British Journal of Pharmacology (2000) 131, 1468–1474

www.nature.com/bjp

Relevance of the C-terminal Arg-Phe sequence in γ_2-melanocyte-stimulating hormone (γ_2-MSH) for inducing cardiovascular effects in conscious rats

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1 The cardiovascular effects by γ_2-melanocyte-stimulating hormone (γ_2-MSH) are probably not due to any of the well-known melanocortin subtype receptors. We hypothesize that the receptor for Phe-Met-Arg-Phe-amide (FMRFa) or Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide (neuropeptide FF; NPFFa), other Arg-Phe containing peptides, is the candidate receptor. Therefore, we studied various Arg-Phe containing peptides to compare their haemodynamic profile with that of γ_2-MSH(6–12), the most potent fragment of γ_2-MSH.

2 Mean arterial pressure (MAP) and heart rate (HR) changes were measured in conscious rats after intravenous administration of γ_2-MSH related peptides.

3 Phe-Arg-Trp-Asp-Arg-Phe-Gly (γ_2-MSH(6–12)), FMRFa, NPFFa, Met-enkephalin-Arg-Phe-amide (MERFa), Arg-Phe-amide (RFa), acetyl-Phe-norLeu-Arg-Phe-amide (acFnLRFa) and desamino-Tyr-Phe-norLeu-Arg-Phe-amide (daYFnLRFa) caused a dose-dependent increase in MAP and HR. γ_2-MSH(6–12) showed the most potent cardiovascular effects ($ED_{50}=12$ nmol kg^{-1} for ΔMAP; 7 nmol kg^{-1} for ΔHR), as compared to the other Arg-Phe containing peptides ($ED_{50}=177-292$ nmol kg^{-1} for ΔMAP; 130–260 nmol kg^{-1} for ΔHR).

4 Peptides, which lack the C-terminal Arg-Phe sequence (Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Pro-Gly (γ_2-pro^{11}-MSH), desamino-Tyr-Phe-norLeu-Arg-[L-1,2,3,4 tetrahydroisoquinoline-3-carboxylic acid]-amide (daYFnLR[TIC]a) and Met-enkephalin (ME)), were devoid of cardiovascular actions.

5 The results indicate that the baroreceptor reflex-mediated reduction of tonic sympathetic activity due to pressor effects is inhibited by γ_2-MSH(6–12) and that its cardiovascular effects are dependent on the presence of a C-terminal Arg-Phe sequence.

6 It is suggested that the FMRFa/NPFFa receptor is the likely candidate receptor, involved in these cardiovascular effects.

British Journal of Pharmacology (2000) **131,** 1468–1474

Keywords: Melanocortin; γ-melanocyte-stimulating hormone; Phe-Met-Arg-Phe-amide; Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide; blood pressure; heart rate

Abbreviations: α-MSH, α-melanocyte-stimulating hormone; β-MSH, β-melanocyte-stimulating hormone; γ_2-MSH, γ_2-melanocyte-stimulating hormone; γ_2-MSH(6–12), Phe-Arg-Trp-Asp-Arg-Phe-Gly; γ_2-pro^{11}-MSH, Lys-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Pro-Gly; ACTH, adrenocorticotropic hormone; acFnLRFa, acetyl-Phe-norLeu-Arg-Phe-amide; daYFnLRFa, desamino-Tyr-Phe-norLeu-Arg-Phe-amide; daYFnLR[TIC]a, desamino-Tyr-Phe-norLeu-Arg-[L-1,2,3,4 tetrahydroisoquinoline-3-carboxylic acid]-amide; FMRFa, Phe-Met-Arg-Phe-amide; HR, heart rate; MAP, mean arterial pressure; MC, melanocortin; ME, Met-enkephalin; Tyr-Gly-Gly-Phe-Met; MERFa, Met-enkephalin-Arg-Phe-amide, Tyr-Gly-Gly-Phe-Met-Arg-Phe-amide; NPFFa, neuropeptide FF; Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide; RFa, Arg-Phe-amide

Introduction

Melanocortins (α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH and adrenocorticotropic hormone (ACTH)) belong to a family of peptides derived from the precursor pro-opiomelanocortin (see Table 1). In addition to their effects on the hormonal system, behaviour, temperature and feeding regulation, γ-MSHs and in particular γ_2-MSH and its shorter fragment γ_2-MSH(6–12) have strong effects on the cardiovascular system (Callahan *et al*., 1985; 1988; Li *et al*., 1996; Sun *et al*., 1992; Van Bergen *et al*., 1995; 1997a). Systemic administration of γ_2-MSH and γ_2-MSH(6–12) to rats causes a dose-dependent increase in mean arterial pressure (MAP) and heart rate (HR). So far, a most likely candidate receptor, involved in these cardiovascular effects, is the melanocortin (MC) receptor. There are indications for a centrally located target for γ-MSH (Sun *et al*., 1992; Van Bergen *et al*., 1998; Fodor *et al*., 1996). As the MC3-4 receptor subtypes are mainly expressed in the brain (Gantz *et al*., 1993; Mountjoy *et al*., 1994), these subtypes are most likely involved in γ-MSH-induced cardiovascular actions. However, there are results that are in contradiction with this hypothesis. α-MSH shows high affinity for and can activate all subtype MC receptors (Adan *et al*., 1994; Gantz *et al*., 1994; Mountjoy, 1994), but systemic administration of this peptide has no influence on the cardiovascular system (Van Bergen *et al*., 1997b). Furthermore, γ_2-MSH(6–12) shows very potent pressor and cardioaccelerator effects (Van Bergen *et al*., 1995), but has no affinity and cannot activate any of

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Table 1 Amino acid sequence of various melanocortins and FMRFa-like peptides

