Endocr (2009) 35:312–324
DOI 10.1007/s12020-008-9141-3

ORIGINAL PAPER

Neuropeptide Y and gamma-melanocyte stimulating hormone (γ-MSH) share a common pressor mechanism of action

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Received: 29 July 2008 / Accepted: 21 October 2008 / Published online: 11 April 2009

Abstract Central circuits known to regulate food intake and energy expenditure also affect central cardiovascular regulation. For example, both the melanocortin and neuropeptide Y (NPY) peptide families, known to regulate food intake, also produce central hypertensive effects. Members of both families share a similar C-terminal amino acid residue sequence, RF(Y) amide, a sequence distinct from that required for melanocortin receptor binding. A recently delineated family of RFamide receptors recognizes both of these C-terminal motifs. We now present evidence that an antagonist with Y1 and RFamide receptor activity, BIBO3304, will attenuate the central cardiovascular effects of both gamma-melanocyte stimulating hormone (γ-MSH) and NPY. The use of synthetic melanocortin and NPY peptide analogs excluded an interaction with melanocortin or Y family receptors. We suggest that the anatomical convergence of NPY and melanocortin neurons on cardiovascular control centers may have pathophysiological implications through a common or similar RFamide receptor(s), much as they converge on other nuclei to coordinately control energy homeostasis.

Keywords γ-MSH · Neuropeptide Y · Hypertensive effects · Central nervous system · RFamide peptides · RFamide receptors · Y1 antagonists · Central vasopressin system

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Introduction

The association between elevated body weight and cardiovascular (CV) disease has become an increasingly important area for investigation, due to the progressively larger percent of the American (and world) population that has inappropriately high body weight or frank obesity [1]. However the basis for this association is complex and incompletely understood. Is the etiology of cardiovascular disease partially linked with mechanisms causing weight gain, or exclusively a pathological consequence of increased body mass, high blood lipids, or diabetes?

Regulation of appetite and energy homeostasis are major factors in weight control [2]. The risk of cardiovascular disease increases proportionately with the increase in body mass [3]. One potential factor contributing to both weight gain and CV disease could be a common initiating mechanism(s), effectively linking the etiology of increased body mass, or the central response to increased body mass, with blood pressure regulation. Delineation of a common factor associated with both blood pressure regulation and energy homeostasis could provide an explanation for how

pathological alterations in the central control of food intake might also affect central cardiovascular mechanisms.

Over the past 10–15 years, several peptide families and their associated receptors have become recognized as important mediators of food intake and energy expenditure. These include the melanocortins [4], neuropeptide Y (NPY) [5], and leptin systems [6]. However, previous attempts to link melanocortin-4 receptor (MC4-R) signaling or leptin with blood pressure regulation have produced relatively minor effects (i.e., 3–10 mmHg increases in MAP [7]. For example, the modest pressor effect of centrally administered α-melanocortin or melanocyte stimulating hormone (MSH) is mediated via the MC4-R [8].

However, there are potent central cardiovascular effects associated with members of the melanocortin and NPY peptide families. The pressor effects of γ-MSH peptides in mouse models (e.g., a 38 mmHg rise in MAP) are not due to an interaction with known central melanocortin receptors, as these effects can be elicited following peripheral administration of γ-MSH in the MC3-R and MC4-R knockout mice [8]. Several characteristics common to both melanocortin and NPY peptides include a centrally mediated increase in sympathetic drive and inhibition of the cardiac baroreceptor reflex [9–13].

We now present evidence that the cardiovascular effects of γ-MSH and NPY are attenuated by administration of BIBO3304, a compound originally developed as a Y1 receptor antagonist. The Y family of receptors is a subset of the more general RFamide family [14]. Recent work indicates that many Y1 antagonists are more general RFamide receptor antagonists [15–19]. Our data support this concept. We used NPY and γ-MSH analogs to provide evidence for BIBO3304-sensitive non-Y1 and non-melanocortin receptor-mediated cardiovascular activity. NPY and melanocortin neurons are known to converge on both energy homeostatic and cardiovascular control centers [4]. Our data provide pharmacological evidence that suggests the cardiovascular center convergence may extend beyond a common anatomical substrate, to involve a common or similar receptor mechanism outside of the melanocortin and Y receptor families.

Materials and methods

Animals

Rats and mice were housed at 21 ± 2°C with ad libitum access to food (Purina Rodent Diet 5001, Purina Mills, MO) and water. The Oregon Health and Science University Animal Care and Use Committee approved these studies, with adherence to the National Institutes of Health guidelines for use of experimental animals.

Rats

Male 300–400 g Sprague Dawley rats (Charles River Laboratories, Wilmington, MD) were prepared with chronic 3rd and 4th intraventricular (ICV) cannulae. The cannulation surgery was performed under xylazine–ketamine anesthesia, using previously described stereotaxic protocols for dual ventricular cannulation [20]. Cannulated rats were allowed at least 5 days of recovery before being used.

For monitoring blood pressure, rats were given a series of urethane injections to slowly titrate them to a state of surgical anesthesia that did not attenuate cardiovascular sympathetic drive. This was assessed by robust pressor responses to intravenous (IV) γ-2 MSH. An initial loading dose of urethane (200 µl/100 g body weight of a 400 mg/ml solution) was given by intraperitoneal injection, the rats allowed to achieve a lightly anesthetized steady state (about 20–30 min post-injection), and then additional 50 µl doses of urethane administered (about 20 min between each dose) until a surgical level of anesthesia was achieved (assessed by pinch reflexes). Each rat was then prepared with femoral artery (PE-50 tubing) and vein (PE-10 tubing) catheters that were filled with heparinized (40 units per ml) 0.9% saline. The arterial catheter was connected to a Statham pressure transducer and the signal analyzed by a Dasy Lab System (Version 6.00.06) running on a PC computer (DasyTech USA, Amherst, MA). This protocol results in a preparation with a mean arterial pressure (MAP) of 90–100 mmHg and a heart rate (HR) of about 300–350 beats per minute (BPM). The preparation is stable, with no further anesthetic doses required, for up to 8 h.

Cardiovascular responses to saline or peptide administration were analyzed as the MAP and HR responses, compared to a 10-minute pre-administration average. The following parameters were measured: peak MAP and HR responses; the relative areas under the response curves (total pressor response [21]); and time from administration to initiation, peak, and resolution of each MAP or HR response. The point of initiation or resolution of each response curve was taken as a 10% change from the respective baseline. To calculate area under each response curve, the height of the response above baseline was multiplied by the curve width (in minutes) at 10% above the baseline for the rise and decay aspects of the curve (height × width or H × W).

