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RESEARCH PAPER

In vitro and *in vivo* pharmacological role of TLQP-21, a VGF-derived peptide, in the regulation of rat gastric motor functions

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Background and purpose: *Vgf* gene expression has been detected in various endocrine and neuronal cells in the gastrointestinal tract. In this study we investigated the pharmacological activity of different VGF-derived peptides. Among these, TLQP-21, corresponding to the 556–576 fragment of the protein was the unique active peptide, and its pharmacological profile was further studied.
Experimental approach: The effects of TLQP-21 were examined *in vitro* by smooth muscle contraction in isolated preparations from the rat gastrointestinal tract and, *in vivo,* by assessing gastric emptying in rats. Rat stomach tissues were also processed for immunohistochemical and biochemical characterization.
Key results: In rat longitudinal forestomach strips, TLQP-21 (100 nmol·L^{-1}–10 μmol·L^{-1}) concentration-dependently induced muscle contraction (in female rats, EC_{50} = 0.47 μmol·L^{-1}, E_{max}: 85.7 ± 7.9 and in male rats, 0.87 μmol·L^{-1}, E_{max}: 33.4 ± 5.3; n = 8), by release of prostaglandin (PG)E_2 and PGF_{2a} from the mucosal layer. This effect was significantly antagonized by indomethacin and selective inhibitors of either cyclooxygenase-1 (S560) or cyclooxygenase-2 (NS398). Immunostaining and biochemical studies confirmed the presence of VGF in the gastric neuronal cells. TLQP-21, injected i.c.v. (2–32 nmol per rat), significantly decreased gastric emptying by about 40%. This effect was significantly ($P < 0.05$) blocked by i.c.v. injection of indomethacin, suggesting that, also *in vivo,* this peptide acts in the brain stimulating PG release.
Conclusions and implications: The present results demonstrate that this VGF-derived peptide plays a central and local role in the regulation of rat gastric motor functions.
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Abbreviations: CGC, cerebellar granule cells; COX, cyclooxygenase; E_{max}, maximally effective concentration; EP, prostaglandin E_2 receptor; FP, prostaglandin $F_{2\alpha}$ receptor; GI, gastrointestinal; PG, prostaglandin; RLF, rat longitudinal forestomach; TTX, tetrodotoxin

Introduction

The *vgf* gene encodes for VGF, a 617 amino acid precursor protein (Levi *et al.*, 1985; Salton *et al.*, 1991) with a tissue-specific pattern of expression limited to neurons in the central and peripheral nervous systems and to specific populations of endocrine cells (Salton, 1991; Ferri *et al.*, 1992; Kanemasa *et al.*, 1995; Salton *et al.*, 2000).

The precursor protein in the rat is processed by the neuroendocrine-specific pro-hormone convertases PC1/3 and PC2, producing a number of peptides that are stored in dense core granules and secreted through the regulated pathway (Trani *et al.*, 1995; 2002; Levi *et al.*, 2004). Recently, in rat brain extracts, a previously uncharacterized VGF-derived peptide, designated as TLQP-21, which spans from residue 556 to residue 576 of the precursor sequence, was identified

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by our group (Bartolomucci *et al.*, 2006). This peptide was able to increase energy expenditure, prevent the early phase of diet-induced obesity in mouse (Bartolomucci *et al.*, 2006), to regulate food intake and body weight in hamsters (Jethwa *et al.*, 2007; Jethwa and Ebling, 2008) and prevent death induced by serum and potassium deprivation, in rat cerebellar granule cells (Severini *et al.*, 2008).

Besides TLQP-21, other VGF-derived peptides were shown to possess biological activity: TLQP-62 and AQEE-30 increased the synaptic charge in hippocampal neurons and produced an anti-depressant action (Alder *et al.*, 2003; Hunsberger *et al.*, 2007; Thakker-Varia *et al.*, 2007), AQEE-30 and LQEQ-19 facilitated penile erection (Succu *et al.*, 2004; 2005), and, more recently, LEGS-26 and QEAE-38 (named NERP-1 and NERP-2) suppressed vasopressin secretion both *in vitro* and *in vivo* assays (Yamaguchi *et al.*, 2007).

In the peripheral nervous system, the *vgf* gene is highly expressed in sympathetic, primary sensory neurons and in myenteric plexus ganglia, with evidence of expression in the glandular portion of the stomach, suggesting the presence of this gene throughout the gastrointestinal (GI) tract (Ferri *et al.*, 1992; Liu *et al.*, 1994; Salton *et al.*, 2000; Snyder *et al.*, 2003). Furthermore, pro-VGF fragments were described in human enterochromaffin-like cells, related hyperplastic and neoplastic cells (Rindi *et al.*, 2007). However, the possible role of the VGF-derived peptides in the regulation of gastric functions has never been investigated.

The present work was designed to explore: (i) if some of the VGF-derived peptides may play a role in the regulation of rat GI functions testing their *in vitro* contractile activity on different parts of the rat GI tract; (ii) the mechanism of action of the unique active VGF-derived peptide (TLQP-21) on the *in vitro* contractile activity of the rat longitudinal forestomach (RLF) strip; (iii) the *in vivo* TLQP-21 central and peripheral effect on rat gastric emptying and its possible action mechanism.

As a consequence of our findings, we now know that, *in vitro,* TLQP-21 stimulated contraction of the RLF strip through the release of prostaglandins (PGs) from cell types within the mucosal layer and, *in vivo,* the peptide exerted a central inhibitory role on gastric emptying, involving PG release.

Methods

Animals

In vitro and *in vivo* studies were conducted according to the guidelines of the Italian Ministry of University and Research (D.L.116, 27/01/92) and the European Communities Council Directive (86/609/EEC). Each experimental protocol was authorized by the Ethics Committee of the Italian Ministry of Health.

In vitro studies

Gastrointestinal contraction. Wistar male and female rats (250–350 g; Charles River, Calco, Italy) were killed by inhalation of 75% CO_2 in air. Different portions of the GI tract (oesophagus, stomach, pylorus, jejunum, proximal and distal colon) were removed and washed in fresh Tyrode's solution as previously described (Severini *et al.*, 2000). For the stomach contractility assays, longitudinal strips were obtained from the RLF region and mounted vertically in a 5 mL organ bath in oxygenated (95% O_2 and 5% CO_2) Tyrode's solution at 37°C. Responses were recorded isotonically by a strain gauge transducer (DY 1, Basile, Milan, Italy) and displayed on a recording microdynamometer (Unirecord, Basile. Milano, Italy). Tissues were allowed to equilibrate for about 60 min and the Tyrode buffer was changed every 10 min.

