Morphological Changes in Pancreatic Islets of KATP Channel-Deficient Mice: The Involvement of KATP Channels in the Survival of Insulin Cells and the Maintenance of Islet Architecture

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Summary. The ATP-sensitive potassium channel (KATP channel) is an essential ion channel involved in glucose-induced insulin secretion. The KATP channel is composed of an inwardly rectifying potassium channel, Kir6.2, and the sulfonylurea receptor (SUR 1); in the pancreas it is reported to be shared by all endocrine cell types. A previous study by our research group showed that Kir 6.2-knockout mice lacked KATP channel activities and failed to secrete insulin in response to glucose, but displayed normal blood glucose levels and only mild impairment in glucose tolerance at younger ages. In some aged knockout mice, however, obesity and hyperglycemia were recognizable. The present study aimed to reveal morphological changes in pancreatic islets of Kir 6.2-knockout mice throughout life. At birth, there were no significant differences in the islet cell arrangement between the knockout mice and controls. At 14 postnatal weeks glucagon cells appeared in the central parts of islets, and this image became more pronounced with aging. In animals older than 50 weeks insulin cells decreased in numbers and intensity of insulin immunoreactivity; most islets in 70- and 80-week-old mice were predominantly composed of glucagon cells and peptide YY (PYY) (PYY)-containing cells. Staining of serial sections and double staining of single sections from these old mice demonstrated the frequent coexpression of glucagon and PYY, which is a phenotype for the earliest progenitor cells of pancreatic endocrine cells. These findings suggest that the KATP channel is important for insulin cell survival and also regulates the differentiation of islet cells.

Extensive study of the differentiation of pancreatic endocrine cells has confirmed that the endocrine cells are derived from the epithelium of the primordial gut and later from growing pancreatic ducts (Pictet et al. 1972). The cell lineage among the different populations of islet cells has long been debated, however. Many immunohistochemical studies have reported glucagon as the first hormone detectable in the developing pancreas (Pictet and Rutter 1972; Rall et al., 1973; Fujii, 1979; Teitelman and Lee, 1987; Hashimoto et al., 1988). On the basis of its colocalization with other hormones, Alpert et al. (1988) proposed that α cells containing glucagon (either alone or together with insulin) may be precursors to all islet endocrine cells in the ontogeny of the mouse pancreas. Another endocrine phenotype expressed early during islet morphogenesis is the PP family, including PP (pancreatic polypeptide), NPY (neuropeptide Y), and PYY (peptide YY) (Herrera et al., 1991; Teitelman et al., 1993; Upchurch et al., 1994). After considerable debate over the issue of which peptide of this family predominates in the developing pancreas, it is now generally accepted that the earliest endocrine cells are multihormonal and coexpress glucagon and PYY (Larsson, 1998).

The ATP-sensitive potassium channel (KATP channel) is an essential regulator of both glucose- and sulfonylurea-induced insulin secretions. Existence of the KATP channel in insulin-containing β cells was physiologically and pharmacologically demonstrated

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by COOK and HALES (1984), and its expression in α cells was later reported by BOKVIST et al. (1999), while SUZUKI et al. (1997, 1999) immunohistochemically demonstrated its existence in all islet cell types of the rat pancreas. Molecular characterization of the KATP channel indicates that it is a complex composed of two subunits: the inwardly rectifying potassium channel (Kir6.2) and sulfonylurea receptor (SUR) 1 (INAGAKI et al., 1995). The critical roles of the KATP channel in different cells encouraged us to produce genetically engineered mice that lacked these molecules. In a previous study by our research group (MIKI et al., 1997), transgenic mice were generated in order to disrupt the KATP channel specifically in β cells. Mice expressing a dominant-negative form of Kir6.2, in which glycine at position 132 was substituted for by serine (Kir6.2 G132S Tg mice), showed a significant decrease in the KATP channel currents associated with elevated basal calcium concentrations in β cells. Such a condition signifies a continuous depolarization of β cell membrane. Neonatal transgenic mice displayed hypoglycemia, due to continuous insulin secretion, while the animals became severely hyperglycemic after 4 postnatal weeks. Histological analysis of the hyperglycemic transgenic mice revealed a conspicuous decrease in the number of β cells, the appearance of α cells in the central regions of islets, and a reduction in islet size (MIKI et al., 1997). Thus, the lack of KATP channel functions in β cells causes the excitotoxicity of β cells by a sustained Ca²⁺ concentration, resulting in stimulated cell death (apoptosis), as observed in cerebellar neurons (HESK, 1996).

