Inhibitory Effect of Relaxin-3 on Insulin Secretion in Isolated Pancreas and Insulinoma

Hiroyuki Yamamoto,* Takeo Arai, Ryota Tasaka, Yasunori Mori, Kazuaki Iguchi, Keiko Unno, and Minoru Hoshino

Laboratory of Bioorganic Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan

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Relaxin-3 is a recently discovered member of the insulin superfamily and is a ligand for three orphan G-protein-coupled receptors: GPCR135, GPCR142 and LGR7 (leucine-rich repeat-containing G-protein-coupled receptor 7). GPCR135 mRNA is expressed in the pancreas, however, it is not known, whether the peptide affects pancreatic islet function. Reverse transcriptase (RT)-PCR and radioreceptor assay have shown that the relaxin-3 receptor (GPCR135) is expressed in pancreatic islets and rat insulinoma, but LGR7 is not expressed. Moreover, relaxin-3 has been revealed to inhibit the secretion of insulin from pancreatic islets. However, we can not detect relaxin-3 in small intestine and pancreas. These results suggest a novel role of relaxin-3 in the regulation of insulin release.

Key words —— relaxin-3, G-protein-coupled receptor (GPCR)135, insulin

INTRODUCTION

Relaxin is a member of the insulin superfamily. The characteristic feature of this superfamily is the presence of two peptide subunits that are connected by two disulfide bonds. Until recently, only a single relaxin gene had been found in both mice (M1)[1] and rats (R1),[2] and two relaxin genes, H1 and H2, had been identified in humans.[3] However, a further relaxin gene, relaxin-3, has now been identified in humans (H3), mice (M3), and rats (R3).[4] Although M1 and R1 mRNAs are widely expressed in mice and rats, relaxin-3 mRNA expression appears to be localized to the brain and testis.[4]

Relaxin has recently been identified as a ligand for two highly related leucine-rich repeat-containing G-protein-coupled receptors (LGRs), LGR7 and LGR8.[5,6] Further studies have revealed that LGR7 is a relaxin-1 receptor, which also binds relaxin-3 with high affinity,[7] and that LGR8 is a receptor of an insulin-like peptide, INSL3.[8] Recently, a further two G-protein-coupled receptors, GPCR135[4] [or somatostatin- and angiotensin-like peptide receptor (SALPR)[9]] and GPCR142,[10] which are activated by relaxin-3, have been identified. In the rat central nervous system (CNS), GPCR135 mRNA is highly expressed in the hypothalamic paraventricular nucleus, supraoptic nucleus, and preoptic area.[4,9] On the other hand, in peripheral tissues, GPCR135 mRNA is expressed in the pancreas, adrenal glands, salivary glands, placenta, mammary glands, and testis.[4,9] It has been reported that, unlike other mammals, rats do not express GPCR142,[11] and rats are a useful model animal for the GPCR135 action analysis.

Recently, intracerebroventricular or paraventricular administration of relaxin-3 was demonstrated to stimulate feeding via GPCR135.[12] Moreover, chronic administration into the cerebral ventricles induced increases in plasma leptin and insulin concentrations.[13] These studies indicate that relaxin-3 might play a role in energy homeostasis. However, the function of relaxin-3 in the peripheral tissues is not well known. In the present study, the expression of GPCR135 and the effects of relaxin-3 on insulin release in the isolated pancreatic islets and insulinoma of rats were examined.

MATERIALS AND METHODS

Animals —— Male Wistar rats (10–15 weeks old; Nihon SLC Co., Hamamatsu, Japan) were used as experimental animals. Prior to the experiments, the rats were housed in a controlled room under conventional conditions of temperature, humidity, and illumination (12 : 12 light-dark cycle) within large cages fitted with wire mesh bottoms to prevent coprophagy. The animals were given free access to laboratory chow and water. All experimental protocols were approved by the University of Shizuoka Laboratory Animal Care Advisory Committee.