Cell lines

The following cell lines were used: CHO (Chinese hamster ovary) cells expressing the rat Y1 receptor for receptor binding studies [22], and HEK tsA201 cells for electrophysiological experiments (kindly provided by

Dr. G. W. Zamponi, University of Calgary, Calgary, Canada).

Peptides and drugs

The following peptides were used: γ-melanocyte stimulating hormone (γ-MSH), NPY (Peptidec Technologies, Pierrefonds, QC, Canada), (D-Arg_{25}) NPY (a Y1 receptor agonist, [23], Bachem, Inc., King of Prussia, PA), and γ-$MSH_{6–12}$ (custom synthesized by Peptides International, Louisville, KY). The latter peptide, a fragment of the native γ-MSH peptide, lacks the essential His residue of the core melanocortin sequence [24], but has previously been demonstrated to retain the parent peptide's pressor activity [25]. Des-$AA_{10–17}$-cyclo-7/21[$Cys_{7,21}$, Pro_{34}] NPY, a centrally-truncated, NPY analog [26], was a gift from Jean Rivier, Salk Institute, La Jolla, CA. BIBO3304 [15] was obtained from Boehringer-Ingelheim (GFR). All peptides and drugs were dissolved in 0.45% saline or deionized water for in vivo administration.

Drug treatment procedures

γ-MSH

To initially examine the effects of central BIBO3304 on cardiovascular responses, three IV 20 nmol doses of γ-MSH were given to a group of rats ($n = 7$), while blood pressure and heart rate were recorded (PRE responses). Each dose of peptide was given in a 30-μl IV infusion over 15 s, followed by an additional 50 μl infusion of 0.9% heparinized saline. Rats were allowed to recover for at least 25–30 min between each peptide dose. The recovery period began when the MAP returned to the pre-injection baseline. Cardiovascular responses to an IV 0.9% saline vehicle were recorded before and after γ-MSH dosing. Following the third dose and recovery period, each rat was given sequential 3rd and 4th ICV 5 μl infusions of BIBO3304, a Y1/RFamide receptor antagonist [18, 19, 27]. Each ICV dose was 5 nmol given over 2 min, with a 5-min period between the 3rd and 4th ventricular infusions.

Following the end of the ICV infusions, rats were allowed a 30-min drug diffusion period before post-treatment (POST) responses to γ-MSH were assessed. An additional three doses of IV γ-MSH were administered in a manner similar to the pre-blockade protocol. Comparisons of γ-2 MSH cardiovascular responses were made PRE and POST ICV saline. Treatment effects on MAP and HR were analyzed by taking the mean of the three MAP peak amplitudes, MAP curve areas, and HR responses for each rat, pre and post BIBO3304 treatment. These results were analyzed by two tailed Student's t test for paired data. Data is presented as mean ± standard error.

NPY

To examine the effects of central BIBO3304, IV doses (5 nmol) of NPY, or a Y1 receptor agonist [(D-Arg_{25}) NPY] were given to a group of rats ($n = 7$), while blood pressure and heart rate were recorded (PRE responses). Both peptides were given PRE and POST ICV BIBO3304 (in a manner similar to γ-MSH above). Saline vehicle doses were interspersed between each peptide dose. In addition, each rat received two times 5 nmol doses of ANG II (ANG II) before the central BIBO3304 infusion and two doses after. The angiotensin MAP response served as a positive control for assessing cardiovascular reflex stability. One ANG II dose was given before each series of NPY peptides/saline vehicle injections, and one dose after (i.e., two PRE doses and two POST doses). The mean of the two PRE ANG II doses was compared to the mean of the two POST responses. All other peptide dosing was randomly ordered.

Statistical analysis was by two approaches. Paired t tests compared the PRE and POST saline and within peptide species MAP and HR peak and area responses. Significance was calculated following a Bonferroni correction for multiple t tests as $P < 0.0125$. A second approach used a Repeated Measures ANOVA to examine the overall effect of saline and peptide treatments. If the null hypothesis was rejected ($P < 0.05$), within group PRE and POST responses were examined with Bonferroni's Multiple Comparison Test (significance taken as $P < 0.05$). All results presented as mean ± standard error.

Cardiovascular-specific analogs of γ-MSH and NPY

The effects of 5 nmol IV or ICV BIBO3304 were examined on the pressor responses of IV γ-$MSH_{6–12}$ and des-$AA_{10–17}$-cyclo-7/21[$Cys_{7,21}$, Pro_{34}] NPY and (D-Arg_{25}) NPY in rats ($n = 7$). Comparisons were made between the analogs and their parent peptides at multiple doses. Each peptide dose was given PRE and POST IV or ICV BIBO3304 administration. Statistical analysis was similar to the NPY study above.

Y1 receptor-binding experiments

Cells for Y1 receptor binding studies were grown in DMEM/Nut Mix F-12 (Gibco BRL) containing 10% fetal calf serum (Biotech Line, AS, USA), 2.4 mM L-glutamine (Gibco BRL) and 0.25 mg/ml G-418 (Gibco BRL), 100 units of penicillin/ml, and 100 μg streptomycin/ml (Gibco BRL) until harvesting. Receptor expression in HEK 293 EBNA-1 was selected for by growth in the presence of 200 μg/ml hygromycin. After harvesting, the cell membranes were frozen in aliquots at −80°C.

Before the binding assays, the thawed membrane aliquots were resuspended in 25 mM HEPES-buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 g/l bacitracin and homogenized using an Ultra-Turrax homogenizer.

In the receptor binding assays, the total radioligand binding and ligand competition were measured. All measurements were for duplicate or triplet samples. The radioligand was ^{125}I-pPYY (Amersham, Uppsala, Sweden) with iodinated tyrosines at positions 21 and 27 and a specific activity of 4000 Ci/mmol. Total binding was defined as the amount of radioactivity remaining bound to the cell homogenate after incubation in presence of 0.1 nM ^{125}I-pPYY. In the competition assay the peptides FMRFamide and γ-MSH (designated for experiments as peptides A and B, thus identities unknown to the experimenter), and the positive control, porcine neuropeptide Y (pNPY), were allowed to compete with the radioligand. Each of the unknown peptides was tested at two concentrations, 10 μM and 100 nM. pNPY was tested at 100 nM.

Incubations were terminated by filtration through GF/C filters (Filtermat A, Wallac Oy, Turku, Finland), which had been presoaked in 0.3% polyethylenimine, using a TOMTEC (Orange, CT, USA) cell harvester. The filters were washed with 50 mM Tris (pH 7.4) at 4°C and dried at 60°C. The dried filters were treated with MeltiLex A (Wallac Oy, Turku, Finland) melt-on scintillator sheets and the radioactivity retained on the filters counted using the Wallac 1450 Microbeta counter.