Saturating concentrations of acetylcholine (ACh, 25 $\mu mol \cdot L^{-1}$) were added every 20 min, washing the tissue after 1 min contact time, until reproducible contractile responses were obtained. After 30 min equilibration time, TLQP-21 non-cumulative concentration–responses curves were constructed by adding increasing concentrations of the peptide to the tissue chamber, until maximum muscular responses occurred. The tissue was washed as soon as the contraction peak had developed, after a contact time with muscle strips of 1–3 min.

In preliminary experiments, peptide applications were performed at different time intervals (5, 10, 15 and 20 min) to establish the appropriate interval between subsequent administrations. As an interval of 20 min was necessary to obtain a complete recovery, that is, to avoid tachyphylaxis, this interval was used for every successive experiment. Only one concentration–response study was performed on each strip. TLQP-21-induced contractions were expressed as mean ± SEM of the percentage of ACh-induced contractions and EC_{50} calculated by interpolation from the appropriate concentration–response curve. Maximally effective concentration (E_{max}) denotes the maximal response achieved by the peptide, expressed as a percentage of that obtained with saturating concentrations of ACh (25 $\mu mol \cdot L^{-1}$).

Antagonists were all at a sufficiently high concentration to block the corresponding agonists under the experimental conditions.

To investigate the contribution of the gastric mucosa on the effect of TLQP-21, additional experiments were performed with mucosa-free strips. These preparations were obtained by removing the mucosa from the RLF with a lancet. The tissues were set up in the organ baths as previously mentioned.

Prostaglandin release. Longitudinal RLF strips were prepared as previously described and tested for standard response to ACh. Tissues were weighed and subsequently transferred to an Eppendorf tube and incubated without (control) or with TLQP-21 (400 $nmol \cdot L^{-1}$, 4 $\mu mol \cdot L^{-1}$) for 90 s at 37°C in 0.75 mL of Tyrode's solution. PGE_2 and $PGF_{2\alpha}$ concentration was measured immediately and in parallel using commercially available EIA kits (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. The reactions were read at 405 nm on a 1420-011 multi-label counter (Wallac Oy, 20101 Turku, Finland).

Immunostaining. Samples of gastric body-forestomach were taken from female adult rats (Wistar, 200–250 g, killed by decapitation: $n = 8$), pinned flat onto pieces of cork, immersion-fixed in paraformaldehyde (40 $g \cdot L^{-1}$, in 0.1 $mol \cdot L^{-1}$ phosphate buffer: 3 h at 0–4°C) and frozen as previously described (Rindi *et al.*, 2007). The rabbit antiserum used was raised against the C-terminal sequence of rat VGF, $VGF_{609-617}$,

and has been previously characterized (Ferri *et al.*, 1995). Rat gastric sections were treated with Triton X-100 (1 mL·L^{-1}, in H_2O for 1 h), incubated overnight with the anti-VGF antiserum (1:2000–10 000), followed by Cy3-conjugated anti-rabbit antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Routine controls included substitution of each layer, in turn, with non-immune serum, or phosphate-buffered saline. Pre-absorption of the antiserum with the relevant peptide (approximately 50 nmol·mL^{-1}) virtually abolished staining. Preparations were examined with a Olympus BX60 fluorescence and transmitted light microscope, equipped with a PM30 photographic system.

Western blot analysis of rat stomach homogenates. Wistar rats (weighing 250–350 g) were killed by inhalation of 75% CO_2 in air. The stomachs were rapidly dissected, minced, boiled for 10 min in 10 mL H_2O·g^{-1} of tissue and further incubated at room temperature for 15 min. Coarse materials were briefly sedimented and the supernatant cleared at 20 000× *g* for 45 min at 4°C. This procedure resulted in both protease inactivation and enriched extraction of low molecular weight peptides (Trani *et al.*, 1995). Approximately 0.03 mg of proteins was loaded on gels according to the protocol for NuPAGE system (Invitrogen, Carlsbad, CA, USA). Western blot analysis was performed by using an anti-$VGF_{609–617}$ serum (previously mentioned) at 1:5000 dilution for 2 h and goat anti-rabbit-HRP conjugated secondary antibody at 1:2000 dilution for 45 min (Pierce Biotecnology, Rockford, IL, USA). Chemiluminescent detection of immunoblots was achieved by using the Supersignal West Dura kit (Pierce Biotecnology, Rockford, IL, USA) according to the manufacturer's instructions.

In vivo studies

Gastric emptying. Adult Wistar rats, weighing 180–200 g (Charles River, Calco, Italy) were used. The animals were housed in single cages with wire net bases to avoid coprophagy. Before the experiments, rats were fasted for 24 h, but had free access to water until the treatment was started. For i.c.v. administration of peptides, a polyethylene cannula (PE10) was implanted into the left lateral ventricle of the brain, 5 days before the experiments as previously described (Netti *et al.*, 1984). At the end of the experiments, dye (0.5% Evans Blue) was injected through the cannula to confirm its position in the ventricle.

The gastric emptying assay, consisting of a phenol red test meal was performed in female and male Wistar rats (Broccardo and Improta, 1990). Briefly, a liquid and acaloric meal (1.5 mL per rat) consisting of a 50 mg phenol red solution in 100 mL aqueous methylcellulose (1.5%) was administered by gavage to conscious rats. TLQP-21 (0.1, 0.4, 2, 8 and 32 nmol per rat) or saline (NaCl 0.9%) were injected i.c.v in a constant volume (5 µL per rat) immediately before meal administration. TLQP-21 (48 or 480 nmol·kg^{-1}) or saline was administered 5 min before meal administration when i.p. injected. Rats were killed by CO_2 (70%) inhalation 10 min after the test meal, when maximal differences between saline and TLQP-21 groups were observed. In each experiment, four non-treated rats were euthanized by CO_2 (70%) inhalation immediately after the administration of the phenol red solution, and this was considered the reference of 0% emptying rate. The stomach and its contents were homogenized with 100 mL of 0.1 N NaOH, protein precipitated with 20% trichloroacetic acid and the supernatant made alkaline with NaOH. The absorbance of the sample was measured at 560 nm (A560) with a spectrophotometer. Gastric emptying (GE) for each rat was calculated according to the following formula: GE (%) = [1 – (A560 sample/mean of A560 reference)] × 100, where A560 sample was the absorption at 560 nm of the gastric content at 10 min and A560 reference was the absorption at 560 nm of the gastric content at zero emptying time. Each value was expressed as percentage change with respect to the percentage gastric emptying in saline-treated rats.

