Connexins protect mouse pancreatic β cells against apoptosis

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Abstract
Type 1 diabetes develops when most insulin-producing β cells of the pancreas are killed by an autoimmune attack. The in vivo conditions modulating the sensitivity and resistance of β cells to this attack remain largely obscure. Here, we show that connexin 36 (Cx36), a trans-membrane protein that forms gap junctions between β cells in the pancreatic islets, protects mouse β cells against both cytotoxic drugs and cytokines that prevail in the islet environment at the onset of type 1 diabetes. We documented that this protection was at least partially dependent on intercellular communication, which Cx36 and other types of connexin channels establish within pancreatic islets. We further found that proinflammatory cytokines decreased expression of Cx36 and that experimental reduction or augmentation of Cx36 levels increased or decreased β cell apoptosis, respectively. Thus, we conclude that Cx36 is central to β cell protection from toxic insults.

Reference

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Type 1 diabetes develops when most insulin-producing β cells of the pancreas are killed by an autoimmune attack. The in vivo conditions modulating the sensitivity and resistance of β cells to this attack remain largely obscure. Here, we show that connexin 36 (Cx36), a trans-membrane protein that forms gap junctions between β cells in the pancreatic islets, protects mouse β cells against both cytotoxic drugs and cytokines that prevail in the islet environment at the onset of type 1 diabetes. We documented that this protection was at least partially dependent on intercellular communication, which Cx36 and other types of connexin channels establish within pancreatic islets. We further found that proinflammatory cytokines decreased expression of Cx36 and that experimental reduction or augmentation of Cx36 levels increased or decreased β cell apoptosis, respectively. Thus, we conclude that Cx36 is central to β cell protection from toxic insults.

Introduction
Pancreatic β cells are functionally heterogeneous (1) and interconnected by channels made of connexin 36 (Cx36) (2–4), a member of the connexin (Cx) protein family (5). Previous studies have implicated this protein in the synchronization of stimulus-induced Ca²⁺ waves between β cells and the electrotonic spread of depolarizing and hyperpolarizing currents within the islets (6–8), as well as in the regulation of basal and stimulated insulin secretion (6–10) and insulin hyperpolarizing currents within the islets (6–8), as well as in the regulation of basal and stimulated insulin secretion (6–10) and insulin waves between Cx36+ and Cx36−/− cells. These experiments showed that loss of Cx36 sensitized β cells to this attack remain largely obscure. Here, we show that connexin 36 (Cx36), a trans-membrane protein that forms gap junctions between β cells in the pancreatic islets, protects mouse β cells against both cytotoxic drugs and cytokines that prevail in the islet environment at the onset of type 1 diabetes. We documented that this protection was at least partially dependent on intercellular communication, which Cx36 and other types of connexin channels establish within pancreatic islets. We further found that proinflammatory cytokines decreased expression of Cx36 and that experimental reduction or augmentation of Cx36 levels increased or decreased β cell apoptosis, respectively. Thus, we conclude that Cx36 is central to β cell protection from toxic insults.

Results
Comparison of mice featuring β cells with different Cx patterns. To test the influence of Cx on the in vivo resistance of β cells, we compared mice whose Cx (a) differed in Cx36 gene dosage (Cx36+/+; Cx36−/−; ref. 7; 8, 13); (b) were specifically targeted, through the control of the rat insulin promoter (RIP), for the expression of transgenic Cx36 (RIP-Cx36+/+; RIP-Cx36−/−; ref. 14); or (c) coexpressed Cx36 with either Cx32 (RIP-Cx32+/+; RIP-Cx32−/−; ref. 11) or Cx43 (RIP-Cx43+/+; RIP-Cx43−/−; ref. 14), whose de novo β cell-specific expression was also directed by RIP. The control mice of all lines expressed Cx36, even though the native levels of this Cx were higher in Cx36−/− mice than in RIP-Cx36+/+, RIP-Cx32−/−, and RIP-Cx43−/− mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI40509DS1). Cx36−/− mice did not express Cx36 (Supplemental Figure 1). RIP-Cx36+/+ and RIP-Cx36−/− mice expressed higher levels of Cx36 than did Cx36+/+ and RIP-Cx36−/− mice (Supplemental Figure 1). RIP-Cx32 and RIP-Cx43 mice expressed Cx32 and Cx43, respectively, in addition to native Cx36 (Supplemental Figures 1 and 2). The international nomenclature of the mouse lines, and the abbreviated names used herein, are shown in Supplemental Figure 3. Experiments were performed with null and transgenic mice that had been backcrossed with C57BL/6J mice for 5–10 (Cx36) or 3–8 (RIP-Cx36, RIP-Cx32, and RIP-Cx43) generations.

