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ORIGINAL ARTICLE

Characterization of the mechanisms involved in the gastric antisecretory effect of TLQP-21, a vgf-derived peptide, in rats

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Abstract TLQP-21, a *vgf*-derived peptide modulates gastric emptying and prevents ethanol-induced gastric lesions in rats. However, it remains to be studied whether or not TLQP-21 affects gastric acid secretion. In this study, we evaluated the effects of central (0.8–8 nmol/rat) or peripheral (48–240 nmol/kg, intraperitoneally) TLQP-21 administration on gastric acid secretion in pylorus-ligated rats. The mechanisms involved in such activity were also examined. Central TLQP-21 injection significantly reduced gastric acid volume and dose-dependently inhibited total acid output (ED_{50} = 2.71 nmol), while peripheral TLQP-21 administration had no effect. The TLQP-21 antisecretory activity was prevented by cysteamine (300 mg/kg, subcutaneously), a depletor of somatostatin, by indomethacin (0.25 mg/rat, intracerebroventricularly), a non-selective cyclooxygenase inhibitor, and by functional ablation of sensory nerves by capsaicin. We conclude that TLQP-21 could be considered a new member of the large group of regulatory peptides affecting gastric acid secretion. The central inhibitory effect of TLQP-21 on gastric acid secretion is mediated by endogenous somatostatin and prostaglandins and requires the integrity of sensory nerve fibres.

Keywords TLQP-21 · Gastric acid secretion · Somatostatin · Prostaglandins · Sensory fibres

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Introduction

Despite our vast knowledge, the understanding of the control of gastric acid secretion is far from complete. Gastric acid secretion, in fact, is regulated by a complex network of afferent and efferent pathways of central and enteric nervous system as well as neuroendocrine and immune cells acting via autocrine, paracrine and hormonal pathways (Schubert and Peura 2008).

In the last years, numerous peptides including leptin, orexin and ghrelin have been shown to be involved in the regulation of gastric acid secretion (Schubert 2008). The main source of these peptides is the gastric mucosa; however, most of them are also produced in the hypothalamus (Lu et al. 2002; Cowley et al. 2003). Within the hypothalamus, there is a complex interaction between several nuclei involved in the regulation of gastric acid secretion including arcuate nucleus (ARC), the paraventricular (PVN), the lateral hypothalamic area (LHA), the ventromedial nucleus (VMH) and the dorsomedial nucleus (DMH) (Taché and Yang 1990). In particular, the ARC is considered one of the most important hypothalamic centres that regulate gastrointestinal functions, since it integrates signals from the periphery as well as from the brain stem nuclei.

Among the various peptides showing a dual localization in the brain and in the gut, recently the attention has been focused on the VGF peptides derived from the *vgf* gene, originally identified as a nerve growth factor responsive gene (Levi et al. 1985; Salton et al. 1991). The *vgf* gene encodes for a polypeptide comprising 615 (humans) and 617 (rodents) amino acids that, upon processing by the prohormone convertase PC1/3 and PC2, yields several peptides that are stored in dense core granules and released through a regulated secretory pathway (Possenti et al. 1989; Trani et al. 2002).

Vgf mRNA has a tissue-specific pattern of expression limited to neurons within the central and peripheral nervous system and to various endocrine cells (Trani et al. 1995; Levi et al. 2004). In the rat brain, VGF immunoreactivity has been detected in the cortex, hippocampus, cerebellum and olfactory system and in various hypothalamic nuclei such as PVN and ARC (Salton et al. 2000; Levi et al. 2004). In the peripheral nervous system, *vgf* mRNA is highly expressed in sympathetic, primary sensory neurons and in myenteric plexus ganglia, with clear evidence of expression in the glandular portion of the stomach, indicating the presence of this gene throughout the gastrointestinal tract (Ferri et al. 1992; Liu et al. 1994; Salton et al. 2000; Snyder et al. 2003).

More recently, it has been reported that primary cultures of rat gastric entero-chromaffin-like cells strongly and specifically express *vgf* (Lambrecht et al. 2006). Furthermore, Rindi and colleagues (2007), have described proVGF-related peptides in human entero-chromaffin-like cells and related hyperplastic and neoplastic cells.

Vgf is regulated in the hypothalamus in response to feeding and/or fasting, and in the dorsal vagal complex of the medulla oblongata in response to duodenal ulcer formation. Vgf expression in the course of ulcerative lesions is up-regulated in brainstem nuclei which control vagal efferent and afferent communications with the stomach (Kanemasa et al. 1995a, b).

