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FMRF-AMIDE AND L-ARG-L-PHE INCREASE BLOOD PRESSURE AND HEART RATE IN THE ANAESTHETISED RAT BY CENTRAL STIMULATION OF THE SYMPATHETIC NERVOUS SYSTEM

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Summary. The neuropeptide FMRFamide (L-Phe-L-Met-L-Arg-L-Phe-NH_2) increases mean arterial blood pressure (MABP) and heart rate (HR) in the anaesthetised rat at concentrations ranging from 10-1000 μg/kg. Here, we demonstrate that peptides containing L-arginyl-L-phenylalanine (L-Arg-L-Phe), the C-terminal sequence of FMRFamide, mimic its haemodynamic effects. L-Arg-L-Phe was approximately 4 fold more potent in increasing MABP and HR than FMRFamide. In 40 different peptides investigated, the following order of potency of the effective compounds was established: L-Arg-L-Phe-L-Ala = L-Arg-L-Phe > FMRFamide > L-Met-L-Arg-L-Phe = L-Arg-L-Trp > L-Arg-L-Tyr > D-Arg-L-Phe = L-Arg-L-Phe-OMe > L-Arg-L-Leu = L-Arg-L-Ile > L-Lys-L-Phe > L-Arg-L-Met. L-Arg-L-Phe or FMRFamide did not cause any pressor response in pithed rats, indicating a central mechanism of action. In anaesthetised rats, intravenous injections of FMRFamide or L-Arg-L-Phe (100 μg/kg) were associated with a 2-3 fold increase in plasma noradrenaline levels, whereas plasma adrenaline levels remained unchanged. Thus, L-Arg-L-Phe may represent the active principle of FMRFamide acting by a central mechanism involving the release of noradrenaline from sympathetic nerve terminals.

L-Phe-L-Met-L-Arg-L-Phe-NH_2 (FMRFamide) is a cardioexcitatory molluscan neuropeptide isolated from extracts of the ganglia of the clam *Macrocallista nimbosa* (1). FMRFamide-like immunoreactive material has been found in the central nervous system of a variety of species (2) including the rat, where it is located in nerve cells and terminals (3,4,5). In addition, FMRFamide has an excitatory effect on rat medullary neurones (6) and, when injected intravenously or intracisternally, increases blood pressure and heart rate in anaesthetised rats by an as yet unknown mechanism (7,8).

While studying the effects of various dipeptides on basal and stimulated release of endothelium-derived relaxing factor (EDRF; 9) *in vivo*, we observed that in anaesthetised rats some of these peptides produced a substantial increase in arterial blood pressure and heart rate of 1-2 min duration. Among some 40 congeners tested, L-Arg-L-Phe was the most potent pressor peptide. L-Arg-L-Phe is part of the amino acid sequence of FMRFamide, so we have studied the structure-activity relationships and mechanism of action of FMRFamide-like peptides in more detail.

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MATERIALS AND METHODS

Materials. Sodium thiopental (Inactin ; 5-ethyl-5-(1methyl-propyl)-2-thio-barbituric acid) was obtained from Byk Gulden (Konstanz, F.R.G.). FMRFamide, L-Arg-L-Phe, L-Arg-L-Phe·OMe, L-Met-L-Arg-L-Phe and L-Arg-L-Phe-L-Ala were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). All other petides were obtained from either Bachem or Sigma Chemical Co. (Poole, Dorset, U.K.).

Surgical procedure. Male Wistar rats (200-300g; Glaxo Laboratories Ltd., Greenford, U.K.) were anaesthetised with sodium thiopental (Inactin ; 120 mg/kg i.p.). The trachea was cannulated to facilitate respiration and body temperature was maintained at 37°C by means of a rectal probe connected to a homeothermic blanket (BioScience, Sheerness, U.K.). The carotid artery was cannulated and connected to a Transamerica type 4-4222-0001 pressure transducer for the measurement of phasic (BP) and mean arterial blood pressure (MABP) and heart rate (HR). All haemodynamic parameters were continuously recorded on a grass model 7D polygraph (Grass Instruments, Quincy, USA). The left jugular vein and the left femoral vein were cannulated for infusions of saline and applications of drugs. All animals were allowed a 30 min stabilisation period subsequent to completion of the surgical procedure. All peptides investigated were given as intravenous bolus injections (total volume of 50-100 μl) apart from L-Arg-L-Phe which was also infused.

