

Loss of Connexin36 Channels Alters β -Cell Coupling, Islet Synchronization of Glucose-Induced Ca^{2+} and Insulin Oscillations, and Basal Insulin Release

Magalie A. Ravier,¹ Martin Güldenagel,² Anne Charollais,³ Asllan Gjinovci,³ Dorothee Caille,³ Goran Söhl,² Claes B. Wollheim,³ Klaus Willecke,² Jean-Claude Henquin,¹ and Paolo Meda³

Normal insulin secretion requires the coordinated functioning of β -cells within pancreatic islets. This coordination depends on a communications network that involves the interaction of β -cells with extracellular signals and neighboring cells. In particular, adjacent β -cells are coupled via channels made of connexin36 (Cx36). To assess the function of this protein, we investigated islets of transgenic mice in which the Cx36 gene was disrupted by homologous recombination. We observed that compared with wild-type and heterozygous littermates that expressed Cx36 and behaved as nontransgenic controls, mice homozygous for the Cx36 deletion (Cx36^{-/-}) featured β -cells devoid of gap junctions and failing to exchange microinjected Lucifer yellow. During glucose stimulation, islets of Cx36^{-/-} mice did not display the regular oscillations of intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$) seen in controls due to the loss of cell-to-cell synchronization of $[\text{Ca}^{2+}]_i$ changes. The same islets did not release insulin in a pulsatile fashion, even though the overall output of the hormone in response to glucose stimulation was normal. However, under nonstimulatory conditions, islets lacking Cx36 showed increased basal release of insulin. These data show that Cx36-dependent signaling is essential for the proper functioning of β -cells, particularly for the pulsatility of $[\text{Ca}^{2+}]_i$ and insulin secretion during glucose stimulation. *Diabetes* 54:1798–1807, 2005

Normal insulin secretion requires the coordinated functioning of β -cells within pancreatic islets. This coordination is achieved through extracellular signals and ligands of the connective matrix as well as through the interaction of β -cells and

their neighboring islet cells. Thus, cell interactions mediated by surface receptors and cell adhesion and junctional molecules have been implicated in the regulation of β -cell functions (1–3). Specifically, β -cells are interconnected by gap junctions, in which connexin channels linking adjacent cells are concentrated (3). The interaction of these channels across the extracellular space allows direct exchanges of low-molecular weight cytoplasmic molecules and ensures electrical coupling. Previous studies have implicated connexins and/or the cell-to-cell exchanges (3) and electrical coupling (4,5) that these proteins permit in the control of β -cell function. However, because of the uncertainty about the connexin species that functionally connects native β -cells (6,7), the in vivo relevance of the connexin-dependent signaling and the nature of the mechanism linking connexin expression to β -cell function has not been resolved.

We recently showed that connexin36 (Cx36) is expressed by primary β -cells (6,7) and insulin-producing cell lines that retain some ability to increase insulin secretion during glucose stimulation (6,8). We further documented that, at variance with most other cell types that coexpress several connexin species (3), β -cells could not be shown to be linked by connexins other than Cx36 (7). These observations have opened the way to a direct experimental test of the function of Cx36. Thus, alterations in the Cx36 content of insulin-producing cells affect their secretion, with both decreases and increases in the amount of Cx36 resulting in an inhibited secretory response to glucose (8–11). However, in view of the limitations of these in vitro approaches, the many differences among cell lines and primary β -cells and the multiple signaling pathways that converge to regulate the function of native pancreatic islets (1–3), the contribution of Cx36 to the in vivo function of the endocrine pancreas remains to be demonstrated. Here we have addressed this issue by studying mutant mice that do not express Cx36 (after targeted deletion of the cognate gene) (12) and feature deficits in the neural and retinal networks of cells that natively express this connexin isoform (12–15).

RESEARCH DESIGN AND METHODS

Transgenic mice. Cx36-deficient (12) and C57BL/6 mice were crossed to obtain heterozygous offspring. Progenies were intercrossed to obtain Cx36^{-/-}, Cx36^{+/-}, and Cx36^{+/+} littermates. Genotyping was done by PCR analysis and Southern blot hybridization (12). Male and female mice age 3–6 months and with an 87.5 (F2) to 97% (F4) C57BL/6 background were used for most experiments. For the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) and insulin-

From the ¹Unit of Endocrinology and Metabolism, University of Louvain Faculty of Medicine, Brussels, Belgium; the ²Institute of Genetics, University of Bonn, Bonn, Germany; and the ³Department of Cell Physiology and Metabolism, Geneva University Medical Center, Geneva, Switzerland.

Address correspondence and reprint requests to Paolo Meda, MD, Department of Cell Physiology and Metabolism, University of Geneva, C.M.U., 1 rue Michel Servet, 1211 Geneva 4, Switzerland. E-mail: paolo.meda@medecine.unige.ch. Jean-Claude Henquin, Unit of Endocrinology and Metabolism, University of Louvain Faculty of Medicine, UCL 55.30, Avenue Hippocrate 55, B-1200 Brussels, Belgium. E-mail: henquin@endo.ucl.ac.be.

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M.A.R., M.G., and A.C. contributed equally to this work. $[\text{Ca}^{2+}]_i$, intracellular calcium concentration; Cx36, connexin36; LY, Lucifer yellow.

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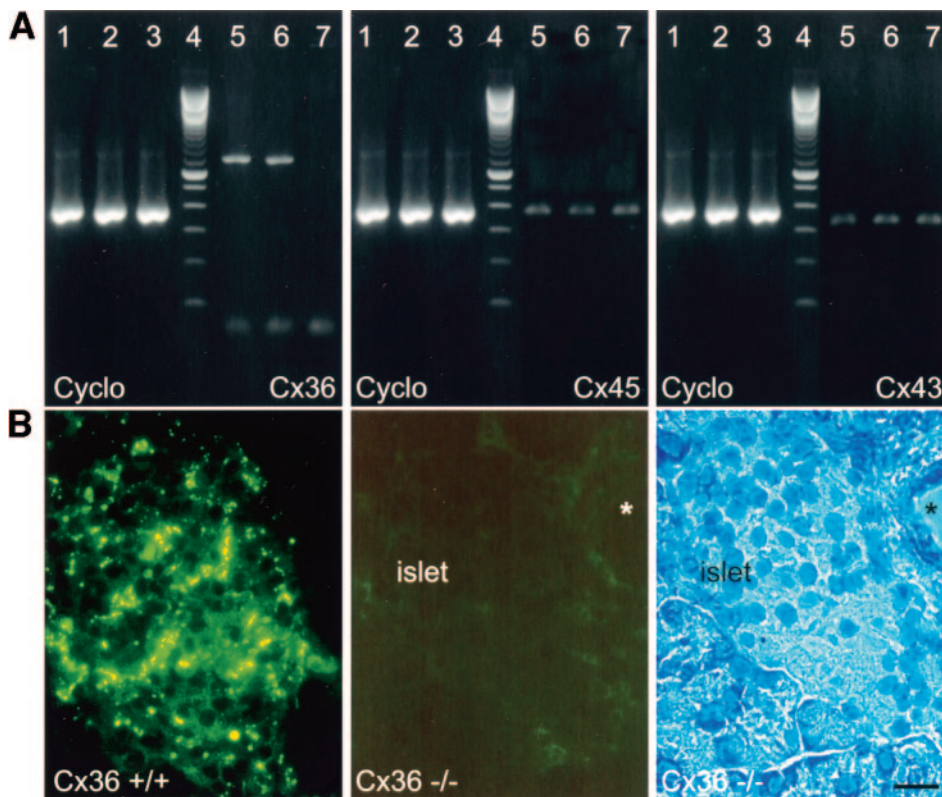


