Increased expression of GAD65 and GABA in pancreatic β-cells impairs first-phase insulin secretion

YUGUANG SHI,† JAMIL KANAANI,† VIRGINIE MENARD-ROSE,† YAN HUI MA,‡ PI-YUN CHANG,‡ DOUGLAS HANAHAN,§ ALLAN TOBIN,§ GEROLD GRODSKY,‡ AND STEINUNN BAEKKESKOV†

†Departments of Medicine, Microbiology and Immunology, and Hormone Research Institute, ‡Department of Biochemistry and Biophysics and Metabolic Research Unit, §Department of Biochemistry and Biophysics and Hormone Research Institute, School of Medicine, University of California, San Francisco 94143; and ‡Department of Biology, University of California, Los Angeles, California 90095

Received 5 August 1999; accepted in final form 25 April 2000

Shi, Yuguang, Jamil Kanaani, Virginie Menard-Rose, Yan Hui Ma, Pi-Yun Chang, Douglas Hanahan, Allan Tobin, Gerold Grodsky, and Steinunn Baekkeskov. Increased expression of GAD65 and GABA in pancreatic β-cells impairs first-phase insulin secretion. Am J Physiol Endocrinol Metab 279: E684–E694, 2000.—The functional role of glutamate decarboxylase (GAD) and its product GABA in pancreatic islets has remained elusive. Mouse β-cells express the larger isoform GAD67, whereas human islets express only the smaller isoform GAD65. We have generated two lines of transgenic mice expressing human GAD65 in pancreatic β-cells (RIP7-hGAD65, Lines 1 and 2) to study the effect that GABA generated by this isoform has on islet cell function. The ascending order of hGAD65 expression and/or activity in β-cells was Line 1 heterozygotes < Line 2 heterozygotes < Line 1 homozygotes. Line 1 heterozygotes have normal glucose tolerance, whereas Line 1 homozygotes and Line 2 heterozygotes exhibit impaired glucose tolerance and inhibition of insulin secretion in vivo in response to glucose. In addition, fasting levels of blood glucose are elevated and insulin is decreased in Line 1 homozygotes. Pancreas perfusion experiments suggest that GABA generated by GAD65 may function as a negative regulator of first-phase insulin secretion in response to glucose by affecting a step proximal to or at the KATP channel.

regulation of insulin secretion; neurotransmitter; pancreas perfusion; glucose intolerance

PANCREATIC β-CHEES in islets of Langerhans express the enzyme glutamate decarboxylase (GAD) and GABA at levels comparable to those encountered in the central nervous system (14, 44, 57). However, the physiological function of these molecules in islets remains unclear (reviewed in 37, 53).

GABA produced in β-cells has been suggested to serve as a functional regulator of pancreatic hormone release or as a paracrine signaling molecule for communication between β-cells and the other endocrine cells in islets of Langerhans (53). These hypotheses have been tested by different investigators by use of in vitro assays and exogenous GABA or its mimics. These investigations have yielded conflicting data. In perfused rat and dog pancreas, GABA was shown to inhibit arginine-stimulated insulin release (20, 27). In contrast, perfusion of rat pancreas with GABA or GABA mimics had no detectable effect on insulin secretion (14, 50). Similarly, conflicting results have been reported on the effect of GABA on glucagon and somatostatin secretion (13, 14, 27, 50, 51). There is convincing evidence to suggest that GABA may have an inhibitory effect on glucagon release in vitro (13, 14, 51). It is not clear, however, whether GABA acts as a signal molecule for glucose-induced inhibition of glucagon secretion (14, 51).

There are at least two distinct GABA receptors for inhibitory neurotransmission in the brain, GABA_A and GABA_B receptors (26, 36). GABA_A receptors, which regulate chloride conductance and can cause cell inhibition by hyperpolarization, are expressed on islet α- and δ-cells but not on β-cells (51, 58). It has been proposed that the inhibitory effect of GABA on glucagon secretion may be mediated by GABA_A receptors (13, 51). Recent results suggest that GABA_B receptors are expressed in pancreatic islets (Chang and Baekkeskov, unpublished results). GABA_B receptors are coupled to G proteins and can mediate inhibition of the closure of K⁺ channels or the opening of Ca²⁺ channels (26 and references therein). A GABA_B receptor agonist has been reported to inhibit both insulin secretion and the rise in cytoplasmic Ca²⁺ of β-cells (20).

