Gastrin releases a blood calcium-lowering peptide from the acid-producing part of the rat stomach

(calcium incorporation/calcium homeostasis/hypocalcemic hormone)

PER PERSSON, ROLF HÅKANSSON*, JAN AXELSON, AND FRANK SUNDLER

Departments of Pharmacology, Surgery, and Medical Cell Research, University of Lund, Lund, Sweden

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ABSTRACT Gastrin-17 induces hypocalcemia in the rat without stimulating calcitonin release. The gastrin-induced hypocalcemia persisted after thyroparathyroidectomy or parathyroidectomy. In contrast, gastrectomy or extirpation of the acid-producing part of the stomach prevented the hypocalcemic effect, suggesting the involvement of the proximal stomach in the gastrin-evoked lowering of blood calcium. The drop in blood calcium upon injection of gastrin-17 did not reflect a loss of calcium via the gastric juice or via the urine. Extracts of the acid-producing mucosa of the rat stomach had a hypocalcemic effect. The extracts were purified by gel chromatography and reversed-phase high-performance liquid chromatography. Digestion with leucine aminopeptidase destroyed the hypocalcemic activity, while trypsin had no effect, suggesting a peptide (or peptides) with an unprotected NH₂ terminus and without basic amino acid residues (or with protected basic amino acids).

Both gastrin-17 and the mucosal extract stimulated the uptake of ⁴⁶Ca into bone (radius and sternum). Gastrin-17 was without effect in rats that had undergone gastrectomy, while the mucosal extract was equally effective in gastrectomized and unoperated rats. We suggest that the effects of gastrin-17 on blood calcium and on calcium transfer into bone are indirect and that gastrin-17 stimulates the release of a peptide hormone, tentatively named gastrocalcin, from the acid-producing mucosa of the stomach. Gastrocalcin stimulates the uptake of ⁴⁶Ca into bone, thereby causing hypocalcemia.

Calcium plays a major role in fundamental biological processes, such as bone mineralization, muscle contraction, and intracellular signal transduction. In vertebrates the control of Ca²⁺ in blood involves the parathyroid hormone, calcitonin, and vitamin D. The parathyroid hormone stimulates the release of Ca⁺⁺ from bone, the reabsorption of Ca²⁺ in renal tubules, and the conversion of inactive 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, which then enhances the absorption of Ca²⁺ from the intestines. The effects of parathyroid hormone are reflected in hypercalcemia. Calcitonin, which is produced in the C cells of the thyroid, suppresses the release of Ca⁺⁺ from bone and causes hypocalcemia.

In 1975, Schulak and Kaplan (1) reported that porcine gastrin evokes hypocalcemia in intact as well as in thyroparathyroidectomized rats, suggesting a mechanism not involving calcitonin. This view is supported by the fact that in the rat neither exogenous gastrin-17 nor hypergastrinemia of endogenous origin stimulated calcitonin release (cf. ref. 2). Gastrectomy or resection of the proximal stomach inhibited the hypocalcemic response to gastrin (1, 3, 4), indicating that the proximal stomach mediated the response. The present study examines the mechanism by which gastrin induces hypocalcemia and the role played by the proximal stomach.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, weighing 150–225 g, were used. The rats were fasted overnight (18–24 hr) before each experiment.

Enzymes and Synthetic Peptides. Synthetic human gastrin-17 (nonsulfated) and human calcitonin were from Peninsula Laboratories. Fresh solutions were prepared each day. The two peptides were dissolved in 0.9% saline and administered subcutaneously (s.c.) or into the jugular vein in a total vol of 1 or 2 ml (s.c.) and 0.2 ml (i.v.). The administration was s.c. unless otherwise stated. Control rats were injected with the appropriate volume of 0.9% saline. Leucine aminopeptidase (microsomal type IV-S, porcine kidney) and trypsin (type III, bovine) were obtained from Sigma.

Surgical Procedures. Operations were performed under light diethyl ether anesthesia. Parathyroidectomy and combined thyroidectomy and parathyroidectomy were performed under an operation microscope. The proximal part of the stomach was resected (fundectomy) as described in detail elsewhere (5). Gastrectomy was performed by resection of the stomach followed by joining the esophagus and duodenum end-to-end. The animals were allowed to recover for 7 days after the operation.

