Ghrelin Is Present in Pancreatic α-Cells of Humans and Rats and Stimulates Insulin Secretion

Yukari Date,[1,2] Masamitsu Nakazato,[1] Suzuko Hashiguchi,[3,4] Katsuya Dezaki,[3] Muhtashan S. Mondal,[1] Hiroshi Hosoda,[2] Masayasu Kojima,[2] Kenji Kangawa,[2] Terukatsu Arima,[4] Hisayuki Matsuo,[2] Toshihiko Yada,[3] and Shigeru Matsukura[1]

Ghrelin, a novel growth hormone–releasing peptide isolated from human and rat stomach, is a 28–amino acid peptide with a posttranslational acylation modification that is indispensable for stimulating growth hormone secretion by increasing intracellular Ca^{2+} concentration. It also functions in the regulation of feeding behavior, energy metabolism, and gastric acid secretion and motility. Using two different antibodies against the NH_2- and COOH-terminal regions of ghrelin, we studied its localization in human and rat pancreas by immunohistochemistry. Ghrelin-immunoreactive cells were identified at the periphery of pancreatic islets in both species. Ghrelin co-localized exclusively with glucagon in rat islets, indicating that it is produced in α-cells. We identified ghrelin and des-acyl ghrelin in the rat pancreas using reverse-phase high-performance liquid chromatography combined with two radioimmunoassays. We also detected mRNA encoding ghrelin and its receptor in the rat pancreatic islets. Ghrelin increased the cytosolic free Ca^{2+} concentration in β-cells and stimulated insulin secretion when it was added to isolated rat pancreatic islets. These findings indicate that ghrelin may regulate islet function in an endocrine and/or paracrine manner. *Diabetes* 51:124–129, 2002

Growth-hormone secretagogues (GHSs) are small synthetic peptides and nonpeptide molecules that stimulate growth hormone (GH) release from the anterior pituitary through the GHS receptor (GHS-R) (1). GHS-R, a G protein–coupled receptor, promotes calcium release from the endoplasmic reticulum (2,3). Ghrelin, a 28–amino acid peptide with an *n*-octanoylated Ser 3, was originally discovered in rat stomach as a cognate endogenous ligand for GHS-R by using an intracellular calcium influx assay on a stable cell line expressing rat GHS-R (4). The Ser 3 *n*-octanoylation is a unique modification necessary for ghrelin's activity. Ghrelin stimulates GH release when administered intravenously or intracerebroventricularly to rats and when applied directly to rat primary pituitary cells (4–6). In addition, ghrelin increases food intake and body weight upon intracerebroventricular administration (7,8). Furthermore, intravenously or intracerebroventricularly administered ghrelin stimulates gastric acid secretion by activating the vagal system (9,10). These findings suggest that ghrelin is secreted in response to altered food intake or some other nutritional states and thereby plays a role in the regulation of feeding behavior, energy metabolism, and digestion. Ghrelin is produced primarily in the enteroendocrine cells of rats and humans (4,11,12). Many types of enteroendocrine cells, including insulin- and glucagon-producing cells of the pancreatic islets, develop from endodermal epithelium. Ghrelin, like insulin and glucagon, may be produced in the islets and involved in the regulation of energy metabolism.

In the present study, we investigated the cellular source of ghrelin in rat and human pancreas by immunohistochemistry. Ghrelin molecules in rat pancreas were characterized by reverse-phase high-performance liquid chromatography (RP-HPLC) combined with radioimmunoassay (RIA). The expression of ghrelin and its receptor was examined by reverse transcription–polymerase chain reaction (RT-PCR). Finally, we studied the effects of ghrelin on the insulin secretion from rat pancreatic islets and the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat pancreatic β-cells, a widely known mediator of a variety of β-cell functions (13–15).

