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Peripheral $GABA_B$ agonists stimulate gastric acid secretion in mice

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1 We characterized the effects of intravenous GABA and preferential $GABA_A$ (muscimol), $GABA_B$ (*R*-baclofen and SKF-97541) and $GABA_C$ agonists (imidazole-4-acetic acid) on gastric acid secretion in urethane-anesthetized mice implanted with a gastric cannula, and determined the role of vagal cholinergic mechanisms, and gastrin and somatostatin by using peptide immunoneutralization, the SSTR2 antgonist, PRL-2903, and SSTR2 knockout mice.

2 The selective $GABA_B$ agonists *R*-baclofen (0.1–3 mg kg^{-1}, i.v.) and SKF-97541 (0.01–0.3 mg kg^{-1}, i.v.) induced a dose-related stimulation of gastric acid secretion. SKF-97541 was about 10 times more potent than *R*-baclofen stimulating gastric acid secretion. Neither GABA (0.1–100 mg kg^{-1}, i.v.) nor muscimol (0.1–3 mg kg^{-1}) nor imidazole-4-acetic acid (0.1–10 mg kg^{-1}) affected basal gastric acid secretion.

3 Stimulatory effects of SKF-97541 (0.1 mg kg^{-1}, i.v.) were blocked by the selective $GABA_B$ antagonist, 2-hydroxysaclofen, cholinergic blockade with atropine, subdiaphragmatic vagotomy or gastrin immunoneutralization.

4 Somatostatin immunoneutralization or SSTR2 blockade with PRL-2903 enhanced the secretory response to SKF-97541 (0.1 mg kg^{-1}, i.v.) by 78 and 105%, respectively.

5 In SSTR2 knockout mice, SKF-97541 (0.1 mg kg^{-1}, i.v.) increased basal gastric acid secretion by 48%. Neither GABA nor muscimol nor imidazole-4-acetic acid modified basal gastric acid secretion in SSTR2 knockout mice.

6 These results indicate that, in mice, stimulation of $GABA_B$ receptors increases gastric acid secretion through vagal- and gastrin-dependent mechanisms. Somatostatin implication might be secondary to the release of gastrin and the increase in gastric luminal acidity.
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Abbreviations: ANOVA, analysis of variance; CNS, central nervous system; DMN, dorsal motor nucleus of the vagus; GABA, γ-aminobutyric acid; KLH, keyhole limpet hemocyanin; NTS, nucleus tractus solitarius; SSTR2, somatostatin type 2 receptor

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS) (Sivilotti & Nistri, 1991). GABA mediates its actions *via* three distinct receptors: the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptors (Bormann, 2000). In addition to its CNS functions, the GABAergic system is also present in peripheral tissues, including the gastrointestinal tract (Gilon *et al*., 1990; 1991; Harty *et al*., 1991; Krantis *et al*., 1994). $GABA_A$ and $GABA_B$ receptor subtypes have been localized in cells of the gastric mucosa and in neuronal components (Erdo *et al*., 1990; Nakajima *et al*., 1996; Castelli *et al*., 1999). In addition, $GABA_B$ receptors have been localized on gastric vagal afferent neurons, where they regulate vagal reflexes (Lehmann *et al*., 1999; Smid *et al*., 2001). However, less is known about the distribution of $GABA_C$ receptors. Different $GABA_C$ receptor subunits have been localized in the myenteric plexus of the rat small and large intestine (Fletcher *et al*., 2001), while their presence in the stomach remains unknown. Taken together, these morphological observations support a role for local GABA in the regulation of gastrointestinal motor and secretory functions.

Previous studies indicate that GABA modulates gastric acid secretion in several species, including humans. Both inhibitory and stimulatory effects of the GABAergic system on gastric acid secretion have been reported in rats, dogs and humans (Goto & Debas, 1983; Pugh *et al*., 1985; Thirlby *et al*., 1988; Blandizzi *et al*., 1991a,b; 1992; 1995; Lin, 1995). Several studies suggest that the stimulatory effects of GABA on acid secretion are vagally mediated, associated to a direct effect on central GABA receptors (Yamasaki & Goto, 1990a,b). According to this, the $GABA_B$ agonist baclofen has been used as a tool to induce a vagal-dependent stimulation of gastric acid secretion in rats (Yang *et al*., 1989; Martinez & Taché, 1996). However, other studies in rats and dogs suggest that the stimulatory effects of parenterally administered baclofen are associated to the stimulation of peripheral $GABA_B$ receptors and are mediated through both vagal and extravagal pathways (Blandizzi *et al*., 1992). $GABA_B$-mediated inhibitory effects on acid secretion have also been described in rats (Del Tacca

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et al., 1990; Blandizzi *et al*., 1991a). On the other hand, the $GABA_A$ agonist muscimol, injected either intracerebroventricularly or systemically in rats, stimulated gastric acid secretion through both vagal-dependent and -independent mechanisms (Del Tacca *et al*., 1990; Blandizzi *et al*., 1991b; Lin, 1995).

Several studies have also demonstrated that the GABAergic system modulates cholinergic neurotransmision and endocrine cell function within the stomach (Harty & Franklin, 1983; 1986; Harty *et al*., 1991; Weigert *et al*., 1998). GABA stimulated the release of acetylcholine from rat antral mucosal/submucosal sections and induced antral gastrin and somatostatin release through stimulation of postganglionic cholinergic neurons (Harty & Franklin, 1986; Harty *et al*., 1991; Weigert *et al*., 1998). These effects were reproduced with muscimol and blocked with bicuculline, suggesting a mediation through peripheral $GABA_A$ receptors (Harty *et al*., 1991). Later studies showed that exogenous GABA stimulates gastrin release and inhibits somatostatin release from the isolated rat stomach *via* $GABA_A$ receptors (Weigert *et al*., 1998).

All these findings support the concept of endogenous GABA being a neurocrine or paracrine modulator in the stomach. However, most of the existing evidence is derived from studies in rats and in several cases in *in vitro* conditions. Therefore, the aims of this study were first to characterize the effects of peripherally administered GABA on gastric acid secretion in mice *in vivo*. Secondly, the relative involvement of $GABA_A$, $GABA_B$ and $GABA_C$ receptors was studied using receptor-preferential agonists. Finally, the mechanisms involved in $GABA_B$ receptors-dependent modulation of acid secretion were also characterized. The role of vagal cholinergic pathways was determined by cholinergic blockade and vagotomy. The relative involvement of gastrin and somatostatin was assessed by *in vivo* immunoneutralization of the endogenous peptides. Moreover, the role of somatostatin was further characterized by pharmacological blockade of somatostatin type 2 (SSTR2) receptors with the selective antagonist PRL-2903 and by using SSTR2 knockout mice (Rossowski *et al*., 1998; Piqueras *et al*., 2003b; 2004).

