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Annexin XI may be involved in Ca^{2+}- or GTP-γS-induced insulin secretion in the pancreatic β-cell

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Abstract The aim of this study was to investigate possible involvement of annexin XI in the insulin secretory machinery. In fluorescence immunocytochemistry, annexin XI was found in the cytoplasm of pancreatic endocrine cells and a pancreatic β-cell line, MIN6, in a granular pattern. MIN6 cells also possessed weak and diffused annexin XI immunoreactivity in the cytoplasm. Immunoelectron microscopy revealed annexin XI in the insulin granules. Insulin secretion from streptolysin-O-permeabilized MIN6 cells was inhibited by anti-annexin XI antibody, when the release was stimulated by either Ca^{2+} or GTP-γS, but not by a protein kinase C-activating phorbol ester. Inhibition of insulin release by anti-annexin XI antibody was reproduced in permeabilized rat islets. These findings suggest that annexin XI may be involved in the regulation of insulin secretion from the pancreatic β-cells. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Annexin; Insulin release; Ca^{2+}; GTP-γS; Pancreatic β-cell

1. Introduction

Although it is widely accepted that an increase in intracellular Ca^{2+} concentrations is a crucial event in triggering hormone secretion, we still have limited knowledge on relevant intracellular Ca^{2+} receptors. Many Ca^{2+}-binding proteins, such as EF-hand proteins and annexins which have respective Ca^{2+}-binding structures, the EF-hand domain and the core domain, have been found in a variety of tissues or cells. These proteins are considered to be responsible for transduction of intracellular Ca^{2+}-signaling [1].

In the pancreatic β-cell, roles of intracellular Ca^{2+} receptors in insulin secretion are not fully understood except certain proteins as calmodulin [2]. Annexins form a superfamily of Ca^{2+}-binding proteins featured by Ca^{2+}-dependent phospholipid-binding activities [3]. This property and preferential distribution of annexins in intracellular vesicles [4] suggest that annexins may be involved in the membrane–membrane interaction and play positive roles in exocytosis, endocytosis, granule aggregation and the structural organization of membranes [5–8]; annexins I and III stimulate aggregation of the neutrophil granules and their fusion to the plasma membranes [9–11]; some members of the annexin family are, in particular, considered to participate in the control of exocytosis. For example, annexin II is required in the secretory machinery in chromaffin cells [12,13].

Annexin XI, initially designated as CAP-50 (calcyclin-associated protein 50 kDa), was first purified from rabbit lung as a protein which binds a two EF-hand Ca^{2+}-binding protein calcyclin (or S100A6 [14]), in a specific manner [15]. Annexin XI is widely distributed in tissues and organs [16]. This annexin is unique, because it possesses the longest N-terminal domains, comprising of 197 amino acids, among all known annexins. Annexin XI is expected to play versatile roles, because it is phosphorylated by a few protein kinases [17,18] and because its long N-terminal domain includes the calcyclin-binding site and proteolytic sites [19,20]. Nevertheless, we do not know much on physiological functions of annexin XI.

In the present study, we investigated possible roles of annexin XI in the regulation of insulin release by a morphological approach and in the secretion study with a membrane permeabilization technique using streptolysin-O (STLO).

2. Materials and methods

2.1. Materials

MIN6 cells were kindly donated by Prof. J.-I. Miyazaki (Osaka University). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was from Wako (Tokyo, Japan). GTP-γS was from Sigma (St. Louis, MO, USA). STLO was from Mullex Biotech (Dartford, UK). Dulbecco's modified Eagle medium (DMEM) was from Nissui (Tokyo, Japan). Bovine serum albumin (BSA) (fraction V) was from Chemicon International Inc. (Temecula, CA, USA). The insulin radioimmunoassay kit was from Eiken (Tokyo, Japan). Rhodamine-conjugated goat anti-guinea pig IgG was from ICN (Costa Mesa, USA). Guinea pig anti-insulin antibody was from Seikagaku Kogyo (Tokyo, Japan). Fluorescein isothianate (FITC)-conjugated goat anti-rabbit IgG was from Vector (CA, USA). 10 nm gold colloidal particle-conjugated goat anti-rabbit IgG and 15 nm gold colloidal particle-conjugated goat anti-guinea pig IgG were from Amersham Japan (Tokyo, Japan). Normal rabbit IgG was kindly provided by Dr. Y. Nishizawa (Nagoya University). Anti-annexin XI serum was obtained in rabbits by injecting annexin XI purified from bovine lung. This serum was specific for annexin XI and did not crossreact with other annexins [16].

