Immunohistochemical Localization of Calbindin-D28k in the Pancreas of Normal and K_{ATP} Channel-deficient Mice: Its Possible Role in the Prevention of Cell Damage to Islet Cells

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ABSTRACT

Calbindin-D28k is a predominant calcium-binding protein contained in the pancreatic islets. It can buffer Ca²⁺ rises following stimulation and thereby protect cells against calcium toxicity. Our previous study of ATP-sensitive K⁺ channel (K_{ATP} channel)-knockout mice showed that β cells decreased in both their number and immunoreactivity for insulin, due to the sustained elevation of intracellular Ca²⁺ concentration, whereas α cells also expressing K_{ATP} channels generally increased in number. The present immunohistochemical study aims to explain the difference in responses of α and β cells to the loss of K_{ATP} channels. The immunoreactivity for calbindin-D28k was localized in α cells in normal mice, but not present in β cells. This finding held true in islet cells of the K_{ATP} channel-knockout mice. An immunohistochemical survey using six rodents including the mouse showed that calbindin-D28k was preferentially localized in α cells in the rat, guinea pig, and mouse, while in the hamster both α and β cells were rich in calbindin-D28k. None of the α and β cells in the squirrel or gerbil pancreas were immunoreactive for calbindin-D28k. This finding may explain how α cells but not β cells in the knockout mice could escape from the calcium toxicity, and shows that the cellular localization of calbindin-D28k in the islets differs even among rodents.

Cytoplasmic proteins which possess high affinity calcium binding sites in their molecules are grouped as calcium-binding proteins (CaBPs), which include calbindin, calmodulin, calretinin, parvalbumin, S 100 protein, troponin C (12, 32, 37) and calcyclin (29). Calbindin was originally identified as a vitamin D-dependent CaBP from the chick duodenum (45). There are two subtypes of calbindins with different molecular weights,

calbindin-D9k and calbindin-D28k. Calbindin-D28k is present in neurons, paraneurons and other cells: neurons of the central and peripheral nervous systems, sensory cells, amine/peptide-producing endocrine cells and urinary tubules of the kidney (4, 13, 15, 16, 31, 33, 38, 44), whereas calbindin-D9k is exclusively present in the mammalian intestinal epithelium (7, 43). The existence of calbindin-D28k in the endocrine pancreas has been studied immunohistochemically in various animals (2, 17, 26, 30, 38). However, the cellular localization of calbindin-D28k in the pancreatic islets remains controversial and appears to be species-dependent (4). Several studies have emphasized its predominant localization in β cells (17, 26, 38), but others have described a more

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intense expression of this CaBP in non- β cells (2, 30).

On the basis of the calcium-binding properties, following functions of calbindins have been proposed; a) transcellular calcium transport in the intestine and kidney (3, 11, 46), b) regulation of secretions from pancreatic islet cells (20, 34, 39) and hypothalamic neuroendocrine cells (21), and c) action as a mobile calcium buffer restricting evoked calcium signals in neurons and sensory cells (5, 36). The last function includes the protection of neurons against calcium toxicity (23).

It is well known that calcium plays an important role in the stimulus-secretion coupling of β cells. The uptake and metabolism of glucose by β cells result in the closure of ATP-sensitive K^+ channels (K_{ATP} channel), causing membrane depolarization and the opening of voltage-dependent Ca^{2+} channels which induces transient rises in the intracellular free calcium concentration ($[Ca^{2+}]_i$). It has been suggested that CaBPs such as calmodulin (35, 40, 47), calcyclin (29) and calbindin-D28k (20) are important mediators of the stimulus-secretion coupling in the β cell. Among these CaBPs in the pancreatic islets, calbindin-D28k may play a major role in the pancreatic islets, referring to its concentrated localization in the islet cells and remarkable stimulation of insulin synthesis and release (4, 34).

Our research group generated K_{ATP} channel-knockout mice to analyse functional significance of K_{ATP} channels (25). The K_{ATP} channel is shared by insulin (β) and glucagon (α) cells (1, 9); histochemical findings suggested that somatostatin (δ) and pancreatic polypeptide (PP)-containing cells also express the K_{ATP} channels (41). In the K_{ATP} channel-knockout mice, β cells decreased in number and in immunoreactivity for insulin, possibly due to the unregulated elevation of $[Ca^{2+}]_i$, but α cells generally increased in number (25, 48). This raises the question as to how α and β cells show a different behavior against the loss of the K_{ATP} channel. We anticipated that α cells would be able to escape cell damage through the buffering action of CaBPs. In this study, we show that calbindin-D28k, a predominant CaBP in the islet cells, is localized in α cells, but not in β cells in the pancreas of normal and K_{ATP} channel-knockout mice. Moreover, we compare the distribution of calbindin-D28k in six rodent species

including the mouse, as a species difference has been suggested.

