Changes in annexin I and II levels during the postnatal development of rat pancreatic islets

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INTRODUCTION

Annexin I and annexin II (also known as lipocortin I and lipocortin II, respectively) are members of the annexin family of structurally related Ca²⁺-dependent membrane-binding proteins (Geisow and Walker, 1986; Geisow et al., 1987; Klee, 1988; Burgoyne and Geisow, 1989), which possess four or eight conserved 70 amino acid repeats of a homologous core domain in which the Ca²⁺- and phospholipid-binding sites are contained (Brugge, 1986). To date, more than 11 annexins have been identified in various tissues and cell types (Moss, 1992; Chang et al., 1992; Schlaepfer et al., 1992). Annexin I (molecular mass 35 kDa, p35) is a substrate for protein kinase C (PKC) and for the epidermal growth factor receptor/kinase (EGFR) (Summers and Creutz, 1985; Khanna et al., 1986; Michener et al., 1986; Fava and Cohen, 1984; Sawyer and Cohen, 1985). Annexin II (molecular mass 36 kDa, p36) is also a major substrate for PKC and for pp60⁹⁰⁶ (Brugge, 1986). Both the tyrosine and serine/threonine phosphorylation sites have been identified in the N-terminal tails of annexin I and annexin II (Haigler et al., 1987; Schlaepfer and Haigler, 1988). These proteins have been reported to bind various secretory granules and promote their aggregation in a Ca²⁺-dependent manner (Burgoyne, 1988; Drust and Creutz, 1989; Ali et al., 1989; Nakata et al., 1990; Handel et al., 1991; Turgeon et al., 1990; Meers et al., 1993; Roth et al., 1993). Although annexins have been detected in a wide variety of tissues and cell types, the distribution patterns of subsets of the family appear to be tissue-specific, and the individual genes may be independently regulated (Pepinsky et al., 1988). However, the biological functions of the annexins have not yet been firmly established and remain to be elucidated.

In the present study, we investigated the dynamic changes in expression of two members of the annexin family that are immunologically related to annexin I and annexin II in the rat pancreatic islets during postnatal development. Developmental changes in levels of protein kinases (PKC, EGFR, pp60⁹⁰⁶) in the islets, which can phosphorylate these annexins, were also investigated. Our results indicate that annexin I and annexin II may have certain important roles in the signal transduction in pancreatic islets.

MATERIALS AND METHODS

Isolation of rat pancreatic islets

Male Sprague-Dawley rats fed ad libitum were anesthetized by
intrapitoneal injection of Nembutal (40-50 mg/kg). Following aseptic extirpation of the pancreas, fat, connective tissues and lymph nodes were separated using forceps and scissors under a dissecting microscope. The cleaned pancreases were minced into small pieces (around 1 mm in diameter) and washed twice with sterilized phosphate buffered saline (PBS). After incubation in PBS containing 2 mg/ml collagenase P (Worthington BC, New Jersey, USA) for 15 minutes at 37 °C pancreatic tissue was washed twice in PBS, transferred to a Petri dish containing RPMI 1640 medium supplemented with 10% fetal bovine serum, and cultured at 37 °C for 16 hours. Aseptic handling was maintained until this step. With mild agitation, the endocrine cells containing the islets rose to the surface of the medium and were collected. These cells were then mixed in a centrifuge tube with 25 ml of Percoll (dialyzed and lyophilized, medium and were collected. These cells were then mixed in a centrifuge tube with 25 ml of Percoll (dialyzed and lyophilized, Pharmacia Co. Ltd Tokyo, Japan) with a specific gravity (r) of 1.075. Another 25 ml of Percoll (r=1.050) was layered onto the mixture. Islets were purified by centrifugation at 850 g for 20 minutes without breaking, after which the majority were separated into the interface (Korbutt and Pipeleers, 1992). The purified islet fraction was analyzed by indirect immunofluorescence staining using a polyclonal anti-insulin antibody to determine its purity. We also confirmed that insulin was secreted from the fraction when stimulated with high concentrations of glucose. The islet fraction was then semi-quantitatively analyzed by western blotting using anti-annexin I and anti-annexin II antibodies. Reference annexin I and annexin II were purified from bovine lung according to the procedure of Khanna et al. (1987).