Peptide	*Structure*
ACTH(1–24)	Ser[1]-Tyr[2]-Ser[3]-Met[4]-Glu[5]-His[6]-Phe[7]-*Arg[8]*-*Trp[9]*-Gly[10]–Pro[24]
α-MSH	Ser[1]–Glu[5]-His[6]-Phe[7]-*Arg[8]*-*Trp[9]*-Gly[10]-Lys[11]-Pro[12]-Val[13]
β-MSH	Ala[1]–Glu[12]-His[13]-Phe[14]-*Arg[15]*-*Trp[16]*-Gly[17]–Asp[22]
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_2-MSH(6–12)	Phe[6]-*Arg[7]*-*Trp[8]*-Asp[9]-**Arg[10]-Phe[11]**-Gly[12]
γ_3-MSH	Try[1]-Val[2]-Met[3]-Gly[4]-His[5]-Phe[6]-*Arg[7]*-*Trp[8]*-Asp[9]-**Arg[10]-Phe[11]**-Gly[12]–Gln[25]
FMRFa	Phe[1]-Met[2]-**Arg[3]-Phe[4]-NH$_2$**
NPFFa	Phe[1]-Leu[2]-Phe[3]-Gln[4]-Pro[5]-Gln[6]-**Arg[7]-Phe[8]**-NH$_2$
MERFa	Tyr[1]-Gly[2]-Gly[3]-Phe[4]-Met[5]-**Arg[6]-Phe[2]**-NH$_2$
RFa	**Arg[1]-Phe[2]**-NH$_2$
acFnLRFa	ac-Phe[1]-norLeu[2]-**Arg[3]-Phe[4]**-NH$_2$
daYFnLRFa	desamino-Tyr-Phe[1]-norLeu[2]-**Arg[3]-Phe[4]**-NH$_2$

the brain MC receptor subtypes *in vitro* (Oosterom, unpublished observations). These studies indicate that the cardiovascular effects of γ-MSH's are probably not due to either of the well-known MC subtype receptors.

The molluscan peptide Phe-Met-Arg-Phe-amide (FMRFa) and its mammalian analogues Tyr-Gly-Gly-Phe-Met-Arg-Phe-amide (Met-enkephalin-Arg-Phe-amide; MERFa) and Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide (neuropeptide FF; NPFFa) were isolated from bovine, rat and human central nervous system (Majane *et al*., 1983; Tang *et al*., 1984; Yang *et al*., 1983; 1985). The chemical structural resemblance (Arg-Phe-amide) between FMRFa-like peptides and γ-MSHs, their comparable haemodynamic profile (increase in MAP and HR) (Allard *et al*., 1995; Barnard & Dockray, 1984a; De Wildt *et al*., 1993; Mues *et al*., 1982; Thiemermann *et al*., 1991; Wong *et al*., 1985) and correspondent anatomical location of these peptides and their receptors within the brain (hypothalamus and nucleus tractus solitarius) (Fodor *et al*., 1996; Kivipelto *et al*., 1989; 1992; Majane *et al*., 1989; O'Donohue *et al*., 1984; Pittius *et al*., 1984), makes the FMRFa/NPFFa/MERFa receptor a very important candidate for mediating the cardiovascular effects of γ-MSHs. Therefore, in the present study various peptides with a C-terminal Arg-Phe structure are tested for their haemodynamic profile in conscious rats. Furthermore, peptides with C-terminal modifications are used to indicate that the C-terminal Arg-Phe structure is indeed essential for bioactivity.

The goal of this study is to examine which structure of γ_2-MSH(6–12), the most potent fragment of γ_2-MSH, is responsible for the cardiovascular effects. This knowledge may lead to a suggestion for a candidate receptor and its related mechanism of action responsible for the cardiovascular effects of γ_2-MSH(6–12).

Methods

Animals

Naive male albino Wistar rats (U:WU/CPB) weighing 230–280 g were used. Two or three rats were housed per Macrolon cage (23 × 17 × 14 cm) containing a layer of woodshavings under conditions of constant ambient temperature (21 ± 1°C), constant humidity (60 ± 15%), and light/dark rhythm (with lights on from 0700 to 1900 h). After surgery, the animals were housed individually in Plexiglas cages (25 × 25 × 25 cm) under presurgical conditions. Food (complete laboratory chow: Hope Farms, Woerden, The Netherlands) and water were accessible *ad libitum* throughout the experiment.

Surgery

Rats were anaesthetized with halothane (O_2/NO_2 1:2; introduction 5%; maintenance 1.3–1.7%; Fluothane®, Zeneca BV, Ridderkerk, The Netherlands). Prior to operation each rat received eye ointment (Caf$^+$, Alpharmo BV, Arnhem, The Netherlands) to protect the eyes against dehydration. During the operation, body temperature of the rats was maintained with a heated pad (K-temp, I.M.S., Helmond, The Netherlands).

For BP and HR measurements, the femoral artery was cannulated with a polyethylene tubing (PE10, i.d. 0.28 mm, o.d. 0.61 mm; Portex, The Hague, The Netherlands), which was melted to another polyethylene tubing (PE50, i.d. 0.58 mm, o.d. 0.96 mm; Portex) with a 180° loop. The latter was melted to third polyethylene tubing (PE100, i.d. 0.86 mm, o.d. 1.52 mm; Portex). The cannula was filled with a heparin solution (50 IU ml^{-1}, Leo Pharmaceutical Products BV, Weesp, The Netherlands) and guided underneath the skin to the head. For i.v. administration of drugs the jugular vein was cannulated according to Steffens (1969). The silastic cannula (i.d. 0.51 mm, o.d. 0.94 mm, Dispo Medical BV, Hattum, The Netherlands) was filled with saline and guided underneath the skin towards the head. Both cannulae were connected to a steel connector with a 90° loop, which in turn was connected to a short polyethylene tubing and closed by a steel stylet. Both cannulae were fixated to the skull with dental cement (Dental Union BV, Nieuwegein, The Netherlands). After the operation the rats received a single injection of saline (1.5 ml, subcutaneously (s.c.)) and were allowed to recover until they regained consciousness in a heated chamber (I.M.S.).