2.2. Cell culture

MIN6 cells (passages 32–39) were cultured in DMEM supplemented with 66 mg/l kanamycin sulfate and 15% fetal calf serum at 37°C in a humidified atmosphere of 95% air/5% CO_2 [21]. The cells

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were passaged and harvested using trypsin/EDTA and the culture medium was replaced every other day.

2.3. Islet isolation

Pancreatic islets were isolated from male Wistar rats with collagenase digestion.

2.4. Fluorescence immunocytochemistry

Pancreases of the Wistar rats (male, 200 g body weight) or MIN6 cells grown on the cover glass were fixed with 2% paraformaldehyde and 0.15% picric acid in 0.1 M phosphate buffer (PB, pH 7.4) for 1–4 h at room temperature. After washing with phosphate-buffered saline (PBS, pH 7.2), pancreases were soaked overnight in 30% sucrose in 0.1 M PB at 4°C, embedded in OCT compound and frozen quickly. 15 μm thick sections were cut by a cryostat and mounted onto poly-L-lysine-coated glass slides. Tissue sections on the slides and MIN6 cells on the cover glass slips were treated with 0.3% Triton X-100 in PBS for 30 min. They were immersed in 5% normal goat serum for 30 min, and incubated with a mixture of anti-annexin XI serum and anti-insulin serum overnight. After washing with PBS, specimens were incubated with FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-guinea pig IgG mixture for 1 h. After mounting with PBS–glycerol containing paraphenilendiamine, cells were examined with a confocal laser scanning microscope MRC-1024 (Bio-Rad) using a krypton–argon laser.

2.5. Immunoelectron microscopy

MIN6 cells were fixed with 2% paraformaldehyde, 0.15% picric acid and 0.1% glutaraldehyde in 0.1 M PB for 1 h at room temperature. After washing with PBS, cells were dehydrated in graded *N,N*-dimethylformamide at progressively lower temperatures and embedded in Lowicryl K4M (TAAB) at −20°C. Resin-embedded specimens were cut with ultramicrotome and mounted on nickel grids. Ultrathin sections were treated with 2% BSA in PBS for 30 min and incubated with a mixture of anti-annexin XI serum and anti-insulin serum for 4 h. After washing with PBS, sections were incubated with 10 nm gold colloidal particle-conjugated anti-rabbit IgG and 15 nm gold colloidal particle-conjugated anti-guinea pig IgG mixture for 1 h. Sections were

