Effect of annexin I on insulin secretion through surface binding sites in rat pancreatic islets

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Abstract This study investigates the effect of extracellular annexin I (Anx I) on regulating insulin secretion in isolated rat pancreatic islets. Results show that Anx I stimulates insulin release in pancreatic islets regardless of the presence or absence of extracellular Ca^{2+}. In particular, confocal microscopy shows that Anx I binds to the surface of islet cells in the absence of extracellular Ca^{2+}. However, insulin secretion through Anx I significantly decreases in trypsin-treated islets. Likewise, there is minimal binding of Anx I to the surface of trypsin-treated islets. Anti-Anx I polyclonal antibody also inhibits the stimulating effect of Anx I on insulin secretion. These results indicate that Anx I is capable of binding to the cell surface receptor, in order to regulate the stimulation of insulin release in rat pancreatic islets.

Key words: Annexin I; Insulin secretion; Exocytosis; Annexin I receptor; Pancreatic islet

1. Introduction

Annexin I (Anx I; also called lipocortin I or annexin A1) is a 37 kDa member of the annexin superfamily, which consists of at least 13 different kinds of proteins in the vertebrate. Annexins can perform reversible Ca^{2+}-dependent binding to anionic phospholipids. They are composed of the divergent N-terminal head domain and the conserved C-terminal core domain. The core structure consists of either four or eight repeats of a conserved 70 amino acid unit [1–3]. Likewise, annexins have been reported to influence various intracellular processes in several cells and tissues such as exocytosis [4–6], membrane trafficking [7], endocytosis [8,9], and anti-inflammation [10–12].

Various annexins play a regulatory role in the exocytosis of secretory vesicles in adrenal chromaffin cells [5], anterior pituitary cells [6], and pancreatic β-cells [13–15]. Studies have shown that intracellular annexins play a role in the regulation of insulin secretion in pancreatic β-cells. In particular, Anx I is localized in insulin-containing vesicles. It may be involved in the regulatory mechanism of glucose-induced insulin secretion via the protein phosphorylation process [13]. On the other hand, the literature suggests that annexin VII may be involved in glucose-induced insulin secretion via Ca^{2+} signaling through IP_3 receptors [14]. Likewise, annexin XI is concentrated in insulin-containing vesicles. It may function in Ca^{2+}- and GTP-γS-dependent insulin release via the mediating membrane fusion [15]. However, the regulatory mechanisms of annexins on the secretory machinery have yet to be fully explained.

Recent studies on the different types of cells have shown that annexins are translocated to the outer cell surface [11,16,17]. Moreover, Anx I binds to binding sites on the surface of the cells. It performs specific physiological functions in blood cells [18,19], fibroblast-like synoviocytes [12], or anterior pituitary cells [20,21]. These reports served as the basis of this study, i.e. whether pancreatic islet cells have surface binding sites for Anx I-mediated insulin secretion.

Specifically, this study demonstrates the stimulating effect of exogenous Anx I on insulin secretion in rat pancreatic islets for the first time. Likewise, biochemical and confocal imaging approaches reveal the involvement of Anx I receptors in insulin secretion.

2. Materials and methods

2.1. Purification of human recombinant Anx I

Human recombinant Anx I was overexpressed in the *Escherichia coli* strain C600 harboring pHT1 [23], which was kindly donated by Dr. Doe Sun Na, College of Medicine, University of Ulsan, South Korea. Recombinant Anx I in the soluble fraction of bacterial cells [23] was purified as previously described [24] with some modifications. Purified Anx I was separated on 12% gel using SDS–PAGE and a single 37 kDa band was detected by protein staining with Coomassie brilliant blue R-250 (Sigma) and Western blotting with anti-Anx I monoclonal antibody (mAb) (Transduction). Purified Anx I was used for all subsequent experiments.

2.2. Isolation of rat pancreatic islets

Islets were isolated from pancreases of male Sprague–Dawley rats (8 weeks old) under aseptic conditions using collagenase digestion [25] and Ficoll density gradient [26] methods. Isolated pancreatic islets were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 5.6 mM D-glucose at 37°C under 5% CO_2 for 16–18 h [27]. Cultured islets were suspended in Krebs–Ringer bicarbonate HEPES buffer (KRBH, pH 7.4) and used for experiments [28]. All other tissue culture reagents were purchased from Sigma.