RESEARCH DESIGN AND METHODS

Animals. Male Wistar rats that weighed 300–350 g (Charles River Japan, Shiga, Japan) were used in all of the experiments. Rats were housed individually in plastic cages at constant room temperature in a 12-h light (0700–1900)/12-h dark cycle and were given standard laboratory diet and water ad libitum. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

RIAs for ghrelin. Two ghrelin-specific RIAs were developed as previously described (16). Two kinds of polyclonal antibodies were raised against the COOH-terminally Cys-extended rat ghrelin (position 1–11) with octanoylated Ser-3 and NH_2-terminally Cys-extended rat ghrelin (position 13–28) in New Zealand white rabbits. An anti–rat ghrelin (1–11) antiserum (#G606) specifically recognized ghrelin with *n*-octanoylated Ser 3 and did not recognize des-acyl ghrelin. An anti–rat ghrelin (13–28) antiserum (#G107) recognized *n*-octanoyl modified and des-acyl ghrelin equally. Both antisera had 100% cross-reactivity with human ghrelin (1–28).

Synthetic rat [Tyr^{29}]-ghrelin (1–28) and [Tyr^{0}]-ghrelin (13–28) were radioiodinated by the lactoperoxidase method (16). Diluted samples or standard

From the [1]Department of Internal Medicine, Miyazaki Medical College, Miyazaki, Japan; [2]National Cardiovascular Center Research Institute, Osaka, Japan; the [3]Department of Physiology, Jichi Medical School, Tochigi, Japan; and the [4]Second Department of Internal Medicine, Faculty of Medicine, Kagoshima University, Kagoshima, Japan.

Address correspondence and reprint requests to Masamitsu Nakazato, PhD, Third Department of Internal Medicine, Miyazaki Medical College, Kiyotake, Miyazaki 889-1692, Japan. E-mail: nakazato@post.miyazaki-med.ac.jp.

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$[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; GH, growth hormone; GHS, growth-hormone secretagogue; GHS-R, GHS receptor; ICV, intracerebroventricularly; IV, intravenously; KRB, Krebs-Ringer bicarbonate buffer; PBS, phosphate-buffered saline; RIA, radioimmunoassay; RP-HPLC, reverse-phase high-performance liquid chromatography; RT-PCR, reverse transcription–polymerase chain reaction; TFA, trifluoroacetic acid.

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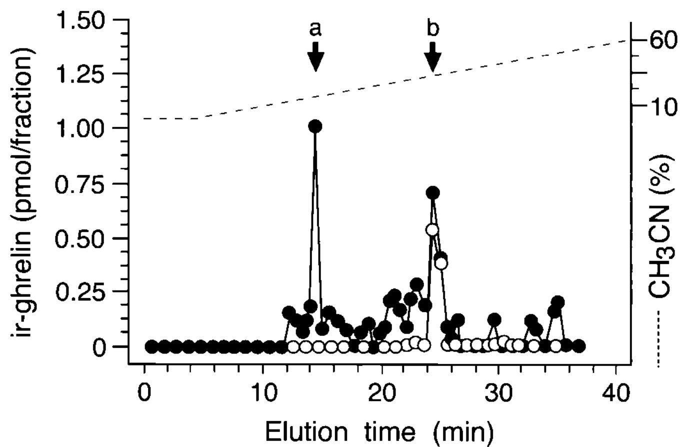

FIG. 1. Representative RP-HPLC profile of ghrelin immunoreactivity in rat pancreas. Open and closed circles represent data from ghrelin NH_2-terminus and COOH-terminus RIA, respectively. Homogenized pancreas (1 g) was separated by RP-HPLC on a TSK ODS SIL 120A column (4.6 × 150 mm). A linear gradient of 10–60% CH_3CN containing 0.1% TFA was run for 40 min at 1.0 ml/min; 0.5 ml elution fractions were collected. Arrows indicate the elution positions of des-acyl rat ghrelin (1–28) (a) and *n*-octanoylated rat ghrelin(1–28) (b).