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). Mice used in the present study were born from different litters; all descendants are born from genotyped littermates obtained through inbreeding. If not stated otherwise, all experiments were carried out in wild-type animals with the same genetic background as the knockout mice, except for the deletion of the *SSTR2* gene. Mice were maintained on a 12 : 12 h light–dark cycle with controlled conditions of temperature (22°C) and humidity (60%), in group cages (5–6 mice per cage) 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.), GABA (Sigma-Aldrich, St. Louis, MO, U.S.A.), *R*-baclofen (Tocris Cookson Inc., Ellisville, MO, U.S.A.), SKF-97541 [3-aminopropyl(methyl)phosphinic acid; Tocris], muscimol (Tocris), imidazole-4-acetic acid (Tocris), atropine methyl bromide (Sigma-Aldrich), 2-hydroxysaclofen [(rs)-3-amino-2-(4-chlorophenyl)-2-hydroxypropyl-sulfonic acid; Tocris] and 2-deoxy-D-glucose (Sigma-Aldrich) 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; Dr D.H. Coy, Tulane University, New Orleans, LA, U.S.A.) (Rossowski *et al*., 1998) was dissolved in 0.01% acetic acid to a concentration of $1\,\mu g\,\mu l^{-1}$, and subsequent dilutions were made in 0.9% saline. All solutions were prepared immediately before each experiment. Purified monoclonal somatostatin antibody (CURE S.6) and monoclonal gastrin antibody (CURE no. 05109.1D) (CURE:Digestive Diseases Research Center, Antibody-RIA Core, UCLA, Los Angeles, CA, U.S.A.) were used for *in vivo* immunoneutralization. Purified monoclonal antibody to keyhole limpet hemocyanin (KLH; CURE:Digestive Diseases Research Center) was used as control. Production, characterization and purification of these antibodies have been described in detail previously (Kovacs *et al*., 1989; Wong *et al*., 1990; Ohning *et al*., 1996). Doses of compounds were selected according to previous studies in mice and rats, and adjusted according to preliminary experiments.

Gastric acid secretion measurements

Procedure Gastric acid secretion was monitored in urethane-anesthetized mice following a technique previously described by us (Martinez *et al*., 1998; Piqueras *et al*., 2003a,b; 2004). Fasted 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, the stomach localized 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 cannula 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 drug administration. Gastric acid secretion was determined over time 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 backtitrated to pH 7.0 (0.001 N NaOH) with an automatic titrator (Radiometer Copenhagen). To minimize the effects of body temperature on the acid-secretory responses to GABA and its analogs (Hara *et al*., 1990a,b), rectal temperature was monitored at regular intervals throughout the experimental time and maintained between 36 and 37°C with a heating system.

Experimental protocols

In all cases, acid output was monitored at 10-min intervals throughout the experimental time.

Effects of GABA and preferential agonists for $GABA_A$, $GABA_B$ and $GABA_C$ receptors on gastric acid secretion After a 30-min basal period, vehicle (saline, 0.1 ml) and GABA (0.1, 0.3, 1.0, 3.0, 10.0 and 100.0 mg kg^{-1}, equivalent to 1–1000 μmol kg^{-1}) or $GABA_A$ (muscimol; 0.1, 0.3, 1.0 and 3.0 mg kg^{-1}; equivalent to 0.9–27 μmol kg^{-1}), $GABA_B$ (*R*-baclofen, 0.1, 0.3, 1.0 and 3.0 mg kg^{-1}, equivalent to 0.5–15 μmol kg^{-1}, and SKF-97541, 0.01, 0.03, 0.06, 0.1 and 0.3 mg kg^{-1}, equivalent to 0.07–2 μmol kg^{-1}) and $GABA_C$ agonists (imidazole-4-acetic acid, 0.1, 0.3, 3.0 and 10.0 mg kg^{-1}, equivalent to 0.6–60 μmol kg^{-1}) were administered as intravenous boli, in cumulative increasing doses, with a 30-min interval between doses, except for *R*-baclofen at 1.0 and 3.0 mg kg^{-1} and SKF-97541 at 0.1 and 0.3 mg kg^{-1} for which a 60-min interval was taken.

In a separate experiment, the effects on basal gastric acid secretion of a single bolus administration of SKF-97541 (0.1 mg kg^{-1}) were characterized. After a 30-min basal period vehicle saline or SKF-97541 was administered as a bolus (0.1 ml, i.v.) and acid secretion determined during the following 90-min. In a series of experiments, mice were pretreated with vehicle (0.1 ml, i.v.) or the $GABA_B$ antagonist, 2-hydroxysaclofen (5 mg kg^{-1}, 0.1 ml, i.v.), 30 min before vehicle (0.1 ml, i.v.) or SKF-97541 (0.1 mg kg^{-1}, i.v.) administration.

Finally, the effects of imidazole-4-acetic acid on pentagastrin- and 2-deoxy-D-glucose-stimulated acid secretion were also studied. After a 30-min basal period, pentagastrin (16 μg kg^{-1} h^{-1}) was infused i.v. (0.1 ml h^{-1}). After 30-min, either imidazole-4-acetic acid (3 mg kg^{-1}) or vehicle (0.1 ml) was administered as an i.v. bolus, and acid secretion monitored for the following hour. In a separate experiment, after a 30-min basal period, 2-deoxy-D-glucose (200 mg kg^{-1}) was given as an i.v. bolus, and 10 min later imidazole-4-acetic acid (3 mg kg^{-1}) was administered as an i.v. bolus, and acid secretion monitored for the following hour.

Effects of cholinergic blockade with atropine and vagotomy on SKF-97541 effects on basal gastric acid secretion After a 30-min basal period, animals were pretreated with atropine (2 mg kg^{-1}, s.c.) or its vehicle (0.1 ml, s.c.). After 30-min, SKF-97541(0.1 mg kg^{-1}) or vehicle (0.1 ml) was administered as an i.v. bolus. Gastric acid secretion was monitored for the next 90-min. In a group of animals, 2-deoxy-D-glucose (200 mg kg^{-1}, i.v.) was administered 30-min after atropine pretreatment (2 mg kg^{-1}, s.c.), and gastric acid acid secretion monitored for the following 90-min.

In a series of experiments, the vagi were cut at a subdiaphragmatic level at the time of implantation of the gastric cannula. Control animals had similar manipulations, but the vagi were left intact (sham vagotomy). In vagotomized and sham vagotomized mice, after a 30-min basal period SKF-97541 (0.1 mg kg^{-1}) was administered as an i.v. bolus and acid secretion determined during the following 90-min.

Effects of in vivo *immunoneutralization of gastrin on SKF-97541 effects on gastric acid secretion* After a 30-min basal period, purified gastrin monoclonal antibody (150 μg/mouse, 0.1 ml) or control antibody (KLH, 150 μg per mouse, 0.1 ml). was injected i.v. and, 30-min later, SKF-97541 (0.1 mg kg^{-1}) was administered as an i.v. bolus. Gastric secretion was monitored for the next 90-min.

Involvement of somatostatin and SSTR2 receptors on SKF-97541 effects on gastric acid secretion The role of somatostatin and SSTR2 receptors on SKF-97541 effects was determined by: (i) *in vivo* peptide immunoneutralization; (ii) pharmacological blockade of SSTR2 receptors with the selective receptor antagonist PRL-2903 and (iii) using SSTR2 knockout mice. For somatostatin immunoneutralization, after a 30-min basal period, animals were injected with purified monoclonal somatostatin antibody (CURE S.6,. 150 μg per mouse, 0.1 ml) and 30-min later received either vehicle (0.1 ml, i.v.) or SKF-97541 (0.1 mg kg^{-1}, i.v.) and gastric acid secretion was monitored for the next 90-min. For the pharmacological blockade of SSTR2 receptors, after a 30-min basal period animals received PRL-2903 (i.v. bolus of 1.5 mg kg^{-1} followed by a continuous i.v. infusion, 1.5 mg kg^{-1} h^{-1}) or vehicle (i.v. bolus of 0.1 ml followed by a continuous i.v. infusion of 0.1 ml h^{-1} for 1 h). At 10-min after starting the infusion of vehicle or PRL-2903, animals received an i.v. injection of SKF-97541 (0.1 mg kg^{-1}) or vehicle (0.1 ml) and acid secretion was monitored for the following 90-min. In SSTR2 knockout mice, after a 30-min basal period either vehicle (0.1 ml) or SKF-97541 (0.1 mg kg^{-1}) was administered as an i.v. bolus, and acid secretion monitored for the next 90-min.