MATERIALS AND METHODS

The generation of K_{ATP} channel-knockout mice has been mentioned in detail by Miki *et al.* (25). The splenic lobes of the pancreas were obtained from seven knockout mice, from 40 to 90 weeks of age, and from control mice of the same age group. The splenic lobes of pancreas were also obtained from normal male rats (Wistar, 200–300 g in body weight), guinea pigs (Hartley, about 350 g), golden hamsters (about 120 g), squirrels (*Eutamias asiaticus lineatus*) and gerbils (*Meriones tristis*). All tissues were fixed in 10% buffered formalin for about 24 h, dehydrated through a graded series of ethanol, and embedded in paraffin according to conventional methods. Serial sections, about 2–3 μ m in thick, were prepared and mounted on gelatin-coated glass slides.

Deparaffinized sections were processed according to the indirect immunoperoxidase method for insulin and the avidin-biotin peroxidase complex (ABC) method for glucagon and calbindin-D28k. After pretreatment with a normal goat serum, the sections were incubated with a guinea pig anti-human insulin serum (Yanaihara Institute, Shizuoka, Japan) diluted to 1:4,000, followed by incubation with a peroxidase-labeled porcine anti-guinea pig IgG (1:200, Dako, Glostrup, Denmark). Adjacent sections were incubated with either rabbit anti-rat brain-derived calbindin-D28k serum (kindly donated by Prof. M. Watanabe, Hokkaido University) (27), diluted to 1:2,000, or rabbit anti-human/rat glucagon serum (Yanaihara Institute), diluted to 1:8,000. They were then incubated with biotin-labeled goat anti-rabbit IgG (Histofine kit, Nichirei, Tokyo) and avidin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, USA). The antigen-antibody reactions were visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3, 3'-diaminobenzidine (DAB) and 0.002% H_2O_2 .

Double immunostaining

The deparaffinized sections were incubated with the rabbit anti-calbindin-D28k serum (1:400), followed by Cy3-labeled donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove,

USA), and then incubated with guinea pig anti-porcine glucagon serum (1 : 400, Progen Immuno-Diagnostica, Heidelberg, Germany), followed by FITC-labeled donkey anti-guinea pig IgG (1 : 200, Jackson ImmunoResearch). The immunostained sections were observed under a confocal laser scanning microscope (Fluoview, Olympus).

RESULTS

Calbindin-D28k in normal and K_{ATP} channel-knockout mice

Immunohistochemical staining of the normal mouse pancreas revealed the presence of calbindin-D28k in islets, but not in exocrine cells or duct cells. The immunoreactivity was restricted to the periphery of islets, forming typical ring structures with an immunonegative central area (Fig. 1B). Their distribution coincided with that of glucagon-containing α cells as compared with serial sections (Fig. 1B, C). Insulin-containing β cells showed no significant immunoreactivity for calbindin-D28k (Fig. 1A, B). Definite colocalization of calbindin-D28k and glucagon was shown by the double immunostaining of the mouse pancreas using anti-calbindin-D28k and glucagon sera (Fig. 2A-C).

In aged Kir6.2-knockout mice, α cells increased in number and were diffusely distributed throughout the islets in a manner differing considerably from the islet architecture seen in the normal mice (Fig. 2D), while β cells decreased in both number and intensity of insulin immunoreactivity (for details, see ref. 48). Calbindin-immunoreactive cells in these knockout mice increased in number, showing the same distributional pattern as α cells. Here again, the colocalization of calbindin-D28k and glucagon was confirmed by the double immunostaining of single sections (Fig. 2D-F).