*To whom correspondence should be addressed: Laboratory of Bioorganic Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan. Tel.: +81-54-264-5734; Fax: +81-54-264-5909; E-mail: yamahiro@mail.u-shizuoka-ken.ac.jp

Chemicals —— Human relaxin-3 was obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA, U.S.A.). Rat and porcine insulins and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.). Rat galanin was synthesized by solid-phase technology. Peptide purity was confirmed by Matrix-assisted laser desorption/ionization Time-of-flight (MALDI-TOF) mass analysis and analytical reverse-phase HPLC. [^{125}I]-Na solution was purchased from the Institute of Isotopes Co., Ltd. (Budapest, Hungary). Ethidium bromide, chloramine-T, and forskolin were purchased from Waco Pure Chemical Industries Ltd. (Osaka, Japan).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) —— Total RNA was prepared by using an SV Total RNA Isolation System (Promega Corporation, Madison, WI, U.S.A.) and cDNA was synthesized using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) according to the manufacturer's instructions. Genomic DNA was removed with DNaseI. The primer sequences used were as follows: rat GPCR135 (5′-GTGACATCCATGAACATGTATGCCAGCGTC-3′ and 5′-GTTGAACTTGATGAGGATGCTCCAGGTGGT-3′ Sigma-Aldrich, Inc.),[4] rat LGR7 (5′-TTGAAGTAAATCAATTTGAT-3′ and 5′-AACACTCTCCATCCACGG-3′ Sigma-Aldrich, Inc.) and rat relaxin-3 (5′-GTATGGTTGGAGCGAGGAAAAT-3′ and 5′-GTATGGTTGGAGCGAGGAAAAT-3′). The following PCR program was used: initial denaturation at 95°C for 5 min and then 40 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. The PCR products were electrophoresed on ethidium bromide-containing agarose gels (1.2%) and visualized under UV illumination.

Radioligand Receptor Assay —— Human relaxin-3 was labeled with [^{125}I]-Na in the presence of chloramine-T.[14] [^{125}I]-labeled relaxin-3 was isolated by gel filtration chromatography. Binding studies were performed using RINm5F cells as described previously.[15,16] Briefly, 2×10^5 cells of rat insulinoma cell line RINm5F, cultured in 24-well microplates, were washed with 50 mM HEPES-KOH (pH 7.4) containing 120 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 15 mM sodium acetate, 10 mM glucose, and 0.2% BSA, and incubated for 6 hr at 15°C with [^{125}I]-relaxin-3 (10000 cpm) and the desired amount of unlabeled relaxin-3. After non-bound [^{125}I]-ralaxin-3 had been removed by aspiration, cell-bound [^{125}I]-ralaxin-3 was absorbed with 1 M potassium hydroxide and the radioactivity was counted.

Radioimmunoassay —— Insulin-like immunoreactivity was measured using a specific radioimmunoassay as described previously.[17] In brief, rat insulin was used as a standard, and [^{125}I]-porcine insulin was prepared using the chloramine-T method.[14] Anti-porcine insulin guinea-pig serum No. 2 (final dilution, 1 : 200000) was used as a primary antiserum. The standard diluent was 0.01 M phosphate buffer (pH 7.4) containing 0.14 M NaCl, 25 mM EDTA, and 0.5% BSA. Free and bound [^{125}I]-porcine insulin were separated by adding normal guinea-pig serum (1 : 300, 0.1 ml), goat anti-guinea-pig γ-globulin serum (1 : 40, 0.1 ml) and 10% (w/v) polyethylene glycol (0.5 ml). After a 3-hr incubation period at 4°C, the mixture was centrifuged at 3000 rpm for 30 min at 4°C; the supernatant was aspirated and the precipitate was counted in a well-type gamma counter.

Isolation of Pancreatic Islets —— Rat pancreas was digested by collagenase and the islets were separated by hand picking under a microscope.[18] Batches of islets were preincubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Thereafter, islets in groups of five were incubated in 400 μl of the DMEM containing 5.6 or 16.8 mM glucose in the presence or absence of relaxin-3 ranging in concentration from 0.01 nM to 100 nM. After a 60-min incubation at 37°C in a normal atmosphere, the medium was collected for the analysis of insulin.

Insulin Secretion from Rat Insulinoma —— Rat insulinoma cell line RINm5F were cultured in DMEM containing 10% fetal bovine serum at 1×10^6 cells per well in a 24-well flat-bottomed culture plate (FALCON; Becton Dickinson Labware, Bedford, MA, U.S.A.) for 24 hr and used for the experiments after reaching confluence. The culture medium was aspirated, and the cells were treated with 400 μl culture medium containing 100 nM forskolin with or without relaxin-3 (0.01–100 nM) for 30 min. As a control, galanin which caused insulin inhibition via cAMP reduction was added to the medium. The medium was subsequently collected for the analysis of insulin.