Loss of Cx36 sensitizes mice to the effects of cytotoxic drugs. To test whether reduced levels of Cx36 modified the in vivo resistance of β cells, mice of the Cx36 line were given a single i.p. injection of 200 mg/kg BW STZ. The drug caused Cx36−/− mice to develop a mild hyperglycemia (Figure 1A). This alteration was more pronounced in Cx36−/− littermates, and even further enhanced in Cx36−/− littermates (Figure 1A), which lack Cx36 (refs. 7, 8, 13, and Supplemental Figure 1). These differences were the result of a larger drop in insulin content and in the mass of residual β cells (Figure 1, B and C). After injection of 60 mg/kg BW AX, a drug that kills β cells by a different mechanism than STZ (11), Cx36−/− mice also became more hyperglycemic than Cx36+/+ and Cx36−/− littermates (Figure 1D). These experiments showed that loss of Cx36 sensitized β cells to the in vivo effects of 2 cytotoxic drugs.

Overexpression of Cx36 protects transgenic mice against cytotoxic drugs. To test whether increased levels of Cx36 could protect β cells, we repeated the experiments in transgenic mice of the RIP-Cx36 line, which we developed to overexpress Cx36 in β cells (Supplemental Figures 3 and 4). RIP-Cx36+/− mice, which had the same Cx36 gene dosage as Cx36−/− mice, expressed lower levels of Cx36 protein (Supplemental Figures 1, 3, and 4) and became more hyperglycemic after injection of STZ (Figure 2A), caused by a marked loss of pancreatic β cells and insulin content (Figure 2, B and C). These alterations were significantly decreased in RIP-Cx36+/- and RIP-Cx36−/− littermates, which featured increased insulin content and levels of Cx36 (Figure 2, A–C, and Supplemental Figures 1 and 4). Comparable observations were made after injection of AX (Figure 2D). These experiments showed that increased expression of Cx36 protected β cells against the in vivo effects of 2 cytotoxic drugs.
Pancreatic islets are protected by Cx36 in vitro. To assess whether the Cx36-dependent protection was an intrinsic property of pancreatic islets, we exposed islets isolated from Cx36 and RIP-Cx36 mice to STZ. After 15 hours of exposure to 2.2 mM (Cx36+/+ line) or 4.4 mM (RIP-Cx36 line) STZ, the islets of Cx36+/+ and RIP-Cx36–/– mice, which had the same Cx36 dosage, featured a comparable proportion of ethidium bromide–labeled (EB-labeled) dead cells (Figure 3, A–C). This proportion was increased in the islets of Cx36–/– mice (Figure 3B). Islets from RIP-Cx36+/– and RIP-Cx36+/+ mice contained mostly living, calcein-labeled cells (Figure 3C). These observations showed that in 2 independent mouse lines, islets differing in Cx36 expression also differed in sensitivity to STZ.

To investigate such a difference, we assessed whether increased levels of Cx36 abolished the expression and function of the GLUT-2 transporter, preventing the uptake of both STZ and AX (11, 17, 18), in islets of RIP-Cx36 mice. Immunolabeling of pancreatic sections showed expression of GLUT-2 in most β cells of all mouse genotypes and proper localization of the protein at the cell membrane (Supplemental Figure 5C). Most β cells also incorporated 6-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-6-deoxyglucose (NDBG), a nonhydrolyzable analog of glucose that enters β cells only via GLUT-2 (ref. 17 and Supplemental Figure 5D). These experiments showed that the resistance of RIP-Cx36 islets against STZ and AX could not be attributed to loss of the transporter whereby these drugs enter β cells.
To test whether Cx36 also protects β cells against endogenous signals implicated in the pathogenesis of type 1 diabetes, we exposed islets to a cytokine mix of 50 U/ml IL-1β, 1,000 U/ml IFN-γ, and 1,000 U/ml TNF-α for a 24-hour period (15, 16, 19, 20) and monitored β cell apoptosis. In islets of RIP-Cx36−/− and Cx36+/+ mice, which had a similar Cx36 gene dosage, TUNEL labeling showed that 2 cytokines in combination — and all 3 even more so — increased the proportion of apoptotic β cells (Figure 3, D–F). Higher numbers of apoptotic β cells were found in islets of Cx36+/− and Cx36−/− mice (Figure 3E). Conversely, lower numbers of apoptotic β cells were found in islets of RIP-Cx36+/− mice (Figure 3F), which showed increased levels of Cx36 compared with RIP-Cx36−/− mice (Supplemental Figures 1, 2, and 4). Our findings indicated that Cx36 protected β cells against the cytokines found in the islet environment at the early stages of an autoimmune attack.

