Inhibition of connexin 36 hemichannels by glucose contributes to the stimulation of insulin secretion

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ISLET β-CELLS ARE STIMULATED specifically to secrete insulin by glucose in a physiological concentration range. The stimulation requires glucose to be metabolized to increase the cytosolic ATP concentration and/or the ATP/ADP ratio, and the β-cells then become depolarized by closure of the ATP-dependent membrane KATP channels (18). Subsequent opening of voltage-dependent Ca2+ channels ensues and allows an increase of the cytosolic Ca2+ concentration that finally triggers an increase in insulin secretion (28). A stimulatory glucose concentration (>5 mM, between 6 and 7 mM) triggers a biphase response initiated by a first phase, followed by a second phase that increases progressively with time or remains flat, depending on the experimental model (rat vs. mouse islets, respectively). The first phase of insulin secretion can be mimicked by depolarizing β-cells with a high K+ solution at a basal non-stimulatory glucose concentration (5 mM), whereas the mechanism responsible for the second phase has been argued to be due to the increase of a so far unknown glucose metabolite or cofactor (17).

β-Cells within the pancreatic islets are not independent of their neighboring cells. They are electrically and chemically coupled through gap junction channels (4, 6, 34). They provide the needed synchronization of the membrane depolarization, spike activity, and cytosolic calcium oscillations among β-cells for an appropriate glucose-induced insulin release. Connexin 36 (Cx36) has been identified as the principal molecular component of gap junction channels between β-cells in both rodents and humans (3). Connexins oligomerize into hexameric connexons or hemichannels in the Golgi organ, and at the plasma membrane of β-cells, they exchange ions, second messengers, and metabolites between the interior of the two coupled cells (3, 24). The relevance of Cx36-based channels for an adequate secretory response to glucose stimulation has been evidenced using transgenic mice with germline knockout (3, 16, 29) or β-cell-specific knockout of the Cx36 gene (38). These studies have shown partially contradictory results concerning glucose-induced insulin secretion since either an increased basal rate of insulin secretion; islets; connexin 36; hemichannels; gap junction channels; glucose

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β-CELLS’ HEMICHANELS ARE CLOSED BY GLUCOSE

secretion (3, 29, 38) and a preserved stimulation by high glucose (3, 29) or a significant reduction (16, 38) with (16) or without (38) glucose intolerance has been reported. Although connexins are generally considered to mediate cell-to-cell communication by forming gap junction channels, many if not all connexin isoforms are also able to form open solitary hemichannels for rapid exchange of ions, second messengers, cofactors, and metabolites between the cell interior and interstitial space (13, 31, 35). In the particular case of Cx36 hemichannels expressed in mouse interneurons, it has been shown that they can release ATP upon depolarization with a high K⁺ solution (33, 37). Pannexins are paralogs of connexins that can only form hemichannels (31). Both types of hemichannels, connexons and pannexons, are opened by membrane depolarization and divalent cation omission or other mechanisms (31).

We have demonstrated previously that rat islet preexposure to KCl depolarization suppresses the subsequent stimulation of insulin secretion by high glucose (27). A similar effect was also observed in mouse islets (15). Moreover, the second phase of stimulation of insulin secretion by rat-perifused islets in response to 10 mM α-ketoisocaproic acid was practically abolished by the simultaneous depolarization with 70 mM KCl, and it was accompanied by a strong release of islet amino acids (mainly GABA and Tau) (26). These previous observations are consistent with the presence of Cx36 hemichannels activated by depolarization in the β-cells. Thus, we have postulated that the two possible functions of connexins, the intercellular and transmembrane exchanges of ions and small molecules, might coexist in β-cells and play different roles in the mechanism of glucose stimulation of insulin secretion. To address this issue, we have studied the electrophysiological properties of hemichannels formed by the expression of Cx36 in Xenopus oocytes, the exchange of adenine nucleotides with the extracellular medium, and the glucose stimulation of insulin secretion in rat and murine islets from wild-type and transgenic mice with a germlinal knock out of the Cx36 gene. A good correlation was found between the expression level/activity of hemichannels and the release or uptake of adenine nucleotides across the plasma membrane depolarized with KCl at 5 mM glucose. ATP changes were antagonized by mefloquine, a connexin inhibitor, and glucose and these two modulating effects were confirmed in Xenopus laevis oocytes expressing Cx36. We postulate that closing of Cx36 hemichannels by high glucose might contribute toward generating an optimal elevation of intracellular ATP (and ATP/ADP ratio) and the subsequent stimulation of insulin secretion. We also conclude that the lack of gap junction channels impairs the in vitro stimulation of insulin secretion by glucose and makes the Cx36−/− animals partially intolerant to glucose.

MATERIALS AND METHODS

Materials. Collagenase P and free fatty acid bovine serum albumin were obtained from Roche Diagnostics (Barcelona, Spain). Bovine serum albumin, mefloquine, enzymes, and coenzymes were obtained from Sigma-Aldrich Quimica (Madrid, Spain). Rat insulin standards were from Linco Research, (St. Charles, MO). Na⁺-125I was obtained from Perkin-Elmer España (Madrid, Spain). Inorganic compounds were obtained from Perkin-Elmer España (Madrid, Spain). Insulin antiserum was kindly provided by Dr. M. Villanueva-Peñacarrillo from the Department of Metabolism, Nutrition & Hormones, Fundación Jiménez Díaz, Madrid, Spain. Islet insulin content was measured radioimmunologically in three batches each of 10 islets of a variable number of animals (n = 6 or 8, as given in RESULTS) after ethanol-acid (77% ethanol + 1% concentrated hydrochloric acid) extraction (50 μl).