In mammals, there are two highly homologous non-allelic isoforms of GAD, GAD65 and GAD67 (11). The two forms differ mainly in their association with the co-enzyme pyridoxal 5'-phosphate (PLP) (22, 35) and may differ in some aspects of subcellular targeting (6, 7, 23, 25, 48, 54). GAD65, which is less saturated with...
co-enzyme (22, 35), is found both in the cytosol and associated with the cytosolic face of the membrane of synaptic-like microvesicles in β-cells (6, 7, and unpublished results). GAD65 is the major GAD isoform in rat islets and the only isoform in human islets (28, 34, 45).

In contrast, mouse β-cells seem to express exclusively the cytosolic and highly PLP-saturated GAD67 isoform, even though both isoforms were detected at mRNA levels (12, 28, 45–47). Perhaps not surprisingly, therefore, GAD65−/− mice exhibit normal glucose homeostasis (24). Targeted disruption of the GAD67 gene in the mouse is more likely to result in a phenotype because of loss of GABA in islets of Langerhans. Neonatal lethality of GAD67−/− mice has, however, precluded analysis of glucose homeostasis and islet cell function in mice deficient in this isoform (2, 8). Targeted disruption of the genes encoding the GAD isoforms has therefore not provided information about the role of GABA in the endocrine pancreas. Thus the role of GAD and GABA in islets of Langerhans remains elusive.

The experiments reported so far on the effect of GABA on hormone release in pancreatic islets have been limited to analyzing the effect of exogenous GABA and GABA mimics, which may function differently from the endogenous transmitter produced in β-cells. Thus the physiological conditions that affect GABA synthesis and release may not be approximated by the in vitro assays. Furthermore, the dose of exogenous GABA used in most of the experiments may exceed the level of endogenous GABA (53).

It is not clear how GABA release from β-cells is regulated in vivo. GABA can be measured in conditioned medium from insulinoma cell lines (13, 40, 55), but not from islets (40), consistent with a paracrine rather than an endocrine function. There are conflicting data as to whether the release from insulinoma cell lines is regulated by glucose (13, 40, 55).

To assess the role of GABA in pancreatic islets in an in vivo setting, we have taken advantage of the low or absent expression of GAD65 in mouse islets and targeted expression of this isoform to β-cells in two lines of transgenic mice. We show in this study that expression of transgenic GAD65 and elevated levels of GABA in pancreatic β-cells result in inhibition of glucose-induced insulin release and impaired glucose tolerance and diabetes in transgenic mice. Based on results of studies of the perfused pancreas of transgenic and control mice, we propose that GABA acts as a specific inhibitor of first-phase insulin release and exerts its effect at a step before or at β-cell depolarization.

MATERIALS AND METHODS

Generation of transgenic mice. A 9.7-kb rat insulin promoter (RIP7)-human glutamate decarboxylase (hGAD65) hybrid gene, which consists of 9.5 kb of the rat insulin II promoter (RIP7), the first intron of the insulin gene, a cDNA encoding the human GAD65, and a downstream polyadenylation site (Poly A). RT-PCR analysis was carried out using a DNA template prepared from nontransgenic littermates (mice 1 and 4), transgenic mice from each line (mice 2 and 3 for Line 1; mice 5 and 6 for Line 2), wild-type βTC3 cells (7), and βTC3 cells transiently transfected with the RIP7-hGAD65 construct. The DNA templates were prepared with (+) or without (−) reverse transcriptase. Arrows, amplified DNA bands from β-tubulin and the RIP7-hGAD65 transgene. Lane M shows a DNA band size marker. Similar results were obtained in 2 independent experiments.