**Th1 cytokines similarly activate apoptotic pathways in β cells featuring different Cx36 expression.** To investigate the molecular mechanism of the β cell protection, we first assessed the production of NO, which is released by β cells exposed to cytokines (19, 20). We found that similar levels of nitrite, a stable product of NO oxidation, accumulated in the media of all islets exposed for 24 hours to IL-1β, IFN-γ, and TNF-α, regardless of the Cx36 levels of their β cells (Figure 4, A and B). Immunolabeling for cytochrome C resulted in the staining of bright spots, compatible with a mitochondrial localization of the enzyme, in both MIN6 cells and primary β cells of untreated Cx36+/+ and RIP-Cx36−/− mice (Supplemental Figure 6, A–C). After exposure...
to the cytokines, which caused β cell apoptosis (Figure 4, C and D), many MIN6 cells and β cells of Cx36+/− and RIP-Cx36−/− islets showed less punctate and more homogeneous labeling (Supplemental Figure 6, A–C), consistent with the release of cytochrome C into the cytosol. The cytokine-induced apoptosis was unaffected by cyclosporin A in both RIP-Cx36−/− and RIP-Cx36+/− islets (Figure 4C), which indicated that under these conditions, cytochrome C was not released through the canonical permeation transition pore (19–22). Furthermore, apoptosis was similarly inhibited in RIP-Cx36−/− (P < 0.01) and RIP-Cx36+/− (P < 0.05) islets by exposure to 50 μM of the pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-FMK (Z-VAD), added 1 hour before exposing the islets to cytokines and maintained throughout the 24-hour duration of the experiments (Figure 4D). These findings indicate that islets featuring different Cx36 levels were similar with regard to the receptors and signaling cascades that lead to caspase-dependent β cell apoptosis.

**Junctional signaling is increased in the protected islets.** To test whether the β cell protection provided by Cx36 depends on the establishment of functional gap junctions, we used the STZ model of β cell toxicity, which has been shown to induce massive apoptosis of β cells (4). Cx36 protects β cells from Th1 cytokines. (A) Islets isolated from C57BL/6 mice showed much more healthy cells (calcein stain; green) than dead cells (EB stain; red). The proportion of dead cells substantially increased after 24 hours exposure to STZ. (B) In islets of Cx36−/− mice, STZ (black) increased the volume density (Vv) of dead cells over that in untreated islets (white) and in islets exposed to citrate buffer (gray). The volume density of dead β cells was lower in the islets of STZ-treated Cx36+/+ mice than in those of Cx36−/− and Cx36+/− littermates. (C) Dead cell volume density was significantly higher in STZ-treated RIP-Cx36−/− islets than in islets of RIP-Cx36−/− and RIP-Cx36+/− littermates. (D) TUNEL labeling showed that Cx36+/+ islets contained rare apoptotic cells (green). The number of apoptotic cells increased after 24 hours exposure to IL-1β, IFN-γ, and TNF-α. (E) Exposure of Cx36−/− islets to either IL-1β and IFN-γ (gray) or IL-1β, IFN-γ, and TNF-α (black) increased the volume density of apoptotic cells over that in untreated mice (white). Apoptosis was lower in the islets of cytokine-treated Cx36+/+ mice than in those of Cx36−/− and Cx36+/− littermates. (F) The volume density of apoptotic cells was higher in RIP-Cx36−/− islets than in islets of RIP-Cx36−/− littermates exposed to the 3 cytokines. Values are medians of the indicated number of experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus respective cognate control (Cx36+/+ or RIP-Cx36−/−).
ment of intercellular channels, we dissociated the cells of isolated islets. Exposure to 1.1 mM STZ for 15 hours resulted in a comparable increase of EB-stained necrotic cells from Cx36+/-, Cx36+/–, and Cx36–/– mice (Figure 5A). Because cytokines induce both necrosis and apoptosis of rodent β cells (23–26), we further isolated islet cells from the Cx36 and RIP-Cx36 lines and exposed them for 36 hours to the 3 cytokines described above. These conditions markedly increased the proportion of annexin V–EGFP– and propidium iodide–stained β cells in Cx36+/+ and RIP-Cx36–/– mice (Figure 5B and Supplemental Figure 7). Similar proportions of apoptotic and necrotic single β cells were found in samples dispersed from islets of Cx36–/– and RIP-Cx36–/– mice (Figure 5B). Our observations indicated that all β cells featured a similar sensitivity to a cytotoxic environment when junctional coupling was abrogated.