Pancreas Perfusion —— Male Wistar rats were fasted for 24 hr with free access to water. Anesthesia was induced with sodium pentobarbital (50 mg/kg). Perfusion of rat pancreas was carried out according to the method of Grodsky and Fanska[19] with slight modification. Briefly, a polyethylene cannula

was inserted into the celiac artery of the pancreas for perfusion, and the outlet cannula was inserted into the portal vein. All other vessels were ligated. The perfusate consisted of Krebs-Ringer bicarbonate buffer (KRBB, pH 7.4) containing 0.2% BSA, 5.8 mM glucose, and 4.0% dextran T-70 (Meito Sangyo, Nagoya, Japan). The vascular perfusate was saturated constantly by bubbling with 95% O_2 and 5% CO_2. The flow rate was maintained at 1.9 ml/min by using a peristaltic pump. The isolated pancreas was transferred to a chamber and suspended in saline at 37°C. After a 30-min preperfusion, sampling was started. Following the initial basal secretion period (10 min), 100 nM relaxin-3 in KRBB without glucose was infused at the rate of 0.1 ml/min for 30 min by means of a Harvard compact syringe pump (Model 975; Harvard Apparatus, South Natick, MA, U.S.A.). As a control, only KRBB without glucose was infused in the same manner. This was followed by a final 10-min equilibration period. The total perfusion time was 80 min. Portal effluents were collected every minute into chilled glass tubes and stored at −20°C until assayed for insulin content.

Statistical Analysis —— All results are expressed as the mean ± S.E.M. Significant differences between two groups were analyzed by either the Student's *t* tests or by a two-way analysis of variance (ANOVA), with post hoc analysis. Differences between two groups were considered to be statistically significant at $p < 0.05$.

RESULTS

Relaxin-3 and Its Receptor Expression

RT-PCR revealed the expression of GPCR135 (relaxin-3 receptor) mRNA in rat insulinoma RINm5F cells and pancreatic islets [Fig. 1(A)]. The size of the resultant PCR product corresponded to an expected size for GPCR135 cDNA (585 bp). The expression of LGR7 mRNA was not detected by RT-PCR (data not shown). Moreover, relaxin-3 mRNA was detected in testis [Fig. 1(B)]. The size of the PCR product corresponded to an expected size for relaxin-3 cDNA (457 bp), whereas no PCR product was detected in small intestine and pancreas even if an increased content of total RNA was used. The radioreceptor assay revealed the expression of GPCR135 in RINm5F cells. Radioactive relaxin-3 bound to RINm5F cells with high affinity, with K_i values of 3 nM, and competitively with relaxin-3 in a concentration-dependent manner [Fig. 1(C)].