Effects of gastrin and 2-deoxy-D-glucose on basal gastric acid secretion in wild-type and SSTR2 knockout mice After a 30-min basal period, vehicle (0.1 ml) followed 30-min later by pentagastrin (16 μg kg^{-1}) were administered as i.v. boli. In a separate experiment, after a 30-min basal period, vehicle (0.1 ml) and 2-deoxy-D-glucose (50, 100 and 200 mg kg^{-1}) were administered as i.v. boli in increasing cumulative doses, with a 60-min interval between consecutive doses.

Statistical analysis

Gastric acid secretion (μmol per time) is expressed as mean $\pm$ s.e.m. Net secretion was calculated by subtracting the estimated basal secretion 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 GABA, $GABA_A$, $GABA_B$ and $GABA_C$ agonists, pentagastrin and 2-deoxy-D-glucose on basal gastric acid secretion

In urethane-anesthetized wild-type mice, basal gastric acid secretion was low (0.07 ± 0.01 μmol (10 min)$^{-1}$, pooled data

from five animals) and stable over a 2-h experimental time. *R*-baclofen (0.1, 0.3, 1.0 and 3.0 mg kg^{-1}, $n=7$) increased basal gastric acid secretion in a dose-related manner. The lower doses tested had no effect, while at 1.0 and 3.0 mg kg^{-1}, acid secretion increased to 2.46 ± 0.32 and 6.99 ± 0.99 μmol h^{-1}, respectively ($P<0.05$ vs basal: 0.63 ± 0.04 μmol h^{-1} or vehicle: 0.58 ± 0.05 μmol h^{-1}; Figure 1a and b). Similar dose-related stimulation of acid secretion was obtained with the compound SKF-97541, although it was about 10 times more potent than *R*-baclofen stimulating basal gastric acid secretion. At 0.1 and 0.3 mg kg^{-1} ($n=6$) SKF-97541 increased acid secretion by 4- (2.92 ± 0.60 μmol h^{-1}) and 12-fold (7.99 ± 0.93 μmol h^{-1}), respectively, compared with basal (0.63 ± 0.04 μmol h^{-1}) or vehicle (0.66 ± 0.06 μmol h^{-1}). Lower doses (0.01, 0.03 and 0.06 mg kg^{-1}) did not modify basal secretion (Figure 1a and c). SKF-97541, given as a single i.v. dose of 0.1 mg kg^{-1} ($n=5$), increased basal secretion by 3.7- and 5.3-fold compared with basal (1.03 ± 0.29 μmol h^{-1}, $P<0.05$) or the secretory rate in vehicle-treated animals (0.71 ± 0.14 μmol h^{-1}, $n=3$, $P<0.05$), respectively (Figure 2). Acid secretion was significantly increased during the 20–30 min period after SKF-97541 administration, reaching a maximum during the 40–50 min period (0.88 ± 034 μmol (10 min)$^{-1}$, $P<0.05$ vs basal or vehicle-treated group; Figure 2). Thereafter, secretion returned toward basal levels and 80-min post-administration the secretory rate was not longer different from basal. Based on this response, an i.v. bolus of SKF-97541 at 0.1 mg kg^{-1} was used in further experiments, and the acid-secretory response was evaluated during the 60-min period after administration.

Pentagastrin (16 μg kg^{-1}, i.v.) increased gastric acid secretion by 3.4-fold over basal or the secretory response to vehicle (Table 1). 2-deoxy-D-glucose (50–200 mg kg^{-1}, i.v.) stimulated basal gastric acid secretion in a dose-related manner. Acid secretion was increased by 2-, 13- and 14-fold over the secretory rate after vehicle administration, for the doses tested (Table 2).

Neither GABA (0.1–100.0 mg kg^{-1}, $n=4$) nor muscimol (0.1–3.0 mg kg^{-1}, $n=4$) affected basal gastric acid secretion (Figure 1a). At the dose of 1.0 mg kg^{-1}, some of the animals receiving muscimol had signs of dyspnea, which was increased at 3.0 mg kg^{-1}. In a preliminary experiment, a higher dose (10 mg kg^{-1}) resulted in 100% mortality within 20-min after administration, therefore higher doses were not tested. Imidazole-4-acetic acid (0.1–10.0 mg kg^{-1}, $n=4$, Figure 1a) had a tendency to inhibit basal gastric acid secretion at the doses of 3.0 (0.36 ± 0.14 μmol h^{-1}, $P=0.054$ vs basal: 0.64 ± 0.11 μmol h^{-1}) and 10.0 mg kg^{-1} (0.35 ± 0.04 μmol h^{-1}, $P=0.068$ vs basal). However, when tested under stimulated conditions of secretion, imidazole-4-acetic acid (3 mg kg^{-1}) did not modify neither the secretory response to pentagastrin (vehicle: 1.14 ± 0.31 μmol h^{-1}, $n=4$, $P>0.05$ vs imidazole-4-acetic acid: 1.52 ± 0.84 μmol h^{-1}, $n=7$) nor the response to 2-deoxy-D-glucose (2-deoxy-D-glucose: 3.40 ± 0.29 μmol h^{-1}, $n=5$, $P>0.05$ vs 2-deoxy-D-glucose + imidazole-4-acetic acid: 3.95 ± 0.26 μmol h^{-1}, $n=4$).

Effect of 2-hydroxysaclofen on SKF-97541-induced stimulation of basal gastric acid secretion

The selective $GABA_B$ antagonist 2-hydroxysaclofen (5 mg kg^{-1}) did not affect basal gastric acid secretion (0.74 ± 0.12 μmol h^{-1}; $P>0.05$ vs basal: 0.63 ± 0.08), but blocked the secretory response to SKF-97541 (0.1 mg kg^{-1}, i.v.) (2-hydroxysaclofen + SKF-97541: 0.98 ± 0.19 μmol h^{-1}, $n=5$, $P<0.05$ vs vehicle + SKF-97541: 1.81 ± 0.14 μmol h^{-1}, $n=3$; Figure 3).