Cardiovascular measurements and intravenous administration

Forty-five min prior to baseline recordings of BP and HR, rats were connected to long (0.5 m) polyethylene tubings (Portex) for stress-free cardiovascular measurements and i.v. administration. The arterial cannula was connected to a pressure transducer (Viggo-Spectramed, disposable DTX/plus, Ohmeda, Bilthoven, The Netherlands) by a PE100 tubing (i.d. 0.86 mm, o.d. 1.52 mm; Portex). The pressure transducer was connected to a DC-preamplifier and biotachometer (Instrument service, Utrecht University, The Netherlands) coupled to a P75-computer. Data were continuously recorded and measured with the HDAS (Haemodynamic Data Acquisition System) (Instrumental Department, University of Limburg, Maastricht, The Netherlands) and DatView program (Instrumental Department, University of

Limburg, Maastricht, The Netherlands). Mean arterial pressure (MAP) was calculated according to the formula: $(2 \times P_d + P_s)/3$, in which P_d is diastolic pressure and P_s systolic pressure. The jugular vein cannula was attached to a syringe (1000 μl, Inacom Instruments, Veenendaal, The Netherlands) and microinfusion pump (Harvard Inc., Massachusetts, U.S.A.) by a PE50 tube (i.d. 0.58 mm, o.d. 0.96 mm, Portex). Systemic injections were performed at 500 μl min^{-1}.

Experimental design

Rats were surgically equipped with a femoral artery and jugular vein cannula under sterile conditions for measurements of blood pressure (BP) and heart rate (HR) and for intravenous (i.v.) administration of drugs, respectively. They were allowed to recover from surgery for 3 days. During the recovery period, the animals were handled daily for weighing and habituation purposes. Cardiovascular measurements and i.v. injections were performed stressfree in conscious, resting rats by a long-line technique. Baseline BP and HR were recorded before i.v. injections. Subsequently, rats were injected i.v. with phenylephrine (5 μg kg^{-1}) to check whether the animals show a significant increase in BP. All rats responded and were used for further measurements. Before drug treatment, rats were injected with saline (200 μl). One drug was injected per rat in a volume of 100 μl and an interval of 15 min was used between each dose of injection to allow stabilization of BP and HR. In order to flush the cannula each infusion of a drug was followed by 100 μl saline.

All experiments were performed in the home cage during the light phase of the circadian cycle between 0900 and 1400 h. After the experiment, all rats were killed by an overdose (0.5 ml) of pentobarbital (160 mg ml^{-1}).

The experiments were approved by the ethical committee for animal experimentation of the Medical Faculty, Utrecht University, The Netherlands.

Cardiovascular effects of Arg-Phe containing peptides

Rats were injected i.v. with increasing doses of γ_2-MSH(6–12) ($n=6$), MERFa ($n=7$), RFa ($n=6$), neuropeptide FF (NPFFa; $n=6$), FMRFa ($n=6$), acetyl-Phe-norLeu-Arg-Phe-amide (acFnLRFa; $n=6$) or desamino-Tyr-Phe-norLeu-Arg-Phe-amide (daYFnLRFa; $n=6$). The dose range for γ_2-MSH(6–12) was 1.5, 5, 15, 50 and 100 nmol kg^{-1}; for MERFa 25, 50, 100, 200, 300, 400 and 500 nmol kg^{-1}; for acFnLRFa 100, 200, 300, 500, 1000 nmol kg^{-1} and for RFa, NPFFa, FMRFa and daYFnLRFa 15, 50, 100, 250, 500 and 1250 nmol kg^{-1}.

Effect of C-terminal modifications on cardiovascular actions

Rats were injected i.v. with increasing doses of γ_2-pro^{11}-MSH ($n=6$), desamino-Tyr-Phe-norLeu-Arg[L-1,2,3,4 tetrahydroisoquinoline-3-carboxylic acid]-amide (daYFnLR[TIC]a; $n=6$) or Met-enkephalin (ME; $n=8$). The dose-range for ME was 25, 50, 100, 200, 300, 400 and 500 nmol kg^{-1}; for γ_2-pro^{11}-MSH and daYFnLR[TIC]a 15, 50, 100, 250, 500 and 1250 nmol kg^{-1}.

Drugs

Phenylephrine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; MERFa, ME, RFa, NPFFa, FMRFa and acFnLRFa from Bachem, Bubendorf, Switzerland; γ_2-MSH(6–12), daYFnLRFa and daYFnLR[TIC]a were synthesized, purified and analysed by mass spectrometry at the National Institute of Public Health and the Environment, Bilthoven, The Netherlands; γ_2-pro^{11}-MSH was kindly donated by Dr R. Adan, Rudolf Magnus Institute for Neurosciences, Utrecht, The Netherlands. All drugs were dissolved in bidestilled water prior to use.

Statistics

The MAP (mmHg) and HR (beats min^{-1}) data are presented as maximal effects (mean changes $\pm$ s.e.mean) to each dose of injection. This maximal effect is the difference between the maximal response to drug injection and the pre-injection value (measured just before i.v. injection). Over these data a non-linear Hill-fit was performed with the formula: $Y = \text{bottom} + (\text{top-bottom}/1 + 10^{((\log ED50 - X)*\text{Hillslope})})$ in which Y is the MAP or HR response, X is the log of the dose, bottom is the Y value at the bottom plateau; top is the Y value at the top plateau, and LogED_{50} is the logarithm of the ED_{50}, the concentration that gives a response halfway between bottom and top. The variable Hillslope controls the slope of the curve, which was fitted over the data points. Only those curves were fitted according to this method when a plateau level (E_{max}, the estimated maximal effect) was reached. The ED_{50} and E_{max} reflect a measure of potency and intrinsic activity, respectively.

