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Peripheral PACAP inhibits gastric acid secretion through somatostatin release in mice

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1 Studies in rats suggest that PACAP modulates gastric acid secretion through the release of both histamine and somatostatin.

2 We characterized the effects of exogenous PACAP on gastric acid secretion in urethane-anesthetized mice implanted with a gastric cannula and in conscious 2-h pylorus ligated mice, and determined the involvement of somatostatin and somatostatin receptor type 2 (SSTR2) by using somatostatin immunoneutralization, the SSTR2 antagonist, PRL-2903, and SSTR2 knockout mice.

3 Urethane-anesthetized wild-type mice had low basal acid secretion ($0.10 \pm 0.01\ \mu$mol $(10\,\text{min})^{-1}$) compared with SSTR2 knockout mice ($0.93 \pm 0.07\ \mu$mol $(10\,\text{min})^{-1}$). Somatostatin antibody and PRL-2903 increased basal secretion in wild-type mice but not in SSTR2 knockout animals.

4 In wild-type urethane-anesthetized mice, PACAP-38 ($3–270\ \mu\text{g}\,\text{kg}^{-1}\,\text{h}^{-1}$) did not affect the low basal acid secretion, but inhibited the acid response to pentagastrin, histamine, and bethanechol.

5 In wild-type urethane-anesthetized mice pretreated with somatostatin antibody or PRL-2903 and in SSTR2 knockout mice, peripheral infusion of PACAP-38 or somatostatin-14 did not inhibit the increased basal gastric acid secretion.

6 In conscious wild-type mice, but not in SSTR2 knockout mice, PACAP-38 inhibited gastric acid secretion induced by 2-h pylorus ligation. The antisecretory effect of PACAP-38 was prevented by immunoneutralization of somatostatin.

7 These results indicate that, in mice, peripheral PACAP inhibits gastric acid secretion through the release of somatostatin and the activation of SSTR2 receptors. There is no evidence for stimulatory effects of PACAP on acid secretion in mice.
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Abbreviations: ANOVA, analysis of variance; ECL, enterochromaffin-like; KLH, keyhole limpet hemocyanin; PACAP, pituitary adenylate cyclase-activating polypeptide; PAC_1, PACAP receptor type 1; SSTR1–5, somatostatin receptor type 1–5; VIP, vasoactive intestinal polypeptide; $VPAC_1$/$VPAC_2$, VIP/PACAP receptor type 1/VIP/PACAP receptor type 2

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family of regulatory peptides. It has two bioactive forms (PACAP-27 and PACAP-38), derived from a common precursor (Vaudry *et al*., 2000), that are equipotent in *in vivo* and *in vitro* systems (Miyata *et al*., 1989). Biological effects of PACAP are mediated through three distinct receptors, pharmacologically classified as PAC_1 (PACAP receptor type 1), $VPAC_1$ (classical VIP receptor) and $VPAC_2$ (previously VIP_2) (Harmar *et al*., 1998). The PAC_1 receptor has high affinity for PACAP peptides but a low affinity for the other family members, whereas $VPAC_1$ and $VPAC_2$ receptors have nearly equal affinity for VIP and PACAP (Pisegna & Wank, 1993; Vaudry *et al*., 2000; Laburthe & Couvineau, 2002). Both PACAP and PACAP receptors are widely expressed in the gastrointestinal tract, the longer form (PACAP-38) being the more abundant in the gut (Sundler *et al*., 1992; Lauffer *et al*., 1999). In particular, in the stomach, PACAP immunoreactivity has been described both in enteric cell bodies and nerve fibers in the gastric smooth muscle layers and mucosa (Sundler *et al*., 1992; Hannibal *et al*., 1998; Miampamba *et al*., 2002). Similarly, PAC_1 receptors have been localized in the gastric corpus both in neuronal elements and in enterochromaffin-like (ECL) cells by immunocytochemistry and RT–PCR (Zeng *et al*., 1999; Miampamba *et al*., 2002). From these morphological observations, it has been suggested that PACAP, acting as a neuropeptide, might play an important role in the regulation of gastric acid secretion.

Although several studies have shown that PACAP modulates gastric acid secretion, the results obtained are limited to a single species, the rat, and are in some cases contradictories.

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Studies *in vitro* in an isolated rat stomach preparation have shown that PACAP stimulates gastric acid secretion through the release of ECL cell histamine (Sandvik *et al*., 2001). This has been confirmed by measuring the release of histamine *in vivo* using microdialysis techniques (Norlen *et al*., 2001) and *in vitro* in ECL cell-enriched cultures (Zeng *et al*., 1999; Lindström & Håkanson, 2001). However, using the same experimental approaches, as well as conscious rats, other reports showed that PACAP inhibits both basal and secretagogues-stimulated gastric acid secretion, and these effects were related to the local release of somatostatin, secretin, and prostaglandins (Mungan *et al*., 1995; Li *et al*., 2000). In addition, *in vitro* studies in isolated mouse stomach showed that VIP produced a weak and transient stimulation of acid secretion, followed by a sustained increase in somatostatin secretion (Schubert, 1991). A direct effect of both PACAP and VIP on D cells has been later demonstrated *in vitro*, probably mediated through VIP/PACAP receptors (Zeng *et al*., 1996; 1999; Zeng & Sachs, 2002). These observations indicate that PACAP exerts a dual action, releasing both a stimulant (histamine) and an inhibitor (somatostatin) of acid secretion, through a direct activation of ECL and D cells, respectively. Therefore, the acid response observed to PACAP administration would result from a balance between the histamine-dependent stimulatory effects and the somatostatin-dependent inhibitory effects. Indeed, Zeng *et al*. (1999) demonstrated that the *in vivo* immunoneutralization of somatostatin busted the acid secretory response elicited by PACAP in the rat, supporting a parallel stimulation of D and ECL cells during peripheral PACAP administration.

Somatostatin actions are mediated through the activation of five different receptor subtypes (SSTR1–SSTR5) (Patel, 1999; Martinez, 2002). Although the five somatostatin receptor subtypes are localized in the stomach (Prinz *et al*., 1994; Le Romancer *et al*., 1996; Krempels *et al*., 1997; Sternini *et al*., 1997; Schindler & Humphrey, 1999), functional *in vivo* studies in rats, dogs, and mice as well as *in vitro* studies in human, rat, and dog antral tissue suggest that somatostatin effects on gastric acid secretion are mediated through the activation of SSTR2 receptors located primarily on ECL cells, where they inhibit the release of histamine (Rossowski *et al*., 1994; Lloyd *et al*., 1995; Zaki *et al*., 1996; Aurang *et al*., 1997; Fung & Greenberg, 1997; Martinez *et al*., 1998; Patel, 1999; Martinez, 2002; Piqueras *et al*., 2003b). In addition, somatostatin also regulates gastrin gene expression and gastrin release from G cells (Karnik *et al*., 1989; Chiba & Yamada, 1994). *In vitro* studies in canine antral G cells suggest that modulatory effects of somatostatin on gastrin release are also mediated through SSTR2 receptors (Lloyd *et al*., 1997).

The objectives of the present studies were first to characterize the *in vivo* effects of peripheral infusion of PACAP on gastric acid secretion in mice by assessing changes in basal gastric acid secretion and in the secretory response to various secretagogues. Second, we examined whether somatostatin is involved in PACAP effects by using *in vivo* immunoneutralization of endogenous somatostatin. Lastly, the role of SSTR2 receptors was investigated using mice with specific deletion of the SSTR2 receptor gene (Zheng *et al*., 1997) and the selective SSTR2 antagonist PRL-2903 (Rossowski *et al*., 1998; Kawakubo *et al*., 1999; Piqueras *et al*., 2003b).

Methods

Animals

Adult male mice (20–30 g, 3–6 months of age) were used. Mice deficient for the SSTR2 receptor were generated by gene targeting in mouse embryonic stem cells using a neomycin cassette with the entire *SSTR2* gene on a 129Sv/C57B16 hybrid background (Zheng *et al*., 1997). The original knockout mice were genotyped to be homozygous $-/-$ SSTR2 mutant or $+/+$ wild-type mice by Southern blot analysis (Zheng *et al*., 1997) and then maintained from the F1 as inbred colonies. Mice used in the present study were born from different litters; all descendants are born from genotyped littermates obtained through inbreeding. The appearance, behavior, and gastrointestinal and brain morphology of knockout mice appeared indistinguishable from those of wild-type mice (Zheng *et al*., 1997; Martinez *et al*., 1998). Mice were maintained in group cages (five to six mice per cage) on a 12 : 12-h light–dark cycle with controlled conditions of temperature (22°C) and humidity (60%), with food (Harlan Ibérica S.A., Spain) and tap water *ad libitum*. All experiments were performed in mice fasted for 16–18 h but with free access to water up to the beginning of the experiments. Animal care and handling were done in accordance with the regulations of the American Physiological Society. All animals were humanely euthanized, following current regulations, at the end of the experiments.