To provide insights into TLQP-21-evoked gastric motor responses, the cyclooxygenase (COX) inhibitor indomethacin was given in different ways to two sets of animals. In the first set, indomethacin was given s.c. (3 mg·kg^{-1}) (Takeuchi *et al.*, 1990), 20 min before i.c.v. TLQP-21. In the second set, indomethacin was given i.c.v. (0.25 mg per rat) (Saperas *et al.*, 1991), 5 min before i.c.v. TLQP-21.

Data analysis. Statistical analysis was performed with a statistic package (GraphPad Prism, GraphPad Software San Diego, CA, USA). All data are represented as the mean ± SEM. Differences between the groups were assessed by one-way analysis of variance (ANOVA) followed by multi-comparison Dunnett's test. The ED_{50} was calculated by linear regression analysis of the concentration–response data.

Chemicals. TLQP-21 (TLQPPASSRRRHFHHALPPAR) and the other VGF-derived peptides were synthesized by PRIMM (Milan, Italy). Stock solutions of peptides (1–10 mmol·L^{-1}) were made in distilled water, stored at –20°C and then diluted in water before use. COX inhibitors (SC560, NS398) and PG receptor antagonists (SC19220 and $PGF_{2\alpha}$ dimethylamide; Cayman Chemical Company, Ann Arbor, MI, USA) were dissolved in DMSO. SR140,333 was provided by Sanofi-Synthelabo Ricerche (Montpellier, France) and tetrodotoxin (TTX) was from Alomone (Jerusalem, Israel). The other reagents were from Sigma (St. Louis, MO, USA).

Results

In vitro activity

Gastrointestinal contraction. We tested the contractile activity of the natural and synthetic VGF-derived peptides reported in Figure 1 on different regions of the rat GI tracts (oesophagus, pylorus, gastric antrum, gastric forestomach, jejunum, proximal and distal colon). Each of the biologically active VGF-derived peptides purified from brain extracts (TLQP-62, TLQP-21, AQEE-30 and LQEQ-19) failed to evoke contractions in the tested GI preparations (25–50 µmol·L^{-1}, $n = 5$, data not shown) with the exception of TLQP-21. This peptide elicited a reproducible and concentration-dependent contractile activity (100 nmol·L^{-1}–6 µmol·L^{-1}) of the RLF smooth muscle (Figure 2) and only weak and not concentration-dependent activity on oesophagus, gastric antrum and circular forestomach muscule strips, even at much higher concentrations (25–50 µmol·L^{-1}, data not shown).

MKTFTLPASVLFCFLLLIRGLGA **APPG**RSDVYPPPLGSEHNGQVAEDAVSRPKDDSVPEVRAAR
NSEPQDQGELFQGVDPRALAAVLLQALDRPASPPAVPAGSQQGTPEEAAEALLTESVR
SQTHSLPASEIQASAVAPPRPQTQDNDPEADDRSEELEALASLLQELRDFSPSNAKRQQETAA
AETETRTHTLTRVNLESPGPERVWRASWGEFQARVPERAPLPPSVPSQFQARMSENVPLPETH
QFGEGVSSPKTHLGETLTPLSKAYQSLSAPFPKVRR**LEGS**FLGGSEAGERLLQQGLAQVEAG
RR**QAEA**TRQAAAQEERLADLASDLLLQYLLQGGARQRDLGGRGLQETQQERENEREEEAE
QERR**GGGE**DEVGEEDEEAAEAEAEAEEAERARQNALLFAEEEDGAEDKRSQEEAPGHRRK
DAEGTEEGGEEDDDDEEMDPQTIDSLIELSTKLHLPADDVVSIIEEVEEKRKRKKK**NAPP**
EPVPPPRAAPAPTHVRSPQPPPPAPARDELPDWNEVLPPWDREEDEVFPPGPYHPFPNYIRPR
TLQPPASSRRRHFHHALPPARHHPDLEAQARRA**QEE**ADAEERRLQEQEELENYIEHVLLHRP

TLQPPASSRRRHFHHALPPARHHPDLEAQARRA**QEE**ADAEERRLQEQEELENYIEHVLLHRP	**TLQP**-62
TLQPPASSRRRHFHHALPPARHHPDLEAQARR	**TLQP**-30
TLQPPASSRRRHFHHALPPAR	**TLQP**-21
TLQPPASSRRR	**TLQP**-11
HFHHALPPAR	**HFHH**-10
AQEEADAEERRLQEQEELENYIEHVLLHRP	**AQEE**-30
LQEQEELENYIEHVLLHRP	**LQEQ**-19

Figure 1 VGF sequence. The upper figure shows the primary sequence of the VGF protein. The leader peptide is shown in italics, and the arrow indicates the cleavage site. VGF fragments that are known to show a biological activity are underlined. VGF-derived peptides, previously purified from brain extracts are, by convention, designated by the four-letter codes of N-terminal amino acids, and the number represents the total number of amino acid residues in the peptide. The VGF-derived peptides tested in this study are listed in the lower figure.

