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# Involvement of capsaicin-sensitive neurons in gastrin release provoked by intragastric administration of bile salts in the rat

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**Abstract:** To clarify the mechanism of gastrin release provoked by the reflux of bile juice into the stomach, we studied the effects of tetrodotoxin (0.08 mg/kg), atropine sulfate (0.5 mg/kg), truncal vagotomy, and chemical denervation of afferent sensory neurons with capsaicin (100 mg/kg) on gastrin release induced by bile salts in the rat ($n = 6$ per group). Sodium taurocholate and deoxycholate (>2.5 mM) significantly increased the serum levels of gastrin. However, sodium tauroursodeoxycholate had no effect. The levels of serum gastrin before and 1 h after administration of 2.5 mM sodium taurocholate were 94.6 ± 10.7 and 211.0 ± 21.1 pg/ml, respectively. Tetrodotoxin and atropine sulfate completely inhibited this sodium taurocholate induced-gastrin increase, while truncal vagotomy was without effect. Capsaicin markedly reduced the increasing effects of sodium taurocholate. These findings suggested that the neuronal pathways involved in gastrin release are probably an intragastric local circuit originating from capsaicin-sensitive afferent sensory neurons and terminating in muscarinic receptors in the postsynaptic efferent cholinergic neuron system.

**Key words:** bile salts, gastrin, capsaicin, muscarinic receptor

## Introduction

The reflux of bile juice into the stomach can cause gastric ulcers[1] and gastritis,[2] although the details of the mechanism involved remain unclear. The effects of bile salts on the gastric mucosa depend on a number of factors, such as concentration, pH, type of salt, and the length and site of exposure. Bile salts can disrupt the gastric mucosal barrier by promoting hydrogen ion back diffusion,[3] or by direct cytotoxicity, including an effect on mucosal energy metabolism.[4] Further, the increase of gastric acid secretion through gastrin release from G cells seems to be facilitated, via stimulation of a parasympathetic pathway, by the intragastric administration of taurocholic acid.[5] Recently, the humoral mechanisms involved in gastrin release have been clarified by in vivo and in vitro experiments. It has been shown that muscarinic and β-adrenergic receptors on antral G cells are involved in the stimulation of gastrin release, while α-adrenergic receptors seem to be inhibitory.[6–8] Although bile salt-mediated gastrin release seems to be mediated via stimulation of the parasympathetic pathways or the cellular microvilli, the precise mechanism involved is still not clear. In this study, we examined the effects of atropine sulfate, capsaicin, tetrodotoxin, and truncal vagotomy on gastrin release provoked by bile salts in the rat, and evaluated the role played by the intragastric nervous system in the reflux of bile salts into the stomach.

## Materials and methods

### *Animals*

Experiments were performed on 14- to 18-week-old male Wistar rats weighing 300–400 g (SLC, Shizuoka, Japan). The animals were fasted overnight, but were allowed water up to the time of the experiments. There were six rats in each experimental group.

### *Drugs*

The following drugs were used: urethane (Nacalai Tesque Inc., Kyoto, Japan), sodium taurocholic acid (TCA; Nacalai Tesque), sodium deoxycholic acid

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(DCA; Nacalai Tesque), sodium tauroursodeoxycholic acid (TUDCA; Nacalai Tesque) atropine sulfate (Tanable Pharmaceutical Co. Ltd., Osaka, Japan), ethanol (Nacalai Tesque), capsaicin (Sigma Chemical Company, St Louis, Mo.), Tween 80 (Nacalai Tesque), tetrodotoxin (Sigma), glycine (Nacalai Tesque), D-mannitol (Nacalai Tesque), carbachol (Sigma), 0.1 mol/l HCl (Nacalai Tesque), sodium hydroxide (Nacalai Tesque), Hank's balanced salt solution (HBSS; Nissui, Tokyo, Japan), kanamycin (Meiji Co. Ltd., Tokyo, Japan), type I collagenase (Sigma), bovine serum albumin (Nacalai Tesque), trypan blue (Sigma), and a gastrin assay kit (Dainabot Co. Ltd., Tokyo, Japan). In addition, $^{51}CrNa_2CO_4$ was purchased from the Japan Radioisotope Association (Tokyo).