In a subsequent study by our research group, mice lacking KATP channels, which are expressed in α, possibly somatostatin (δ) and PP cells as well as β cells, were generated by genetic disruption of Kir6.2 and then analyzed (MIKI et al., 1998). Despite severe defects in glucose- and tolbutamide-induced insulin secretion, glucose tolerance was impaired only slightly in the younger knockout mice. Our preliminary histological examination (MIKI et al., 1998) revealed an abnormal architecture of islets, apparently similar to that of the Kir6.2-transgenic mice: many α cells appeared in the central regions of the islets. Unlike the transgenic mice, however, the number of β cells did not significantly change in the younger knockout mice examined. Interestingly, the aged knockout mice displayed mild obesity and hyperglycemia. To understand the precise nature of the change in pancreatic islets in the Kir6.2-knockout mice, histological analysis was extended to the entire life span of the knockout mice in the present study. Here we show that, in aged Kir6.2-knockout mice, β cells exhibit decreased numbers and immunoreactivity for insulin, while α cells coexpressing PYY increase in number, suggesting that the KATP channel is indispensable for the normal architecture of islets as well as insulin secretion.

MATERIALS AND METHODS

Methods for targeting the Kir6.2 gene and production of Kir6.2-deficient mice were described in our previous paper (MIKI et al., 1998). In the present study, pancreatic tissues were collected from homozygous Kir6.2−/− mice at day 0, weeks 2, 4, 8, 12, 16, 20, 40, 50, 60, 70 and 80, and from age-matched control mice (Kir6.2+/−). The mice were sacrificed by decapitation, and the pancreas removed and immersed in 10% buffered formalin for 24 h. Due to differences in the architecture of pancreatic islets between duodenal and splenic parts (ORCI and PERRELET, 1981; ORCI, 1982), the tissue samples were always obtained from the splenic portion of the pancreas. The fixed tissues were processed according to conventional procedures and finally embedded in paraffin. Serial sections, about 2 μm in thickness, were prepared, deparaffinized and processed for immunohistochemistry by using polyclonal antisera against insulin, glucagon, somatostatin, PP, and PYY. In all steps for washing and dilution of antisera, we used 0.01 M phosphate-buffered saline (PBS), pH 7.2.

Immunoreactivity for insulin was detected by the indirect immunoperoxidase method. After pretreatment with normal goat serum, the sections were incubated with guinea pig anti-human insulin serum (1:4,000; Y370, Yanaihara Institute, Shizuoka) overnight at room temperature. They were then incubated with peroxidase-labeled porcine anti-guinea pig IgG (DAKO Japan, Kyoto) for 45 min, and the peroxidase activities were visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine (DAB) and 0.002% H₂O₂.