2.3. Measurement of insulin secreted into medium

The amount of insulin secreted into medium from islets was measured by ELISA (enzyme-linked immunosorbent assay) in triplicate as described in [29]. First, the 96-well plates were coated with the 1:1000 diluted rabbit anti-guinea pig IgG (Sigma) in the carbonate–bicarbonate buffer (50 mM, pH 9.6). The plates were washed with phosphate-

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Abbreviations: Anx I, annexin I; FITC, fluorescein isothiocyanate

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buffered saline (PBS) (pH 7.2) containing 0.05% Tween 20 and incubated with the 1:10 000 diluted anti-insulin polyclonal antibody (Dako) in the phosphate buffer (40 mM, pH 7.4) containing 0.1% bovine serum albumin (BSA). After washing the plates, the standards or the samples diluted with PBS (pH 7.2) containing 6% BSA were added to the plates and incubated at 4°C overnight. The peroxidase-conjugated insulin (2.5 mU/ml) (Sigma) was added to each well and incubated to assay the free residual antibody-binding activity in the mixture. The absorbance of developed reactions was read at 490 nm after 15 min at 37°C from the addition of *o*-phenylenediamine. All other incubation steps were carried out at 37°C for 2 h. Bovine insulin (RIA grade from Sigma) was used as the standard.

2.4. Analysis of the surface binding sites of Anx I by laser scanning confocal microscopy

Fluorescence labeling was performed using fluorescein isothiocyanate (FITC; Sigma) as described in [17]. Anx I or BSA (2 mg) was incubated in 1 ml of coupling buffer (50 mM bicarbonate, pH 8.5) containing 0.1 mg of FITC with slow stirring for 2 h at 4°C in the dark for the conjugation of FITC. FITC-conjugated Anx I and FITC-conjugated BSA were separated from free FITC by dialysis in PBS. For the analysis of the surface binding sites of Anx I, isolated islets were incubated in KRBH (pH 7.4) or KRBH containing trypsin (0.3 μg/ml) at 37°C for 5 min and washed with KRBH. Washed islets were incubated in KRBH containing 1 μM of FITC-conjugated BSA or 1 μM of FITC-conjugated Anx I at 37°C for 1 h. Islet cells were fixed with 2% of paraformaldehyde dissolved in PBS. The fixed islet cells were washed, resuspended in PBS, and transferred to a glass slide together with antifade [30]. The images were acquired through a Zeiss LSM 510 laser scanning confocal microscope.

2.5. Detection of Anx I secreted into medium

Isolated islets (200 islets) were incubated in 0.5 ml of KRBH (pH 7.4) in the presence or absence of 20 mM glucose at 37°C for 60 min. After discarding islets by centrifugation, 0.1 ml of medium was used to measure the amount of insulin and 0.4 ml of medium was concentrated by adding 0.4 ml of 20% cold trichloroacetic acid (TCA). Samples were centrifuged at 10 000 × *g* for 15 min. The precipitates were washed with ethanol:ether (1:1, v/v) three times. Concentrated samples were separated on 12% gel using SDS–PAGE for Western blotting with anti-Anx I mAb.

2.6. Statistical analysis

Data were presented as the mean ± S.E.M. and statistical significance was analyzed by one-way analysis of variance (ANOVA) or Student's *t*-test. In all cases $P < 0.05$ was taken as significant.

3. Results

3.1. Anx I stimulates insulin secretion from rat pancreatic islets

The role of intracellular annexins in insulin secretion has been reported in rat pancreatic islets [13–15]. Likewise, the presence of Anx I surface binding sites has been demonstrated in several cell types [12,19,21]. Thus, we sought to investigate the external effect of Anx I on insulin secretion in rat pancreatic islets. Isolated islets were incubated in KRBH (pH 7.4) containing varying concentrations of Anx I in the presence or absence of 0.1 mM Ca^{2+}. Surprisingly, results showed that exogenous Anx I prominently enhanced insulin release in pancreatic islets. In addition, the stimulating effect of Anx I was the same regardless of the presence or absence of extracellular Ca^{2+} (Fig. 1). These results suggested that Anx I binding sites might exist on the surface of islet cells and they might be protein receptors.

