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Connexin 30.2 is expressed in mouse pancreatic beta cells ☆

C. Coronel-Cruz [a,b], B. Hernández-Tellez [b], R. López-Vancell [a], Y. López-Vidal [c], J. Berumen [a,d], A. Castell [b], E.M. Pérez-Armendariz [a,b,*]

[a] Unidad de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM), México, D.F. 04510, México and Hospital General de México, México, D.F. 06726, Mexico
[b] Departamento de Biología Celular y Tisular, Facultad de Medicina, UNAM, México, D.F. 04510, Mexico
[c] Departamento de Microbiología y Parasitología, Facultad de Medicina, UNAM, México, D.F. 04510, Mexico
[d] Unidad de Medicina Genómica, Facultad de Medicina, UNAM and Hospital General de México, México, D.F. 06726, Mexico

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ABSTRACT

Nowadays, connexin (Cx) 36 is considered the sole gap junction protein expressed in pancreatic beta cells. In the present research we investigated the expression of Cx30.2 mRNA and protein in mouse pancreatic islets. Cx30.2 mRNA and protein were identified in isolated islet preparations by qRT-PCR and Western blot, respectively. Immunohistochemical analysis showed that insulin-positive cells were stained for Cx30.2. Confocal images from double-labeled pancreatic sections revealed that Cx30.2 and Cx36 fluorescence co-localize at junctional membranes in islets from most pancreases. Abundant Cx30.2 tiny reactive spots were also found in cell cytoplasms. In beta cells cultured with stimulatory glucose concentrations, Cx30.2 was localized in both cytoplasms and cell membranes. In addition, Cx30.2 reactivity was localized at junctional membranes of endothelial or cluster of differentiation 31 (CD31) positive cells. Moreover, a significant reduction of Cx30.2 mRNA was found in islets preparations incubated for 24 h in 22 mM as compared with 3.3 mM glucose. Therefore, it is concluded that Cx30.2 is expressed in beta and vascular endothelial cells of mouse pancreatic islets.

1. Introduction

Gap junctions (GJ) are conglomerates of intercellular channels. In vertebrates they are mainly formed by a family of homologous transmembrane proteins (~20) termed connexins (Cxs). These channels allow the direct intercellular transfer of molecules and second messengers between adjacent cells. A connexon or hemichannel is an hexamer. Docking of two connexons forms an intercellular channel. Since cells, from most tissues, express more than one Cx subtype, intercellular channels may be homotypic (both connexons formed by the same Cx subtype), heterotypic (each connexon formed by a different Cx subtype) or heteromeric (each connexon formed by more than one Cx). Moreover, hemichannels at the cell membrane may be active [1]. Alterations in Cxs are involved in human pathology and their deficiency is critical for cell development [2,3].

Diabetes is a chronic disease that involves different metabolic disorders where subjects have in common high blood glucose levels. Its world prevalence has increased around ten times in the last 4 decades [4]. Among the different hormones that regulate glucose homeostasis, insulin is the only one that decreases glucose levels. Insulin is produced in beta cells that, in mouse, constitute most of the islet central mass (~80%). The glucose insulin release response curve from isolated perfused islets is well fitted by a sigmoidal function, with a threshold at ~6 mM followed by a linear increase that reaches it saturating point at ~22 mM [5]. Insulin secretion depends critically on electrical activity of beta cells induced by glucose. This electrical activity consists of bursts of action potentials which happen synchronously and in phase in most beta cells from a single islet. This high electrical synchrony produces cyclic changes in the interstitial $[K^+]$ and $[Ca^{2+}]$ [6] and in the intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations [7,8] in the different islet regions. The latter determines pulses of insulin release [8–10].

Electrical coupling [11] through GJ [12] intercommunicates most pancreatic beta cells [13,14]. Cx36 is localized at junctional membranes of beta cells in rodent [14–17] and human islets [18]. Isolated islets cells from Cx36-deficient mice lack synchronization in their $[Ca^{2+}]_i$ oscillations and exhibit loss of pulses of

* Corresponding author. Address: Laboratorio de sinapsis eléctricas, Unidad de Medicina Experimental y Departamento de Biología Celular y Tisular, Torre de Investigación, 5to piso, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, 3000, Copilco Universidad, Mexico, D.F. 04510, Mexico.

E-mail addresses: emperezarmendariz@aol.com, mperezarmendariz@aol.com (E.M. Pérez-Armendariz).

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insulin release induced by glucose [8]. Moreover, Cx36-deficient mice are glucose intolerant [19]. These alterations have been explained to result from beta cells uncoupling since neither GJ particles [8] nor electrical or chemical coupling [20,21] were detected in islet cells from these animals.

From above, Cx36 is considered the only Cx expressed in beta cells. However, besides Cx36, the mRNA of Cx43 and Cx45 have been identified in rodent beta cells purified by cell sorting [22] and of Cx30.3, Cx31, Cx31.1, Cx31.9, Cx37, Cx43 and Cx45 in human islets by RT-PCR [18]. However the cell-specific distribution of most of these Cxs remains to be determined.