Calbindin-D28k distribution in the normal pancreas of other rodent species

The immunoreactivity for calbindin-D28k was found in pancreatic islets of all species examined, except for the squirrel. In the rat, intense reactivity was localized to mantle cells of islets, while some endocrine cells occupying the central region of islets, namely β cells, were weakly positive and variable in intensity (Fig. 1E). In the guinea pig, calbindin-positive cells formed small cell groups throughout the islets, showing a more

predominant distribution at the periphery of the islets (Fig. 1G). Observations of serial sections from the rat and guinea pig pancreas revealed that intensely immunoreactive cells for calbindin-D28k corresponded to α cells (Fig. 1D-H).

In contrast, immunohistochemistry for calbindin-D28k in the hamster pancreas stained almost all islet cells containing at least α and β cells (Fig. 3B). The calbindin immunoreactivity appeared to be slightly more intense in the central region than in the peripheral region. Serial sections indicated that β cells were more intensely immunoreactive for calbindin-D28k than α cells (Fig. 3A-C).

In the gerbil, islets contained a few calbindin-positive cells present mainly at the peripheral region (Fig. 3E). These immunoreactive cells did not coincide to α and β cells (Fig. 3D-F), but possibly were identical to PP or δ cells, judging from comparison on serial sections. The islets of the gerbil were also characterized by a dense distribution of calbindin-positive fine nerve fibers which surrounded sinusoidal capillaries (Fig. 3E). No islet cells in the squirrel pancreas were immunoreactive to the anti-calbindin-D28k serum (Fig. 3H). However, perivascular nerve fibers and ganglion cells in the pancreas were intensely immunoreactive to the calbindin antiserum, suggesting a sufficient cross-reactivity of the antiserum to calbindin-D28k of the squirrel.

DISCUSSION

Early immunohistochemical studies for the vitamin D-dependent CaBP, calbindin-D28k, in pancreatic islets have emphasized its existence in β cells. Morrissey *et al.* (26) described how a majority of islet cells, possibly containing β cells at least, stained positively with the antiserum against human kidney calbindin-D28k in the dog, cat, rat, mouse, and chick. Immunostaining by use of the antiserum against chick intestinal calbindin-D28k demonstrated its selective localization in β cells of the chick pancreas (38), although a later immunohistochemical study on the chick pancreas reported the existence of calbindin-D28k in α cells as well as β cells (30). The localization of calbindin-D28k in β cells may be supported by physiological data showing that glucose-induced insulin secretion is impaired in vitamin D-deficient rats, and that 1, 25-(OH)₂D₃ (the active metabolite of vitamin D)

Fig. 1 Immunostaining of three serial sections from the mouse (A-C), rat (D-F) and guinea pig pancreas (G, H) by the use of antisera against insulin (A, D), calbindin-D28k (B, E, G), and glucagon (C, F, H).

In the mouse, immunoreactivity for calbindin-D28k (B) is found in peripheral islet cells that coincide with glucagon cells (C) but not with insulin cells (A). Intensely calbindin-positive cells in the rat pancreatic islet are located in the outer rim of the islet (E), and correspond to glucagon cells (F). The central β cell region in the rat contains some cells with weak immunoreactivity for calbindin-D28k (D, E). In the guinea pig, calbindin-immunoreactive cells (G) are largely identical to glucagon cells (H). Immunostaining of insulin in the guinea pig is not available, due to use of the anti-insulin serum raised in this species. Bar = 50 μ m

Fig. 2 Double immunostaining of single sections from the pancreas of normal (A–C) and KATP channel-knockout mice (D–F; 55 weeks old) by the use of antisera against glucagon (A, D) and calbindin-D28k (B, E). Fig. C and F show merged images.

In the normal mouse, glucagon cells are located at the periphery of the islet, and coincide with calbindin-containing cells. Glucagon cells in the KATP channel-deficient mouse increase in number and are distributed diffusely throughout the islet. The increased glucagon cells also contain calbindin immunoreactivity. Bar = 50 μ m

treatment improves the insulin secretion (18, 19, 28, 42). In contrast, Pochet *et al.* (30) and Bourlon *et al.* (2) reported a preferential localization of calbindin-D28k in α cells of the rat pancreas, using antisera against chick intestinal and rat renal calbindin-D28k, respectively. This finding is in agreement with the semi-quantitative analysis showing that the calbindin-D28k content is higher in non- β cells than β cells isolated from the rat pancreas (22, 30). Furthermore, Bourlon *et al.* (2) noted a marked influence of vitamin D

deficiency and 1, 25-(OH) $_2$ D $_3$ treatment on calbindin immunoreactivity in α cells, while the immunoreactivity in β cells remained unchanged after these treatments. On the other hand, Buffa *et al.* (4), who stained the pancreas of several mammalian species using antiserum raised against the chick duodenal calbindin-D28k, noted that the immunoreactivities were detected in α , β , and PP cells of the dog, cat and rat; α and PP cells (but not β cells) in the guinea pig; and only PP cells in humans. Thus, the cellular