peptide solutions (100 μl) were incubated for 24 h with 100 μl of the antiserum diluent (final dilution of anti–ghrelin (1–11) antiserum, 1:620,000; final dilution of anti–ghrelin (13–28) antiserum, 1:20,000). A tracer solution (16,000 cpm in 100 μl) was added, and the mixture was incubated for another 24 h. The bound and free ligands were separated using a second antibody (200 μl). All of the procedures were performed at 4°C, and the samples were assayed in duplicate. Half-maximal inhibition by rat ghrelin (1–28) on the standard RIA curve with anti–ghrelin (1–11) antiserum was 3.8 fmol/tube and with anti-ghrelin (13–28) antiserum was 80 fmol. The intra- and interassay coefficients of variation for NH_2-terminus RIA were 3.5 and 3.2%, respectively, and those for COOH-terminus RIA were 3.7 and 3.3%, respectively.

Quantification and chromatographic characterization of immunoreactive ghrelin in rat pancreas. Peptide extracts from three rat pancreata were loaded onto Sep-Pak C-18 cartridge (Waters, Milword, MA), and the bound peptides were eluted with 60% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). A fraction of the eluate was subjected to two RIAs for ghrelin, and the remainder was used for RP-HPLC under the conditions described in Fig. 1. All of the HPLC fractions were quantified by the ghrelin RIA. *n*-Octanoylated rat ghrelin (1–28) and des-acyl rat ghrelin (1–28) were chromatographed using the same HPLC system as standards.

Immunohistochemistry. Three Wistar rats were perfused transcardially with 0.1 mol/l phosphate buffer (pH 7.4) and then with 4% paraformaldehyde in a 0.1 mol/l phosphate buffer. Their pancreata were cut at −20°C with a cryostat into 12-μm-thick slices. Serial sections were incubated overnight at 4°C first with anti–ghrelin (1–11) antiserum (final dilution, 1:5,000), anti–ghrelin (13–28) antiserum (final dilution, 1:5,000), or anti-glucagon antiserum (NICHIREI, Tokyo, Japan; dilution 1:500), and then with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or Alexa Fluor 568 goat anti-rabbit IgG. The slides were observed by fluorescence microscopy (BH2-RFC; Olympus, Tokyo, Japan).

Human pancreata obtained at autopsy from three patients who had died of cardiovascular disease were immersed in the same fixative for 24 h at 4°C. They were cut at −20°C with a cryostat into 12-μm-thick slices, then incubated overnight at 4°C with anti–ghrelin (1–11) antiserum (final dilution, 1:5,000), anti–ghrelin (13–28) antiserum (final dilution, 1:5,000), or anti-glucagon antiserum (dilution, 1:500). The slides were stained by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) as described previously (11).

Immunohistochemical double-staining. The rat pancreatic sections were incubated overnight at 4°C with sheep anti-glucagon antiserum (Biogenesis, England, U.K.; dilution, 1:1,000) and then with Alexa Fluor 594 donkey anti-sheep IgG (Molecular Probes). Next, the sections were incubated with anti–ghrelin (13–28) antiserum (final dilution, 1:3,000) and then with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). The slides were observed by fluorescence microscopy (BH2-RFC; Olympus).

RT-PCR for ghrelin and ghrelin receptor. Total RNA was individually extracted from the pancreata and the isolated islets of Langerhans of Wistar rats by the acid guanidinium thiocyanate-phenol-chloroform method (17). First-strand cDNA was synthesized from 2.5 μg of RNA and oligo $(dT)_{18}$ primer using ReverTra Ace-α (Toyobo, Osaka, Japan) and then subjected to PCR amplification using primers specific for ghrelin or the ghrelin receptor as described elsewhere (11). The PCR products were electrophoresed on a 2% agarose gel (FMC BioProducts, Rockland, ME). Some portions of the PCR products of ghrelin and the ghrelin receptor were digested with *Pst*I and *Hinf*I, respectively, then electrophoresed. The other portions of the PCR products were purified with Magic PCR DNA Purification System (Promega, Madison, WI) and then sequenced by BigDye Terminator Cycle Sequencing System (Applied Biosystems, Foster City, CA).

