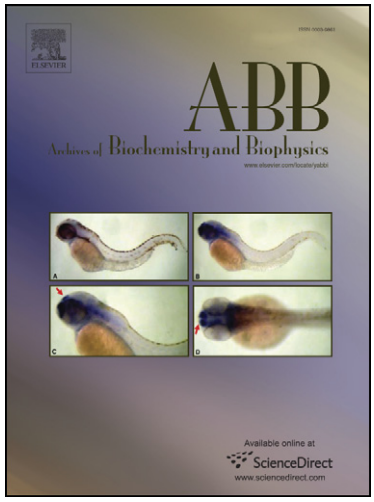

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Changes in islet plasma membrane calcium-ATPase activity and isoform expression induced by insulin resistance

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Short running title: INSULIN RESISTANCE AND ISLET PMCAs

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Abstract

We studied the effect of insulin resistance (IR) induced by administration of a fructose-rich diet (FRD) to normal Wistar rats for 21 days, upon islet plasma membrane calcium ATPases (PMCAs) and insulin secretion. FRD rats showed significantly higher triglyceride and insulin levels, insulin:glucose ratio and HOMA-IR index than controls. FRD islets released significantly more insulin in response to glucose and showed a) marked changes in PMCA isoform protein content (decreased PMCA2 and increased PMCA3), b) a decrease in total PMCAs activity, and c) higher levels of cytosolic calcium $[Ca^{2+}]_i$. The lower PMCAs activity with the resultant increase in $[Ca^{2+}]_i$ would favor the compensatory greater release of insulin necessary to cope with the IR state present in FRD rats and to maintain normal glucose homeostasis. Thus, changes in PMCAs activity and isoform expression play a modulatory role upon insulin secretion during long-term adaptation to an increased hormone demand.

Keywords: Calcium pumps; PMCAs; Insulin resistance; Insulin secretion; PMCA isoforms; Pancreatic islets

Glucose-induced insulin secretion is regulated by a complex process that includes increase in islet glucose metabolism and ATP/ADP ratio [1], closure of the KATP-dependent channel [2], depolarization of the plasma membrane, opening of Ca^{2+} channels, and increase of the cytosolic Ca^{2+} concentration that finally triggers insulin release [3].

Ca^{2+} homeostasis during cellular activity involves its removal from the cell by two different mechanisms, the Na^{+}/Ca^{2+} exchanger (NCX) [4] and the plasma membrane calcium adenosin triphosphatase transporters (ATPases) (PMCAs) [5,6]. PMCAs belong to a family of ionic ATPases of the P-type and the P2 subfamily (subtype 2B) [7,8] with high affinity-low capacity for Ca^{2+} extrusion through the plasma membrane and responsible for the fine-tuning of intracellular Ca^{2+} concentration (0.1-0.2 μM) [5,9]. While PMCAs 1 and 4 are expressed in most tissues [10], PMCAs 2 and 3 display a more restricted expression; in adult animals they are predominantly expressed in brain and muscle, and in smaller amounts in uterus, liver, kidney, pancreas and mammary gland [11].

Several authors have reported the presence of PMCAs in rat islets [12-18]; in β-cells, a unique combination of PMCAs subtypes is present [17-19] and their amount is 10 times higher than in the exocrine pancreas [19]. Our group has demonstrated that while glucose produces a transient and dose-dependent inhibition of islet PMCAs activity, this activity is increased by insulin secretion blockers [16]. Therefore, islet PMCAs activity is modulated by insulin secretagogues or blockers, and consequently participates in the short-term regulation of insulin secretion [13,16,20,21].

Despite the extensive knowledge about islet PMCAs patterns and regulatory role upon insulin secretion under normal conditions, there are scarce reports on their possible changes and participation in the pathogenesis of diseases affecting islet function, such as type 2 diabetes, (T2DM) and impaired glucose tolerance (IGT). Considering that insulin resistance (IR) is an early functional abnormality that precedes the development of T2DM, knowledge about PMCAs state in that condition could help to understand their possible participation in the pathogenesis of that disease.