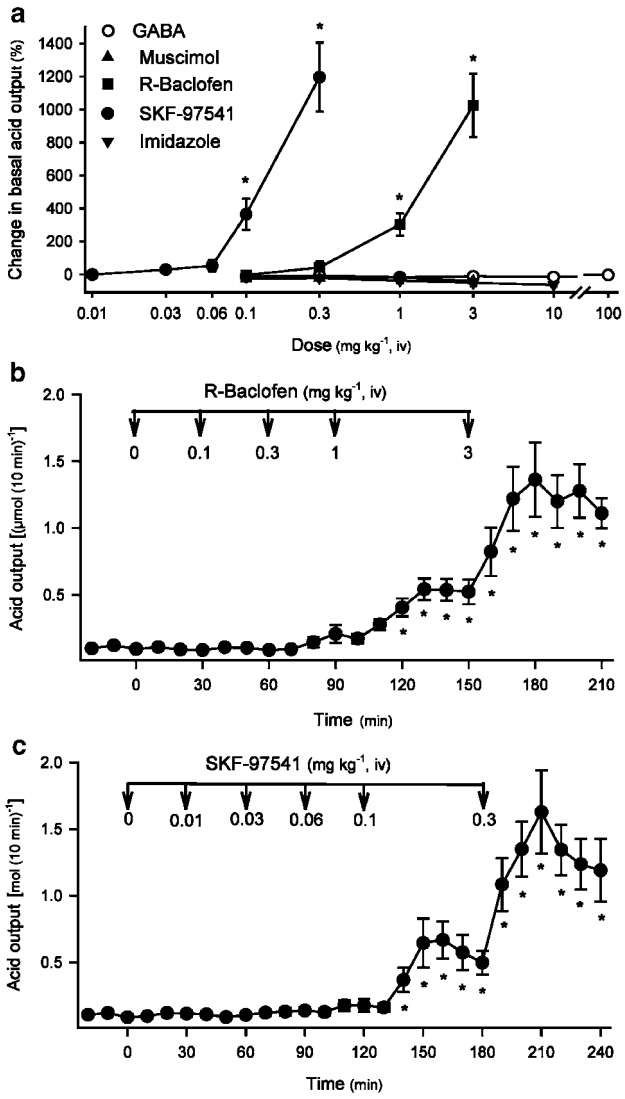

Figure 1 Effects of GABA, and preferential $GABA_A$, $GABA_B$ and $GABA_C$ agonists on basal gastric acid secretion in mice. (a) In urethane-anesthetized mice GABA (0.1, 0.3, 1.0, 3.0, 10.0 and 100.0 mg kg^{-1}) or preferential $GABA_A$ (muscimol: 0.1, 0.3, 1.0 and 3.0 mg kg^{-1}), $GABA_B$ (*R*-baclofen: 0.1, 0.3, 1.0 and 3.0 mg kg^{-1}; SKF-97541: 0.01, 0.03, 0.06, 0.1 and 0.3 mg kg^{-1}) and $GABA_C$ agonists (imidazole-4-acetic acid: 0.1, 0.3, 3.0 and 10.0 mg kg^{-1}) were administered as an i.v. bolus in increasing cumulative doses. Acid secretion was monitored at 10-min intervals throughout the experiment. Data are mean ± s.e.m. for the changes in gastric acid output (% from basal) for the different compounds tested. *$P<0.05$ vs basal secretion. (b, c) Time course changes in basal gastric acid secretion, at 10-min intervals, in animals receiving cumulative doses of *R*-baclofen (0.1, 0.3, 1.0 and 3.0 mg kg^{-1}, b) or SKF-97541 (0.01, 0.03, 0.06, 0.1 and 0.3 mg kg^{-1}, c).

Effects of cholinergic blockade and vagotomy on SKF-97541-induced stimulation of basal gastric acid secretion

Peripheral cholinergic blockade with atropine (2 mg kg^{-1}, s.c.) did not modify basal gastric acid secretion (basal:

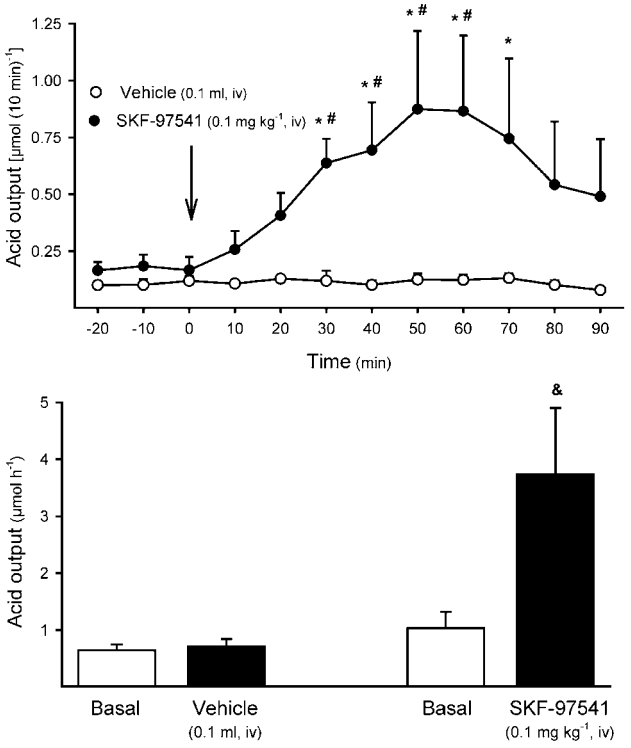

Figure 2 Effect of a single bolus administration of SKF-97541 on basal gastric acid secretion in mice. In urethane-anesthetized mice, after a 30-min basal period, either vehicle (0.1 ml) or SKF-97541 (0.1 mg kg^{-1}) was administered as an i.v. bolus. Acid secretion was monitored at 10-min intervals throughout the experiment. The upper panel shows time course changes in gastric acid secretion at 10-min intervals and the lower panel the cumulative acid response. *$P<0.05$ vs basal secretory rate; $^{\#}P<0.05$ vs secretory rate in the vehicle-treated group at the same time point; $^{\&}P<0.05$ vs basal or the secretory response to vehicle.

Table 1 Effects of pentagastrin on basal gastric acid secretion in wild-type and SSTR2 knockout mice[a]

	Wild type (N = 5)	*SSTR2 −/−* (N = 8)
	Acid output (μmol h^{-1})[b]	
Basal	0.74 ± 0.07	8.36 ± 1.12
Vehicle (0.1 ml, i.v.)	0.73 ± 0.06	8.87 ± 0.70
Pentagastrin (16 μg kg^{-1}, i.v.)	2.51 ± 0.67*	14.60 ± 1.33*

[a]After a 30-min basal period, vehicle (0.1 ml) was administered i.v., followed 30-min later by a bolus of pentagastrin (16 μg kg^{-1}). Acid output was determined at 10-min intervals all along the experiment.
[b]Data represent the mean ± s.e.m. of cumulative acid output (μmol h^{-1}) for the number of animals indicated (N).
*$P<0.05$ vs respective basal or secretory rate after vehicle administration (repeated-measures ANOVA).

0.64 ± 0.09 μmol h^{-1}, $P>0.05$ vs atropine: 0.61 ± 0.12 μmol h^{-1}, $n=6$). The increase in gastric acid secretion elicited by SKF-97541 (0.1 mg kg^{-1}, i.v.) was completely blocked in atropine-pretreated animals (atropine + SKF-97541: 0.75 ± 0.12 μmol h^{-1}, $n=6$, $P<0.05$ vs vehicle + SKF-97541: 1.78 ± 0.23 μmol h^{-1}, $n=4$, Figure 4a). Similarly, atropine pre-treatment also blocked the secretory response elicited by 2-deoxy-D-glucose (vehicle + 2-deoxy-D-glucose: 3.40 ± 0.29 μmol h^{-1}, $P<0.05$ vs atropine + 2-deoxy-D-glucose: 0.80 ± 0.11 μmol h^{-1}, $n=5$ for each; Figure 4b).