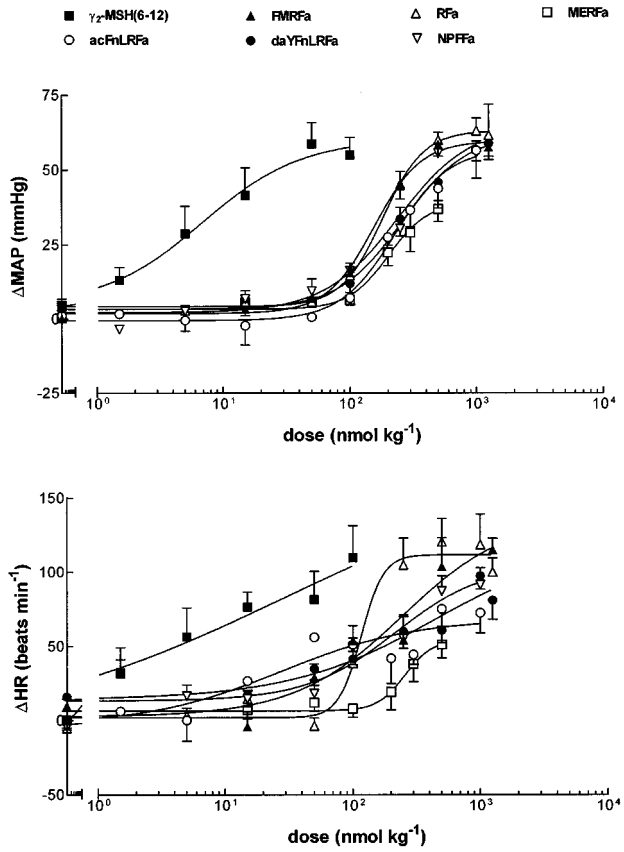

Figure 1 Dose-response relationship for γ_2-MSH(6–12) and various FMRFa-like peptides with respect to their effects on mean arterial blood pressure (MAP) (upper panel) and heart rate (HR) (lower panel) after intravenous administration to conscious, freely moving rats. The results are expressed as absolute maximal change from pre-administration values and as mean $\pm$ s.e.mean ($n=6$–7).

Baseline MAP and HR (recorded before phenylephrine injection), ED_{50} and E_{max} levels were analysed by a one-way Analysis of Variance (ANOVA) and *post-hoc* Tukey HSD test. A value of $P<0.05$ was considered significant.

Results

Cardiovascular effects of Arg-Phe containing peptides

Figure 1 shows the MAP and HR response to i.v. injection of γ_2-MSH(6–12), MERFa, RFa, NPFFa, FMRFa, acFnLRFa and daYFnLRFa and the fitted dose-response curve for each peptide. All the tested peptides showed a dose-dependent increase in MAP and HR. The dose-response curves of γ_2-MSH(6–12) was shifted to the left in relation to the other peptides.

Table 2 shows the basal levels of MAP and HR prior to drug administration. Baseline MAP and HR were not significantly different between the 10 groups of rats. ED_{50} and E_{max} levels of ΔMAP and ΔHR are shown in Table 2. γ_2-MSH(6–12) showed the most potent cardiovascular effects as compared to the other Arg-Phe containing peptides. daY-FnLRFa showed a significant decrease of potency on MAP as compared to FMRFa. The intrinsic activity of MERFa was significantly lower than that of the other Arg-Phe containing peptides.

Effect of C-terminal modifications on cardiovascular actions

To indicate that the C-terminal Arg-Phe structure is indeed essential for bioactivity, this sequence was modified by substitution of the C-terminal Phe in γ_2-MSH with Pro (γ_2-pro^{11}-MSH), substitution of the C-terminal Phe in daY-FnLRFa with L-1,2,3,4 tetrahydroisoquinoline-3-carboxylic acid (daYFnLR[TIC]a), or by removal of the Arg-Phe-amide structure in MERFa (ME).

Figure 2 shows the MAP and HR response to i.v. injection of γ_2-MSH(6–12), γ_2-pro^{11}-MSH, daYFnLRFa, daY-FnLR[TIC]a, MERFa and ME and the fitted dose-response curve for each peptide. The Arg-Phe containing peptides showed a dose-dependent increase in MAP and HR, whereas peptides with a C-terminal modification (γ_2-pro^{11}-MSH, daYFnLR[TIC]a and ME) had no significant effect on the cardiovascular system.

ED_{50} and E_{max} levels of ΔMAP and ΔHR for the tested peptides are shown in Table 2. The ED_{50} levels of the peptides with a C-terminal modification was not measurable. The intrinsic activity (E_{max} levels) of these peptides was significant lower than that of the Arg-Phe containing analogues.

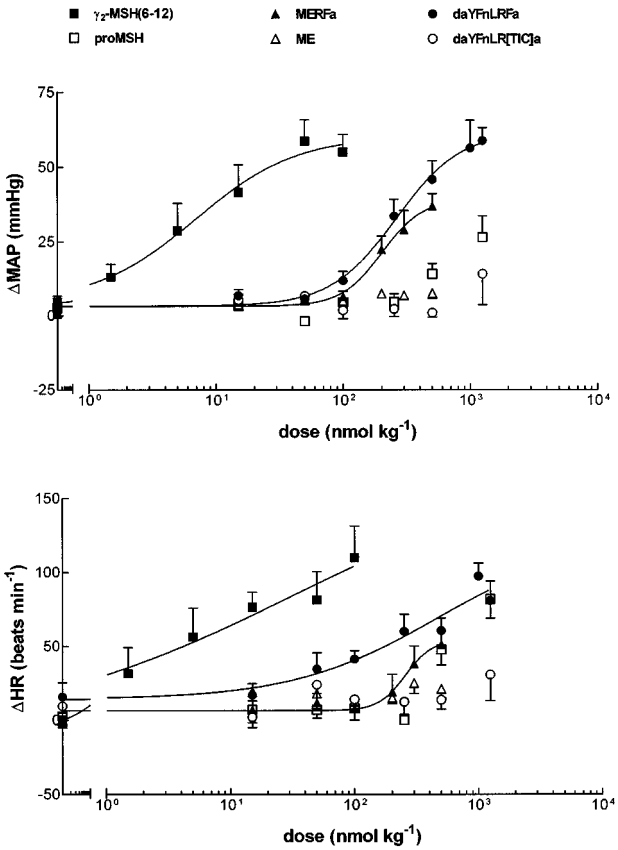

Figure 2 Dose-response relationship for γ_2-MSH(6–12), γ_2-pro^{11}-MSH, MERFa, ME, daYFnLRFa and daYFnLR[TIC]a with respect to their effects on mean arterial blood pressure (MAP) (upper panel) and heart rate (HR) (lower panel) after intravenous administration to conscious, freely moving rats. The results are expressed as absolute maximal change from pre-administration values and as mean ± s.e.mean ($n=6-8$).