Blood Sampling. Rats were placed in Bollman-type restraining cages and injected in the proximal part of the tail with lidocain (Xylocain; Astra, Södertälje, Sweden) 1 hr before the start of the experiment and every hour thereafter. Blood was drawn from the tip of the tail. Each rat was sampled at least four times.

Ca in Blood. The effect of s.c. or i.v. injection of gastrin-17 or s.c. injection of an extract of acid-producing (oxyntic) mucosa (for details on the preparation of the extract, see below) was studied by measuring the levels of circulating calcium before and 20, 60, and 180 min after injection. For determination of ionized calcium, 150 µl of fresh whole blood was collected in heparinized capillary tubes and analyzed at once using a Ca²⁺ selective electrode (ICA1; Radiometer, Copenhagen). The pH of the blood was measured by the same electrode. If the pH was below 7.36 the samples were not taken into account. Total calcium was determined spectrophotometrically in 25 µl of serum with a commercially available kit based on the conjugation of calcium with o-creosolphthalein (Calcium C; Wako Chemicals, Neuss, F.R.G.).

Ca in Gastric Juice and Urine. Fistulas were generated by implantation of plastic tubes in the rumen of the stomach under diethyl ether anesthesia. The rats were allowed to recover for 2 weeks. Before the start of each experiment, the gastric fistula was opened, and the stomach was rinsed with warm saline and allowed to drain freely for 1 hr. Gastrin-17 or saline was injected and gastric juice was collected in 30-min portions. The volumes were measured, the acid output was determined by titration with 0.02 M NaOH, and

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*To whom reprint requests should be addressed: Department of Pharmacology, Solvegatan 10, 223 62 Lund, Sweden.
the total calcium output was determined spectrophotometrically with 50 \( \mu l \) of gastric juice. Plastic fistulas were implanted in the urinary bladder under diethyl ether anesthesia and the rats were allowed to recover for 3 days. Before the start of each experiment, the fistula was opened, and the bladder was rinsed with warm saline and allowed to drain freely for 1 hr. Gastrin-17 or saline was injected and urine was collected in 15-min portions. The volumes were measured, and the total calcium output was determined by the spectrophotometric method with 50 \( \mu l \) of urine.

**Uptake of Peroral \( ^{45} \text{Ca} \) in Blood.** \( ^{45} \text{CaCl}_2 \) (5 \( \mu \)Ci per kg of body weight; specific activity, 4–50 Ci/g; 1 Ci = 37 GBq; NEN-Chemicals, DuPont, Dreieich, F.R.G.) was administered in 1 ml of 0.9% saline orally via a gastric tube. Injection of gastrin and sampling of blood was made 15 min later. Blood samples (50 \( \mu l \)) were then collected at various time intervals in capillary tubes and added to an aliquot of solubilizer [150 \( \mu l \) of Soluene-350/isopropanol (1:2)]. The mixture was incubated for 1 hr at 40\(^\circ\)C. Decolorization was achieved by adding 200 \( \mu l \) of 30% hydrogen peroxide and leaving the mixture for 2 hr at room temperature (6). After decolorization, 10 ml of Picofluor-40 (Packard Instrument) cocktail was added to each sample. The vials were capped, vortex mixed, and left standing overnight at room temperature before determination of radioactivity (LKB \( \beta \)-counter Wallac 1214 Rackbeta). Also, \( ^{45} \text{Ca} \) in deproteinized blood was analyzed; 50 \( \mu l \) of blood was added to 100 \( \mu l \) of 5% trichloroacetic acid in microcentrifuge tubes. The samples were centrifuged for 10 min at 1000 \( \times \) g. A 10-ml Picofluor-40 cocktail was added to each supernatant, and the radioactivity of the deproteinized samples was determined after overnight equilibration.

**Uptake of \( ^{45} \text{Ca} \) into Bone.** For the study of \( ^{45} \text{Ca} \) transfer into bone, the rats were killed by an overdose of diethyl ether 55 min after the peroral or s.c. administration of \( ^{45} \text{Ca} \). The rats had received gastrin-17 (usually 100 \( \mu g/kg \)) or oxyntic mucosal extract (1 mg per kg of body weight in 1 ml of 0.9% saline s.c.) 40 min before sacrifice. (For details on the preparation of the extract, see below.) The radius and sternum were dissected out, cleaned, and placed in an oven at 800\(^\circ\)C for 24 hr. The resulting ash was weighed and dissolved in 1 M HCl. Picofluor-40 (10 ml) was added. The radioactivity of the samples was counted after overnight equilibration.