Specific binding was calculated from the difference between the total and the competitive binding. The binding assays were repeated at least three times for each peptide–receptor combination. Data were first subjected to an ANOVA. Post hoc analysis compared all experimental groups to a control group using Dunnett's Multiple Comparison Test. A significant effect was taken to be at the $P < 0.05$ level.

Electrophysiological recording

Activation of G_i/G_o-coupled NPY receptors has been shown to mediate the inhibition of N-type voltage-dependent calcium channels (VDCC). To examine the effects of NPY and γ-MSH peptides on Y1 receptor-mediated intracellular events, HEK tsA201 cells transiently expressed the Y1 receptor and N-type VDCC subunits. Barium currents, a surrogate for calcium conductance through the N-type VDCCs, were measured to determine the effect of NPY and γ-MSH peptides on Y1 receptor signaling. For these experiments, HEK tsA201 cells were maintained at 37°C (5% CO_2) in standard Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 1% penicillin–streptomycin (Invitrogen). The cells were plated on glass coverslips at 50% confluency and transiently transfected with cDNA constructs encoding the VDCC subunits (α_{1B}, $\alpha_2\delta$, and β_{1b}; kindly provided by Drs. G. W. Zamponi and T. P. Snutch, University of Calgary, Calgary, Canada) and Y1 receptor linked to Enhanced Green Fluorescent Protein (EGFP; kindly provided by Dr. A. G. Beck-Sickinger, University of Leipzig, Leipzig, GFR) using the standard calcium phosphate protocol [28, 29]. Following transfection, the cells were moved to a 30°C (5% CO_2) incubator where they were maintained for up to 4 days.

During electrophysiological recording sessions, the cells were placed in a recording chamber that was continuously perfused with external recording solution composed of (in mM) 20 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 40 tetraethylammonium (TEA) chloride, 10 glucose, 65 CsCl (adjusted to pH 7.20 with TEA-OH; 250–256 mOsm/l) at room temperature (22°C). Only cells demonstrating green fluorescence were considered for patching. Patch pipettes (BF 150-86-15; Sutter Instruments borosilicate glass) show typical resistances of 5–6 MΩ when back-filled with internal solution composed of (in mM) 108 $CsCH_3SO_4$, 4 $MgCl_2$, 9 EGTA, 9 HEPES, 1 Na-GTP (adjusted to pH 7.20 with CsOH; 240–245 mOsm/l). The recording pipette was attached to the headstage of an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA) used in voltage-clamp mode and linked to a computer equipped with Clampex 8.2 software (Axon Instruments). Membrane currents were evoked every 30 s by a 14 ms, 100 mV depolarizing test voltage pulse from a holding potential of −80 mV. Peptides (50 nM γ-MSH, 50 nM γ-MSH_{6-12}, or 50 nM NPY) were added to the external recording solution immediately prior to application and perfused. The peptide application was followed by at least a 10-min washout of the peptide.

Statistical analyses were conducted in two ways. A paired t test compared each peptide to a control response (set at 100%). An ANOVA compared N-type VDCC current changes between treatment groups, with post hoc analysis by Dunnett's Multiple Comparison Test. A significant effect was taken to be at the $P < 0.05$ level.

Data analysis programs

Data were analyzed using GraphPad Prism^R software (GraphPad Software, San Diego, CA) and procedures. Electrophysiological data were analyzed offline using Clampfit 9.2 software (Axon Instruments).

Results

Quantitative comparison of the γ-MSH and NPY cardiovascular responses

γ-MSH administration produced both pressor and cardioaccelerator effects, with no evidence of a baroreceptor-mediated bradycardia (Fig. 1a). In contrast, the NPY pressor response (Fig. 1b) lasted significantly longer than that of γ-MSH for a relatively similar change in peak MAP ($P < 0.05$, $n = 7$). The baseline MAPs and HR for the γ-MSH and NPY administration groups were similar. Additionally, NPY produced a distinctive series of effects on HR (Fig. 1b). Initially there was a decrease in HR that qualitatively resembled a baroreceptor-mediated bradycardia. However, during the mid-point of the pressor response the bradycardia reversed to a cardioaccelerator response.

A quantitative summary of the time course of the MAP and HR effects for γ-MSH and NPY are seen in Tables 1, 2, 3, and 4. Peptide administration is designated as t_o, and subsequent time points are measured from it. The MAP effect was analyzed at an initiation time point (t_i; where MAP was 10% above the baseline), the peak of the response (t_p), and a resolution point (t_r: where the response decreased by 90%). The HR responses presented a more complicated picture. Since γ-MSH produced only an increase in HR, the same time points for MAP were used to

Table 1 Time course of the γ-MSH cardiovascular responses (15 nmol IV)

Parameter	t_i (s)	t_p (min)	t_r (min)
MAP: t_o	21.58 ± 2.01	38.50 ± 2.30	4.50 ± 0.60
HR: t_o	22.00 ± 3.26	38.83 ± 7.05	5.93 ± 0.97

The time course of the cardiovascular effects of γ-MSH and NPY administration are shown in Tables 1, 2, and 3. The selected time points are measured from the time of administration (t_o); and include the initiation of the response (t_i), peak(s) of the response (t_p), and the resolution of the response (t_r)