Preparation of islets and single β-cells. Wistar rats aged 10–12 weeks were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg body wt). After the abdomen was opened, the common bile duct proximal to the pancreas was first ligated, and then collagenase (3 mg/ml) dissolved in 5 mmol/l Ca^{2+}-containing Krebs-Ringer bicarbonate buffer (KRB) was injected into the common bile duct at the distal end. The pancreas was resected and incubated at 37°C for 17 min. Islets were hand-collected under a microscope and either used for the study of insulin release or dispersed into single cells by treatment with Ca^{2+}-free KRB + 0.1 mmol/l EGTA. The single cells were plated on coverslips and maintained in a short-term culture for up to 2 days in Eagle's minimum essential medium containing 5.6 mmol/l glucose supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 mU/ml penicillin at 37°C in a 95% air and 5% CO_2 atmosphere. The cells during this culture period responded to test agents in a consistent manner. β-Cells were selected by immunostaining with anti-insulin antiserum or by morphological and physiological criteria, as reported previously (18,19).

Measurement of insulin release from islets. Insulin release from rat pancreatic islets was examined as described previously (19,20). Briefly, groups of seven isolated islets were first incubated for 30 min in KRB containing 2.8 mmol/l glucose for stabilization. The islets then were incubated at 37°C for 30 min in KRB containing 2.8 or 8.3 mmol/l glucose, either with or without the addition of 10^{-12} mol/l ghrelin. At the end of the incubation period, aliquots of the medium were collected for the measurement of insulin concentration with an enzyme immunoassay kit (Morinaga, Yokohama, Japan).

Measurement of $[Ca^{2+}]_i$ in single β-cells. $[Ca^{2+}]_i$ was measured by dual-wavelength fura-2 microfluorometry combined with digital imaging as reported previously (20). Briefly, cells on coverslips were incubated with 2 mmol/l fura-2 acetoxymethyl ester in KRB containing 2.8 mmol/l glucose for 30 min at 37°C. They were then mounted in a chamber and superfused with KRB at a rate of 1 ml/min at 37°C. The cells were excited alternately at 340 and 380 nm every 2.5 s, emission signals at 510 nm were detected with an intensified charge-coupled device camera, and ratio images were produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). Ratio values were converted to $[Ca^{2+}]_i$ according to calibration curves (20).

Statistical analysis. All the data are presented as means ± SE, and comparisons between groups were performed by unpaired *t* test. The level of significance was set at $P < 0.05$.

RESULTS

Characterization of pancreatic ghrelin content. RP-HPLC coupled with two separate RIAs was used to analyze ghrelin molecules in rat pancreas. In the RIA for the ghrelin COOH-terminus, two major peaks were observed, eluting at positions consistent with des-acyl (1–28) and *n*-octanoylated rat ghrelin (1–28) (Fig. 1). RIA for the ghrelin NH_2-terminus revealed only one major ghrelin-immunoreactive peak eluting at a position consistent with *n*-octanoylated rat ghrelin (1–28). The amount of ghrelin in the pancreas as measured by COOH-terminus RIA was 9.43 ± 0.76 pg/mg (mean ± SE), and that measured by NH_2-terminus RIA was 2.74 ± 0.71 pg/mg.

Immunohistochemistry. Ghrelin-immunoreactive cells were found in the mantle of the pancreatic islets, just like glucagon-producing α-cells (Fig. 2*A*–*C* and *E*–*H*). In rat islets, ghrelin-immunoreactive cells co-localized with glucagon in immunofluorescence double-staining (Fig. 2*D*). In humans, ghrelin-immunoreactive cells also had the same distribution as α-cells (Fig. 2*E*–*H*).

