

Tamás Rószér · Éva Kiss-Tóth · Mihály Petkó ·
A. József Szentmiklósi · Gáspár Bánfalvi

Phe-met-arg-phe (FMRF)-amide is a substrate source of NO synthase in the gastropod nervous system

Received: 12 July 2005 / Accepted: 1 February 2006 / Published online: 13 April 2006
© Springer-Verlag 2006

Abstract The possible involvement of the L-arginine-containing Phe-met-arg-phe (FMRF)-amide (FMRFa) in neuronal nitric oxide (NO) biosynthesis was studied in a gastropod species. We found NADPH-diaphorase-positive neurons and FMRFa-containing fibers in close proximity in the enteric nervous system. Administration of L-arginine and FMRFa induced quantitatively similar nitrite production in both intact intestinal tissues and tissue homogenates. These changes could be prevented by the presence of NOARG (an NO synthase inhibitor). Neither chemically modified FMRFa (D-arginine instead of L-arginine) nor amino acid constituents of FMRFa (methionine, phenylalanine) affected basal nitrite production. FMRFa-induced alterations were reduced in the presence of Na⁺ channel blockers (tetrodotoxin, amiloride, lidocaine), the Na⁺/K⁺ ATPase inhibitor ouabain, or protease inhibitors (leupeptine, pepstatin-a). FMRFa and its amino acid constituents were analyzed by paper chromatography. When FMRFa was added to tissue homogenates, the peptide was eliminated within 1–2 min, whereas methionine, phenylalanine, arginine, and citrulline levels were elevated simultaneously. We tested the effects of FMRFa, L-

arginine, and NOARG on intestinal contractile activity. FMRFa relaxed the intestine for 1–2 min and then induced contractions for 20–40 min. In the presence of NOARG, no relaxant effect of FMRFa was recorded. As administration of L-arginine strongly inhibits the mechanical activity of the intestinal muscle, NO production presumably plays a substantial role in the action of FMRFa, at least in the initial phase. Our biochemical data indicate a direct involvement of FMRFa in NO biosynthesis. FMRFa might be hydrolyzed by extracellular peptidases and then the locally released arginine might be transported into the cells and broken-down to produce NO. Depolarization-induced NO production attributable to the activation of amiloride-sensitive Na⁺ channels might also be involved.

Keywords Neuropeptides · NADPH diaphorase · Nitric oxide synthase · FMRFamide-related peptides · Invertebrates · Gastropods · *Cepaea nemoralis* (Mollusca)

É. Kiss-Tóth · G. Bánfalvi
Department of Animal Anatomy & Physiology,
Faculty of Science, Debrecen University,
P.O. Box 15, H-4010 Debrecen, Hungary

M. Petkó
Institute of Anatomy, Histology and Embryology,
Medical and Health Science Center, Debrecen University,
Debrecen, Hungary

A. J. Szentmiklósi
Department of Pharmacology and Pharmacotherapy,
Medical and Health Science Center, Debrecen University,
Debrecen, Hungary

T. Rószér (✉)
Department of Biochemistry and Molecular Biology,
Medical and Health Science Center, Debrecen University,
P.O. Box 6, H-4012 Debrecen, Hungary
e-mail: roszer@freemail.hu
Tel.: +36-52-3166662336
Fax: +36-52-3166663008

Introduction

The molluscan cardioexcitatory Phe-met-arg-phe (FMRF)-amide-related peptides (FARPs) have been found throughout the animal kingdom. FARPs are oligopeptides containing an RFamide-like (arginine, phenylalanine amide) motif at the C terminus. FMRFamide (FMRFa) was first purified from clam (*Macrocalista nimbosa*) ganglia. Subsequently, FMRFa and FMRFa-related peptides have been isolated from a wide variety of species, ranging from primitive metazoans to mammals (Santana and Benjamin 2000; Nichols et al. 2002; Mercier et al. 2003). These oligopeptides are considered as the first neurohormones in the lower metazoa (Cazzamali and Grimmelhuijzen 2002). In bivalvian and gastropod species, FMRFa-like peptides are known as direct modulators of heart rate and intestinal muscle contractions (Muneoka and Kobayashi 1992).

Actions of FARPs are mediated by seven transmembrane-domain-containing G-protein-coupled receptors, although in snails and mammals, FMRFa peptides can also directly activate amiloride-sensitive sodium channels

(Perry et al. 2001; Cazzamali and Grimmelikhuijzen 2002) by an extracellular binding site. This activation can be inhibited by amiloride or benzamil. FARP-gated sodium channels show low sequence homology to mammalian epithelial acid-sensing ion channels. The activation of amiloride-sensitive sodium channels by FMRFa contributes to hypertension in mammals (Yang and Majane 1990). FMRFa can also inhibit opiate receptors in the vertebrate brain; therefore, it can play a role in pain modulation (Tang et al. 1984).

In a previous paper, we have described FMRFa-containing fibers and NADPH-diaphorase-positive neurons lying in close proximity in the central ganglia of the snail *Helix lucorum* (Röszer et al. 2004a). The application of synthetic FMRFa might enhance the NO synthesis of central neurons. This effect is reduced by amiloride hydrochloride (AH).

The free radical nitric oxide (NO) is a widely distributed signal molecule in the invertebrate nervous system (Huang et al. 1998) and is liberated from nitrergic cells during the oxidation of L-arginine to L-citrulline, which is catalyzed by NO synthase (NOS, EC 1.14.13.39; Mayer and Andrew 1998). The synthesized NO is diffusible, readily enters adjacent target cells, and enhances the production of the second messenger cGMP from guanosine triphosphate within the cytosol (Wood and Garthwaite 1994; Olgart et al. 2000).

The balance between enzymatic NO production and NO liberation must be a crucial step in NO signaling (Olgart et al. 2000). Although a number of regulators of neuronal NO biosynthesis have been identified, FMRFa is the first described extracellular neurosignal leading to enhanced NO liberation in the invertebrate nervous system. The effect of FMRFa on neuronal NO synthesis can be reduced by AH; therefore, amiloride-sensitive sodium channels are candidates as possible target sites of FARPs in the regulation of NOS.