Treatments

Pentagastrin (Peptavlon; Ayerst Laboratories, New York, NY, U.S.A.), histamine (Sigma-Aldrich, St Louis, MO, U.S.A.), bethanechol (Sigma-Aldrich), somatostatin-14 (Peptides International Inc., Louisville, Kentucky, U.S.A.) and PACAP-38 (Peptides International Inc.) were dissolved in 0.9% saline. The selective SSTR2 antagonist PRL-2903 (Fpa-c[D-Cys-Pal-D-Trp-Lys-Tle-Cys]-Nal-NH_2; also known as DC 41-33; provided by Dr D.H. Coy, Tulane University, New Orleans, LA, U.S.A.) (Rossowski *et al*., 1998) was dissolved in 0.9% saline. All solutions were prepared immediately before each experiment. Purified monoclonal somatostatin antibody (CURE S.6) and purified monoclonal antibody to keyhole limpet hemocyanin (KLH) (provided by CURE:Digestive Diseases Research Center, UCLA, Los Angeles, CA, U.S.A.) were used for *in vivo* immunoneutralization. Production, characterization, and purification of the monoclonal antibodies have been described in detail previously (Kovacs *et al*., 1989; Wong *et al*., 1990). Doses of compounds were selected according to previous studies in mice (Martinez *et al*., 1998; Piqueras *et al*., 2003a, b).

Gastric acid secretion in urethane-anesthetized mice

Gastric acid secretion was monitored in urethane-anesthetized mice, as previously described (Martinez *et al*., 1998; Piqueras *et al*., 2003a, b). Fasted wild-type ($+/+$) and SSTR2 knockout ($-/-$) mice were anesthetized with urethane (1.25 g kg^{-1}, about 0.2 ml, i.p.). The trachea was cannulated to ensure a clear airway and the esophagus ligated. Thereafter, the abdomen was opened and the pylorus ligated. An incision was made in the nonglandular portion of the stomach, the

gastric lumen was rinsed until clean with warm 0.9% saline, and a double-lumen gastric cannula was inserted through the forestomach incision. A catheter [30-gauge needle inserted into polyethylene E-10 tubing (Baxter, Irvine, CA, U.S.A.)] was placed into the ileal vein for constant intravenous infusion of saline (0.1 ml h^{-1}) and administration of substances. Gastric acid secretion was determined by continuous intragastric perfusion with warm saline (pH 7.0, 0.3 ml min^{-1}). After the surgery, a 30–45 min period was allowed for stabilization and thereafter the effluents were collected at 10 min intervals and back titrated to pH 7.0 (0.001 N NaOH) with an automatic titrator (Radiometer Copenhagen).

Gastric acid secretion after pylorus ligation in conscious mice

Pylorus ligation was performed as previously described in rats (Kato *et al*., 1995) with appropriate modifications to mice. Under a 5–6 min halothane anesthesia (Fluothane®, AstraZeneca), a small laparotomy was performed and the pylorus was localized and ligated with 4-0 silk. Then the abdominal cavity was closed by suture with 4-0 silk. The animals regained consciousness in a 4–5 min period, and were maintained undisturbed in their home cages, without food or water, for a 2 h period. Thereafter, animals were euthanized by cervical dislocation followed by thoracotomy, the stomach dissected, the gastric content collected and measured with a micropipette and the gastric lumen washed with 3.0 ml of saline solution (pH 7.0). The gastric content plus the washout solution were centrifuged (3500 r.p.m., 10 min, 4°C) and the supernatant used for pH measurement and determination of gastric acid output (μmol $(2\,h)^{-1}$) by titration with 0.01 N NaOH. Acid concentration (mmol l^{-1}) was calculated by dividing the 2-h acid output by the volume of secretion.

Experimental protocols

In urethane-anesthetized mice, gastric acid secretion was monitored every 10 min for 30–60 min before treatments and for an additional 30 min period after ending the infusion of peptides. Vehicle or peptide infusion was performed using an i.v. infusion rate of 0.1 ml h^{-1}.

Effects of i.v. infusion of PACAP-38 in basal and/or secretagogues-stimulated gastric acid secretion in wild-type and SSTR2 knockout mice In urethane-anesthetized wild-type mice with gastric cannula, after a 30 min basal period, saline (0.1 ml) was infused for 1 h, thereafter, PACAP-38 (3, 7, 22, 45, 135 or 270 μg kg^{-1}, corresponding to 0.66–59.5 nmol kg^{-1}) was infused, 1 h for each dose, in increasing cumulative doses. In other studies, after a 30 min basal gastric secretion, pentagastrin (16 μg kg^{-1} h^{-1}), histamine (5 mg kg^{-1} h^{-1}) or bethanechol (0.6 mg kg^{-1} h^{-1}) was infused i.v. and, 30 min later, either saline or PACAP-38 (45, 135 or 270 μg kg^{-1}) was infused i.v. for 1 h. In a control group, only vehicles (saline solution) were administered, following the same protocol. In urethane-anesthetized SSTR2 knockout mice, after a 1 h basal period, saline (0.1 ml) was infused for 1 h, thereafter PACAP-38 (3, 7, 22, 45, 135 or 270 μg kg^{-1}) was infused, 1 h for each dose, in increasing cumulative doses. In a separate group of SSTR2 knockout mice, the effects of a single 1-h infusion of PACAP-38 (135 μg kg^{-1}) or vehicle (0.1 ml) on basal gastric acid secretion were also determined.

Effects of i.v. infusion of somatostatin-14 on pentagastrin-stimulated gastric acid secretion in wild-type mice and on basal secretion in SSTR2 knockout mice In urethane-anesthetized wild-type mice with gastric cannula, after a 30 min basal period, pentagastrin (16 μg kg^{-1} h^{-1}) was infused i.v, and 30 min later, a 1 h infusion of somatostatin-14 (20 μg kg^{-1}) or vehicle saline (0.1 ml h^{-1}) was started. In urethane-anesthetized SSTR2 knockout mice, after a 1 h basal period, either somatostatin-14 (20 μg kg^{-1} h^{-1}) or vehicle saline (0.1 ml h^{-1}) was infused for 1 h.

Effects of i.v. infusion of PACAP-38 on gastric acid secretion in wild-type and SSTR2 knockout mice pretreated with somatostatin monoclonal antibody In wild-type urethane anesthetized mice with a gastric cannula, after a 30-min basal period, purified somatostatin monoclonal antibody (CURE S.6, 150 μg per mouse, 0.1 ml) was administered i.v. After 30 min either saline, somatostatin-14 (20 μg kg^{-1} h^{-1}) or PACAP-38 (135 μg kg^{-1} h^{-1}) was infused i.v. for a 1 h period. In urethane-anesthetized SSTR2 knockout mice, after a 1 h basal period, either somatostatin monoclonal antibody (150 μg per mouse) or control antibody (anti-KLH, 150 μg per mouse) was administered i.v. (0.1 ml).

Effects of the selective SSTR2 antagonist PRL-2903 on basal secretion in wild-type and SSTR2 knockout urethane anesthetized mice In wild-type animals, after a 30 min basal period, PRL-2903 was administered as a bolus (1.5 mg kg^{-1}, 0.1 ml, i.v.) followed by a continuous i.v. infusion (1.5 mg kg^{-1} h^{-1}) during a 2-h period (total dose administered: 4.5 mg kg^{-1}). At 10 min after starting PRL-2903 infusion, either somatostatin-14 (20 μg kg^{-1} h^{-1}), PACAP-38 (135 μg kg^{-1} h^{-1}) or vehicle saline (0.1 ml h^{-1}) was infused i.v. for 1 h. In SSTR2 knockout mice, after a 30 min basal period, PRL-2903 was administered as a bolus (1.5 mg kg^{-1}, 0.1 ml, i.v.), followed by a 2 h infusion (1.5 mg kg^{-1} h^{-1}).