In all perifusion and incubation media used in islet experiments, Ca²⁺ omission means that the extracellular [Ca²⁺] was 0 mM and that 100 μM EGTA is added to the KRBH. Depolarization with KCl (15, 30, and 70 mM) was always accompanied by addition of 250 μM diazoxide; the increment of extracellular KCl above the standard value of KRBH (5.9 mM) was compensated routinely with a reduction of NaCl osmolality.

Islet adenine nucleotide measurement. Total islet (rat or mouse) ATP content was measured with the luciferin/luciferase system. Three groups of 25 islets each were incubated at 37°C for 60 min in 25 μl of KRBH. The incubation was stopped on acetone chilled with dry ice, and 20 μl of 1.35 M perchloric acid (PCA) was added. PCA was neutralized and precipitated with 15 μl of 0.1 M Tris + 2.8 M KHCO₃, Supernatant aliquots (10 μl) of samples and ATP (or ADP) standard solutions (0–10 μM) treated in the same way were then mixed with 100 μl of α-luciferin solution (0.1 mM) in a 96-well plate. The emitted light was measured in a microplate reader (Synergy-2; Biotek) after the addition of 10 μl of luciferase (0.1 mg/ml). ADP was converted to ATP by pyruvate kinase (10 IU/ml) in the presence of 1 mM phosphoenolpyruvate and its concentration calculated by subtracting the ATP measured initially before the conversion. Further experimental details are given in a previous study (27).

ATP release from incubated islets could not be detected as nucleotide accumulation in the incubation medium because of the high activity of membrane ectonucleotidases that were resistant to two available specific inhibitors (100 and 200 μM sodium polystyrene tartrate and 100 μM 6N,N-diethyl-d,β-p-dibromoethylethyl ATP, trisodium salt; results not shown). However, the initial rate of ATP release was measured in real time as the amount of luminescence emission after reaction of the extracellular adenine nucleotide with the luciferin/luciferase system present in the incubation medium. For that purpose, isolated mouse islets were cultured overnight at 37°C in RPMI 1640 containing 10% heat-inactivated fetal calf serum and 11.1 mmol/l of d-glucose. Groups of 40 islets each were then transferred to the individual wells of a 24-well plate containing 200 μl of KRB and either 5 or 20 mM glucose and 30 μl of luciferin-luciferase mixture (LLM; see below). Plates inserted into a microplate reader (FluoStar Optima; BMG) underwent an experimental protocol that read the
luminescence every 4 s, with a total number of 30 readings/well (2 min/well) at 37°C. At second 20, 327 µl of high-potassium Krebs-Ringer bicarbonate (HKKRB; see below) containing either 5 or 20 mM glucose was resuspended in 2.5 ml of IR and desalted in a 10-ml, 10-DG column. On the other hand, 10 mg of d-luciferin (10 mg) was resuspended in 1.5 ml of ultrapure (Bio-Rad Laboratories) equilibrated with IR. The solution was placed in a water- and pH-adjusted with 7.5. In the free divalent cations solution, CaCl2 and MgCl2 were omitted and pH adjusted to 7.4 with NaOH. The LLM was prepared by diluting 30 µl of d-luciferin into 100 µl of luciferase solution. Oocyte preparation and electrophysiology. DNA fragments encoding the full-length mouse Cx36 (966bp, GenBank accession no. AF016190) were inserted into the StuI site of a pBSXG vector (23). Capped RNA for oocyte injection was transcribed from the linearized plasmids using T7 RNA polymerase. The preparation of Xenopus oocytes was performed as described previously (2). Stage V–VI oocytes were coinjected with in vitro-transcribed cRNA (0.5 µg/µl, 50 nl/oocyte) and an antisense oligonucleotide directed against Xenopus Cx38 mRNA that completely blocked endogenous expression (13) or only antisense as a negative control. The transmembrane currents (Im) of single oocytes were recorded by the conventional two-electrode voltage clamp method. Microelectrodes were filled with 2 M KCl and 10 mM EGTA at pH 7.20 and had a low tip resistance (0.5–1 MΩ). The normal bath solution (ND96) was as follows (in mM): 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, adjusted to pH 7.5. In the free divalent cations solution, CaCl2 and MgCl2 were not added. Macroscopic currents were filtered at 200 Hz and sampled at 1 kHz. For data acquisition and analysis, we used a Digidata interface and pClamp (Axon Instruments). For dye uptake experiments, 1 mM propidium iodide (Sigma-Aldrich Quimica) dissolved in ND96 with or without divalents was added for 30 min. After washout, confocal images of oocytes (×10 objective, MRC-1024; Bio-Rad) were collected at emission wavelengths of 598/40 nm using a 568-nm line excitation.

In vivo experiments: glucose and insulin tolerance tests. For the intraperitoneal glucose tolerance test (IPGTT), animals were fasted overnight for 12 h, and blood samples were obtained from the tail vein. Animals were injected intraperitoneally with 2 g glucose/kg body wt and blood samples taken at the indicated intervals (0, 15, 30, 60, and 120 min). For the intraperitoneal insulin tolerance test, fed animals were injected intraperitoneally with soluble insulin at 0.75 IU/kg body wt. Blood glucose was measured in each sample after 0, 15, 30, 45, and 60 min using an Accu-Chek compact glucometer (Roche Farma, Madrid, Spain). Plasma insulin levels were quantified in fasted state and measured by enzyme-linked immunosorbent assay (Crystal Chem, Downers Grove IL).