**Preparation of Oxyntic Mucosal Extract.** Altogether, six preparations were made: 100–120 rats were killed for each preparation. The stomachs were dissected out and opened. The antrum was cut away. The mucosa was scraped off the oxyntic gland area with a scalpel, weighed (the wet weight of the pooled material varied between 70 and 80 g), and homogenized in ice-cold acetone (10 ml per g of tissue). The sediment was collected, washed with ice-cold acetone, followed by acetone-diethyl ether (50:50), and diethyl ether. The sediment was then taken up in 500 ml of 1 M acetic acid, boiled for 30 min, and left overnight. Undissolved material was sedimented by centrifugation at 6000 \( \times \) g at +4\(^\circ\)C for 20 min. The supernatants were freeze-dried and taken up in 0.2 M acetic acid, boiled for 30 min, and left standing overnight. Precipitated material was sedimented by centrifugation at

<table>
<thead>
<tr>
<th>Operation</th>
<th>( \text{Ca}^{2+} ) in whole blood, mM</th>
<th>Total Ca in serum, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>No operation</td>
<td>1.29 ± 0.01 (10)</td>
<td>2.34 ± 0.03 (8)</td>
</tr>
<tr>
<td>Gastrectomy</td>
<td>1.28 ± 0.02 (5)</td>
<td>2.33 ± 0.03 (3)</td>
</tr>
<tr>
<td>Fundectomy</td>
<td>1.28 ± 0.01 (3)</td>
<td>2.35 ± 0.03 (5)</td>
</tr>
<tr>
<td>Parathyroidectomy</td>
<td>0.71 ± 0.01 (5)</td>
<td>1.14 ± 0.04 (5)</td>
</tr>
<tr>
<td>Thyroparathyroidectomy</td>
<td>0.69 ± 0.01 (6)</td>
<td>1.09 ± 0.02 (6)</td>
</tr>
<tr>
<td>Gastrectomy +</td>
<td>0.67 ± 0.02 (6)</td>
<td>—</td>
</tr>
</tbody>
</table>

Levels were measured 1 week after the operations. Results are expressed as means ± SEM. Numbers in parentheses represent number of rats.

Fig. 1. Influence of gastrectomy (GX), fundectomy (FX), parathyroidectomy (PTX), thyroparathyroidectomy (THX), and THX+GX on the effect of gastrin-17 (100 \( \mu g/kg \)) on whole blood ionized calcium (\( \text{Ca}^{2+} \)) (A and C) and serum total calcium (\( \text{Ca}_{\text{tot}} \)) (B and D) in fasted rats. Operations were 1 week before the experiment. Gastrin-17 or saline was injected s.c. at arrow. Results are expressed as means ± SEM. Basal calcium levels are given in Table 1. (A and B) Time course of the hypocalcemic response (expressed as change over starting value) to gastrin-17. Fundectomy prevented the effect. (C and D) Integrated blood calcium changes after injection of gastrin-17. Calculations are made on results such as those shown in A and B (*, \( P < 0.05; **, P < 0.01 \) by Student's \( t \) test). NS, not significant. Numbers in parentheses represent number of rats.
25,000 × g for 15 min. The supernatant was freeze-dried, weighed, and taken up in 0.2 M acetic acid. At this stage, the weight of the freeze-dried material was 5–6 g. Portions of 2.5–3 g in 10–20 ml were passed through a Sephadex G-50 fine column (90 × 2.6 cm), equilibrated, and developed with 0.2 M acetic acid. Proteins/peptides were monitored (7) with bovine serum albumin (United States Biochemical) used as standard. Fractions of 2–3 ml were collected, freeze-dried, and weighed; 100–200 μg of each fraction was reconstituted in 0.2 ml of 0.9% saline and tested for calcium-lowering activity after i.v. injection. The fractions containing such activity were pooled and purified further by repeated reversed-phase HPLC using a μBondapak C18 column (7.8 × 300 mm). The solvent was 0.08% trifluoroacetic acid with 0–50% acetonitrile (as specified). The flow rate was 1 ml/min. In the first, but not in the subsequent, HPLC steps the column was overloaded. The overloading of the column was reflected in a >10% change in the elution volume for the fractions containing hypocalcemic activity. The HPLC system consisted of a Waters model 680 automated gradient controller equipped with a model U6K injector, a 6000 pump, and a model 440 absorbance detector (214 nm). Fractions of 0.5 ml were collected and freeze-dried. The dry residues were redissolved in 0.9% saline for assessment of calcium-lowering activity.