In a previous work, we have described the distribution of NOS within the midintestinal autonomic nervous system of several pulmonate snails (Röszer et al. 2004b). The intrinsic neural network containing NOS innervates the circular muscle layer of the cecum wall and several myenteric neurons. We have proposed that enteric NO liberation plays a significant role in the control of intestinal contractile activity. In the present work, we have examined whether the L-arginine-containing FMRFa is a possible substrate for NOS. The tetrapeptide might be hydrolyzed around or within the NOS-containing nerve cells, and the formed L-arginine might be converted to NO by NOS. To evaluate this possibility, we have investigated the anatomical interactions of NOS- and FMRFa-containing cells and have studied NO-derived nitrite formation in a simple neuronal network of the helcid snail *Cepaea nemoralis*.

Materials and methods

Care of animals

Our research was performed under ethical guidelines established by the 28th Act (1998) of Parliament of the Republic of Hungary.

Snails (*Cepaea nemoralis*) were collected in April or late June and kept under +25°C in wet chambers. Results reported herein were derived from measurements carried out during the active periods of snails in April 2003, April 2004, September–October 2004, and October 2005. Animals were fed with fresh vegetables and anesthetized by halothane or ether prior to dissection.

Colorimetric detection of NO-derived nitrite in neural tissues

Breakdown products of NO, nitrate (NO₃⁻), and nitrite (NO₂⁻) can be easily detected by photometric means. In our assay, nitrate was converted to its nitrite form, and the total nitrite content of test and control solutions was measured by a modified Griess-reaction (Marzinzig et al. 1997; Guevara et al. 1998; Borcherdig et al. 2000). This method was useful as an indirect means of NO detection in the snail nervous system.

Tissue samples Midintestinal segments (cecum with proximal intestine) or central ganglia were removed (under a microscope) and immediately immersed in a control solution of 100 µl 0.1 M phosphate-buffered saline pH 7.4 (PBS). After a 20-min incubation, tissues were immersed in test solutions and incubated again for 20 min (see below).

Control solutions Tissues were immersed in 0.1 M PBS pH 7.4.

Test solutions We tested the effects of L-arginine (Sigma), N-ω-nitro-L-arginine (NOARG, Sigma), FMRFa (Sigma), tetrodotoxin (TTX, Sigma), AH (Sigma), lidocaine (Richter), ouabain (Sigma), pepstatin-A (Sigma), leupeptine (Sigma), D-arginine containing FMRFa (D-FMRFa, Sigma), and anti-FMRFa immunoglobulin (anti-FMRFa IgG; DiaSorin). Chemicals were dissolved in PBS or in 10% dimethylsulfoxide (DMSO) in PBS (AH solutions).

Incubation and measurement Tissues were immersed in control or test solutions for 20 min at +25°C. Samples of control or test media (50 µl) were then incubated with 10 µl 1 U/ml *Aspergillus* nitrate reductase (Sigma) and 10 µl 10 mM β NADPH (Sigma) in MOPS-glycine buffer (pH 7.2) for 60 min at +37°C. Nitrate reductase activity was controlled during the protocol (for details and validation controls, see Röszer et al. 2004b).

Samples and nitrite standards (0.2–50 µM) were added to microplate wells and incubated for 20 min with 100 µl modified Griess reagent (Sigma). Finally, the optical

density was measured against 50 μ l untreated samples with 100 μ l distilled water as blanks at 540 nm in a microplate reader (iEMS Reader Labsystems). We constructed nitrite reference curves and calculated the nitrite contents of samples by extrapolation.

Tissues were weighed after the procedure and the calculated nitrite concentrations were expressed in nanomol per gram per minute. We also determined the changes of nitrite levels as a percentage of control values.

Measurement of nitrite levels in tissue homogenates

After isolation, intestinal tissues were homogenized with 800 μ l PBS plus 100 μ l 10 mM β NADPH and 100 μ l 2 U/ml nitrate reductase. Homogenates were centrifuged (12,500 rpm, 15 min, 4°C), and the supernatant was added in 50- μ l aliquots to microplate wells. Incubation was started by the addition of 50 μ l PBS, L-arginine, or FMRFa to the samples (concentrations were 0, 5, 50, 500 μ M L-arginine or FMRFa). Measurements were carried out as described above.

Statistical tests Results of the bioassay were presented as mean values (\pm SEM). The significance of the results was determined by paired Student's *t*-tests. Values of $P < 0.05$ were considered to be statistically significant.

NADPH diaphorase histochemistry

Midintestinal segments were rinsed in PBS, stretched in paraffin-coated dishes, and fixed with 4% paraformaldehyde diluted in PBS at +4°C for 3 hs. After fixation, samples were washed in 0.1 M TRIS-HCl buffer pH 8.1 with 0.3% Triton X-100 (Tris-TX) for 60 min before histochemistry. Standard NADPH diaphorase (NADPHd) enzyme histochemistry (Huang et al. 1997) was performed by adding 1 mM β NADPH (Sigma) and 0.2 mM nitroblue tetrazolium (Sigma) in Tris-TX to samples, which were then incubated for 45 min at +25°C protected from light. The enzymes of the mitochondrial respiratory chain and the "nothing dehydrogenase" effect could be excluded as being responsible for NADPHd staining (for details of controls of the method, see Röszer et al. 2004b).

FMRFa immunocytochemistry

The FMRFa immunolabeling procedure was carried out on the NADPHd-stained samples. PBS-TX buffer (PBS containing 0.3% Triton X-100 and 0.1% sodium azide) was used during all the steps described.

To prevent non-specific background staining, samples were incubated overnight with 3% normal horse serum (Vector). Preparations were then incubated with a polyclonal rabbit antiserum directed against synthetic FMRFa tetrapeptide (DiaSorin) for 24 h at room temperature at a dilution of 1:500. After incubation with the primary

antibody, samples were incubated with a biotinylated pan-specific horse IgG (Vector), at a dilution of 1:40 for 2 h and then with fluorescein-isothiocyanate-conjugated avidin (Sigma), at a dilution of 1:1,000 for 1 h at room temperature. Between the incubation steps, tissues were washed in PBS-TX. To control the specificity of the immunolabeling, primary antiserum was omitted or replaced with 3% normal horse serum. Both treatments completely abolished specific immunostaining. As a positive control, the immunocytochemical procedure was carried out on specimens derived from *Caenorhabditis elegans* N2 strains (gift of Theresa Stiernagle, Caenorhabditis Genetics Center, University of Minnesota). Samples were mounted in glycerol:PBS (1:1) medium and examined without dehydration.

Measurement of intestinal muscle motility

Isolated longitudinal cecum muscles of snails were immersed in organ chambers (TSZ-04, Experimetria) filled with 10 ml Krebs solution. The composition of the nutrient solution was: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1 mM NaH₂PO₄, 1.2 mM MgCl₂, 24.9 mM NaHCO₃, 0.004 mM EDTA, 0.11 mM ascorbic acid, 11.5 mM glucose, pH 7.5).