RESULTS

In vivo characterization of Cx36-KO mice. An intraperitoneal glucose tolerance test showed significantly greater glucose excursions in plasma glucose at 15 (P < 0.01) and 30 min (P < 0.001) in Cx36−/− mice than in Cx36+/+ animals (Fig. 1A). The corresponding area under the curve of the glucose excursion was significantly higher in Cx36−/− than in wild-type mice (25,435 ± 1,237.08, n = 8 vs. 21,126.3 ± 1,130.4 arbitrary units, n = 6; P < 0.029). Basal plasma insulin values were significantly lower in Cx36−/− than in wild-type mice (0.31 ± 0.15, n = 10, vs. 0.65 ± 0.33 ng/ml, n = 6; P < 0.014). No difference between the relative plasma glucose concentrations of Cx36−/− and Cx36+/+ animals was observed after an intraperitoneal insulin tolerance test (Fig. 1B).

Glucose-induced insulin secretion in perfused mouse islets. The glucose intolerance observed in mice lacking Cx36 correlated with a defect in insulin secretion of their corresponding isolated islets. Upon an increase in the perfusate glucose concentration from 5 to 20 mM, Cx36−/− islets responded with a characteristic biphasic insulin response (Fig. 2A). Compared with wild-type, Cx36−/− islets showed diminished insulin secretion at basal levels (5 mM) and a marked decrease of the first and second phases of glucose-induced insulin secretion (20 mM) (Fig. 2A). As expected from a half-reduction of Cx36 expression in the heterozygous Cx36+/− mice, the insulin secretion profile in Cx36+/− islets was between that of Cx36+/+ and Cx36−/−.
Cx36+/− responses (Fig. 2A). At a slightly suprathreshod glucose concentration (8 mM), wild-type islets responded with a small biphasic stimulation of insulin secretion (Fig. 2B). By contrast, Cx36−/− islets showed a diminished basal rate of release (at 5 mM glucose) and no significant stimulation by 8 mM glucose (Fig. 2B). Islet insulin content was statistically similar in all three mouse phenotypes (381.4 ± 23.6, n = 8, 427.8 ± 34.2, n = 6, 312.1 ± 48.4 ng/islet, n = 8, in Cx36+/+, Cx36−/+−, and Cx36−/− islets, respectively).

The simultaneous addition of 50 μM mefloquine, a high-affinity blocker of Cx36 gap junction channels (9, 21), together with 20 mM glucose diminished only the second phase of insulin secretion and did not modify the first one in wild-type islets (Fig. 3A). Preperfusion with 50 μM mefloquine for 15 min at 5 mM glucose did not further decrease the secretory response to 20 mM glucose in the presence of the drug (unpublished results). By contrast, mefloquine (50 μM) showed a trend to reduce the basal rate of insulin secretion at 5 mM glucose in wild-type islets. In islets from Cx36+/− mice, the simultaneous combination of 50 μM mefloquine together with 20 mM glucose reduced the magnitude of the first- and second-phase response to glucose (Fig. 3B). However, mefloquine had no effect on the already strongly diminished secretory response of Cx36−/− islets (Fig. 3C). Detailed dose response studies of mefloquine effects on basal and glucose-stimulated insulin secretion as well as on insulin content in rat islets in the range 25 to 250 μM were performed. Mefloquine concentrations of 25 and 50 μM were without any significant effect on basal insulin secretion, whereas 100 and 250 μM stimulated insulin release significantly at 5 mM glucose (unpublished results). The highest concentration (250 μM) diminished within 40% islet insulin content during 1 h of incubation at 5 mM glucose, but no significant effect was found at lower concentrations (unpublished results). Two-hundred fifty micromolar mefloquine induced a strong stimulation of insulin secretion in perifused islets independently of the glucose concentration (5 or 20 mM) that was maintained after withdrawal of the drug (unpublished results). The stimulation of insulin secretion by mefloquine (100 and 250 μM) at 5 mM glucose was not counteracted by 0.25 mM diazoxide, discarding the closure of KATP channels as the responsible mechanism (unpublished results). In all islet experiments, mefloquine was always assayed in the presence of 250 mM diazoxide together with KCl. It was also found that 50 μM mefloquine had only a marginal effect (−30%) on the monophasic stimulation of insulin release by 70 mM KCl in rat islets, which speaks against a strong inhibition of mefloquine on L-type calcium channels (unpublished results).

Electrophysiological properties of murine Cx36 hemichannels expressed in Xenopus oocytes. The electrophysiological properties of mouse Cx36 hemichannels had not been characterized previously, and they were studied in the Xenopus oocyte system. We found that overexpression of Cx36 hemichannels in solitary oocytes significantly increased plasma membrane permeability, as revealed by the uptake of extracellular propidium iodide tracer at normal conditions (i.e., at the resting membrane potential, −42 ± 5 mV; and normal extracellular divalent cation concentration, 1.5 mM Ca2+ and 1 mM Mg2+); permeability was further enhanced by lowering extracellular divalent cation concentrations (Fig. 4A). Cx36 hemic-
channel activity is under strict control of membrane potential since it was activated upon depolarization of membrane potential and deactivated by returning to hyperpolarized potentials (Fig. 4B). Voltage activation of hemichannels was partially diminished at normal extracellular concentrations of divalent cations and almost completely suppressed by 10 μM mefloquine (Fig. 4B and C). We also found that glucose blocked hemichannel currents activated by depolarization in a dose-dependent (sigmoidal) manner (Fig. 4C), with an IC₅₀ close to 8 mM and ~85% of inhibition at 20 mM glucose. Depolarization with 70 mM KCl in the absence of Ca²⁺ and Mg²⁺ stimulated oocyte depletion of endogenous ATP that was prevented by 50 μM mefloquine and 20 mM glucose (unpublished observations).