3.2. Inhibitory effects of trypsin treatment and anti-Anx I polyclonal antibody (pAb) on Anx I-stimulated insulin secretion

To characterize the property of Anx I binding sites, rat pancreatic islets were incubated in KRBH (pH 7.4) containing

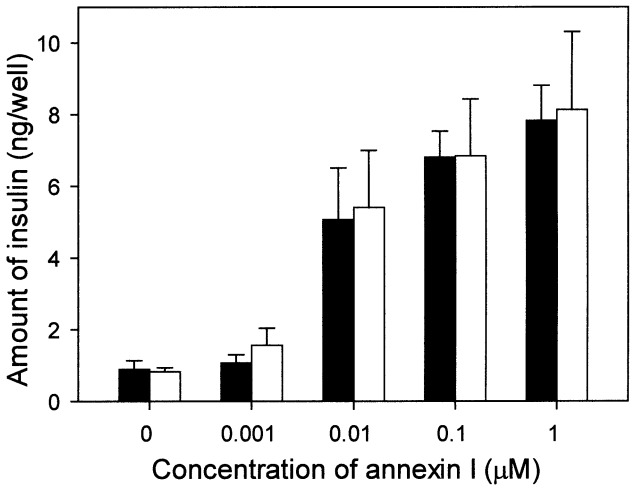

Fig. 1. Effect of Anx I on insulin secretion from rat pancreatic islets. Isolated islets (20 islets/well) were incubated in KRBH (pH 7.4) containing varying concentrations of Anx I in the presence (black) or absence (white) of 0.1 mM Ca^{2+} at 37°C for 90 min. The amount of insulin secreted into medium was measured by ELISA. Results are means ± S.E.M. from four independent experiments. Each experiment was conducted in triplicate. $P > 0.05$ presence of 0.1 mM Ca^{2+} versus absence of Ca^{2+} (ANOVA). $P < 0.05$ control (no Anx I-treated) versus Anx I-treated in the presence (black) or absence (white) of 0.1 mM Ca^{2+} (ANOVA).

varying concentrations of trypsin. They were then washed and treated with Anx I to determine whether a trypsin-sensitive receptor was involved in the binding of Anx I to mediate insulin secretion. As expected, the effect of Anx I on insulin secretion was significantly decreased in pancreatic islets treated with trypsin on a dose-dependent basis (Fig. 2).

On the other hand, rat pancreatic islets were treated with anti-Anx I pAb and Anx I solution to inhibit the binding of Anx I to the cell surface receptor. Similarly, anti-Anx I pAb inhibited the Anx I-stimulated insulin secretion on a dose-dependent basis (Fig. 3).

3.3. Anx I binds to the surface of islet cells

To further confirm the existence and the property of the surface receptor of Anx I, isolated pancreatic islets were treated with FITC-conjugated BSA or FITC-conjugated Anx I. Trypsin-treated islets were also treated with FITC-conjugated Anx I. The specimens were then examined under confocal microscopy. In the control, the fluorescence was minimal (Fig. 4A). In contrast, in the samples treated with FITC-conjugated Anx I, the fluorescence image showed that Anx I bound to the peripheries of islet cells (Fig. 4C). However, the fluorescence image of the trypsin-treated islets showed that trypsin treatment significantly eliminated the fluorescence on the peripheries of islet cells (Fig. 4E).

3.4. Glucose causes the secretion of Anx I from pancreatic islets

Results obtained from this study gave rise to the problem of whether Anx I would be secreted to the outside of islet cells to bind to the surface binding sites, hence necessitating the detection of Anx I secreted to the outside of islets. Results showed that 20 mM glucose increased not only insulin release but also Anx I secretion to the extracellular space of islet cells (Fig. 5).

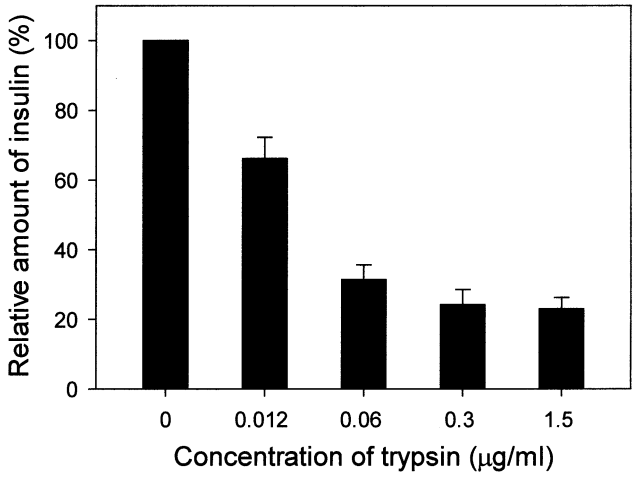

Fig. 2. Effect of Anx I on insulin secretion from pancreatic islets treated with trypsin. Isolated islets (20 islets/well) were treated with varying concentrations of trypsin at 37°C for 5 min, washed, and incubated in KRBH (pH 7.4) containing 1 μM Anx I at 37°C for 90 min. The amount of insulin secreted into medium was measured by ELISA and presented as percentages of the control, which was treated with buffer instead of trypsin. Results are means ± S.E.M. from three independent experiments. Each experiment was conducted in triplicate. The average of controls was 5.8 ± 0.9 ng (mean ± S.E.M.). $P < 0.05$ control (no trypsin-treated) versus trypsin-treated (*t*-test).