The mechanical activity of muscles was recorded for 10 min in control (normal Krebs solution) and test solutions (Krebs solution with L-arginine, NOARG, or FMRFa) by an isometric mechano-electric transducer system (SG-01D, Experimetria). The frequency and force of contractions was determined.

Detection of amino acids and FMRFa by paper chromatography

The amino acids (L-arginine, L-citrulline, L-methionine, DL-phenylalanine) and FMRFa of control solutions and tissue homogenates were separated by vertical paper chromatography (Whatman no. 4 and no. 1 filter paper). All experiments were carried out in a chromatography laboratory with controlled air flow, humidity (30 \pm 5% relative moisture), and temperature (+22 \pm 2°C). We used the following eluents: *n*-butanol/ethanol/acetic acid/water (6/1.5/0.5/2 v/v/v; eluent A), methanol/water/pyridine (8/1/1 v/v/v; eluent B), *n*-propanol/formic acid/water (8/1/1 v/v/v; eluent C), acetone/urea/water (6/0.5/4 v/w/v; eluent D). Chromatograms were dried in a hot air flow, sprayed with ninhydrin dissolved in acetone, and air-dried at +80°C.

Controls Amino acids and FMRFa were dissolved in water (concentration range: 0.5–1000 μ M).

Samples Isolated midintestinal tissues were homogenized in 500 μ l distilled water and centrifuged at 5000 rpm. The supernatants were collected, and 200- μ l volumes of homogenates were added to Eppendorf tubes, together

with 200 μl distilled water or 2 mM FMRFa dissolved in distilled water. The tubes were incubated at $+22^\circ\text{C}$. Samples were obtained and applied to the chromatography paper by capillary glass tubes and air-dried at 0 s, 30 s, 60 s, 5 min, 10 min, and 15 min after the beginning of incubation.

For qualitative analysis of amino acids and FMRFa, we determined the R_f values of the controls and each detected sample spots. The developed chromatograms were photographed by a digital camera, and the luminosity of spots was determined by Adobe Photoshop software. For quantitative analysis, we created reference curves and calculated the amino acid/FMRFa content of samples by interpolation. All chromatographic analyses were carried out three times by two independent operators.

Results

Interaction of FMRFa-containing and NADPHd-reactive nerve cells in the intestinal nervous system

We found 35–50 FMRFa-immunolabeled unipolar neurons in the stomach wall and 25–40 cells in the myenteric network of the midintestine (Fig. 1). Labeled nerve fibers were richly ramified in the myenteric, submucosal, and mucosal layers of the whole alimentary tract, with the highest density at the gastric-midintestinal junction. Nerve branches of the intestinal nerve originating from the visceral ganglion and fibers of the anterior and posterior gastric nerves originating from the buccal ganglia also contained FMRFa. NADPHd activity was found in the salivary nerves originating from the buccal ganglia. Like our previous results, peripheral NADPHd reactivity was confined to unipolar neurons of the midintestinal myenteric plexus. In the cecum wall, most of the FMRFa-containing neurons were in close proximity to NADPHd-reactive cell bodies (Fig. 1). FMRFa-immunoreactive terminals were seen around several NADPHd-stained perikarya and initial axon segments (Fig. 1). Both NADPHd-stained and FMRFa-immunolabeled terminals were found in the midintestinal muscle layers. FMRFa-containing fibers formed varicose terminals in the muscle and mucosal layers, whereas NADPHd-reactive fibers ran parallel to muscle cells without arborization or varicosities.

Nitrite production of isolated midintestine

Effects of L-arginine and NOARG

Gut segments of *C. nemoralis* were incubated with PBS for 20 min. During this incubation period, the basal rate of tissue nitrite production was 0.95 ± 0.3 nmol/g per minute ($n=5$). Administration of 1, 10, and 20 mM L-arginine evoked a dose-dependent enhancement of nitrite production to 3.00 ± 0.9 ($n=4$), 3.70 ± 0.2 ($n=4$), and 14.35 ± 6.4 ($n=4$) nmol/g per minute, respectively. In the presence of 1

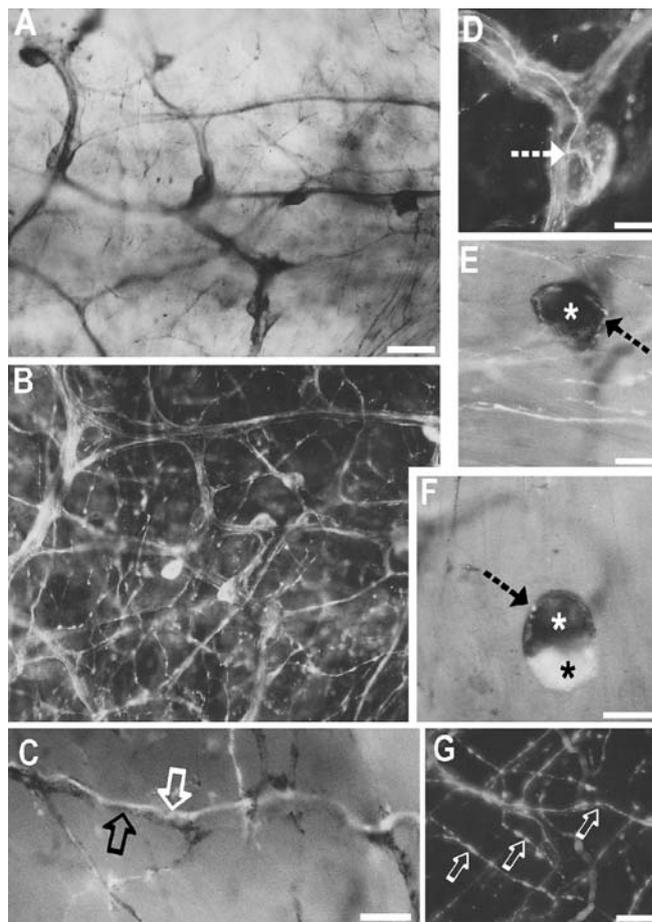


Fig. 1 Localization of NADPHd-reactive and FMRFa-containing elements in the midintestinal enteric network. **a,b** Structure of NADPHd-positive and FMRFa-containing myenteric network in the midintestine, respectively. Bar 75 μm . **c** FMRFa-containing (white arrow) and NADPHd-reactive (black arrow) fibers in the wall of stomach. Bar 34 μm . **d** FMRFa-containing fibers (arrow) around a myenteric nerve cell. Bar 30 μm . **e** NADPHd-stained neuron (asterisk) with FMRFa-immunoreactive fibers surrounding the cell surface (arrow). Bar 30 μm . **f** FMRFa-immunolabeled (black asterisk) and an adjacent NADPHd-stained soma (white asterisk) in the cecum (arrow FMRFa-containing terminal). Bar 45 μm . **g** FMRFa-containing varicosities within the intestinal muscle layer. Bar 15 μm

or 2 mM NOARG, endogenous nitrite formation was reduced to 0.56 ± 0.2 ($n=4$) and 0.45 ± 0.2 ($n=4$) nmol/g per minute, respectively.