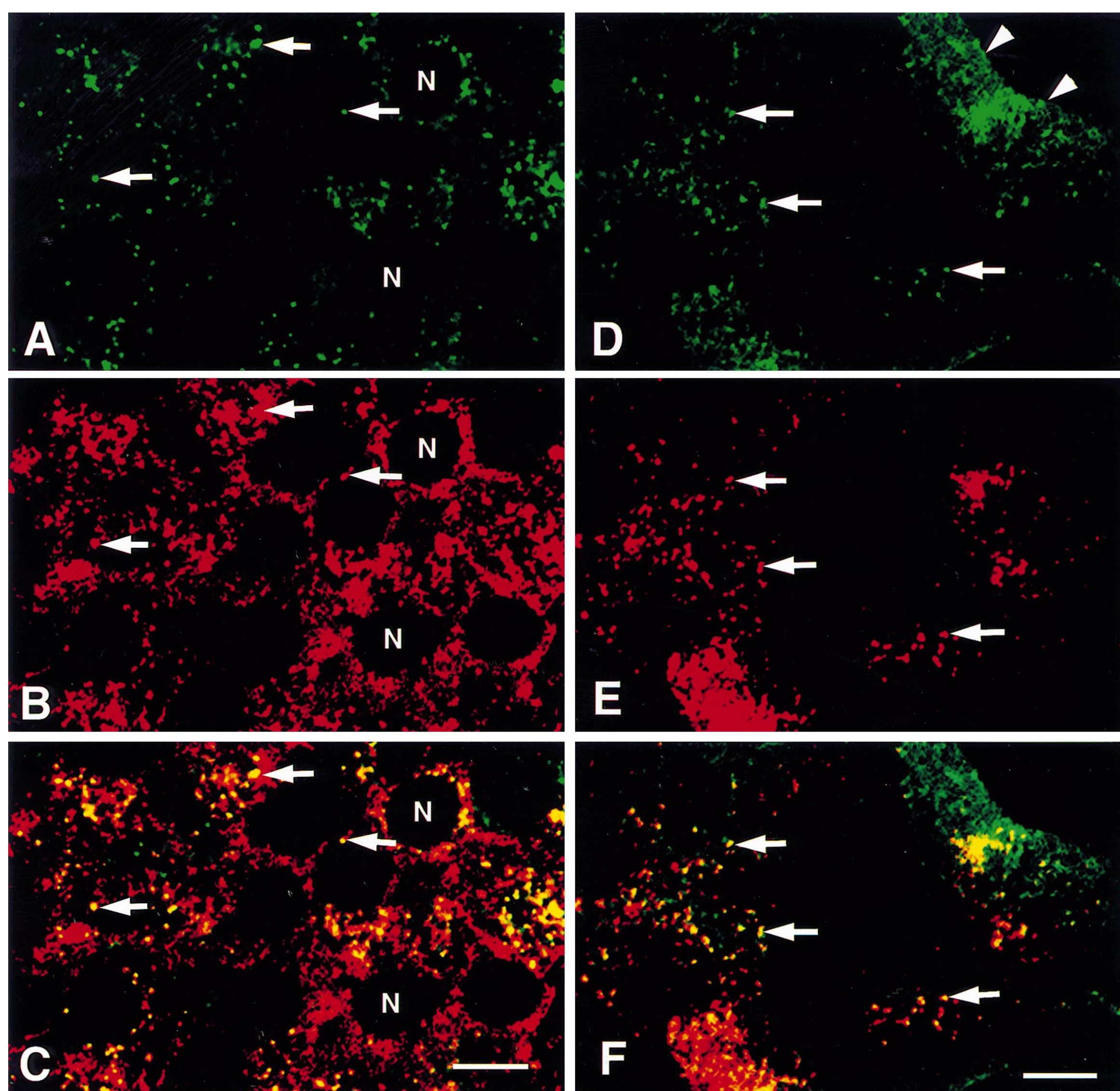

Fig. 1. Distribution of annexin XI and insulin immunoreactivities in rat pancreatic islets and MIN6 cells. 15 μm thick sections of the rat pancreas or MIN6 cells were double-stained with anti-annexin XI (A and D) and anti-insulin (B and E) antibodies, and examined with confocal laser scanning microscopy. A, B and C: Double-stained images of the rat pancreatic islet. Annexin XI-immunopositive granular structures (arrows in A) were mostly positive for insulin immunoreactivity (arrows in B) and shown in yellow in double-stained images (arrows in C). Note that there are some insulin granules without annexin XI immunoreactivity and that the nuclei (N) were never stained with anti-annexin XI antibody. D, E and F: MIN6 cells contained annexin XI immunoreactivity in the insulin granules and cytoplasm (C). Arrows show double-stained granules. Some cells showed diffused distribution of annexin XI in the peripheral cytoplasm (arrowheads in D). Bars = 10 μm.

counterstained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 electron microscope.