**Western blot analysis**

Isolated pancreatic islets were homogenized in 10 volumes of homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Tween-20, 1 μg/ml leupeptin, 200 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 3 mM benzamidine and 0.1 mg/ml soybean trypsin inhibitor using a Polytron homogenizer with three 30-second bursts (interval time, 15 seconds). After centrifugation at 8,000 g for 10 minutes, the supernatant was centrifuged at 100,000 g for 60 minutes. The resultant supernatant was dialyzed against distilled water at 4 °C overnight and the pellet was homogenized in a small volume of distilled water. Protein concentration was determined using the method of Bradford (1976). Fifty micrograms of each sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoretic transfer of proteins from the gels onto nitrocellulose membranes, they were incubated with anti-human annexin I polyclonal antibody (6.5 μg/ml) and anti-human annexin II monoclonal antibody (2 μg/ml) at room temperature overnight. Membranes were washed and incubated with anti-rabbit IgG or anti-mouse IgG peroxidase-conjugated, respectively, for 2 hours at room temperature. Color development was done using 4-chloro-1-naphthol (Wako Pure Chemical Industries, Osaka, Japan) as the chromogenic substrate for the peroxidase reaction.

**Immunohistochemistry**

Rats were perfused intracardially with PBS, followed by perfusion with 4% paraformaldehyde in PBS. Pancreases were removed, embedded in paraffin, and thin sections (5 μm thickness) were cut on a microtome. Monoclonal antibodies against annexin I, annexin II, αPKC and EGFR, and polyclonal antibodies against pp60, insulin, glucagon and actin were used for adjacent sections, according to the Vectastain-ABC system immunostaining method (Vector Lab. Inc., Burlingame, USA), using diaminobenzidine tetrahydrochloride (DAB) as a substrate for the peroxidase reaction. Sections were counterstained with Mayer’s-hematoxylin eosin.

**Antibodies**

Anti-annexin I and anti-annexin II monoclonal antibodies, and polyclonal antibodies were provided by the Nippon-Shinyaku Co. Ltd, Kyoto, Japan. Anti-insulin and glucagon polyclonal antibodies and anti-EGFR monoclonal antibody were purchased from DAKO Japan Co. Ltd, Tokyo, Japan. Anti-PKC monoclonal antibody, which is specific for the α-isoform, was purchased from Amersham Co. Ltd, Tokyo, Japan. Anti-pp60 and anti-actin polyclonal antibodies were obtained from MBL Co. Ltd, Nagoya, Japan, and Biochemical Technologies Inc., Massachusetts, USA, respectively.

**RESULTS**

Two bands immunoreactive with the anti-annexin I antibody were detected at apparent molecular masses of 41 kDa and 39 kDa. The intensity of lower molecular mass band (39 kDa) was transiently reduced at 4 weeks, but both peptides were abundant in adult rat pancreatic islets (Fig. 1A). As purified bovine lung annexin I comigrated with the lower molecular mass 39 kDa immunoreactive band, this is likely to be an intact annexin I molecule. When the polyclonal antibody was blocked with excess annexin I purified from bovine lung, the immunoreactivity of both bands was significantly reduced (data not shown). The higher molecular mass band (41 kDa) might be an isoform of annexin I in rat pancreatic islets. It is also possible that the lower band is also an isoform of annexin
Annexin I and II levels in pancreatic islets with the same molecular mass as purified bovine lung annexin I. The amino acid sequence of short peptides from both bands is currently under investigation to allow definite identification. A single immunoreactive band was observed with anti-annexin II antibody with an apparent molecular mass of 36 kDa (Fig. 1B). The purified annexin II from bovine lung comigrated to the same position as this band. The level of annexin II in pancreatic islets also increased with postnatal development. To confirm the results obtained by western blot analysis, and to examine whether there were dif-
ferences in distribution between different islet cell types, we immunohistochemically investigated annexin I and annexin II in rat pancreatic islets.

Fig. 2 shows the immunostaining patterns with anti-annexin I and anti-annexin II monoclonal antibodies of pancreatic islets from 1-week-old to adult rats. The density of immunostaining (brown color) for annexin I became much stronger with the development of islets from 1-week-old to adult. Pancreatic islets of 1-week-old and 4-week-old rats contained almost no immunoreactivity (Fig. 2A, B; arrow), but strong immunostaining with anti-annexin I antibody in adult rat islets was observed (Fig. 2C; arrow). This immunohistochemical data supported the results of western blot analysis. A similar immunohistological result was obtained with the polyclonal antibody used for western blot analysis in Fig. 1A (data not shown).