Measurement of plasma catecholamines. The plasma levels of noradrenaline and adrenaline were measured according to the procedure of Bouloux *et al.* (10). Briefly, the catecholamines were extracted from plasma by adsorption onto alumina followed by desorption into phosphoric acid. Separation of catecholamines was achieved by high-performance liquid chromatography followed by electrochemical detection. The sensitivity of the assay for both catecholamines was 0.4 nmol/l and the intra-assay coefficient of variation was 8% at 1 nmol/l.

Statistical analysis. The data are expressed as mean $\pm$ s.e.mean of *n* observations. Student's unpaired t-test was used to determine the significance of differences between means and a *P*-value of less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Haemodynamic effects. Intravenous bolus injections of L-Arg-L-Phe caused a rapid, transient and dose-dependent rise in both MABP and HR in anaesthetised rats at doses ranging from 10-1000 μg/kg (Fig. 1). Infusions of the dipeptide had no significant haemodynamic effects at 10-100 μg/kg/min, but at 1-20 mg/kg/min produced a substantial and prolonged rise

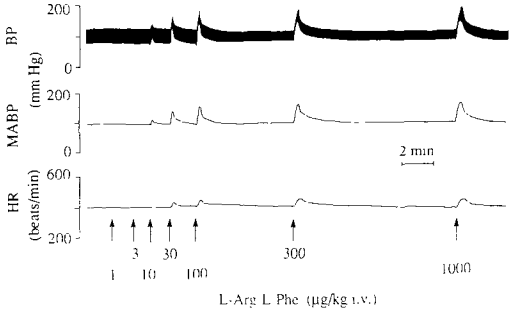


Figure 1. *Bolus injections of L-Arg-L-Phe cause a dose-dependent, transient increases in arterial blood pressure and heart rate in the anaesthetised rat.* Depicted is an original trace from n=6 individual experiments showing the effects of intravenous bolus injections of L-Arg-L-Phe on phasic blood pressure (BP), mean arterial blood pressure (MABP) and heart rate (HR) in the anaesthetised rat.

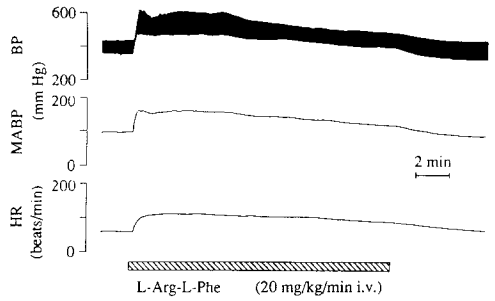


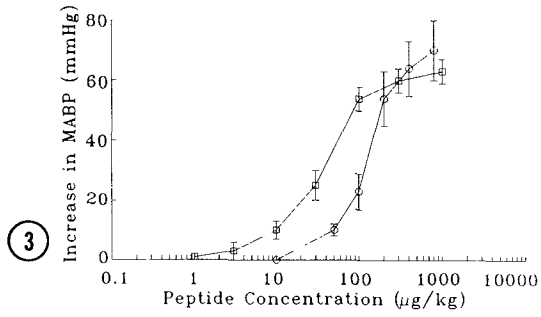
Figure 2. *Infusions of L-Arg-L-Phe cause a sustained rise in blood pressure and heart rate in the anaesthetised rat.* Depicted is an original trace from n=3 individual experiments showing the effects of an infusion of L-Arg-L-Phe (20 mg/kg/min i.v.; hatched bar) on phasic blood pressure (BP), mean arterial blood pressure (MABP) and heart rate (HR) in the anaesthetised rat.

in both MABP and HR over the course of the infusion (Fig. 2). Among some 40 different di-, tri- and tetrapeptides investigated, L-Arg-L-Phe and L-Arg-L-Phe-L-Ala were the most potent pressor peptides (Table 1). They were of similar potency (EC_{50} for the increase in MABP: 30 μg/kg (n=6) for L-Arg-L-Phe and 20 μg/kg (n=5) for L-Arg-L-Phe-L-Ala), 4-6 times more potent than FMRFamide (EC_{50} 120 μg/kg; n=3; Fig. 3), and 6-10 times more potent than the FMRFamide fragment L-Met-L-Arg-L-Phe (EC_{50} 200 μg/kg; n=3; Table 1). This pattern of potency was similar for the effects of these peptides on HR (Fig. 4). Interestingly, both L-Arg-L-Phe and FMRFamide failed to produce any haemodynamic changes in anaesthetised rabbits at concentrations up to 1 mg/kg (n=3).