FIG. 1. Pancreatic islets of Cx36^{-/-} mice selectively lack the Cx36 isoform. A: RT-PCR revealed a transcript for Cx36 in islets of Cx36^{+/+} (lane 5) and Cx36^{+/-} (lane 6) mice but not of Cx36^{-/-} littermates (lane 7). Cx45, Cx43, and cyclophilin (Cyclo) were amplified in all samples. **B:** Immunostaining showed Cx36 in β -cells of Cx36^{+/+} (left panel) but not Cx36^{-/-} (middle panel; phase-contrast in right panel) mice. Scale bar, 20 μ m.

release studies on isolated islets, mice age 5–11 months of both sexes were used. Because no obvious difference was seen as a function of sex or genetic background, data from F2–F4 male and female mice were pooled. All studies were conducted in accordance with the regulations of our state committees on animal welfare.

Connexins. Connexin transcripts were screened in total pancreas RNA, as previously described (6,8). The following primers were used: 5'-GAGCCAG GCCAAGAGGAAGTC-3' and 5'-GGCATGCTGAAGGGGAGAAAT-3' for Cx36 (amplicon = 559 bp); 5'-CGGCGGCTTCACTTT CATT-3' and 5'-AGAACACATGGGCCAAGTAC-3' for Cx43 (amplicon = 334 bp); 5'-GGCAA ACCAATTCCACCACC-3' and 5'-CTCGTTTGGGATACGGTACGC-3' (amplicon = 379 bp) for Cx45; and 5'-GGTCAACCCACCCGTGTCT-3' and 5'-GCC ATCCAGCCACTCAGTCT-3' for cyclophilin (amplicon = 387 bp). The cycle used was 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, for 35 cycles. PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Connexin proteins were immunostained in pancreas sections, as previously described (6,8). Sections were exposed for 3 min to -20°C cold acetone and incubated for 2 h at room temperature with one of the following rabbit sera: anti-Cx36, diluted 1:100 (6); anti-Cx43, diluted 1:500 (Zymed, San Francisco, CA); and anti-Cx45, diluted 1:400. After being rinsed, sections were exposed for 1 h at room temperature to sheep antibodies against rabbit immunoglobulins, diluted 1:400.

Gap junctions. Islets were isolated by collagenase digestion (16), fixed for 60 min in a 2.5% glutaraldehyde solution, and processed for freeze-fracture (9,10) using a Balzers BAF 060 (Balzers High Vacuum, Balzers, Liechtenstein). Replicas were examined with a Philips CM10 electron microscope.

Junctional coupling. Isolated islets were attached to Sylgard- and poly-L-lysine-coated dishes for iontophoretic microinjection (5 min) of 4% Lucifer yellow (LY) into individual islet cells (16). The injected field was photographed and the islets were fixed in 4% paraformaldehyde, embedded in Epon, serially sectioned, and photographed under ultraviolet illumination. The incidence and extent of coupling were determined as previously described (16).

Morphometry. Pancreata of four mice per genotype were fixed in Bouin's solution, embedded in paraffin, and processed for immunolabeling of insulin (16). The volume and numerical densities of pancreatic islets and the distribution of islet sizes were evaluated by morphometry, as previously reported (16).

NADPH and Ca²⁺. Islets were isolated by collagenase digestion and cultured 18–24 h in RPMI medium containing 10 mmol/l glucose. For NADPH measurements, individual Cx36^{+/+} and Cx36^{-/-} islets were incubated for 2 h in a Krebs medium containing 10 mmol/l glucose and then simultaneously evaluated for NADPH autofluorescence during perfusion with 3 or 15 mmol/l glucose (17).

For [Ca²⁺]_i measurements, a similar procedure was applied to islets loaded with fura-PE3 for 2 h in a Krebs medium containing 10 mmol/l glucose. [Ca²⁺]_i was also measured in small clusters of 5–20 cells that were prepared, cultured for 48 h, and monitored, as previously described (5,17,18). In all these experiments and in the measurements of insulin secretion described below, the concentration of CaCl₂ in the perfusion medium was 2.5 mmol/l.

Insulin secretion and content. In some experiments, insulin secretion by a single islet was measured every 30 s, while monitoring [Ca²⁺]_i changes (18,19). In other experiments, batches of 25 cultured islets were perfused and stimulated by stepwise increases in the glucose concentration (20). Effluent medium was collected every 2 min for insulin measurement. At the end of the experiment, the islets were extracted in acid-ethanol to determine the insulin content.

Perfusion of the whole pancreas was performed *in situ*, as previously reported (16). The pancreas was perfused at 37°C with 1 ml/min modified Krebs-Ringer HEPES buffer supplemented with the indicated concentrations of glucose, a flux that was maintained in all mice using a pressure column pump (16). The perfusate contained the glucose concentrations indicated in the text, each applied for 20 min. During the first 20-min equilibration period, the medium contained 1.4 mmol/l glucose and no effluent was sampled. Thereafter, aliquots were collected every minute for insulin measurement (16). Differences in insulin secretion among animals and groups were assessed by ANOVA and Scheffe's test, which compared the areas under the secretion curve (*I*) from the 6th min of each period, to avoid the first phase of insulin secretion, to the 1st min of the successive period (the time required for a full change of the glucose concentration; this 15-min interval evaluated the 2nd phase of insulin secretion); 2) for the first 5 min of each period to evaluate the 1st phase; and 3) for the last 5 min of exposure to 1.4 mmol/l glucose to evaluate basal insulin release. To measure insulin content, the whole pancreas was extracted in acid-ethanol before the radioimmunoassay (16).

RESULTS

Cx36^{-/-} mice no longer express Cx36 or gap junctions. Total RNA was extracted from pancreatic islets isolated from wild-type (Cx36^{+/+}), heterozygous (Cx36^{+/-}), and homozygous (Cx36^{-/-}) transgenic littermates. RT-PCR amplification of Cx36^{+/+} RNA revealed the transcript of Cx36 and faint expression of mRNAs coding for Cx45 and Cx43 (Fig. 1A). Immunostaining showed abundant localization of Cx36 at β -cell interfaces (Fig. 1B), but failed