Moreover, we have previously proposed that GJ channels in mouse beta cells are heteromeric [14]. This was based on the characterization of the biophysical properties of the junctional currents recorded in dual voltage clamped freshly isolated beta cell pairs [13,14]. Intercellular channels in beta cells are weekly voltage sensitive and have a tiny main unitary conductance (γ_j, ~6 pS) [14]. In the present research, we looked for the expression of Cx30.2 because it is the other member from the Cx family, besides Cx36, whose biophysical properties are most compatible with those recorded in isolated beta cell pairs.

2. Materials and methods

2.1. Animals

CD1. mice were maintained and handled in accordance with the national and international Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction. Animals were anaesthetized and sacrificed by decapitation before tissue dissection.

2.2. Western blot

About 500 islets were isolated from 12 pancreases and homogenized by sonication. Proteins were extracted using buffer (RIPA #20–188, Millipore Corporation, Billerica, MA, USA) with protease inhibitors (P8340, Sigma, St. Louis, MO, USA). Gels were loaded with 50 μg of total protein. The electrophoretic run and transfer protein protocols were performed as previously described [23]. The membranes were incubated overnight at 4 °C with rabbit anti-Cx30.2 (Invitrogen, CA, USA; 1:100). After rinsed, they were incubated for 1 h at room temperature with a horseradish peroxidase (HRP) goat secondary antibody (1:5000). Blots were revealed with chemiluminescence (SuperSignal West Pico, Pierce, Milwaukee, WI, USA).

2.3. Immunohistochemistry

(IHC). Pancreases (10 mice) were fixed in 4% buffered paraformaldehyde for 24 h. After antigens were retrieval with citrate buffer and endogenous peroxidase was blocked, serial sections were incubated with anti-Cx30.2 (1:50), anti-Cx36 (Invitrogen, CA, USA; 1:50) or guinea pig anti-insulin (Invitrogen, CA, USA; 1:2000) for 18 h at 4 °C. After rinsed, sections were incubated for 30 min at 37 °C with an HRP secondary antibody (1:500), and immunoreactivity was revealed by incubation in 3,3′-diaminobenzidine tetrahydrochloride (Zymed, CA. USA). As negative controls, sections were incubated only with a secondary antibody.

2.4. Immunofluorescence

Pancreatic cryosections were co-incubated overnight at 4 °C with anti-Cx30.2 (1:100) and monoclonal anti-Cx36 (1:100), or anti-cluster of differentiation 31 (CD31, BD Pharmingen, San Diego, USA; 1:500). Isolated cultured beta cells were also co-incubated with anti-Cx30.2 and anti-insulin (1:100). After rinsed, sections and cells were revealed with their corresponding secondary antibodies. Images were acquired using a confocal laser-scanning microscope (Olympus model FV1000, Japan). For each experimental condition, at least seven different pancreases were analyzed. As negative controls, sections were incubated only with the corresponding secondary antibody.

2.5. Islets and beta cells cultures

Islets of Langerhans were dissociated from minced pancreases using collagenase 3 mg/ml in Krebs ringer solution with 3% albumin, as previously described [13]. Collected islets were incubated overnight in a DMEM medium (GIBCO, Grand Island, NY, USA) with 11 mM D-glucose. The next day, islets were incubated for 24 h in the DMEM containing 3.3 or 22 mM glucose. Beta cells were dispersed from batches of 150 islets following a protocol previously described [13] and cultured in RPMI with 11 or 22 mM glucose overnight before processed for immunofluorescence studies. Islets and cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. All culture media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% of fetal bovine serum (FBS).

2.6. RNA Isolation

Total RNA was isolated from five pools of isolated islets (~450), collected from 12 pancreases for each culture condition (3.3 or 22 mM glucose), using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The quality and purity of RNA was confirmed as previously described [23]. Traces of DNA were removed by digestion with RNase-free DNase I (Invitrogen, CA, USA) following the manufacture's protocol.

2.7. Real-time quantitative retrotranscription

PCR (qRT-PCR). Reverse transcription of total RNA was performed as previously reported [23]. The expression of Cx30.2 and Cx36 genes was explored in islets incubated in 3.3 or 22 mM glucose with qRT-PCR using TaqMan probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. TaqMan gene expression assays were used (Cx30.2, Mm00731344_s1, Cx36 Mm00439121_m1 and GAPDH, Mm99999915_g1; Applied Biosystems). The cDNA from five different pools were explored in duplicates in a final volume of 20 μl, including 100 ng of cDNA template, 10 μl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl of 20X TaqMan Gene Expression Assay, and 7 μl of RNase-free water. The cycling program was run in a 7500 real time PCR system (Applied Biosystems, Foster City, USA), which was set as follows: an initial PCR activation step at 50 °C for 2 min followed by 95 °C for 10 min, then 45 cycles of melting at 95 °C for 30s and annealing/extension at 62 °C for 1 min. Measurement of gene expression was based on relative standard curves constructed from a 10-fold serially diluted pool of islets cDNAs ranging from 500 to 0.05 ng. Curves for GAPDH gene were tested in three different experiments ran in duplicate. The correlation coefficient (r) average was higher than 0.98. The expression of target genes was calculated based on the GAPDH standard curve and normalized in each experiment to the intensity of the internal reference (GADPH) using a previously described method [29]. The normalized intensity values were measured in ng/μl. The statistical significance of the differences between the groups was calculated with the t-test.