RT-PCR amplification of the ghrelin and ghrelin receptor transcripts. Ghrelin and ghrelin receptor tran-

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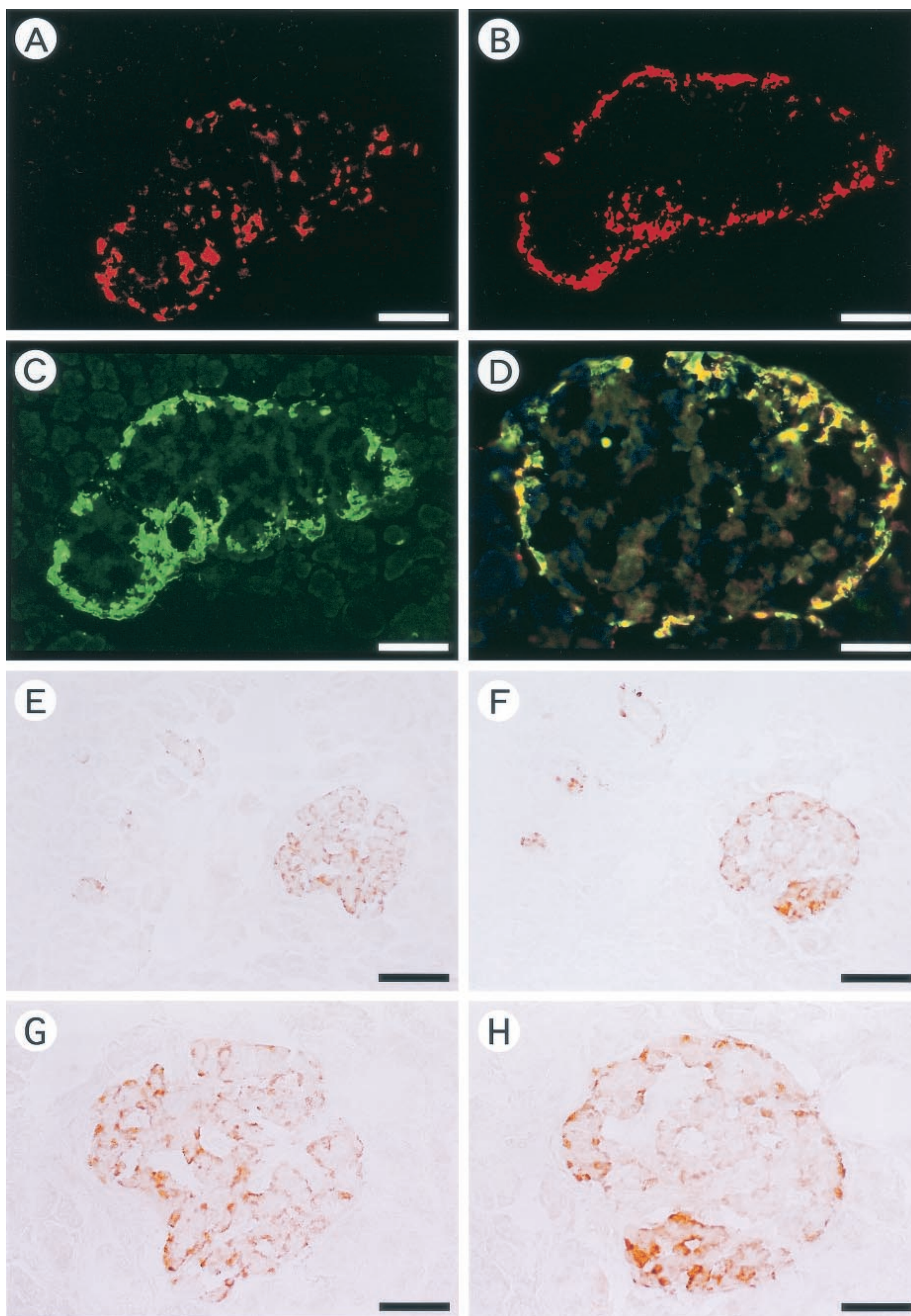