Immunostaining for glucagon, somatostatin, PP

**Fig. 1.** Immunostaining of insulin and glucagon cells using serial sections from the pancreas of a control mouse at 80 weeks (a, b) and Kir6.2-knockout mice at 4 (c, d), 16 (e, f), 40 (g, h), 70 (i, j) and 80 weeks (k, l) of age. Glucagon cells are diffusely distributed throughout the islet in the knockout mice, differing from normal islet architecture, and conspicuously increase in number in aged mice (j, l). In contrast, the staining ability of β cells by the insulin antiserum decreases in intensity in the older animals (k). Scale bar = 100 μm.
Fig. 1. Legend on the opposite page.
and PYY was conducted by the avidin-biotin complex (ABC) method. After pretreatment with normal goat serum, sections were incubated with rabbit antisera against human/rat glucagon (1 : 10,000; YP040, Yanaihara Institute), human/rat somatostatin (1 : 4,000; YP020, Yanaihara Institute), human PP (18–36) (1 : 8,000; Y080, Yanaihara Institute) or human PYY (1 : 20,000; Y072, Yanaihara Institute) overnight at room temperature. The antigen-antibody reactions were detected by incubation with biotin-labeled goat anti-rabbit IgG (Histofine; Nichirei, Tokyo), followed by streptavidin-peroxidase complex (Histofine; Nichirei). Peroxidase activities were detected by incubation in the Tris-HCl buffer containing DAB and H₂O₂.

Specificity of the immunoreaction was checked by preincubation of the antisera with the corresponding antigen (10μg/diluted antiserum). Furthermore, the anti-PP serum was preincubated with PYY and NPY, and the anti-PYY serum with PP (18–36) and NPY. All peptides were purchased from the Yanaihara Institute, Shizuoka, Japan.

**Double staining**

After preincubation with normal goat serum, deparaffinized sections were incubated with anti-PYY serum (diluted to 1 : 4,000), followed by Cy3-labeled donkey anti-rabbit IgG serum (1 : 200; Jackson Immunoresearch Laboratories, Pennsylvania, USA). After rinsing in PBS, the sections were incubated with guinea pig anti-porcine glucagon serum (1 : 400; Progen Biotechnik, Heidelberg, Germany), followed with fluorescein isothiocyanate (FITC)-labeled donkey anti-guinea pig IgG (1 : 200; Jackson Immunoresearch Laboratories). The sections were mounted with cover slips using glycerin-PBS and observed under a confocal laser scanning microscope (Fluoview, Olympus, Tokyo).

**Statistical evaluation**

For evaluation of α and β cell numbers, serial sections from one mouse of each age group and a control mouse (80 weeks) were alternatively immunostained for glucagon and insulin. Alpha and β cells possessing nuclei were counted under a light microscope for 5 islets randomly selected in each animal. The ratios of α and β cell populations among all islet cells were calculated and statistically compared by using analysis of variance and the Duncan multiple range test.

**RESULTS**

Pancreatic islets become gradually larger with age under normal conditions, reaching a plateau in size at sexual maturation (White et al., 1999), but in the Kir6.2-knockout mice they increased in size even after passing the maturation stage. Especially in aged knockout mice older than 40 weeks, the islets were much larger than those in controls, and were frequently lobulated, displaying an irregular outline of islet cells (Fig. 1k, l). This change was in contrast to Kir6.2-transgenic mice, in which islet size conspicuously decreased at the onset of diabetes at around 4 postnatal weeks and thereafter (MiKi et al., 1997). The islet architecture of Kir6.2-knockout mice was characterized by decreases in both the number and the intensity of insulin immunoreactivity of β cells, and by an increase in the number of α cells. Since a comparison of α and β cells helps afford an understanding of essential morphological changes in islets, time-course changes of α/β cells in the splenic portion of the pancreas will be described first.

**Insulin (β) cells and glucagon (α) cells**

Immunostaining of newborn (day 0) knockout mice showed distributions of insulin and glucagon cells similar to those in controls. Glucagon cells formed one or two cell lines surrounding the central insulin cell mass. In 2- and 4-week-old mice, some glucagon cells appeared in the central regions of pancreatic
Fig. 3. Immunostaining of PYY, glucagon and PP using three serial sections from a control mouse at 16 weeks and KATP channel-deficient mice at 16, 70, and 80 weeks. In normal conditions, about half of the glucagon cells contain PYY (a, b). In the knockout mice, the PYY cells increase in number in parallel with glucagon cells. A few PP cells, which are immunoreactive to anti-human PP serum preabsorbed with PYY, are also diffusely distributed in the islet (f, i, l). Scale bar = 100 μm