### *$^{51}Cr$ Release cytotoxic assay*

To evaluate the critical concentration of bile salts for direct cytotoxicity, a $^{51}Cr$ release cytotoxic assay was performed, according to a modification of the method of Terano et al.[9] In brief, the rats were anesthetized with urethane (1.2 g/kg, i.p.) and the gastric mucosa was excised from the stomach and minced into pieces. These pieces were incubated in HBSS containing 0.1% collagenase and 100 mg/ml kanamycin in a shaking bath at 37°C in 5% $CO_2$–95% $O_2$. The tissues were then pipetted several times and filtered through a nylon mesh. The filtrate was washed by centrifugation at 600 rpm for 5 min in HBSS. Cell viability was shown to be more than 95% by the trypan blue dye exclusion test. Cells were then incubated in HBSS containing 100 μCi of $^{51}CrNa_2CO_4$ for 1 h at 37°C. The labeled cells were then washed three times with HBSS to remove any excess isotope, and 1 ml of HBSS (37°C) containing bile salts was added to the cell monolayer. After incubation for varying periods, the medium was decanted and the monolayers were washed with 1 ml of HBSS. The medium and the washings were combined and centrifuged at 2000 rpm for 15 min. The pellet thus obtained was combined with the washed cell monolayer and allowed dissolve to in 1 N NaOH for 1 h. The radioactivity of the cells and the supernatant was then counted with an automatic gamma counting system and the percentage of $^{51}Cr$ released per sample was expressed as follows:

$$\text{Specific release (\%)} = \{(\text{cpm of supernatant})/(\text{cpm of supernatant} + \text{cpm of cells})\} \times 100$$

### *Animal experiments*

The experiments were done according to the method of Yokotani et al.[10] Briefly, while the rats were anesthetized with urethane (1.2 g/kg, i.p.), the esophagus was ligated and the trachea was cannulated through a cervical incision. The femoral vein was also cannulated for the infusion of saline. Then the abdomen was opened via a midline incision, and a round-tipped polyethylene cannula (3.5 cm in length and 0.4 cm in diameter) was inserted into the stomach via an incision in the duodenum. The cannula was held in place by two ligatures around the duodenum, one oral and another caudad to the cannula, and the abdominal incision was closed. The stomach was washed with saline, after which 2.0 ml of a gastric solution prewarmed at 37°C was instilled; this was replaced at 15-min intervals. This solution consisted of a 1/5 (v/v) mixture of glycine and mannitol adjusted to 300 mOsmol and pH 3.5 by the addition of 0.1 N HCl according to the method of Blair et al.[7] One h was allowed before the beginning of each experiment for stabilization of the basal acid output. Atropine sulfate (0.5 mg/kg, i.m.), or carbachol (0.4 mg/kg, i.m.) was injected 30-min before the administration of bile salts. Control rats received an injection of saline (1 ml, i.m.) 30-min before the intragastric administration of each bile salt. After another 30 min period, gastric acid secretion and gastrin release were measured. Gastric acid output was determined by titration with 0.01 N NaOH and expressed as mEq/15 min. The serum gastrin level was determined by radioimmunoassay, using a commercially available gastrin assay kit (Dainabot Co. Ltd., Tokyo, Japan), as reported previously.[11] The antibody used was specific for gastrin 17 and did not cross-react with the larger gastrin polypeptide. Values were expressed as picogram equivalents of synthetic human gastrin 17.

### *Effect of truncal vagotomy*

To determine the influence of the centrally-mediated autonomic nervous system on gastrin release following the intragastric administration of bile salts, six rats were bilaterally vagotomized in the cervical region before the experiments described above were performed. In a preliminary study, the effect of truncal vagotomy was confirmed by the injection of 2-deoxy-D-glucose (50 mg/kg) into the left carotid artery, as reported previously:[12,13] gastric acid and gastrin release were not changed after the injection of 2-deoxy-D-glucose in vagotomized rats, but were increased in untreated rats (data not shown).

### *Tetrodotoxin pretreatment*

To determine whether the stimulation of gastrin release by bile salts was mediated via the nervous system, six rats were mechanically ventilated and injected with tetrodotoxin (0.08 mg/kg, i.v.) before the effects of bile salts were assessed.

*Capsaicin pretreatment*

Capsaicin was dissolved in saline containing 10% ethanol and 10% Tween 80 and was given to rats for 2 days, as described by Okuma et al.[14] On the 1st day, the rats received 40 mg/kg, s.c. and on the 2nd day, they received 60 mg/kg, s.c. (total dose: 100 mg/kg). Two weeks later, the effectiveness of the treatment was tested by assessing the blink reflex.[15] The effects of bile salts on gastric acid secretion and gastrin release were then studied as described above.