Administration of a fructose-rich diet (FRD) to normal rats induces IR together with other metabolic abnormalities similar to those observed in human IGT, T2DM and the metabolic syndrome, *i.e.*, IR, dyslipidemia, hypertension, and hyperinsulinemia [22-24]. On the other hand, FRD-induced hyperglycemia and dyslipidemia are associated with an increased production of reactive oxygen species (ROS) and a reduction of antioxidant defenses, thus promoting an oxidative stress [25,26]. Since pancreatic islets have a low concentration of antioxidant enzymes, long-time feeding of a FRD causes β-cell damage [25-28], as shown by the development of T2DM [23]. Under such circumstances, islet PMCAs activity could undergo modifications due to a direct inhibitory effect of ROS upon the pump´s activity [29,30], while islet membrane nonenzymatic glycosylation, consecutive to hyperglycemia, could also contribute to decrease its activity [31,32].

In an attempt to explain the mechanism of the process of β-cell adaptation/failure to an increased demand of insulin, we studied the effect of IR and IGT induced by FRD administration to normal rats upon islet PMCA transcription, expression and activity, and the relationship between these changes and insulin secretion.

Materials and methods

Materials

PMCA antibodies were generously provided by Dr. J.T. Penniston and Dr. E.E. Strehler (Mayo Clinic, Rochester, Minnesota, USA). Anti-mouse and anti-rabbit IgG antibodies were obtained from Vector (Burlingame, CA, USA). PCR reagents were from Invitrogen Argentina. All other highly purified reagents were obtained from Sigma (St. Louis, MO, USA).

Animals

Normal adult male Wistar rats weighing 180-200 g were housed in climate-controlled conditions and received either a standard commercial diet and tap water (control group, C) or the same food plus 10% fructose (Invertose 026430 Productos de Maiz S.A., Argentina) added to the drinking

water (FRD). Twenty-one days after this treatment, the animals were killed to perform the studies described below.

Plasma determinations

Before sacrifice, blood samples were drawn from the retroorbital plexus under light isoflurane anesthesia, and collected in heparinized tubes to measure plasma glucose (One Touch Ultra, Johnson & Johnson, USA), triglyceride (enzymatic kit, BioSystems S.A., Barcelona, Spain) and insulin (radioimmunoassay, RIA) [33] levels. IR was assessed with the HOMA-IR index, calculated as insulin (μU/ml) x glucose (mmol/L)/22.5 [34].

Islet isolation

Pancreatic islets were isolated by collagenase digestion (Roche, Mannheim, Germany) [35]. The islets were collected and resuspended in specific buffers to study insulin secretion and PMCAs expression and activity.

Insulin secretion studies

Groups of 5 islets isolated from C and FRD rats were incubated for 1 h in 0.6 ml of Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4 (gassed with 95% O_2, 5% CO_2), with different glucose concentrations (2, 4, 8, 12, 16 and 20 mM). Insulin released to the incubation media was measured by RIA [33].

Islet insulin content

Groups of 30 islets were homogenized in 400 μl of distilled water and the insulin content was determined by RIA [33].

RNA preparation and RT-PCR

Total RNA was isolated from C and FRD islets using Trizol reagent (Gibco) following the manufacturer´s protocol [36]. RNA integrity was verified by electrophoresis on agarose-formaldehyde gels. Total RNA (1 μg) was reverse-transcribed and cDNA was submitted to PCR