To test whether the protection dependent on Cx36 could be provided by other types of Cx channels, we tested the RIP-Cx32 and RIP-Cx43 lines. After injection of STZ, control RIP-Cx32–/– and RIP-Cx43–/– mice became hyperglycemic; in contrast, the corresponding heterozygous and homozygous littermates remained normoglycemic throughout the experiment (Figure 5, F and G). These observations suggested that other Cx isoforms can protect β cells in vivo in the presence of native levels of Cx36.

To test whether Cx-mediated coupling is a critical event in this protection, we microinjected isolated islets with either EB or Lucifer Yellow (LY), which differently permeate Cx36, Cx32, and Cx43 channels (27, 28). In islets of Cx36+/+ and RIP-Cx36–/– mice, coupling of β cells was larger when evaluated with EB than LY (Figure 6). This coupling was absent in islets of Cx36–/– mice. When tested with EB, the extent of coupling was greater in the islets of RIP-Cx36+/+ mice. β cells of RIP-Cx43–/– and RIP-Cx32–/– mice also showed a control coupling pattern. In contrast, homozygous littermates of each line featured much larger territories of coupled β cells when tested with LY (Figure 6). The incidence of coupling varied little in the different islet groups, except in the Cx36–/– mice, in which it was nil (Supplemental Figure 8).

TH1 cytokines decrease Cx36 expression, and experimental alterations in Cx36 expression modulate the cytokine-induced death of insulin-producing cell lines. We further tested whether the conditions eliciting β cell apoptosis affected the expression of Cx36. Using the insulin-producing MIN6 and INS1E cell lines, which share with β cells the native expression of Cx36 (6, 10), we found that the mix of cytokines mentioned above also elicited apoptosis and release of cytochrome C (Figure 5C and Supplemental Figure 6). Under these conditions, the levels of Cx36 mRNA and of the cognate protein...
Figure 5
β cell protection requires cell contact and Cx expression. (A) The proportion of dead cells was similar in all control islet cell suspensions. STZ similarly increased this proportion in all groups, n as indicated. (B) Islet cells of control and homozygous mice of both Cx36 and RIP-Cx36 lines showed increased apoptosis (white) and necrosis (gray) after exposure to the cytokine mix of IL-1β, IFN-γ, and TNF-α. (C) The cytokine mix increased apoptosis of INS1E cells and control C57BL/6 islets. (D) The cytokine mix decreased Cx36 mRNA in mouse islets and in MIN6 and INS-1E cells. Values are expressed relative to the L27 gene level. (E) The cytokine mix also decreased Cx36 in extracts of INS1E and MIN6 cells. Values are shown relative to the tubulin signal, normalized to control. Cx36 and actin Western blot immunolabeling, from which the quantitative data were generated, is also shown (inset). **P < 0.01, ***P < 0.001 versus corresponding control. (F) STZ injection induced hyperglycemia in RIP-Cx32−/− mice, but not in RIP-Cx32+/− and RIP-Cx32+/+ littermates. (G) RIP-Cx43−/− mice also became hyperglycemic after STZ injection, whereas RIP-Cx43−/− and RIP-Cx43+/− littermates did not. Data are mean ± SEM of 4–6 experiments (A), of 3–5 experiments (B–E), or of 3–12 mice (F and G).
were reduced in both cells (Figure 5, D and E, and Supplemental Figure 9). At least in cell lines, this decrease was mostly caused by IL-1β (Figure 5D), the cytokine that predominantly causes β cell apoptosis (24-26). Thus, the cytokines that are cytotoxic to β cells downregulated the levels of Cx36.