Effects of FMRFa, amino acids, antiFMRFa IgG, and D-FMRFa

To test the effects of FMRFa, freshly isolated midintestinal segments were incubated with PBS for 20 min. The basal rate of nitrite production was 0.45 ± 0.05 nmol/g per minute ($n=5$). Application of 0.8 mM FMRFa elevated the nitrite formation to 0.95 ± 0.2 nmol/g per minute ($n=4$). Incubation with 1.6 mM FMRFa caused a further elevation of nitrite release to 3.2 ± 0.3 nmol/g per minute ($n=4$). FMRFa elicited an increase of endogenous nitrite formation by

110% (0.8 mM FMRFa) and 610% (1.6 mM FMRFa; Fig. 2a). In the presence of 1 mM NOARG, FMRFa was not able to increase nitrite production (Fig. 2a). In tissue homogenates ($n=3$), both L-arginine and FMRFa evoked a dose-dependent increase in nitrite production (Fig. 2b). In contrast, D-FMRFa (1.6 mM) failed to influence nitrite liberation (Fig. 2a).

Similarly, incubation of tissues with 1.6 mM phenylalanine or 1.6 mM methionine did not alter nitrite formation ($n=4$).

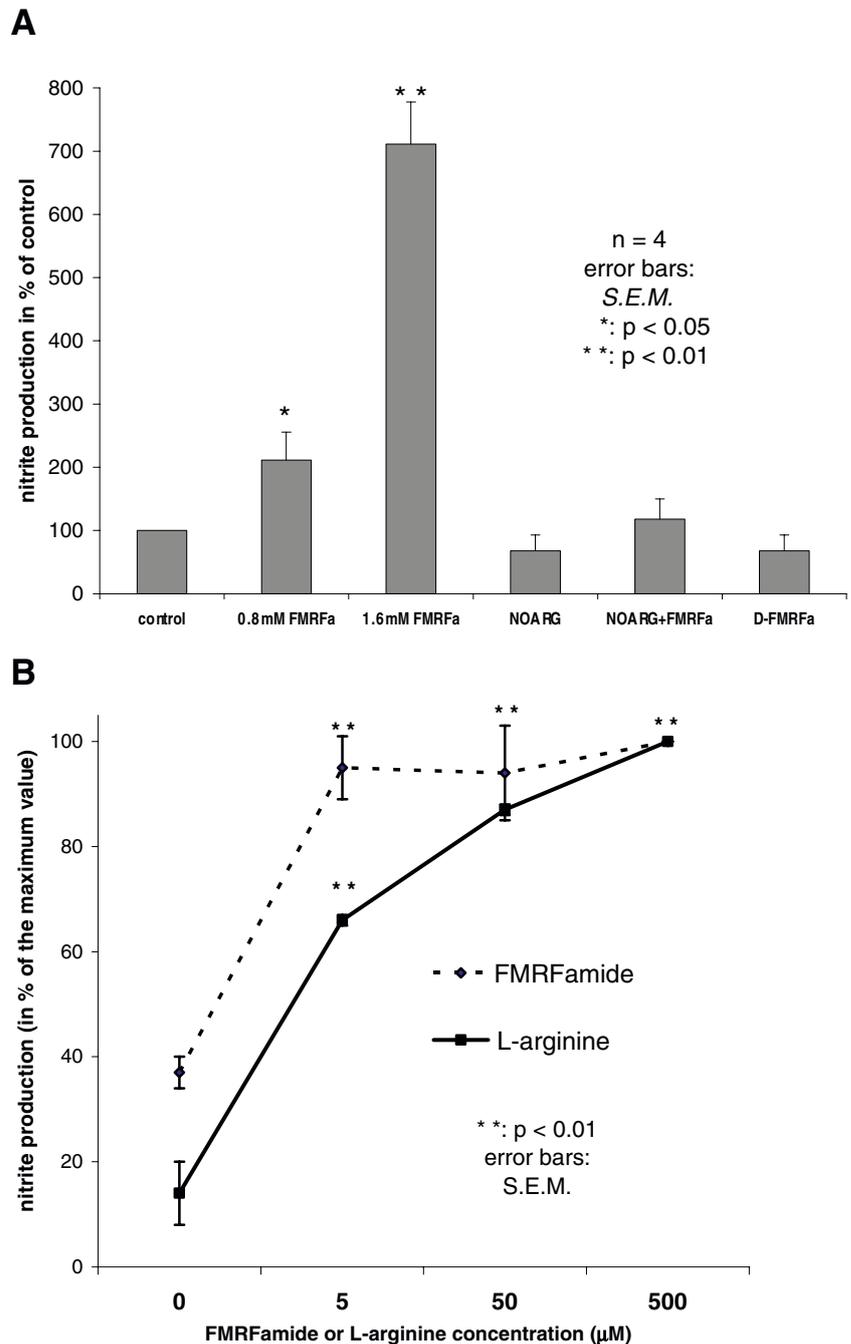
Anti-FMRFa IgG reduced nitrite production both in ganglia and in intestinal tissues (Fig. 3a). The nitrite levels decreased to $46.5\pm 19.6\%$ (IgG dilution of 1:5,000) and

$23.2\pm 12.8\%$ (IgG dilution of 1:1,000) in the midintestine, respectively. In the ganglia, IgG reduced the nitrite formation to $57.4\pm 6.6\%$ (1:5,000) and $56.5\pm 7.5\%$ (1:1,000), respectively ($n=4$).

Effects of protease inhibitors and sodium channel blockers

Incubation with 2 mM leupeptine or 1.4 mM pepstatin-A resulted in a reduced nitrite production by $19.4\pm 10.5\%$ and $52.1\pm 10.1\%$, respectively ($n=4$).