Fig. 3 Immunostaining of three serial sections from the hamster (A-C), gerbil (D-F), and squirrel pancreas (G-I) by the use of antisera against insulin (A, D, G), calbindin-D28k (B, E, H) and glucagon (C, F, I). In the hamster, all islet cells are immunoreactive for calbindin-D28k (B), while in the islet of the gerbil there are a few calbindin-immunoreactive cells (E) which do not coincide with insulin and glucagon cells. Arrowheads in Fig. 3E show immunoreactive nerve fibers. An islet in the squirrel contains no calbindin-immunoreactive cells (H). Bar = 50 μ m

localization of calbindin-D28k in pancreatic islets appears to be contradictory even among rodents.

The present study using antiserum raised against calbindin-D28k from the rat brain demonstrated the generally consistent localization of calbindin-D28k in α cells in the pancreas of rodents, except for the squirrel and gerbil. Rodents can be classified into three types according to the stainability of β cells with the calbindin-D28k antiserum: no reactivity (mouse, guinea pig, squirrel, gerbil), weak reactivity (rat), or intense reactivity (hamster). The present immunohistochemical data suggest that, in the rat, β cells contain a low amount of calbindin-D28k, in agreement with the findings by Pochet *et al.* (30) and Bourlon *et al.* (2), but the mouse β cells almost lack this molecule. Noteworthy, physiological studies on the relation between vitamin D and insulin release were carried out using only the rat pancreas (2, 6, 8, 14, 18, 19, 28, 42). In studies seeking to determine the role of calbindin-D28k in the β cells, calbindin-D28k was overexpressed in the rat β cell line by transfection in the expression vector (34, 39). Thus, the localization and function of calbindin-D28k in β cells may have been overestimated, owing to earlier immunohistochemical findings in the chicken and rat.

Calbindin-D28k is capable of buffering calcium in response to a rapid increase in $[Ca^{2+}]_i$ induced by calcium ionophore treatment, as shown in the rat β cell line engineered to overexpress calbindin-D28k (34). Interestingly, calbindin, even though overexpressed, did not affect basal $[Ca^{2+}]_i$ in these cells (34). This fact has been confirmed in cultured hippocampal and sensory neurons expressing calbindin-D28k under normal conditions (5, 23). One of main roles of calbindin-D28k in the excitatory cells may be the inhibition of excessive and sustained elevations of $[Ca^{2+}]_i$; the CaBP can return $[Ca^{2+}]_i$ quickly to the resting level (5, 34, 36) and thereby protect cells from calcium excitotoxicity. In calbindin-D28k-expressing neurons and lymphocytes, there is a clear correlation between the presence of calbindin-D28k and resistance to cell death following exposure to the Ca^{2+} ionophore and glutamate in neurons (23), or exposure to the glucocorticoid, cAMP and Ca^{2+} ionophore in lymphocytes (10).

Physiological and pharmacological studies have demonstrated the existence of KATP channels

in α and β cells (1, 9), although morphological studies have reported its localization in all islet cell types (41). It is believed that a loss of the KATP channel, equal to the closure of the channel, induces chronic membrane depolarization, resulting in unusual and sustained elevations of $[Ca^{2+}]_i$. The elevated Ca^{2+} levels induce a secretion of pancreatic hormones, although the involvement of the KATP channel in the secretion of pancreatic hormones other than insulin remains unclear. Dominant-negative KATP channel-transgenic mice, in which only β cells lost their KATP channel function, developed hyperinsulinemia in neonates and then induced the apoptosis of β cells, resulting in a conspicuous decrease of β cells in number from 4 weeks after birth (24). The KATP channel-knockout mice, in which KATP channels of α and β cells at least were defected, displayed different changes between α and β cells: β cells decreased in number, while α cells instead increased in number (25, 48). These differing effects from the loss of KATP channels may be explained by the preferential occurrence of calbindin-D28k in α cells; β cells lacking the calcium-reducing system might fail to resist the high Ca^{2+} concentration, resulting in the numerical decrease of β cells.

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