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Gaegurin-6 stimulates insulin secretion through calcium influx in pancreatic β Rin5mf cells

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ABSTRACT

Gaegurin-6, an antimicrobial peptide that belongs to the alpha-helix family, was isolated from the skin of *Rana rugosa*. Gaegurin-6 contains a hydrophobic motif at the N-terminus and a helical region at the C-terminus. Although gaegurin-6 has been implicated in cell signaling, the precise role in insulin secretion is currently unknown. We have attempted to determine whether gaegurin-6 affects insulin secretion and tried to elucidate the relationship between the structural motifs and biological activity. In this study, we have shown that gaegurin-6 stimulates insulin secretion and also increases the intracellular calcium concentration in pancreatic β Rin5mf cells. Moreover, a corollary study revealed that both the hydrophobicity of the N-terminus and the disulfide bridge of the C-terminus of gaegurin-6 are critical for its effects on insulin secretion. Membrane pore-forming ability is also observed in gaegurin-6, but not in the linear form or the N-terminus hydrophobic amino acid-deleted form. We further showed that these regions of gaegurin-6 are responsible for calcium influx in pancreatic β Rin5mf cells. Taken together, these results indicate that gaegurin-6 can affect insulin secretion in pancreatic β cells through the modulation of calcium influx.

1. Introduction

Membrane-active host defense molecules have been isolated from various sources [1–3], showing antimicrobial activity against bacteria and fungi. These peptides vary in chain length and hydrophobicity. The mode of action of these peptides is not fully understood, but it is suggested that peptide binding and enhancement of membrane permeability play a critical role in their function [4,5].

Gaegurin-6 (brevinin-1EMb) [6,7] is an antimicrobial peptide that has been isolated from the skin of the Korean frog, *Rana rugosa* [8]. All gaegurin-6s contain two invariant cysteine residues, one at the C-terminus and the other at the seventh position from the C-terminus. The heptapeptide motif containing these two cysteine residues was linked by an intra-residue disulphide bridge, which was conserved in the antimicrobial peptides derived from others of the genus *Rana*, such as brevinins [9] and ranalexin [10].

Insulin is stored in large dense core vesicles and released by exocytosis. Exocytosis is a multifarious and complex process, which involves the transport of vesicles to the plasma membrane, docking, priming, and fusion with the plasma membrane. The secretory response in pancreatic β cells requires the concerted action of nutrients, coupled with hormones, acting on G-protein coupled receptors [11]. The molecular nature of the activators and effectors involved in the process has not been fully identified. One study has demonstrated that the rise in calcium concentration in response to nutrient secretagogues is coupled to pulsatile insulin release in mouse pancreatic islets [12]. Therefore, calcium is likely to be the main constituent of the framework for pulsatile insulin release. Besides antimicrobial peptides, the skin secretions of *Rana* species have yielded the insulinotropic peptides [13,14]. The discovery of these insulin stimulatory peptides, such as rugosin-A like peptide and brevinin-1 in the frog skin has provided a stimulus to study frog skin peptides.

In this study we found that gaegurin-6 stimulated insulin secretion. We further showed that both hydrophobic regions of the N-terminus and helical structure of C-terminus are essential for this biological activity. Our finding of the calcium-inducing effect of the gaegurin-6 supports the notion of its role in insulin secretion. These results therefore provide novel insight into the structural characteristics of gaegurin-6 necessary for insulin secretion in pancreatic β islet cells.

2. Materials and methods

2.1. Reagents

Synthetic gaegurin-6, linear gaegurin-6, deleted gaegurin-6, and brevinin-1 were synthesized in the Hormone Research Center of Jeon Nam National University (Kwangju, Korea). EDTA and (YO-PRO) were obtained from Calbiochem (EMD Biosciences Inc., San Diego, CA). Insulin RIA kits were purchased from Linco Research Inc. (St. Charles, MO).

Abbreviations: PKC, Protein kinase C; PMA, Phorbol 12-myristate 13-acetate.

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2.2. Cell line and culture conditions

The pancreatic β islet Rin5mf cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO™, Auckland, NZ) supplemented with 10% heat-inactivated FBS (vol/vol; HyClone Laboratories, Logan, UT) plus 100 μg/ml of streptomycin and 100 units/ml of penicillin at 37 °C and 5% CO_2. The cells were maintained at the concentration of 4500 mg/L D-glucose. PC12, A431, OVCAR, and COS7 cells were also maintained under appropriated conditions.

2.3. Measurement of calcium concentration with fluo-3

The calcium concentration was measured via confocal microscopy (Zeiss LSM 510 Meta; Oberkochen, Germany) using the calcium-sensitive indicator, fluo-3 AM. In brief, Rin5mf cells were loaded with 5 μM fluo3-AM in normal incubation medium at room temperature for 45 min. After washing with the corresponding culture medium, the cells were further incubated for 15 min in the absence of Fluo-3 AM in order to completely de-esterify the dye. We then placed the culture dishes onto a thermostatted stage of an inverted confocal microscope, and observed the cells with the 20× objective. Fluo-3 AM was excited by the 488-nm line of an argon laser and the emission range was 515 nm. In order to preclude the possible effects of dye loading, we normalized the cells with saponin at the end of the experiments.