*Statistical analysis*

All data are presented as means ± SEM. Analysis of variance, the Wilcoxon matched pairs signed ranks test, and the Mann-Whitney test were used for statistical analyses. With all statistical analyses, an associated probability ($P$ value) or $<0.05$ was regarded as indicating a significant difference.

## Results

*Effects of bile salts on serum gastrin levels*

The serum gastrin level was not altered by the intragastric administration of TUDCA (Fig. 1). One h after the intragastric administration of 2.5 mM TCA and 2.5 mM DCA, serum gastrin levels increased significantly, from 94.6 ± 10.7 to 211.0 ± 21.1 and 241.2 ± 15.6 pg/ml, respectively. Increasing the concentration of TCA and DCA to 5 and 10 mM did not enhance these bile salts-induced changes. TUDCA was without effect even at the highest concentration (10 mM).

*$^{51}Cr$ Release assay*

Assessment of cytotoxicity was determined by $^{51}Cr$ release assay for a 3-h incubation period. The cytotoxicity of TCA and DCA increased in a concentration-dependent manner. After the 3-hour incubation period, the specific $^{51}Cr$ release provoked by 10 mM TCA (43%) or 10 mM DCA (54%) had increased significantly compared with that in the control rats (15%), as shown in Fig. 2. At 2.5 mM, DCA induced a slight but significant increase in the specific $^{51}Cr$ release, while TCA was without effect. Thus, DCA was more cytotoxic than TCA. TUDCA did not increase the $^{51}Cr$ release, even at 10 mM. In the following experiments, therefore, we used DCA, TCA, and TUDCA at doses lower than 5 mM, at which doses these agents had no, or less, cytotoxic effect on the gastric cells.

*Effects of bile salts on serum gastrin and gastric acid sercretion*

The serum gastrin level did not increase after the intragastric administration of 2.5 mM TUDCA, and gastric acid secretion also showed no increase for the entire experimental period (3 h) (Fig. 3, upper).

Following the administration of 2.5 mM TCA, the serum gastrin level increased progressively, from 96.8 ± 6.0 pg/ml to 141.5 ± 13.2 pg/ml, after 30 min. Gastric acid output also increased progressively, from 0.11 ±

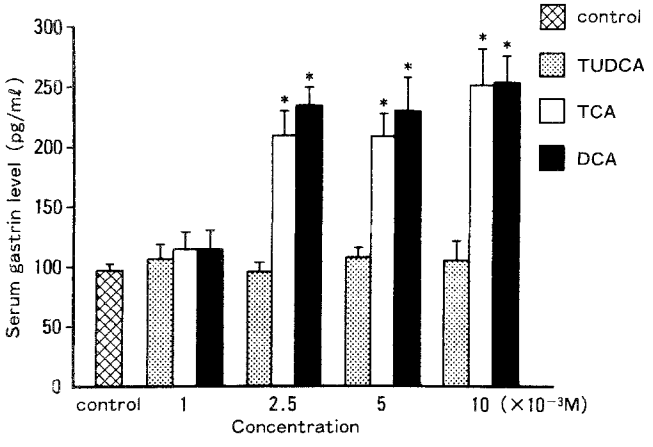


**Fig. 1.** Effects of bile salts on serum gastrin levels. Serum gastrin levels were measured 1 h after administration of each bile salt. Each point represents the mean ± SEM of six rats. Mann-Whitney test (two-tailed) was used for statistical analysis. $^*P < 0.05$ vs control. *TUDCA*, Sodium tauroursodeoxycholic acid; *TCA*, sodium taurocholic acid; *DCA*, sodium deoxycholic acid