FIG. 2. Localization of ghrelin-immunoreactive cells in rat and human pancreas. Antisera for ghrelin (1–11) (*A*), ghrelin (13–28) (*B*, *D*, *E*, and *G*), and glucagon (*C*, *D*, *F*, and *H*) were used. Ghrelin-producing (*A* and *B*) and glucagon-producing (*C*) cells are present at the periphery of the rat pancreatic islet. *D*: Immunofluorescence double-staining of ghrelin and glucagon in rat pancreas. Co-localization of ghrelin and glucagon is shown in yellow. In human pancreas, ghrelin-producing (*E* and *G*) and glucagon-producing (*F* and *H*) cells are present at the periphery of the pancreatic islets. Bar = 100 μm in *A*–*C*, *E*, and *F* and 50 μm in *D*, *G*, and *H*.

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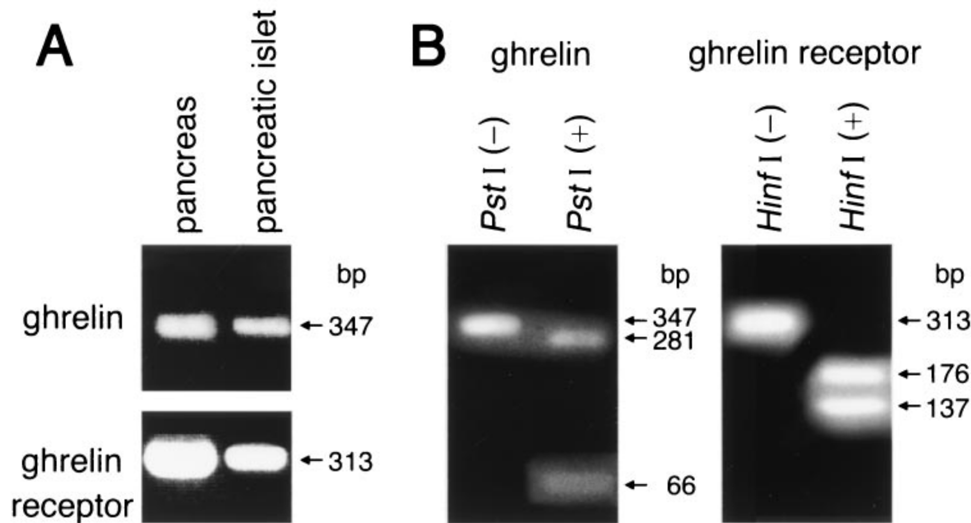

FIG. 3. ***A*****: Representative electrophoretic analysis patterns of the RT-PCR products of ghrelin (top) and ghrelin receptor mRNA (bottom) in rats.** ***B*****: The PCR product of ghrelin digested with *Pst*I [*lane Pst*I (+)] or not [*lane Pst*I (−)] and that of the ghrelin receptor digested with *Hinf*I [*lane Hinf*I (+)] or not [*lane Hinf*I (−)].**

scripts corresponding to predicted sizes of 347 and 313 bp, respectively, were found in the pancreas and the isolated rat islet (Fig. 3). The PCR product of ghrelin was cleaved into 281- and 66-bp fragments on *Pst*I digestion, the sizes expected (Fig. 3*B*). The product of the ghrelin receptor was cleaved into 176- and 137-bp fragments on *Hinf*I digestion, the sizes expected (Fig. 3*B*). The PCR products of 347 and 313 bp were also confirmed to correspond to ghrelin and the ghrelin receptor, respectively, by direct sequencing (data not shown).

Stimulation of insulin release from islets by ghrelin. Insulin release from isolated rat islets under static incubation condition was stimulated by 8.3 mmol/l glucose. The glucose-stimulated insulin release was significantly ($P < 0.05$) increased by the addition of 10^{-12} mol/l ghrelin. In contrast, 10^{-12} mol/l ghrelin had no effect on the basal insulin release in the presence of 2.8 mmol/l glucose (Fig. 4).