Effects of PACAP-38 and pentagastrin on gastric acid secretion in wild-type and SSTR2 knockout conscious mice with pylorus ligation for 2 h In wild-type or SSTR2 −/− mice under short halothane anesthesia, either pentagastrin (16 μg kg^{-1}), PACAP-38 (135 μg kg^{-1}) or vehicle saline (0.1 ml) was injected i.v., through the ileal vein, immediately before pylorus ligation. Mice regained the righting reflexes within 5 min and gastric acid secretion was assessed 2 h later. A separate group of SSTR2 +/+ mice received a bolus injection of somatostatin monoclonal antibody (CURE S.6, 150 μg per mouse, 0.1 ml, i.v.) immediately before the administration of either PACAP-38 (135 μg kg^{-1}, i.v.) or vehicle (0.1 ml, i.v.). Thereafter, the pylorus was ligated and gastric acid secretion assessed 2 h later.

Statistical analysis

Gastric acid secretion (μmol per time) is expressed as mean ± s.e. Net secretion was calculated by subtracting the mean basal secretion for 1 h from the secretion during the period of interest. Differences between two groups were determined by paired or unpaired Student's *t*-test, as

appropriate. Differences between multiple groups were determined by analysis of variance (ANOVA) followed, when necessary, by a Student–Newman–Keuls multiple comparisons test. Within-group differences in acid secretion over time were assessed by repeated-measures ANOVA followed, when necessary, by a Student–Newman–Keuls multiple comparisons test. Data were considered statistically significant when P was $\leqslant 0.05$.

Results

Effects of PACAP-38 on basal and secretagogues-stimulated gastric acid secretion in wild-type mice and on basal secretion in SSTR2 knockout mice

Wild-type urethane-anesthetized mice had a low basal gastric acid secretion (0. $69 \pm 0.08\ \mu mol\ h^{-1}$, $n=6$), which was not modified by the i.v. infusion of vehicle or repeated injections of PACAP-38 at increasing doses (3, 7, 22, 45, 135 and $270\ \mu g\ kg^{-1}\ h^{-1}$) at 1 h intervals (Figure 1). However, PACAP-38 ($135\ \mu g\ kg^{-1}\ h^{-1}$) inhibited the acid secretory response to either pentagastrin, histamine or bethanechol (Figure 2). Pentagastrin infusion increased gastric acid secretion reaching a plateau two-fold over basal values within 20–30 min (mean plateau value: $0.21 \pm 0.01\ \mu mol\ (10\ min)^{-1}$; $P<0.05$ vs basal: $0.11 \pm 0.01\ \mu mol\ (10\ min)^{-1}$; $n=7$); with an acid output of $0.60 \pm 0.14\ \mu mol\ h^{-1}$ ($n=7$) during the plateau response (40–100 min period). The infusion of PACAP-38 (45, 90 or $135\ \mu g\ kg^{-1}\ h^{-1}$, 1 h), 30 min after starting the pentagastrin infusion, inhibited pentagastrin-stimulated acid secretion with a net reduction of $26.7 \pm 14.1\%$ ($0.41 \pm 0.12\ \mu mol\ h^{-1}$, $n=5$; $P>0.05$), $47.2 \pm 16.7\%$ ($0.37 \pm 0.08\ \mu mol\ h^{-1}$, $n=5$; $P>0.05$) and $91.1 \pm 8.2\%$ ($0.04 \pm 0.06\ \mu mol\ h^{-1}$, $n=6$; $P<0.05$; $F(4,22)=6.582$, $P=0.001$), respectively, compared with the pentagastrin + vehicle group (Figure 2a). Histamine ($5\ mg\ kg^{-1}\ h^{-1}$) stimulated gastric acid secretion and the plateau response reached values 10–13-fold (mean plateau value: $1.17 \pm 0.07\ \mu mol\ (10\ min)^{-1}$) over basal ($0.10 \pm 0.01\ \mu mol\ (10\ min)^{-1}$, $P<0.05$) after 40–50 min. The infusion of PACAP-38 ($135\ \mu g\ kg^{-1}\ h^{-1}$) inhibited the net histamine response by $42.8 \pm 8.6\%$ (histamine + PACAP-38: $3.64 \pm 0.55\ \mu mol\ h^{-1}$, $n=5$; $P<0.05$ vs histamine + vehicle: $6.95 \pm 1.11\ \mu mol\ h^{-1}$, $n=5$; $F(2,11)=16.464$, $P<0.001$; Figure 2b). Bethanechol ($0.6\ mg\ kg^{-1}\ h^{-1}$) stimulated basal secretion by 12-fold (mean plateau value: $1.09 \pm 0.07\ \mu mol\ (10\ min)^{-1}$) over basal values ($0.09 \pm 0.01\ \mu mol\ (10\ min)^{-1}$), reaching a secretory plateau within 40 min after starting the infusion. PACAP-38 ($135\ \mu g\ kg^{-1}\ h^{-1}$) inhibited the net secretory response to bethanechol by $63 \pm 8.3\%$ ($2.24 \pm 0.51\ \mu mol\ h^{-1}$, $n=5$; $P<0.05$ vs behanechol + vehicle: $6.07 \pm 0.74\ \mu mol\ h^{-1}$, $n=5$; $F(2,11)=30.267$, $P<0.001$; Figure 2c).

In urethane-anesthetized SSTR2 knockout mice, basal secretion was between 9 and 11 times higher than that observed in wild-type animals (SSTR2 knockout: $0.93 \pm 0.07\ \mu mol\ (10\ min)^{-1}$, $n=6$; wild-type: $0.10 \pm 0.01\ \mu mol\ (10\ min)^{-1}$, $n=6$; $P<0.05$; Figure 1). PACAP-38, infused in cumulative doses (3–$270\ \mu g\ kg^{-1}\ h^{-1}$), had a tendency to increase basal secretion, although no statistical significance was reached due to the variability in the data. Percent changes from basal secretion were 3.2 ± 18.8, 15.5 ± 22.8, 40.9 ± 27.8, 43.8 ± 33.1, and $20.2 \pm 24.9\%$ for the doses of 7, 22, 45, 135, and $270\ \mu g\ kg^{-1}\ h^{-1}$, respectively ($n=6$; Figure 1). A single infusion of PACAP-38 ($135\ \mu g\ kg^{-1}\ h^{-1}$, i.v.) did not modify acid secretion in SSTR2 −/− mice ($10.35 \pm 1.68\ \mu mol\ h^{-1}$, $n=4$) when compared with basal acid secretion ($10.52 \pm 1.78\ \mu mol\ h^{-1}$) or the vehicle-treated control group ($10.02 \pm 2.55\ \mu mol\ h^{-1}$, $n=4$).

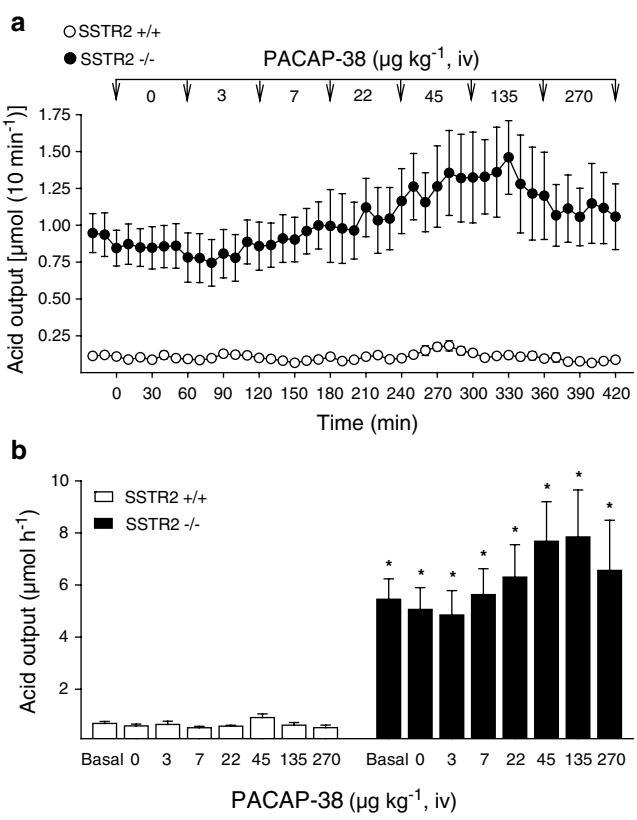

Figure 1 Effects of PACAP-38 on basal gastric acid secretion in urethane-anesthetized wild-type and SSTR2 knockout mice. After a 30 min basal period, vehicle ($0.1\ ml\ h^{-1}$) and PACAP-38 (3, 7, 22, 45, 135 and $270\ \mu g\ kg^{-1}\ h^{-1}$) were administered i.v. in increasing cumulative doses, and acid secretion monitored at 10 min intervals throughout the experiment. (a) Time course changes in gastric acid secretion. (b) Cumulative gastric acid output at 1 h intervals. $^*P<0.05$ vs secretory rate in wild-type mice.