Basal gastric acid secretion was similar in vagotomized (0.55 ± 0.03 μmol h^{-1}, $n=5$) and sham vagotomized animals (0.87 ± 0.17 μmol h^{-1}, $n=4$; $P>0.05$ vs vagotomy; Figure 4c). In vagotomized mice, SKF-97541 (0.1 mg kg^{-1}, i.v.) did not

Table 2 Effects of 2-deoxy-D-glucose on basal gastric acid secretion in wild-type and SSTR2 knockout mice

Dose (mg kg^{-1}, *i.v.*)[a]	*Wild type* (N = 5)	*SSTR2 −/−* (N = 4)
	Acid output (μmol h^{-1})[b]	
Basal	0.35 ± 0.04	8.19 ± 2.49
Vehicle	0.43 ± 0.06	8.33 ± 2.13
2-Deoxy-D-glucose		
50	0.86 ± 0.19	11.12 ± 1.40
100	5.85 ± 0.30*	11.98 ± 1.60
200	6.07 ± 0.69*	12.16 ± 2.74*

[a]After a 30-min basal period, vehicle (0 mg kg^{-1}) and 2-deoxy-D-glucose were given i.v. in increasing cumulative doses with a 1 h interval between consecutive doses. Acid output was determined at 10-min intervals all along the experiment.
[b]Data represent the mean ± s.e.m. of cumulative acid output (μmol h^{-1}) for the number of animals indicated (*N*).
*$P<0.05$ vs respective basal or secretory rate after vehicle administration (repeated-measures ANOVA).

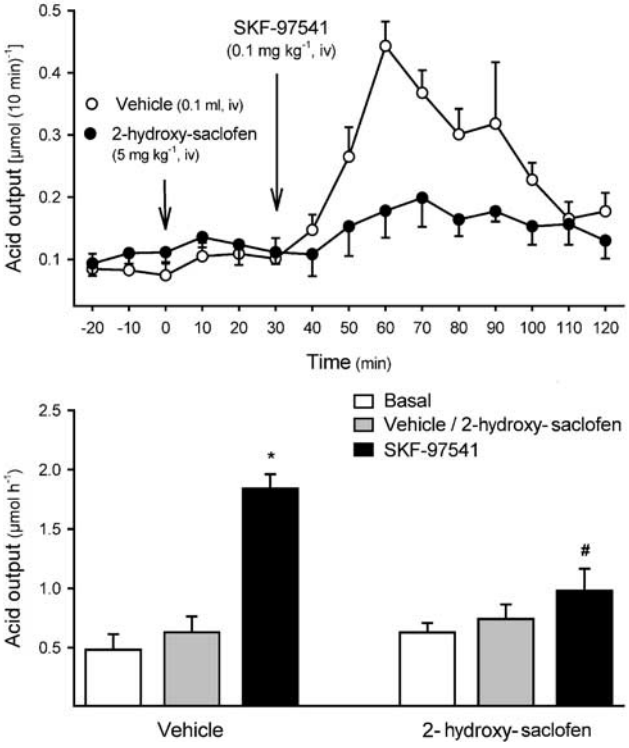

Figure 3 Effect of 2-hydroxy-saclofen on SKF-97541-induced stimulation of gastric acid secretion in mice. In urethane-anesthetized mice, after a 30-min basal period, either vehicle (0.1 ml, i.v.) or the $GABA_B$ antagonist 2-hydroxy-saclofen (5 mg kg^{-1}, 0.1 ml, i.v.) were administered. After 30-min, animals received a single bolus injection of SKF-97541 (0.1 mg kg^{-1}). Acid secretion was monitored at 10-min intervals throughout the experimental time. The upper panel shows time course changes in gastric acid secretion and the lower panel the cumulative acid response. *$P<0.05$ vs basal secretion; $^{\#}P<0.05$ vs the secretory response to SKF-97541 in vehicle-treated mice.

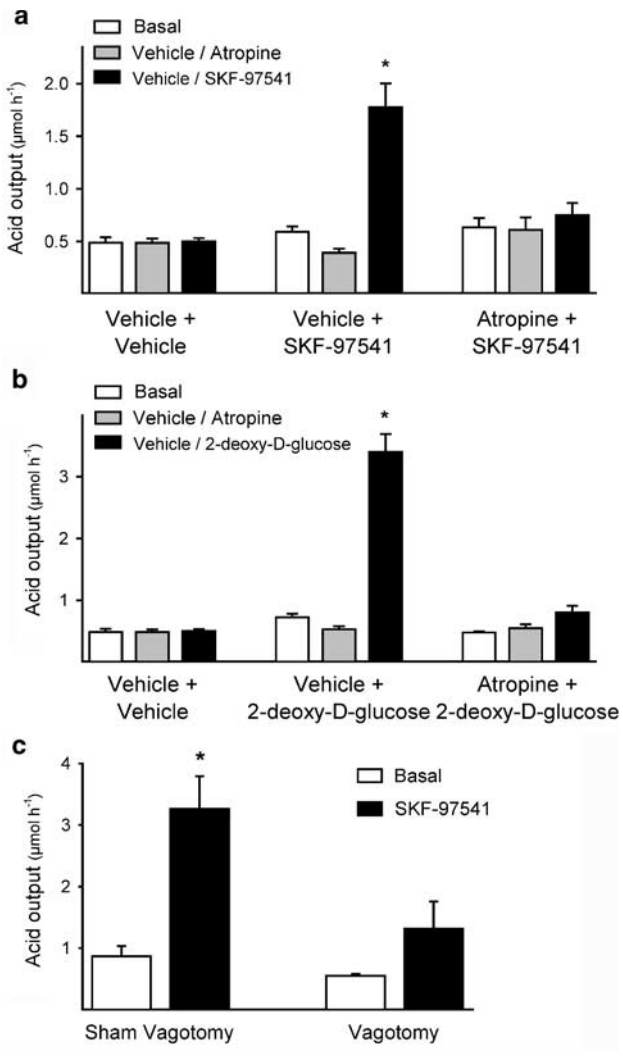

Figure 4 Effect of cholinergic blockade and vagotomy on SKF-97541- and 2-deoxy-D-glucose-induced stimulation of gastric acid secretion in mice. (a, b) Secretory responses to SKF-97541 (a) and 2-deoxy-D-glucose (b) in atropine pretreated mice. After a 30-min basal period, animals were pretreated with atropine methyl bromide ($2\,mg\,kg^{-1}$, s.c.) or vehicle (0.1 ml, s.c.). After 30-min later, vehicle (0.1 ml), SKF 97541 ($0.1\,mg\,kg^{-1}$, a) or 2-deoxy-D-glucose ($200\,mg\,kg^{-1}$, b) was administered as an i.v. bolus. Data represent cumulative acid output for the different treatments. $^*P<0.05$ vs other experimental groups. (c) Secretory response to SKF-97541 in vagotomized and sham vagotomized mice. In sham vagotomized and vagotomized mice, after a 30-min basal period, SKF-97541 ($0.1\,mg\,kg^{-1}$) was administered as an i.v. bolus (0.1 ml) and acid secretion was monitored for the next 90-min. Data represent cumulative acid output for the different treatments. $^*P<0.05$ vs other experimental groups.

stimulate gastric acid secretion longer ($1.32\pm0.44\,\mu mol\,h^{-1}$, $n=5$) when compared with the secretory response in sham vagotomized animals ($3.26\pm0.53\,\mu mol\,h^{-1}$, $n=4$; $P<0.05$ vs vagotomy; Figure 4c).

Effects of in vivo *immunoneutralization of gastrin on SKF-97541-induced stimulation of basal gastric acid secretion*

In vivo immunoneutralization of gastrin did not modify basal gastric acid secretion compared with the basal secretory rate (basal: $0.48\pm0.03\,\mu mol\,h^{-1}$; gastrin antibody: $0.55\pm0.05\,\mu mol\,h^{-1}$; pooled data from eight antibody-treated animals; Figure 5) or the secretory rate in control antibody-treated animals ($0.75\pm0.13\,\mu mol\,h^{-1}$, pooled data from nine control antibody-treated mice; Figure 5). In gastrin antibody-treated mice, SKF-97541 ($0.1\,mg\,kg^{-1}$, i.v.) did not longer stimulate acid secretion when compared with the secretory response in control antibody-treated mice (control antibody: $2.05\pm0.32\,\mu mol\,h^{-1}$, $n=6$, $P<0.05$ vs gastrin antibody: $0.52\pm0.07\,\mu mol\,h^{-1}$, $n=5$; Figure 5).