2.6. Secretion study with permeabilized cells

MIN6 cells were seeded at a density of 2×10^4 cells per well into a 48-MultiWell (Sumitomo, Tokyo, Japan) 2–3 days prior to an experiment. On the day of experimentation, MIN6 cells were preincubated for 1 h at 37°C in 0.5 ml of HEPES–Krebs buffer (20 mM HEPES, 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.0 mM $NaHCO_3$, pH = 7.4) supplemented with 5 mg/ml BSA. After washing twice with 0.5 ml of the glutamate buffer containing (mM): K-glutamate 100, Na-glutamate 42, HEPES (pH 7.0) 16, MgATP 3, EGTA 1 and 5 mg/ml BSA with 10 nM Ca^{2+}, the cells were further incubated for 30 min in 0.5 ml of the glutamate buffer at 10 nM to 10 μM Ca^{2+} plus 250 U/l STLO and various substances and anti-annexin XI antibody. $CaCl_2$ was added to the glutamate buffer to give an arbitrary concentration of free Ca^{2+}, and we verified the free Ca^{2+} concentration by a Ca^{2+}-fluorescent dye. At the end of the 30 min incubation, the medium was spun briefly to sediment any detached cells, and the supernatant was retained for insulin assay. For islet secretion study, groups of size-matched five isolated islets were preincubated for 1 h in BSA-containing HEPES–Krebs buffer with 3 mM glucose. Islets were, then, washed twice with basal glutamate buffer (Ca^{2+} 0.1 μM) and further incubated for the glutamate buffer supplemented with various substances and 250 U/l STLO. After 1 h incubation, insulin released into the media was assayed by radioimmunoassay.

3. Results

3.1. Fluorescence immunocytochemistry

In rat pancreatic islets, numerous cells contained annexin XI immunoreactivity (Fig. 1A–C). Some of islet cells possessed annexin XI-immunopositive granular structures. It must be noted that none of the nuclei exhibited annexin XI immunoreactivity (N, in Fig. 1A–C). Double staining with anti-annexin XI and anti-insulin antibodies revealed preferential distribution of annexin XI in the insulin granules (Fig. 1C). Annexin XI immunoreactivity, also distributed in glucagon- or somatostatin-immunopositive cells (not shown), was restricted to the pancreatic islet cells and was not observed in pancreatic acinar cells and fibroblasts (not shown). Intracellular distribution of annexin XI was also examined in the insulin-secreting cell line MIN6 (Fig. 1D–F). Annexin XI immunoreactivity in the cytoplasm exhibited a granular or a diffused pattern (Fig. 1D). Most of annexin XI-immunopositive granular structures in MIN6 cells showed insulin immunoreactivity (Fig. 1F). The immunoreactivity disappeared when anti-annexin XI antibody was pre-absorbed with excess of the antigen.

3.2. Immunoelectron microscopy

To verify co-localization of annexin XI and insulin, we carried out immunoelectron microscopy of MIN6 cells. As shown in Fig. 2, MIN6 cells possessed electron dense large granules which showed insulin immunoreactivity (15 nm gold particles). 10 nm gold particles representing annexin XI immunoreactivity were accumulated in these granules. Annexin XI immunoreactivity was also distributed in the cytoplasm although this reaction was weaker. In MIN6 cells, nuclei and other organelles were, as in islet cells, free from the immunoreactivity.

3.3. Insulin secretion from permeabilized MIN6 cells and rat pancreatic islets

Fig. 3 depicts effects of anti-annexin XI antibody on insulin secretion permeabilized with STLO. Inhibition of Ca^{2+}-induced insulin release by anti-annexin XI antibody is demonstrated in Fig. 3A. In permeabilized MIN6 cells, a significant increase in insulin release was observed with Ca^{2+} at concentrations higher than 10 nM. This threshold for Ca^{2+} to initiate insulin release agrees to other reports using pancreatic islets or β-cell lines with other permeabilization techniques [22,23]. Anti-annexin XI antibody (×250) inhibited insulin release with 10 μM Ca^{2+} (38.5% inhibition of stimulated release) without affecting those at lower concentrations of Ca^{2+}. This is compatible with general properties of annexin-binding to cell membranes; half maximal binding of annexins IV, VI or VII to cell membranes requires Ca^{2+} within a low μM range [24]. We also investigated the effects of annexin XI antibody on insulin release from permeabilized MIN6 cells by GTP-γS and the protein kinase C (PKC) activator TPA,