Among the various VGF-derived peptides, we focused our attention on a new peptide designated as TLQP-21, which spans from the residue 556 to residue 576 of the precursor sequence. This peptide, in fact, represents one of the major peptide fragments present in the brain (Bartolomucci et al. 2006) and has been shown to exert a role in the control of gastrointestinal motility and metabolic functions (Bartolomucci et al. 2008; Severini et al. 2009). Furthermore, the recent evidence that TLQP-21 is involved in gastric mucosal defence mechanisms (Sibilia et al. 2010), prompted us to investigate the possible role of TLQP-21 in the regulation of gastric acid secretion. TLQP-21 was administered both centrally (i.c.v.) or peripherally to conscious pylorus-ligated rats.

To clarify the mechanism(s) involved in the effects of TLQP-21 on gastric acid secretion, we first examined the involvement of somatostatin (SRIF). Reportedly, SRIF participates in the inhibitory control of gastric acid secretion. For this purpose, TLQP-21 was administered i.c.v. in rats pre-treated with Cysteamine (Cys), which is a specific depletory of SRIF in central and peripheral rat tissues (Szabo and Reichlin 1981; Sagar et al. 1982). On the basis of the results obtained, we examined the effects of TLQP-21 on the expression of SRIF mRNA in the hypothalamus. We then studied the neuronal mechanisms conveying the inhibitory effect of TLQP-21 from the brain to the stomach. In particular, we examined whether or not sensory afferent fibres and prostaglandin system (PGs) are involved on TLQP-21-antisecretory activity, as previously reported for TLQP-21-induced gastroprotection (Sibilia et al. 2010).

Methods

Animals

Male Sprague–Dawley rats, weighing 200–250 g (Charles-River, Calco, Italy) were housed in single cages which had wire net bottoms to avoid coprophagy. Before starting the experiments, all rats were deprived of food for 24 h, but allowed free access to tap water until the beginning of the treatments.

For the i.c.v. administration of peptides, a polyethylene cannula (PE10) was implanted into the left lateral ventricle of the brain, 5 days before the experiment, as previously described (Netti et al. 1984). At the end of experiments, dye (0.5% Evans blue) was injected through the cannula to confirm its position in the ventricle.

All procedures were performed in accordance with the Italian Guidelines for the use of animals in Medical Research and conformed with the European Community Directive of November 1986 (86/609/EEC), and were approved by the Institutional Animal Care and Use Committee.

Drugs

TLQP-21 (TLQPPASSRRHFHHALPPAR) synthesized by PRIMM (Milano, Italy) was dissolved in saline immediately before the experiment and was injected in a volume of 5 µl/rat, i.c.v., 2 ml/kg intraperitoneally (i.p.).

Cysteamine hydrochloride (Cys, Sigma-Aldrich, UK) was dissolved in double distilled water and titrated to pH 7 with NaOH solution 1 N and given subcutaneously (s.c.) at a dose of 300 mg/kg in a volume of 2 ml/kg.

Indomethacin (Indo, Liometacen; Promedica, Parma, Italy) was suspended in saline and administered i.c.v. at the dose of 0.25 mg/rat.

Capsaicin (Capsa, Sigma-Aldrich, UK) was dissolved in an appropriate vehicle (8% EtOH and 6% tween 80 in physiological saline) and administered s.c. at the dosing volume of 2 ml/kg.

Theophylline and atropine (Sigma-Aldrich, UK) were dissolved in saline and administered i.p. at the dosing volume of 2 ml/kg

Experimental procedures

Gastric acid secretion

Acid secretion studies were performed using the pylorus ligation method as modified by Shay et al. (1954). Under light, short duration ether anaesthesia, ligation of pyloric sphincter was carried out through a small midline incision with minimal trauma to the stomach; thereafter, the abdominal wall was closed. In all the experiments, the animals were awake within 5 min after pylorus ligation and freely moving. The animals were euthanized with CO_2 inhalation 3 h after pylorus ligation, the oesophageal-gastric junction ligated so that the complete stomach could be removed and the contents collected and centrifuged. The volume of supernatant was measured and a sample (0.5 ml) taken and diluted appropriately (1:20) for titration with 0.01 N standard sodium hydroxide solution (Analar BDH Italia, Milan) to pH 7. The results were expressed as total volume secreted (ml/3 h) and total acid output (uEq/3 h).