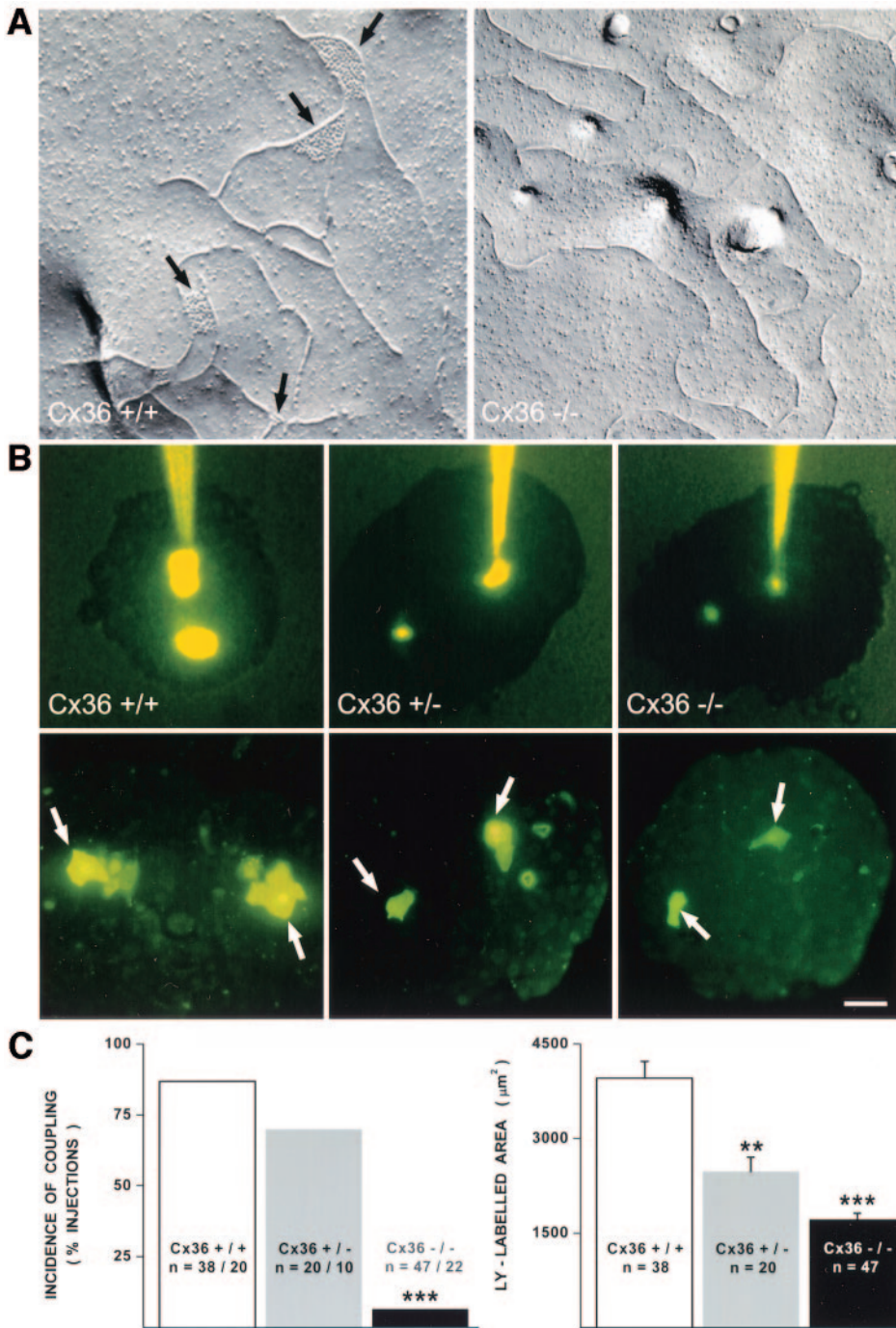


FIG. 2. Cx36 deletion resulted in loss of gap junction plaques and coupling among β -cells. **A:** Gap junctions (arrows) connected β -cells of Cx36^{+/+} but not Cx36^{-/-} littermates. Scale bar, 100 nm. **B:** LY diffused among β -cells of Cx36^{+/+} but not Cx36^{-/-} islets. Scale bar, 100 μ m and 15 μ m for islets (upper panels) and sections (lower panels), respectively. **C:** The coupling incidence and extent were higher in Cx36^{+/+} than in Cx36^{-/-} islets and intermediate in Cx36^{+/-} islets. ***P* < 0.01, ****P* < 0.001 for Cx36^{-/-} vs. Cx36^{+/+} and Cx36^{+/-}. Values of *n* within the columns are the number of injections/number of injected islets.

to detect Cx45 and Cx43 at these sites when antibodies that revealed these proteins in large pancreatic vessels and myocardium were used (data not shown). Similar observations were made in islets of Cx36^{+/-} littermates that carried one Cx36 wild-type allele (Fig. 1A). In contrast, no Cx36 transcript or protein was detected in islets of Cx36^{-/-} littermates, which showed normal levels of Cx43 and Cx45 mRNAs (Fig. 1A and B). Thus, targeting of the native Cx36 gene resulted in the loss of the cognate protein within pancreatic islets, which was not associated with overexpression of other connexins.

To assess whether the loss of Cx36 affected the formation of β -cell gap junctions, islets isolated from the three mouse genotypes were processed for freeze-fracture. Ultrastructural analysis of β -cell membranes in islets of

Cx36^{+/+} and Cx36^{+/-} mice revealed numerous gap junctions associated with tight junctions (Fig. 2A and Table 1). In contrast, no gap junctions were observed in Cx36^{-/-} islets, despite the persistence of tight junctions (Fig. 2A

TABLE 1
 β -cells of Cx36^{-/-} mice are not connected by gap junctions

Genotype	Junctional regions*	Type of junction			Gap junctions	
		Tight	Tight + gap	Gap	With tight junctions	Alone
Cx36 ^{+/+}	77 (120)	31	40	6	68	49
Cx36 ^{+/-}	30 (50)	10	18	2	16	20
Cx36 ^{-/-}	109 (155)	109	0	0	0	0

Data are *n* of junctional regions (*n* of β -cell membranes examined).