Table 2 Basal levels of MAP (mmHg) and HR (beats min^{-1}) prior to phenylephrine administration and ED_{50} and E_{max} levels of ΔMAP and ΔHR for γ_2-MSHs and FMRFa-like peptides

	Basal levels		*ΔMAP*		*ΔHR*	
Peptide	*MAP* (mmHg)	*HR* (beats min^{-1})	*ED_{50}* (nmol kg^{-1})	*E_{max}* (mmHg)	*ED_{50}* (nmol kg^{-1})	*E_{max}* (beats min^{-1})
γ_2-MSH(6–12) ($n=6$)	103±2	424±9	12±4*	61±8	7±3	106±27
γ_2-pro^{11}-MSH ($n=6$)	102±4	365±12	nm	30±6§	nm	83±12
MERFa ($n=7$)	105±3	389±9	202±1	38±3‡	260±1**	52±4†
ME ($n=8$)	100±4	382±10	nm	10±8†	nm	21±1§
RFa ($n=6$)	105±3	404±13	200±24	65±3	130±20	124±11
NPFFa ($n=6$)	103±2	396±16	217±40	62±4	169±43**	92±13
FMRFa ($n=6$)	108±7	415±13	177±24	62±5	152±29	119±8
acFnLRFa ($n=6$)	108±4	396±7	211±14	55±4	156±64	93±10
daYFnLRFa ($n=6$)	113±2	384±8	292±32#	63±6	118±43	97±10
daYFnLR[TIC]a ($n=6$)	105±4	388±14	nm	11±8†	nm	22±10§

Data are expressed as mean ± s.e.mean; nm = not measurable; *$P<0.005$ significantly different from all other peptides, **$P<0.05$ significantly different from γ_2-MSH(6–12), †$P<0.05$ significantly different from all Arg-Phe containing peptides, ‡$P<0.05$ significantly different from all other peptides except γ_2-pro^{11}-MSH, §$P<0.05$ significantly different from all other peptides except MERFa, #$P<0.05$ significantly different from FMRFa.

Discussion

The strong cardiovascular effects of γ_2-MSH(6–12) in conscious rats as described in the present study were consistent with those reported previously by our laboratory (Van Bergen *et al*., 1995). In that study it was shown that γ_2-MSH(6–12) was more potent than γ_2-MSH, whereas shortening of the C-terminal site of γ_2-MSH resulted in loss of cardiovascular effects, indicating that the bioactivity of γ_2-MSH is carried by the C-terminal site. It has been postulated that the cardiovascular effects of γ_2-MSH in rats are dependent on the C-terminal Arg-Phe sequence (Klein *et al*., 1985). This agrees with studies of our laboratory and others showing that α-MSH, β-MSH and γ_3-MSH, which do not contain the C-terminal Arg-Phe structure, are devoid of cardiovascular activity in conscious rats (Gruber & Callahan, 1989; Van Bergen *et al*., 1997b). On the other hand, peptides other than γ-MSHs, which contain the C-terminal Arg-Phe sequence, e.g. the molluscan peptide Phe-Met-Arg-Phe-amide (FMRFa) and mammalian analogues Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide (NPFFa) and Met-enkephalin-Arg-Phe-amide (MERFa) increase MAP and HR in anaesthetized rats (Allard *et al*., 1995; Barnard & Dockray, 1984a; Mues *et al*., 1982; Thiemermann *et al*., 1991; Wong *et al*., 1985). These studies further suggest that the C-terminal Arg-Phe structure in γ-MSHs is essential for cardiovascular actions. It has to be mentioned that the cardiovascular effects of the FMRFa-like peptides in the latter studies were found in anaesthetized animals. To avoid any effect of anaesthesia on the cardiovascular response, we systemically injected γ_2-MSH(6–12), FMRFa, NPFFa, MERFa and RFa in conscious rats. GTP-γ-S assays and radioligand displacement studies showed that elongation (desamino-Tyr-Phe-norLeu-Arg-Phe-amide (daYFnLRFa)) or modification (acetyl-Phe-norLeu-Arg-Phe-amide (acFnLRFa)) of Phe at position 1 in FMRFa leads to increased efficacy and potency for the FMRFa receptor in the invertebrate brain (Chin *et al*., 1994; Heyliger *et al*., 1998; Payza, 1987). We were interested whether these analogues of FMRFa also showed a stronger effect *in vivo* and tested these ligands for their cardiovascular action in conscious rats.

Cardiovascular effects of Arg-Phe containing peptides

The results demonstrate that all the tested peptides showed a dose-dependent increase in MAP and HR. Elongation (daYFnLRFa) or modification (acFnLRFa) of the N-terminal site of FMRFa did not lead to a stronger effect on the cardiovascular system. daYFnLRFa even showed a significant decrease of potency on MAP ($ED_{50}=292$ nmol kg^{-1}) as compared to FMRFa ($ED_{50}=177$ nmol kg^{-1}). These results are in contradiction with those of the above-mentioned binding studies on the FMRFa receptor in invertebrates (Chin *et al*., 1994; Heyliger *et al*., 1998; Payza, 1987). On the other hand, our results are supported by radioligand displacement studies by Payza *et al*. (1993), who indicated that daYFnLRFa shows a decreased efficacy and potency for the NPFFa receptor in rat spinal cord. To our knowledge, there is no literature on the binding and activity capacities of FMRFa-like peptides in the rat brain, which makes it difficult to relate the cardiovascular effects of the present study to specific binding of either the FMRFa or NPFFa receptor. As we found a similar intrinsic activity and potency on the cardiovascular system for FMRFa and NPFFa, we postulate that these peptides share the same receptor. Future binding studies in rat brain tissue are necessary to confirm this hypothesis.