islets, and thus showed a more complex distribution of glucagon cells (Fig. 1d). The number of glucagon cells appeared to increase significantly in these younger stages, as compared with control mice. Evaluation of the knockout mice at various ages from 8 to 80 weeks indicated that the population of glucagon cells increased with age (Fig. 1f, h, j, l). A large number of glucagon cells occurred in the central parts of islets (Fig. 1j, l), which are typically occupied only by insulin cells in normal mice (Fig. 1a, b). Statistically, the total number of glucagon cells sharply increased after 60 weeks; glucagon cells in each islet were either equal in number to insulin cells or more predominant than insulin cells (Fig. 2). In the oldest mice examined (80 weeks), the percentage of glucagon cells to all islet cells reached about 65% (versus 16.8±2.8% in controls at the same age).

The morphological changes in insulin cells did not
become significant until 20 weeks (Fig. 1c, e). After 40 weeks, the stainability of insulin cells tended to decrease prominently (Fig. 1 g, i, k); there were only a few intensely insulin-immunoreactive cells in each islet of 80-week-old mice. Statistical evaluation demonstrated a conspicuous decrease in the number of insulin cells after 60 weeks, in parallel with the decrease in the immunoreactivity for insulin (Fig. 2). The reduction in insulin immunoreactivity was more remarkable as compared with the numerical changes of insulin cells.

Glucagon cells expressing PYY

Our preliminary immunostaining of Kir6.2-knockout mice revealed a conspicuous increase in α cells that were immunoreactive to an antiserum against bovine PP, indicating the coexpression of glucagon and PP (SEINO et al., 2000). However, some researchers have pointed out a frequent crossreactivity of anti-PP sera with PYY as mentioned below. When we preincubated the anti-bovine PP serum with PYY, a major population of the PP-immunoreactive cells disappeared, suggesting the crossreactivity of our anti-PP serum to PYY. In the present study, therefore, we used an antiserum against human PP, which had low crossreactivity to PYY and also was preincubated with PYY and NPY, for demonstration of true PP cells.

A small number of somatostatin cells and PP cells were scattered at the periphery of pancreatic islets in newborn mice. At 4 postnatal weeks, these cells appeared not only in the peripheral areas but also in the central regions of islets. From 8 to 80 weeks, somatostatin cells and PP cells were dispersed throughout the islets, but their number did not significantly increase (for PP cells, Fig. 3f, i, l).

PYY immunoreactivity in adult control mice was found in several endocrine cells present at the periphery of islets (Fig. 3a). The PYY-immunoreactive cells were largely identical to glucagon cells (Fig. 3a, b), but less than half of the glucagon cells coexpressed PYY, as reported by UPCHURCH et al. (1994). In the knockout mice, PYY cells showed the same distributional and numerical changes as glucagon cells: they increased in number with age, appearing in the central parts of islets (Fig. 3d, g, j). At 60, 70 and 80 weeks, the population of PYY cells in most pancreatic islets was comparable to or higher than that of glucagon cells (Fig. 3). Observation of serial sections, which were alternatively stained using different antisera against four islet hormones, showed that glucagon cells also expressed immunoreactivity for PYY (Fig. 3). Double immunostaining for glucagon and PYY confirmed that most glucagon cells contained PYY, while a small number of cells were immunoreactive only for either glucagon or PYY (Fig. 4). We did not observe any distinct colocalization of insulin and glucagon or insulin and PYY. The PYY immunoreactivity was not influenced by preincubation of the antiserum with PP and NPY, and it disappeared only when incubated with PYY.
DISCUSSION