Initial ATP release via Cx36 hemichannels in β-cells. As indicated in MATERIALS AND METHODS, islet ATP release could
Fig. 4. Functional characterization of mouse Cx36 hemichannels expressed in Xenopus oocytes. Oocytes were injected with an antisense oligonucleotide to block endogenous Cx38 (control) alone and in combination with murine Cx36 cRNA 24 h before experiments. A: propidium iodide (IP; 1 mM) uptake after 30 min of incubation. A, i: confocal images showing that murine Cx36 (mCx36) but not control oocytes exhibited some permeability to PI in normal medium (ND96 solution with 1.8 mM CaCl2 and 1 mM MgCl2) that was further enhanced by lowering divalent cation concentration (omission of Ca2+ and Mg2+). Scale bars, 200 μm. A, ii: quantification of PI uptake calculated as mean values of straight histogram in arbitrary units (AU; segmented lines). B, i: macroscopic currents (Im) elicited by depolarization of the membrane potential (Vm). B, ii: Im/Vm relationships in control (△ and ▧) and mCx36 oocytes (○ and ●) recorded at normal solution (△ and ▧) and low divalent cation concentration (○ and ●). Activation of mCx36 hemichannels increased monotonically with the degree of depolarization across the entire negative and positive voltage and deactivated upon hyperpolarization. At low divalent cation concentration, the amplitude of mCx36 hemichannel currents activated by voltage increased significantly. C: MFQ (i) and 20 mM glucose (ii) inhibited mCx36 hemichannel currents activated by depolarization. C, iii: dose-response curve for glucose. Hemichannel current was isolated from total transmembrane current by subtracting the component inhibited by 10 μM MFQ and the fraction of MFQ-sensitive current that was blocked by glucose represented in percentage. Normalized average data (means ± SE; n = 5) were fitted to a Hill equation of the form $I_{m} = I_{m_{\text{max}}} + (I_{m_{\text{max}}}-I_{m_{\text{min}}})/\left(1 - ([\text{glucose}]/IC_{50})^{n}\right)$ and the following fitting parameters: $IC_{50} = 7.66$ mM, Hill coefficient $n = 5$, and $I_{m_{\text{min}}} = 17.81\%$. 
not be evaluated by measuring its medium accumulation at times long enough to obtain significant differences due to the high activity of membrane ectonucleotidases. However, the initial release of ATP by incubated islets could be evaluated following light emission by medium luciferin-luciferase (Fig. 5). Islets of wild-type mice released ATP after being depolarized with 70 mM KCl at 5 mM glucose [mean of the peak amplitude: 42 ± 15 arbitrary units (AU) of light; n = 4]. The addition of 20 mM glucose to the extracellular solution almost completely suppressed ATP release from the same islets (mean of the peak amplitude: 1 ± 1.45, n = 4, vs. 42 ± 15 AU, n = 4; P < 0.035). To test whether the release of ATP required Cx36 expression, we performed the same experiment with Cx36−/− mouse islets. Interestingly, Cx36−/− islets did not release ATP after 70 mM KCl depolarization at any glucose concentration (mean of the peak amplitudes: 1 ± 0.3 AU at 5 mM and 0.42 ± 0.15 AU at 20 mM glucose, n = 4; P < 0.035 compared with Cx36+/+ islets at 5 mM glucose). The results confirm the presence of open Cx36 hemichannels in β-cells that are able to release ATP when they are activated by depolarization and that this ATP release is inhibited by high glucose.

Attempts to demonstrate the opening of β-cells’ hemichannels in rat islets following the uptake of several fluorescent dyes (YoPro-1, Lucifer Yellow, fura, calcein, etc.) at a wide concentration range were fruitless; an intense labeling of the intercellular space impeded us from seeing any specific intracellular or nuclear dye accumulation.

Changes in β-cell ATP content and ATP/ADP ratio in rat and murine islets from wild-type (Cx36+/+) or Cx36-KO (Cx36−/− and Cx36−/−) mice. Because of the rapid degradation by islet membrane ectonucleotidases of released ATP, as illustrated by the real-time measurements above (Fig. 5), the magnitude of islet ATP depletion was measured routinely and considered the most accurate procedure for the evaluation of ATP release. At basal glucose (5 mM), a gradual decrease in intracellular ATP content in rat islets was obtained as larger depolarizations were induced (15, 30, and 70 mM KCl) without the ATP/ADP ratio being modified (Table 1). Interestingly, ATP depletion occurred when islets were incubated in a Ca2+-free solution, excluding the possibility that ATP release was mediated by exocytosis. In fact, the ATP drop was blocked by 50 μM mefloquine; 20 mM glucose increased islet ATP content, and the corresponding ATP/ADP ratio to values that are stimulatory for secretion (Table 1). However, at variance with 5 mM, 20 mM glucose prevented the drop of ATP content and ATP/ADP ratio during incubation with 70 mM KCl in a Ca2+-free solution. Other nutrient secretagogues, like α-ketoglutarate and semialdehyde succinic acid, that increased the ATP content and the ATP/ADP ratio to the same levels as 20 mM glucose failed to maintain islet ATP content under the same depolarizing conditions. Fifty micromolar dibenzoyl-ATP, a strong P2X7 receptor agonist, did not modify islet ATP content at 5 mM glucose (3.23 ± 0.18 pmol/islet, n = 8, vs. 3.10 ± 0.11 pmol/islet, n = 8; not significant).