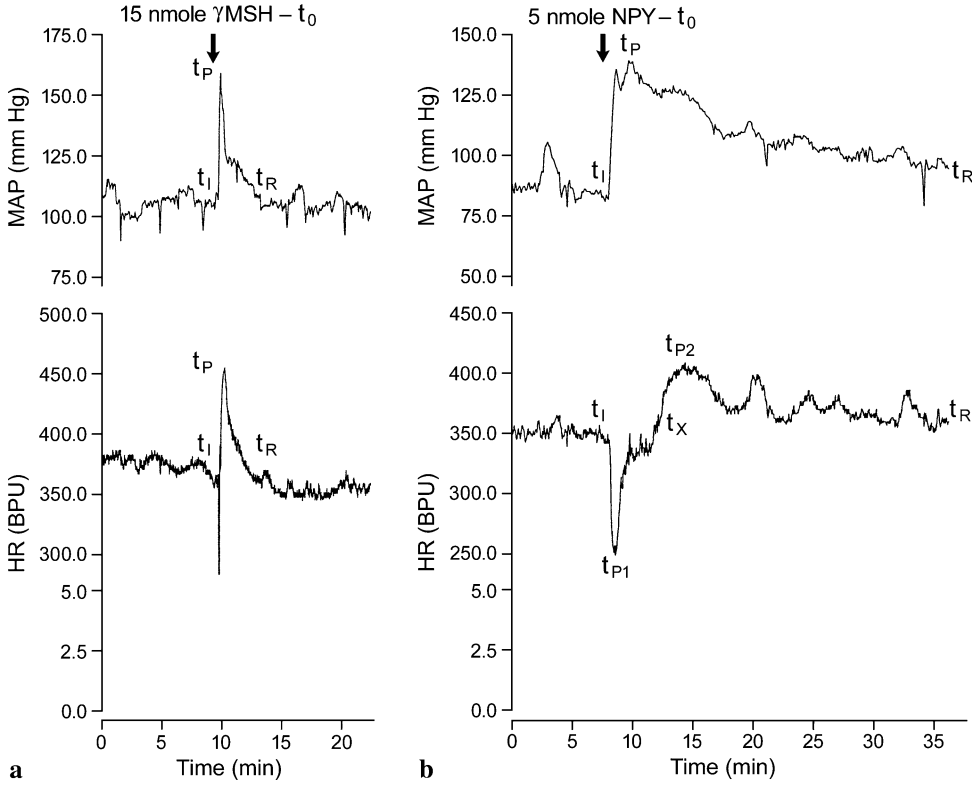

Fig. 1 Individual MAP and HR responses to the IV administration of 15 nmol of γ-MSH (**a**) and 5 nmol of NPY (**b**). The *arrow* designates time point of peptide administration (t_o). The subsequent time points define the response initiation, peak, and resolution. **a** γ-MSH produces a marked pressor and tachycardia response. Note the lack of a bradycardia response during the pressor response. The *downward spike* in the HR tracing following peptide administration is not a true decrease in rate (i.e., an increase in interbeat interval), but rather is due to skipped heart beats. This is consistent with catecholamine-mediated tachycardia. **b** NPY produces prolonged pressor and HR responses, although its relative MAP peak is similar to that of γ-MSH. This effectively produces a much greater MAP curve area for NPY compared to γ-MSH (or other RFamide peptides). The NPY HR response is bi-phasic; bradycardia followed by tachycardia. The time points for the MAP and HR tracings are defined in the "Results" section

Table 2 Time course of the NPY pressor response (5 nmol IV)

Parameter	t_i (s)	t_p (min)	t_r (min)
MAP: t_o	32.0 ± 8.0	2.7 + 0.2	23.1 + 6.0

Table 3 Time course of the NPY cardiac response (5 nmol IV)

Parameter	t_i (s)	t_{p1} (min)	t_x (min)	t_{p2} (min)	t_r (min)
HR: t_o	27.0 ± 5.0	1.8 ± 0.3	4.8 ± 1.0	12.1 ± 3.9	18.5 ± 4.0

Table 4 Peak bradycardia and tachycardia responses to NPY (5 nmol IV)

Parameter	Baseline HR	Bradycardia	Tachycardia
BPM	360 ± 11	−53 ± 18	49 ± 14

describe this effect. However, NPY administration produced a sequential bradycardia and tachycardia (see Fig. 1b). Thus, there are two HR peaks for NPY, t_{p1} is for the peak decrease in HR, while t_{p2} is at the maximum cardioaccelerator response. t_x designates the crossover point between the two HR responses.

γ-MSH and BIBO3304

ICV administration of the Y1/RFamide antagonist BIBO3304 [28] produced a 40–50% attenuation of the IV γ-MSH MAP and HR responses ($n = 7$, Fig. 2a, b). In addition, there was a >60% attenuation in the total pressor response (the relative MAP curve area calculated as peak height × peak width; Fig. 2c). Substituting vehicle for the ICV administration of drug produced no change in the pre versus post γ-MSH responses (data not shown). Central or peripheral administration of BIBO3304 alone produced no significant changes in any baseline cardiovascular parameters ($n = 7$). In a subsequent study comparing several IV doses of γ-MSH and γ-$MSH_{6–12}$, ICV BIBO3304 attenuated >50% of the MAP response of either peptide (Fig. 5a).

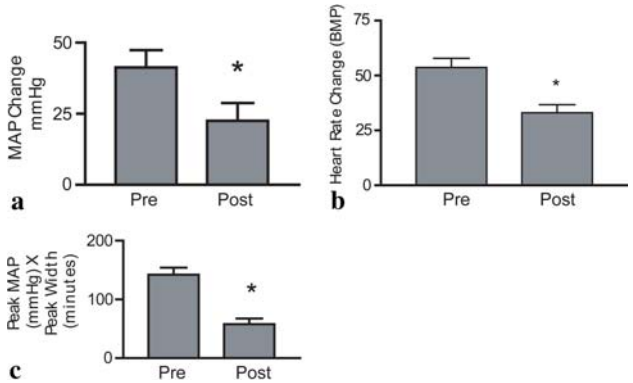

Fig. 2 The IV γ-MSH cardiovascular response PRE and POST ICV BIBO3304 treatment. The evaluated parameters include the change in peak MAP response (**a**), increase in HR (**b**), and area under the MAP curve (**c**, HxW). All values are reported as mean ± standard error of mean. Significant post treatment reductions in all measured parameters are seen when compared to the pre-treatment response (*denotes $P < 0.05$)

Following these findings, we sought to determine if γ-MSH acted by binding directly to the Y1 receptor, since this receptor is known to mediate the pressor effects of NPY [30]. Receptor binding studies showed that γ-MSH (at concentrations of 10 and 100 nM) failed to compete with iodinated PYY for binding to the rat Y1 receptor (Table 5). FMRFamide also failed to bind to these receptors, providing a negative control. In contrast, as a positive control, porcine NPY (100 nM) competed away 29–70% of the radioligand binding.

We also determined if γ-MSH was altering Y1 receptor signaling without altering peptide binding to the Y1 receptor. Activation of Y1 receptors inhibits the activation of N-type VDCCs, measured as a decrease in barium current (I_{BA}). Whole-cell recordings showed a failure of the melanocortin peptides to affect Y1 receptor-mediated inhibition of N-type VDCCs. In the positive control, application of 50 nM NPY significantly reduced I_{BA} by almost 50% (Fig. 3). However in contrast, 50 nM γ-MSH or γ-$MSH_{6–12}$ reduced I_{BA} by only 3–9% (Fig. 3, $P > 0.05$). These relatively minor current decreases were consistent with a natural run down of N-type VDCC current over time. As might be expected, there were no significant differences in effects between the two melanocortin peptides. The receptor binding and electrophysiological experiments suggested that γ-MSH does not affect Y1 receptor agonist binding capacity or signaling.