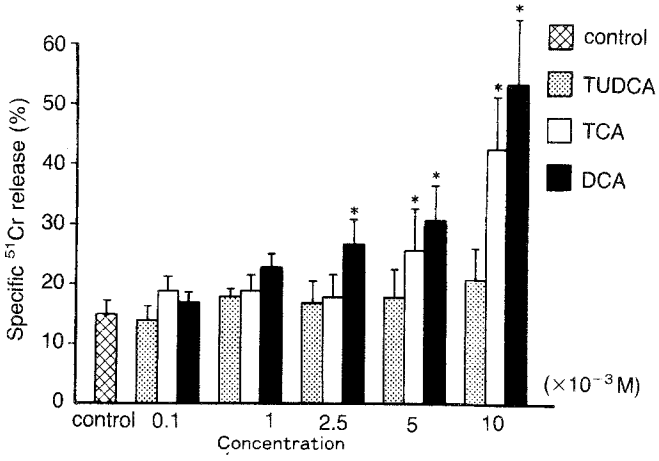


**Fig. 2.** $^{51}Cr$ Release assay to determine bile salt cytotoxicity in gastric cells after 3-h incubation. Each point represents the mean ± SEM of six rats. Mann-Whitney test (two-tailed) was used for statistical analysis. $^*P < 0.05$ vs control. *TUDCA*, Sodium tauroursodeoxycholic acid; *TCA*, sodium taurocholic acid; *DCA*, sodium deoxycholic acid

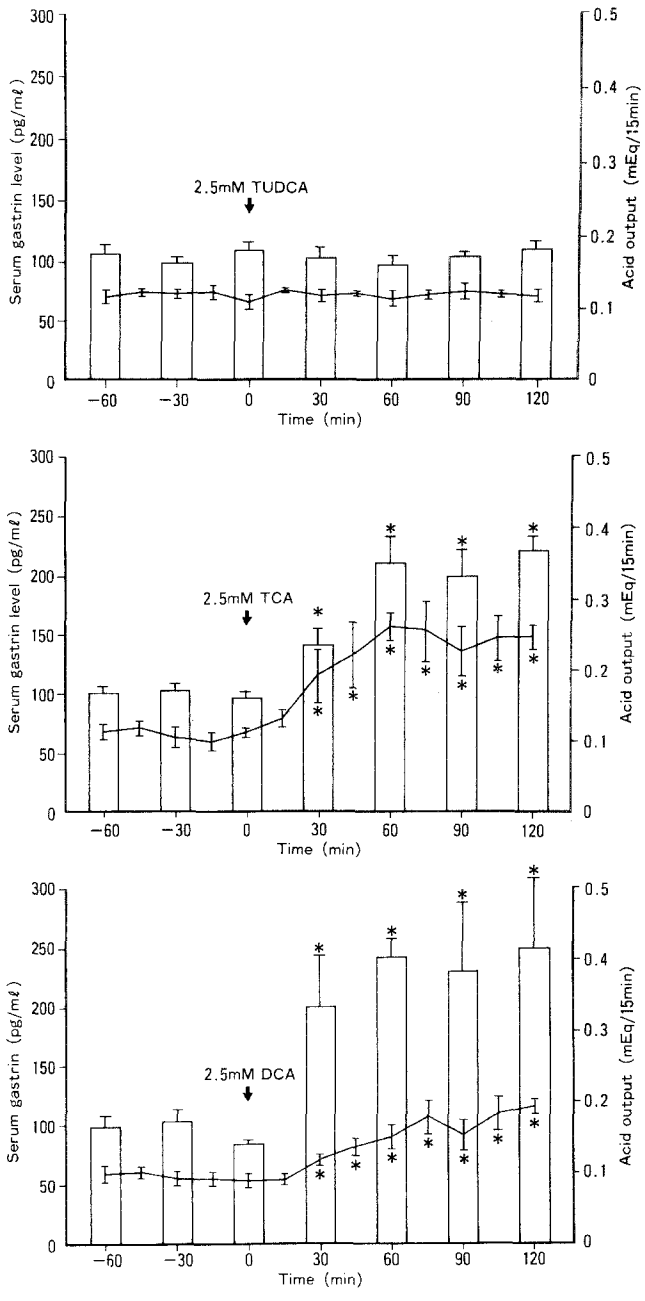


**Fig. 3.** Effects of bile salts on serum gastrin and gastric acid secretion. After the intragastric administration of each bile salt (2.5 mM), serum gastrin levels (*columns*) for 30-minute periods were measured and gastric acid output (*lines*) for 15-minute periods was measured. Each point represents the mean ± SEM of six rats. Wilcoxon matched pairs signed ranks test (two-tailed) was used for statistical analysis. $^*P < 0.05$ vs level at 0 min. *TUDCA*, Sodium tauroursodeoxycholic acid; *TCA*, sodium taurocholic acid; *DCA*, sodium deoxycholic acid

0.01 mEq/15 min to 0.19 ± 0.04 mEq/15 min, after 30 min. These effects reached the maximum after 1 h and plateau levels were then maintained for the remainder of the experimental period (Fig. 3, middle).