In one series of experiments, fractions from the Sephadex G-50 column, containing calcium-lowering activity, were collected, freeze-dried, and subjected to digestion with trypsin or leucine aminopeptidase. Trypsin (5 μg; 50 units) was dissolved in 600 μl of 0.1 M Tris buffer (pH 8.0) and incubated with 200 μg of the freeze-dried material for 6 hr at 37°C. For control purposes, 200 μg of the material was incubated under identical conditions but without trypsin, and trypsin was incubated without freeze-dried material. The digestion was terminated by boiling for 1 hr. Digestion with leucine aminopeptidase (10 units) was carried out as described above, with 0.1 M NH4CO3 buffer (pH 8.0) for 16 hr. Controls included material incubated without enzyme and enzyme without hypocalcemic material. The incubation mixtures were injected s.c. (four rats in each group).

RESULTS AND COMMENTS

Effect of Surgery on Blood Calcium. One week after gastrectomy or fundectomy, the blood calcium levels were

![Graph showing calcium levels after surgery](image)

**Fig. 3.** Gel chromatography of an extract of rat oxyntic mucosa. Freeze-dried material (2.5 g), prepared from 35 g of oxyntic mucosa (see text for details), was dissolved in 20 ml of 0.2 M acetic acid and passed through a Sephadex G-50 fine column. The column was developed with 0.2 M acetic acid. Fractions (3 ml) were collected and freeze-dried. The drawn curve represents protein/peptides. The column was calibrated with blue dextran (void volume) and 3H2O (total volume). The elution volumes of corticotropin-releasing factor (CRF) and [Met]enkephalin are indicated. The hatched areas represent material with hypocalcemic activity (tested as described in Fig. 4). Each lyophilized fraction was dissolved in 1 ml of 0.9% saline; 100–200 μl was injected i.v. Blood Ca responses were monitored. The pooled hypocalcemic material weighed 300 mg after lyophilization and was purified by reversed-phase HPLC (for details, see text). Eight milligrams were applied in each run; this overloaded the column. The column was developed with an isotropic elution using 0.08% trifluoroacetic acid and 10% acetonitrile for 40 min, followed by a linear gradient of 10–50% acetonitrile for 30 min. Fractions of 0.5 ml were collected and freeze-dried. Two zones with hypocalcemic activity were found using one-fifth of each fraction for i.v. injection. The two zones were repurified by HPLC. Elution was with 0.08% trifluoroacetic acid and a linear gradient of 0–16.5% acetonitrile for 60 min. Fractions of 0.5 ml were collected, freeze-dried, and tested for hypocalcemic activity with one-tenth of each fraction for i.v. injection. The purification procedure was applied on six preparations, each starting with 70–100 g of mucosa. The results presented are from a typical run.
unchanged. Parathyroidectomy and thyroparathyroidectomy, on the other hand, lowered the blood calcium levels (Table 1).

**Hypocalcemic Effect of Gastrin-17.** Gastrin-17 induced hypocalcemia in unoperated, thyroparathyroidectomized, and parathyroidectomized rats but not in gastrectomized or fundectomized rats (Fig. 1). The drop in total calcium was greater than that in ionized calcium and more long-lasting; the responses for total calcium and for ionized calcium peaked 60 and 20 min, respectively, after the injection of gastrin-17. The hypocalcemic response was dose dependent (Fig. 2). Intravenous administration of gastrin-17 produced a 60% greater peak response than subcutaneous injection; the time courses of the responses were identical (data not shown).

**Effect of Gastrin-17 on Calcium in Gastric Juice and Urine.** Gastrin-17 (100 μg/kg s.c.) produced a marked acid response in the five fistula rats studied but the stimulated acid secretion was not accompanied by an increased output of calcium in the gastric juice and was not accompanied by an increased output of calcium in the urine (data not shown). Five control rats received 0.9% saline.

**Hypocalcemic Effect of Oxyntic Mucosal Extract.** An acetic acid extract, prepared from oxyntic mucosa, was passed through a Sephadex G-50 column and the fractions were analyzed for hypocalcemic activity (Figs. 3 and 4). Digestion with leucine aminopeptidase degraded the hypocalcemic activity but trypsin was without effect (data not shown). The fractions that contained hypocalcemic activity were pooled and purified further by repeated HPLC. At least two separate peaks displayed hypocalcemic activity (Fig. 3).