In the first series of experiments, it was examined the effect of central (0.8–8 nmol/rat, i.c.v.) or peripheral (48 and 240 nmol/kg i.p) TLQP-21 administration on gastric acid secretion. Control rats received an equal amount of saline. TLQP-21 or saline was injected at the time of pylorus ligation.

SRIF and TLQP gastric effect

In the second series of experiments, it was considered the possible involvement of SRIF on the gastric effects of TLQP-21. For this purpose, we examined the effects of TLQP-21 (8 nmol/rat, i.c.v.) on acid gastric secretion in rats pretreated (4 h before) with Cys (300 mg/kg, s.c.) which depletes endogenous SRIF stores.

For PCR studies, two groups of six rats each were used; the rats were injected i.c.v. with TLQP-21 (8 nmol/rat) or saline. Rats were killed under ether anaesthesia by decapitation 3 h after i.c.v. injection and brain immediately removed. The hypothalamus was quickly dissected, placed in RNAlater (Qiagen SpA, Italy) and frozen at −20°C until processed. Total RNA was extracted from cells using TRIzol-like reagent, it is an improvement to the single-step, RNA isolation method developed by Chomczynski and Sacchi (1987). The integrity of RNA extracted from cells was examined by electrophoresis. Three thousands nanogram of total RNA were incubated with rDNase I (Ambion) for 20 min at 37°C to digest contaminating genomic DNA. Four hundred nanogram total RNA of each sample were subjected to reverse transcription with MMLV (Invitrogen, Carlsbad, CA, USA) followed by amplification using specific primers based on the published sequence of rat SRIF (forward primer 5′-ATGCTGTCCTGCCGTC TC-3′; reverse primer 5′-AGCCTCATCTCGTCCTG CT-3′, 252 bp fragment). Semiquantitative PCR analysis of total RNA yielded a DNA fragment of the expected length for all specific mRNAs. To normalize results for differences in RNA sampling, an aliquot of the same RT reaction was used to amplify a glyceraldehyde-6-phosphate (forward primer 5′-GCCATCAACGACCCCTTCATTG-3′; reverse primer 5′-TGCCAGTGAGCTTCCCGTTC-3′, 600 bp fragment). Negative controls of the PCR reaction were made omitting the specific primers from the reaction mixture.

To assure that PCR was performed in the linear amplification range, samples were initially analyzed after 15, 17, 20, 25, 27, 30, 35 and 40 cycles (data not shown). For each factor, we choose the cycle number that gave half of the maximal amplification.

COX inhibition and TLQP gastric effect

To explore the possible involvement of PGs in the gastric effect of TLQP-21, rats were pretreated with the non-selective COX inhibitor, Indo (0.25 mg/rat, i.c.v.) 5 min before TLQP-21 (8 nmol/rat, i.c.v.). Additional groups of rats received only TLQP-21 or Indo. Control rats were treated i.c.v. with an equal volume of saline. The dose of Indo was chosen on the basis of the previous studies (Saperas et al. 1991; Severini et al. 2009).

Capsaicin-sensitive fibres and TLQP-21 gastric effect

Under ether anaesthesia, rats were treated s.c. with increasing doses of 10, 20, 30 and 50 mg/kg Capsa on 4 consecutive days as previously described (Izbéki et al. 2002). To counteract the respiratory impairment associated with Capsa injection, the rats were pretreated i.p. with theophylline (5 mg/kg) and atropine (2 mg/kg) as previously reported (Sann et al. 1995). Control rats received an equal volume of vehicle for Capsa, theophylline and atropine. The effectiveness of the Capsa denervation was assessed 7 days after the last Capsa administration by instilling a drop of 1% NaOH solution into the left eye of each rat. Only rats with reduced wiping movements were

used. The animals were challenged with TLQP-21 (8 nmol/rat, i.c.v) or saline as described above.

Statistical analysis

Statistical analysis was performed with a statistic package (GraphPad Prism, GraphPad Software San Diego, CA). All data are represented as the mean ± SEM. Differences between the groups were assessed by one-way ANOVA followed by multicomparison Bonferroni test. The ED_{50} was calculated by linear regression analysis of the dose–response data on inhibition of gastric acid output. A probability of $p < 0.05$ was considered significant.