Structure-activity relationships. Changes in the configuration of L-Arg-L-Phe had profound effects on its biological activity. The D-isomer D-Arg-L-Phe (EC_{50} 400 μg/kg; n=3; Fig. 5) or the methyl ester of L-Arg-L-Phe were less than 10 times as active as the L-isomer free acid (Table 1). An exchange of L-Arg against another basic amino acid, L-His or L-Lys, led either to a complete loss (L-His-L-Phe) or a 30-40 fold reduction in activity (EC_{50} of L-Lys-L-Phe for the increase in MABP was 1000 μg/kg; n=3). Interestingly, the reversed dipeptide, L-Phe-L-Arg, was also inactive.

Table 1. *Structure-activity relationships of L-Arg-L-Phe and related peptides*

Peptide	EC_{50} MABP (μg/kg)	n
L-Arg-L-Phe-L-Ala	20	5
L-Arg-L-Phe	30	6
FMRFamide	120	3
L-Met-L-Arg-L-Phe	200	3
L-Arg-L-Trp	200	3
L-Arg-L-Tyr	300	3
D-Arg-L-Phe	400	3
L-Arg-L-Phe·OMe	400	3
L-Arg-L-Leu	500	4
L-Arg-L-Ile	500	3
L-Lys-L-Phe	1000	3
L-Arg-L-Met	>1000	3



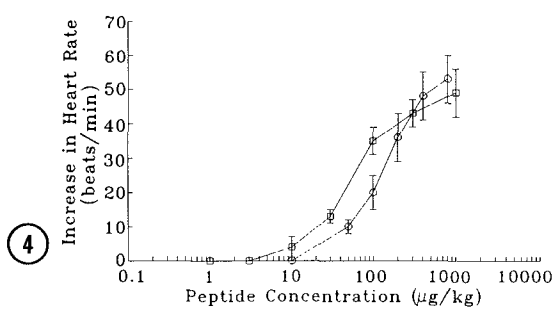
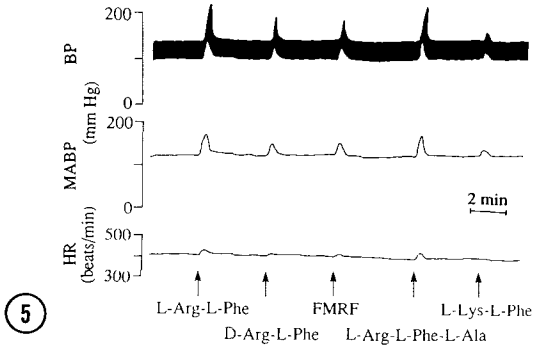


Figure 3. *Comparison of the potency of L- Arg-L-Phe and FMRFamide in increasing arterial blood pressure.* Intravenous bolus injections of L-Arg-L-Phe (open squares; n=6) and FMRFamide (open circles; n=3) produce dose-dependent increases in mean arterial blood pressure (MABP) in anaesthetised rats.

Figure 4. *Comparison of the potency of the positive chronotropic effects of L-Arg-L-Phe and FMRFamide.* Intravenous bolus injections of L-Arg-L-Phe (open squares; n=6) and FMRFamide (open circles; n=6) produce dose-dependent increases in heart rate (HR) in anaesthetised rats.

As mentioned above, extending the sequence by one amino acid at the N- or C-terminal resulted either in a substantial decrease (L-Met-L-Arg-L-Phe) or a slight increase (L-Arg-L-Phe-L-Ala) in biological activity. A modification of the N-terminal, however, does not necessarily lead to a decrease in potency, for other peptides comprising part of the FMRFamide sequence, such as the chicken brain peptide, L-Leu-L-Pro-L-Leu-L-Arg-L-Phe-NH_2 (LPLRFamide; 11), or L-Tyr-Gly-Gly-L-Phe-L-Met-L-Arg-L-Phe-NH_2 (YGGPMRF) are of the same or similar potency as FMRFamide (7,8).