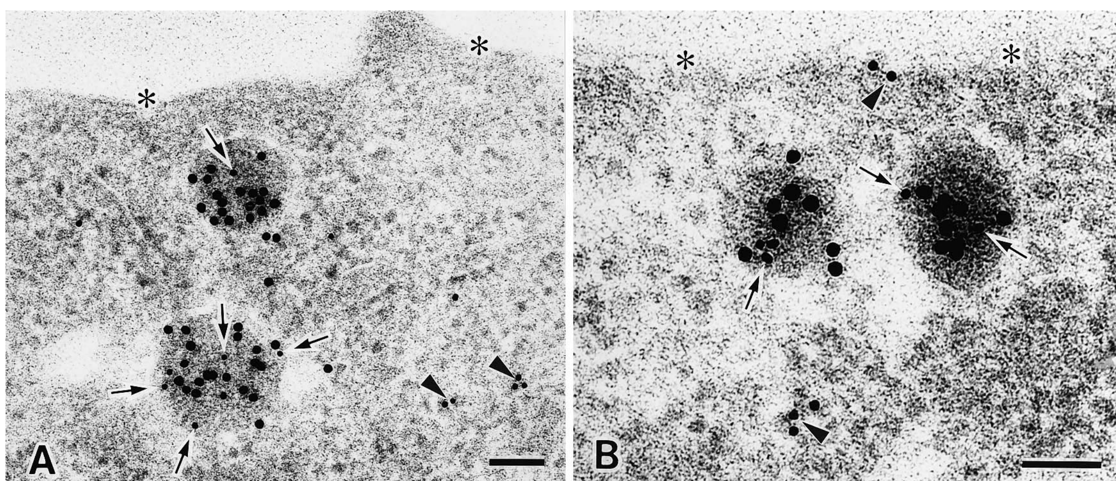

Fig. 2. Immunoelectron micrographs of MIN6 cells with anti-annexin XI and anti-insulin antibodies. Ultrathin sections of resin-embedded MIN6 cells were double-stained with the immunogold method and observed with electron microscopy. Some of insulin-immunoreactive (large dense particles) granules contained annexin XI immunoreactivity (small dense particles, arrows). Annexin XI immunoreactivity also distributed in the cytoplasm (arrowheads). Asterisks show cell membranes. Bars = 1 nm.

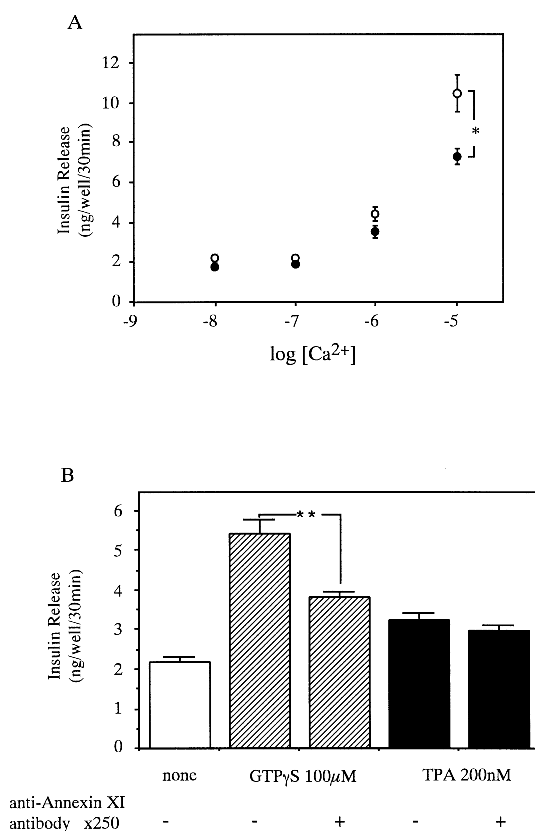

Fig. 3. Inhibition by anti-annexin XI antibody of insulin release from STLO-treated MIN6 cells. A: Effects of anti-annexin XI antibody on Ca^{2+}-induced insulin release from STLO-permeabilized MIN6 cells. After 1 h preincubation, MIN6 cells (2×10^4 cells/well) were permeabilized with 250 U/l STLO and incubated for 30 min with Ca^{2+}/EGTA glutamate buffer at different concentrations of Ca^{2+} in the presence (closed circles) or absence (open circles) of anti-annexin XI antibody (×250). Each symbol represents mean ± S.E.M. for six observations from two independent experiments. $*P<0.05$. B: Effects of anti-annexin XI antibody on GTP-γS or TPA-induced insulin release from STLO-permeabilized MIN6 cells. MIN6 cells were incubated with STLO-containing glutamate buffer at 10^{-7} in the presence of GTP-γS (100 μM) or TPA (200 nM) with or without anti-annexin XI antibody. $**P<0.005$.