Results

Effects of TLQP-21 on gastric acid secretion

Figure 1 shows the effects of central TLQP-21 administration on gastric acid secretion in pylorus-legated rats. TLQP-21 significantly inhibited gastric acid secretion reaching a maximal inhibition at the highest (8 nmol/rat) dose used. This decrease in gastric acid secretion results from a dose-dependent reduction in acid concentration and to a lesser extent to a fall in the volume of gastric secretion. The ED_{50} value calculated from the log dose–response curve on inhibition of gastric acid output was 2.71 nmol. When peripherally administered TLQP-21 did not significantly modify gastric acid secretion even if administered at the high dose of 240 nmol/kg. Total gastric volume were 4.7 ± 0.41 in saline-treated rats; 4.85 ± 0.59 and 4.99 ± 0.56 in rats treated i.p. with TLQP-21 at the dose of 48 or 240 nmol/kg, respectively.

The role of SRIF in the inhibitory effect of centrally administered TLQP-21 on gastric acid secretion

In order to examine the role of SRIF in the inhibitory action of TLQP-21 on gastric acid secretion, rats were pretreated with Cys. As expected, Cys significantly increased (+53%) gastric acid volume compared with that detected in saline-treated rats and completely prevented the inhibitory action of TLQP-21 both on gastric acid volume and acid output. Even if in Cys-TLQP-21-treated rats there was a tendency to increase the stimulatory activity of Cys on gastric acid secretion, this increase did not reach statistical significance (Fig. 2a, b).

The hypothalamic SRIF mRNA levels were not influenced by central TLQP-21 injection (data not shown).

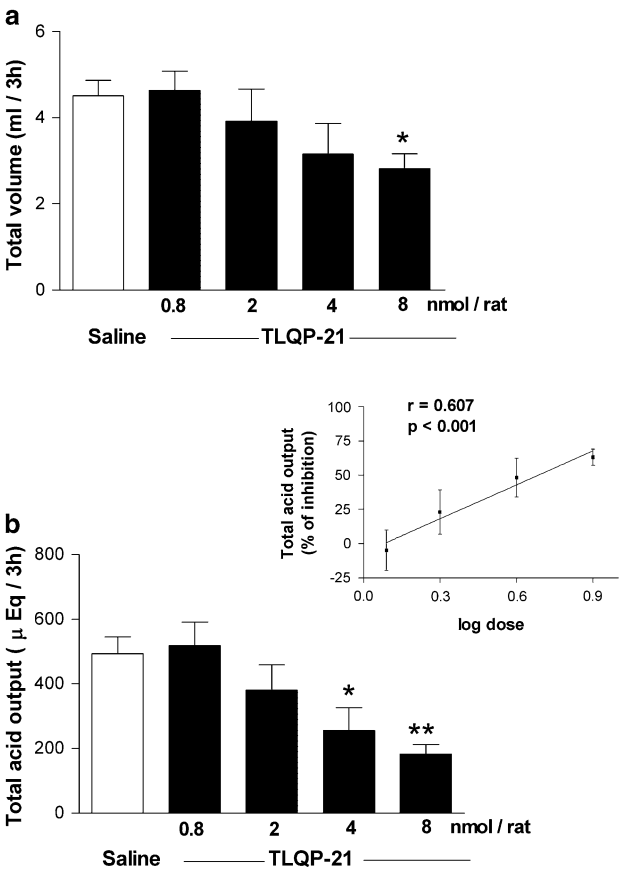

Fig. 1 Effect of different doses of TLQP-21 (0.8–8 nmol/rat) administered i.c.v. on gastric acid secretion (volume **a**, and total acid output **b**) in conscious pylorus-ligated rats, 3 h after pylorus ligation. Each value is the mean ± SEM of 9–11 animals. $*P < 0.05$, $**P < 0.01$ versus saline group. Inset figure represents the log dose–response curve for the antisecretory activity of TLQP-21. Data are expressed as a percentage of inhibition of total acid output of control saline-treated rats

Effect of capsaicin denervation and COX inhibition on TLQP-21-induced antisecretory activity

To examine the possible involvement of capsaicin-sensitive sensory fibres in the inhibitory action of centrally administered TLQP-21 (8 nmol/rat), rats were pretreated with Capsa. Capsa denervation slightly increases both gastric acid volume (11%) and acid output (7%) compared with saline-treated rats and completely removed the inhibitory effect of TLQP-21 on gastric acid secretion (Fig. 3a, b).