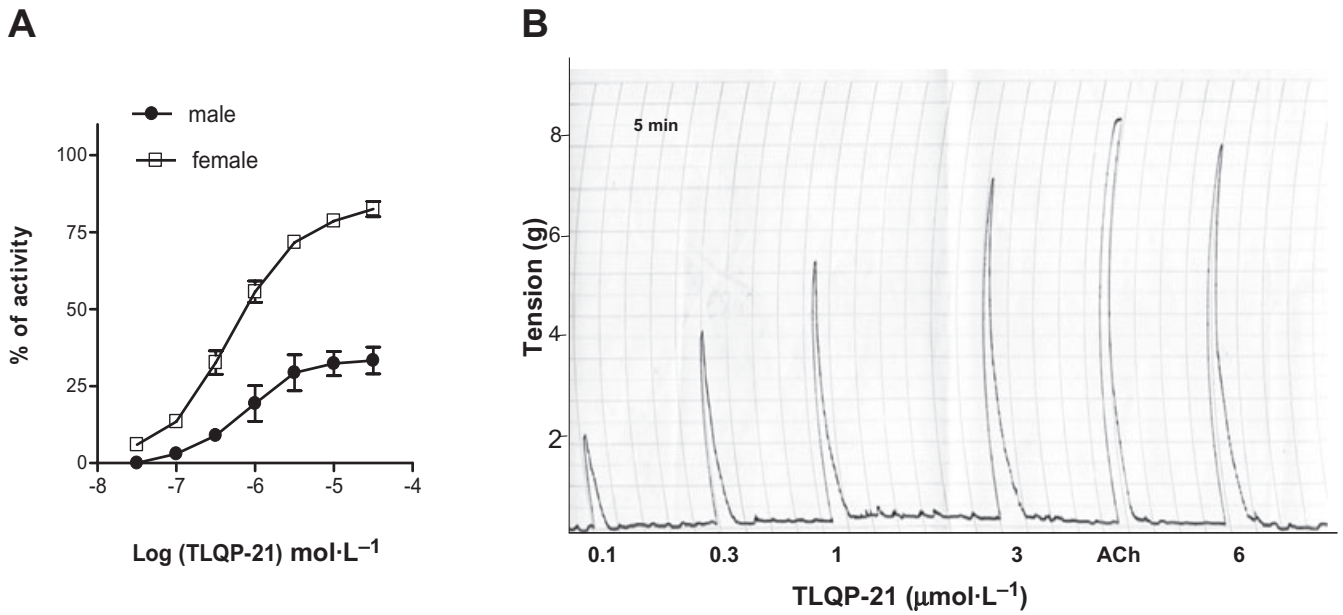

Figure 2 Contractile activity of TLQP-21 on rat longitudinal forestomach (RLF) strips. (A) TLQP-21 concentration–response curve. The figure shows comparative activity on male and female RLF strips. Each point represents the mean and the vertical bars the SEM of eight different determinations. Abscissa: –log of the peptide molar concentration. Ordinate: peptide activity as a percentage of the maximum effect obtained with 25 µmol·L^{-1} acetylcholine (ACh). (B) Qualitative example of the contractile responses evoked in female rats by increasing peptide concentrations (0.1, 0.3, 1, 3 and 6 µmol·L^{-1}). Contractile activities are compared with the maximum response produced by 25 µmol·L^{-1} ACh.

In addition, we tested on RLF strips, the contractile action of the synthetic peptides TLQP-11, HFHH-10 and TLQP-30, corresponding to fragments or an extension of the TLQP-21 sequence. In view of the presence of specific cleavage sites in the VGF protein, these peptides could be generated *in vivo* by endogenous peptidases. Nevertheless, these peptides failed to contract RLF strips (25–50 µmol·L^{-1}, $n = 5$, data not shown).

TLQP-21 was more potent on contracting RLF strips from female rats than those from male rats. As shown in Figure 2A, TLQP-21 (100 nmol·L^{-1}–10 µmol·L^{-1}) concentration-dependently contracted the RLF strips from male rats with E_{max} value of 33.4 ± 5.3% and EC_{50} value of 0.87 µmol·L^{-1} ($n = 8$ at each concentration), while in female rats E_{max} was equal to 85.7 ± 7.9% and the EC_{50} value of 0.47 µmol·L^{-1} ($n = 8$ at each concentration). In light of these sex differences, we used RLF strips from female rats for the subsequent *in vitro* pharmacological studies. A representative concentration–response trace is shown in Figure 2B. The effect of TLQP-21

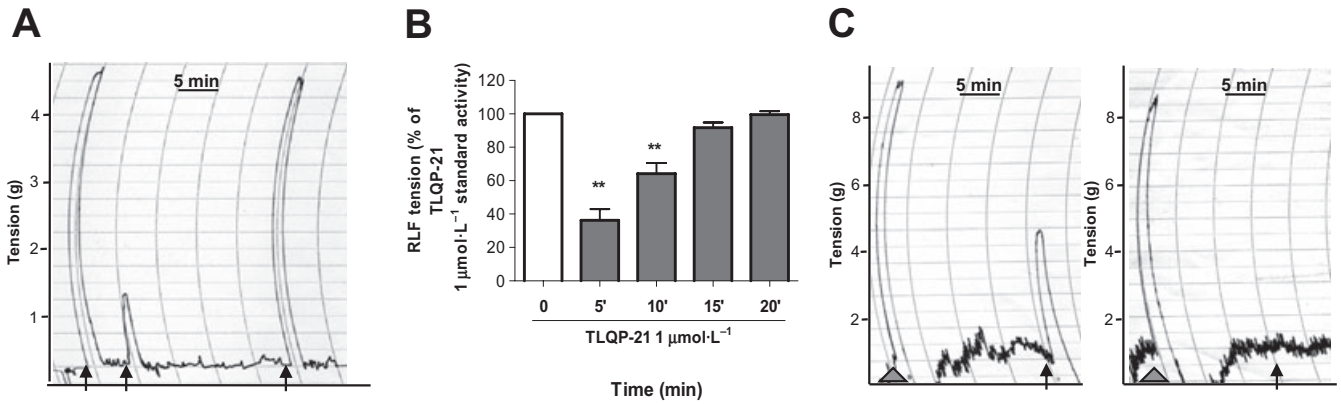

Figure 3 Tachyphylaxis to TLQP-21. (A) A representative trace of contractile responses produced by TLQP-21 (1 µmol·L^{-1}, arrows) in rat longitudinal forestomach (RLF) strips. Note that tachyphylaxis was induced when a second application of TLQP-21 was made, 5 min after the first. (B) TLQP-21 (1 µmol·L^{-1}) was given at different time intervals (5, 10, 15 and 20 min) after the initial application. Contractile effects of TLQP-21 show that an interval of 15–20 min was necessary to obtain a complete recovery. (C) Representative tracings acquired from two strips obtained from the same RLF: the left indicates an intact strip and the right, a strip after the removal of the mucosal layer. Arrows indicate the addition of TLQP-21 (1 µmol·L^{-1}); arrowheads identify responses to 25 µmol·L^{-1} acetylcholine. Each point represents the mean ± SEM from five experiments expressed as percentage change versus the TLQP-21 initial activity (Time = 0). Statistically significant differences were calculated by one-way analysis of variance (ANOVA) for repeated measures followed by Dunnett's test, analysing data from different time intervals and the corresponding control (0) (**$P < 0.01$).