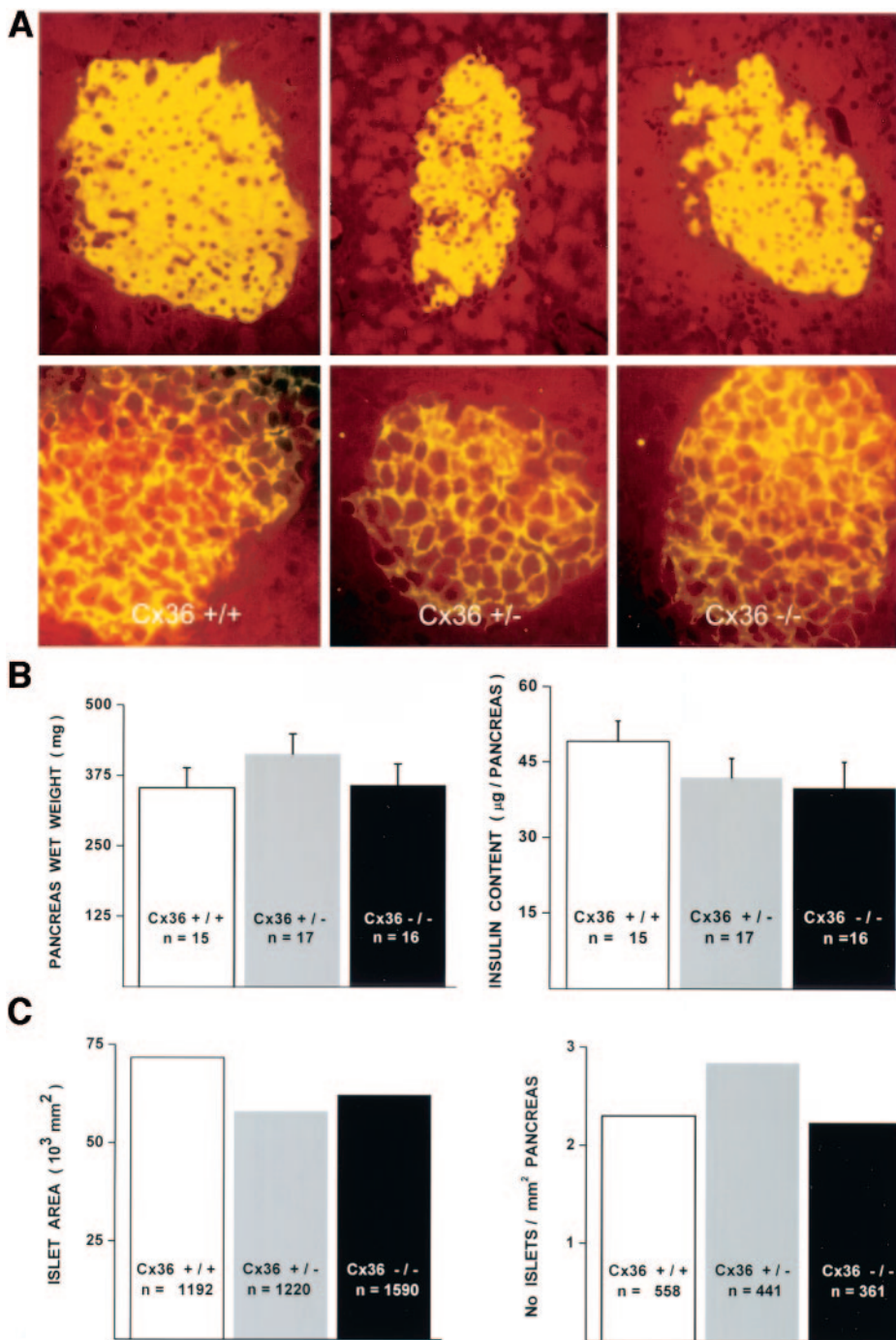


FIG. 3. The loss of Cx36 did not affect islet structure or β -cell differentiation. **A:** All islets were similarly immunostained for insulin (*upper panels*) and GLUT-2 (*lower panels*). Scale bar, 10 (*upper panels*) and 15 (*lower panels*) μm . Magnification: 25 \times (*upper panels*) and 230 \times (*lower panels*). **B and C:** Pancreas weight, insulin content (means \pm SE of the indicated number of pancreata), size, and numerical density of islets (medians of the indicated number of islets) were similar in all groups.

and Table 1). Thus, the loss of Cx36 resulted in the disappearance of gap junctions among β -cells.

To assess whether the loss of gap junctions altered β -cell coupling, individual cells were microinjected with LY in the central region of the islets. After a 5-min injection, the tracer diffused between 5 and 10 β -cells in Cx36^{+/+} islets (Fig. 2*B* and *C*). Coupling was still observed in Cx36^{+/-} islets, although at reduced levels (Fig. 2*B* and *C*). In contrast, LY consistently remained within the injected cell in Cx36^{-/-} islets (Fig. 2*B* and *C*). Thus, the absence of gap junctions made of Cx36 resulted in the loss of dye transfer among β -cells.

Mice lacking Cx36 feature morphologically normal pancreatic islets. To assess whether the lack of Cx36 affected the structure of the islets, pancreas sections were

immunostained for insulin and GLUT2. We observed that most β -cells were intensively stained for these two proteins, with no obvious difference among the three mouse genotypes (Fig. 3*A*). There was also no difference among the mice with regard to weight and insulin content of pancreas (Fig. 3*B*), size of individual islets, and number of islets per unit volume of pancreas (Fig. 3*C*). Therefore, the absence of Cx36 was not detrimental to the correct development and differentiation of β -cells nor to their clustering into morphologically normal islets.

Islets lacking Cx36 no longer show synchronous $[\text{Ca}^{2+}]_i$ oscillations in β -cells during glucose stimulation. To assess whether the lack of Cx36 affected β -cell synchronization, we monitored $[\text{Ca}^{2+}]_i$ within intact isolated islets. Continuous stimulation with 12 mmol/l glu-

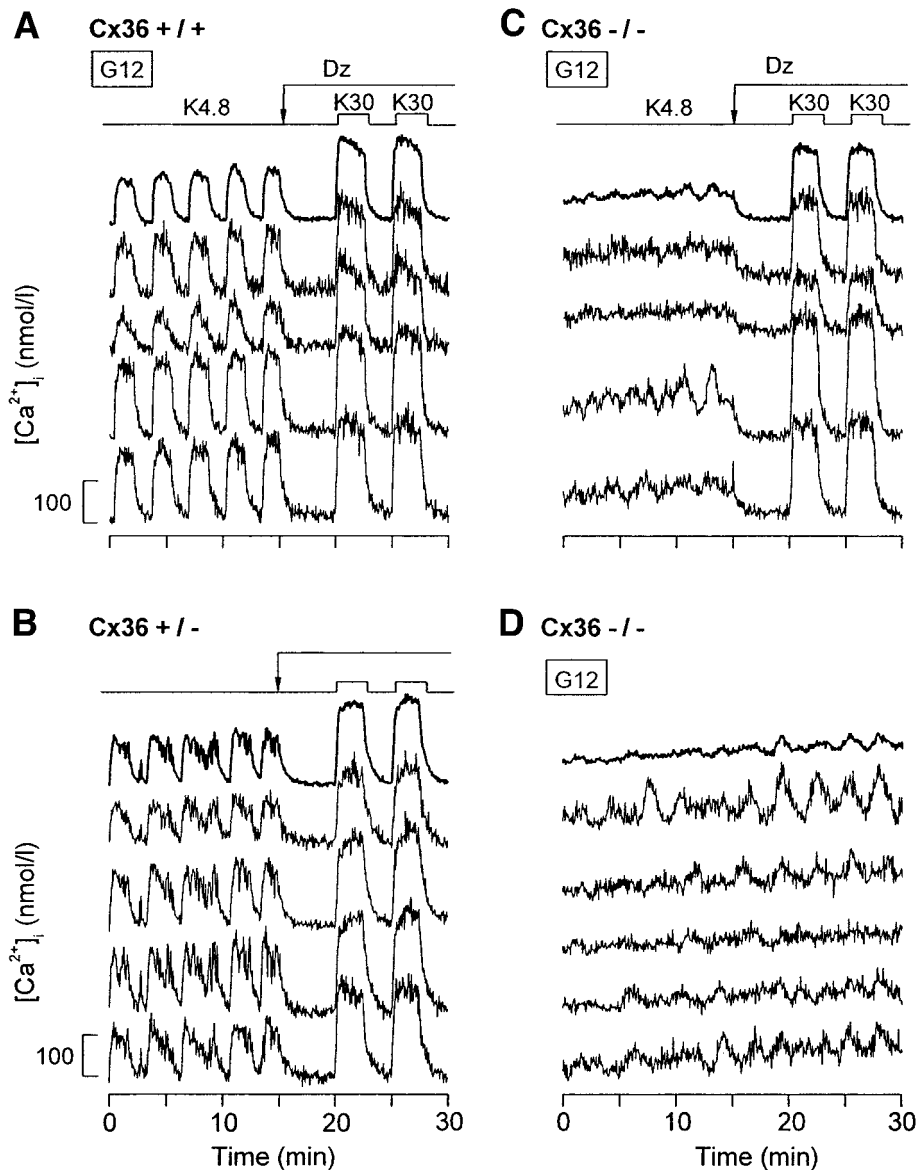


FIG. 4. The loss of Cx36 abolished the synchronization of glucose-induced $[Ca^{2+}]_i$ oscillations in β -cells. Islets were perfused with 12 mmol/l glucose (G12) before 100 μ mol/l diazoxide (Dz) and 4.8–30 mmol/l KCl (K4.8 and K30) were added, as indicated. In each panel, the upper trace shows the global response of the islet and the four to five lower traces show the responses in noncontiguous regions of the same islet. Islets of Cx36^{+/+} (A) and Cx36^{+/-} (B) mice showed regular $[Ca^{2+}]_i$ oscillations (upper trace) due to synchronization of all islet regions (lower traces). C and D: These oscillations were not observed in Cx36^{-/-} islets. Traces representative of 10 experiments.