The finding that L-Arg-L-Phe-L-Ala is somewhat more potent than L-Arg-L-Phe was interesting, for the cardioexcitatory activity of FMRFamide in molluscs is not significantly



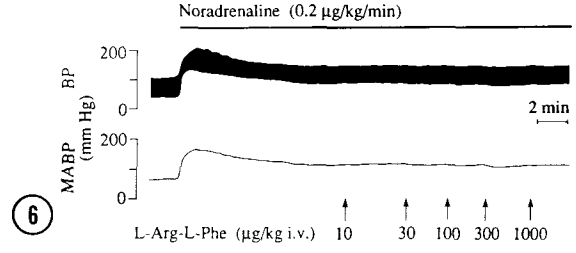


Figure 5. *Structure-activity relationships of various FMRFamide-related peptides.* Depicted is an original trace from n=3 individual experiments showing the changes in phasic blood pressure (BP), mean arterial blood pressure (MABP) and heart rate (HR) in the anaesthetised rat in response to intravenous bolus injections of L-Arg-L-Phe (100 μg/kg), D-Arg-L-Phe (300 μg/kg), FMRFamide (100 μg/kg), L-Arg-L-Phe-L-Ala (100 μg/kg) and L-Lys-L-Phe (1 mg/kg).

Figure 6. *L-Arg-L-Phe is inactive in the pithed rat.* Depicted is an original trace from n=4 individual experiments showing the effects of intravenous bolus injections of L-Arg-L-Phe on phasic blood pressure (BP), mean arterial blood pressure (MABP) and heart rate (HR) in the pithed rat. Noradrenaline (0.2 μg/kg/min) was infused to obtain blood pressure values which were not different from those in anaesthetised rats.

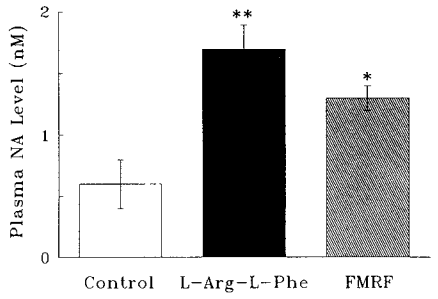


Figure 7. *L-Arg-L-Phe and FMRFamide increase plasma noradrenaline levels in the anaesthetised rat.* Depicted are the changes in plasma noradrenaline (NA) levels in the anaesthetised rat after intravenous bolus injection of L-Arg-L-Phe (100 μg/kg; n=4) and FMRFamide (100 μg/kg; n=4). Blood samples were obtained when the maximum increase in blood pressure in response to either peptide was established; NA was measured by HPLC/electrochemical detection analysis.

affected by removal of the N-terminal L-Phe (*ie* L-Met-L-Arg-L-Phe), but is dramatically reduced by modifying the C-terminal amide (*ie* free carboxyl group instead of the amide; 1). Further support for this notion comes from a previous study showing that there is no significant difference in potency when comparing the pressor responses of L-Arg-L-Phe-OH and L-Arg-L-Phe-NH_2 or FMRF-OH and FMRF-NH_2 (7). Extending the FMRF molecule by one amino acid, Gly, however, substantially reduces its potency, suggesting that L-Arg-L-Phe-L-Ala is a better fit to the "FMRFamide receptor".