Table 1 Effect of various antagonists on the contraction of rat longitudinal forestomach strips induced by TLQP-21 and by the corresponding selective agonists

Antagonists (mol·L^{-1})	*Agonist (mol·L^{-1})*	*Absence of antagonist*	*Presence of antagonist (%)*
Tetrodotoxin (1 µmol·L^{-1})	TLQP-21 (1 µmol·L^{-1})	100	98 ± 5
Atropine (1 µmol·L^{-1})	TLQP-21 (1 µmol·L^{-1})	100	97 ± 4
	Acetylcholine (10 µmol·L^{-1})	100	0*
Methysergide (2.5 µmol·L^{-1}) + ketanserin (2.5 µmol·L^{-1})	TLQP-21 (1 µmol·L^{-1})	100	96 ± 8
	5-HT (10 µmol·L^{-1})	100	0*
Mepyramine (1 µmol·L^{-1}) + cimetidine (1 µmol·L^{-1})	TLQP-21 (1 µmol·L^{-1})	100	101 ± 3
	Histamine (10 µmol·L^{-1})	100	0*
SR140.333 (100 nmol·L^{-1})	TLQP-21 (1 µmol·L^{-1})	100	99 ± 6
	Substance P (10 nmol·L^{-1})	100	0*

Contractile effects of TLQP-21 (1 µmol·L^{-1}) and with the other selective agonists (acetylcholine, 5-HT, histamine and substance P) in the absence of antagonists are reported as 100% of activity. Results obtained in the presence of different antagonists are expressed as % of the activity of the corresponding agonists (mean ± SEM from six independent experiments).
TLQP-21 was given at 20 min intervals to avoid tachyphylaxis, and the incubation time of the antagonists with the tissues was 15 min.
*$P < 0.05$ versus the corresponding control (absence of antagonist).

(1 µmol·L^{-1}) was determined against contractions induced by ACh, at a concentration previously determined to be maximally effective (25 µmol·L^{-1}, 1 min contact).

Contractile activity exerted by TLQP-21 showed a clear tachyphylaxis, as a second application of the peptide (1 µmol·L^{-1}) at time intervals shorter than 20 min resulted in a reduced response, while complete reproducibility was obtained on reapplying the peptide after 20 min, as shown in Figure 3A and 3B. In addition, manual removal of the mucosal layer from the RLF strips prevented TLQP-21-induced contractile activity (3 ± 4% of the ACh-induced contraction, $n = 5$), while ACh-induced contraction was unmodified, when compared with the corresponding contraction of intact RLF preparations, relative to the weight of tissue used (Figure 3C).

Mechanisms of TLQP-21-induced contractions of RLF strips. To characterize the possible mediator(s) of TLQP-21 action, we tested different compounds, capable of inhibiting substances known to stimulate gastric motility (see Table 1). The addition of atropine, SR140,333, a mixture of methysergide and ketanserin, and a mixture of mepyramine and cimetidine did not modify the response elicited by TLQP-21, while it prevented or reduced any contraction evoked by their respective agonists ACh, substance P, 5-HT and histamine. TTX also failed to influence the effect of TLQP-21, suggesting that a neuronal action potential is not involved.

On the other hand, 15 min pretreatment with the non-specific COX inhibitor indomethacin (from 100 nmol·L^{-1} to10 µmol·L^{-1}), the specific COX-1 inhibitor (SC560, from 10 nmol·L^{-1} to 10 µmol·L^{-1}) or the specific COX-2 inhibitor (NS398, from 10 nmol·L^{-1} to 10 µmol·L^{-1}) dose-dependently prevented TLQP-21-induced contractions (1 µmol·L^{-1}), as shown in Table 2.

To compare the TLQP-21 contractile activity on RLF strips with that of $PGF_{2\alpha}$ and PGE_2, non-cumulative concentration–responses curves of these PGs were made. The EC_{50} value for

Table 2 Effect of different COX inhibitors and PG receptor antagonists on the contractions of RLF strips induced by TLQP-21 and by PGE_2 and $PGF_{2\alpha}$

Inhibitor (mol·L^{-1})		*Agonist (mol·L^{-1})*	*Absence of inhibitor*	*Presence of inhibitor (%)*
Indomethacin	10 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	6 ± 2*
	1 μmol·L^{-1}		100	58 ± 7*
	100nmol·L^{-1}		100	81 ± 6
S560 (COX-1)	10 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	15 ± 4*
	1 μmol·L^{-1}		100	40 ± 8*
	100 nmol·L^{-1}		100	57 ± 7*
	10 nmol·L^{-1}		100	89 ± 11
NS398 (COX-2)	10 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	0*
	1 μmol·L^{-1}		100	20 ± 3*
	100 nmol·L^{-1}		100	75 ± 5*
	10 nmol·L^{-1}		100	92 ± 8
SC19220 (EP antagonist)	100 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	30 ± 7*
		PGE_2(5 nmol·L^{-1})	100	24 ± 5*
		$PGF_{2\alpha}$(11 nmol·L^{-1})	100	98 ± 3
	10 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	83 ± 6
	1 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	96 ± 6
$PGF_{2\alpha}$ dimethylamide (FP antagonist)	100 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	50 ± 7*
		$PGF_{2\alpha}$(11 nmol·L^{-1})	100	60 ± 9*
		PGE_2(5 nmol·L^{-1})	100	86 ± 6
	10 μmol·L^{-1}	TLQP-21 (1 μmol·L^{-1})	100	75 ± 6

Contractile effects obtained with TLQP-21 (1 μmol·L^{-1}) and with PGs (PGE_2 and $PGF_{2\alpha}$) in the absence of inhibitors are reported as 100% of activity. Results obtained in the presence of different doses of COX inhibitors and PG antagonists are expressed as % of the activity of the corresponding agonists (mean ± SEM from six independent experiments).
TLQP-21 was given at 20 min intervals to avoid tachyphylaxis and the incubation time of the antagonists with the tissues was 15 min.
*$P < 0.05$ versus the corresponding control (absence of inhibitor).
COX, cyclooxygenase; EP, prostaglandin E_2 receptor; FP, prostaglandin $F_{2\alpha}$ receptor; PG, prostaglandin; RLF, rat longitudinal forestomach.