Increase in $[Ca^{2+}]_i$ in single β-cells by ghrelin. Ghrelin at 10^{-12} mol/l increased $[Ca^{2+}]_i$ in single rat pancreatic β-cells in the presence of 8.3 mmol/l glucose (Fig. 5*A*), whereas it had no effect on $[Ca^{2+}]_i$ in the presence of 2.8 mmol/l glucose (Fig. 5*B*). In the β-cells that exhibited glucose-induced $[Ca^{2+}]_i$ oscillations, the peak of the $[Ca^{2+}]_i$ increase in response to ghrelin administration was much higher than the peaks of $[Ca^{2+}]_i$ oscillations (Fig. 5*A*). This effect could be considered the potentiation of glucose-induced $[Ca^{2+}]_i$ oscillations. Ghrelin at 10^{-12} mol/l either elicited $[Ca^{2+}]_i$ increases or potentiated $[Ca^{2+}]_i$ oscillations in 20 of 59 (34%) β-cells at 8.3 mmol/l glucose. The time lag until the onset of $[Ca^{2+}]_i$ responses ranged from 1 to 4 min.

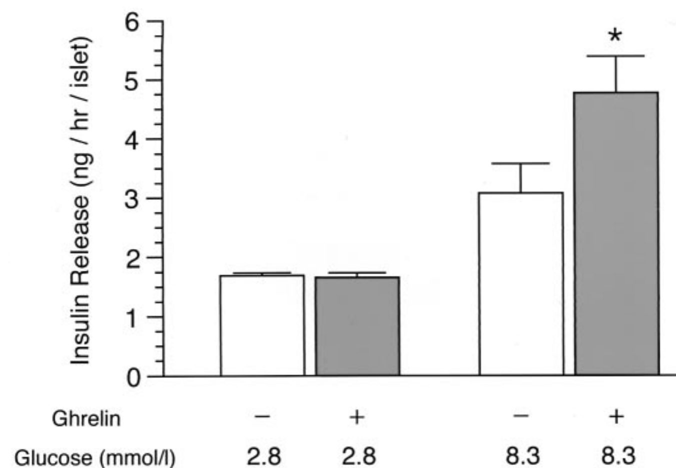

FIG. 4. Effect of ghrelin on insulin release from rat islets. Ghrelin at 10^{-12} mol/l stimulated insulin release in the presence of 8.3 mmol/l glucose but not 2.8 mmol/l glucose. The results are expressed as the mean ± SE for 10 experiments in each group. *$P < 0.05$ versus 8.3 mmol/l glucose alone.

DISCUSSION

The pancreas and liver develop from a common precursor (the hepatopancreatic ring) that is derived from the gastrointestinal tract (the anterior primitive intestine). In the most highly evolved vertebrates, a close functional con-

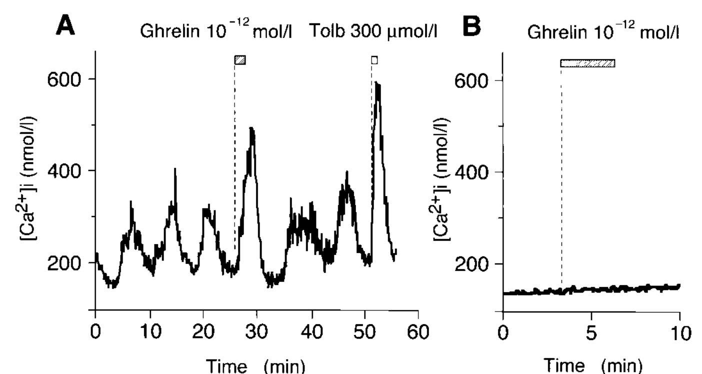

FIG. 5. Effect of ghrelin on $[Ca^{2+}]_i$ in single rat pancreatic β-cells. Ghrelin at 10^{-12} mol/l evoked an increase in $[Ca^{2+}]_i$ in a single pancreatic β-cell in the presence of 8.3 mmol/l glucose (*A*) but not 2.8 mmol/l glucose (*B*). The cells also responded to 300 µmol/l tolbutamide (Tolb). The results shown are representative of 20 cells from two independent experiments in *A* and of 10 cells from two independent experiments in *B*. Dotted lines indicate the beginning of the superfusion with the agents specified.