Effects of in vivo *immunoneutralization of somatostatin and blockade of SSTR2 receptors on SKF-97541-induced stimulation of basal gastric acid secretion*

The somatostatin monoclonal antibody CURE.S6 (150 μg per mouse, i.v.) increased basal gastric acid secretion 20-min after administration, reaching a secretory plateau by 30-min. The plateau phase represented a four- to five-fold increase over basal secretory rates and lasted for the next 60 min. SKF-97541 ($0.1\,mg\,kg^{-1}$, i.v., $n=4$), administered during the plateau phase, increased acid secretion to $3.93\pm0.28\,\mu mol\,h^{-1}$

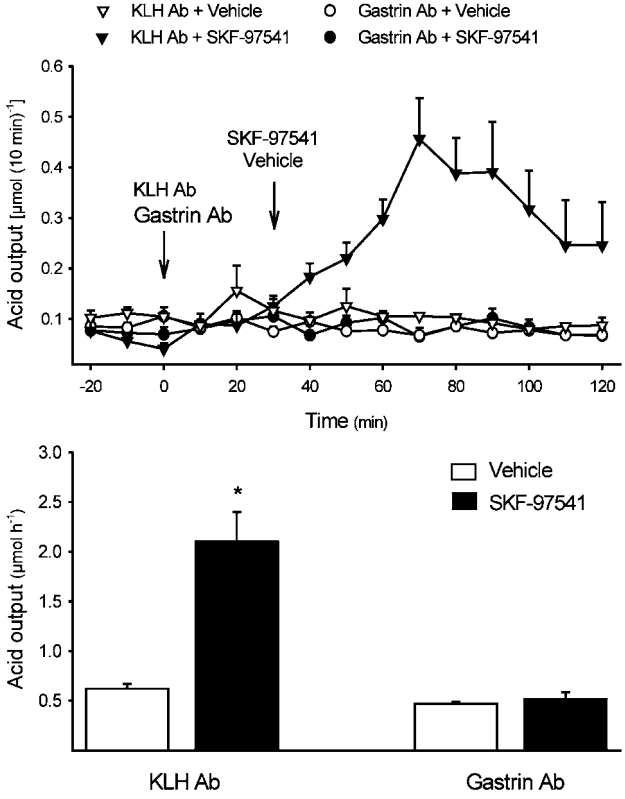

Figure 5 Effects of *in vivo* immunoneutralization of gastrin on SKF-97541-induced stimulation of gastric acid secretion in mice. After a 30-min basal period, either purified gastrin monoclonal antibody (Gastrin Ab, 150 μg per mouse, 0.1 ml) or control antibody (KLH Ab, 150 μg per mouse, 0.1 ml) was injected i.v. and, 30-min later, either SKF-97541 ($0.1\,mg\,kg^{-1}$) or vehicle (0.1 ml) was administered as an i.v. bolus. Acid secretion was monitored at 10-min intervals throughout the experiment. The upper panel shows time course changes in acid secretion. The lower panel shows the cumulative acid output for the 1-h period after vehicle or SKF-97541 administration for the different experimental groups. $^*P<0.05$ vs all other groups.

($P<0.05$ vs vehicle: $2.05\pm0.32\,\mu mol\,h^{-1}$, $n=4$). The net secretory response elicited by SKF-97541 in somatostatin antibody-treated mice ($1.82\pm0.28\,\mu mol\,h^{-1}$) was about 78% higher than that in control antibody-treated animals ($1.02\pm0.38\,\mu mol\,h^{-1}$; Figure 6a).

The SSTR2 antagonist PRL-2903 increased acid secretion to a stable secretory plateau with a mean secretory rate of $1.62\pm0.06\,\mu mol\ (10\,min)^{-1}$ (pooled data from four animals; Figure 6b). When SKF-97541 ($0.1\,mg\,kg^{-1}$, i.v.) was administered in the presence of PRL-2903, the secretory response reached $20.91\pm5.09\,\mu mol\,h^{-1}$ ($n=5$; $P<0.05$ vs PRL-2093 + vehicle: $7.86\pm3.02\,\mu mol\,h^{-1}$, $n=4$; Figure 6b). The net secretory response to SKF-97541 in the presence of PRL-2903 ($13.73\pm5.09\,\mu mol\,h^{-1}$) was higher than that in animals treated with vehicle + SKF-97541 (1.30 ± 0.31, $n=3$, $P<0.05$).

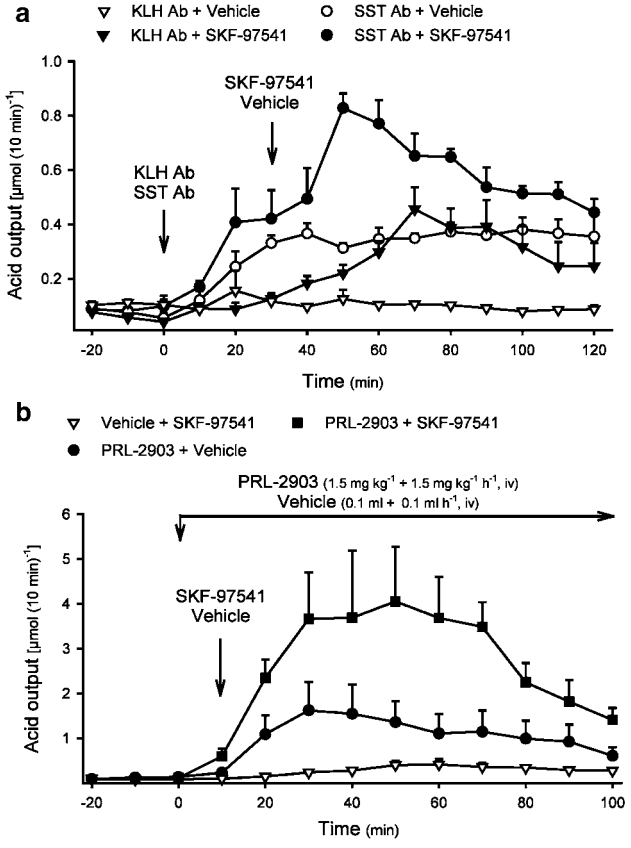

Figure 6 Effects of *in vivo* immunoneutralization of somatostatin and blockade of SSTR2 receptors on SKF-97541-induced stimulation of gastric acid secretion in mice. (a) Effect of somatostatin immunoneutralization on SKF-97541-induced stimulation of acid secretion. After a 30-min basal period, purified somatostatin monoclonal antibody (SST Ab, CURE S.6, $150\,\mu g$ per mouse, 0.1 ml) or control antibody (KLH, $150\,\mu g$ per mouse, 0.1 ml) was injected i.v. and, 30-min later, either SKF-97541 ($0.1\,mg\,kg^{-1}$) or vehicle (0.1 ml) was administered as an i.v. bolus. Acid secretion was monitored at 10-min intervals throughout the experiment. (b) Effect of SSTR2 receptor blockade with PRL-2903 on SKF-97541-induced stimulation of acid secretion. After a 30-min basal period, PRL-2903 was administered as a bolus ($1.5\,mg\,kg^{-1}$), followed by a continuous i.v. infusion ($1.5\,mg\,kg^{-1}\,h^{-1}$). After 30-min, later SKF-97541 ($0.1\,mg\,kg^{-1}$) or vehicle (0.1 ml) was administered as an i.v. bolus. Acid secretion was monitored at 10-min intervals throughout the experiment.