4. Discussion

This study presented experimental evidence that extracellular Anx I stimulated insulin secretion by binding to a putative cell surface receptor in rat pancreatic islets. While Anx I has been reported to function via cell surface receptors in other cell types [12,19,21], this is the first report on the function of Anx I surface binding sites in pancreatic islet cells to date.

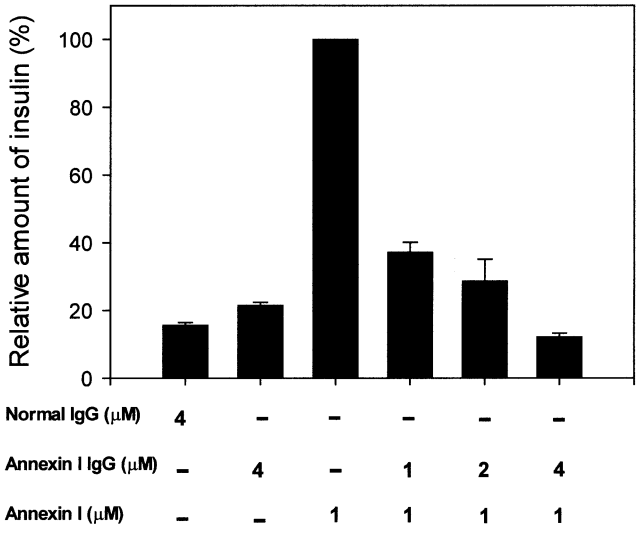

Fig. 3. Inhibition of Anx I-induced insulin secretion from pancreatic islets by anti-Anx I antibody. Isolated islets (20 islets/well) were incubated in KRBH (pH 7.4) containing anti-Anx I pAb and Anx I, which were premixed at various ratios, at 37°C for 90 min. The amount of insulin secreted into medium was measured by ELISA and presented as percentages of the control, which was treated with only Anx I. Results are means ± S.E.M. from three independent experiments. Each experiment was conducted in triplicate. The average of controls was 6.5 ± 0.7 ng (mean ± S.E.M.). $P < 0.05$ control (only Anx I-treated) versus IgG-treated (*t*-test).

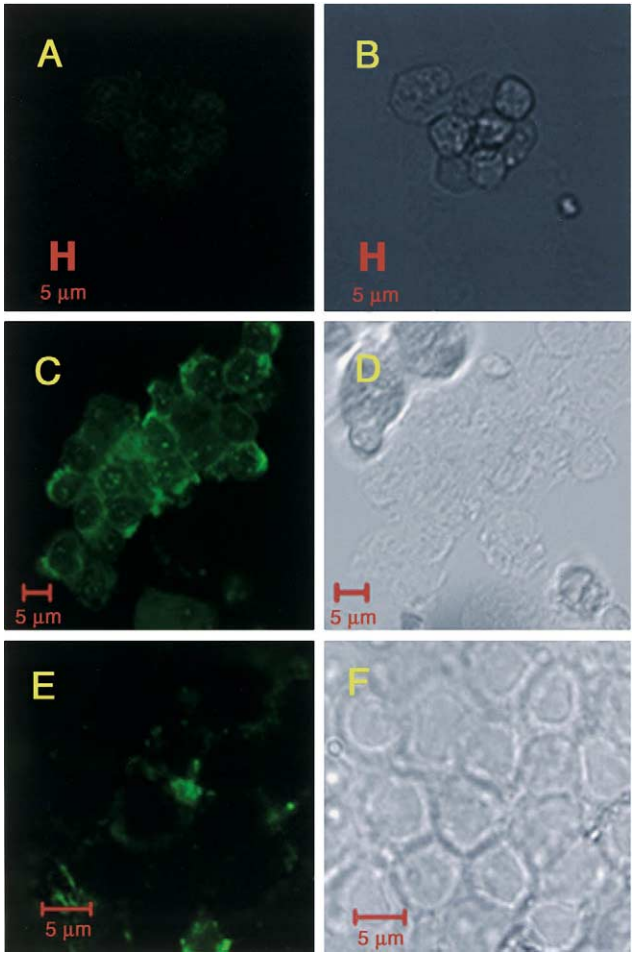

Fig. 4. Binding of Anx I to the surface of pancreatic islet cells. Isolated islets were incubated in KRBH (A,B and C,D) or KRBH containing trypsin (0.3 μg/ml) (E,F) at 37°C for 5 min and washed with KRBH. Each sample was incubated with 1 μM of FITC-conjugated BSA (A,B) or 1 μM of FITC-conjugated Anx I (C,D and E,F) at 37°C for 60 min, washed with KRBH, fixed with 2% paraformaldehyde, and transferred to the slide glass. Stained samples were examined with a Zeiss LSM 510 confocal microscope (×650). Panels A, C, and E represent fluorescence images; panels B, D, and F represent phase contrast images.