To test whether experimental down- and upregulation of Cx36 affected cytokine-induced apoptosis, we first transfected INS1E cells with 2 different siRNAs specifically targeting Cx36 mRNA or with an irrelevant control siRNA that did not alter the cytokine-induced downregulation of Cx36 (Supplemental Figure 9). Compared with this control, the 2 Cx36-specific siRNAs reduced by about 50% the levels of Cx36 (Supplemental Figure 9). Under these conditions, different combinations of IL-1β, TNF-α, and IFN-γ increased the incidence of INS1E cell apoptosis compared with both the control levels observed in the absence of the cytokines and the levels observed in cells exposed to these cytokines after transfection with the control siRNA (Supplemental Figure 9). In a second set of experiments, WT MIN6 cells were compared with a clone of companion cells that we selected for their spontaneously higher expression of Cx36, which was shown to be stable for years, and to a clone of MIN6 cells transfected with an antisense cDNA for mCx36 (1). Immunostaining of both intact cells and protein extracts revealed that the 3 clones featured markedly different levels of Cx36 protein (in decreasing order of expression: overexpressing MIN6 cells, WT MIN6 cells, and antisense MIN6 cells), which were reduced after overnight exposure of each clone to the Th1 cytokines mentioned above (Supplemental Figure 9). Under such conditions, the incidence of total cell death resulting from both apoptosis and necrosis significantly increased after exposure to the cytokines, and this increase was inversely related to the levels of Cx36 (in decreasing order of expression: antisense MIN6 cells, WT MIN6 cells, overexpressing MIN6 cells; Supplemental Figure 9). When only apoptosis was scored, the clone of MIN6 cells overexpressing Cx36 was significantly less affected by cytokines than either the control or the antisense clone (Supplemental Figure 9). These observations indicate that the experimental down- and upregulation of Cx36 increased and decreased, respectively, the cytokine-induced death of different lines of insulin-producing cells.

Discussion
In the present study, we found that Cx36, the protein that forms gap junctions of native pancreatic islets (1-9), protected the insulin-producing β cells against both cytotoxic drugs and the Th1 cytokines that predominate in the islet environment at the onset of type 1 diabetes (15, 16, 19-22). Thus, β cells lacking Cx36 were more sensitive to the lethal effects of these molecules, whereas β cells overexpressing Cx36 were protected against proapoptotic conditions both in vivo and in vitro.

Although different mouse strains are known to have different sensitivities to β cell–toxic insults (29, 30), within each mouse line studied herein, we found no obvious intrinsic difference in the behavior of individual β cells obtained from WT, heterozygous, and homozygous null animals. Thus, in all the animal models investigated, β cells expressed similar key molecules and pathways, including the GLUT-2 transporter, which is required for the uptake of STZ and AX (11, 18, 31); produced similar levels of NO when exposed to cytokines; and activated the caspasases, which are the main mediators of cytokine-induced β cell apoptosis (15, 16, 19). Accordingly, disassociated β cells of all mice were equally sensitive to the cytokine-activated apoptotic mechanisms. In contrast, intact islets isolated from the native vascular, neural, and exocrine pancreas environment revealed that the levels of Cx36 modulated the sensitivity of β cells to identical cytotoxic conditions, implying an islet-autonomous difference.

This difference can first be accounted for by a negative effect of Th1 cytokines on Cx36 expression. Thus, the mix of IL-1β, IFN-γ and TNF-α, which promoted β cell apoptosis, also markedly decreased the levels of Cx36. Specifically, the cytokine IL-1β, the predominant trigger of β cell apoptosis (16, 19-26), also had the largest effect on decreasing the levels of Cx36 transcript and protein. Conversely, the in vitro down- and upregulation of Cx36 expression inversely related to the proapoptotic effect of the Th1 cytokines. The mechanism underlying this relationship remains to be unraveled and may involve any intermediate of the complex cascade of signal molecules that ultimately elicits a (pro)apoptotic effect. Whatever this intermediate, our data are consistent with the view that β cells expressing high levels of Cx36 and exposed to cytokines can retain sufficient levels of the Cx to somewhat resist immunological aggression, whereas β cells featuring low levels of Cx36 can become essentially deprived of this protein and thus be sensitized to the aggressive conditions.