The intrinsic activity of MERFa ($E_{max}=38$ mmHg (ΔMAP) and 52 beats min^{-1} (ΔHR)) was significantly lower than that of the other Arg-Phe containing peptides ($E_{max}=55-65$ mmHg (ΔMAP) and 92–124 beats min^{-1} (ΔHR)). This may be due to the fact that the Met-enkephalin part of MERFa binds on opioid receptors, mediating opposing effects (hypotension and bradycardia) on the cardiovascular system (Douglas & Kitchen, 1992; Hao & Rabkin, 1997), whereas the C-terminal Arg-Phe structure can bind to the FMRFa/NPFFa receptor.

γ_2-MSH(6–12) showed the most potent cardiovascular effects ($ED_{50}=12$ nmol kg^{-1} for ΔMAP; 7 nmol kg^{-1} for ΔHR), as compared to the other Arg-Phe containing peptides ($ED_{50}=177-292$ nmol kg^{-1} for ΔMAP; 130–260 nmol kg^{-1} for ΔHR). It has been shown that α-MSH contains two message sequences for the same biological action (Eberle & Schwyzer, 1976; Schwyzer & Eberle, 1977). Eberle and Schwyzer (1976) reported that the message elements necessary for triggering melanotropic responses are contained in the central tetrapeptide sequence *His-Phe-Arg-Trp* and C-terminal tripeptide sequence *Lys-Pro-Val*. Gruber & Callahan (1989) postulated that γ_2-MSH also has dual message sequences: *Arg-Trp* (at positions 7 and 8) and *Arg-Phe* (at positions 10 and 11), which are responsible for enhancing cardiovascular activity. This hypothesis agrees with our previous finding that peptides with a single C-terminal *Arg-Trp* message sequence (γ_2-MSH(1–10), γ_2-MSH(1–8) and fragments of ACTH: ACTH(4–10) and ACTH(4–9)) showed a reduction or even complete loss of cardiovascular action and agrees with the present finding that peptides with a single C-terminal *Arg-Phe* message sequence (FMRFa, NPFFa, MERFa, RFa, acFnLRFa and daYFnLRFa) showed a reduced potency as compared to γ_2-MSH(6–12), which contains both the *Arg-Trp* and *Arg-Phe* structure.

Payza (1987) reported that replacement of the C-terminal Arg-Phe sequence by a Arg-Trp sequence increased the binding potency of FMRFa on the FMRFa receptor in invertebrates. Replacement of the aromatic hydrophobic amino acid by a non-aromatic hydrophobic amino acid (Leu or Pro) decreased the binding potency of FMRFa on the molluscan FMRF receptor (Chin *et al*., 1994; Payza, 1987). In agreement with findings of Mues *et al*. (1982), the present study shows that the smallest fragment of FMRFa required for cardiovascular activity was RFa. It is likely that the cardiovascular effects of γ_2-MSH in rats are dependent on the Arg-aromatic hydrophobic amino acid sequence located at or near its C-terminus (Klein *et al*., 1985), due to binding properties of this structure on the FMRFa/NPFFa receptor.

Relevance of the C-terminal Arg-Phe structure

To indicate that the C-terminal Arg-Phe structure is indeed essential for bioactivity, this sequence was modified by substitution of the C-terminal Phe in γ_2-MSH with Pro (γ_2-pro^{11}-MSH), substitution of the C-terminal Phe in daYFnLRFa with a rigid analogue of Phe, L-1,2,3,4 tetrahydroisoquinoline-3-carboxylic acid (TIC) (daYFnLR[TIC]a) or by removal of the Arg-Phe structure in MERFa (ME). Systemic injection of these C-terminal modified peptides did not result in significant cardiovascular changes. These results further indicate that cardiovascular effects of γ_2-MSHs and FMRFa-like peptides are dependent on the presence of a C-terminal Arg-Phe sequence.

Inhibition of the baroreceptoreflex

The dose-dependent increase in MAP induced by γ_2-MSH(6–12) is accompanied with a dose-dependent tachycardia. A similar haemodynamic profile is found for the other Arg-Phe containing peptides. This suggests that the baroreceptor reflex-mediated reduction of tonic sympathetic activity due to pressor effects is inhibited by these peptides. This agrees with findings of Callahan *et al.* (1985; 1988), who reported that the pressor response to γ_2-MSH in conscious rats was blocked by the ganglionic blocking agent chlorisondamine or by blockade of the catecholamine release from sympathetic nerve terminals with bretylium tosylate. Others showed that pressor responses to Arg-Phe containing peptides in anaesthetized rats was reduced by the ganglionic blocking agent hexamethonium (Barnard & Dockray, 1984b). Furthermore, Thiemermann *et al.* (1991) showed that systemic injections of FMRFa were associated with an increase in norepinephrine levels, whereas plasma epinephrine levels remained unchanged. These findings indicate that γ_2-MSH and FMRFa-like peptides induce a centrally mediated activation of preganglionic sympathetic efferents. In addition, it is plausible that Arg-Phe containing peptides have direct positive chronotropic action on the heart, as application of FMRFa, MERFa and acFnLRFa on isolated molluscan heart preparations caused cardioacceleration (Painter *et al.*, 1982). However, application of γ-MSH on isolated molluscan (Greenberg *et al.*, 1988) or rat (Van Bergen *et al.*, 1998) heart preparations caused no cardiac effects and in the pithed rat systemically administered γ_2-MSH (Sun *et al.*, 1992; Van Bergen *et al.*, 1998) or FMRFa (Thiemermann *et al.*, 1991) does not exert cardiovascular effects. These findings suggest that γ_2-MSH does not induce cardiovascular action *via* a peripheral site of action. Further studies have to be performed to understand the exact site and mechanism of cardiovascular actions of Arg-Phe containing peptides.

The results from the present study strongly suggest that γ_2-MSH(6–12), FMRFa and NPFFa share an identical receptor involved in cardiovascular regulation.

