropins has been carried out with (synthetic) γ_2-MSH, we deemed it necessary to investigate the cardiovascular actions of its endogenous counterpart. Lys-γ_2-MSH, i.v. administered to conscious rats, was as potent as γ_2-MSH in inducing acute, dose-dependent increases in mean arterial pressure and heart rate.

The strong cardiovascular effects of γ_2-MSH as described in the present study were consistent with those reported previously (Klein et al., 1985; Sun et al., 1992; De Wildt et al., 1993). The ED_{50} value of γ_2-MSH was 30 ± 5 nmol/kg body weight, which was also observed in an earlier study from our laboratory (De Wildt et al., 1993).

In the present study we attempted to determine the region(s) of the (Lys-)γ_2-MSH/ACTH-(4–10) molecule that is essential for its cardiovascular actions. As previously mentioned, the undecapeptide γ_1-MSH, which lacks Gly^{12} of γ_2-MSH, induces strong cardiovascular actions (Mues et al., 1982; Gruber and Eskridge, 1986). γ_1-MSH is as potent as γ_2-MSH, when administered to conscious rats (Klein et al., 1985; Gruber et al., 1985) or less potent than γ_2-MSH in conscious SHR (Sun et al., 1992). Our data show that further C-terminal shortening of γ_2-MSH resulted in γ-MSH-peptides which had no effect on mean arterial pressure and heart rate. Taken together, these results suggest that the intrinsic activity of γ_2-MSH is carried by the C-terminal γ-MSH fragment Asp^9-Arg^{10}-Phe^{11}. It has been postulated that the cardiovascular effects of γ-MSH are dependent on the Arg-hydrophobic amino acid sequence located at or near its C-terminus (Klein et al., 1985). In accordance with this hypothesis is the fact that α-MSH, γ_3-MSH and Org 2766, which do not contain this sequence in the C-terminus, are devoid of cardiovascular activity in conscious rats (Gruber et al., 1985; Klein et al., 1985; De Wildt et al., 1993). Peptides other than γ-MSHs, which contain the dipeptide sequence Arg-Phe, e.g. the putative [Met^5]enkephalin precursor, [Met^5]enkephalin-Arg-Phe-NH_2 (Mues et al., 1982), and the molluscan peptide, Phe-Met-Arg-Phe-NH_2, and related peptides (Raffa, 1988) display cardiostimulatory actions. It is of interest to note that Lys-γ-MSH-(1–8)-NH_2 caused a small, but significant increase in pressor activity at the highest dose as compared to Lys-γ-MSH-(1–8). Mues et al. (1982) showed that the presence of a carboxyl terminal carboxamide instead of the carboxylic acid within a peptide causes stronger increases in mean arterial pressure in urethane-anesthetized rats.

For the sake of completeness, it should be mentioned that under some pathophysiological conditions, e.g. hypovolemic shock in the rat, α-MSH and other ACTH fragments improve cardiovascular function by as yet unknown mechanisms (Bertolini et al., 1986; Coppi and Falcone, 1992).

Stepwise N-terminal chain shortening by deleting the first three amino acids in γ_2-MSH slightly affected the potency, i.e. the dose-response curves shifted to the right. Since Tyr^1, Val^2 and Met^3 are considered to be hydrophobic amino acids, each of these γ-MSH fragments might bind to the receptor in a similar, equipotent manner. Removal of the tetrapeptide Tyr^1-Val^2-Met^3-Gly^4 significantly lowered the potency with no effect on efficacy. Therefore, it was expected that removal of an additional His^5 would lead to a further reduction in potency. Surprisingly, γ-MSH-(6–12) was significantly more potent than γ_2-MSH as measured by a leftward shift of the dose-response curve. A possible explanation is that His^5 hinders the Arg^7-hydrophobic sequence. Since His^5 is not lipophilic, solely Arg^{10}-Phe at the C-terminal site can account for the pressor and tachycardic effects. Removal of His^5 unmasks the second Arg^7-hydrophobic sequence; now both amino acids surrounding Arg^7 (Phe^6 and Trp^8) make γ-MSH-(6–12) highly lipophilic. This could explain the increased potency of this γ-MSH fragment. Thus, our results demonstrate that Tyr^1, Val^2, Met^3, Gly^4, and His^5 are essential for potency, i.e. affinity for the receptor.