The \textit{KATP} channel in pancreatic $\beta$ cells is a key regulator that stimulates insulin secretion in response to glucose: elevation of the ATP concentration caused by glucose metabolism closes the \textit{KATP} channel, resulting in membrane depolarization, which is linked to voltage-gated Ca\textsuperscript{2+} channels (LARSSON \textit{et al.}, 1996). This is supported by our analysis of \textit{KATP} channel-deficient mice showing the unresponsiveness of $\beta$ cells to glucose and tolbutamide, a potent insulin secretagogue (MIKI \textit{et al.}, 1998). Although it has been suggested that \textit{KATP} channels are shared by all islet cell types, their roles in other cell types remain unknown. In the present study, the morphology of pancreatic islets in Kir6.2-knockout newborn mice showed no significant difference in comparison with that in controls, indicating that all endocrine cell types could differentiate without any harm until the early postnatal period. However, the reduced immunoreactivity of $\beta$ cells to insulin antibodies and the decrease in number of $\beta$ cells became prominent from 40 weeks, and especially so at weeks 60–80. The attenuation of $\beta$ cells in the aged knockout mice may be caused by cell damage and accelerated cell death (apoptosis), since a histochemical method was able to detect some apoptotic islet cells in the knockout mice (SEINO \textit{et al.}, 2000). Thus, we can highlight a consistent relationship between the loss of the \textit{KATP} channel and the decrease in the $\beta$ cell population in both transgenic and knockout mice. However, it is noteworthy that the decrease in number of $\beta$ cells was less severe and appeared in later stages in contrast to the transgenic mice, in which a conspicuous decrease of the $\beta$ cell population was recognized as early as four weeks of age (MIKI \textit{et al.}, 1997). This finding correlates to the difference in the change of islet size: islets in the transgenic mice decreased in size, while those in knockout mice were rather enlarged, indicating the stimulated growth of pancreatic islets. This islet enlargement may be attributable to the numerical increase of glucagon and/or PYY-containing cells beyond the decrease in $\beta$ cell number, suggesting that \textit{KATP} channels affect not only the $\beta$ cell population but also other islet cell types in different manners.

The opposite effects—caused by a lack of the \textit{KATP} channel—between $\alpha$ cells and $\beta$ cells raise a question. Loss of \textit{KATP} channels induces an unusual elevation of the intracellular Ca\textsuperscript{2+} level, as shown in Kir6.2-transgenic and knockout mice (MIKI \textit{et al.}, 1997, 1988). Calcium, when its cellular level is high, may act as a toxin by activating Ca\textsuperscript{2+}-dependent proteases and endonucleases (ORRENIUS \textit{et al.}, 1989; GAIDO and CIDLOWSKI, 1991). It is reported that the existence of calcium-binding proteins (CaBP) can prevent cell damage or apoptosis via the regulation of cytosolic Ca\textsuperscript{2+} levels in the cell. Cultured hippocampal neurons containing a CaBP, calbindin-D28k, are resistant to excitotoxicity induced by either glutamate or a calcium ionophore, possibly due to its ability to reduce free intracellular calcium levels (MATTSON \textit{et al.}, 1991). Moreover, overexpression of calbindin-D28k in a lymphocyte cell line dramatically attenuates the apoptotic effects of dexamethasone, forskolin and a calcium ionophore (DOWD \textit{et al.}, 1992). It has been reported that in the rat pancreas $\alpha$ cells are intensely immunoreactive for calbindin-D28k, while $\beta$ cells are weakly positive (POCHET \textit{et al.}, 1987; BOURLON \textit{et al.}, 1996). Our immunostaining using the mouse pancreas also demonstrated the preferential occurrence of calbindin-D28k in $\alpha$ cells; $\beta$ cells showed a negative or weakly positive reaction (WINARTO \textit{et al.}, 2000). Therefore, $\beta$ cells—not $\alpha$ cells—lacking the regulatory mechanism of intracellular calcium levels may be damaged in \textit{KATP} channel-deficient mice.