The authors thank J. van de Haar, for her skilled technical assistance and Dr R. Adan, Rudolf Magnus Institute for Neurosciences, Utrecht, the Netherlands, for kindly donating $\gamma_2$pro^{11}-MSH. The research project was financially supported by Solvay Pharmaceuticals, Hannover, Germany.

References

ADAN, R.A.H., CONE, R.D., BURBACH, J.P.H. & GISPEN, W.H. (1994). Differential effects of melanocortin peptides on neural melanocortin receptors. *Mol. Pharmacol.,* **46,** 1182–1190.

ALLARD, M., LABROUCHE, S., NOSJEAN, A. & LAGUZZI, R. (1995). Mechanisms underlying the cardiovascular responses to peripheral administration of NPFF in the rat. *J. Pharmacol. Exp. Ther.,* **274,** 577–583.

BARNARD, C.S. & DOCKRAY, G.J. (1984a). Increases in arterial blood pressure in the rat in response to a new vertebrate neuropeptide, LPLRFamide, and a related molluscan peptide, FMRFamide. *Regul. Pept.,* **8,** 209–215.

BARNARD, C.S. & DOCKRAY, G.J. (1984b). Increases in arterial blood pressure in the rat in response to a new vertebrate neuropeptide, LPLRFamide, and a related molluscan peptide, FMRFamide. *Regul. Pept.,* **8,** 209–215.

CALLAHAN, M.F., KIRBY, R.F., JOHNSON, A.K. & GRUBER, K.A. (1988). Sympathetic terminal mediation of the acute cardiovascular response of γ_2-MSH. *J. Auton. Nerv. System,* **24,** 179–182.

CALLAHAN, M.F., KIRBY, R.F., WOLFF, D.W., STRANDHOY, J.W., LYMANGROVER, J.R., JOHNSON, A.K. & GRUBER, K.A. (1985). Sympathetic nervous system mediation of acute cardiovascular actions of γ_2-melanocyte-stimulating hormone. *Hypertension,* **S I,** 145–150.

CHIN, G.J., PAYZA, K., PRICE, D.A., GREENBERG, M.J. & DOBLE, K.E. (1994). Characterization and solubilization of the FMRFamide receptor of squid. *Biol. Bull.,* **187,** 185–199.

DE WILDT, D.J., KRUGERS, H., KASBERGEN, C.M., DE LANG, H. & VERSTEEG, D.H. (1993). The hemodynamic effects of gamma 2-melanocyte-stimulating hormone and related melanotropins depend on the arousal potential of the rat. *Eur. J. Pharmacol.,* **233,** 157–164.

DOUGLAS, H. & KITCHEN, I. (1992). Mechanisms involved in the cardiovascular responses to opioid products of proenkephalin in the anaesthetised rat. *Gen. Pharmacol.,* **23,** 269–277.

EBERLE, A. & SCHWYZER, R. (1976). Hormone-receptor interactions. The message sequence of alpha- melanotropin: demonstration of two active sites. *Clin. Endocrinol. (Oxf),* **5** (Suppl): 41S–48S.

FODOR, M., SLUTTER, A., FRANKHUIJZEN-SIEREVOGEL, A., WIEGANT, V.M., HOOGERHOUT, P., DE WILDT, D.J. & VERSTEEG, D.H. (1996). Distribution of Lys-γ_2-melanocyte-stimulating hormone-(Lys-γ_2-MSH)-like immunoreactivity in neuronal elements in the brain and peripheral tissues of the rat. *Brain Res.,* **731,** 182–189.

GANTZ, I., KONDA, Y., TASHIRO, T., SHIMOTO, Y., MIWA, H., MUNZERT, G., WATSON, S.J., DELVALLE, J. & YAMADA, T. (1993). Molecular cloning of a novel melanocortin receptor. *J. Biol. Chem.,* **268,** 8246–8250.

GANTZ, I., SHIMOTO, Y., KONDA, Y., MIWA, H., DICKINSON, C.J. & YAMADA, T. (1994). Molecular cloning, expression, and characterization of a fifth melanocortin receptor. *Biochem. Biophys. Res. Com.,* **200,** 1214–1220.

GREENBERG, M.J., PAYZA, K., NACHMAN, R.J., HOLMAN, G.M. & PRICE, D.A. (1988). Relationships between the FMRFamide-related peptides and other peptide families. *Peptides,* **9,** 125–135.

GRUBER, K.A. & CALLAHAN, M.F. (1989). ACTH-(4–10) through γ-MSH: evidence for a new class of central autonomic nervous system-regulating peptides. *Am. J. Physiol.,* **257,** R681–R694.

HAO, J.M. & RABKIN, S. (1997). Increased cardiac ppENK mRNA in cardiac hypertrophy and effects on blood pressure of its peptide products. *Am. J. Physiol.,* **272,** H2885–H2894.

HEYLIGER, S.O., PAYZA, K. & ROTHMAN, R.B. (1998). The effect of FMRFamide analogs on [^{35}S]GTP-γ-S stimulation in squid optic lobes. *Peptides,* **19,** 739–747.

KIVIPELTO, L., AARNISALO, A.A. & PANULA, P. (1992). Neuropeptide FF is colocalized with catecholamine-synthesizing enzymes in neurons of the nucleus of the solitary tract. *Neurosci. Lett.,* **143,** 190–194.

KIVIPELTO, L., MAJANE, E.A., YANG, H.-Y.T. & PANULA, P. (1989). Immunohistochemical distribution and partial characterization of FLFQPQRFamidelike peptides in the central nervous system of rats. *J. Comp. Neurol.,* **286,** 269–287.

KLEIN, M.C., HUTCHINS, P.M., LYMANGROVER, J.R. & GRUBER, K.A. (1985). Pressor and cardioaccelerator effects of gamma MSH and related peptides. *Life Sci.,* **36,** 769–775.

LI, S.J., VARGA, K., ARCHER, P., HRUBY, V.J., SHARMA, S.D., KESTERSON, R.A., CONE, R.D. & KUNOS, G. (1996). Melanocortin antagonists define two distinct pathways of cardiovascular control by alpha- and gamma-melanocyte-stimulating hormones. *J. Neurosci.,* **16,** 5182–5188.