Effects of somatostatin-14 on acid secretion in wild-type and SSTR2 knockout mice

In wild-type animals, pentagastrin infusion ($16\ \mu g\ kg^{-1}\ h^{-1}$) stimulated gastric acid secretion, reaching a plateau response within 30 min ($0.20 \pm 0.\ 01\ \mu mol\ (10\ min)^{-1}$, $n=4$, pooled data from groups treated with vehicle for somatostatin-14). This represents a 2.4-fold increase when compared with basal values ($0.08 \pm 0.01\ \mu mol\ (10\ min)^{-1}$; $P<0.05$). Somatostatin-14 ($20\ \mu g\ kg^{-1}\ h^{-1}$), infused 30 min after the start of pentagastrin infusion, reduced the acid secretory response to pentagastrin by $57.2 \pm 8.0\%$ ($0.53 \pm 0.03\ \mu mol\ h^{-1}$, $n=4$; $P<0.05$ vs pentagastrin + vehicle: $1.24 \pm 0.05\ \mu mol\ h^{-1}$, $n=4$; Figure 3a). In SSTR2 knockout mice, the infusion of somatostatin-14 ($20\ \mu g\ kg^{-1}\ h^{-1}$, $n=4$) did not modify the high basal acid secretion observed under urethane anesthesia (Figure 3b).

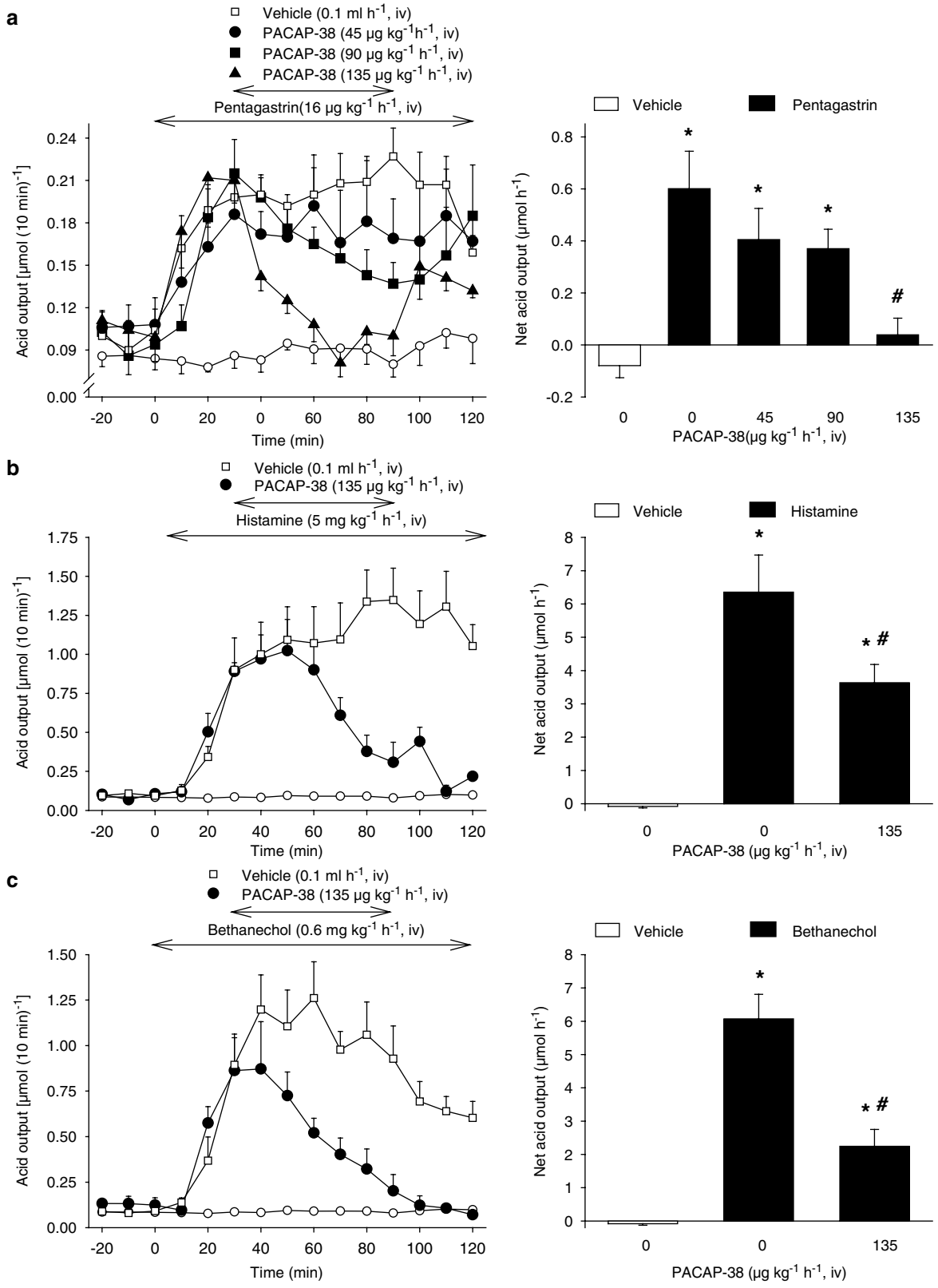

Figure 2 Effects of PACAP-38 on secretagogues-stimulated gastric acid secretion in urethane-anesthetized wild-type mice. After a 30 min basal period, gastric acid secretion was stimulated with either pentagastrin (a), histamine (b) or bethanechol (c). At 30 min after starting secretagogues administration PACAP-38 (45, 90 or 135 μg kg^{-1}) was infused i.v. for 1 h. Acid secretion was monitored at 10 min intervals throughout the experiment. Left panels represent time-course changes in gastric acid output in 10 min intervals. The open-circles graph in the three panels represents time-course changes in acid secretion in animals receiving only an i.v. infusion of vehicle saline through the experimental time. Right panels show the net change in cumulative acid output for the 1 h period of PACAP-38 or vehicle infusion. *$P<0.05$ vs non-stimulated secretion; #: $P<0.05$ vs respective secretagogue + vehicle group.