Effects of GABA, $GABA_A$, $GABA_B$ and $GABA_C$ agonists, pentagastrin and 2-deoxy-D-glucose on basal gastric acid secretion in SSTR2 knockout mice

Urethane-anesthetized SSTR2 knockout mice had a basal secretion 12–15 times higher than that observed in wild-type animals (SSTR2 knockout: $1.14\pm0.10\,\mu mol\ (10\,min)^{-1}$; wild type: $0.08\pm0.01\,\mu mol\ (10\,min)^{-1}$; pooled data from basal secretion monitored during a 2-h period in six and five animals, respectively; $P<0.05$).

SKF-97541 ($0.01–0.30\,mg\,kg^{-1}$, i.v.; $n=8$), showed a tendency to increase acid secretion at the doses of 0.06 and $0.1\,mg\,kg^{-1}$, although statistical significance was not reached (Figure 7a). Similar results were obtained with *R*-baclofen ($0.1–3.0\,mg\,kg^{-1}$, i.v., $n=4$; data not shown). When SKF-97541 was administered as a single i.v. bolus ($0.1\,mg\,kg^{-1}$, $n=7$), acid secretion increased to $10.38\pm2.52\,\mu mol\,h^{-1}$ ($P<0.05$ vs basal: $6.99\pm1.00\,\mu mol\,h^{-1}$), while no changes were observed in vehicle-treated animals (basal: $7.74\pm0.99\,\mu mol\,h^{-1}$; vehicle: $7.88\pm1.02\,\mu mol\,h^{-1}$, $n=4$; Figure 7b and c).

Pentagastrin ($16\,\mu g\,kg^{-1}$, i.v.) increased gastric acid secretion by 75 and 65% over basal or the secretory response to vehicle, respectively (Table 1). 2-deoxy-D-glucose (50, 100 and

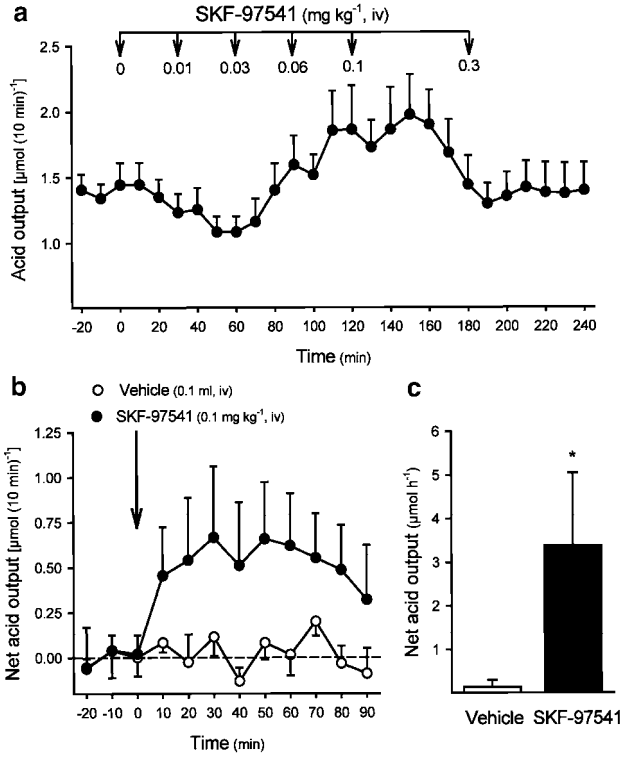

Figure 7 Effects of SKF-97541 on basal gastric acid secretion in SSTR2 knockout mice. In urethane-anesthetized SSTR2 knockout mice, after a 30-min basal period, vehicle and increasing cumulative doses of SKF-97541 (0.01, 0.03, 0.06, 0.1 and $0.3\,mg\,kg^{-1}$) or a single dose ($0.1\,mg\,kg^{-1}$) were administered as an i.v. bolus. Acid secretion was monitored at 10-min intervals throughout the experiment. (a) Effect of increasing cumulative doses of SKF-97541 on basal gastric acid secretion. (b) Net changes on basal gastric acid secretion after a single dose of SKF-97541. (c) Net secretory response for the 1-h period after vehicle or SKF-97541 ($0.1\,mg\,kg^{-1}$) administration. $^*P<0.05$ vs vehicle.

200 mg kg^{-1}, i.v.) increased basal acid secretion by 35% ($P>0.05$), 46% ($P>0.0$ 5) and 48% ($P<0.05$), respectively (Table 2).

Neither GABA nor muscimol nor imidazole-4-acetic acid modified basal gastric acid secretion in SSTR2 knockout mice (data not shown).

Discussion

The present studies provide evidence for a GABAergic-dependent modulation of gastric acid secretion in mice in *in vivo* conditions. Several reports suggest that the GABAergic system may be a physiological modulator of gastrointestinal functions, including gastric acid secretion. Both GABA and GABA receptors have been identified within the gastrointestinal tract, including the gastric mucosa (Erdo *et al*., 1990; Gilon *et al*., 1990; 1991; Harty *et al*., 1991; Krantis *et al*., 1994; Nakajima *et al*., 1996; Castelli *et al*., 1999). Functional data showed that GABA or preferential $GABA_A$ and $GABA_B$ agonists, administered peripherally, affect gastric acid secretion in rats, dogs and humans (Goto & Debas, 1983; Pugh *et al*., 1985; Thirlby *et al*., 1988; Blandizzi *et al*., 1991a,b; 1992; 1995; Lin, 1995). In the present study, the peripheral administration of preferential $GABA_B$ agonists, *R*-baclofen and SKF-97541 resulted in a dose-related stimulation of acid secretion in mice, while the stimulation of $GABA_A$ or $GABA_C$ receptors was devoid of any effect. Interestingly, when the endogenous ligand GABA, with similar affinity for the three receptor subtypes, was administered, no secretory changes were observed. Lack of effects of GABA might suggest the existence of compensatory mechanisms associated to the simultaneous stimulation of different receptor subtypes. These observations contrast with previous data showing that peripherally administered GABA stimulated gastric acid secretion (Goto & Debas, 1983; Pugh *et al*., 1985; Thirlby *et al*., 1988; Yamasaki & Goto; 1989; 1990a). Moreover, previous studies in rats implicate both $GABA_A$ and $GABA_B$ receptors in the secretory effects of peripherally administered GABA (Blandizzi *et al*., 1992; 1995; Lin, 1995). In the present study, the $GABA_C$ agonist imidazole-4-acetic acid showed a tendency to inhibit basal gastric acid secretion. Under urethane anesthesia, inhibitory effects may be dampened by the low secretion associated to somatostatin release (Yang *et al*., 1990). However, when the effects of imidazole-4-acetic acid were tested under stimulated conditions of secretion, with pentagastrin or by vagal stimulation with 2-deoxy-D-glucose, no inhibitory responses were observed. Together, these observations suggest the existence of species-related differences in the role of GABA and GABA receptors in the control of gastric acid secretion. The results obtained suggest that a selective activation of $GABA_B$ receptors is necessary to elicit changes in acid output in mice, while both $GABA_A$ and $GABA_B$ receptors seem to be involved in the GABAergic effects in rats (Blandizzi *et al*., 1992; 1995; Lin, 1995). According to the reported higher binding affinity for $GABA_B$ receptors for SKF-97541 than baclofen (Howson *et al*., 1993; Froestl *et al*., 1995), SKF-97541 was about 10 times more potent than *R*-baclofen stimulating acid secretion *in vivo*.