cose induced $[Ca^{2+}]_i$ oscillations in Cx36^{+/+} islets due to the synchronization of noncontiguous β -cells (Fig. 4A). Diazoxide-induced repolarization abolished the glucose-induced $[Ca^{2+}]_i$ oscillations, whereas depolarizations by pulses of 30 mmol/l KCl evoked synchronous $[Ca^{2+}]_i$ elevations in all islet regions (Fig. 4A). Similar results were obtained in Cx36^{+/-} islets (Fig. 4B). In contrast, during glucose stimulation of Cx36^{-/-} islets, the average $[Ca^{2+}]_i$ was elevated, as shown by a decrease in $[Ca^{2+}]_i$ after the addition of diazoxide (Fig. 4C), but only low or no oscillations were seen (Fig. 4C and D). These oscillations were still rare and irregular when the analysis was restricted to areas of contiguous β -cells. (Figure 4D shows the largest oscillations recorded in one such region.) Within small clusters, the resolution of the spectrofluorometric recording was sufficient to see that some cells prepared from Cx36^{-/-} islets displayed distinct $[Ca^{2+}]_i$ oscillations during glucose stimulation (Fig. 5, traces 1 and 2), whereas other cells did not (Fig. 5, traces 3 and 4). However, in no case were these oscillations synchronized with those that were simultaneously recorded in nearby cells. In contrast, repetitive depolarizations by 30 mmol/l KCl induced well-

synchronized $[Ca^{2+}]_i$ changes throughout Cx36^{-/-} islets (Fig. 4C). Thus, Cx36 is not required for the generation of $[Ca^{2+}]_i$ oscillations in individual cells, but is necessary to ensure their synchronization among the numerous β -cells that form an islet.

Islets lacking Cx36 no longer show pulsatile insulin secretion in response to glucose. To assess the impact of this desynchronization on β -cell function, $[Ca^{2+}]_i$ and insulin secretion were monitored simultaneously in single islets. Glucose-induced $[Ca^{2+}]_i$ oscillations and KCl-induced $[Ca^{2+}]_i$ pulses were accompanied by synchronous pulses of insulin secretion in Cx36^{+/+} islets (Fig. 6A and B). In contrast, no oscillations of insulin secretion were detected in most Cx36^{-/-} islets (Fig. 6C). Small fluctuations of insulin secretion that paralleled fluctuations of $[Ca^{2+}]_i$ were seen in only a small number of these islets during stimulation by glucose (data not shown) or glucose plus forskolin (Fig. 6D). However, depolarizations of Cx36^{-/-} islets by KCl triggered pulses of insulin secretion that were synchronous with the $[Ca^{2+}]_i$ elevations (Fig. 6C). Thus, the loss of Cx36 abolishes the oscillations of both $[Ca^{2+}]_i$ and insulin secretion during glucose stimula-

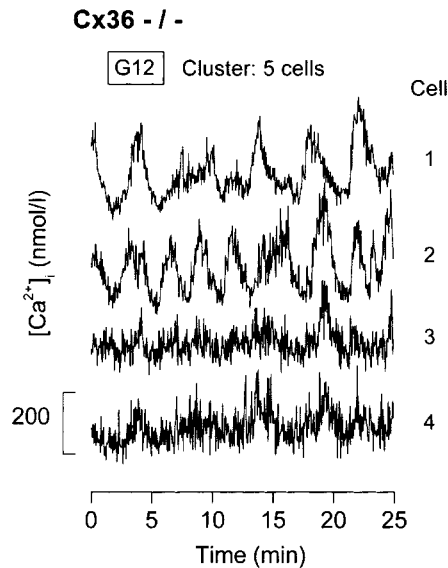


FIG. 5. The loss of Cx36 did not abolish $[Ca^{2+}]_i$ oscillations in individual β -cells. A cluster of five cells prepared from a $Cx36^{-/-}$ islet was perfused with 12 mmol/l glucose (G12). Cells 1 and 2 showed distinct but asynchronous $[Ca^{2+}]_i$ oscillations, whereas cells 3 and 4 did not show regular oscillations.

tion, but does not affect the entrainment of islets by depolarizations externally imposed with KCl.

Islets lacking Cx36 respond to glucose stimulation by an increase in metabolism, cytosolic Ca^{2+} , and insulin secretion. To assess whether the lack of Cx36 affected the glucose metabolism of β -cells, we measured NADPH fluorescence in pairs of $Cx36^{+/+}$ and $Cx36^{-/-}$ islets. In the

presence of 3 mmol/l glucose, basal NADPH fluorescence was similar in the two types of islets, and stimulation with 15 mmol/l glucose caused a similar 40% increase in this fluorescence (data not shown). Thus, the lack of Cx36 did not notably affect the metabolism of glucose by β -cells.

To quantify $[Ca^{2+}]_i$ and insulin responses in the three mouse genotypes, isolated islets were stimulated by stepwise increases in glucose concentration. Stimulation with 8.4 and 25.2 mmol/l glucose induced a concentration-dependent increase in $[Ca^{2+}]_i$ in both $Cx36^{+/+}$ and $Cx36^{-/-}$ islets (Fig. 7A). This increase was more stable in $Cx36^{-/-}$ islets, in which $[Ca^{2+}]_i$ did not oscillate, than in $Cx36^{+/+}$ islets. Integration of $[Ca^{2+}]_i$ during the last 5–10 min of exposure to each glucose concentration resulted in a sigmoidal dosage-response relation (Fig. 7B). Statistically significant differences among the three groups were seen in the presence of 1.4 mmol/l glucose, under which condition $[Ca^{2+}]_i$ was higher in $Cx36^{-/-}$ than in $Cx36^{+/-}$ or $Cx36^{+/+}$ islets ($P < 0.05$). Also, in the presence of 8.4 mmol/l glucose, $[Ca^{2+}]_i$ was higher in $Cx36^{-/-}$ and $Cx36^{+/-}$ islets than in $Cx36^{+/+}$ islets ($P < 0.05$).