**DISCUSSION**

Gastrin produces hypocalcemia in the rat. Neither exogenous nor endogenous gastrins seem to stimulate the release of calcitonin in the rat (2), and the hypocalcemic response evoked by gastrin-17 must therefore have another explanation. Schulak and Kaplan (1) and Kaplan et al. (3) were first to report that thyroparathyroidectomy and parathyroidectomy had no effect on the gastrin-evoked hypocalcemia while gastrectomy or fundectomy prevented it. Krishnamra and Limlomwongse (8) and Limlomwongse and Krishnamra (9) suggested that neither gastric secretion of calcium nor renal
elimination of calcium could explain the gastrin-evoked hypocalcemia. They also reported that gastrin suppressed efflux of calcium from bone (9) and proposed that gastrin exerted a direct action on bone calcium. Our results suggest that the hypocalcemic response to gastrin-17 is indirect, involving the release of hypocalcemic agents from the oxyntic mucosa of the stomach. The gastrin-induced hypocalcemia reflected neither the loss of calcium in the gastric juice nor the loss of calcium in the urine. Also, it appeared unlikely that the gastrin-induced hypocalcemia reflected an impaired uptake of calcium from the intestines for the simple reason that the rats were fasted before the experiments and, consequently, not much calcium could be absorbed from the intestines under the circumstances. In fact, one series of experiments was run in which gastrin-17 induced the same degree of hypocalcemia after prolonged fasting (48 hr) as after overnight fasting (data not shown). Finally, we could show that gastrin-17 enhanced the uptake of \(^{45}\)Ca into bone (radius and sternum), which could account for the gastrin-17-evoked hypocalcemia. Conceivably, gastrin might act directly on bone or indirectly by releasing an agent that enhanced calcium transfer. An indirect action was in fact suggested by the finding that gastrin-17 was without effect on calcium uptake into bone in gastrectomized rats, whereas extracts of oxyntic mucosa stimulated calcium transfer equally well in unoperated and gastrectomized rats. These results may be interpreted to mean that the hypothetical hypocalcemic agent that is released by gastrin-17 emanates from the oxyntic mucosa of the stomach.

![Diagram A](image1)

**Fig. 6.** (A) Effects of gastrin-17 (100 \(\mu\)g/kg s.c.) or a mucosal extract (1 mg/kg s.c. of freeze-dried material from the Sephadex G-50 column) on the uptake of \(^{45}\)Ca into bone (radius and sternum) of unoperated rats. \(^{45}\)CaCl\(_2\) was given orally 15 min before injection of gastrin-17 or extract and 35 min before sacrifice. The incorporation of \(^{45}\)Ca was enhanced by gastrin-17 and by the mucosal extract. (B) Gastric digestion prevented the enhancing effect of gastrin-17 on the incorporation of \(^{45}\)Ca into bone, but not that of the mucosal extract. \(t, P < 0.05; **, P < 0.01; *** P < 0.001\) by Student's \(t\) test.) NS, not significant. Numbers in parentheses represent number of rats.

![Diagram B](image2)

**Fig. 7.** Dose-response curves illustrating the effects of gastrin-17 on the uptake of \(^{45}\)Ca into radius and sternum. \(P\) values for the regression coefficients are as follows: radius, \(< 0.05\); sternum, \(< 0.01\). Each value is the mean of five determinations. Vertical bars give SEM.

An extract of oxyntic mucosa, purified by combined gel chromatography and reversed-phase HPLC, could be shown to mimic the effects of gastrin-17 on blood calcium and on calcium uptake into bone. The chromatographic behavior of the oxyntic mucosal agent and the loss of hypocalcemic effect on digestion with leucine aminopeptidase suggest that it is a small peptide with an unprotected NH\(_2\) terminus. Its resistance to trypsin digestion suggests a lack of basic amino acid residues (or protected basic amino acids). We propose the name gastrocalcin for this putative peptide hormone, which is produced in the oxyntic mucosa and released by gastrin. It accelerates calcium uptake into bone, thereby explaining the hypocalcemia evoked by gastrin. To the three functionally interacting factors controlling Ca\(^{2+}\) concentration in blood (parathyroid hormone, calcitonin, and vitamin D), we wish to add a fourth—namely, gastrocalcin.

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