Neuropeptide Y and BIBO3304

ICV administration of BIBO3304 produced a significant decrease in the IV NPY cardiovascular response (Fig. 4a). Since ICV BIBO3304 did not change the MAP response of D-Arg_{25} NPY (a Y-1 agonist), this effectively acted as a control for non-specific inhibition of cardiovascular responses, as well as evidence for a Y-1 receptor-independent mechanism of action. In contrast, the NPY-induced MAP response was reduced >40% by ICV BIBO3304 and the total pressor response decreased by >70% (Fig. 4b). As a control for diminishing cardiovascular responses over time or following ICV infusions, we compared peak MAP responses to IV AII, PRE and POST ICV BIBO3304 administration. PRE and POST responses (66 ± 6 vs. 54 ± 2 mmHg) were not significantly different.

Table 5 Assessment of the binding of NPY, FMRFamide, and γ-MSH to the rat and human Y1 receptor

Specific binding (cpm)[a] rat Y1 receptor	% Specific binding rat Y1 receptor	Specific binding (cpm) human Y5 receptor	% Specific binding human Y5 receptor
NPY			
2432	75	1222	51
1854	30	1449	47
988	41	842	35
945	29		
FMRFamide			
0	0	22	1
125	6	0	0
0	0	0	0
γMSH			
229	7	0	0
0	0	39	2
0	0	0	0

[a] Specific binding values are in counts per minute (cpm), an average of triplicates

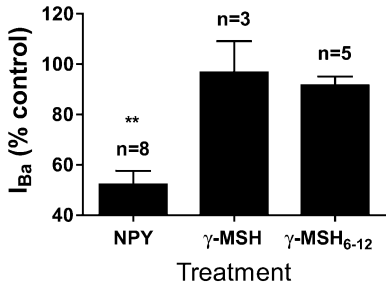

Fig. 3 Effect of 50 nM NPY, γ-MSH, and γ-$MSH_{6–12}$ on N-type channel barium currents (I_{Ba}).The responses for each group are the differences in I_{Ba} prior to and after peptide application (** denotes $P < 0.001$). The decrease in I_{Ba} following the application of NPY was significantly different from the effects of γ-MSH or γ-$MSH_{6–12}$. Application of γ-MSH or γ-$MSH_{6–12}$ did not produce a significant inhibition of I_{Ba} relative to control ($P > 0.05$)

Non-melanocortin and non-Y1 receptor-mediated pressor activities

The IV pressor activity of 20 nM or 100 nM γ-$MSH_{6–12}$, was similar to the native γ-MSH peptide (Fig. 5b). IV or ICV BIBO3304 significantly attenuated the pressor responses of γ-MSH and the γ-MSH analog (compare Fig. 5a, b).

Peripheral or central BIBO3304 treatment did not significantly affect the MAP response or total pressor response of a Y1 receptor agonist (D-Arg_{25}) NPY, (compare Figs. 4a, 5b). Des-$AA_{10–17}$-cyclo-7/21[$Cys_{7,21}$, Pro_{34}] NPY (NPY-CVa) is a NPY Y1 (in vitro) agonist without orexigenic activity, but retaining pressor activity comparable to that of NPY (26 ± 3 mmHg increase in MAP for NPY-CVa versus 41.9 ± 4 mmHg for NPY, compare Figs. 5a with 4b). However treatment with central or systemic BIBO3304 significantly reduced the IV pressor response of this NPY analog, at either 5 or 20 nM doses, by up to 55% (Fig. 5).

Discussion

Our data provide evidence that a significant percent of the cardiovascular effects of γ-MSH and NPY may be due to effects at a common or pharmacologically related receptors. In retrospect, an overlapping cardiovascular mechanism of action for two RFamide class peptides should not be surprising.

Gruber and co-workers [10, 11] first described the central cardiovascular actions of the gamma melanocortins (γ-1 and 2 MSH). They suggested the existence of a cardiovascular system-regulating class of peptides, with an essential C-terminal motif of an Arg-hydrophobic sequence [R-F/W/Y-amide or R-F/W/Y-G, [31, 32]. Peptides with these motifs are referred to as "RFamide" peptides, after the most commonly occurring sequence. Numerous publications have confirmed the central cardiovascular actions of the γ-MSHs [33], as well as similar cardiovascular effects of other RFamide peptides [34, 35]. Thus, both NPY (C-terminal RYamide) and γ-1 or 2 MSH (RFamide or RFG) are members of a common class of peptides with demonstrated central cardiovascular actions.

The cardiovascular response to γ-MSH has been pharmacologically defined. Callahan et al. [9–11] showed that the MAP and HR effects are due to an increase in preganglionic sympathetic drive, with no contribution of adrenal medullary catecholamine release. These studies, as well as a subsequent confirmatory report [33], also suggest a total inhibition of the baroreceptor reflex during the γ-MSH cardiovascular response. Similar cardiovascular effects are seen in other RFamide peptides [34, 35].

In the peripheral nervous system, NPY released from sympathetic nerve terminals potentiates the vasoconstrictive effects of catecholamines through post-junctional Y1 receptors [36, 37]. This has led to the presumption that the