Increasing glucose to >3 mmol/l stimulated insulin secretion by $Cx36^{+/+}$ islets in a dosage-dependent manner (Fig. 7C). $Cx36^{-/-}$ islets also responded to the stepwise glucose stimulation by a rapid increase in insulin secretion (Fig. 7C). The rate of secretion induced by 7–30 mmol/l glucose was not statistically different in $Cx36^{-/-}$, $Cx36^{+/-}$, or $Cx36^{+/+}$ islets (Fig. 7C). However, in the presence of 3 mmol/l glucose, $Cx36^{-/-}$ islets showed a higher rate of secretion than $Cx36^{+/-}$ or $Cx36^{+/+}$ islets ($P < 0.05$) (Fig. 7C). The insulin content of isolated islets was similar in all

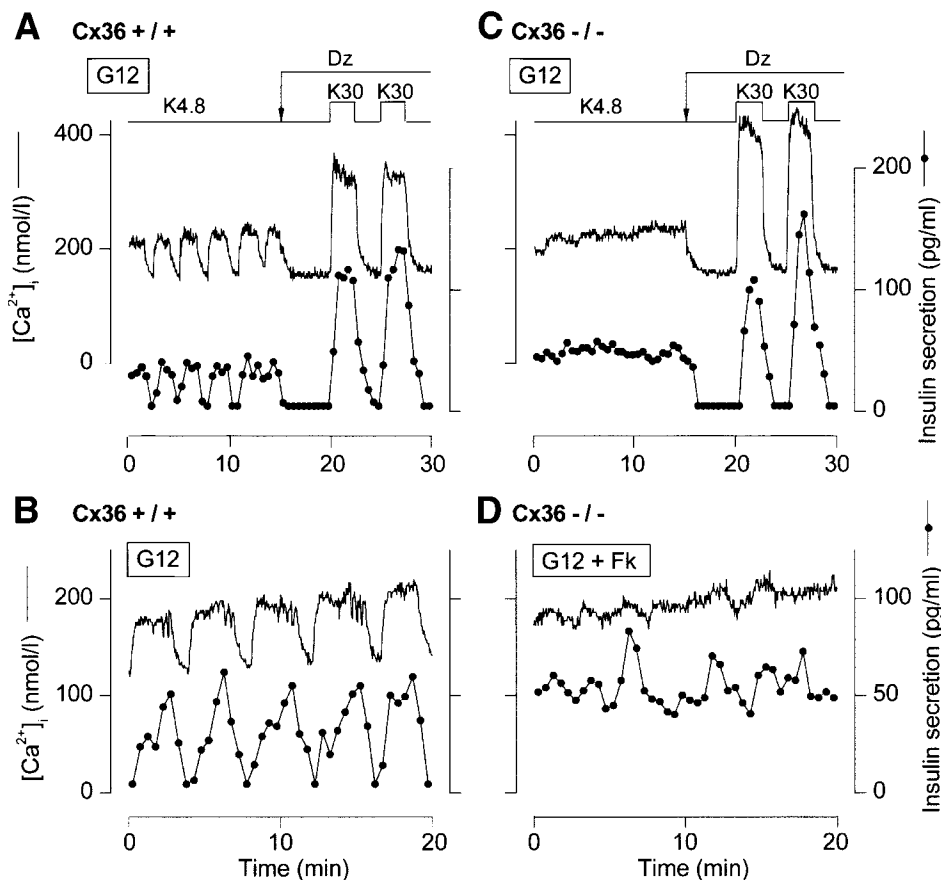


FIG. 6. The loss of Cx36 abolished glucose-induced oscillations of insulin secretion. Single islets were simultaneously monitored for $[Ca^{2+}]_i$ (upper traces) and insulin secretion (lower traces). A and B: $Cx36^{+/+}$ islets showed regular oscillations of insulin secretion corresponding to $[Ca^{2+}]_i$ oscillations. C and D: These oscillations of insulin secretion were either absent or small and irregular in $Cx36^{-/-}$ islets, except during $[Ca^{2+}]_i$ pulses imposed by KCl. Records are representative of 5 (D) and 10 (A–C) experiments. Dz, diazoxide; G12, 12 mmol/l glucose; K4.8, 4.8 mmol/l KCl; K30, 30 mmol/l KCl.

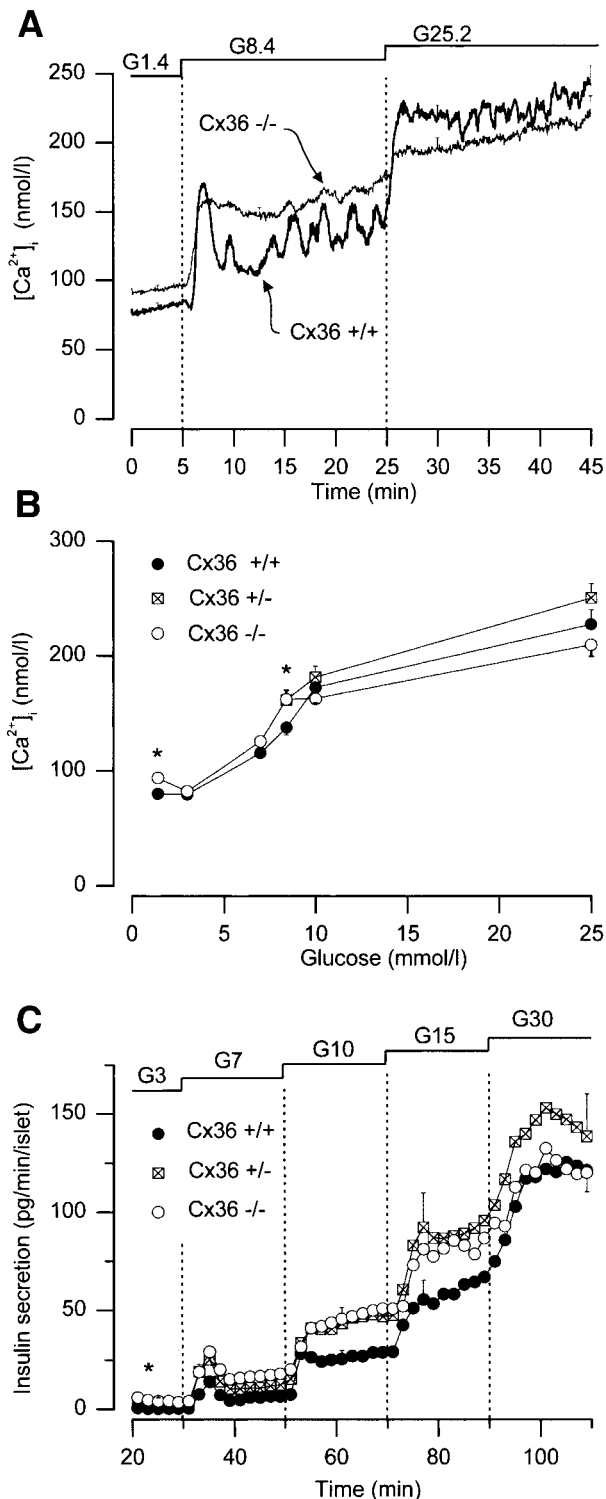


FIG. 7. The loss of Cx36 did not prevent the glucose-induced increase in cytosolic Ca^{2+} and insulin secretion of isolated islets. **A** and **B:** Glucose similarly increased $[Ca^{2+}]_i$ in a concentration-dependent way in Cx36^{+/+}, Cx36^{+/-}, and Cx36^{-/-} islets. **B:** Data are derived from experiments similar to those shown in **A**. $[Ca^{2+}]_i$ was averaged during the last 10 min of the 20-min application of each glucose concentration (steady state). Data are means \pm SE of 12–22 islets. * $P < 0.05$ for Cx36^{-/-} vs. Cx36^{+/+} and Cx36^{-/-} or Cx36^{+/-} vs. Cx36^{+/+}. **C:** Stepwise increases in glucose-stimulated insulin release of all islets. Data are means \pm SE for 8–9 experiments. * $P < 0.05$: basal release (3 mmol/l glucose) of Cx36^{-/-} islets was higher than that of Cx36^{+/+} or Cx36^{+/-} islets.