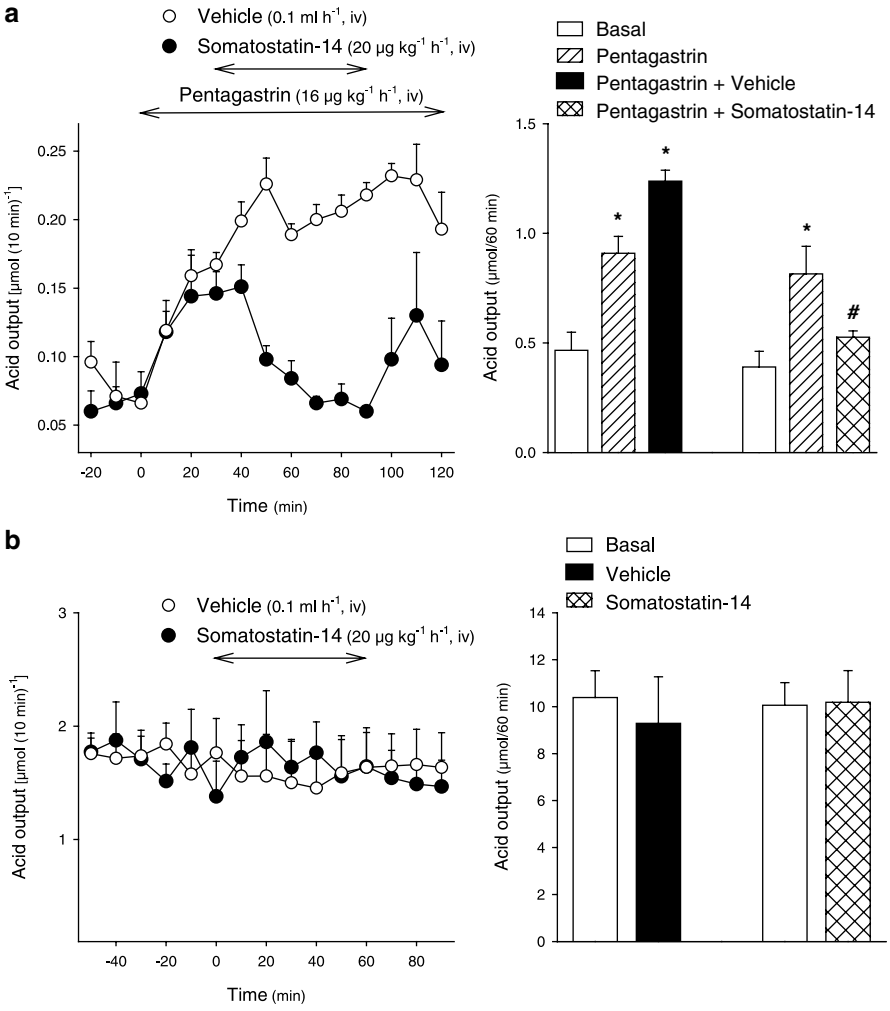

Figure 3 Effects of somatostatin-14 on pentagastrin-stimulated gastric acid secretion in wild-type mice (a) and on basal secretion in SSTR2 knockout mice (b). In urethane-anesthetized wild-type mice (a) after a 30 min basal, acid secretion was stimulated with pentagastrin and 30 min later either somatostatin ($20\,\mu g\,kg^{-1}$) or vehicle (0.1 ml) was infused i.v. for 1 h. Acid secretion was monitored at 10 min intervals throughout the experimental time. In SSTR2 knockout mice (b), due to the high basal secretion under urethane anesthesia, the effects of somatostatin were determined in basal conditions. After a 60 min basal period, either somatostatin ($20\,\mu g\,kg^{-1}$) or vehicle (0.1 ml) was infused i.v. for 1 h. Acid secretion was monitored at 10 min intervals throughout the experimental time. Left panels represent time-course changes in gastric acid output in 10 min intervals. Right panels show the cumulative acid response. $^*P<0.05$ vs respective basal. $\#P<0.05$ vs pentagastrin.

Effects of PACAP-38 and somatostatin-14 on gastric acid secretion in wild-type and SSTR2 knockout mice pretreated with somatostatin monoclonal antibody

In urethane-anesthetized wild-type mice, the somatostatin monoclonal antibody CURE S.6. (150 μg per mouse, i.v.) increased basal gastric acid secretion within 30 min after its administration, reaching a 3-4 fold plateau increase ($0.31\pm0.01\,\mu mol\,(10\,min)^{-1}$, pooled data from 7 animals) over basal secretory rates ($0.08\pm0.01\,\mu mol\,(10\,min)^{-1}$; $P<0.05$; Figure 4), that lasted for the next 90 min experimental period. Infusion of PACAP-38 ($135\,\mu g\,kg^{-1}\,h^{-1}$, i.v.) during the plateau phase, 30 min after monoclonal antibody administration, did not modify acid secretion ($2.19\pm0.28\,\mu mol\,h^{-1}$, $n=5$) when compared with vehicle-treated animals ($1.99\pm0.11\,\mu mol\,h^{-1}$, $n=7$; $P>0.05$; Figure 4). Under the same experimental conditions, the i.v. infusion of somatostatin-14 ($20\,\mu g\,kg^{-1}\,h^{-1}$), 30 min after the antibody administration, failed also to modify gastric acid secretion ($2.18\pm0.13\,\mu mol\,h^{-1}$, $n=4$; $P>0.05$ *vs* vehicle; $F(2,13)=0.40$, $P=0.678$; Figure 4). The control antibody (KLH, 150 μg per mouse, i.v.) did not influence basal gastric acid secretion (basal: $0.42\pm0.02\,\mu mol\,h^{-1}$; KLH: $0.40\pm0.03\,\mu mol\,h^{-1}$; $n=4$).

The somatostatin monoclonal antibody CURE S.6 (150 μg per mouse, i.v.) did not modify the basal gastric acid output in urethane-anesthetized SSTR2 knockout mice when compared with non-treated or control antibody (KLH)-treated animals (Table 1).

Effect of the SSTR2 antagonist PRL-2903 on basal secretion in wild-type and SSTR2 knockout mice, and on PACAP-38 and somatostatin-14 actions in wild-type mice

In wild-type mice, PRL-2903 administered as a bolus ($1.5\,mg\,kg^{-1}$) followed by a continuous 2 h i.v. infusion ($1.5\,mg\,kg^{-1}\,h^{-1}$; total dose administered: $4.5\,mg\,kg^{-1}$; $n=3$) stimulated acid secretion reaching a secretory plateau at

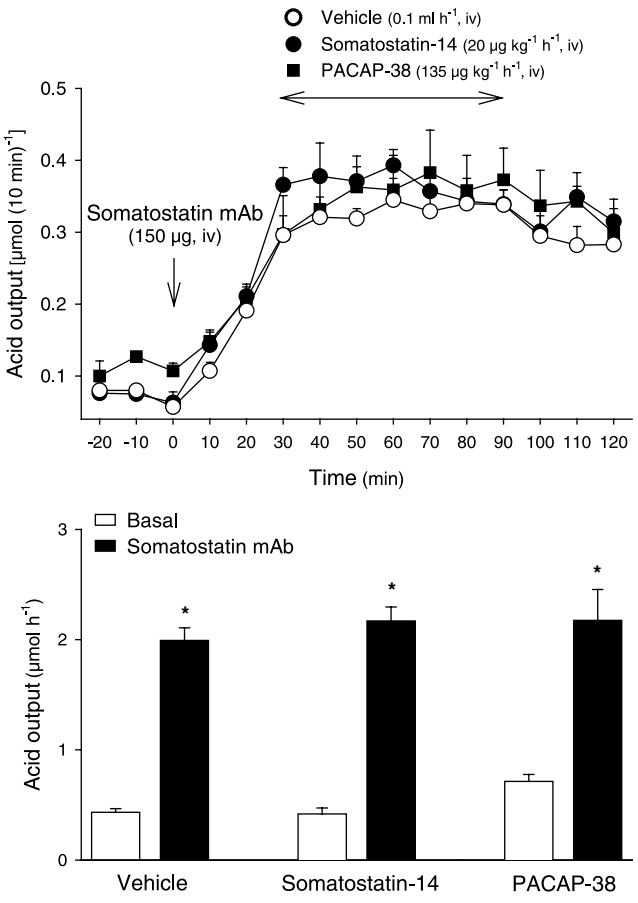

Figure 4 Effects of *in vivo* somatostatin immunoneutralization on somatostatin-14 and PACAP-38 effects on gastric acid secretion in wild-type mice. In urethane-anesthetized wild-type mice, after a 30-min basal, somatostatin (CURE.S6) monoclonal antibody (mAb; 150 μg per mouse) was administered i.v. After 30 min, either somatostatin-14 (20 $\mu g\,kg^{-1}$), PACAP-38 (135 $\mu g\,kg^{-1}$) or vehicle (0.1 ml) was infused i.v. during a 1 h period. Acid secretion was monitored at 10 min intervals throughout the experimental time. The upper panel shows time course changes in gastric acid secretion at 10 min interval. The lower panel shows the cumulative acid response for the different treatments. $^*P<0.05$ vs respective basal secretion.

Table 1 Effect of somatostatin monoclonal antibody (mAb) on basal gastric acid secretion in urethane-anesthetized SSTR2 knockout mice[a]

	No treatment	*Control Ab (KLH)*	*Somatostatin mAb*
Basal	7.79±1.49	5.57±0.32	7.31±1.42
0–1 h post-treatment	7.71±0.90	5.86±0.36	6.49±0.78
1–2 h post-treatment	8.00±0.53	6.43±0.80	8.20±1.93
N	5	4	4

[a]After a 1 h basal period, either the somatostatin mAb CURE S.6 (150 μg per mouse) or control Ab (KLH, 150 μg per mouse) was administered i.v. and acid secretion monitored for the next 2 h. Data represent accumulated gastric acid output in $\mu mol\,h^{-1}$, and are expressed as mean±s.e. for the number of animals indicated for each treatment (N).