Fig. 1. Generation of transgenic mice and RT-PCR analysis of RIP7-hGAD65 expression. A: rat insulin promoter (RIP7)-human glutamate decarboxylase (hGAD65) hybrid gene, which consists of 9.5 kb of the rat insulin II promoter (RIP7), the first intron of the insulin gene, a cDNA encoding the human GAD65, and a downstream polyadenylation site (Poly A). B: expression analysis at mRNA levels of the RIP7-hGAD65 transgene in heterozygous Line 1 mice, heterozygous Line 2 mice, and transiently transfected βTC3 cells. RT-PCR analysis was carried out using a DNA template prepared from nontransgenic littermates (mice 1 and 4), transgenic mice from each line (mice 2 and 3 for Line 1; mice 5 and 6 for Line 2), wild-type βTC3 cells (7), and βTC3 cells transiently transfected with the RIP7-hGAD65 construct. The DNA templates were prepared with (+) or without (−) reverse transcriptase. Arrows, amplified DNA bands from β-tubulin and the RIP7-hGAD65 transgene. Lane M shows a DNA band size marker. Similar results were obtained in 2 independent experiments.

cohesive ends for ligation, the BamHI fragment was partially filled with dGTP, dATP, and dTTP, and the ClaI site with dCTP, by use of reverse transcriptase. The transgenic construct, RIP7-hGAD65, was linearized with SalI and tested for transient expression in a βTC3 cell line (10) in parallel with transgenic constructs generated by use of shorter versions of the rat insulin promoter, RIP1 and RIP5 (9, 30). The RIP7-hGAD65 construct was shown to mediate the highest expression of GAD65. The distribution of the enzyme between cytosolic and membrane fractions was similar to that of islets (6) (results not shown). Two transgenic founder mice, RIP7-hGAD65 mouse 1 and mouse 2, were generated by microinjection of the RIP7-hGAD65 cDNA at a concentration of ~2 ng/ml into B6D2/F2 mouse embryos, according to established procedures (21). RIP7-hGAD65 mouse 1 and mouse 2 were backcrossed into C57Bl/6 mice. Genetic transmission of the transgene was assessed by Southern blot and by polymerase chain reaction (PCR) analyses with DNA isolated from tail biopsies. Physiological studies were carried out on third- and fourth-generation backcrossed heterozygous 10- to 15-wk-old Line 1 and Line 2 mice.
After nine backcrosses into C57Bl/6 mice, Line 1 was bred to homozygosity, and homozygous mice were subjected to physiological studies at 8–15 wk of age. Control mice for all experiments with heterozygote transgenic mice were the transgene-negative littermates of the mice analyzed. Control mice for homozygous Line 1 mice were wild-type C57Bl/6 mice of the same age and sex.

**PCR analysis of transgenic expression.** mRNA expression of the human GAD65 transgene in the transgenic line was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) using a 5′ primer from the 5′ untranslated sequences of RIP7 (AA GTGACGCTACAGTCGG3′) and a 3′ primer from the coding region of the human GAD65 gene (AGCAGGTCGGTGCATGGAG). The tubulin gene was used as a control for the RT-PCR analyses. Total RNAs were isolated from freshly removed mouse pancreases using RNAzol (Tel-Test, Friendswood, TX), followed by digestion with RNase-free DNase I (Promega, Madison, WI) to remove contaminated genomic DNA. PCR amplification conditions were 94°C (2 min), followed by 35 cycles of 94°C (1 min), 60°C (1 min), 72°C (1 min), and finally 72°C for 10 min. The amplified DNA fragments (350 bp) were resolved on a 1.5%-agarose gel.

**Immunohistochemical analysis.** Immunohistochemical analysis of protein expression of the transgene was performed on frozen sections of mouse pancreases, as previously described (28), using a mixture of three human monoclonal antibodies to GAD65, MICA 2, 4, and 6 (49).

For immunohistochemical analysis of GABA expression, 25 μl of fixative containing 4% paraformaldehyde (Polysci ence, PA) and 0.1% glutaraldehyde (Sigma, St. Louis, MO) in PBS, pH 7.3, were perfused directly into the left ventricle of transgenic mice and negative littermates. After perfusion, the pancreases were main stead and finally 72°C for 1 min. The amplified DNA fragments (350 bp) were resolved on a 1.5%-agarose gel.

**Glucose tolerance tests and measurement of insulin and glucagon secretion in vivo.** Glucose tolerance tests were conducted in overnight-fasted animals, as previously described (30). Serum insulin levels were measured by radioimmunooassay with a kit (Binax, South Portland, MA) according to the manufacturer’s instructions. Serum glucagon levels were measured by a double antibody radioimmunoassay with a kit from Diagnostic Products (Los Angeles, CA) according to the manufacturer’s instructions.