Following the administration of 2.5 mM DCA, the serum gastrin level increased progressively, from 84.9 ± 2.2 pg/ml to 201.1 ± 41.9 pg/ml, after 30 min, and gastric acid output increased slightly but significantly, from 0.09 ± 0.01 mEq/15 min to 0.12 ± 0.01 mEq/15 min, after 30 min (Fig. 3, bottom).

### *Effects of atropine on TCA-induced gastrin release*

Treatment of rats with atropine 0.5 mg/kg, i.m. did not affect the serum gastrin level during the first 1-h period (Table 1). Intragastric administration of 2.5 mM TCA markedly increased the serum gastrin level after 1-h. These increasing effects of TCA were completely blocked by pretreatment with atropine. The effects of atropine on DCA-induced gastrin release were very similar to those of TCA (data not shown).

### *Effects of tetrodotoxin on TCA-induced gastrin release*

Tetrodotoxin (0.08 mg/kg, i.v.) alone did not affect the basal level of serum gastrin in the control rats during the first 1-h period (Table 2). The effect of TCA in increasing the level of gastrin was, however, completely inhibited by pretreatment with tetrodotoxin.

### *Effects of TCA on gastrin release in vagotomized rats*

As shown in Table 3, truncal vagotomy did not affect the serum level of gastrin during the first 1-h period. The TCA-induced increase in the serum level of gastrin was not affected by truncal vagotomy.

**Table 1.** Effects of atropine on sodium taurocholic acid (*TCA*)-induced gastrin release

| | | | Serum gastrin (pg/ml) | | | |
|---|---|---|---|---|---|---|
| Atropine pretreatment | | | 0 Min | | 60 Min | |
| Vehicle[a] | (+) | ($n = 6$) | 98.5 ± 12.5 | NS | 94.5 ± 6.0 | * |
| TCA | (−) | ($n = 6$) | 94.6 ± 10.7 | * | 211.0 ± 21.1 | * |
| TCA | (+) | ($n = 6$) | 98.5 ± 12.5 | NS | 92.3 ± 11.3 | |

$^*P < 0.05$ vs control; *NS*, not significant

Results are the means ± SEM of six rats. Atropine was given i.m. 30 min before intragastric administration of vehicle[a] or 2.5 mM TCA. Serum gastrin levels were measured before and 1 h after intragastric administration of buile salt. Analysis of variance and Wilcoxon matched pairs signed ranks test (two-tailed) were used for statistical analysis

+, Rats pretreated with atropine; −, rats that received saline (1 ml, i.m.) alone

[a] Vehicle, A 1/5 (v/v) mixture of glycine and mannitol (300 mOsmol, pH 3.5)

**Table 2.** Effects of tetrodotoxin on TCA-induced gastrin release

| Tetrodotoxin pretreatment | | | Serum gastrin (pg/ml) 0 Min | | 60 Min | |
|---|---|---|---|---|---|---|
| Vehicle[a] | (+) | ($n = 6$) | 117.7 ± 17.9 | NS | 133.5 ± 20.8 | * |
| TCA | (−) | ($n = 6$) | 94.6 ± 10.7 | * | 211.0 ± 21.1 | * |
| TCA | (+) | ($n = 6$) | 117.7 ± 17.9 | NS | 103.0 ± 3.2 | |

* $P < 0.05$; *NS*, not significant
Results are the means ± SEM of six rats. Tetrodotoxin was given i.v. 30 min before intragastric administration of vehicle[a] or 2.5 mM TCA. Serum gastrin levels were measured before and 1 h after intragastric administration of TCA. Wilcoxon matched pairs signed ranks test (two-tailed) was used for comparison of rats before and after intragastric administration of vehicle or 2.5 mM TCA. Analysis of variance was used for comparisons of differences between two groups.
+, Rats pretreated with tetrodotoxin; −, rats that received saline (1 ml, i.m.) alone
[a] Vehicle, A 1/5 (v/v) mixture of glycine and mannitol (300 mOsmol, pH 3.5)