30 min, that was maintained throughout the following 90 min infusion period (Figure 5). The plateau acid response reached mean values (1.30±0.06 μmol $(10\,min)^{-1}$, $n=3$) that represented a 14- and 10-fold increase over basal secretion

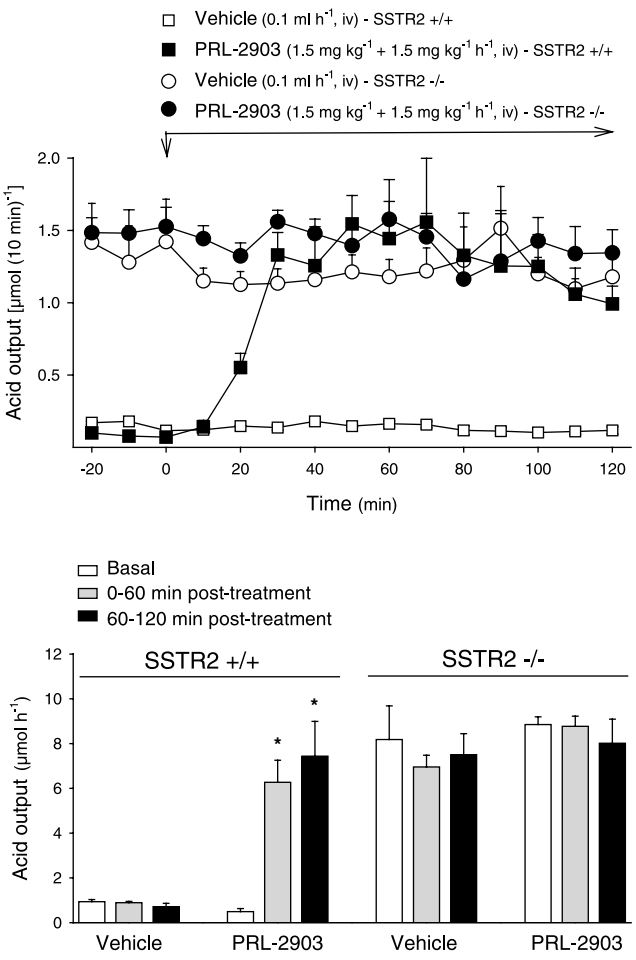

Figure 5 Effects of the SSTR2 antagonist PRL-2903 on basal gastric acid secretion in wild-type and SSTR2 knockout mice. In urethane-anesthetized wild-type (SSTR2 +/+) and SSTR2 knockout (SSTR2 −/−) mice, after a 30-min basal, PRL-2903 was administered as an i.v. bolus (1.5 $mg\,kg^{-1}$), followed by a 2 h continuous infusion (1.5 $mg\,kg^{-1}\,h^{-1}$) (total dose administered: 4.5 $mg\,kg^{-1}$). Gastric acid secretion was monitored at 10 min intervals throughout the experimental time. The upper panel shows time course changes in acid secretion at 10 min intervals. The lower panel shows the cumulative acid response for the different treatments. $^*P<0.05$ vs respective basal secretion or the secretory rate in wild-type mice treated with vehicle.

(0.09±0.01 μmol $(10\,min)^{-1}$, $n=3$; $P<0.05$) and vehicle-treated group, respectively (0.13±0.01 μmol $(10\,min)^{-1}$; $n=3$ $P<0.05$). By contrast, in SSTR2 knockout mice, similar PRL-2903 administration did not modify basal gastric acid secretion. During PRL-2903 infusion, cumulative acid secretion was 16.79±1.39 μmol $(2\,h)^{-1}$ ($n=3$), which was not different from basal (17.70±1.19 μmol $(2\,h)^{-1}$) or secretion in vehicle-treated animals (14.46±1.38 μmol $(2\,h)^{-1}$, $n=3$; Figure 5).

In wild-type mice, when somatostatin-14 (20 $\mu g\,kg^{-1}\,h^{-1}$) was infused in the presence of PRL-2903, the secretory response was not changed, indicating that the inhibitory effect of the peptide was completely blocked (PRL-2903 + somatostatin: 5.70±0.47 $\mu mol\,h^{-1}$, $n=3$; $P>0.05$ *vs* PRL-2093 + vehicle: 7.53±1.16 $\mu mol\,h^{-1}$, $n=5$; Figure 6). Similarly, the inhibitory effect of PACAP-38 (135 $\mu g\,kg^{-1}\,h^{-1}$, i.v.) was also no longer observed when the peptide was infused in the presence of PRL-2903 (7.89±0.68 $\mu mol\,h^{-1}$, $n=6$;

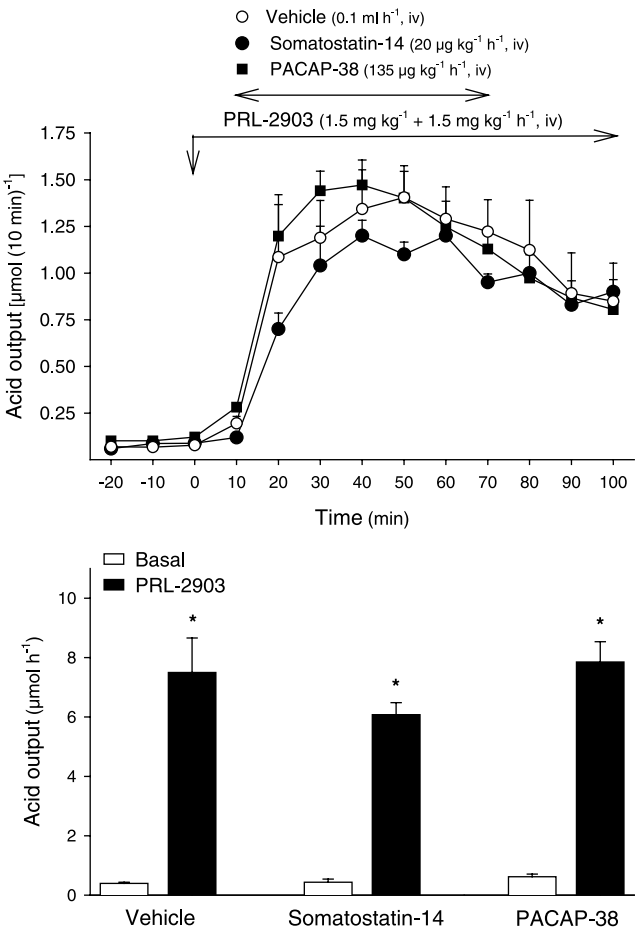

Figure 6 Effects of somatostatin-14 and PACAP-38 on PRL-2903-stimulated gastric acid secretion in wild-type mice. In urethane-anesthetized wild-type mice, gastric acid secretion was stimulated with the SSTR2 antagonist PRL-2903 (bolus of 1.5 mg kg^{-1} + infusion of 1.5 mg kg^{-1} h^{-1} for 2 h). At 10 min after starting the i.v. infusion of PRL-2903, a 1 h infusion of either somatostatin-14 (20 μg kg^{-1}), PACAP-38 (135 μg kg^{-1}) or vehicle (0.1 ml) was started. Acid secretion was monitored at 10 min intervals throughout the experimental time. The upper panel shows time-course changes in gastric acid secretion at 10 min intervals. The lower panel shows cumulative acid output for the different treatments. *$P<0.05$ vs respective basal secretion.

$P>0.05$ *vs* PRL-2903 + vehicle; $F(5,22)=34.19$, $P<0.001$; Figure 6).

Effects of PACAP-38 and pentagastrin on gastric acid secretion in wild-type and SSTR2 knockout conscious mice with pylorus ligation for 2 h

In conscious 2-h pylorus-ligated wild-type mice, i.v. injection of pentagastrin or monoclonal somatostatin antibody (CURE S.6, 150 μg per mouse, i.v.) significantly reduced gastric pH and increased acid concentration and acid output, while not influencing gastric secretory volume compared with vehicle treated mice (Table 2). By contrast, PACAP-38 (135 μg kg^{-1}, i.v., $n=6$) increased gastric pH, reduced secretory volume and acid output by 70±5% ($P<0.05$) and 86±3% ($P<0.05$) respectively, and had also a tendency to reduce acid concentration ($P=0.057$), when compared with vehicle-treated animals (Figure 7). In somatostatin antibody-pretreated wild-type mice, the rise in gastric pH and reduction in acid secretion induced by i.v. PACAP-38 were no longer observed, while the decrease in gastric secretory volume was not modified (Figure 7). In these conditions, acid concentration reached values similar to those observed in animals treated with the somatostatin antibody alone (Figure 7 and Table 2).