Similar results were obtained in wild-type mouse islets. The basal ATP content and ATP/ADP ratio recorded in Cx36+/+ islets at 5 mM glucose were increased twofold by changing the sugar concentration to 20 mM (Fig. 6, A and B). Exposure to 70 mM KCl at 5 mM glucose also reduced approximately threefold their ATP content without changing the ATP/ADP ratio.

Fig. 5. Inhibition of ATP release induced by 70 mM KCl depolarization at 5 mM by 20 mM glucose and by genetic deletion of Cx36 expression. A: luminescence recording traces representative of each experimental condition. **: summary histogram of the mean of the luminescence amplitudes after the peak of ATP release was assessed in each experimental condition. **P < 0.035 and NS compared with the wild-type control at 5 mM glucose; n = 4.
Table 1. ATP content and ATP/ADP ratio of rat islets incubated with G3 and G20, 10 mM α-KIC, and 10 mM SSA

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<tr>
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<th>ATP Content, pmol/islet</th>
<th>ATP/ADP Ratio</th>
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<tr>
<td><strong>Control</strong></td>
<td></td>
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<tr>
<td>G3</td>
<td>2.82 ± 0.14 (18)</td>
<td>1.19 ± 0.06 (18)</td>
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<tr>
<td>G20</td>
<td>4.64 ± 0.09 (12)</td>
<td>2.19 ± 0.22 (12)</td>
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<tr>
<td><strong>15 KCl</strong></td>
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<tr>
<td>G3</td>
<td>2.09 ± 0.06 (4); P = &lt;0.03</td>
<td>1.18 ± 0.04 (4); P = NS</td>
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<tr>
<td>G20</td>
<td>4.09 ± 0.04 (4); P = &lt;0.0001</td>
<td>2.27 ± 0.48 (8); P = NS</td>
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<tr>
<td><strong>30 KCl</strong></td>
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<tr>
<td>G3</td>
<td>1.81 ± 0.26 (4); P = &lt;0.005</td>
<td>0.90 ± 0.04 (4); P = NS</td>
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<tr>
<td>G20</td>
<td>3.12 ± 0.23 (3); P = NS</td>
<td>2.69 ± 0.40 (3); P = NS</td>
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<td><strong>70 KCl</strong></td>
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<tr>
<td>G3</td>
<td>1.19 ± 0.13 (4); P = &lt;0.0001</td>
<td>1.04 ± 0.07 (4); P = NS</td>
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<tr>
<td>G20</td>
<td>2.12 ± 0.06 (8); P = NS</td>
<td>1.21 ± 0.06 (8); P = NS</td>
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<tr>
<td><strong>70 KCl + 0 Ca2</strong></td>
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<tr>
<td>G3</td>
<td>1.28 ± 0.14 (12); P = &lt;0.0001</td>
<td>0.99 ± 0.08 (12); P = NS</td>
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<tr>
<td>G20</td>
<td>4.13 ± 0.38 (3); P = NS</td>
<td>1.32 ± 0.23 (3); P = NS</td>
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<td><strong>50 μM MFO</strong></td>
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<tr>
<td>G3</td>
<td>2.35 ± 0.13 (4); P = NS</td>
<td>2.32 ± 0.17 (8); P = &lt;0.02</td>
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<tr>
<td>G20</td>
<td>1.21 ± 0.27 (8); P = NS</td>
<td>1.04 ± 0.27 (8); P = NS</td>
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Values are means ± SE. KIC, α-ketosioacproic acid; SSA, semialdehyde succinic acid; G3, 3 mM glucose; G20, 20 mM glucose; MFO, mefloquine; NS, not significant. Changes induced by Ca2⁺ omission (0 mM CaCl₂ + 100 μM EGTA = 0 Ca2⁺) and depolarization with 70 mM KCl (70 KCl + 250 μM diazoxide = 70 KCl), depolarization alone (70, 30, and 15 mM KCl in the presence of 250 μM diazoxide), and 50 μM MFO (an inhibitor of connexins 36 and 50). Three groups each of 25 rat islets were incubated in 25 μl of Krebs-Ringer bicarbonate-HEPES buffer for 1 h at 37°C with the additives indicated. The tubes containing islets and medium were frozen, plunging them in acetone chilled with dry ice. Frozen medium plus islets were treated with perchloric acid and then neutralized to characteristic values for secretion in the presence of 70 mM KCl (Fig. 7, A and B). Again, 20 mM glucose did not only prevent the islet ATP fall but increased further both islet ATP content and ATP/ADP ratio to stimulatory values for secretion in the presence of 70 mM KCl (Fig. 8, A and B).

By contrast, Cx36⁻/⁻ islets showed abnormally elevated ATP levels at 5 mM glucose, similar to those reached at 20 mM glucose in Cx36⁺/⁺ islets, but an ATP/ADP ratio characteristic of nonstimulated islets (Fig. 8, A and B); 20 mM glucose further increased the ATP content and ATP/ADP ratio (Fig. 8, A and B). Basal and stimulated ATP content and under a physiological KCl concentration, and the ATP/ADP ratio was increased significantly above the value obtained at 5 mM glucose and 70 mM KCl (Fig. 7, A and B). Again, 20 mM glucose did not only prevent the islet ATP fall but increased further both islet ATP content and ATP/ADP ratio to stimulatory values for secretion in the presence of 70 mM KCl (Fig. 8, A and B).