$PGF_{2\alpha}$ was 11.5 nmol·L^{-1} ($n = 4$ at each concentration), and the EC_{50} value for PGE_2 was 5.5 nmol·L^{-1} ($n = 4$ at each concentration), as compared with the TLQP-21 EC_{50} value of 0.87 μmol·L^{-1}.

In the light of these findings, we investigated the identity of the PGs released in response to TLQP-21 using antagonists for the FP and the EP receptors (prostaglandin E_2 and $F_{2\alpha}$ receptors, nomenclature follows Alexander *et al.*, 2008). Either PGF_{2a} dimethylamide (antagonist for the FP receptor, 10–100 μmol·L^{-1}) or SC19220 (antagonist for the EP receptor, 1–100 μmol·L^{-1}) significantly reduced the TLQP-21-induced contraction as well as the activity of the corresponding agonists $PGF_{2\alpha}$ and PGE_2 used at EC_{50} concentrations (Table 2). To confirm the release of these PGs from RLF strips, these strips were incubated with different concentrations of TLQP-21 (400 nmol·L^{-1}, 4 μmol·L^{-1}) and the PG content of the incubation medium assayed by EIA. As shown in Figure 4, a dose-dependent increase in the concentration of PGE_2 and $PGF_{2\alpha}$ was detected in the medium following incubation with TLQP-21, when compared with the corresponding basal levels. On the contrary, no increase in PG production was detected by using the same tissues after mechanical removal of the mucosa (data not shown).

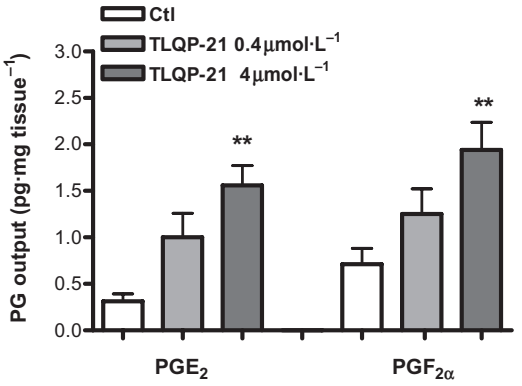

Figure 4 Prostaglandin (PG) release by rat longitudinal forestomach (RLF) strips. RLF strips were incubated without (Ctl) or with TLQP-21 (0.4 and 4 μmol·L^{-1}) at 37°C. PG production was determined 60 s after peptide addition and detected by EIA (see *Methods*). Results are expressed as PG released corrected for the weight of the strip (pg·PG·mg^{-1} wet tissue) from five independent experiments (mean ± SEM). Statistically significant differences were calculated by one-way analysis of variance (ANOVA) for repeated measurements followed by Dunnett's test, analysing data from treated samples and the corresponding controls (Ctl). (**$P < 0.01$).

VGF expression and processing in the rat stomach. We studied the presence and distribution of VGF-containing nerve structures in the stomach by means of immunocytochemistry using antibodies raised against the C-terminal region of VGF (Figure 5) (Ferri *et al.*, 1995). As shown in Figure 5A, a number of strongly labelled, VGF C-terminus-immunoreactive axons were shown in the different layers of the forestomach (Figure 5A, panels a-c). Scattered bundles of beaded axons were seen in the mucosa, running in the lamina propria between the gastric glands (Figure 5A, panel a). The VGF-containing innervation of the muscularis externa was especially rich, with very numerous varicose axons running in both the circular and the longitudinal layers (Figure 5A, panel a). Immunoreactive terminals were also shown in myenteric ganglia, largely depicting non-labelled cell bodies (Figure 5, panels a and c). Weak but distinct labelling was seen in a few perikarya (Figure 5A, panel b).

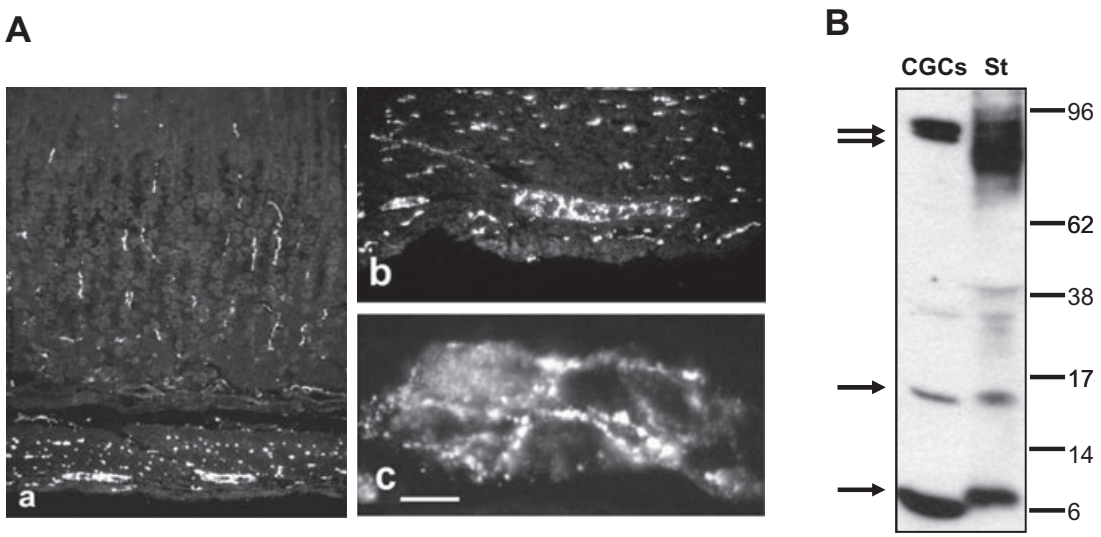

Figure 5 VGF localization. (A) VGF immunostaining. (a) A dense VGF-immunoreactive innervation was found in circular and longitudinal muscle layers, while small numbers of beaded fibres run in the mucosal lamina propria (bar scale = 100 μm). (b) Several VGF-immunoreactive terminals depict non-reactive cell bodies in myenteric ganglia (bar scale = 50 μm) and (c) an occasional immunolabelled perikaryon (bar scale = 10 μm) (bar scale = 40 μm). (B) VGF peptides in rat stomach. Western blot analysis performed on extracts from primary cultures of rat cerebellar granule cells (CGCs) maintained for 8 days *in vitro* and from rat stomach (St), as described in the *Methods* section. The apparent molecular weights in kDa are indicated on the right. Arrows point to the main VGF peptides: two arrows indicate the high MW precursors, while one arrow marks the peptides NAPP-129 and TLQP-62, previously described in rat neurons.