groups (91 ± 11 , 94 ± 8 , and 93 ± 8 ng/islet for Cx36^{+/+}, Cx36^{+/-}, and Cx36^{-/-} islets, respectively; $n = 8$ –9 experiments). Thus, the loss of Cx36 and synchronized $[Ca^{2+}]_i$ oscillations did not impair the glucose-stimulated insulin secretion of isolated islets but slightly raised basal release. **Islets lacking Cx36 also feature increased basal insulin secretion in situ.** To assess whether the same conclusions applied to the in situ situation, the whole pancreas was perfused with various glucose concentrations. We observed that Cx36^{+/+} pancreata had a low basal release of insulin in the presence of 1.4 mmol/l glucose and were progressively stimulated as a function of glucose concentration (Fig. 8A). Thus, insulin secretion was already significantly ($P < 0.01$) higher in the presence of 6.0 mmol/l glucose and increased over 10-fold ($P < 0.001$) in the presence of 16 mmol/l glucose (Fig. 8B). Secretion readily returned to basal levels when the glucose concentration was lowered to 1.4 mmol/l (Fig. 8A and B). Similar observations were made in Cx36^{+/-} pancreata (Fig. 8A and B). In contrast, Cx36^{-/-} pancreata showed a significantly ($P < 0.01$) higher basal release of insulin, both at the beginning and at the end of the experiment (Fig. 8A and B). During the last 5 min of the initial period in 1.4 mmol/l glucose, the cumulated basal output of Cx36^{-/-} pancreata (2.9 ± 0.7 a.u.; $n = 12$), which was significantly higher than that of Cx36^{+/+} (0.7 ± 0.1 a.u., $n = 11$; $P < 0.006$) and Cx36^{+/-} (1.0 ± 0.2 , $n = 12$; $P < 0.02$) pancreata. The corresponding numbers for the basal period that concluded the perfusion experiment were 3.3 ± 0.6 (Cx36^{-/-}; $n = 12$), 1.2 ± 0.1 (Cx36^{+/+}; $n = 12$; $P < 0.003$ vs. Cx36^{-/-}), and 1.9 ± 0.2 a.u. (Cx36^{+/-}; $n = 11$; $P < 0.04$ vs. Cx36^{-/-}).

When challenged with 4 mmol/l glucose, Cx36^{-/-} pancreata showed a first phase of insulin release of 5.3 ± 1.4 a.u. ($n = 12$), which was not statistically different from that of either Cx36^{+/+} (5.4 ± 1.5 a.u., $n = 11$) or Cx36^{+/-} (9.1 ± 3.8 a.u., $n = 12$) pancreata. Similarly, during stimulation by 6.0, 8.0, or 16.0 mmol/l glucose, the first phase of insulin release was similar in all animals, despite a progressive increase in amplitude (Fig. 8A). Thus, in the presence of 16 mmol/l glucose, the first phase was 26.1 ± 6.5 (Cx36^{-/-}; $n = 12$), 32.0 ± 7.7 (Cx36^{+/+}; $n = 12$), and 27.3 ± 5.3 a.u. (Cx36^{+/-}; $n = 11$). The data indicate that the islets of all mice responded similarly to stimulatory concentrations of glucose. However, taking into account the higher basal secretion of Cx36^{-/-} mice, their steady-state insulin secretion was not significantly increased in the presence of glucose concentrations < 8 mmol/l (Fig. 8A and B). In the presence of 16.7 mmol/l glucose, the insulin secretion of these mice was raised to the levels observed in Cx36^{+/+} and Cx36^{+/-} littermates (Fig. 8A and B).

DISCUSSION

We found that targeting the Cx36 gene resulted in the deletion of the cognate protein within pancreatic islets. This deletion caused the disappearance of gap junctions among β -cells, showing that Cx36 is necessary for the establishment of such structures. Cx36 deletion also abolished the native ability of β -cells to exchange LY (3,16), showing that other connexin isoforms did not functionally replace the lost Cx36 channels. The persistence of Cx43 and Cx45 in islets of Cx36^{-/-} mice is consistent with the

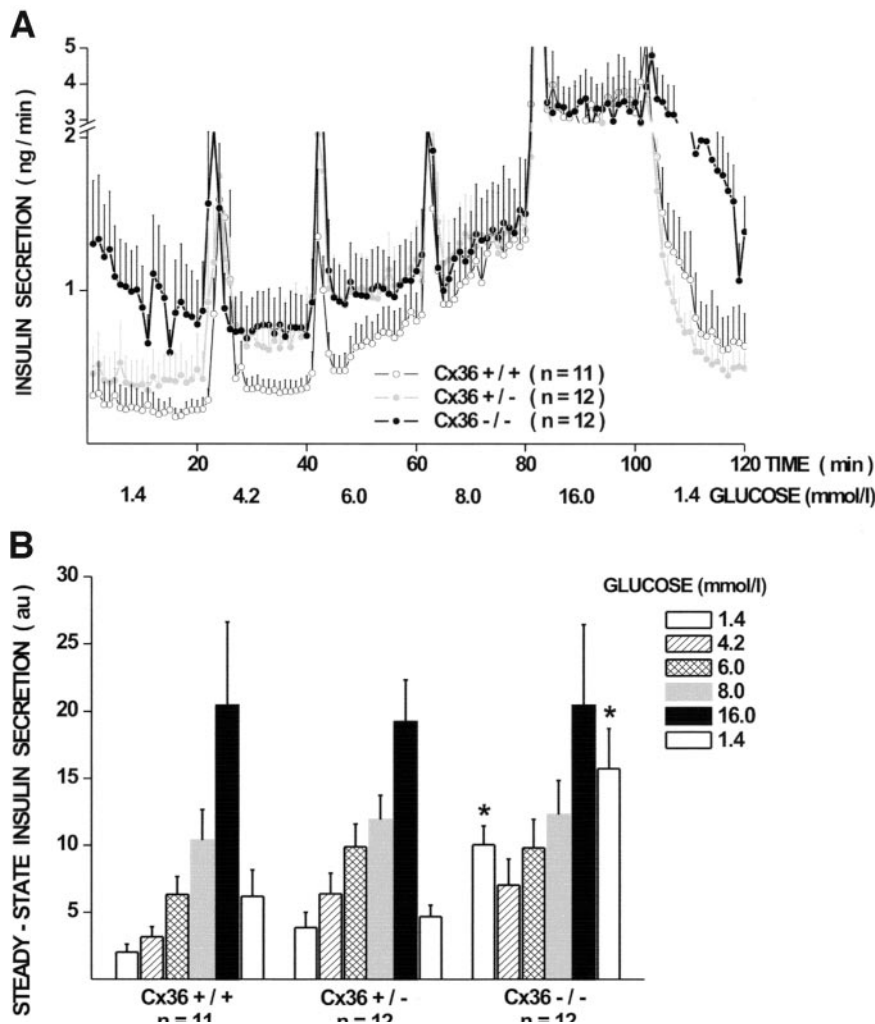


FIG. 8. The loss of Cx36 alters basal insulin secretion by the perfused pancreas. **A:** Pancreas of Cx36^{+/+} (○) and Cx36^{+/-} mice (◐) showed a low basal insulin output (1.4 mmol/l glucose), which increased with glucose stimulation. Pancreas of Cx36^{-/-} mice (●) showed a higher basal release. Glucose (≤ 8 mmol/l) induced a first phase of secretion in Cx36^{-/-} pancreata, but did not increase the second phase. Secretion was stimulated by 16 mmol/l glucose in all groups. **B:** Basal insulin release was higher in Cx36^{-/-} than in Cx36^{+/+} or Cx36^{+/-} pancreata. Data are means \pm SE of the indicated number of experiments. * $P < 0.01$ for Cx36^{-/-} vs. Cx36^{+/+}.

distribution of these two connexins between the connective and vascular cells of the islets (6,7). Together, these findings show that Cx36 is necessary to functionally couple pancreatic β -cells.