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By contrast, Cx36⁻/⁻ islets showed abnormally elevated ATP levels at 5 mM glucose, similar to those reached at 20 mM glucose in Cx36⁺/⁺ islets, but an ATP/ADP ratio characteristic of nonstimulated islets (Fig. 8, A and B); 20 mM glucose further increased the ATP content and ATP/ADP ratio (Fig. 8, A and B). Basal and stimulated ATP content and under a physiological KCl concentration, and the ATP/ADP ratio was increased significantly above the value obtained at 5 mM glucose and 70 mM KCl (Fig. 7, A and B). Again, 20 mM glucose did not only prevent the islet ATP fall but increased further both islet ATP content and ATP/ADP ratio to stimulatory values for secretion in the presence of 70 mM KCl (Fig. 8, A and B).

and this ATP drop was prevented by 50 μM mefloquine (Fig. 6, A and B). At 20 mM glucose, 70 mM KCl did not decrease islet ATP content (Fig. 6, A and B). To rule out that 20 mM glucose might counteract the ATP loss through an increased metabolism, we checked the effect of nonmetabolized (L-glucose, 3-O-methyl-D-glucose) or poorly metabolized (D-galactose) structural glucose analogs. Each of the analogs, used at 15 mM in combination with 5 mM glucose, prevented the drop of islet ATP induced by 70 mM KCl (Fig. 6C). Noticeably, the ATP values shown in Fig. 6D. The preventive effect of glucose on KCl-induced loss of islet ATP was already observed at 8 mM (Fig. 7A); islet ATP was restored to the control level recorded at 5 mM glucose.
ATP/ADP ratio in heterozygous (Cx36+/−) islets were intermediate of the corresponding values in control and Cx36−/− islets (unpublished results). At 20 mM glucose, islets from the three different mouse phenotypes showed similar increases in their ATP content and ATP/ADP ratio above the basal values (Fig. 6, A and B, and unpublished results).

KCl-induced ATP loss in wild-type islets was reversed by 5 mM extracellular ATP; both the ATP content and the ATP/ADP ratio increased well above the control values at 5 mM glucose, reaching the levels observed at 20 mM glucose in nondepolarized islets (Fig. 8, C and D). However, homozygous Cx36−/− islets did not modify their abnormally high basal ATP content or normal ATP/ADP ratio by 70 mM KCl-induced depolarization at 5 mM glucose in either the absence or presence of 5 mM extracellular ATP (Fig. 8, C and D).

Similarly to adenine nucleotides, islet amino acids were also released by KCl depolarization and/or extracellular Ca2+ omission in rat islets (unpublished results). Mouse islets’ amino acids, mainly GABA and Tau, were also released, and their islet content was correspondingly depleted by KCl depolarization in wild-type but not in Cx36−/− islets (unpublished results).

Effect of extracellular ATP on insulin secretion in Cx36+/+, Cx36+/−, and Cx36−/− mouse islets. In the presence of a basal stimulatory glucose concentration (5 mM), mouse Cx36+/+ islets responded to 70 mM KCl with only a transient peak of insulin release that faded out after about 10 min. The amplitude of this “first-phase-like” peak was similar to that induced by raising the glucose concentration from 5 to 20 mM glucose (compare Fig. 2A with Fig. 9A). Compared with wild-type controls, Cx36−/− islets showed a marked reduction of the
first-phase-like secretion peak in response to KCl depolarization (6.2 ± 0.5, n = 6, vs. 16.7 ± 1.4 ng insulin/40 islets × 10 min, n = 4; P < 0.0001; Fig. 9B), and Cx36+/− islets showed a similar decrease (6.5 ± 0.8, n = 5, vs. 16.7 ± 1.4 ng insulin/40 islets × 10 min, n = 4; P < 0.0002; Fig. 9C).

Interestingly, the addition of 5 mM extracellular ATP in Cx36+/− islets during the KCl depolarization period, shown previously to result in the elevation of intracellular ATP and ATP/ADP ratios to levels similar to those induced by 20 mM glucose (Fig. 8, C and D), did not modify significantly the
dynamics of the first-phase-like response but induced a second phase that doubled the total amount of insulin secreted during the last 20 min of depolarization (Fig. 9A). This effect was not accounted for by activation of purinergic receptors since the blockade of either P2 (50 μM suramin) or A1 (50 μM CGS 15943) receptors in rat islets did not decrease or increase 10 or 5 mM ATP-induced insulin secretion, respectively; however, stimulation by 10 mM ATP was almost completely suppressed by 50 μM mefloquine (unpublished observations). However, in Cx36+/− islets, 5 mM ATP induced a significant stimulation of both the first and second phases that were quite smaller than in wild-type islets (11.2 ± 1.3, n = 5, vs. 20.6 ± 30.9 ng insulin/40 islets × 10 min, n = 4, P < 0.02; and 11.8 ± 0.8, n = 5, vs. 32.7 ± 7.4 ng insulin/40 islets × 20 min, n = 4, P < 0.02, respectively; Fig. 9B). By contrast, the addition of 5 mM ATP to Cx36−/− islets did not modify the first-phase-like response, nor did it induce a second phase (Fig. 9C). Fifteen micromolars 3-O-methyl-D-glucose did not modify by itself the first-phase-like response to 70 mM KCl depolarization in wild-type islets but partially suppressed the stimulation of a second phase by 5 mM ATP (Fig. 9D).