To characterize which of VGF-derived peptides, previously purified by brain extracts (Trani *et al.*, 2002) were present, Western blot analysis was performed on extracts from rat stomach, comparing bands with those from primary cultures of rat cerebellar granule cells.

As shown in Figure 5B, in addition to high-form precursors (double arrows), two bands detected in stomach samples could be identified as NAPP-129 and TLQP-62 (single arrows) on the basis of their electrophoretic point. The presence of other peptides of lower molecular weight is difficult to detect by Western blot analysis.

In vivo *studies*

Gastric emptying. I.c.v. injections of TLQP-21 (from 0.1 to 32 nmol per rat) delayed gastric emptying of a phenol red meal, measured both in male and female rats, as reported in Figure 6A. TLQP-21 decreased the gastric emptying significantly, but not in a dose-related manner (2, 8 and 32 nmol per rat) in comparison with gastric emptying values in rats given i.c.v. saline (value marked 0). The maximal gastric emptying inhibitory response was about 40% both in male (Figure 6A) and female rats (data not shown). The effect of i.c.v. administration of TLQP-21 on gastric emptying was evaluated after i.p. and i.c.v. injection of the COX inhibitor indomethacin at dose levels, which had no effect on gastric emptying. Indomethacin (3 mg·kg^{-1}), when given i.p., did not modify the delay of gastric emptying induced by i.c.v. administration of TLQP-21 (8 nmol per rat). However, when indomethacin was given i.c.v. (0.25 mg per rat), before i.c.v. TLQP-21, it totally blocked the inhibitory effect of the peptide (Figure 6B). TLQP-21, when administered peripherally (48 and 480 nmol·kg^{-1} i.p.), did not affect gastric emptying of a phenol red meal measured in male (Figure 6C) and female rats (data not shown).

Discussion and conclusions

As previously reported, the *vgf* gene is expressed in the GI tract and, during rat development, its expression was detected by *in situ* hybridization as early as embryonic day 15.5 in the developing rat GI tract and oesophageal lumen (Snyder *et al.*, 2003). Intense VGF immunoreactivity was observed in subpopulations of neurons of the enteric plexus and enteroendocrine cells in the adult rat (Ferri *et al.*, 1992), indicating a possible role of the VGF-derived peptides in the regulation of GI smooth muscle functions in the rat.

In the present study, analysis of the contractile activity of the VGF-derived peptides, biologically active in other systems, showed that only TLQP-21, corresponding to the 556–576 fragment of the precursor protein, was able to influence GI contraction *in vitro*.

In the light of these findings, we assessed the biological relevance of TLQP-21 by characterizing it pharmacologically and elucidating its role on *in vitro* and *in vivo* motor GI functions.

Analysis of some *in vitro* GI test assays revealed that the TLQP-21 activity was limited to the RLF musculature. This peptide evoked, in RLF strips, a concentration- and gender-dependent contractile activity, showing a greater effect in the response intensity in female rather than in male rats. This difference in response might be ascribed to a quantitatively different distribution of TLQP-21 receptors between male and female RLF smooth muscle, but such a difference would be difficult to demonstrate, as the receptor has not yet been

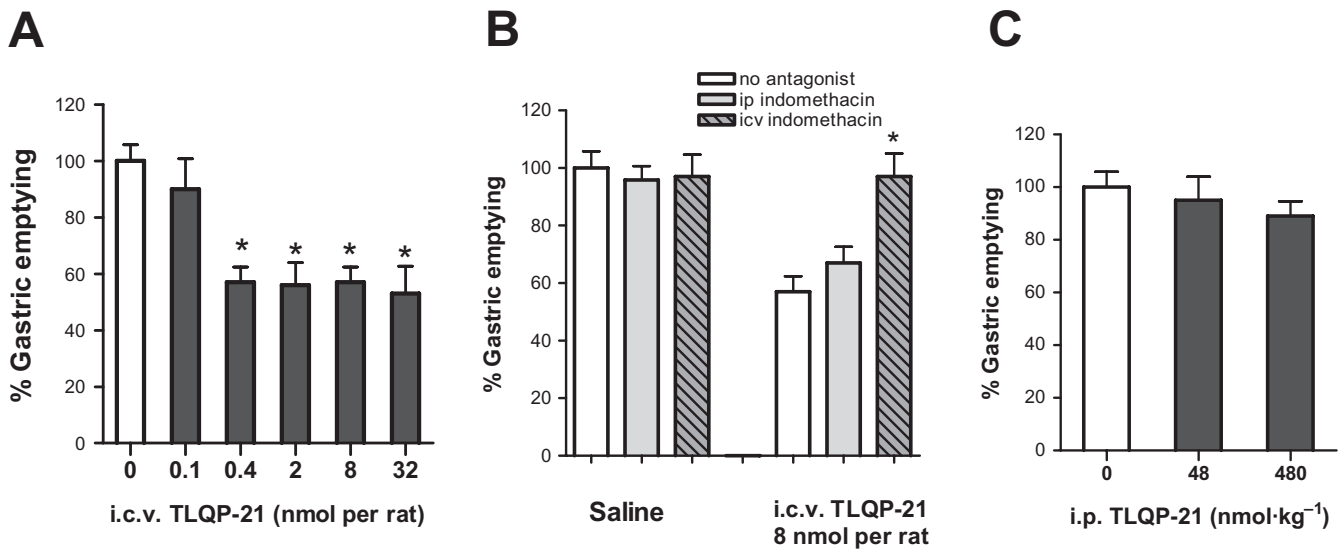

Figure 6 Gastric emptying. (A) Effect of different doses of TLQP-21 injected i.c.v. (0.4–32 nmol per rat) on gastric emptying of a phenol red meal. Each point represents the mean ± SEM of gastric emptying values from eight rats expressed as a percentage change versus saline-treated rats (0.739 ± 0.08 = 100%). *$P < 0.05$ in comparison with the group injected with saline. (B) Effect of peripheral (3 mg·kg^{-1}, i.p.) and central (0.25 mg per rat, i.c.v.) indomethacin pretreatment on gastric emptying – response to i.c.v. saline or TLQP-21 injection (8 nmol per rat). Each point represents the mean ± SEM of gastric emptying values from eight male rats expressed as a percentage change versus saline-treated rats (0.744 ± 0.01 = 100%). *$P < 0.05$ in comparison with the group injected with only TLQP-21. (C) Effect of different doses of TLQP-21 (48 and 480 nmol·kg^{-1}) injected i.p. on gastric emptying. Each point represents the mean ± SEM of gastric emptying values from eight rats expressed as a percentage change versus saline-treated rats (0.745 ± 0.11 = 100%).

identified. Another possible explanation could be a different gender-related COX distribution in gastric smooth muscle but, to our knowledge, no data are reported on this matter.