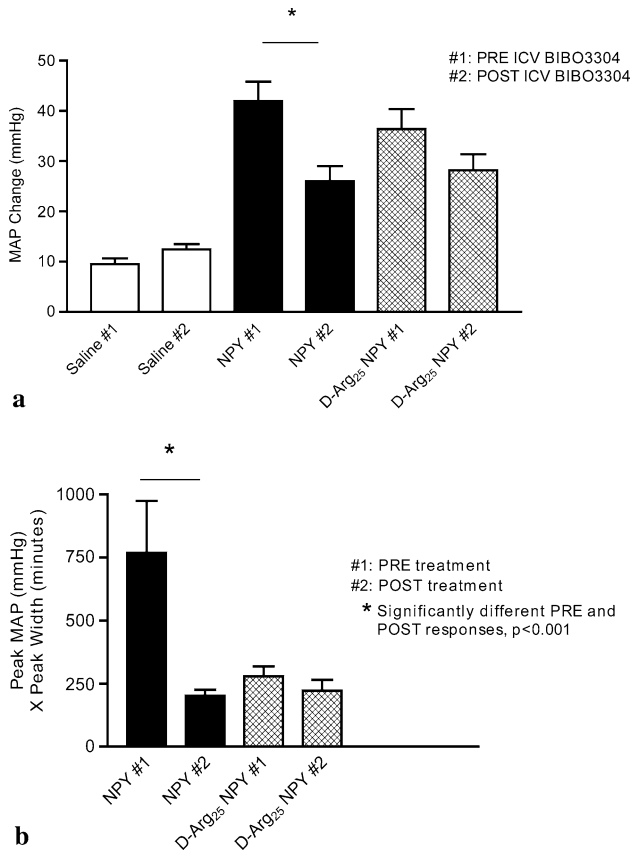

Fig. 4 The cardiovascular effects of NPY, selected analogs, and angiotensin II prior to and following treatment with BIBO3304. **a** Shows the absolute increases in MAP produced by IV administration of NPY and related compounds (all at 5 nmol doses), and saline, prior to (PRE) and after (POST) ICV administration of BIBO3304. The blood pressure responses of NPY are significantly reduced following BIBO3304. PRE and POST BIBO3304 pressor responses to (D-Arg^{25}) NPY (a peripheral Y1 receptor agonist) are not significantly different. **b** Shows the area under the MAP response curves for all NPY agonists' tested, prior to and after ICV administration of BIBO3304. Only the area under the NPY MAP response curve is reduced by drug treatment. The differences in PRE MAP areas of the Y1 agonist compared to NPY, reflect their relative MAP peak amplitudes and response duration times. Values reported as mean ± standard error of mean (* denotes $P < 0.05$)

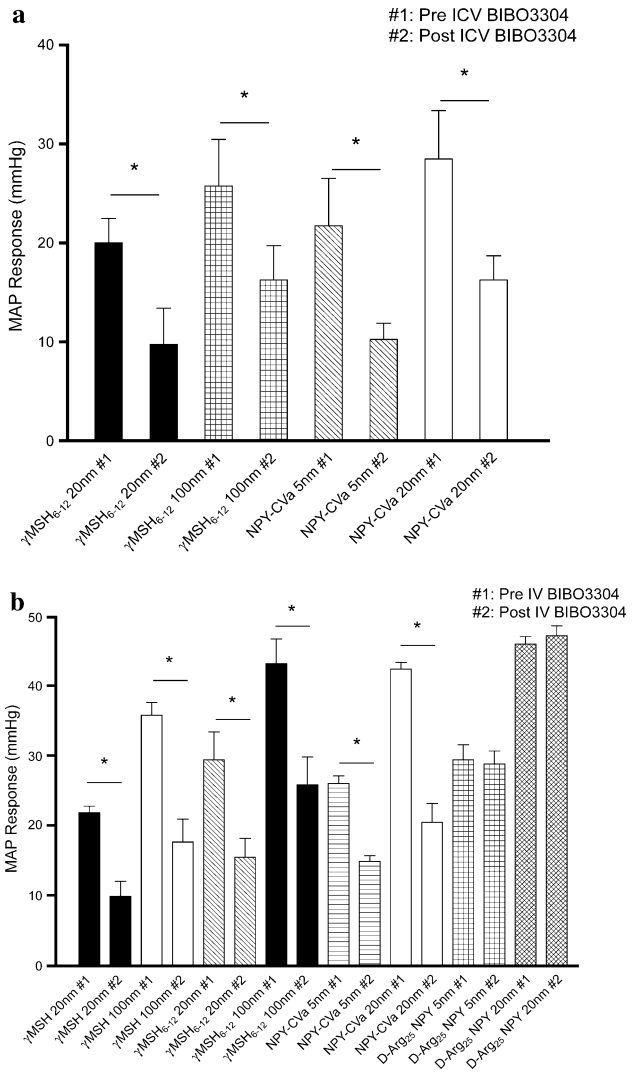

Fig. 5 The cardiovascular effects of γ-MSH and NPY analogs following ICV or IV BIBO3304 treatment. **a** Shows the effects on MAP produced by ICV BIBO3304 for two graded doses of γ-MSH$_{6–12}$ and NPY-CVa. BIBO3304 produced significant MAP reductions for both doses of either peptide. **b** Shows the effects on MAP produced by IV BIBO3304 for two graded doses of γ-MSH, γ-MSH$_{6–12}$, NPY-CVa, and D-Arg$_{25}$ NPY. BIBO3304 produced significant reductions in MAP for both doses of γ-MSH$_{6–12}$ and NPY-CVa, while having no significant effect on D-Arg$_{25}$ NPY (a Y1 receptor-specific agonist).The results demonstrate that either IV or ICV BIBO3304 can attenuate the pressor response of IV γ-MSH$_{6–12}$ or NPY-CVa. The inability of IV BIBO3304 to significantly reduce the pressor response of D-Arg$_{25}$ NPY is consistent with the data reported in Fig. 4 for ICV BIBO3304 and D-Arg$_{25}$ NPY

NPY-dependent pressor/hypertensive effects are solely due to effects of the peptide at the peripheral sympathetic nervous system. Other peripheral cardiovascular effects of NPY include a Y2 receptor-mediated attenuation of cardiac cholinergic drive and a contribution to post-junctional neurogenic vasoconstriction [38, 39] Similar effects on the cardiac baroreceptor reflex have been reported for the Y4 receptor [40]. However, Y1 receptor antagonists (e.g., BIBO3304 or BIBP3226) are not reported to have significant effects at other Y receptors [15]. While there are previous reports of central cardiovascular actions of NPY, these have been produced following central administration [12, 41]. Thus, our attenuation of the IV NPY cardiovascular response by ICV BIBO3304 is the first demonstration of a central component to the cardiovascular effects of peripherally administered NPY. However, since peripherally administered Y receptor ligands have effects on central feeding mechanisms (see below), the possibility of central cardiovascular effects following IV administration of NPY was predictable.

The cardiovascular effects of NPY initially appear quite different from γ-MSH or other RFamide peptides (compare Fig. 1a, b). However, this may be due to the fact that NPY has both peripheral and central cardiovascular actions. NPY produces a prolonged increase in MAP, necessitating a curve area comparison to other RFamide peptides for a true appreciation of its pressor effects. In addition, during its pressor response, there is an initial decrease in HR. However, unlike a typical baroreceptor-mediated bradycardia, the HR effect reverses during the pressor response to become a frank cardioaccelerator effect (Fig. 1b and Tables 1, 2, 3, 4). Several factors may be responsible for these actions.