Annexin II was also demonstrated to exist in rat pancreatic islets (Fig. 2D, E and F). Annexin II was expressed at very low

Fig. 3. Distribution of annexin I-positive cells in adult pancreatic islets. Localization of annexin I, insulin and glucagon in rat pancreatic islets was analyzed using the respective antibodies as described in Materials and Methods. All sections are adjacent, and were stained with anti-annexin I monoclonal antibody (A), anti-insulin polyclonal antibody (B), anti-glucagon polyclonal antibody (C), non-immune mouse IgG (D) or non-immune rabbit IgG (E). Bars, 50 μm.
level in pancreatic islets from 1-week-old rats (Fig. 2D; arrows), but moderate staining for annexin II was observed in islets from 4-week-old rats (Fig. 2E; arrow), and a marked increase in those from adults (Fig. 2F; arrow). These immunohistochemical findings support the developmentally enhanced expression of annexin II shown by western blot analysis. In addition, it is noted that annexin I and annexin II are specifically expressed in adult pancreatic islets, but are absent or expressed at very low levels in exocrine tissues (Figs 2, 3). The immunostaining patterns of annexin I and annexin II were diffuse and homogeneous in adult islet cells, and no specific distributional differences were observed between different islet cell types. Localization of α cells and β cells was confirmed by immunohistochemistry using anti-insulin and anti-glucagon.

Fig. 4. Distribution of αPKC, EGFR and pp60
c in pancreatic islets and their developmental changes. Immunohistochemical analysis of αPKC, EGFR and pp60
c in pancreatic islets was performed using anti-αPKC monoclonal, anti-EGFR monoclonal or anti-pp60
c polyclonal antibodies, respectively. Localization of αPKC (A,B), EGFR (C,D) and pp60
c (E,F) was demonstrated in 4-week-old (A,C,E) and adult rats (B,D,F). Bars, 50 µm.
polyclonal antibodies, respectively (Fig. 3B,C). β Cells are located in the central part of the islets, whereas α cells show a peripheral distribution. It is, therefore, evident that annexin I and annexin II are present in all islet cells including α and β cells (Figs 2, 3).

Furthermore, we also qualitatively analyzed, by immunohistochemical methods, developmental changes in the expression of various protein kinases (αPKC, EGFR, pp60src) capable of phosphorylating annexin I and annexin II (Fig. 4). These kinases showed similar staining patterns to annexin I and annexin II, and adult rat pancreatic islets showed much stronger staining for all kinases than those from 4-week-old animals. The distribution patterns of these kinases in the islets were diffuse and homogeneous, and were similar to those of annexin I and annexin II. These data indicate that the three kinases are present at much higher levels in endocrine than in exocrine cells, and they may play important roles in some specific functions of islet cells.

Thus, the expression patterns of annexin I, annexin II and the three kinases in islets were similar during postnatal development. To exclude the possibility that these postnatal changes may be a general phenomenon, we performed an immunohistochemical study for actin, which was present in both exocrine and endocrine tissues in pancreas during developmental stages (Fig. 5).

Immunoreactivity of actin was evenly distributed in both exocrine tissues and islet cells as well as connective tissues in adult rat pancreas. The same staining patterns were observed in 1-week-old and 4-week-old rat pancreases. Furthermore, the staining intensity of actin did not change during postnatal development. This result indicates that annexin I, annexin II and the three kinases are abundantly present in endocrine tissues and synergistically increase in amount during postnatal development.

Therefore, annexin I and annexin II, as substrates of these kinases, may exert certain physiological roles that can be regulated by phosphorylation by these kinases.

**DISCUSSION**

There are not many reports of the existence and functional roles of annexins in pancreatic islets. Moreover, Iida et al. (1992) reported that they could not detect annexin II or annexin VI in pancreatic islet cells. Clark et al. (1991) showed the expression of annexin VI in some endocrine organs including human pancreatic islet cells. There have been no reports that show the existence of either annexin I or annexin II in pancreatic islets. In the present study, we have produced the first direct evidence of the existence of both annexin I and annexin II in pancreatic islets.
II in pancreatic islets, and of dynamic changes in their content during postnatal development.

Developmental studies of annexins have been performed in various tissues such as brain (Burgoyne et al., 1989), liver (Masaki et al., 1994) and testis (our unpublished data), and their levels have been shown to decrease with age in these organs. These findings may imply that annexins have certain functional roles related to cellular proliferation or early processes of differentiation. According to Clark et al. (1991), annexin VI expression appears to be developmentally regulated in B- and T-lymphocyte differentiation.

In the present study, levels of annexin I and annexin II in rat pancreatic islets increased during the developmental process. Although the biological or physiological functions of the annexins have not been firmly established, our results suggest that, in the endocrine glands of rat pancreas (islets of Langerhans), annexin I and annexin II have physiological roles other than involvement in cell differentiation and/or proliferation.

In general, there is not much difference in the growth pattern of pancreatic islets between the sexes at any age, and islet volume continues to increase with increasing body weight (McEvoy, 1981).