In addition, the lack of activity of other fragments or extensions of TLQP-21, which can be generated *in vivo*, indicated that the biological activity is restricted to the TLQP-21 primary sequence.

With respect to the mechanism of action of TLQP-21 *in vitro*, the absence of any effect by TTX treatment suggested that this peptide does not function through the local nervous system. Moreover, manual removal of the mucosal layer from the RLF strips prevented TLQP-21- but not ACh-induced contraction, indicating that the stimulatory effect of this peptide on gastric smooth muscle was due to its direct action on receptors, localized in the gastric mucosa.

To provide further insights into the action mechanism of TLQP-21, we evaluated its effect in the presence of specific inhibitors of ACh, substance P, 5-HT and histamine, demonstrating that these mediators were not involved in its contractile activity.

However, the activity of TLQP-21 was affected by inhibitors of PG biosynthesis, regulated by the two isoforms of COX (Süleyman *et al.*, 2007). In the stomach, the constitutive isoform (COX-1) is dominantly expressed while the mitogen-inducible isoform (COX-2) is negligibly expressed in normal stomach (Kargman *et al.*, 1996, Schmassmann *et al.*, 1998), but over-expressed during the healing of gastric lesions (Sun *et al.*, 2000). In the present work, we demonstrated that both COX-1 and COX-2 inhibitors concentration-dependently antagonized the contractile effects of TLQP-21.

As PGs generated via the COX-1 pathway are essential for physiological functions, whereas those formed via the COX-2 pathway have a role in pathophysiological events, this VGF-derived peptide may represent a new approach in the understanding and treatment of gastric dysfunction.

In addition, by the use of selective antagonists for the FP and EP receptors and additional EIA studies, performed by incubating RLF strips with TLQP-21, we confirmed that this VGF-derived peptide stimulated the release of PGE_2 and $PGF_{2\alpha}$ from cell types within the mucosal layer. It is well known that gastric mucosa synthesizes large amounts of PGs, among which $PGF_{2\alpha}$ and PGE_2 (Hawkey and Rampton, 1985) are able to contract the longitudinal smooth muscles (Takeuchi *et al.*, 1990) through FP and EP1 receptors present in the muscle layer respectively (Shimomura *et al.*, 1994; Sametz *et al.*, 2000). All these findings suggest that TLQP-21, through unidentified receptors present in the gastric mucosa, stimulates the release of the PGs responsible for the muscle layer contraction.

To support a possible physiological role exerted by TLQP-21 in the regulation of gastric functions, in the present work we demonstrated an intense VGF immunoreactivity, prominent in axons in different layers of the forestomach. Furthermore, although detection of low molecular weight peptides from gut tissues still remains elusive, we identified in stomach homogenates, by Western blot analysis, higher molecular weight precursors and two bands with an electrophoretic profile, consistent with NAPP-129 and TLQP-62, which represent the most abundant VGF peptides in neuronal cells.

The second step of our study was to investigate the central and peripheral effects of TLQP-21 on rat gastric emptying *in vivo*. Our data showed that central but not peripheral administration of TLQP-21 evoked a significant but not gender- or dose-related delay of gastric emptying. First of all,

these results clearly indicated that the gastric motor effect induced by centrally administered TLQP-21 is central nervous system-mediated. The lack of activity of TLQP-21 on gastric emptying after peripheral administration may be due to its different and lower peripheral bioavailability with respect to its central administration. Moreover, our *in vivo* results failed to confirm the gender-dependent gastric contractile effect, clearly observed *in vitro*. We would suggest that the receptor distribution in the brain areas involved in the TLQP-21 modulation of gastric emptying is quantitatively similar in both female and male rats. However, further information regarding the TLQP-21 pharmacokinetics and its receptor distribution will be necessary.

Finally, the lack of concentration dependence of the central inhibitory effect of TLQP-21 on gastric emptying could be explained as the consequence of the integrated action with other neurotransmitter pathways/release involved in the control of this function. It is well established that neuropeptides play an important role in the central control of gastric motility *in vivo,* via a mechanism that requires PGs (Takeuchi *et al*., 1990; Robert *et al*., 1991; Bakke, 1993; Shimomura *et al*., 1994; Sütö *et al*., 1996; Sekiguchi *et al*., 2006). To better characterize the mechanisms underlying the central effects of TLQP-21 on gastric motor function, we tested the action of this peptide after pretreatment with the PG synthesis inhibitor indomethacin. This inhibitor *in vitro* had been shown to abolish the gastric contractile effect of TLQP-21. The finding that the gastric emptying delay induced by central TLQP-21 was blocked by central but not peripheral injection of indomethacin permitted us to speculate that TLQP-21 affects gastric emptying by stimulating central PG pathways. Our hypothesis is strongly supported by recent reports indicating that activation of EP_4 signalling in the central nervous system delayed gastric emptying and suppressed food intake in mice (Ohinata *et al*., 2006). In addition, the inhibitory effect of centrally administered TLQP-21 on food intake and body weight in hamsters (Jethwa *et al*., 2007) involves PG production in the brain areas controlling these activities.

The specific brain sites involved in the inhibitory control of gastric emptying by TLQP-21 remain to be elucidated. However, the presence of immunoreactivity for VGF in various hypothalamic nuclei such as paraventricular nucleus and arcuate nucleus, involved in the control of GI functions (Tebbe *et al*., 2001), suggests that TLQP-21 could be active in these areas.

In conclusion, among the VGF-derived peptides tested, only TLQP-21 played a role in the local and central regulation of gastric motor functions through a PG-dependent mechanisms.

In view of the primary role that stomach plays in feeding, the gastric anti-emptying effect induced by TLQP-21 certainly represents a signal of satiety which, together with the peptide-induced increase of energy expenditure previously reported (Bartolomucci *et al*., 2006), could be a further mechanism by which this new brain-gut neuropeptide could prevent weight gain and obesity.

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Conflict of interest

The authors state no conflict of interest.

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