**Pancreatic perfusion.** In vitro pancreas perfusion was performed as previously described for the mouse or Chinese hamster pancreas (17, 30, 32). Perfusate consisted of bicarbonate-phosphate-calcium buffer (17) containing 0.2% purified, “stabilized” bovine albumin and 3% T-40 Dextran (Al drich Chemical, Milwaukee, WI). Perfusate was introduced into the celiac artery at 1 ml/min, and effluent was collected at 1- to 2-min intervals from the portal vein after a single passage through the pancreas. To minimize loss of soluble oxygen during the transit time from oxygenator to pancreas at these low flow rates (17), glass was employed for the influx tubing. As described (17), perfuse pressure was continuously monitored. Surgical integrity of the system was also evaluated by comparing perfusate inflow rate to efflux rate from the portal vein into the collection tubes. Experiments in which efflux rate was <80% of inflow were discarded. As previously described (31), the pancreas was perfused in each experiment for 20 min without glucose to test for nonstimulated leakage or washout of insulin, a potential problem in perfusion experiments using mouse pancreas. This period was also used to establish whether the transgenic mouse could deliver insulin in the presence of perfusate.
pancreas released insulin in a constitutive manner (31). Agents, including glucose, IBMX (Aldrich Chemical), and KCl (Sigma), were added as indicated in the individual experiments. Insulin levels in pancreatic perfusate were measured by solid-phase radioimmunoassay (29), with rat insulin as the reference standard and antiporcine insulin antibody (Linco Research, Eureka, MO). Data analyses were carried out using Student’s t-test for paired as well as unpaired values.

RESULTS

Heterozygous RIP7-hGAD65 transgenic mice express elevated levels of GAD65 and GABA, and Line 2 has the highest expression. The RIP7 transgenic vector directs high levels of β-cell specific expression (41). An RIP7-hGAD65 construct was generated (Fig. 1A) and used for microinjection of mouse embryos. Transgenic RIP7-hGAD65 lines were established from two founder mice identified by Southern blot and PCR analyses. RT-PCR (Fig. 1B), immunohistochemical analysis (Fig. 2, a and b), and immunoblot and enzyme assays (Fig. 3, A and B) established expression of the transgene in Lines 1 and 2. Thus immunofluorescence studies using human GAD65 specific monoclonal antibodies that recognize the mouse and human protein equally well (28) revealed significant expression of GAD65 in islets of both RIP7-hGAD65 Lines 1 and 2 heterozygous mice (Fig. 2, a and b). At the heterozygote stage, the expression of GAD65 was highest in the RIP7-hGAD65 Line 2 islets. As reported earlier (28), no expression of endogenous GAD65 was detected in normal mouse pancreas by immunofluorescence analysis (results not shown).

Wild-type mice express GAD67 in pancreatic β-cells, and GABA generated by this isoform can be detected by immunofluorescence staining (52, 56). To analyze whether transgenic expression of GAD65 resulted in increased levels of GABA in pancreatic β-cells, pancre-

Fig. 2. Immunofluorescence analysis of GAD65 and GABA expression in heterozygous RIP7-hGAD65 transgenic and control mice. a and b: Immunofluorescence micrographs of frozen unfixed sections of RIP7-hGAD65 Line 1 (a) and Line 2 (b) pancreas incubated with a pool of human monoclonal antibodies specific for GAD65 (dilution 1:200), followed by a secondary incubation with an FITC-conjugated monoclonal mouse anti-human IgG antibody (dilution 1:300). Micrographs are representative of results obtained with 4 sets of transgenic and control animals. c and d: Immunofluorescence micrographs of fixed sections of RIP7-hGAD65 Line 2 pancreas (d) and a nontransgenic littermate (c) incubated with a GABA-specific rabbit antibody (dilution 1:7,500), followed by incubation with an FITC-conjugated goat anti-rabbit IgG. Panels e and f show staining for insulin on sections serial to sections shown in c and d, respectively. For panels c-f, similar results were obtained with 3 sets of transgenic and control animals.
atic sections from transgenic and nontransgenic littermates of Line 2 were subjected to incubations with serial dilutions of a GABA antiserum to test whether high dilutions could reveal differences in expression between wild-type and transgenic mice. Dilutions of 1:7,500 and 1:10,000 revealed clear differences in GABA levels between wild-type and transgenic islets (Fig. 2, c and d). At those dilutions, only about one-third of wild-type islets stained for GABA, and the staining was weak (Fig. 2, c). In contrast, 65–75% of transgenic islets were strongly positive for GABA at the same dilutions (Fig. 2, d), and the remainder was moderately or weakly positive. Thus expression of RIP7-hGAD65 results in a significant increase in expression of GAD65 as well as GABA in pancreatic β-cells.