**DISCUSSION**

Rat and mouse islets lose ATP when they are depolarized with KCl, and this effect is not impaired by the omission of extracellular Ca²⁺, excluding exocytosis as the main mechanism of release. The ATP loss occurred without any significant change in the ATP/ADP ratio, suggesting that it was attributable to a proportionate release of both ATP and ADP and not to a decrease in the phosphorylation potential. Islet ATP depletion could be reversed by the extracellular uptake of the nucleotide. Mefloquine partially suppressed the loss of ATP, as well as its uptake, in both rat and mouse islets, supporting the hypothesis that the opening of Cx36 hemichannels in the β-cells mediated the effect. The drug inhibits gap junctional coupling between interneurons in brain slices at 25 μM with minimal nonspecific actions (9). The erythro-mefloquine racemate mixture used in this study (Sigma Aldrich Quimica) is 10,000 times less potent than that from other sources on pannexin 1-mediated currents in Neuro2A cells (IC₅₀ = 483.8 ± 37.5 μM) (21). This, together with the lack of effect of 50 μM dibenzyol-ATP, a strong P2X7 receptor agonist, on islet ATP strengthens the idea that connexons and not pannexons are mainly responsible for the observed ATP movements in islets. This conclusion was strongly substantiated in islets from mice homozygous for a germline deletion of the Cx36 gene; islet ATP was exchanged with the extracellular medium only in wild-type and not in Cx36−/− islets. Although the formation of Cx36 gap junction channels between pancreatic β-cells is well known, the ability of Cx36 to form functional hemichannels is questionable. No evidence of open Cx36 hemichannels was obtained in either insulin-producing MIN6 cells or freshly isolated mouse islets, using dye tracers as a marker of Cx36 hemichannel permeability (30). By contrast, however, another study in neurons reported that the opening of Cx36 hemichannels upon depolarization with 100 mM KCl induced ATP release, which was prevented by blocking Cx36 gene expression with siRNA (33). A decrease in islet ATP content by 30 mM KCl has been reported before and attributed to membrane depolarization-dependent stimulation of the Na⁺-K⁺-ATPase; however, the ATP drop could not be rescued by ouabain, which by itself diminished the nucleotide content at 3, 10, or 20 mM glucose (11).

In the present study, we provide for the first time electrophysiological evidence that Cx36, when exogenously expressed in Xenopus Laevis oocytes, forms open Cx36 hemichannels that enhance plasma membrane permeability. They are under strict functional control of the membrane potential, and their activity increases upon depolarization and decreases by hyperpolarization (Fig. 4). Our results also show that glucose is a potent inhibitor of voltage-activated Cx36 hemichannel currents in oocytes. According to the dose-response curve, glucose in the range of 5–20 mM can act as a modulator of hemichannel activity since its IC₅₀ is close to 8 mM, which is slightly above the threshold for the stimulation of insulin secretion (Fig. 4). Hemichannels’ currents were not measured directly in islet β-cells, but their intensity is probably much lower than in Cx36-transduced oocytes due to great differences in connexin expression. Islet ATP loss, taken as an indirect indicator of depolarization-induced hemichannel opening in β-cells, is also suppressed by glucose already at 8 mM (Fig. 7).

Glucose-dependent closing of Cx36 hemichannels may play a physiological role in the mechanism of stimulation of insulin secretion in β-cells. Increasing KCl concentrations (15, 30, 70 mM) above the physiological value induced a progressive reduction of islet ATP content (Table 1). Fifteen micromolar KCl already caused a significant islet ATP drop, and the corresponding depolarization (~21 mV) drove the membrane potential to the level of the plateau phase reached by high glucose (15); at 40 mM, KCl depolarized the membrane by 40 mV, and the potential stabilized at the peak level of the Ca²⁺-induced spikes that are shot from the plateau phase of the electrical burst activity induced by high glucose (15). Therefore, KCl-driven plasma membrane depolarizations of magnitude similar to those induced by glucose might cause ATP depletion by the opening of Cx36 hemichannels in β-cells. The antagonistic effect of stimulatory glucose concentrations might serve the additional role of reducing islet ATP loss as a subsidiary effect to the increased glucose metabolism. Both effects might contribute to increase and maintain sufficiently high islet ATP content and ATP/ADP ratio. This duality of glucose was experimentally nondissociable, as inhibition of metabolism by 10 mM n-mannoheptulose; results not shown) almost completely suppressed islet ATP, and no modulation by KCl depolarization or glucose on islet ATP loss could be detected. However, 20 mM glucose completely blocked the initial rate of ATP release in depolarized wild-type islets (Fig. 5), and nonmetabolized glucose analogs (l-glucose, D-galactose, and 3-O-methyl-D-glucose) mimicked the blocking effect of glucose (Fig. 6). Moreover, glucose dose-dependently inhibited hemichannels’ currents in oocytes overexpressing Cx36 (Fig. 4). As proof of glucose specificity, other nutrient secretagogues like α-ketoisocaproic acid or semialdehyde succinic acid were not able to completely block the loss of ATP induced by KCl depolarization (Table 1). Although it has not been demonstrated directly in β-cells, we speculate, according to the overall accumulated experimental evidence, that glucose might exert a regulatory role on Cx36 hemichannels. The poor permeability of oocyte membrane to glucose (5, 22) suggests that it is probably interacting with an extracellular domain of Cx36 hemichannels. Similarly, the preventive effect of l-glucose on
depolarization-induced ATP loss is also exerted extracellularly, as this glucose diastereoisomer is restricted to the extracellular space. This postulated inhibitory effect of glucose does not seem to be exerted on gap junction conductance (34), suggesting that its interaction with Cx36 is hindered in gap junction channels.