**Table 3.** Effects of vagotomy on TCA-induced release of gastrin

| Vagotomy | | | Serum gastrin (pg/ml) 0 Min | | 60 Min | |
|---|---|---|---|---|---|---|
| Vehicle[a] | (+) | ($n = 6$) | 102.2 ± 13.5 | NS | 98.6 ± 8.9 | * |
| TCA | (−) | ($n = 6$) | 101.8 ± 8.9 | * | 225.5 ± 33.4 | NS |
| TCA | (+) | ($n = 6$) | 94.6 ± 10.7 | * | 211.0 ± 21.1 | |

* $P < 0.05$; *NS*, not significant
Results are the means ± SEM of six rats. Serum gastrin levels were measured before and 1 h after intragastric administration of the vehicle[a] or 2.5 mM TCA. Wilcoxon matched pairs signed ranks test (two-tailed) was used for comparison of rats before and after intragastric administration of vehicle or 2.5 mM TCA. Analysis of variance was used for comparison of differences between two groups
+, Vagotomized rats, −, non-vagotomized rats
[a] Vehicle, A 1/5 (v/v) mixture of glycine and mannitol (300 mOsmol, pH 3.5)

*Effects of TCA on gastrin release in capsaicin-pretreated rats*

As shown in Table 4, carbachol (0.4 mg/kg, i.m.) induced a marked and significant increase in the serum gastrin level. This increasing effect of carbachol was not affected by pretreatment with capsaicin. It is therefore likely that pretreatment with capsaicin did not injure antral G cells.

In capsaicin-pretreated rats, the increasing effect of TCA on the serum level of gastrin was markedly reduced (91%), as shown in Table 4.

## Discussion

Duodenogastric reflux of bile has been suggested as an important pathogenetic factor for both gastric and duodenal ulcers.[16,17] Rhodes et al.[1] reported that bile acid concentration in gastric juice was elevated in patients with gastric ulcer disease compared with normal individuals. The mechanism whereby bile salts cause mucosal damage is thought to involve (a) an increase of hydrogen ion back diffusion, (b) direct cytotoxicity, and (c) an increase of gastric acid output. Davenport[3] found that detergents, such as bile salts and dodecylsulfates, injured the gastric mucosal barrier. Hydrogen ion back diffusion and the changes in potential difference were greatest under acidic conditions.

TCA, the taurine conjugate of the bile salt cholic acid, is synthesized in the liver and secreted into the bile. In the intestine, bacterial action can result in its deconjugation to form cholic acid (CA), deoxylation to form taurodeoxycholic acid (TDCA), or both deconjugation and deoxylation, to form DCA. The cytotoxicity of bile salts varies according to their hydrophobicity and is also concentration-dependent.

**Table 4.** Effects of capsaicin on carbachol- and TCA-induced release of gastrin

| Capsaicin | | | Serum gastrin (pg/ml) 0 Min | | 60 Min | |
|---|---|---|---|---|---|---|
| (−) | Carbachol | ($n = 6$) | 91.6 ± 12.6 | * | 211.8 ± 11.2 | NS |
| (+) | Carbachol | ($n = 6$) | 98.1 ± 9.7 | * | 203.0 ± 13.4 | |
| (−) | TCA | ($n = 6$) | 94.6 ± 10.7 | * | 211.0 ± 21.1 | * |
| (+) | TCA | ($n = 6$) | 128 ± 16.6 | NS | 142.0 ± 15.3 | |

* $P < 0.05$; *NS*, not significant
Results are the means ± SEM of six rats. Capsaicin was given to rats over a 2-day period (total 100 mg/kg, s.c.). Serum gastrin levels were measured before and 1 h after treatment with carbachol (0.4 mg/kg, i.m.) or the intragastric administration of 2.5 mM TCA. Wilcoxon matched pairs signed ranks test (two-tailed) was used for comparison of rats before and after intragastric administration of 2.5 mM TCA, or before and after treatment with carbachol. Analysis of variance was used for comparisons of differences between two groups
+, Rats pretreated with capsaicin; −, rats that received saline (1 ml, i.m.) alone

Hydrophobic bile salts damage cell membranes more severely than hydrophilic salts, the order of cytotoxicity being: DCA > CDCA > CA > UDCA, and free bile salts > glycine conjugates > taurine conjugates.[18,19] Cytotoxicity has been reported to be increased by a higher concentration of bile and is also related to pH.[20] Bile salts begin to precipitate from the solution as the pH falls to a point near the dissociation constant (pKa). In the present study, the $^{51}Cr$ release assay showed that TCA cytotoxicity was detectable at concentrations higher than 2.5 mM, while no cytotoxicity was found in the case of TUDCA.