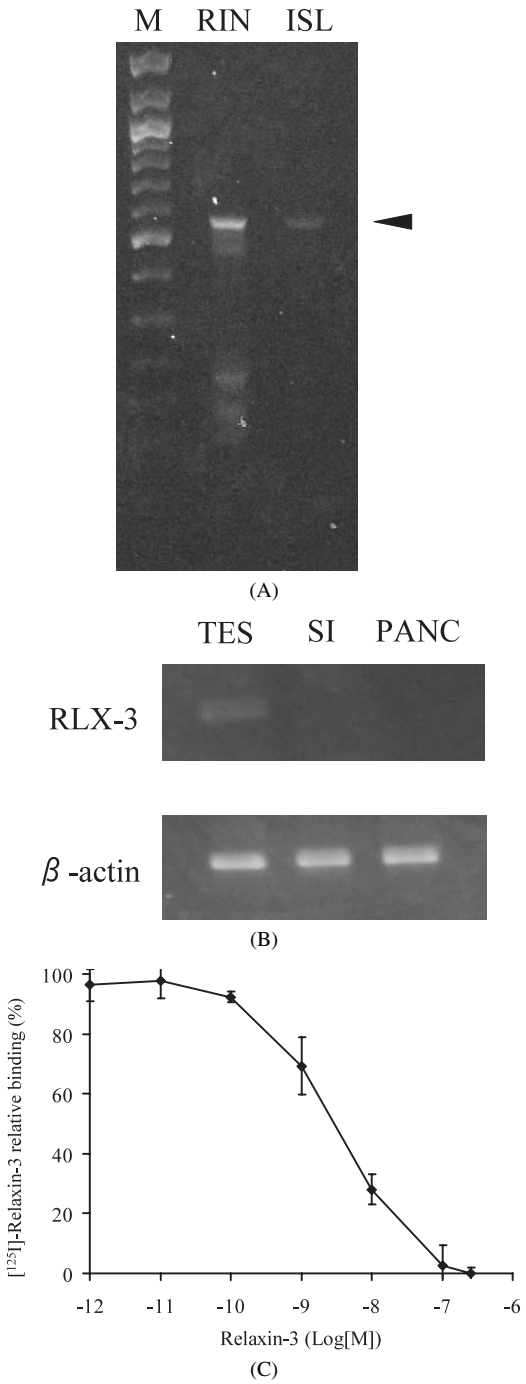

Fig. 1. Expression of Relaxin-3 and Relaxin-3 Receptors

(A) RT-PCR of GPCR135 mRNA. RIN, insulinoma RINm5F; ISL, isolated pancreatic islet; M, markers. The arrow indicates the PCR product for GPCR135. (B) RT-PCR of relaxin-3 mRNA. TES, testis; SI, small intestine; PANC, pancreas; RLX-3, relaxin-3. PCR products were visualized by ethidium bromide staining on a 1.2% agarose gel. (C) Radioreceptor assay of relaxin-3 in insulinoma RINm5F. Unlabeled relaxin-3 (500 nM) was added for non-specific binding. The data represent the mean ± S.E.M. of three experiments performed in duplicate.

Effect of Relaxin-3 on Insulin Secretion

RINm5F cells were incubated in the presence

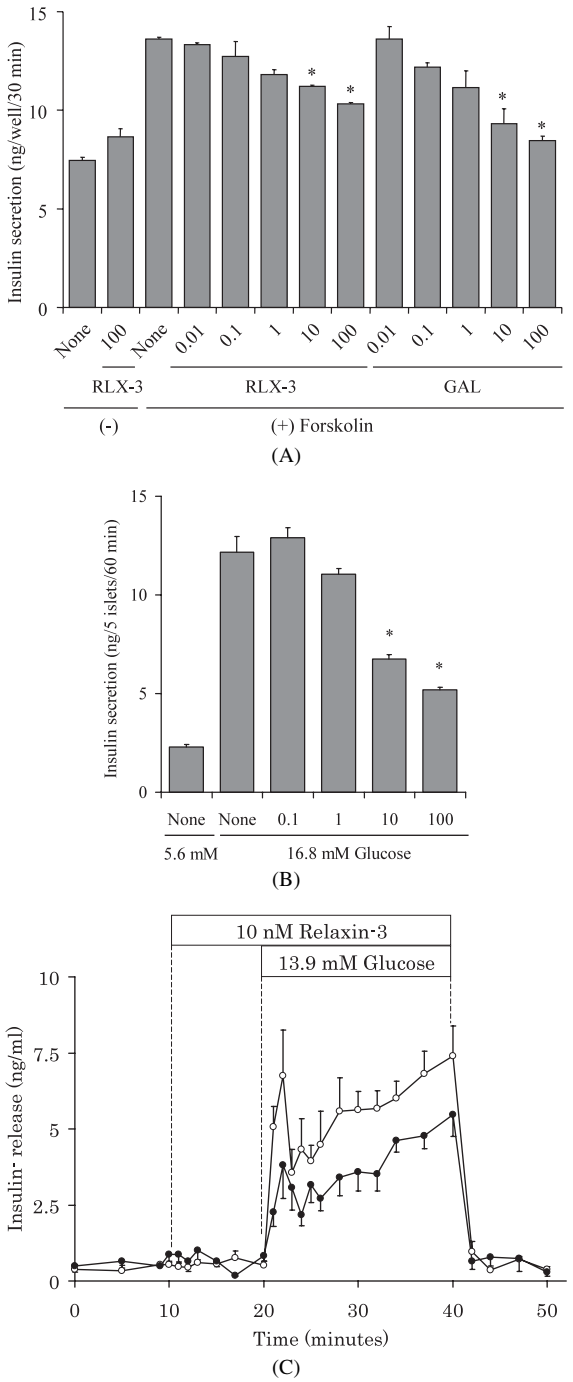

Fig. 2. Effects of Relaxin-3 on Insulin Secretion

(A) RINm5F insulinoma cells were incubated with relaxin-3 or galanin (0.01–100 nM) in the presence of forskolin (100 nM) for 30 min. RLX-3, relaxin-3; GAL, galanin. (B) Isolated pancreatic islets were incubated with relaxin-3 (0.01–100 nM) in the presence of 16.8 mM glucose for 60 min. Basal secretion of insulin was observed in the presence of 5.6 mM glucose. (C) Isolated pancreatic perfusion was carried out. Insulin secretion was induced by 13.9 mM glucose for 20 min. Perfusion of relaxin-3 (10 nM) was started at 10 min before the commencement of glucose perfusion and continued for 30 min. Perfusate fractions of 2 min were collected. Closed circles indicate perfusion of relaxin-3, open circles, glucose alone. Insulin contents in medium and perfusate were determined by radioimmunoassay. The data represent the mean ± S.E.M. of three to five experiments performed in duplicate. Asterisks indicate significant differences ($p < 0.05$) via forskolin alone (A) or 16.8 mM glucose alone (B).