As a corollary to this proposal, 70 mM KCl stimulation of a single monophasic release of insulin at 5 mM glucose (Fig. 9) might be ascribed to depolarization-induced loss of adenine nucleotides through open Cx36 hemichannels. This interpretation is supported by the generation of a second phase of sustained insulin secretion by addition of 5 mM extracellular ATP in wild-type islets that was progressively suppressed by half and complete reduction of Cx36 expression in Cx36+/− and Cx36−/− islets, respectively (Fig. 9, B and C). Extracellular ATP did not only stimulate a second phase of secretion but also reinforced the first-phase response due to KCl depolarization in Cx36+/− but not Cx36−/− islets. The potentiation of both phases of insulin secretion by an increase in islet ATP strengthens its role as a second messenger not only during the amplifying (second) but also possibly the triggering (first) phase of insulin release (17). This is not to deny that other messengers and cofactors like cAMP acting via Epac2 (20) and mitochondrial GTP (36) might also be implicated in the triggering and amplifying phases of glucose-induced stimulation of insulin secretion.

Cx36+/− islets exhibit a higher ATP content at basal (5 mM) glucose that is similar to that reached in wild-type islets at 20 mM glucose but a lower ATP/ADP ratio. It probably results from the deletion of hemichannels that might prevent a low background (nonstimulated) “leakage” of nucleotides at basal hyperpolarized conditions. Although islet ATP levels are high, basal insulin secretion was significantly reduced compared with wild-type controls. The magnitude of the first and second phases of the insulin secretory response to 20 mM glucose was progressively decreased in heterozygous and homozygous Cx36−/− islets, respectively. The diminution of the second-phase response is probably mediated by the loss of β-cells’ synchrony due to the half-reduction or complete absence of gap junction channels in Cx36+/− and Cx36−/− islets, respectively. If it is assumed that the first-phase response is caused predominantly by depolarization-induced elevation of cytosolic Ca2+, it is not easy to understand why it is decreased by Cx36 ablation. It is known that cytosolic Ca2+ elevations induced by repetitive pulses of 30 mM KCl are well synchronized among different β-cells of Cx36+/− islets (28). Therefore, Cx36 deletion might additionally cause an impairment of the coupling mechanism between plasma membrane depolarization by glucose and voltage-dependent calcium entry in the β-cells. A milder inhibition of both phases of glucose-induced insulin secretion in wild-type and Cx36+/− islets was also reproduced by 50 μM mefloquine (Fig. 3). It may be concluded that this drug, at variance with glucose, blocks Cx36 function in both hemichannels and gap junction channels because it suppresses intracellular adenine nucleotide exchange with both the medium and nutrient-induced insulin secretion.

In two different models of Cx36 gene-knockout mice, germinal (3, 29) and β-cell-specific (38) pancreatic islets and perfused pancreas systematically showed an increased basal rate of insulin release, whereas in another mouse model of germinal Cx36-knockout, basal secretion was unaffected (16). The reduced basal insulin secretion observed in our transgenic islet preparation coincided with a diminished plasma insulin concentration in the corresponding Cx36−/− mice. The rearrangement of both phases of the secretory response to glucose (at 8 and 20 mM) in islets from Cx36−/− mice in our study confirms the results obtained in the perfused pancreas of β-cell-specific knockout mice at 6.0, 8.0, and 16.4 mM (38). Whereas in Head et al. (16) and the present work some significant elevations of the glycaemia were found in the oral glucose tolerance test or IPGTT, no modification was found in the β-cell-specific knockout mice (38). Given the similarity of the genetic mouse models used in all these studies, the observed differences might be ascribable to methodological variations. The metabolic changes associated with aging might perhaps make a difference in the phenotypic expression of Cx36 deletion since the age of the transgenic mice used varied from 5 to 11 (29) mo to 4 (16) or 3 mo in the present study. One may thus conclude that the insulin secretory defects caused by Cx36 suppression in β-cells (Refs. 16 and 38 and the present work) induce a certain degree of glucose intolerance in the whole animals without any sign of insulin resistance, as judged by the pioneering work of Bavamian et al. (3) and later confirmed by Head et al. (16) and the present work.

In summary, the participation of Cx36 in both hemichannels and gap junction channels makes it difficult to separate their respective functions in the regulation of insulin secretion (37). Our work has partially confirmed previous results on the effect of the genetic ablation of Cx36 on nutrient-induced insulin secretion by β-cells that are attributable to the participation of gap junction channels between β-cells. Mefloquine has also been characterized as a relatively specific inhibitor of Cx36 roles in islet secretory function. Moreover, our results have uncovered the possibility that Cx36 hemichannels’ closure by glucose would contribute to increase and maintain islet ATP content and the ATP/ADP ratio at the levels required for a sustained stimulation of insulin secretion. Cx36 is the prominent connexin in human β-cells (32), and its encoding gene is located on chromosome 15q14, a locus associated with type 2 diabetes (25). It has been speculated that a reduction in Cx36 function might also occur in humans and lead to loss of β-cell function (8). Indeed, exposure of islets to high peripheral levels of glucose and fatty acids, as occurs in prediabetic states, may induce a decreased Cx36 content and impair β-cell/β-cell coupling (1, 7, 14).

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DISCLOSURES
None of the authors have any conflicts of interests, financial or otherwise.

AUTHOR CONTRIBUTIONS
REFERENCES