To explore the role of PGs in the inhibitory action of TLQP-21 on gastric acid secretion, rats were pretreated with Indo (0.25 mg/rat, i.c.v.), a non selective COX inhibitor. Indo, per se, did not modify gastric acid secretion but significantly reduced (−30%) the inhibitory effect of central injection of TLQP-21 (8 nmol/rat, i.c.v.) on gastric acid volume and acid output (Fig. 4a, b).

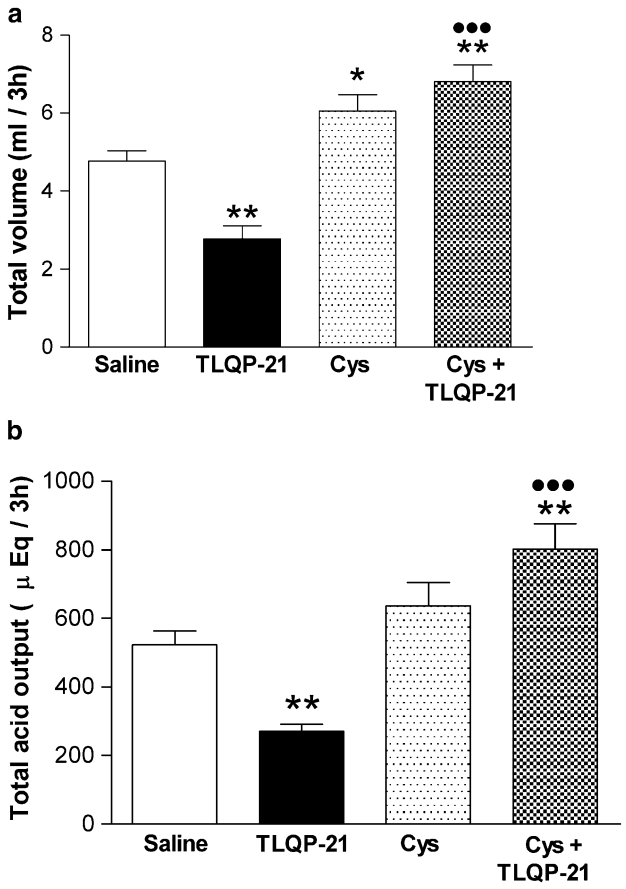

Fig. 2 Effect of central TLQP-21 (8 nmol/rat) administration on gastric acid secretion (volume **a**, and total acid output **b**) in conscious pylorus-ligated rats pretreated (4 h before) with cysteamine (Cys, 300 mg/kg, s.c.) Each value is the mean ± SEM of 8–10 animals. $^{*}P < 0.05$; $^{**}P < 0.01$ versus saline; $^{\bullet\bullet\bullet}P < 0.001$ versus TLQP-21

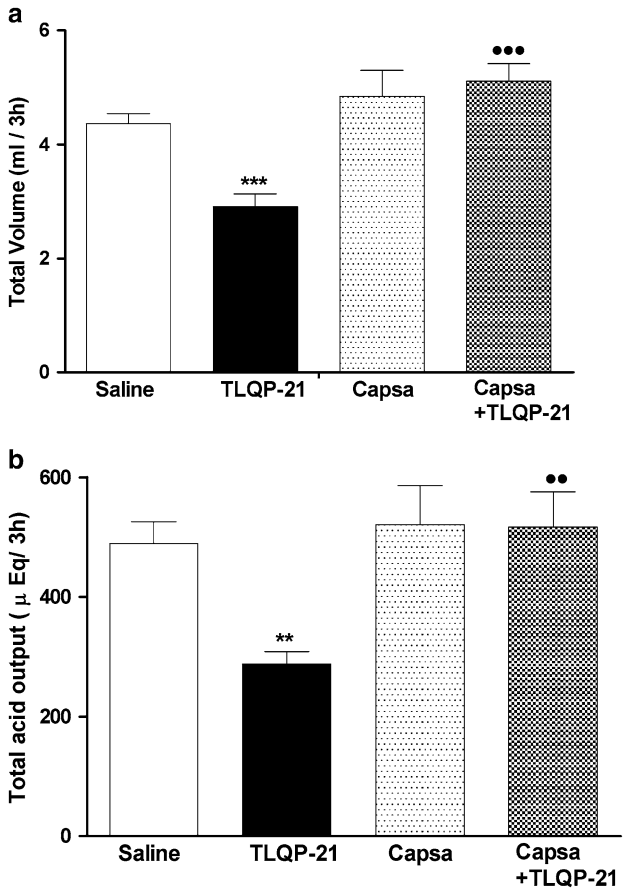

Fig. 3 Effect of central TLQP-21 (8 nmol/rat) administration on gastric acid secretion (volume **a**, and total acid output **b**) in conscious pylorus-ligated rats with intact sensory nerves or with Capsa-induced sensory denervation. Each value is the mean ± SEM of 8–10 animals. $^{**}P < 0.01$, $^{***}P < 0.001$ versus saline; $^{\bullet\bullet}P < 0.01$ $^{\bullet\bullet\bullet}P < 0.001$ versus TLQP-21