Fig. 2 Effect of FMRFa on intact neurons in the midintestine (a) and on midintestinal tissue homogenates (b). **a** Intact tissue samples were incubated with PBS for 20 min. Nitrite production was then measured and taken as the control value. Effects of treatments were compared with the basal (control) nitrite levels. All experiments were carried out on samples derived from four individuals (NOARG 2 mM NOARG, NOARG+FMRFa 2 mM NOARG and 1.6 mM FMRFa, D-FMRFa 1.6 mM D-FMRFa). **b** L-arginine or FMRFa were added to tissue homogenates, and nitrite formation was followed



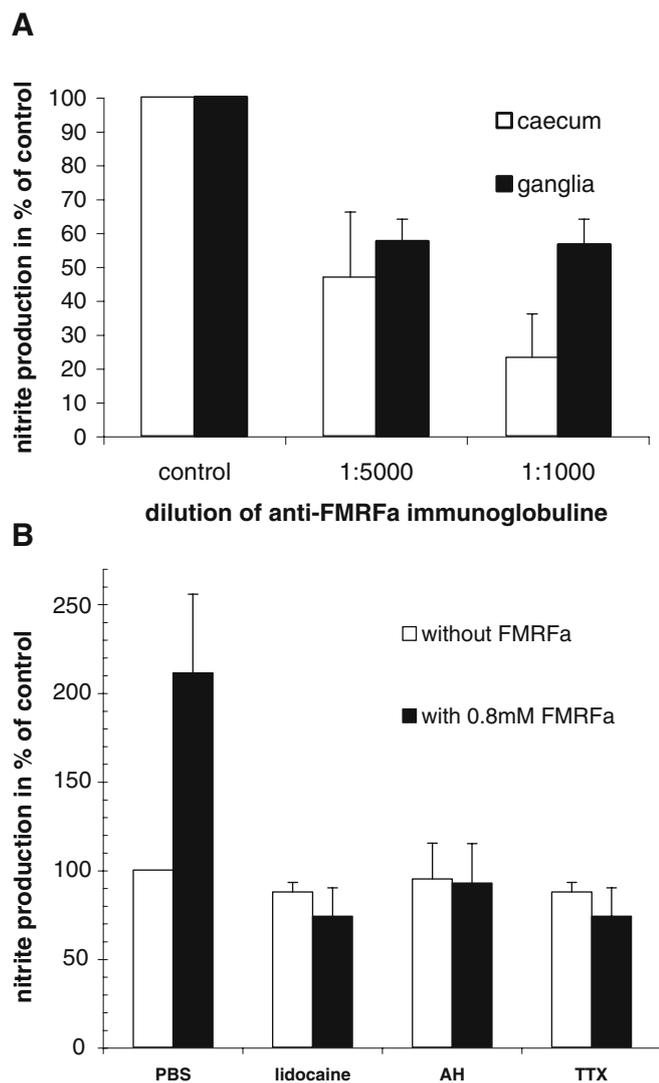


Fig. 3 Effects of anti-FMRFa IgG (a) and sodium channel blockers (b). a Isolated ganglia (black columns) or midintestinal samples (clear columns) were incubated with PBS and then with anti-FMRFa IgG molecules for 20 min. Nitrite levels measured in PBS were taken as control values. b Intestinal samples were incubated with PBS for 20 min and the nitrite levels were measured (controls). Samples were then incubated with sodium channel blockers diluted in PBS (clear columns) or with a mixture of FMRFa and sodium channel blockers (black columns). All experiments were repeated three times (TTX 0.3 μ M TTX, AH 2 mM AH, lidocaine 0.2 mM lidocaine)

Isolated gut segments were treated with sodium channel inhibitors (AH, lidocaine, and TTX). The tissues were

incubated with PBS and then with 2 mM AH (the stock solution of AH contained 10% DMSO as a stabilizer), 0.2 mM lidocaine, or 0.3 μ M TTX. These experiments were also carried out in the presence of 0.8 mM FMRFa (Fig. 3b). AH reduced the nitrite levels to $95 \pm 2\%$, lidocaine to $87.6 \pm 5.4\%$, TTX to $87.6 \pm 5.4\%$ ($n=4$). FMRFa elicited a $211 \pm 44.4\%$ increase in nitrite production, but no enhancement was detected in the presence of FMRFa plus sodium channel inhibitors ($n=4$).

PBS-activated gut segments were incubated with 0.25 or 0.5 mM ouabain alone or with a mixture of 0.5 mM ouabain and 0.8 mM FMRFa. Ouabain (0.25 and 0.5 mM) reduced the nitrite production to $50.7 \pm 8.8\%$ and $26.1 \pm 6.81\%$, respectively ($n=4$). In the presence of FMRFa, ouabain induced only a $2.3 \pm 3.8\%$ reduction in nitrite production ($n=4$).

Qualitative and quantitative analysis of amino acids and FMRFa in midintestinal tissues

We used four different eluents to separate FMRFa and its amino acids from tissue homogenates. The determined R_f values are listed in Table 1.

The most effective separation was carried out by the use of eluent A (for R_f values, see Table 1). Arginine and citrulline were clearly differentiated by the use of eluent B (Table 1). The limit of detection was 0.25 μ M for arginine and phenylalanine, 0.5 μ M for methionine, 5 μ M for citrulline, and 50 μ M for FMRFa. The concentration and luminosity of the spots was linear between 0.5 μ M and 1 mM for arginine and phenylalanine, between 5 μ M and 500 μ M for citrulline, between 50 μ M and 500 μ M for methionine, and between 500 μ M and 1 mM for FMRFa.

We found that midintestinal tissues contained detectable arginine, citrulline, methionine, and phenylalanine. The average concentration values were 5 ± 2 μ M for L-methionine, 8 ± 2 μ M for L-citrulline, 10 ± 4 μ M for L-arginine, and 10 ± 3 μ M for DL-phenylalanine. The methionine and phenylalanine concentrations were constant during the whole incubation period, whereas the arginine and citrulline concentrations of tissue homogenates were elevated by 2–3 μ M/min (Fig. 4a).