While our study has not identified the mechanism whereby Cx36 protects β cells, it indicates that the β cell–to–β cell transfer of gap junction–permeant moieties may be key to this protection. First, all the pancreatic islets that were protected against both pharmacological and immunological attacks featured significantly greater β cell coupling than did normally sensitive native islets, in which this coupling is of modest amplitude (1-4, 7-9). Conversely, the islets of Cx36-/- mice, in which this coupling is absent (7-9), were more sensitized to the same conditions. Second, in the presence of native Cx36 levels, the protection of β cells was increased by the de novo expression of either Cx32 or Cx43, 2 isoforms that are not natively expressed by β cells (1, 2, 4) and form channels with biophysical and regulatory characteristics different from those made of Cx36 (5, 27, 28). It would be important to validate this tentative conclusion using drugs that specifically and reversibly block Cx36 channels. At this time, however, the available drugs all have pleiotropic effects, and cannot sustain cell uncoupling for a time sufficient to observe the eventual effects on cell function and death without inducing multiple confounding effects (32, 33).

It is worth stressing that the Cx36-dependent protection was effective against molecules that activate a variety of signaling pathways within β cells, including DNA alkylation, depletion in NAD+ and ATP, liberation of NO, production of reactive oxygen species, and [Ca2+] alterations (11, 18, 19-26, 31). Several of the second messengers, which are central to these pathways and converge to modulate β cell apoptosis, permeate Cx channels (5, 27, 28). Therefore, it is conceivable that toxic molecules generated by a proapoptotic trigger, or their metabolites, may be more efficiently diluted within the increased volume of cytoplasm that results from enlarged Cx-dependent cell coupling. This dilution would be impeded within both single cells, which cannot establish functional Cx channels, and β cells that are uncoupled from their neighbors, as a result of downregulation or loss of Cx36 (7-9). This mechanism does not exclude the parallel possibility that increased β cell communication could also allow a healthy β cell to rescue an adjacent dying cell with an as-yet putative survival factor that could be transferred through Cx36 channels. Indeed, a unique feature of Cx channels is the permeability to multiple moieties and the bidirectional diffusion of these molecules (5, 27, 28), specifi-
Intra-islet Cx36 functions as a prosurvival molecule via a mechanism independent of the formation and function of gap junctions, without affecting the exchange of cationic and anionic tracers. We show that Cx36 expression and junctional coupling in islets of Cx36+/– and RIP-Cx36+/– mice were greater in islets resistant to cytotoxic insults. Cx36+/– mice were protected against a variety of insults, including STZ and AX treatment, in contrast to Cx36–/– mice. RIP-Cx36+/– mice protected against STZ and AX treatment. Figure 6 shows the increased junctional coupling in islets resistant to β cell–toxic conditions. (A–E) Microinjection of islets with LY showed limited β cell coupling in islets of Cx36+/+ and RIP-Cx36+/+ mice (A and C), no coupling in islets of Cx36–/– mice (B), and greater coupling in islets of RIP-Cx43+/+ and RIP-Cx32+/+ mice (D and E). (F–J) Microinjection with EB showed sizable coupling in islets of Cx36+/+, RIP-Cx43+/+, and RIP-Cx32+/+ mice (F, I, and J), no coupling in islets of Cx36–/– mice (G), and greater coupling in islets of RIP-Cx36+/– animals (H). Scale bar: 100 μm. (K and L) Coupling extent in islets from Cx36+/+, Cx36+/–, RIP-Cx43–/–, and RIP-Cx32–/– mice. Values show medians of the indicated number of islet microinjections. *P < 0.05, **P < 0.01, ***P < 0.001, median test.
levels of Cx36, whereas the latter ones lacked this Cx. These data, which are consistent with the differential sensitivity of different mouse lines to the effects of both STZ and AX (29, 30), indicate that several factors in addition to Cx36 contribute to control β cell resistance, and thus the levels of circulating glucose.