Discussion

The present study shows, for the first time, that centrally administered TLQP-21 exerts a significant inhibition of gastric acid secretion in pylorus-ligated rats. When peripherally administered TLQP-21 did not significantly modify gastric acid secretion.

The TLQP-21-antisecretory activity is in line with our previous studied showing a central TLQP-21 protective action on the gastric mucosa exposed to the noxious agent ethanol (Sibilia et al. 2010), indicating for the peptide a role in gastrointestinal defense. Supporting this hypothesis, VGF is induced in response to fasting in the ARC (Hahm et al. 1999) and during fasting a decrease in acid secretion is important for the maintenance of gastric mucosal integrity.

The specific brain sites involved in the inhibitory control of gastric acid secretion by TLQP-21 remain to be elucidated. However, the presence of immunoreactive VGF in hypothamic nuclei such as PVN and ARC, involved in the control of gastric acid secretion (Tebbe et al. 2001), suggests that these nuclei could represent one of the brain sites in question.

Other brain areas which could be involved in the antisecretory activity of TLQP-21 are NTS and DMV. Previous studies, in fact, have reported that ulcerative lesions induced by Cys, induce VGF mRNA in neurons of both NTS and DMV which directly project to the stomach (Kanemasa et al. 1995a; Salton et al. 2000). Considering that the NTS/DMV complex plays a key role in the integration of vagal afferent and efferent fibres that mediate gastric secretory activity, it is possible that central TLQP-21 could reduce vagal activity, thus resulting in inhibition of gastric acid secretion. It is well known that cholinergic neurons stimulate acid secretion directly or indirectly by inhibiting SRIF secretion which is the principal inhibitor of acid gastric secretion (Schubert 2003).

Several lines of evidence indicate a link between the SRIF system and the inhibitory control of gastric acid

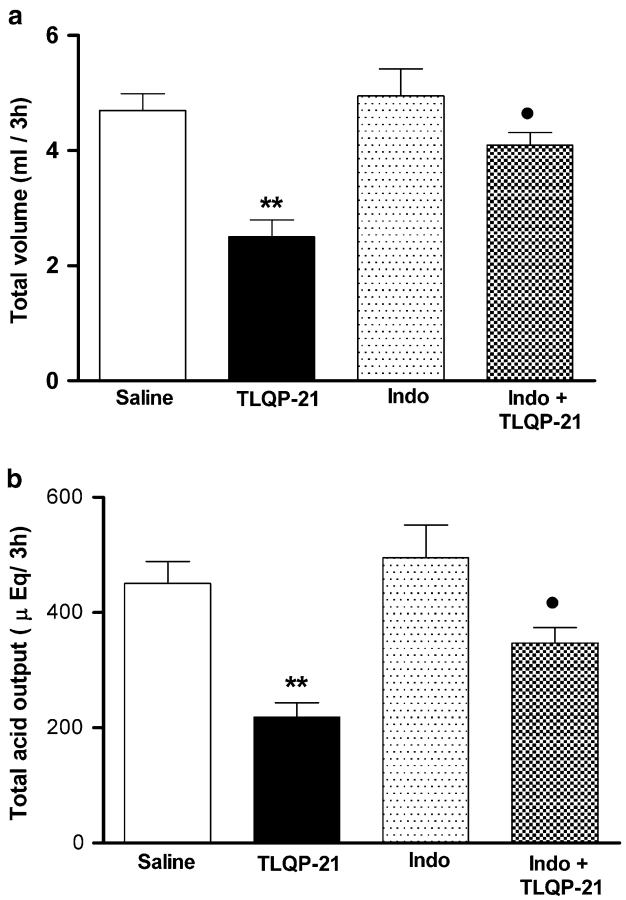

Fig. 4 Effect of central TLQP-21 (8 nmol/rat) administration on gastric acid secretion (volume **a**, and total acid output **b**) in conscious pylorus-ligated rats pretreated (5 min before) with Indo (0.25 mg/rat, i.c.v.) Each value is the mean ± SEM of 8–10 animals. $^{**}P < 0.01$ versus saline; $^{\bullet}P < 0.05$ versus TLQP-21