**Line 1 homozygotes express the highest levels of GAD65.** After breeding of Line 1 to homozygosity, islets of Langerhans were isolated from Line 1 and Line 2 heterozygotes, from Line 1 homozygous mice, and from wild-type C57Bl/6 mice for analysis of GAD65 expression and activity. Immunoblot analysis was performed...
using the antibody 1701, which recognizes GAD65 and GAD67 equally well. In some experiments, equal amounts of protein were loaded from transgenic and wild-type islets (Fig. 3A), whereas in other experiments up to a fivefold excess of wild-type islets was loaded on the gel (results not shown). The analyses revealed a high expression of GAD65 in Line 1 and Line 2 transgenic mice, which was severalfold higher than expression of endogenous GAD67 in wild-type and transgenic mice (Fig. 3A). Overloading of protein and prolonged exposures of autoradiograms did not reveal expression of endogenous GAD65 in islets of wild-type mice (results not shown). Quantitative immunoblot analysis (a representative blot is shown in Fig. 3A) revealed that expression of GAD65 was highest in Line 1 homozygous mice, followed by Line 2 heterozygotes and then Line 1 heterozygotes. The levels of transgenic GAD65 exceeded endogenous GAD67 levels by ~8.7-fold (Line 1 homozygotes), 7.5-fold (Line 2 heterozygotes), and 4.5-fold (Line 1 heterozygotes). Thus Line 1 homozygotes, Line 2 heterozygotes, and Line 1 heterozygotes represent three distinct levels of GAD65 expression.

We next analyzed GAD enzyme activity in isolated islets from Line 1 heterozygotes, Line 2 heterozygotes, Line 1 homozygotes, and wild-type C57Bl/6 mice to confirm that the increased expression levels in transgenic mice also reflect increased enzyme activity in islets, and therefore a potential for GABA production. GAD65 provides the majority of PLP-inducible apoenzyme activity in brain but is also present as a holoenzyme (35). Enzyme analysis in the presence and absence of exogenous PLP revealed no PLP-inducible enzyme activity in wild-type C57Bl/6 mice, consistent with the absence of GAD65 in normal mouse islets. Approximately 1.6-fold and 3.4-fold increases in holoenzyme activity over wild-type mice were observed in Line 2 heterozygotes and in Line 1 homozygotes, respectively, whereas no increase was detected in Line 1 heterozygotes (Fig. 3B). Addition of PLP resulted in ~7-fold, 11-fold, and 15-fold increases in GAD activity in Line 1 heterozygotes, Line 2 heterozygotes, and Line 1 homozygotes, respectively. Thus transgenic GAD65 contributes to holoenzyme activity in the two highest expressing lines but most significantly enhances the apoenzyme pool in all three lines.

Exhibition of impaired glucose tolerance and elevated blood sugars in vivo in RIP7-hGAD65 transgenic mice. We addressed the question of whether increased levels of GAD65 and GABA affect glucose homeostasis. Blood glucose levels were analyzed in transgenic mice after an overnight fast and at different time points after glucose administration (Fig. 4). Fasting blood glucose levels in transgenic and control mice were not significantly different for Line 2 heterozygotes (Fig. 4B) or in Line 1 heterozygotes (not shown). However, they were significantly elevated in Line 1 homozygotes (Fig. 4A). Analyses of blood glucose levels after intraperitoneal administration of glucose revealed impaired glucose tolerance in Line 1 homozygotes and in Line 2 heterozygotes (Fig. 4), whereas Line 1 heterozygotes remained normal (results not shown). Blood glucose levels maximized at 20 min in control mice and approached basal values by 90 min. In contrast, Line 1 homozygotes and Line 2 heterozygotes showed prolonged high blood glucose levels. Whereas Line 2 mice reached normal blood glucose levels at 150 min, glucose levels in Line 1 were still elevated at 180 min (Fig. 4). Thus impairment of glucose tolerance is most severe in Line 1 homozygotes, which express the highest levels of antigen, and these mice also exhibit elevated fasting blood sugar.