Vehicle-treated SSTR2 knockout mice with pylorus ligation for 2 h had a lower intragastric pH and an increased acid production compared with wild-type animals, while the secretory volume was not changed (Figure 7). In SSTR2 knockout mice, PACAP-38 (135 μg kg^{-1}, i.v., $n=6$) did not modify the gastric pH or acid production when compared with vehicle-treated animals (Figure 7), while having a tendency to reduce the volume of secretion. In SSTR2 knockout mice, i.v. pentagastrin (16 μg kg^{-1}, $n=3$) significantly increased acid concentration when compared with the vehicle-treated group (vehicle: 75.41±3.76 mmol l^{-1}; pentagastrin: 146.87±15.94 mmol l^{-1}, $n=3$; $P<0.05$).

Discussion

The present study established for the first time that peripheral infusion of PACAP inhibits the gastric acid response to pentagastrin, histamine, and bethanechol in urethane-anesthetized mice, while not influencing the low basal acid secretion observed under these experimental conditions. These data agree with the inhibitory effect of intravenous infusion of PACAP on secretagogues-stimulated gastric acid secretion in conscious rats with pylorus ligation or chronic gastric fistula (Mungan *et al*., 1992; 1995) or in an isolated luminally perfused rat stomach preparation (Li *et al*., 2000). PACAP was reported to have no effect (Mungan *et al*., 1992) or to inhibit basal secretion in conscious rats (Mungan *et al*., 1995; Li *et al*., 2000). In the present study, we found that the peptide did not influence basal acid secretion in urethane-anesthetized mice. This may be related to the low basal secretion observed under urethane anesthesia (about 1.0 μmol h^{-1}) compared with conscious rats (about 200 μmol h^{-1}) (Mungan *et al*., 1995) or the secretory rate in *in vitro* conditions (about 16 μmol h^{-1}) (Li *et al*., 2000). We also show that intravenous infusion of PACAP (135 μg kg^{-1}, equivalent to 30 nmol kg^{-1}) inhibited

Table 2 Effects of pentagastrin and somatostatin immunoneutralization on gastric acid secretion in 2-h pylorus-ligated conscious wild-type mice[a]

Treatment	N	*Volume (ml)*	*pH*	*Acid output (μmol 2 h^{-1})*	*Acid concentration (mmol l^{-1})*
Vehicle	9	0.48±0.06	5.04±0.49	13.87±4.26	22.59±4.78
Pentagastrin	3	0.45±0.02	3.31±0.09*	22.57±3.31*	50.61±7.64*
Anti-somatostatin	4	0.38±0.11	3.09±0.39*	24.83±6.94	66.49±6.60*

[a]Under short halothane anesthesia (5–6 min), animals were injected i.v. with either pentagastrin (16 μg kg^{-1}), anti-somatostatin monoclonal Ab (CURE S.6, 150 μg, i.v.) or vehicle (saline, 0.1 ml); thereafter, the pylorus was ligated and acid secretion determined 2 h later. Data are expressed as mean±s.e. for the number of animals indicated for each treatment (*N*). *$P<0.05$ vs vehicle-treated group.

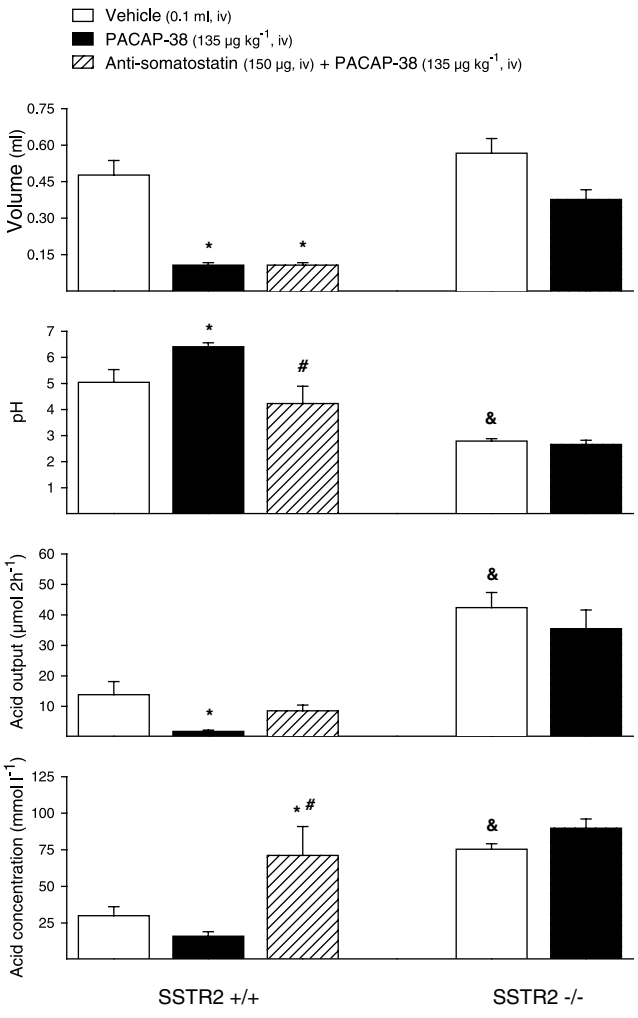

Figure 7 Effects of PACAP-38 on gastric acid secretion in 2-h pylorus-ligated wild-type and SSTR2 knockout mice. Under short halothane anesthesia, wild-type (SSTR2 +/+) and SSTR2 knockout mice (SSTR2 −/−) were injected i.v. with either PACAP-38 (135 μg kg^{-1}) or vehicle (saline, 0.1 ml). A group of wild-type mice was pre-treated with anti-somatostatin monoclonal Ab (CURE S.6, 150 μg, i.v.) immediately before PACAP-38 administration. Thereafter, the pylorus was ligated and the volume of secretion, pH, gastric acid output and acid concentration determined 2 h later. Data represent the mean ± s.e. of four to nine animals per group. *$P<0.05$ vs the respective vehicle-treated group; $^{\#}P<0.05$ vs PACAP-38-treated SSTR2 +/+ mice; $^{\&}P<0.05$ vs vehicle-treated SSTR2 +/+ mice.

gastric acid output by 86% in 2-h pylorus-ligated conscious mice. In conscious rats, PACAP, infused intravenously at a three fold lower dose than used in our study (5 nmol kg^{-1} h^{-1} for 2 h), did not modify gastric acid output monitored 2 h after pylorus ligation, while inhibiting the acid secretory response to pentagastrin and histamine but not to carbachol (Mungan *et al*., 1995). These variations may be related to differences in the doses of PACAP, although species- and experimental models-related differences can not be ruled out.

The different effects of PACAP on acid secretion in rats have been attributed to a combined stimulation of ECL and D cells, inducing the release of histamine and somatostatin, respectively (Zeng *et al*., 1999; Li *et al*., 2000; Lindström *et al*., 2001; Sandvik *et al*., 2001). In our experimental conditions, the intravenous infusion of PACAP inhibited secretagogues- and pylorus ligation-stimulated gastric acid secretion in urethane-anesthetized and conscious mice, respectively. These observations indicate a predominance of somatostatin inhibitory pathways over the stimulatory ones. We showed that intravenous infusion of somatostatin has similar inhibitory effects as intravenous infusion of PACAP-38 on pentagastrin-stimulated gastric acid secretion in wild-type mice. In addition, immunoneutralization of somatostatin prevented PACAP antisecretory effect. Efficacy of the antibody treatment is demonstrated by the increase in basal gastric secretion observed in wild-type mice, as well as by the blockade of intravenous somatostatin-14-induced inhibition of gastric acid secretion (Yang *et al*., 1990; Piqueras *et al*., 2003b; Martinez *et al*., 1995). Lastly, although somatostatin release was not directly monitored in the present conditions, previous studies in isolated mouse stomach demonstrated that exogenous VIP, known to have similar affinity as PACAP on $VPAC_1$ receptors located on D cells (Zeng *et al*., 1999; Vaudry *et al*., 2000; Laburthe & Couvineau, 2002), stimulates somatostatin secretion (Schubert, 1991; Schubert & Makhlouf, 1993). Likewise, in the isolated vascularly perfused rat stomach, celiac artery infusion of PACAP results in a rise in somatostatin in the portal effluent (Li *et al*., 2000). Other studies in isolated D cells demonstrate that both VIP and PACAP stimulate calcium signaling and somatostatin release with almost equal efficacy (Zeng *et al*., 1996; Zeng & Sachs, 2002).