Although the site of action of the $GABA_B$ agonists tested was not specifically studied, the stimulatory responses observed may be centrally mediated, probably at the level of the braim stem, where the dorsal vagal complex is localized (Rogers *et al*., 1996). The results obtained suggest that secretory responses to $GABA_B$ agonists might be mediated through the central stimulation of vagal cholinergic outflow to the stomach. First, the acid responses elicited with *R*-baclofen and SKF-97541 were similar in magnitude to those elicited by the central vagal stimulant 2-deoxy-D-glucose (Hirschowitz & Sachs, 1965). Secondly, the secretory responses to SKF-97541 were prevented by subdiaphragmatic vagotomy or peripheral cholinergic blockade, suggesting an action through the modulation of vagal cholinergic outflow to the stomach. A centrally mediated effect is also possible, taking into account that the compounds used readily cross the blood-brain barrier (van Bree *et al*., 1991; Howson *et al*., 1993). This characteristic may also explain the lack of effects of GABA, that only crosses the blood–brain barrier at very high doses, which produce severe side effects (Kuriyama & Sze, 1971; Frey *et al*., 1979). However, as stated earlier, a compensatory effect associated to the simultaneous stimulation of several peripheral receptors cannot be ruled out. Morphological studies also support a central mechanism of action for $GABA_B$ receptors modulating acid secretion. Immunohistochemical studies showed the presence of $GABA_B$ receptors in the dorsal vagal complex, both in preganglionic neurons throughout the dorsal motor nucleus of the vagus (DMN) as well as within the nucleus tractus solitarius (NTS), in regions related to the control of upper GI reflexes, and particularly in regions that contain vagal-myenteric cholinergic excitatory neurons (Margetamitrovic *et al*., 1999; McDermott *et al*., 2001). According to these observations, electrophysiological data showed that systemic baclofen increases vagal efferent activity in the rat (Yamasaki & Goto, 1989; 1992). Additional pharmacological data also support a central site of action for baclofen. In rats, intracisternal baclofen stimulated acid secretion at doses 100–500 times lower than those needed to elicit a secretory response upon peripheral administration (Yamasaki & Goto, 1990a,b).

Gastric acid response to vagal stimulation may result from an interplay of different peripheral mechanisms recruited by vagal cholinergic activation (Taché, 2002). The present results support the involvement of gastrin in the acid response to the stimulation of $GABA_B$ receptors. First, secretory responses elicited by SKF-97541 and pentagastrin were similar in magnitude. Moreover, the secretory response to SKF-97541 was completely absent after the immunoneutralization of endogenous gastrin. Previous studies showed that immunoneutralization of gastrin in conscious rats, with the same antibody used in the present study, also reduced the acid-secretory response to vagal cholinergic activation during sham feeding (Martinez *et al*., 2002) or to chemical vagal stimulation with thyrotropin-releasing hormone (TRH) and baclofen (Yang *et al*., 1989). However, in rats, gastrin immunoneutralization reduced the secretory response to baclofen only by 33% (Yang *et al*., 1989), while completely blocking the secretory response to SKF-97541 in mice (present study). These observations suggest, again, the existence of species-related differences between rats and mice in the mechanism of action of the GABAergic system modulating gastric acid secretion. The effects of peripheral cholinergic blockade, vagotomy and gastrin immunoneutralization imply that cholinergic stimulation along with circulating gastrin are important to convey the full acid response to GABAergic-dependent vagal stimulation of acid secretion, as previously

suggested in rats for the cephalic phase of gastric acid secretion (Martinez *et al*., 2002). Previous observations suggest a role for gastrin in mediating the acid-secretory effects elicited by stimulation of GABA receptors. Studies in dogs showed that the nonselective $GABA_{A/B}$ agonist progabide increased gastric acid secretion and stimulated gastrin release (Thirlby *et al*., 1988). Moreover, studies *in vitro* have shown that GABA increased gastrin release from rat gastric antral mucosal preparations and isolated stomach, linking this phenomenon to a peripheral mechanism of action (Harty & Franklin, 1983; 1986; Weigert *et al*., 1998). These hormonal effects have been associated to the activation of peripherally located $GABA_A$ receptors (Harty & Franklin, 1986; Weigert *et al*., 1998). However, this mechanism seems unlikely to be taking place in mice in *in vivo* conditions, since no effects on secretion were observed when GABA or the preferential $GABA_A$ agonist muscimol were administered. These observations, again, suggest the existence of species-related differences in the acid-secretory responses, the receptor subtypes involved, as well as their location (central vs peripheral). A final confirmation of the role of gastrin in mice would require the measurement of gastrin during the administration of GABA agonists.

Gastric acid secretory responses to SKF-97541 were increased when the somatostatin-dependent inhibitory mechanisms were blocked (immunoneutralization of the endogenous peptide and pharmacological blockade or genetic deletion of SSTR2 receptors) (Martinez *et al*., 1998; Piqueras *et al*., 2003b; 2004). This might imply that stimulation of the GABAergic system also mediates the release of somatostatin, that in turn has a trend to reduce the acid secretory responses. These observations contrast with previous studies in dogs and rats, suggesting that GABA inhibits somatostin release. In conscious dogs, peripherally administered GABA or the nonselective $GABA_{A/B}$ agonist progabide did not modify or had a tendency to reduce circulating levels of somatostatin, while increasing acid and gastrin secretion (Thirlby *et al*., 1988). Moreover, *in vitro* studies in isolated rat gastric mucosa or in an isolated rat stomach preparation showed that GABA inhibited somatostatin release (Harty & Franklin, 1983; 1986; Koop & Arnold, 1986; Guo *et al*., 1989; Weigert, 1998), probably contributing to the acid secretory responses observed. However, these effects were elicited by GABA or muscimol and blocked by the selective $GABA_A$ receptor antagonist bicuculline, suggesting a $GABA_A$-mediated mechanism (Koop & Arnold, 1986; Weigert, 1998). As previously mentioned, a similar mechanism is unlikely to be present in the mouse, since neither GABA nor muscimol elicited any change in acid secretion. It is likely that, under the present conditions, the apparent release of somatostatin might be an indirect effect, associated to feedback control mechanisms between gastrin and luminal acid concentration and somatostatin (Schubert *et al*., 1988; Shulkes & Read, 1991).

In summary, the results obtained suggest that, in mice, the selective stimulation of $GABA_B$ receptors increases gastric acid secretion through vagal cholinergic- and gastrin-dependent mechanisms. However, from the present studies, the site of action cannot be clearly specified and a combination of peripheral and central mechanisms might be necessary to fully elicit the stimulatory responses observed. Although the somatostatin-dependent inhibitory system seems to be also implicated in the responses observed, the role of somatostatin might be secondary to the release of gastrin and the increase in luminal acidity. These observations suggest that, in addition to its demonstrated role in the control of the lower esophageal sphincter (Lehmann *et al*., 1999; McDermott *et al*., 2001; Smid *et al*., 2001; Zhang *et al*., 2002), the GABAergic system can be also a modulator of gastric acid secretion. A physiological role for the GABAergic system modulating gastric vagal outflow has been proposed. Therefore, the possible involvement of GABA in the ongoing regulation of vago-vagal reflexes modulating gastric function, in addition to mediating vagal responses to specific stimuli, (Rogers *et al*., 1996) cannot be ruled out.

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