The larger molecular weight and tertiary structure of NPY (>4,400 compared to about 1,000 for other RFamide peptides with cardiovascular effects) could affect receptor access and removal. This could limit the production of certain cardiovascular effects, produce different time-sequences of effects, or extend time-courses. An appropriate pharmacological dissection of the NPY cardiovascular response would require at least seven different antagonists (two adrenergic, one cholinergic, one ganglionic, one peripheral Y1, one peripheral Y2, and one peripheral Y4) used singly and in combination. Such an investigation is beyond the scope of our current studies. However, the lack of an appropriate analysis of the NPY cardiovascular response does not negate the core evidence for a non-Y1 receptor-mediated component (this report), apparently of central origin. Rather, our evidence helps to define the parameters that need to be controlled in future studies of NPY cardiovascular activity.

The major findings of our current investigations can be summarized as follows:

(i) The IV γ-MSH and NPY cardiovascular responses were attenuated by central or peripheral administration of relatively low doses of BIBO3304;
(ii) Systemic BIBO3304 affects central cardiovascular regulation;
(iii) Low doses of BIBO3304 did not attenuate the cardiovascular effects of a Y1 receptor agonist, suggesting that the cardiovascular actions of low dose BIBO3304 are not at a Y1 receptor;
(iv) γ-MSH did not bind to or allosterically regulate the Y1 receptor; and
(v) BIBO3304 will inhibit the cardiovascular effects of a γ-MSH analog that lacks melanocortin receptor activity, and a NPY analog that is specific for pressor activity (i.e., lacking orexigenic effects).

Each major finding will be discussed in light of previous reports on the cardiovascular effects of RFamide peptides and the actions of BIBO3304.

The ability of central or peripheral administration of BIBO3304 to attenuate the cardiovascular effects of a systemically administered peptide suggests a central nervous system locus mediating these effects. Such an anatomical site would need to be accessible to both blood and CSF. A circumventricular organ (CVO) site is a potential candidate for such a locus, since the cells in these structures are receptive to both blood and CSF-borne substances. In support of this site of action for BIBO3304, forebrains CVOs are reported to mediate the cardiovascular effects of γ-MSH. The supporting data include a rightward 10-fold shift in the γ-MSH pressor dose–response curve, when comparing carotid versus femoral artery routes of administration [31], and an attenuation of the IV γ-MSH cardiovascular response following forebrain CVO lesions [42]. With this in mind, a parallel can be drawn between the current data and the previous report of central or peripheral AII antagonists attenuating the central cardiovascular and dipsogenic effects of peripherally administered AII [43]. Forebrain CVOs were subsequently shown to be the anatomical site for these effects of AII and AII antagonists [44].

Peripheral administration of BIBO3304 (or BIBP3226, a structurally related NPY/RFamide receptor antagonist) affects central regulation of feeding behavior [15, 45]. Despite this evidence and the many reports of central cardiovascular effects of systemic RFamide peptides [31, 34, 35], cardiovascular studies of NPY-related peptides have always presumed a peripheral site of action for the systemically administered peptide [36–39, 46–48]. Our current data with central or peripheral BIBO3304 suggests that a significant component of the systemic NPY cardiovascular effect is due to activation of central cardiovascular mechanisms. Using relatively low doses of this receptor antagonist, there was not a significant reduction in the cardiovascular effects of a Y1 agonist. Thus, the effects of BIBO3304 in our studies may not at a Y1 receptor. An alternative receptor for these cardiovascular effects is suggested by the evidence that BIBO3304 and BIBP3226 have nanomolar-binding efficacy at NPFF receptors [16–19], RFamide receptors that are closely related to the Y1 receptor. These Y1 antagonists attenuate both the cardiovascular and orexigenic effects of NPFF [16, 49] and QRFP [50]. Thus, these Y1 antagonists appear to act as "mixed" RFamide receptor antagonists, rather than acting as specifically at the Y-1 receptor. This is not surprising, since Y receptor specificity is dependent on the tertiary structure of NPY/PPY peptides (MW > 4,000) [51]. The antagonists have molecular weights of about 600.

One potential criticism of our experiments is our use of an anesthetized preparation. Unfortunately, the time course of many of our studies precluded the use of a conscious rat

model due to ethical considerations. We are acutely aware of the potentially disruptive effects of anesthesia on cardiovascular measurements. In 1989, we first reported inhibitory effects of certain forms of anesthesia on the γ-MSH cardiovascular response [31]. The physiological basis for this is that many anesthetics are inhibitors of central sympathetic drive [52]. Since the cardiovascular effects of γ-MSH and several other RFamide peptides are dependent on central sympathetic drive, sympatholytic anesthetics would inhibit their cardiovascular effects. However, our choice of urethane as an anesthetic obviates this problem, since it preserves cardiovascular reflexes [53]. In addition, rather than using one large dose to induce surgical anesthesia, we titrated each preparation to an acceptable anesthetic state. This produced an extremely stable (6–8 h) cardiovascular assay. The ability of our model to produce robust pressor responses to γ-MSH was a positive control for a reactive central sympathetic nervous system. These results are identical to those we previously reported in conscious rats [31].

To address the possibility that cardiovascular responses were attenuated by experimental techniques, we used several pre hoc and post hoc controls. Maintenance of cardiovascular responses after ICV saline infusion provided initial evidence that the experimental procedures were not affecting cardiovascular responses. Additional positive controls were the maintenance of AII and Y-1 agonist cardiovascular effects following ICV BIBO3304 administration.

An important question raised by the inability of BIBO3304 to attenuate the cardiovascular effects of (D-Arg_{25}) NPY is, why previous investigations were able to use this compound as an effective Y1 antagonist in vivo [38, 45, 54–56]. The administered dose may be an important factor. Despite similar affinities of BIBO3304 or BIBP3226 for the Y1 receptor compared to NPY [15, 57], previous in vivo NPY cardiovascular studies typically used 100 to >10,000-fold molar excess of BIBO3304 or BIBP3226 (e.g., micromoles per kg body weight compared to nanomoles or picomoles of NPY) to block peripheral or central Y1 receptors [54–56, 58]. However, we used 10 nmol of BIB03304 to attenuate the cardiovascular effects of 5–20 nmol of NPY or 20–100 nmol of γ-MSH. In support of our central mechanism of action data, picomole amounts of systemic BIBO3304 lower blood pressure in hypertensive animal models with enhanced central sympathetic drive [47].

Central administration of BIBO3304 inhibited the peak MAP and HR responses to several IV γ-MSH doses by ~50%. Following central BIBO3304, there was also a 75% reduction in the total γ-MSH pressor response. These data confirm our previous reports of a central site of action for peripherally administered γ-MSH [9–11, 31].