of 100 nM forskolin with increasing concentrations of relaxin-3 or galanin. Forskolin stimulated insulin secretion by approximately two fold during the 30-min static incubation [Fig. 2(A)]. The addition of relaxin-3 (100 nM) without forskolin had no affect on insulin secretion. However, in the presence of forskolin, relaxin-3 inhibited insulin secretion dose dependently. The maximal effect was observed at 100 nM relaxin-3, which gave rise to a 47% reduction in secretion ($p < 0.05$). On the other hand, insulin secretion of 100 nM galanin were 17% of control.

In isolated pancreatic islets, a high concentration of glucose (16.8 mM) resulted in a marked stimulation of insulin secretion [Fig. 2(B)]. Relaxin-3 also inhibited glucose-induced insulin secretion dose dependently at concentrations of 10–100 nM ($p < 0.05$).

In the pancreas perfusion, relaxin-3 significantly reduced the insulin release induced by a high concentration of glucose (13.9 mM) throughout the infusion [Fig. 2(C)]. In the presence of glucose, the total insulin release induced by relaxin-3 (119.3 ± 11.5 ng) was approximately 57.2% of the control (208.4 ± 30.6 ng).

DISCUSSION

Relaxin-3, a member of the insulin superfamily, has been demonstrated to be a ligand for GPCR135.[4] The distribution of relaxin-3 is restricted to the nucleus incertus, the laterally adjacent central gray area of the pons, and the hippocampus in the brain.[20] In the peripheral tissues, however, the distribution is limited to the testis.[4] In the CNS, GPCR135 mRNA is expressed at high levels in the substantia nigra and pituitary, and in other regions such as the amygdala, caudate nucleus, hippocampus, and spinal cord at moderate levels.[4,9] In the peripheral tissues, a relatively high expression is observed in the adrenal glands.[4,9] Intracerebroventricular administration of relaxin-3 has also been demonstrated to stimulate food intake via GPCR135 in rats.[12]

Using RT-PCR, we have demonstrated that GPCR135 is expressed in pancreatic islet β cells and the RINm5F insulinoma cell line, but LGR7 is not expressed. Furthermore GPCR142 is a pseudogene in rat, which implies that only GPCR135 is expressed in rat β cells. In radioreceptor assay, radioactive relaxin-3 bound to RINm5F cells with

high affinity, with K_i values of 2.6 nM. However, the K_i value reported in relaxin-3 binding to GPCR135 expression cells is 0.25 nM.[4] It is possible that difference of binding affinity originated species or binding assay method. Relaxin-3 was revealed to inhibit insulin secretion from pancreatic islets and RINm5F cells. These findings suggest a direct effect of relaxin-3 on β cells. Many physiologically active peptides are known to regulate insulin secretion in order to maintain plasma glucose homeostasis. In particular, the peptides released from the gastrointestinal tract are of marked importance for insulin release both as stimulators, such as glucagon-like peptide-1 (GLP-1),[21] and inhibitors, including galanin.[22] Effects of relaxin-3 were weaker than galanin though relaxin-3 (10 nM) significantly inhibited forskolin-stimulated insulin release. In addition, relaxin-3 exhibited no inhibitory effect at a low concentration of glucose (5.6 mM). Since GPCR135 has been reported to couple to Gi protein, which is sensitive to pertussis toxin and inhibits cAMP production,[4] relaxin-3 might inhibit insulin release via the Gi protein.

In conclusion, the present study has demonstrated the expression of the relaxin-3 receptor GPCR135, and the inhibitory effect of ralaxin-3 on insulin release using isolated pancreatic islets and an insulinoma cell line. The results strongly suggest that relaxin-3 is involved in the novel regulation of islet function. However, although relaxin-3 is expressed in the brain, its distribution among peripheral tissues is restricted to the testis. Moreover, we can not detected relaxin-3 in small intestine and pancreas where many modulators of insulin release are present. Furthermore, the plasma levels of relaxin-3 have yet to be reported. Further investigations of the peripheral distribution and release regulation of relaxin-3 are therefore required in order to corroborate the inhibitory effect on insulin release that has been demonstrated in isolated pancreatic islets and an insulinoma cell line.

Since the peripheral distribution of relaxin-3 is reported to be limited to the testis, it remains totally unclear how relaxin-3 can reach the pancreas to regulate insulin secretion under the physiological condition.

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