The cell arrangement of pancreatic islets in the Kir6.2-knockout mouse is characterized by the unique distribution of $\alpha$ cells, which were dispersed throughout the islets. In the normal pancreas of rodents, $\alpha$ cells, $\delta$ cells and PP cells are situated at the periphery of islets, forming a thin external mantle one or two cells in thickness (ORCI and PERRELET, 1981; ORCI, 1982). In the adult Kir6.2-knockout mice, $\alpha$ cells increased in number and appeared randomly throughout the islets, and $\delta$ and PP cells were also scattered in the islets. This condition markedly differs from the normal islet architecture, suggesting disordered differentiation or kinetics of islet cells with the loss of the \textit{KATP} channel.

Another characteristic of Kir6.2-knockout mice is a conspicuous increase in the number of PYY cells and frequent coexpression of glucagon and PYY, as observed in the aged knockout animals. Colocalization of pancreatic hormones has been repeatedly reported in fetuses in relation to the cell-lineage relationship between islet cell types (ALI-RACHED \textit{et al.}, 1984; HASHIMOTO \textit{et al.}, 1988; HERRERA \textit{et al.}, 1991). HERRERA \textit{et al.} (1991) and Teitelman \textit{et al.} (1993) described the coexpression of PP and NPY in $\alpha$ cells in the developing mouse pancreas. However, the localization of authentic PP in fetal $\alpha$ cells was denied by UPCHURCH \textit{et al.} (1994), and “PP-like” and also “NPY-like” immunoreactivities in early stages of islet cell differentiation might be explained by crossreactivity of the antisera to PYY (ALI-RACHED \textit{et al.}, 1984; UPCHURCH \textit{et al.}, 1994; JACKEROTT \textit{et al.}, 1996; MYREN-AXCRONA \textit{et al.}, 1997) which is richly
contained in \( \alpha \) cells during ontogeny. In contrast, some researchers insist on the involvement of PP and PP genes in the differentiation of pancreatic endocrine cells (Herrera et al., 1991, 1994; Herrera 2000). Although there is still a discrepancy in views concerning the lineage of pancreatic islet cells, it is generally believed that cells coexpressing glucagon and PYY appear during the initial stages of islet organogenesis, possibly representing progenitor cells for all islet cell types (Upchurch et al., 1994; Jackerott et al., 1996; Myrskyn-Acrona et al., 1997; Larsson, 1998). PYY mRNA was first identified on day 15 of gestation in the rat pancreas, earlier than glucagon mRNA; PYY gene expression reaches its highest level on fetal day 18, and declines dramatically after birth (Krasinski et al., 1991). Immunohistochemically, several PYY cells are found at the periphery of islets even in the adult pancreas, especially in rodents. It has been reported that a small subpopulation of \( \alpha \) cells and a larger subpopulation of \( \delta \) and PP cells also contain PYY in adult rats (Jackerott et al., 1996) and that, in adult mice, less than half of \( \alpha \) cells coexpress PYY (Upchurch et al., 1994). There may be some discrepancies between these findings and the immunohistochemical data from Böttcher et al. (1993), who detected immunoreactive PYY in a major subpopulation of \( \alpha \) cells in the adult pancreas of the rat and mouse. Despite limited information, we can conclude that the differentiation of islet cells, possibly the differentiation from progenitor cells, can not be precisely managed in \( \text{K}^+\text{A} \) channel-deficient animals, resulting in the increase in \( \alpha \) cells and the considerably consistent coexpression of glucagon and PYY.

In conclusion, the deficiency of the \( \text{K}^+\text{A} \) channel induces damage to \( \beta \) cells and/or suppresses the insulin production, while it upregulates the proliferation of cells expressing glucagon and PYY. These findings suggest that the \( \text{K}^+\text{A} \) channel is involved in both the survival of \( \beta \) cells and the differentiation of islet progenitor cells. In addition, we also noted that Kir6.2-knockout mice showing mild diabetes with age are a good animal model for diabetes research, and useful for analysis of the islet architecture and differentiation of islet cells.

REFERENCES