Following the addition of FMRFa to the tissue homogenates, the concentration of FMRFa was reduced by 10.5 ± 1 μ M/min, whereas methionine, phenylalanine, and arginine concentrations were simultaneously elevated by 8 ± 3

Table 1 R_f values of amino acids and FMRFa for vertical chromatography, at 22°C and 30% relative air humidity (p/f/w 8/1/1 n-propanol/formic acid/water, m/w/p 8/1/1 methanol/water/pyridine, b/e/a/w 6/1.5/0.5/2 n-butanol/ethanol/acetic acid/water, a/u/w 6/0.05/4 acetone/urea/water)

Eluents	L-citrulline	L-arginine	L-methionine	DL-phenylalanine	FMRFa
C (p/f/w)	0.62	0.64	0.67	0.78	1
B (m/w/p)	0.90	0.50	1	0.97	1
A (b/e/a/w)	0.19	0.21	0.52	0.62	0.84
D (a/u/w)	0.89	0.52	0.91	0.91	0.91

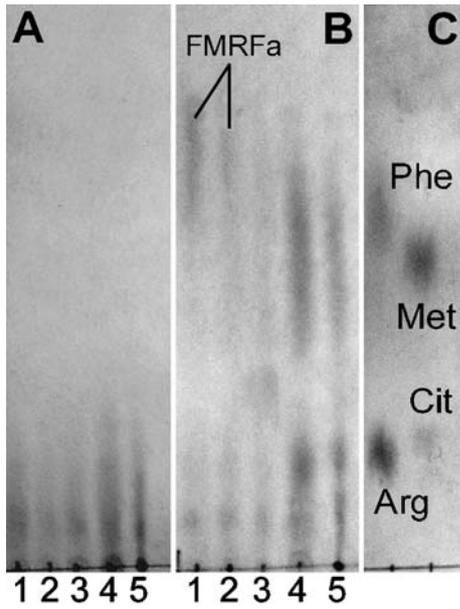


Fig. 4 Vertical paper chromatograms of tissue homogenates. **a** Untreated tissue homogenates. Midintestinal tissues were homogenized and incubated at 22°C. Samples were withdrawn at 0 s, 30 s, 1 min, 5 min, and 15 min and analyzed by chromatography with the ethanol/butanol/acetic acid water eluent (1 0 s, 2 30 s, 3 1 min, 4 5 min, 5 15 min). **b** Tissue homogenates were treated with 1 mM FMRFa, and the incubation and chromatographic separation was repeated (1 0 s, 2 30 s, 3 1 min, 4 5 min, 5 15 min). **c** Chromatogram of DL-phenylalanine (*Phe*), DL-methionine (*Met*), L-arginine (*Arg*), and DL-citrulline (*Cit*). Eluent A; ninhydrin staining

µM/min. The concentration of citrulline was elevated by 2–3 µM/min (Figs. 4b, 5).

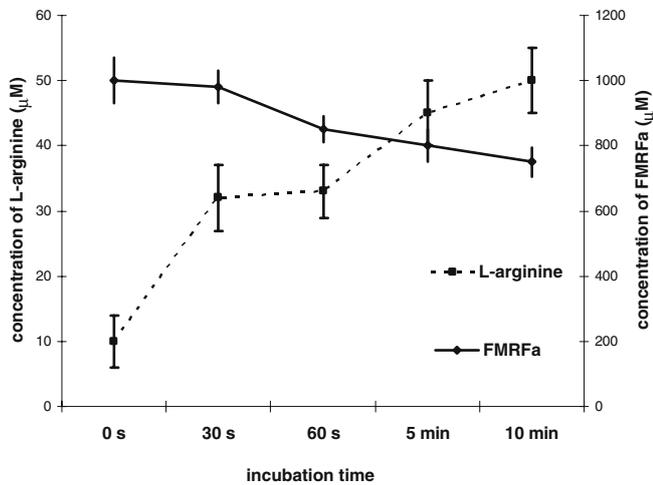


Fig. 5 Quantitative analysis of L-arginine and FMRFa content of midintestinal tissue homogenates following 1 mM FMRFa administration. The concentration of FMRFa was reduced by 10.5±1 µM/min, whereas methionine, phenylalanine, and arginine concentrations were simultaneously elevated by 8±3 µM/min

Effects of L-arginine, NOARG, and FMRFa on midintestinal motility

Isolated cecum segments showed a periodic contractile activity. The mechanical activity of the muscles consisted of an active and a resting period with slight contractile activity. In the active period (4–5 min), contraction waves were recorded with a 0.15±0.5 mN amplitude and 2.6/min frequency (Fig. 6a). The resting phase also lasted 4–5 min, during which only weak contractions could be observed with a 0.5±0.2 mN amplitude and 2.6/min frequency.

Administration of 5–50 µM D-FMRFa did not alter the frequency, amplitude, or periodicity of contractions (Fig. 6b). Following application of 5–50 µM L-arginine to the organ bath, the muscles relaxed within 2 min (Fig. 6c). In the presence of NOARG (1 mM), however, no resting phase could be observed, only continuous prominent contractions (Fig. 6d). FMRFa (5 µM) elicited a transient decrease in the force of contractions for about 2–3 min followed by prominent long-lasting (20–30 min) contractions characterized by a 0.2 mN force and 2–3.9/min frequency. Control mechanical activity of muscles was recovered within 40–60 min. When NOARG was added prior to FMRFa administration, contractility was not affected by FMRFa (Fig. 6d–g).

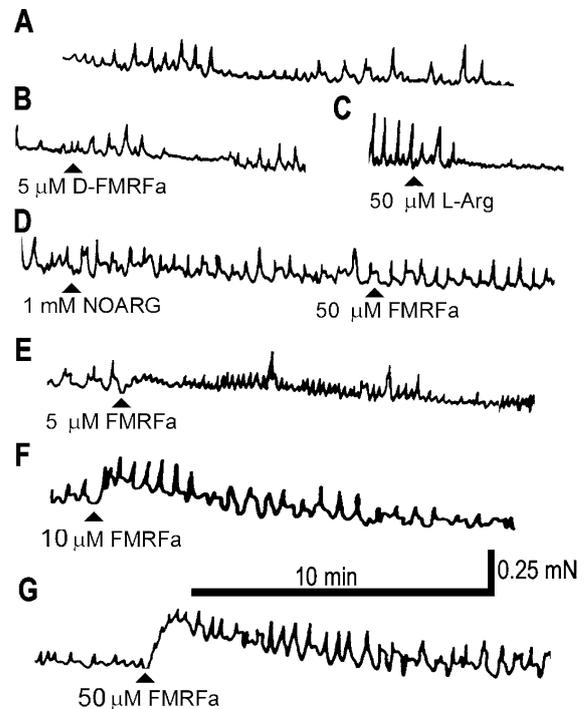


Fig. 6 Effects of L-arginine, FMRFa, D-FMRFa, and NOARG on midintestinal contractile activity. **a** Normal contractile activity, recorded in Krebs solution at 25°C. **b** Myogram recorded following D-FMRFa administration. **c** Effect of L-arginine. **d** Effects of NOARG and FMRFa. **e–g** Effects of various concentrations of FMRFa

Discussion

We have revealed that FMRFa-immunolabeled fibers and NADPHd-positive neurons are in close proximity in the midintestinal enteric nervous system of *C. nemoralis*. This finding provides a morphological confirmation of the interaction between NO-liberating and FMRFa-containing neurons in helioid snails.