The exogenous Anx I was demonstrated to stimulate insulin secretion in pancreatic islets in a Ca^{2+}-independent manner (Fig. 1). Annexins are considered to bind in a Ca^{2+}-dependent manner to anionic phospholipids of membranes for most of their physiological roles [1,2]. Likewise, Anx I-stimulated insulin secretion significantly decreased in islets treated with trypsin (Fig. 2), suggesting that binding sites might be a membrane protein. The high ratio of anti-Anx I pAb to Anx I (4:1) completely blocked the response of Anx I-stimulated insulin secretion (Fig. 3). These observations suggest that extracellular Anx I may mediate insulin secretion via a protein receptor in islet cells. Although Ca^{2+}-independent phospholipid binding properties of annexins have been reported in recent years, a low pH is required for Ca^{2+}-independent phospholipid binding [3]. However, our experiments were conducted at pH 7.4.

Recent studies suggest that intracellular annexins may be

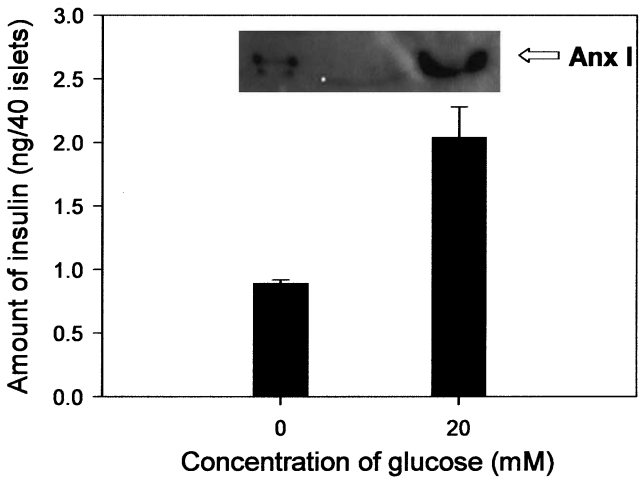

Fig. 5. Secretion of insulin and Anx I from pancreatic islets by glucose. Isolated islets (200 islets) were incubated in 0.5 ml of KRBH (pH 7.4) in the presence or absence of 20 mM glucose at 37°C for 60 min. Secreted Anx I was concentrated by TCA precipitation and a single Anx I band was detected by Western blot analysis using anti-Anx I mAb. The amount of insulin secreted into medium was measured by ELISA. Results are means ± S.E.M. from three independent experiments. Each experiment was conducted in triplicate. $P < 0.05$ control versus 20 mM glucose (t-test).

involved in regulating insulin secretion in islet cells, since annexins increase with insulin secretion due to the high concentration of glucose or Ca^{2+} [13,15]. However, results obtained in the present study do not discount the involvement of intracellular annexins in the regulatory mechanism of insulin secretion in islet cells; rather, they suggest that Anx I may have dual functions on insulin secretion in pancreatic islets, due to the pleiotropic physiological effects of most annexins.

Fluorescence induced by the binding of FITC-conjugated Anx I to the peripheries of islet cells and its elimination by trypsin treatment (Fig. 4C and 4E) indicate that extracellular Anx I binds to a protein receptor on the cell surface. In addition, extracellular Anx I was detected by anti-Anx I mAb on the outside of the islet cells (Fig. 5). Immunoelectron microscopy studies have shown that annexins are localized on insulin-containing vesicles [13–15]. Although it is still unknown whether secretion of annexins can occur, intracellular annexins are reportedly released to the outer cell surface and extracellular annexin activities have recently been described [3,6,11,16,31,32]. Thus, Anx I is probably secreted from the islet cells during exocytosis of the insulin-containing vesicles. These results suggest that Anx I probably acts as an autocrine signal to stimulate insulin secretion in pancreatic islets.

In conclusion, the present study suggests that Anx I may play a role as an extracellular signal via the surface receptor of islet cells, in order to initiate the signal transduction pathway for insulin secretion.

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