The finding that this protein is expressed and functions in the islets of the human pancreas (3), in a pattern like that seen in rodent islets (2, 4, 7, 8), strengthens the interest to search for pharmacological tools promoting Cx36 expression and/or Cx36 channel function (1, 32). From the results of this study and previous reports (7–10), the prediction would be that these agents could foster both the survival of β cells and their insulin secretion. In this context, it is interesting to note that bilendamide, one of the rare molecules that increases gap junctions (32, 33), promotes the Cx36-dependent coupling of rodent β cells in vivo (27, 32, 37, 38). This sulphonylurea is largely used to stimulate insulin release from the glucose-insensitive islets of type 2 diabetic patients (39) and appears to have some antiapoptotic effect on human islets (40).

The search for novel drugs specifically targeting Cx36 and inducing larger and more selective effects now requires the development of devoted methods and innovative algorithms (41). Hopefully, some of these drugs could block the apoptosis that decreases β cell mass in both type 1 and type 2 diabetes (1, 32). Given that Cx36 is mostly restricted to pancreatic β cells, neurons, and few related neuron-endocrine cells (5, 13, 32, 42), such pharmacological tools may also be of value in a variety of neurodegenerative diseases.

Methods

Animals. Mice of the Cx36 line were generated by homologous recombination (13). The RIP-directed lines were generated by microinjecting a Cx36 (12), Cx36, or Cx43 (14) transgene into zygotic pronuclei (Supplemental Figures 2 and 5 and Supplemental Methods). Genotype of littermates was determined by PCR amplification of tail DNA (7, 9, 12–14), 3- to 6-month-old mice backcrossed with C57BL/6 controls were used in all experiments. Experiments in RIP-Cx32, RIP-Cx36, and RIP-Cx43 lines were initiated with mice of the third generation and repeated with mice of the eighth generation; experiments in the Cx36 line were initiated with mice of the seventh generation and repeated with mice of the tenth generation. No significant difference was observed between generations for the initial and repeat experiments (data not shown).

In vivo treatments. STZ (Sigma-Aldrich) was freshly dissolved in 10 mM citrate buffer, pH 4.5, and 200 mg/kg BW was immediately injected i.p. (11, 18, 31). AX (Sigma-Aldrich) was freshly dissolved in 0.9 % NaCl containing 1 mM HCl, and 60 mg/kg BW (Cx36 mice) or 70 mg/kg BW (RIP-Cx36 mice) was immediately injected i.v. (11, 18, 31). Levels of circulating glucose were measured in tail blood (7, 9, 12, 14).

In vitro experiments. RPMI 1640 medium was supplemented with one of the following: 10 mM citrate buffer (all controls); 1.1 mM (single islet cells), 2.2 mM (islets of the Cx36 line), or 4.4 mM (islets of the RIP-Cx36, RIP-Cx32, and RIP-Cx43 lines) STZ for 15 and 24 hours (single islet cells and isolated islets, respectively); 50 μM IL-1β (R&D Systems) plus 1,000 U/ml IFN-γ (Biosciences) and 1,000 U/ml TNF-α (R&D Systems) for 24 and 36 hours (islets and islet cells, respectively); 10 μM cyclosporin A (Calbiochem); 50 μM Z-VAD (Enzyme System Products). The latter 2 drugs were added 1 hour before addition of the cytokines and maintained thereafter throughout the experiment.

Other methods. Procedures for islet and cell isolation, culture, transfection, immunofluorescence, biochemistry, apoptosis, and necrosis tests and dye coupling experiments were as previously reported (2–4, 6–10, 12, 14, 27). See Supplemental Methods for details.

Statistics. Values are expressed as either mean ± SEM or median (depending on whether they had normal or non-Gaussian distribution, respectively) of the indicated number of experiments. Comparison of means was made by 1-way ANOVA, whereas comparison of medians was made using a median test. The additional nonparametric Kolmogorov-Smirnov, Mann-Whitney, and χ2 tests were further used to assess differences between non-Gaussian distributions. All tests were run using SPSS (Windows version; SPSS Inc.). P values less than 0.05 were considered significant.

Study approval. All animal experiments were conducted as per the regulations of the Geneva veterinary office (Geneva, Switzerland; authorization no. 1034/3552/1).

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