The NOS substrate L-arginine elevates the nitrite production of enteric tissues, whereas the NOS inhibitor NOARG (Mayer and Andrew 1998) reduces it. Administration of FMRFa causes a significant enhancement of nitrite formation, and this effect is inhibited by NOARG. When FMRFa is removed from the extracellular space by FMRFa-reactive IgGs, nitrite levels are reduced in the enteric networks and in the central ganglia, whereas the D-arginine-containing FMRFa analog fails to influence nitrite production. Methionine and phenylalanine (amino acids of FMRFa) do not alter nitrite levels. These results indicate that the endogenous substrate L-arginine and the peptide FMRFa elevate NO synthesis in the enteric network. Since D-arginine, L-methionine, and L-phenylalanine do not alter NO production, the L-arginine content of the peptide must be essential for the elevation of NO synthesis. The binding ability of FARPs to sodium channels is associated with their arginine content, with the positively charged arginine residues presumably interacting with the histidine residues of the channel proteins (Ostrovskaya et al. 2004).

To analyze the role of sodium channels in FMRFa-mediated signaling, various sodium channel inhibitors were applied to snail preparations. AH, lidocaine, and TTX all blocked the influence of FMRFa on NO synthesis. AH has been described to affect sodium channels gated by FMRFa (Cottrell 1997; Perry et al. 2001). FMRFa and amiloride-sensitive FMRFa receptor sodium channels have previously been found to play a substantial physiological role in the central ganglia of the snail *Helix lucorum* (Röszer et al. 2004a). FMRFa evokes NO liberation from NOS-containing neurons in *Helix* ganglia, but this effect can be diminished by amiloride (Röszer et al. 2004b). FMRFa receptor sodium channels, however, are not sensitive to TTX or lidocaine. The marine toxin TTX and lidocaine block sodium channels by binding to channel receptors near the extracellular surface and inhibit the propagation of electrical impulses in excitable membranes (Miller and Hondeghem 1995). Our experiments indicate that the general blockade of voltage-gated sodium channels (by TTX or lidocaine) diminishes the effect of FMRFa but cannot reduce basal NOS activity. The inhibitory action of amiloride on the FMRFa-induced NO production suggests that FMRFa opens amiloride-sensitive sodium channels, which can lead to the depolarization and consequently to the activation of voltage-gated sodium channels and to the generation of action potentials. Depolarization also activates L-type voltage-sensitive calcium channels and subsequently increases the intracellular calcium (Johnson et al. 1992). NO is known to be produced in neurons in a calcium-dependent manner by NO synthases (Bredt and Snyder 1994). Our present findings are consistent with our previous

findings on the action of FMRFa (Röszer et al. 2004b) and raise the possibility that the depolarization-induced NO generation attributable to the interaction of FMRFa and amiloride-sensitive sodium channels might be an additional mechanism in FMRFa-induced NO production.

On the other hand, both L-arginine and FMRFa administration induce significant nitrite production in homogenized tissues. This finding suggests that FMRFa can regulate NOS activity in a pure cell extract and does not require ongoing neural activity or intact neuronal cell membranes. Thus, the target of FMRFa might be the NOS molecule itself.

We have used analytical techniques to follow the metabolism of FMRFa in tissue homogenates. After the addition of FMRFa to tissue homogenates, the peptide is eliminated within 1–2 min, whereas methionine, phenylalanine, and arginine levels are elevated simultaneously. Under control conditions (tissues without FMRFa), the levels of methionine and phenylalanine remain almost unchanged, whereas arginine and citrulline levels continuously increase. The continuous elevation of the citrulline concentration can be related to the catalytic activity of NOS. These findings suggest that FMRFa is hydrolyzed to amino acids and metabolized by the midintestinal tissues.

In intact tissues, the effect of FMRFa is diminished by the Na^+/K^+ ATPase blocker ouabain. Ouabain not only inhibits FMRFa-provoked NO generation, but also effectively reduces basal NO synthesis in a dose-dependent manner.

Since Na^+/K^+ ATPases are required for carrier-mediated transport of many molecules, including L-amino acids (Wiseman 1974), L-arginine transport is presumably impaired by ouabain. Arginine transporters exist in cell membranes of enterocytes and of NOS-containing endothelial and neural cells (Meng et al. 2005; Rasmusen et al. 2005). In some NOS-containing cells, the intracellular concentration of L-arginine can be well above the K_m of NOS. Several mechanisms have been proposed to explain this arginine paradox: the co-localization of arginine transporters and NOS, the intracellular regeneration of arginine from citrulline, a balance between arginase and NOS activity, and the regulation of transport-system mRNA levels by NO concentration (Schmidlin et al. 2000; Rasmusen et al. 2005).

Our findings support the hypothesis that the L-arginine content of FMRFa can be used by NOS to produce NO. The FMRFa must be hydrolyzed to its constituent amino acids before being coupling to NO biosynthesis. In this case, the inhibition of cellular or extracellular peptidases must lead to reduced NO generation. In our study, peptidase inhibitors have been shown significantly to reduce the NO production of enteric tissues. The tested protease inhibitors diminish the FMRFa effect, possibly via the inhibition of extracellular proteases. Leupeptin is a more potent inhibitor of NO liberation than pepstatin-A. Leupeptin (acetyl-leucine-leucine-arginal) more easily penetrates into tissues than the larger pepstatin-A (isovaleryl-valine-valine-statine-alanine-statine-4-amino-3-hydroxy-6-methylheptone acid); this might explain the difference in their actions. FMRFa, like other

neuropeptides, might be inactivated by extracellular peptidases, and the locally released arginine might be transported into the cells via a ouabain-sensitive mechanism and broken-down for NO synthesis.

We have also studied the actions of FMRFa, arginine, and NOARG on midintestinal contractile activity. FMRFa exerts a biphasic action on the regulation of intestinal motility with an initial short-lasting inhibition followed by a long-term increase of contractile activity. D-FMRFa is ineffective, and the inhibition of NOS by NOARG prevents the contractile actions of FMRFa. As administration of L-arginine strongly inhibits the mechanical activity of the intestinal muscle, we suggested that, at least in the initial phase, NO production has a substantial role in the action of FMRFa.