Several *in vivo* and *in vitro* studies have also shown that PACAP may stimulate gastric acid secretion in the rat and that this effect is associated to the release of histamine (Norlen *et al*., 2001; Sandvik *et al*., 2001). A direct effect of PACAP on ECL cells is supported by the localization of PAC_1 receptors in rat gastric ECL cells (Zeng *et al*., 1999; Zeng & Sachs, 2002). In the present study, PACAP-38 administered intravenously, as cumulative doses or as a single-dose infusion, did not increase the low basal gastric acid secretion in wild-type urethane-anesthetized mice. Moreover, when acid secretion was stimulated with secretagogues only inhibitory responses were observed, suggesting the prevalence of somatostatin inhibitory actions over the stimulatory effect of histamine. Therefore, it should be expected that elimination of the somatostatin-dependent mechanisms may reveal an acid-secretory response associated to histamine release. However, the present results show that PACAP-38 did not alter the high basal gastric acid secretion induced by the blockade of SSTR2 receptors either by deletion of the gene (SSTR2 knockout mice) or the use of the selective SSTR2 antagonist PRL-2903 in wild-type mice. It is to mention that, under these conditions, gastric acid secretion reached levels similar to those induced by a maximal dose of histamine, making unlikely that any change in histamine release induced by PACAP could be manifested by a further increase in acid secretion. However, during somatostatin antibody treatment, that resulted in a lower rise in basal acid secretion, gastric acid values were not modified by PACAP, arguing against a stimulatory action of PACAP on gastric acid secretion in mice. Moreover, the lack of stimulatory effect of PACAP in SSTR2 knockout mice does not seem to be related to a maximal secretory state in these animals, due to a sustained lack of somatostatin inhibitory action. Additional acid secretion can be induced by exogenously administered gastrin and histamine

in SSTR2 knockout mice (present study and data not shown). Lastly, although PACAP elicited a trend to stimulate basal secretion in urethane-anesthetized SSTR2 knockout mice, this was not confirmed in conscious mice with pylorus ligation or when the somatostatin effects were blocked in wild-type animals.

It has been suggested that anesthetics, including urethane, impair the secretion of ECL-cell histamine in rats (Norlen *et al*., 2000). This might account for the lack of stimulatory effects of PACAP in urethane-anesthetized mice. However, no urethane-associated effects on histamine release were observed in isolated rat ECL cells in primary culture (Norlen *et al*., 2000) or on acid secretion and histamine release in an isolated vascularly perfused rat stomach preparation (Cui *et al*., 2002). This agrees with the view that urethane inhibits acid secretion by stimulating somatostatin release (Yang *et al*., 1990; Martinez *et al*., 1998; Kawakubo *et al*., 1999), rather than by affecting directly ECL-cell histamine release. The lack of stimulatory effects of PACAP in conscious 2-h pylorus-ligated mice, even after the elimination of the somatostatin-dependent inhibitory component, further suggests that the absence of any stimulatory response to PACAP in urethane-anesthetized mice is not due to an impaired histamine release.

Somatostatin exerts its biological actions through the activation of five different receptor subtypes (SSTR1–SSTR5) (Patel, 1999; Martinez, 2002). Consistent with our previous reports (Martinez *et al*., 1998; Piqueras *et al*., 2003b), results obtained here further strengthen the important role of SSTR2 receptors mediating somatostatin antisecretory effects in mice. In the present study, convergent findings demonstrate that somatostatin-dependent inhibition of gastric acid secretion induced by peripheral PACAP is mediated by the activation of SSTR2 receptors. The infusion of PACAP at doses effective in inhibiting acid secretion in wild-type animals did not influence the high acid secretion characteristic of SSTR2 knockout mice. Furthermore, PACAP antisecretory effects were also abolished by the pharmacological blockade of SSTR2 receptors with the somatostatin analog PRL-2903 in wild-type animals. These observations establish that functional SSTR2 receptors are necessary for peripheral PACAP-induced somatostatin-dependent inhibitory effects on gastric acid secretion in mice.

Recent studies using SSTR2 knockout/lacZ knockin mice revealed that the vast majority of epithelial cells in the middle portion of the corpus expressed SSTR2 receptors. Most of these cells were also H^+K^+ ATPase-positive being, therefore, identified as parietal cells (Allen *et al*., 2002). In addition, many of the ECL cells in the corpus and pylorus were also shown to express SSTR2 receptors (Allen *et al*., 2002). Therefore, it is likely that the SSTR2-mediated antisecretory effect of peripheral PACAP in mice results from both a direct effect on the parietal cells as well as from inhibition of histamine release from ECL cells (Prinz *et al*., 1994). This is supported by the demonstration that intravenous infusion of PACAP inhibits gastric acid secretion stimulated by various secretagogues, acting either directly on ECL (gastrin) or on parietal cells (bethanechol and histamine) (Pfeiffer *et al*., 1990; Prinz *et al*., 1999; Lindström *et al*., 2001). Studies in the isolated mouse stomach have shown that somatostatin induces a prompt decrease in histamine secretion and that immunoneutralization of endogenous somatostatin results in a rise in histamine release (Vuyyuru & Schubert, 1997). Similarly, *in vivo* microdialysis studies in rats showed that somatostatin potently inhibits gastrin-stimulated histamine secretion (Norlen *et al*., 2001; Bernsand *et al*., 2003), supporting a direct action of somatostatin in ECL cells. In addition, previous functional studies *in vivo* in mice support a dual action of somatostatin inhibiting histamine release from ECL cells, as well as a direct action on parietal cells (Komasaka *et al*., 2002; Piqueras *et al*., 2003b). It should be noticed that PACAP significantly inhibited the secretory response to direct stimulation of parietal cells with histamine, while somatostatin was only partially effective in the same experimental conditions (Piqueras *et al*., 2003b). This might suggest that other inhibitory mechanisms in addition to the somatostatin-releasing effect of PACAP may also play a role. In rats, other endogenous inhibitory mediators (namely prostaglandin E2 and secretin) have been implicated in the antisecretory effects of PACAP (Li *et al*., 2000).

In summary, results obtained using immunoneutralization of somatostatin by monoclonal antibody, pharmacological blockade of SSTR2 receptor and SSTR2 knockout mice showed that intravenous infusion of PACAP inhibits gastric acid secretion in mice and that this effect is mediated through the release of somatostatin and the activation of SSTR2 receptors, likely localized on ECL and parietal cells. Although no direct measurements of somatostatin release from the stomach were possible *in vivo*, the results obtained are consistent with earlier *in vitro* studies in mice and rats that demonstrated the release of somatostatin by PACAP and its related neuropeptide VIP (Schubert, 1991; Schubert & Makhlouf, 1993; Li *et al*., 2000).

Other neuropeptides that inhibit gastric acid secretion also release somatostatin or have a somatostatin-dependent mechanism of action. For instance, it has been shown that glucose-dependent insulinotropic polypeptide (GIP)-, glucagon-like polypeptide (GLP)-, amylin-, and calcitonin gene-related peptide (CGRP)-induced inhibition of acid secretion in rats and bombesin-induced inhibition of acid secretion in mice are mediated through somatostatin release (Taché, 1992; Rossowski *et al*., 1998; Zaki *et al*., 2002; Piqueras *et al*., 2003b). These observations, together with the present results, suggest that gastric D cells may function as a common target for a variety of inhibitory gut peptides, which input is translated into the release of somatostatin and in turn the activation of SSTR2 receptors on ECL and parietal cells, leading to an inhibition of acid output. Whether or not other neuropeptides or inhibitory mechanisms also depend on somatostatin to act as an integrative inhibitory factor requires further studies using similar functional approaches to those used in this work (pharmacological blockade, immunoneutralization, and/or use of genetically modified animal models).

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