Our study identified three alterations of β -cells without Cx36. First, although $[Ca^{2+}]_i$ was found to oscillate in individual β -cells from Cx36^{-/-} mice, the islets of these animals no longer showed the synchronous oscillations of $[Ca^{2+}]_i$ that normally occur during glucose stimulation (17–22). These oscillations are caused by a depolarization-mediated, periodic influx of Ca^{2+} into β -cells (22,23), as shown by their temporal relation with membrane potential changes (17,21) and their abolition by the omission of extracellular Ca^{2+} , blockers of L-type Ca^{2+} channels, or membrane repolarization (17,22). Synchronization of glucose-induced oscillations of membrane potential (4), and hence of $[Ca^{2+}]_i$ oscillations (17,23), has been attributed to the electrical coupling of β -cells connected by gap junctions. Several studies measuring $[Ca^{2+}]_i$ in whole islets or clusters of islet cells have indirectly supported this interpretation (5,17,24,25) but could not conclusively prove it, mainly because specific inhibitors of connexin channels are not yet available (26). Alternative explanations attributing synchronization to extracellular signals have thus been put forward (27,28). Our data provide the first direct evidence that gap junctions made of Cx36 channels are

essential to ensure the synchronization of glucose-induced $[Ca^{2+}]_i$ oscillations within intact islets.

Second, islets lacking Cx36 did not secrete insulin in a pulsatile manner during glucose stimulation. In normal mouse islets, oscillations of insulin secretion accompany the oscillations of $[Ca^{2+}]_i$, whether these occur spontaneously during glucose stimulation or are induced by KCl depolarizations (18–20,29). Insulin secretion virtually stops oscillating when $[Ca^{2+}]_i$ is kept at a high and steady level by pharmacological means (20,23). The residual oscillations of insulin secretion that are observed under such conditions (29,30) are irregular and of small amplitude compared with those accompanying the normal $[Ca^{2+}]_i$ oscillations (31). The fluctuations of insulin secretion that we observed in a few Cx36^{-/-} islets during stimulation with glucose and forskolin also paralleled the transient $[Ca^{2+}]_i$ increases, suggesting that a weak synchronization among some β -cells may persist after the loss of Cx36, presumably as a result of the signaling achieved by diffusible factors (27,28,32). The absence of insulin pulsatility observed in glucose-stimulated Cx36^{-/-} islets cannot be attributed to defects in the action of Ca^{2+} on exocytosis, because oscillations of $[Ca^{2+}]_i$ imposed by KCl pulses were able to trigger synchronized pulses of insulin secretion. Thus, the observation that the loss of glucose-induced $[Ca^{2+}]_i$ oscillations is associated with the loss of

pulsatility of insulin secretion supports the view of a moment-to-moment regulation of insulin secretion by changes in $[Ca^{2+}]_i$ within β -cells (33). In contrast with the loss of oscillations, the levels of $[Ca^{2+}]_i$ were only slightly affected in glucose-stimulated Cx36^{-/-} islets. Glucose induces a concentration-dependent rise in $[Ca^{2+}]_i$ in islets and clusters of islet cells from normal mice (34,35). A similar sigmoidal relation characterized the effects of increasing glucose concentrations on $[Ca^{2+}]_i$ in islets of all mice, indicating that uncoupled β -cells retain the ability to gradually increase the signal triggering insulin secretion. Also, the loss of Cx36 did not impede overall insulin output, which was similar in the three groups of islets at all stimulatory glucose concentrations. Previous studies have shown that alterations in the level of Cx36 impair glucose-induced insulin secretion in vitro (8–11). Our observations on mice generated in the absence of Cx36 challenge this view, presumably implying that under such conditions, the lost connexin signaling was somehow compensated. Functional compensation has been documented in neurons lacking Cx36, presumably explaining the subtle neurophysiological phenotype that results after the total loss of connexin (15). It is therefore conceivable that one or more of the many factors that modulate insulin secretion (1–3) counterbalances the effects resulting from the lack of Cx36 in β -cells.

Third, islets lacking Cx36 showed a significant increase in the basal release of insulin, which was observed when both isolated islets and perfused pancreas were exposed to nonstimulatory glucose concentrations (1.4–3 mmol/l). The selectivity of this alteration, contrasting with the preserved glucose-responsiveness of the same islets and the previous observations of increased basal release of insulin from β -cells lacking junctional contacts (36,37) supports a causal role for the loss of Cx36 in altered basal release. Basal $[Ca^{2+}]_i$ was not sufficiently elevated to account for the increase in insulin secretion in the presence of low glucose. It is conceivable, however, that a continuous elevation rather than periodic elevations of $[Ca^{2+}]_i$ in Cx36^{-/-} islets during stimulation by nutrients may affect gene expression (38,39) or induce functional alterations of β -cells (40,41), two effects that could account for increased basal insulin. At this stage, our study provides evidence that the mechanism leading to this increase does not involve alterations in the development of the endocrine pancreas or the differentiation of β -cells. Eventually, this mechanism is not operative in Cx36^{+/-} mice, which were phenotypically similar to control littermates for all the parameters we evaluated despite a 50% decrease in coupling. These data indicate that native levels of Cx36 provide β -cells with a safety factor that permits them to preserve normal function, including proper synchronization of glucose-induced calcium and insulin transients, until >50% of the connexin protein is lost.

Several studies have shown that in humans, plasma insulin concentrations oscillate independently of changes in plasma glucose and that these oscillations are caused by pulsatile insulin secretion (42,43). It has also been reported that these oscillations are disorganized in patients with type 2 diabetes and relatives with mild glucose intolerance (42,44–46). Glucose-induced $[Ca^{2+}]_i$ changes are also perturbed in islets of *db/db* (47) and *ob/ob* mice

(18). However, the Cx36^{-/-} mice were not glucose intolerant nor did they spontaneously develop hyperglycemia with aging and increasing transgenic generations (data not shown). Thus, the relation between $[Ca^{2+}]_i$ oscillations and insulin secretion of individual islets on the one hand and whole-body glucose homeostasis on the other hand is not as linear and obligatory as previously thought (23,43,46,47). First, by analogy with the human situation, it has been surmised, but not demonstrated, that oscillations of plasma insulin concentrations also exist in mice and have an impact on the glucose tolerance of the animals. Second, it remains unclear how the pulsatility of individual islets in situ is coordinated to result in the pulsatility of insulin secretion by the whole pancreas. Third, in the general deletion mouse model that we studied, Cx36 was also absent in many neuronal populations (12,15,48), some of which presumably contribute to the control of blood glucose (49).

In summary, our data provide the first direct evidence that communication via Cx36 channels is a central event in the synchronization of Ca^{2+} oscillations among β -cells within individual pancreatic islets and that disruption of this connexin-dependent synchronization results in the loss of the insulin pulses normally elicited by glucose and in increased basal release of the hormone.

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