Overholser (1925) reported that the increase in number of islets in the rat pancreas ceases around day 50 after birth, and thereafter each islet increases in size. If annexin I and annexin II contribute only to the regulation of cell growth and proliferation in pancreatic islets, the levels of these proteins would, therefore, be expected to decrease with age, especially after day 50. However, in the present study, the levels of annexin I and annexin II increased with postnatal development of the islets. These results strongly suggest a possible correlation between the physiological roles of annexin I and annexin II, and certain functions of endocrine glands in pancreatic islets. In fact, fetal or early neonatal islets are capable of biosynthesis and storage of hormones (Asplund, 1973; Heinze et al., 1975). McEvoy (1981) examined the concentrations of both insulin and glucagon in plasma during postnatal development of rats, and reported that their concentrations reached adult levels by approximately day 50. Few authors have investigated the functional relationship between the biosynthesis of hormones and the islet developmental stage. The synchronous increase in the contents of annexin I and annexin II with age strongly suggests that the amount of both annexins is correlated with hormone biosynthesis, secretion or both, in pancreatic islets. Since no cell type-specific localization of either annexin was observed in islets, annexin I and annexin II appear to have certain functions in common in all those cells.

In pancreatic islets, for example, insulin is released from β cells by glucose stimulation. Responding to stimulation with high levels of glucose, β cells showed a rise in concentration of cytosolic Ca²⁺ and phosphorylation of several membrane-associated proteins, followed by exocytosis of insulin secretory granules (Calle et al., 1992). Annexins are involved in Ca²⁺-dependent aggregation of chromaffin granules (Drust and Creutz, 1989) and exocytosis in adrenal chromaffin cells (Ali et al., 1989). Thus, it is possible that annexin I and annexin II have similar functions in pancreatic islets.

The reason why annexin I and annexin II are present specifically in islets but not in exocrine tissues is not obvious. Other annexins might be present in endocrine as well as in exocrine cells, or there may exist other isoforms of annexin I and annexin II.

The relationship between development of pancreatic islets and expression of protein kinases (PKC, EGFR, pp60⁶⁵⁶) is unknown.

The existence of serine/threonine kinases such as PKC (Calle et al., 1992; Load and Ashcroft, 1984; Metz, 1988), A-kinase (Thams et al., 1984; Harrison and Ashcroft, 1982) and calcium-calmodulin kinases (CaM-kinases) (Thams et al., 1984; Colca et al., 1983, 1985; Watkins, 1991; Landt et al., 1992) in pancreatic islets has been reported previously, while tyrosine kinases such as pp60⁶⁵⁶ and EGFR in islets have not been observed. Recently, reports of tyrosine kinase activity of several oncogene products and growth factor receptors have focused attention on tyrosine phosphorylation as an important regulatory mechanism of cell growth and differentiation. Two tyrosine kinases, pp60⁶⁵⁶ and EGFR, therefore, are assumed to contribute to the regulatory functions in islet cells.

In the present study we also observed by immunohistochemistry that expression of these protein kinases was enhanced during postnatal development in pancreatic islets. These data suggest a possible mechanism for the regulation of annexin I and annexin II through phosphorylation by these kinases.

PKC and A-kinase have been reported to be involved in exocytosis of insulin-containing granules (Load and Ashcroft, 1984; Metz, 1988). In addition, annexins have been shown to possess the fusogenic functions of exocytotic as well as endocytic vesicle membranes and this activity of annexins is regulated by phosphorylation (Drust and Creutz, 1989; Ali et al., 1989; Nakata et al., 1990; Oshry et al., 1991; Wang and Creutz, 1992; Emans et al., 1993; Futter et al., 1993). Thus, annexin I and annexin II are likely to play a role in exocytosis and endocytosis in the endocrine cells of pancreatic islets.

The existence of tyrosine kinases in pancreatic islets was also demonstrated. Although PKC and A-kinase have already been suggested to play a role in hormone secretion in islets, the involvement of tyrosine kinases in exocytosis of hormonal granules has not been reported. The physiological substrates in pancreatic islets for tyrosine kinases remain to be elucidated.

In conclusion, annexin I and annexin II are present specifically in endocrine cells of rat pancreas but not in exocrine cells, and the content of annexin I and annexin II increased during the postnatal development of rat pancreatic islets. Annexin I and annexin II, in addition to the protein kinases (PKC, EGFR and pp60⁶⁵⁶), which can phosphorylate them, colocalize and show similar developmental enhancement of expression in pancreatic islets. These results strongly suggest that annexin I and annexin II are involved in the functional maturation of hormone biosynthesis and secretion in pancreatic islet cells.

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REFERENCES


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