Replacing the C-terminal L-Phe by another amino acid always resulted in a decrease in potency or a complete loss of activity. Among the peptides retaining some biological activity, it was interesting to note that those with a hydrophobic amino acid, *ie* L-Arg-L-Trp or L-Arg-L-Tyr, were better substitutes (EC_{50} 200-300 μg/kg; n=3) than those with aliphatic side chains, *ie* L-Arg-L-Leu or L-Arg-L-Ile (EC_{50} = 500 μg/kg; n=3-4). The fact that L-Arg-L-Trp or L-Arg-L-Tyr were biologically active implies that the "FMRFamide receptor" recognises a basic amino acid, L-Arg or L-Lys, attached to a hydrophobic, electron-rich amino acid, L-Phe, L-Trp or L-Tyr, with L-Arg-L-Phe representing the best fit. It is more difficult to explain why apart from L-Arg-L-Leu or the closely related L-Arg-L-Ile all other dipeptides containing an aliphatic amino acid were inactive. Moreover, the reversed dipeptide, L-Leu-L-Arg, was not active, although it is part of the sequence of LPLRFamide which is as potent a pressor agent as FMRFamide (8). L-Arg-L-Met was the weakest among the peptides expressing biological activity (EC_{50} >1000 μg/kg; n=3). However, the fact that it was active together with the activity of L-Arg-L-Leu suggests that the "FMRFamide receptor" recognises not only the C-terminal sequence, but also the dipeptide in the middle. Taken together, the structure-activity relationship data indicate that the putative "FMRFamide receptor" responsible for the haemodynamic effects of this neuropeptide is not specific for FMRFamide, but for L-Arg-L-Phe or a larger peptide with an N-terminal L-Arg-L-Phe sequence.

Mechanism of action. In pithed rats, with (Fig. 6) or without (data not shown) infusion of noradrenaline to raise their blood pressure to control levels, intravenous bolus injections of

L-Arg-L-Phe, L-Arg-L-Phe-L-Ala or FMRFamide over the same dose range as in anaesthetised rats did not produce any significant increases in MABP or HR (n=4), implying an effect on the central nervous system. Standard pharmacological interventions, as described previously (7), failed to reveal the receptor-type responsible for the pressor effects seen with L-Arg-L-Phe or FMRFamide. However, both guanethidine and phentolamine significantly reduce the increases in arterial blood pressure induced by LPLRFamide or FMRFamide (8), suggesting that a release of noradrenaline from sympathetic nerve terminals is the underlying mechanism for the effect of these peptides. Therefore, we have investigated the possible correlation between plasma adrenaline or noradrenaline levels and the pressor responses induced by L-Arg-L-Phe or FMRFamide. Indeed, at 100 μg/kg, intravenous bolus injections of either peptide caused a significant increase in plasma noradrenaline (Fig. 7). In contrast, neither peptide had any effect on plasma adrenaline levels (n=4; data not shown). Moreover, adrenalectomy failed to influence the haemodynamic effects of L-Arg-L-Phe or FMRFamide (n=3).

Our data strongly suggest that the pressor effects of these peptides are exerted by activation of the peripheral sympathetic nerves, rather than by a direct action on the post synaptic receptors; administration of exogenous post-synaptic adrenergic agonists would be expected to reduce heart rate and plasma noradrenaline concentrations by activating the baroreflex, whereas the reverse was observed in our experiments. The lack of a pressor effect in pithed rats suggest that the action of the peptides may involve the cardiovascular centres of the brain, resulting in activation of the peripheral sympathetic nerves and resetting of the baroreflex. Even if these peptides do not cross the blood brain barrier, they can be expected to be accessible to the permeable circumventricular organs in the region of the cardiovascular centres, including the area postrema and the median eminence.

An alternative mechanism of action for these peptides is that they may act peripherally by blocking the pre-synaptic α_2-adrenoceptors that inhibit the release of noradrenaline, resulting in an increase in heart rate and blood pressure. Such mechanism would require intact central cardiovascular centres, as bloackade of pre-synaptic adrenoceptors only leads to noradrenaline release when the noradrenegic neurones are activated, and not under conditions of low noradrenaline release, such as in a pithed animal (12,13). Another peripheral site of action requiring intact brain cardiovascular centres may be interruption of the impulses in the afferent nerves of the baroreflex loop, which can be expected to lead to elevation of heart rate and blood pressure. The detailed mechanism of action of these peptides will be the subjecte of further studies.

We conclude that in the rat, L-Arg-L-Phe or a similar C-terminally modified, small peptide mimics the cardiovascular effects of FMRFamide and, thus, may be a neuropeptide in this species. Moreover, our findings demonstrate that the underlying mechanism for the haemodynamic effects of these two peptides involves the release of noradrenaline from sympathetic nerve terminals via a central mechanism.

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