both of which induce insulin release independently of changes in intracellular Ca^{2+} [23,25]. GTP-γS (100 μM) and TPA (200 nM) increased insulin release from permeabilized MIN6 cells, and addition of anti-annexin XI antibody decreased only the former by 48.8% (Fig. 3B).

Inhibition of Ca^{2+}-induced insulin release by anti-annexin XI antibody was more pronounced in STLO-permeabilized islets (3.09 ± 0.23 vs. 0.58 ± 0.12 ng/five islets/h for 10 μM Ca^{2+} and 10 μM Ca^{2+} plus anti-annexin XI antibody, respectively, $n=5$, $P<0.001$). GTP-γS (100 μM)-induced insulin release from permeabilized islets was also inhibited by anti-annexin XI antibody (2.93 ± 0.17 vs. 1.96 ± 0.05 ng/five islets/h for 100 μM GTP-γS and GTP-γS plus anti-annexin XI antibody, respectively, $n=5$, $P<0.01$). TPA-induced insulin release was, as in MIN6 cells, not affected by anti-annexin XI antibody (1.91 ± 0.1 vs. 1.59 ± 0.11 ng/five islets/h for 200 nM TPA and TPA plus anti-annexin XI antibody, respectively, $n=5$, n.s.). Normal rabbit IgG (up to 32 μg/ml) failed to decrease insulin release by Ca^{2+} or GTP-γS (not shown).

4. Discussion

In the β-cells, annexins I and II have also been demonstrated to exist in islet cells in the rat pancreas [26]. Annexin I is distributed on the insulin granule membranes, and its phosphorylation was shown to be increased by glucose [27]. It has been suggested that annexin I may participate in the regulation of glucose-induced insulin release via protein phosphorylation, because glucose-induced serine phosphorylation of annexin I was inhibited by a PKC inhibitor H-7, which also suppressed insulin release. However, there is as yet no direct evidence available on the influences of annexins on the secretory machinery of the pancreatic β-cell.

The present findings suggest to us that annexin XI may be involved in the regulation of insulin release. It is, however, unlikely that its PKC activation is necessary for annexin XI-dependent regulation of insulin release, because (i) anti-annexin XI antibody failed to affect TPA-induced insulin release and (ii) annexin XI was, in our attempts, not phosphorylated by PKC in vitro (T. Sudo, unpublished data). Inhibition of Ca^{2+}- and GTP-γS-dependent insulin release by anti-annexin XI antibody reminds us of a previous report that both Ca^{2+}- and GTP-γS-induced membrane fusion of chromaffin granules was accelerated by addition of synexin (annexin VII) [28], suggesting similar roles of annexins VII and XI in the chromaffin cell and the pancreatic β-cell, respectively. Possible regulation of annexin functions by nucleotides including GTP has recently been reviewed [29].

It is interesting that certain EF-hand proteins and annexins interact with each other in a specific manner and may exert their roles. The S100-binding sites of some annexins show similar hydrophobic residue clusters [19,30]. Annexins I and XI have respective Ca^{2+}-dependent binding activities to calgizzarin (S100C) and calcyclin (S100A6) [15,19,31,32], while binding of annexin II to p11 (S100A10) does not require Ca^{2+} [33]. Binding of p11 to annexin II plays an important role in catecholamine release via regulating intracellular distribution of the annexin [13], and a similar role has also been suggested in artery endothelial cells [34]. We previously reported that calcyclin may play a positive role in insulin release [22]. Although further direct evidence is necessary, the annexin XI/calcyclin complex may function in insulin secretion in a co-operative manner. In conclusion, the present findings strongly indicate that annexin XI is involved in the secretory processes in the β-cells.

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