The heptapeptide ACTH-(4–10), which is partially homologous with the center part of γ_2-MSH, also displays cardiovascular activity. In the present report ACTH-(4–10) was approximately 10 times less potent than γ_2-MSH. Previous studies observed a 5 times (De Wildt et al., 1993) and 10 times (Klein et al., 1985) lower potency. Similar to γ_2-MSH the C-terminal part of ACTH-(4–10) (Gly^{10}) carried the intrinsic activity of this heptapeptide, and the N-terminal part (Met^4-Glu^5) the potency. The shortest fragment of γ_2-MSH/ACTH-(4–10) exhibiting cardiovascular activity was His-Phe-Arg-Trp, also known as the traditional MSH 'core' for melanotropic activity (Eberle, 1988). Previous studies showed that γ-MSH-(3–9) is equipotent to ACTH-(4–10) in causing pressor and tachycardic effects in conscious rats (Klein et al., 1985; Gruber and Callahan, 1989), which supports the concept that C-terminal elongation from the MSH 'core' carries the intrinsic activity. It is interesting to note that, consistently with our results, several structure-activity studies on the melanoma dispersing activity of α-MSH have demonstrated that the MSH 'core', and especially Phe, Arg, and Trp, is essential for binding and triggering of the biological response (Eberle, 1988; Sahm et al., 1994b), and that the C-terminal amino acids of α-MSH are more important than those in the N-terminus (Sahm et al., 1994a).

Recently five melanocortin (MC) receptors have been cloned and identified (see Hol et al., 1995). Among these the melanocortin MC_3- and MC_4-subtype receptors have been implicated in the neural control of cardiovascular function (Low et al., 1994). γ_1-MSH and γ_2-MSH possess a higher affinity and efficacy for the MC_3 than MC_4 receptor as measured by intracellular cAMP production of transfected cell lines (Gantz et al., 1993; Roselli-Rehfuss et al., 1993; Adan et al., 1995). Furthermore, the distribution of γ-MSH immunoreactivity corresponds well with that of MC_3 receptor mRNA expression in the brain (Low et al., 1994). However, the pharmacological profile of this receptor in vitro is not consistent with the in vivo cardiovascular actions of γ_2-MSH/ACTH-(4–10). [Nle^4,D-Phe^7]α-MSH, α-MSH, and γ_3-MSH bind and activate the MC_3 receptor in the same order of potency as γ_2-MSH (Roselli-Rehfuss et al., 1993; Sahm et al., 1994c; Adan et al., 1995). In contrast, these melanotropins are devoid of cardiovascular actions after i.v. administration to rats (Klein et al., 1985; De Wildt et al., 1993, 1994). These observations negate any correlation between the melanocortin MC_3 receptor and the cardiovascular actions of γ_2-MSH. Therefore, we suggest that an as yet unknown subtype of this receptor mediates these effects. This is supported by the results of a recent study in which we tested the ability of various γ-MSH/ACTH fragments to bind and activate the melanocortin MC_3 receptor (to be published). The results are in favor of our hypothesis.

In conclusion, the message site essential for cardiovascular actions resides in the γ-MSH/ACTH fragment His-Phe-Arg-Trp and, presumably, in fragment Phe-Arg-Trp. Proper C-terminal elongation of this message site is required for full expression of cardiovascular activity of γ_2-MSH, as the sequence Asp^9-Arg^{10}-Phe^{11} appears to play an important role in establishing intrinsic activity; N-terminal elongation of the message site affects only the potency of γ_2-MSH. It remains to be elucidated which melanocortin receptor subtype is mediating these effects.

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