Exhibition of decreased insulin secretion in vivo in transgenic mice. To assess whether the hyperglycemia in homologous Line 1 mice and heterologous Line 2 mice was associated with abnormal secretion of islet hormones, serum insulin and glucagon levels were monitored in separate glucose stimulation experiments. No significant differences were found in serum
glucagon levels between the transgenic and control mice (data not shown). As shown in Fig. 5A, fasting serum insulin levels were significantly lower in Line 1 homozygotes [0.33 ± 0.02 (SD) ng/ml] compared with control C57Bl/6 mice of the same age and sex (0.42 ± 0.02 ng/ml; P < 0.01) and remained significantly lower after administration of glucose. The increment over basal in Line 1 homozygous mice was also significantly different between transgenic and control animals, at both 30 min: 0.15 ± 0.15 (transgenic) vs. 0.47 ± 0.18 ng/ml (controls) (P < 0.025) and at 90 min: 0.31 ± 0.22 (transgenic) vs. 0.84 ± 0.19 ng/ml (controls) (P < 0.001). These results suggest that the fasting hyperglycemia observed in Line 1 homozygotes and the hyperglycemia measured during a glucose tolerance test in both lines are caused by a decrease in glucose-induced insulin secretion.

GAD65 is an important target of autoimmunity in type 1 diabetes in humans (3) and in the nonobese diabetic mouse (59). We speculated that increased expression of GAD65 could target β-cells for infiltration by mononuclear lymphocytes and autoimmune destruction. However, hematoxylin-eosin staining (results not shown) and immunostaining for insulin in pancreatic sections from RIP7-hGAD65 Line 1 (results not shown) and Line 2 mice (Fig. 2, compare panels e and f) at different ages revealed no insulitis and no loss of β-cells. Thus the decrease in insulin secretion and abnormal glucose tolerance in transgenic mice are not the result of autoimmune processes and pancreatic β-cell destruction.

RIP7-hGAD65 Line 2 mice exhibit a selective inhibition of first-phase insulin secretion in vitro. To identify the nature of the inhibition of insulin secretion in transgenic mice, the kinetics of insulin secretion during stimulation with glucose and IBMX were examined using in vitro pancreas perfusion of Line 2 heterozygotes. An initial perfusion without glucose (31) showed no evidence of leakage or constitutive release of insulin. Glucose at 7 mM was selected for the first stimulatory step, because it approximates the basal glucose levels in those mice (Fig. 6). It also is the level at which a submaximal first-phase insulin release is consistently detected (15) and can be used to assess either enhancement or impairment of insulin secretion. This concentration of glucose elicited a sharp first-phase insulin secretion from control pancreases (Fig. 6), as previously reported (32). In contrast, the first-phase insulin response of transgenic pancreases was significantly lowered (17.38 ± 5.07 ng/ml in nontransgenic littermates; Fig. 6). Only a single time point at 38 min was significantly suppressed during second-phase release in transgenic mice. The perfused pancreas of both transgenic and control mice responded to subsequent ascending levels of 11 and 22 mM glucose. The declining insulin responses to higher glucose steps observed for both transgenic and control mice (Fig. 6) have been previously shown (15, 30) and were ascribed to a packet storage of insulin with differing sensitivities to glucose, or alternatively to feedback inhibition (43), also termed time-dependent inhibition (42). In the presence of 22 mM glucose, IBMX, an inhibitor of cAMP phosphodiesterase, potentiated the response to glucose with a large and rapid increase in insulin secretion that was identical in transgenic and control C57Bl/6 mice of the same age and sex (0.42 ± 0.007 vs. 0.87 ± 0.08; P < 0.001). Although there was a tendency for lower fasting serum insulin levels in Line 2 heterozygotes compared with nontransgenic littermates, the differences were not statistically significant either in the set of animals shown in Fig. 5B or in a set of 10 transgenic and 10 control animals subsequently analyzed. The combined data for basal insulin levels obtained for 15 transgenic Line 2 mice and 15 nontransgenic littermates of the same sex and age were 0.20 ± 0.10 (SD) ng/ml for transgenic mice vs. 0.26 ± 0.17 ng/ml for control mice (P < 0.15). Administration of glucose, however, elicited significantly lower blood insulin levels in Line 2 mice at both 30 and 90 min (Fig. 5B). The increment above basal in Line 2 heterozygotes was also significantly different between transgenic and control animals, at both 30 min: 0.15 ± 0.15 (transgenic) vs. 0.47 ± 0.18 ng/ml (controls) (P < 0.025) and at 90 min: 0.31 ± 0.22 (transgenic) vs. 0.84 ± 0.19 ng/ml (controls) (P < 0.001). These results suggest that the fasting hyperglycemia observed in Line 1 homozygotes and the hyperglycemia measured during a glucose tolerance test in both lines are caused by a decrease in glucose-induced insulin secretion.
control pancreases (Fig. 6). Hence, inhibition of glucose-induced insulin secretion in RIP7-hGAD65 Line 2 heterozygous mice primarily affects the first phase of insulin secretion in response to physiological levels of glucose. The single time point that was significantly suppressed during second-phase release could indicate an additional minor effect on this phase.