We used relative area under the MAP and HR curves as additional indices of cardiovascular effects, measuring peak height × peak width at 10% of peak height. The validity of this measurement approach for peak area has been demonstrated using peak widths at 10–75% of peak height [59]. This method quantified the differences in the NPY and γ-MSH pressor curves seen in Fig. 1, since NPY produces a much longer time course effect for a relatively similar MAP peak change. Thus, there is a greater cardiovascular effect associated with a dose of NPY compared to an equivalent (MAP change) dose of γ-MSH. A similar logic is used in calculating MAP, the mean of the areas under the systolic and diastolic pressure curves.

Direct evidence that γ-MSH does not interact with the Y1 receptor was provided by competitive binding studies and electrophysiological activation studies. Neither γ-MSH nor FMRFamide (a model RFamide peptide) competed with iodinated PYY for binding to the rat Y1 receptors. However, a non-competitive or allosteric effect was still possible. To address this, we examined the effects of NPY, γ-MSH, and γ-$MSH_{6–12}$ (a melanocortin fragment that retains cardiovascular but not MCR activity [24, 25] on a recombinant Y1 receptor coupled to N-type VDCC. We found significant decreases in N-type VDCC activity following NPY treatment. However, neither γ-MSH nor γ-$MSH_{6–12}$ affected VDCC activity. Thus, the cardiovascular effects of melanocortin peptides are not due to allosteric modulation of the Y1 receptors.

A well known problem associated with pro-opiomelanocortin-derived peptides is their pleiotropic nature; each peptide may contain multiple receptor binding motifs [60, 61]. An example of this is γ-MSH, which contains both melanocortin and RFamide receptor-binding motifs [31, 62]. Peptides containing the melanocortin-binding motif (e.g., γ-MSH) are reported to have cardiovascular effects that are in part dependent on the melanocortin-3 receptor (MC3R) [8]. We controlled for MC3R effects in our studies by testing γ-$MSH_{6–12}$. This peptide lacks the His_5 residue that is essential for agonist activity at the MC3-R [24], but does contain the RFamide receptor-binding motif. In our studies its pressor effects were equivalent to the parent peptide (Fig. 5a, b).

To further address the hypothesis of a non-Y1 receptor-mediated cardiovascular effect of NPY, we used a sterically restricted NPY derivative, Des-$AA_{10–17}$-cyclo-7/21[$Cys_{7,21}$, Pro_{34}] NPY (NPY-CVa). Although NPY-CVa was designed as a NPY Y1 agonist, it lacks Y1-like orexigenic activity [23, 26]. While poor bioavailability has been suggested as an explanation for this lack of a Y1 biological effect [23], the significant pressor activity of NPY-CVa [26] indicates that bioavailability is not the underlying cause. We showed that IV NPY-CVa cardiovascular activity was sensitive to central or peripheral

BIBO3304 administration. While NYP-CVa also shows significant binding to other Y receptors, none of these receptors shows significant binding of BIBO3304 [23].

Evidence for additional, i.e., undefined, Y receptors has been presented [63]. However, these experiments were performed in the presence of antagonists to all known Y receptors to exclude their potential contribution, and included the use of BIBO3304. Thus, the possibility of an additional class of Y receptors that are insensitive to a Y1 antagonist is not germane to our studies. Therefore, we interpret our data as providing additional support for the hypothesis of a BIBO3304-sensitive cardiovascular receptor(s) outside the Y receptor family, activated by both γ-MSH and NPY.

To insure that our general experimental procedures and/or BIBO3304 treatment were not producing a non-specific inhibition of cardiovascular reflexes, we prospectively included an unrelated (to either the γ-MSH or NPY families) peptide that could be administered peripherally, and would have significant centrally mediated cardiovascular effects. Systemic ANG II produces both a direct peripheral vasoconstrictive effect and a centrally mediated increase in sympathetic drive [64]. The latter effects are mediated by ANG II receptors located in forebrain circumventricular organs [64]. Thus, peripheral ANG II appeared to be an ideal control to insure the specificity of our central drug treatment effects. Pressor responses to ANG II were not significantly different at pre and post central BIBO3304 administration. Retrospectively, the lack of a significant difference in the PRE and POST cardiovascular responses of D-Arg_{25} NPY provide further evidence against non-specific cardiovascular inhibition following BIBO3304 treatment.

Non-Y family RFamide receptors bind either or both γ-MSH and NPY [65, 66]. For example, the sensory neuron-specific receptor (SNSR1) is activated by both γ-MSH and γ-$MSH_{6–12}$ in the 40 nM range, and shows nanomolar NPY binding. In addition to the melanocortins and NPY, there are several other RFamide peptides that also regulate feeding behavior, energy homeostasis, and possess cardiovascular activity (e.g., prolactin-releasing peptide and neuropeptide FF [67]. Thus, energy homeostatic RFamide peptides also have "hypertensive" effects. These cardiovascular actions are dose-dependent and produce greater than a 50 mmHg increase in MAP [11, 34, 68].

The cardiovascular similarity between RFamide peptides goes beyond a common centrally mediated increase in arterial pressure. There is a pattern of increased cardiovascular sympathetic drive and an inhibition of cardiac parasympathetic drive [11, 32]. In addition, both γ-MSH and NPY show a distinctive pattern of regional blood flow changes during their cardiovascular responses [11, 69]—preservation of renal blood flow at the expense of skeletal muscle and mesenteric flow. Whether these hypertensive effects are solely a pharmacological or pathological effect, or whether there is an element of physiological regulation, is yet not known.

In summary, we present evidence for a common or related central mechanism of the peripheral NPY and γ-MSH-mediated cardiovascular responses. The cardiovascular responses to γ-MSH are similar to that of NPY and both can be attenuated by application of BIBO3304, a mixed Y1/RFamide receptor antagonist. However, as demonstrated via binding and electrophysiological data, γ-MSH does not affect NPY Y1 receptor signaling. We further demonstrated that a significant component of the NPY pressor response is Y1 receptor independent. NPY and γ-MSH have biological activities that bear striking parallels to each other and to other energy homeostatic RFamide peptides (e.g., FMRFamide, NPFF, and PIF). Common or closely related RFamide receptor(s) may underlie the shared cardiovascular activities of these peptides.

Acknowledgments This research was supported by NIH R01 DK51730 and NIH R01 DK070332 (RDC), NIH RO1 DK62179 (W.F), and Canadian Institutes of Health Research grant MT 10520 (W. F. Colmers). W. F. Colmers is a Medical Scientist of the Alberta Heritage Foundation for Health Research. MJSC was supported by a 75th Anniversary Award from the Faculty of Medicine and Dentistry, University of Alberta. Kenneth A. Gruber was supported by R01 DK51730-S1.

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