MAJANE, E.A., IADAROLA, M.J. & YANG, H.Y. (1983). Distribution of Met5-enkephalin-Arg6, Phe7 in rat spinal cord. *Brain Res.,* **264,** 336–339.

MAJANE, E.A., PANULA, P. & YANG, H.-Y.T. (1989). Rat brain regional distribution and spinal cord neuronal pathway of FLFQPQRF-NH2, a mammalian FMRF-NH2-like peptide. *Brain Res.,* **494,** 1–12.

MOUNTJOY, K.G. (1994). The human melanocyte stimulating hormone receptor has evolved to become 'super-sensitive' to melanocortin peptides. *Prep. Biochem.,* **257,** 1248 – 1251.

MOUNTJOY, K.G., MORTRUD, M.T., LOW, M.J., SIMERLY, R.B. & CONE, R.D. (1994). Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. *Mol. Endocrinol.,* **8,** 1298 – 1308.

MUES, G., FUCHS, I., WEI, E.T., WEBER, E., EVANS, C.J., BARCHAS, J.D. & CHANG, J.-K. (1982). Blood pressure elevation in rats by peripheral administration of TRY-GLY-GLY-PHE-MET-ARG-PHE and the invertebrate neuropeptide, PHE-MET-ARG-PHE-NH_2. *Life Sci.,* **31,** 2555 – 2561.

O'DONOHUE, T.L., BISHOP, J.F., CHRONWALL, B.M., GROOME, J. & WATSON, W.H. (1984). Characterization and distribution of FMRFamide immunoreactivity in the rat central nervous system. *Peptides,* **5,** 563 – 568.

PAINTER, S.D., MORLEY, J.S. & PRICE, D.A. (1982). Structure-activity relations of the molluscan neuropeptide FMRFamide on some molluscan muscles. *Life Sci.,* **31,** 2471 – 2478.

PAYZA, K. (1987). FMRFamide receptors in *Helix aspersa*. *Peptides,* **8,** 1065 – 1074.

PAYZA, K., AKAR, C.A. & YANG, H.-Y.T. (1993). Neuropeptide FF receptors: structure-activity relationship and effect of morphine. *J. Pharmacol. Exp. Ther.,* **267,** 88 – 94.

PITTIUS, C.W., SEIZINGER, B.R., PASI, A., MEHRAEIN, P. & HERZ, A. (1984). Distribution and characterization of opioid peptides derived from proenkephalin A in human and rat central nervous system. *Brain Res.,* **304,** 127 – 136.

SCHWYZER, R. & EBERLE, A. (1977). On the molecular mechanism of alpha-MSH receptor interactions. *Front Horm. Res.,* **4,** 18 – 25.

STEFFENS, A.B. (1969). A method for frequent sampling of blood and continuous infusion of fluids in the rat without disturbing the animal. *Physiol. Behav.,* **4,** 833 – 836.

SUN, X.Y., FENG, Q.P., GONG, Q.L., EDVINSSON, L. & HEDNER, T. (1992). Cardiovascular and renal effects of gamma-MSH in spontaneously hypertensive and normotensive Wistar Kyoto rats. *Am. J. Physiol.,* **262,** R77 – R84.

TANG, F., TANG, J., CHOU, J. & COSTA, E. (1984). Age-related and diurnal changes in Met5-Enk-Arg6-Phe7 and Met5-enkephalin contents of pituitary and rat brain structures. *Life Sci.,* **35,** 1005 – 1014.

THIEMERMANN, C., AL-DAMLUJI, S., HECKER, M. & VANE, J.R. (1991). FMRFamide and L-ARG-L-PHE increase blood pressure and heart rate in the anaesthetised rat by central stimulation of the sympathetic nervous system. *Biochem. Biophys. Res. Com.,* **175,** 318 – 324.

VAN BERGEN, P., DE WINTER, T.Y., DE WILDT, D.J. & VERSTEEG, D.H. (1997a). Influence of blockade of alpha 1-adrenoceptors, beta 1-adrenoceptors and vasopressin V1A receptors on the cardiovascular effects of gamma 2-melanocyte-stimulating hormone (gamma 2-MSH). *Naunyn Schmiedebergs Arch. Pharmacol.,* **355,** 720 – 726.

VAN BERGEN, P., JANSSEN, P.M., HOOGERHOUT, P., DE WILDT, D.J. & VERSTEEG, D.H. (1995). Cardiovascular effects of gamma-MSH/ACTH-like peptides: structure-activity relationship. *Eur. J. Pharmacol.,* **294,** 795 – 803.

VAN BERGEN, P., KLEIJNE, J.A., DE WILDT, D.J. & VERSTEEG, D.H. (1997b). Different cardiovascular profiles of three melanocortins in conscious rats; evidence for antagonism between gamma 2-MSH and ACTH-(1-24). *Br. J. Pharmacol.,* **120,** 1561 – 1567.

VAN BERGEN, P., VLEEMING, W., VAN HEIJST, B.G., VERSTEEG, D.H. & DE WILDT, D.J. (1998). A study on possible modulating and direct effects of gamma2-MSH and ACTH-(1-24) on the cardiovascular system of the rat. *Naunyn Schmiedebergs Arch. Pharmacol.,* **358,** 220 – 229.

WONG, T.M., GREENBERG, M.J. & TSE, S.Y.H. (1985). Cardiovascular effects of intraventricular injection of FMRFamide, Met-enkephalin and their common analogues in the rat. *Comp. Biochem. Physiol.,* **81C,** 179 – 179.

YANG, H.Y., FRATTA, W., MAJANE, E.A. & COSTA, E. (1985). Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. *Proc. Natl. Acad. Sci. U.S.A.,* **82,** 7757 – 7761.

YANG, H.Y., PANULA, P., TANG, J. & COSTA, E. (1983). Characterization and location of Met5-enkephalin-arg6-phe7 stored in various rat brain regions. *J. Neurochem.,* **40,** 969 – 976.

(Received March 16, 2000
Revised June 20, 2000
Accepted September 12, 2000)