A defect before membrane depolarization of the pancreatic β-cell. Insulin secretion in response to glucose involves coordinated steps, including 1) elevation of intracellular ATP levels as a result of glucose metabolism; 2) closure of sulfonylurea-sensitive K\textsubscript{ATP} channels, and β-cell depolarization; 3) activation of Ca\textsuperscript{2+} channels, and oscillation of cytoplasmic Ca\textsuperscript{2+} levels; and 4) induction of exocytosis (1, 39). To assess whether the defect in first-phase insulin secretion in transgenic mice is at a step before or after depolarization of the β-cell, K\textsuperscript{+} was used to artificially depolarize the β-cell and bypass the closure of K\textsubscript{ATP} channels in Line 2 heterozygotes. It has been shown that stimulation of the pancreas with depolarizing concentrations of K\textsuperscript{+} alone primarily results in first-phase insulin secretion (16). In such experiments, glucose has an additive potentiating effect on insulin release (18). Because we had found the response to glucose to be atypical (Fig. 4), glucose was excluded in the experiments with K\textsuperscript{+} to eliminate it as a variable and to allow a direct measurement of the effect of depolarization on insulin release in the transgenic and the normal pancreas. K\textsuperscript{+} at 20 mM stimulated a sharp first-phase insulin response and a low prolonged insulin secretion that were identical in transgenic and control mice (Fig. 7). A subsequent stimulation with physiological glucose (7 mM) at 50 min again revealed a similar defective first-phase insulin response in transgenic mice, much as when glucose was used as the initial stimulus (compare Fig. 7 with Fig. 6). Thus, in the same pancreas, both the normal response to K\textsuperscript{+} and the impaired response to glucose are demonstrable.

The results show that the β-cell secretory machinery, distal to the β-cell depolarization step, is normal in transgenic mice, suggesting that the defect in first-phase insulin secretion is at a step either before or involving the K\textsubscript{ATP} channel itself.

DISCUSSION

In this study, we show that expression of the smaller isoform of the GABA synthesizing enzyme GAD in pancreatic β-cells of RIP7-hGAD65 transgenic mice results in elevated GAD enzyme activity and increased GABA levels in β-cells. Line 1 heterozygotes, which have the lowest expression of transgenic GAD65, do not display a phenotype. However, homozygous mice of this line, which express almost twice the levels of the enzyme, have the most severe phenotype and exhibit elevated fasting blood glucose, impaired glucose tolerance, and inhibition of fasting as well as glucose-induced insulin secretion. Line 2 heterozygotes, which express ~1.6-fold the levels of Line 1 heterozygotes, have a more subtle phenotype, which includes impaired glucose tolerance, inhibition of insulin secretion in response to glucose, but normal fasting blood glucose...
and insulin levels. Thus increasing levels of GAD65 expression in the transgenic lines correlate with severity of the phenotype. Line 2 heterozygotes do not develop chronic hyperglycemia, diabetes, or obesity over a lifespan of 2.5 yr (not shown). Line 1 homozygous mice are currently being monitored over a prolonged period to assess these parameters.