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nection persists between the gastrointestinal endocrine cells and the pancreatic islets via the "enteroinsular" axis (21). We identified the cellular origin and distribution of ghrelin in the gastrointestinal tracts of rats and humans by in situ hybridization histochemistry, immunohistochemistry, electron microscopy, RIA, and RT-PCR (11). Ghrelin production in the rat stomach has been localized to "previously described" X cells, which are round to ovoid, with round, compact, electron-dense granules. Although these cells, which account for ~20% of all endocrine cells in the oxyntic mucosae of both rats and humans, represent a major endocrine cell population in the oxyntic gland (22,23), their hormonal product and physiological functions have not previously been clarified. The present study shows that ghrelin is present in the α-cells of rat and human pancreas. X cells also have been called A(α)-like cells; although they do not exhibit glucagon-immunoreactivity, they do share some morphological features with pancreatic α-cells, including the presence of compact and dense secretory granules. Although α-cells are lacking or scarce in the gastric fundus of mammals except for dogs and cats, they are fairly abundant in the fundic mucosa of human fetuses (24,25). These findings suggest that pancreatic α-cells and gastric ghrelin cells may originate from the same endodermal progenitor cells.

The pancreatic islets vary in size and cellularity but generally are composed of four main cell types: α (20% of the total), β (68%), δ (10%), and PP (2%) (26). Insulin-producing β-cells are located in the center of the islet, and glucagon-producing α-cells are found at the periphery, with somatostatin-producing δ-cells interposed between the two. One widely accepted model of the vascular supply in the islets proposes that arterial blood is first carried to the β-cell–rich center before passing on to the α- and δ-cells in the periphery (27). Both ghrelin and its receptor are present in the pancreatic islets. Therefore, ghrelin in α-cells may affect islet functions via the systemic circulation. There is another possibility that ghrelin may interact with insulin, somatostatin, or both in a paracrine manner independent of blood flow, because α-, β-, and δ-cells are often in intimate contact within the islets and gap junctions exist between these cells.

Ghrelin at 10^{-12} mol/l stimulated insulin release and increased $[Ca^{2+}]_i$ in rat islet β-cells in the presence of a stimulatory (8.3 mmol/l) but not basal (2.8 mmol/l) glucose concentration. Ghrelin at a higher concentration of 10^{-8} mol/l showed lesser effects on both insulin release and $[Ca^{2+}]_i$ increase, and at 10^{-14} mol/l it had no effects (data not shown). Ghrelin promotes calcium release after it binds to the ghrelin receptor (4). These findings suggest that ghrelin is a novel potentiator of insulin release and that Ca^{2+} may be a messenger signal for ghrelin in β-cells. Considering that the plasma level of *n*-octanoylated ghrelin in rats is 4×10^{-12} mol/l (16), the effects of ghrelin at 10^{-12} mol/l on insulin release and $[Ca^{2+}]_i$ increase are thought to be physiological. Ghrelin may serve as a physiological regulator of insulin release. Because increases and oscillations of $[Ca^{2+}]_i$ have been implicated not only in insulin release but also in insulin synthesis and gene expression (13–15), ghrelin might serve as a trophic factor for islet β-cells, although additional studies are definitely needed.

Ghrelin stimulates insulin secretion, whereas ghrelin secretion from α-cells may be regulated by the plasma concentration of glucose and/or insulin. We recently showed that ghrelin mRNA expression in the rat stomach is upregulated upon fasting and insulin-induced hypoglycemia (28). These findings suggest that ghrelin may function as an anabolic signal molecule during energy depletion. The presence of ghrelin and its receptor in the pancreatic islets provides a new clue for our understanding of the regulation of energy homeostasis.

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