In conclusion, we have revealed a new mechanism of the action for FMRFa-induced physiological alterations in the midintestinal nervous system of a gastropod species, *C. nemoralis*. On the basis of our findings, we propose a new mechanism of FMRFa action on NOS activity. The peptide is metabolized by nerve cells and is directly involved in NO biosynthesis. The extracellular proteases possibly digest the oligopeptide, and its L-arginine content can be utilized by NOS. FMRFa and the cognate peptides might therefore serve as alternative substrate sources of NOS within nerve cells. In addition, the oligopeptide may act on sodium channel receptors. Thus, the depolarization-induced NO generation attributable to the interaction of FMRFa and amiloride-sensitive sodium channels might be an additional mechanism in FMRFa-induced NO production. Furthermore, we provide novel morphological, biochemical, and physiological data for a better understanding of the signaling mechanism and the regulatory functions of FMRFa in invertebrate neuronal networks.

Acknowledgements The skillful help of Mr. D. Rózsa and Mr. I. Schmelczér is gratefully acknowledged. We thank our colleagues for their amiable support of our daily travail.

References

- Borcharding H, Leikefeld S, Frey C, Diekmann S, Steinrucke P (2000) Enzymatic microtiter plate-based nitrite detection in environmental and medical analysis. *Anal Biochem* 282:1–9
- Bredt DS, Snyder SH (1994) Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem* 63:75–195
- Cazzamali G, Grimmelikhuijzen CJP (2002) Molecular cloning and functional expression of the first insect FMRFamide receptor. *Proc Natl Acad Sci USA* 99:12073–12078
- Cottrell GA (1997) The first peptide gated ion channel. *Exp Biol* 18:2377–2386
- Guevara I, Iwanejko J, Dembinska-Kiec A, et al (1998) Determination of nitrite/nitrate in human biological material by the simple Griess reaction. *Clin Chim Acta* 274:177–188
- Huang S, Kerschbaum HH, Engel E, Hermann A (1997) Biochemical characterization and histochemical localization of nitric oxide synthase in the nervous system of the snail, *Helix pomatia*. *J Neurochem* 69:2516–2528
- Huang S, Kerschbaum HH, Hermann A (1998) Nitric oxide-mediated cGMP synthesis in *Helix* neural ganglia. *Brain Res* 780:329–336
- Johnson EM Jr, Koike T, Franklin J (1992) A “calcium set-point hypothesis” of neuronal dependence on neurotrophic factor. *Exp Neurol* 115:163–166
- Marzinzig M, Nussler AK, Stadler J, et al (1997) Improved methods to measure end products of nitric oxide in biological fluids: nitrite, nitrate, S-nitrosothiols. *Nitric Oxide* 1:177–189
- Mayer B, Andrew P (1998) Nitric oxide synthase: catalytic function and progress towards selective inhibition. *Nauny-Schmiedeberg Arch Pharmacol* 358:127–133
- Meng O, Choudry HA, Souba WW, Karinch AM, Huang J, Lin C, Vary TC, Pan M (2005) Regulation of amino acid arginine transport by lipopolysaccharide and nitric oxide in intestinal epithelial IEC-6 cells. *J Gastrointest Surg* 9:1276–1285
- Mercier AJ, Friedrich R, Boldt M (2003) Physiological functions of FMRFamide-like peptides (FLPs) in crustaceans. *Microsc Res Tech* 60:313–324
- Miller RD, Hondeghem LM (1995) Local anesthetics. In: Katzung BG (ed) *Basic and clinical pharmacology*. Appleton and Lange, Norwalk, pp 399–400
- Muneoka Y, Kobayashi M (1992) Comparative aspects of structure and action of molluscan neuropeptides. *Experientia* 48:448–456
- Nichols R, Bendena WG, Tobe SS (2002) Myotropic peptides in *Drosophila melanogaster* and the genes that encode them. *J Neurogenet* 16:1–28
- Olgart C, Gustafson LE, Wiklund NP (2000) Evidence for nonvesicular nitric oxide release evoked by nerve activation. *Eur J Neurosci* 12:1303–1309
- Ostrovskaya O, Moroz L, Krishtal O (2004) Modulatory action of RFamide-related peptides on acid-sensing ionic channels is pH dependent: the role of arginine. *J Neurochem* 91:252–255
- Perry SJ, Straub VA, Schofield MG, Burke JF, Benjamin PR (2001) Neuronal expression of an FMRFamide-gated Na⁺ channel and its modulation by acid pH. *J Neurosci* 21:5559–5567
- Rasmussen C, Cynober L, Couderc R (2005) Arginine and statins: relationship between the nitric oxide pathway and the atherosclerosis development. *Ann Biol Clin* 63:443–455
- Röszer T, Jenei Zs, Gáll T, et al (2004a) A possible stimulatory effect of FMRFamide on neuronal nitric oxide production in the central nervous system of *Helix lucorum* L. *Brain Behav Evol* 63:23–33
- Röszer T, Czimmerer Zs, Szentmiklósi AJ, Bánfalvi G (2004b) Nitric oxide synthesis is blocked in the enteral nervous system during dormant periods of the snail, *Helix lucorum*. *Cell Tissue Res* 316:255–262
- Santana N, Benjamin PR (2000) Gene expression and function of FMRFamide-related neuropeptides in the snail *Lymnaea*. *Microsc Res Tech* 49:547–556
- Schmidlin A, Fischer S, Wiesinger H (2000) Transport of L-citrulline in neural cell cultures. *Dev Neurosci* 22:393–398
- Tang J, Yang H-YT, Costa E (1984) Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe-Met-Arg-Phe-NH₂ like immunoreactivity. *Proc Natl Acad Sci USA* 81:5002–5005
- Yang HY, Majane EA (1990) Mammalian Phe-Met-Arg-Phe-NH₂-like peptides: structure, biological activity and distribution. *Prog Clin Biol Res* 342:86–91
- Wiseman G (1974) Active absorption of amino acids in vivo. In: Mason LA (ed) *Bioembranes*, vol 4A. Plenum, London, New York, pp 380–384
- Wood J, Garthwaite J (1994) Models of the diffusion spread of nitric oxide: implications for neural nitric oxide signaling and its pharmacological properties. *Neuropharmacology* 33:1235–1244