secretion by TLQP-21. First, it has been reported the presence of VGF immunoreactivity in SRIF cells in the pancreas, hypothalamus and stomach (Cocco et al. 2007). Second, in the present study we have shown that pretreatment with Cys, a specific depletory of endogenous (SRIF) in central and peripheral rat tissues (Szabo and Reichlin 1981; Sagar et al. 1982), completely prevented the inhibitory effect of TLQP-21 on gastric acid secretion.

It is unlikely that TLQP-21 could directly stimulate SRIF secretion from D cells since, when peripherally administered, the peptide has no effect on gastric secretion.

Also the possibility that TLQP-21 could modify brain SRIF tone seems to be ruled out since previous (Bartolomucci et al. 2007) and present data indicate a lack of effect of central TLQP-21 on hypothalamic SRIF mRNA levels. Taken together, these observations suggest that TLQP-21 could decrease vagal efferent activity thus increasing SRIF secretion in the stomach leading to an inhibition of gastric acid secretion.

Another possible explanation for the effectiveness of Cys in preventing the TLQP-21 antisecretory effect, could be the ability of central TLQP-21 to activate primary sensory fibres thus resulting in an increased SRIF tone. Capsaicin has been widely used to investigate the possible role of afferent sensory nerves in various physiological and pathological conditions. The action of Capsa consists in an initial short-lasting stimulation, followed by the desenzitation of afferent sensory nerves.

It has been reported, that acute Capsa inhibits gastric acid secretion in pylorus-ligated rats (Abdel Salam et al. 1995), while long-term Capsa injection causes hyperacidity in the same experimental condition (Mózsik et al. 2001). A link between Capsa sensory nerves and SRIF is suggested by the evidence that peptides such CGRP and SRIF, which are involved in the inhibitory control of gastric acid secretion, are released from capsa-sensitive nerve endings (Horie et al. 2004; Szolcsányi and Barthó 2001). CGRP, in turn, releases SRIF from D cells (Kawashima et al. 2002) and it has been reported a decrease in gastric SRIF mRNA levels in rats pretreated with sensory neurotoxic Capsa (Sandvik et al. 1993). Furthermore, Cys administration depletes not only SRIF but also CGRP in that rat stomach and this effect was absent in Capsa-desensitized rats (Evangelista et al. 1992).

The present results showing the disappearance of TLQP-21-induced antisecretory activity in Capsa-pretreated rats are in line with the previous studies showing that central TLQP-21 injection exerts a gastroprotective effect against ethanol-induced gastric lesions that requires the integrity of sensory fibres (Sibilia et al. 2010).

The observation that i.c.v administered PGE2 inhibited vagally stimulated gastric secretion in conscious rats (Yokotani et al. 1988; Yokotani et al. 1996), together with the evidence that central Indo prevents the inhibitory activity of TLQP-21 on gastric emptying (Severini et al. 2009), lead us to examine whether or not central PGs could be participate in the inhibitory role of the peptide in the control of gastric acid secretion. Reportedly, central and peripheral PGs are involved in the regulation of gastric acid secretion (Sautereau et al. 1991).

In agreement with the previous data on gastric emptying, we have shown that Indo pretreatment significantly reduced the antisecretory activity of TLQP-21. Thus, it is possible that TLQP-21, by interacting with central PG receptors could reduce vagal activity resulting in inhibition of gastric acid secretion.

It remains to be clarified whether or not peripheral PGs could be involved in the antisecretory effect of TLQP-21. In vitro studies performed in rat longitudinal forestomach strips have shown a stimulatory effect of TLQP-21 on PG release due to the direct action on PG receptors (Severini et al. 2009). Furthermore, previous in

vivo studies have reported that central TLQP-21 increases PGE2 content in the gastric mucosa exposed to EtOH (Sibilia et al. 2010).

Overall, these findings indicate that TLQP-21 is a member of the large group of regulatory peptides involved in the central inhibitory control of gastric acid secretion. The TLPQ-21 antisecretory activity is mediated by endogenous SRIF and PG and requires the integrity of Capsa-sensitive afferent fibres.

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