Our in vitro studies, using the perfused pancreas in Line 2 heterozygotes, suggest that an inhibition of insulin secretion occurs primarily at the first phase of insulin release stimulated at physiological glucose levels. A single time point, however, was significantly suppressed during second-phase release. Thus the inhibitory effect may not be strictly limited to the first phase of insulin secretion. These results are clearly distinct from a more general inhibition of the insulin secretory pathway seen in perfusion studies of some transgenic models, which overexpress membrane proteins in pancreatic β-cells (19). The normal potentiating effect of IBMX in RIP7-hGAD65 mice suggests that the inhibition of first-phase insulin secretion does not involve cAMP-mediated signaling pathways.

Whereas the in vivo analysis of RIP7-hGAD65 Line 2 mice showed a consistently impaired insulin release at all glucose levels during glucose challenge, the in vitro pancreas perfusion analysis detected impairment only at the first step of glucose stimulation (7 mM) but not at subsequent steps of 11 mM and 22 mM glucose. It is possible that, in vitro, the inhibitory effect of the elevated GABA levels is obscured by the nature of stepwise increments in glucose concentration and the lack of feedback mechanisms by circulating inhibitors and potentiators (15, 42, 43).

It was shown previously that stimulation of the pancreas with depolarizing concentrations of K\(^+\) in the absence of glucose causes mainly first-phase insulin secretion (16). In the present study, we found that the response to K\(^+\) in the transgenic pancreas is normal. These results indicate that the β-cell machinery distal to β-cell depolarization, including Ca\(^{2+}\) channels and exocytosis of secretory vesicles, is intact in RIP7-hGAD65 transgenic mice. Thus inhibition of first-phase insulin secretion probably results from events occurring before membrane depolarization, in the glucose metabolic cascade, or at the K\(^+\) ATP channel (1, 39).

How does GAD65/GABA expressed in β-cells exert an inhibitory effect on first-phase insulin secretion? At least two possible mechanisms can be proposed. First, recent evidence suggests that glutamate may act as a messenger that enters secretory vesicles and induces their priming to become part of a readily releasable pool of granules (33). The levels of GAD65 in RIP7-hGAD65 Line 2 islets are similar to the endogenous levels of the protein in rat and human islets, in which the protein is still relatively rare (4). It is possible, however, that transgenic GAD65 significantly affects levels of glutamate (substrate), resulting in an inhibition of priming. Such inhibition would, however, be predicted to affect mainly second-phase insulin release, which is inconsistent with the observations in RIP7-hGAD65 mice. The second possibility is that the phenotype of RIP7-hGAD65 mice is mediated by the elevated levels of GABA synthesized by GAD65. Metabolism of GABA in the tricarboxylic acid cycle via the GABA shunt (38) seems to be excluded, because this process is likely to generate ATP and stimulate insulin secretion. Alternatively, GABA accumulated in vesicles and secreted from the β-cell may mediate a paracrine or autocrine inhibitory effect on insulin secretion via GABA receptors. GABA\(_A\) receptors are expressed on α- or δ-cells (51), and GABA\(_B\) receptors are expressed on β-cells (Chang and Baekkeskov, unpublished results). One hypothesis is that GABA secreted by β-cells inhibits first-phase insulin secretion in an autocrine fashion via G protein-linked GABA\(_B\) receptors.

Earlier studies, using exogenous GABA, have reported a wide variety of effects on β-cell function, including a general inhibition of both phases of insulin secretion, a stimulation of insulin secretion, or no effect at all (37, 53 for review). The inherent contradiction of the studies using exogenous GABA is not understood, but it may involve differences in GABA uptake and/or transport in the different experimental systems. Our results, with a transgenic model, which increases GAD65 and GABA levels in β-cells, suggest that the action of the endogenous transmitter synthesized in these cells is primarily, although perhaps not exclusively, to regulate the first phase of insulin secretion elicited by glucose at step(s) proximal to or at the K\(^+\) ATP channel.

We thank John Kim, Ann Neill, Raquel Nagal, and Mary Ann Jones for excellent technical assistance, and Patti Keefe for help with references.

This study was supported by National Institutes of Health Grants R01 DK-47043 (to S. Baekkeskov) and P01 DK-41822 (to S. Baekkeskov and D. Hanahan), the Nora Eccles Treadwell Foundation (to S. Baekkeskov), an American Diabetes Association Mentor-Based Fellowship (to S. Baekkeskov), and a Fellowship Award from the Juvenile Diabetes Foundation International (to Y. Shi).

Present address of Y. Shi: Diabetes Research, Endocrine Division, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46285.

REFERENCES