2.4. Measuring the insulin secretion

Rin5mf cells were seeded in six-well plates until confluent. On the day of each experiment, the cells were washed with KRB supplemented with 0.2% bovine serum albumin and then incubated for 60 min at 37 °C in the KRB solution. At the end of incubation, the solutions were replaced with fresh KRB containing 1 μM gaegurin-6, and were incubated for 1 h. The incubation medium was sampled and centrifuged in order to remove cells, and the supernatant was used in the insulin radioimmunoassay. Immunoreactive insulin levels in the medium were measured with insulin RIA kits.

2.5. YO-PRO staining

Cells were plated in 24 multi-wells at an equal density (2000 cells/mL) and cultured overnight. Cells were then stimulated with gaegurin-6 and other derivatives for 30 min in Krebs–Ringer solution containing 1 μm Yo-Pro-1 iodide dye. Following membrane activation under different experimental conditions, the cells were rinsed and dye uptake was quantified by a confocal microscope (485 nm excitation and 535 nm emission).

2.6. MTT assay

MTT assay was performed as a crude measure of cell viability. MTT was converted by metabolically-active cells into a colored water-insoluble formazan salt. The Rin5mf cells was seeded at a density of 5×10^4/ml in 96-well plates, and allowed to grow for 24 h. The growth media was replaced with serum-free media for 24 h prior to treatment. Subsequently, MTT reagents (7.5 mg/ml in PBS) were added to the cells (10 μl/well), and the culture was incubated for 30 min at 37 °C. The reaction was then stopped via the addition of acidified triton buffer (0.1 M HCl, 10% [v/v] Triton X-100; 50 μl/well), and the tetrazolium crystals were dissolved by 20 min of mixing on a plate shaker at room temperature. The samples were then measured on a plate reader (Bio-Rad 450; Richmond, CA) at a 595 nm test wavelength and a 650 nm reference wavelength. The results were representative of experiments were repeated at least in triplicate.

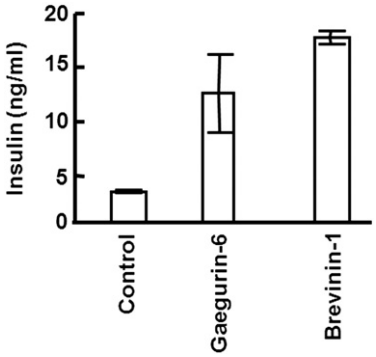

Fig. 1. Gaegurin-6 stimulates insulin secretion in Rin5mf cells. Pancreatic β Rin5mf cells (1.8×10^6/ml) were cultured in 12-well tissue culture plates with medium or in medium containing gaegurin-6 or brevinin-1 for 1 h. The release of insulin from Rin5mf was measured in the supernatants using a RIA kit for insulin. The cells were maintained at the concentration of 4500 mg/L d-glucose. Values are expressed as the means ± SEM of three separate experiments. Statistical analysis was carried out by comparison with the control value ($P<0.001$).

2.7. Data analysis

Data are shown as the means ± SEM. Statistical analysis was carried out using SigmaStat (SPSS, Inc., Chicago, IL). A difference was considered significant if $P<0.05$. Comparison between basal and stimulated status were analyzed using ANOVA with Holm–Sidak comparisons.

3. Results

3.1. Gaegurin-6 stimulates insulin secretion in Rin5mf cells

In order to determine whether gaegurin-6 effects insulin secretion, we investigated the effects of gaegurin-6 on insulin secretion in pancreatic β islet cells. Addition of gaegurin-6 stimulated insulin release (Fig. 1). To understand the signal pathways involved in insulin secretion, we pretreated with brevinin-1, a known insulinotropic peptide, and then measured the insulin level. The concentration of insulin was significantly increased by treatment with brevinin-1 (Fig. 1). Taken together, these results clearly indicate that gaegurin-6 specifically increases insulin secretion in pancreatic β islet cells.

3.2. Gaegurin-6 triggers calcium influx in pancreatic β islet Rin5mf cells

To characterize the signal transduction pathway, we analyzed the increase of calcium induced by gaegurin-6. The application of gaegurin-6 increased the concentration of intracellular calcium (Fig. 2A). This increase was not observed in the presence of EDTA, an extracellular calcium chelator. Protein kinase C (PKC) α is known to be activated and translocated to the plasma membrane under calcium signaling. After transiently expressed with enhanced green fluorescence protein (EGFP)-PKCα in Rin5mf cells, we investigated the subcellular location. Addition of gaegurin-6 stimulated the translocation of PKCα into the plasma membrane (Fig. 2B). Phorbol 12-myristate 13-acetate (PMA) is a specific activator of PKC. PMA exerts its stimulatory effect by translocation of PKCα from cytosol to the plasma membrane and used as a positive control for PKCα translocation. These results strongly suggest that gaegurin-6 induces insulin secretion via calcium influx in pancreatic β islet cells.

3.3. Helical structure is critical for insulin secretion

In order to determine what structural characteristics of gaegurin-6 are critical for insulin secretion, we then synthesized the cyclic–helix disrupted linear form and the N-terminus 14 amino acids of gaegurin-6