Gastrin, which stimulates gastric acid secretion, is secreted from antral G cells. The mechanisms that regulate gastric acid secretion and gastrin release have been studied in detail. Gastrin release is stimulated by amino acids and calcium in the gastric lumen, by the activation of nervous reflexes, by circulating catecholamines and bombesin, and by increases in gastric pH exceeding 6.5.[6,21] Gastrin release is inhibited by decreases of the gastric pH below pH 3, by somatostatin, and by certain prostaglandins.[6,22,23] The release of gastrin is stimulated by TCA applied to the gastric mucosa at pH 3 or 7.[23] The present study demonstrated that antral perfusion with sodium TCA or DCA at pH 3.5 stimulated gastrin release, a finding which confirms previous observations.[5,23] By contrast, TUDCA had no effect on the release of gastrin or on the secretion of gastric acid; however, further studies are necessary to determine why only hydrophobic bile salts increase gastrin release. It has recently been shown that a dual vagal-cholinergic mechanism causes both stimulation (pH > 6.5) and inhibition of gastrin release (pH < 2), presumably by activating separate intermediary mechanisms via the gastrin-releasing peptide (bombesin).[6] Thus, the intermediary bombesin pathway may not be a major regulatory mechanism with respect to gastrin release by bile salts at pH 3.5. In the present study, the plasma somatostatin level was not altered by the intragastric administration of bile salts (data not shown).

Grossman[24] suggested a cholinergic pathway that modulates gastrointestinal hormone release caused by chemical stimulation or by the ingestion of meat. In the present study, the intragastric administration of TCA increased gastrin release in rats, while pretreatment of rats with tetrodotoxin completely blocked this increase. Tetrodotoxin blocks neuronal conduction.[25] Increases in gastrin release provoked by TCA are therefore induced by the activation of a nervous system. We also found that gastrin release and gastrin acid secretion were completely inhibited by atropine sulfate. Parietal cells also have muscarinic receptors, and gastric acid secretion from parietal cells may be increased both by gastrin and by direct stimulation of muscarinic receptors on the parietal cells. However, it is interesting to note that the TCA-induced increase in gastrin release was not affected by truncal vagotomy. These findings clearly demonstrate that the responses were mediated via intragastric neuronal pathways and not via central pathways. Furthermore, the present results corresponded well with a report that gastrin release was induced by perfusion of the denervated gastric antrum with 10 mM taurocholic acid.[23]

Capsaicin is a sensory neurotoxin that has been extensively used to investigate the role of afferent C fibers[26] in physiological and pharmacological processes. Systemic treatment with capsaicin causes prolonged functional impairment of unmyelinated afferent nerve fibers in adult rats and the degeneration of these fibers in neonatal rats.[26,27] Local application of capsaicin to a nerve reproduces many of the functional and morphological effects of systemic treatment in adults, but has the advantage of affecting a specific nerve pathway and avoiding the central nervous system effects.[27] Capsaicin-sensitive vagal afferent fibers contribute to the non-cholinergic increase in gastric mucosal blood flow induced by prolonged electrical stimulation of the vagus in rats.[27,28] In addition, capsaicin-sensitive vagal afferent fibers mediate the vagal portion of the secretory response to gastric distention, and these afferents also play a role in the gastric acid secrtory response to histamine.[29] The role of capsaicin-sensitive vagal afferents in the regulation of gastrin release is still not clear. Capsaicin-sensitive afferent fibers form a particularly dense plexus around the gastric submucosal region.[30–32] These nerve fibers contain a variety of peptides, such as substance P and calcitonin gene-related peptide and their receptors.[33,34] In the present study, TCA-induced gastrin release was significantly inhibited by pretreatment with capsaicin. The capsaicin-sensitive afferent fibers were therefore probably involved in the regulatory mechanism of gastrin release in response to intragastric bile salts.

In conclusion, the intragastric administration of hydrophobic bile salts stimulates gastrin release from antral G cells in the rat. The pathways involved are probably intragastric local reflexes originating from capsaicin-sensitive afferent neurons and terminating in muscarinic receptors in the postsynaptic efferent cholinergic neuron system.

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