[37]. Specific pairs of primers were designed to detect the different isoform mRNAs based on the rat´s PMCA cDNA sequences: PMCA1 (GenBank accession no. **J03753**) forward, 5′-GAA AAC ATC TCC CAA TGA AGG-3′; reverse, 5′-ACC TGA AAG AAG CAA GGG GT-3′; PMCA2 (GenBank accession no. **J03754**) forward, 5′GCC TCA AAA CCT CTC CTG TT-3′; reverse, 5′-ATG TCC CCA ACC ACA ATC TC-3′; PMCA3 (GenBank accession no. **J05087**) forward, 5′-CAC AGC CTT CAA TGA CTG-3′; reverse, 5′-CCT TCC ATG ACA TGA GTG-3′; and PMCA4 (GenBank accession no. **U15408**) forward, 5′-AGC GTA GAC TTG TTT TTG GG-3′; reverse, 5′-CCC TTC AAT CCA GCC AGT T-3′. β-actin (GenBank accession no. **V01217**: forward, 5′-CGT AAA GAC CTC TAT GCC AA-3′; reverse, 5′-AGC CAT GCC AAA TGT CTC AT-3′) was used as internal standard. A semi-quantitative RT-PCR method was performed to determine the levels of each PMCA cDNA by co-amplification with β-actin. Optimal conditions to co-amplify each isoform with β-actin were determined by testing the number of cycles that correspond to the exponential phase of the PCR amplification (25 to 39 cycles). Accordingly, we chose 32 cycles for PMCA 1, 2 and 4, and 35 cycles for PMCA 3. All experiments were performed with equimolar primer concentrations (0.5 μM). At the end of the amplification, a 20 μl aliquot was subjected to electrophoresis in 1.5% (w/v) agarose gel with ethidium bromide in 1x TBE buffer. The intensity of the bands was quantified by densitometry using a Kodak DC290 digital camera and the Kodak 1D Image Analysis Software (Eastman Kodak Company). Quantitation was done by comparing the intensity of a given band towards that of its internal standard (β-actin).

Preparation of islet microsomal fractions

Islets were collected and washed in PBS containing 1 mM EDTA. Tissue was homogenized after 15 min equilibration in a solution of 10 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, 0.1 mM PMSF, 4 μg/ml aprotinin and 2 mM DTT. An equal volume of 10 mM Tris-HCl, pH 7.5, containing 0.3 M KCl, 0.5 M sucrose and 2 mM DTT was added to the homogenates and the mixture was further homogenized. The homogenate was centrifuged at 4000 × g for 15 min to discard unbroken cells, tissue debris and nuclei. The supernatant was centrifuged at 100,000 × g for 90 min, and the

pellet resuspended in 10 mM Tris/HCl, pH 7.2, 120 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1 mM PMSF, 0.1 mM benzamidine, and 8 μg/ml aprotinin. The microsomal fraction was kept in liquid nitrogen until further use.

Electrophoretic transfer and imnmunoblot analysis of islet membrane samples

Twenty microliters of islet membrane samples (equivalent to 600 islets) from C and FRD islets were loaded on a 4% acrylamide stacking gel. Electrophoresis was performed on 7.5% polyacrylamide gels in the presence of SDS [38]. Proteins were then transferred onto PVDF membranes (Biorad). Membranes were blocked with 10% non-fat dry milk in PBS and incubated overnight with the specific antibody: 5F10 monoclonal anti- (human erythrocyte) Ca^{2+}-ATPase antibody (1:1000), JA9 monoclonal anti-PMCA4, polyclonal PMCA1 (NR1), PMCA2 (NR2) or PMCA3 (NR3) (1:200) antibody. After washing with PBS containing 0.05 % (v/v) Tween 20, membrane filters were incubated for 30 min with anti-mouse or anti-rabit antibody (Vector; 1:2,000) and another 30 min with streptavidine (1:2000). Bound antibodies were visualized by addition of DAB (Sigma). The density of the bands was measured using Kodak 1D Image Analysis Software (Eastman Kodak Company).

PMCA activity

PMCA activity was determined by measuring the release of phosphate from ATP, following the Baginski modified method [39]. Islet homogenates (10 and 15 μg of protein) from C and FRD islets were incubated in Tris-HCl buffer (pH 7.2 at 37 ºC), 2 mM ATP, and sufficient $CaCl_2$ to attain 1.7 μM of free calcium.

Ca^{2+}-ATPase activity was expressed as the difference between the activity measured in the aforementioned medium with and without calcium; under these conditions, the activity measured pertains to the expression of PMCA isoforms [40]. After 60 min incubation, the tubes were transferred to an ice/water bath and further incubated for 6 min in the presence of 250 μl of 33mg/ml ascorbic acid/0.5N HCl/0.1% ammonium heptamolybdate. Incubation was stopped by the

addition of 750 µl of acetic acid, 20% Na^+ arsenite and Na^+ citrate. Pump activity was determined by spectrophotometry at 750 nm. Free calcium was measured with a Ca^{2+}-sensitive electrode.

To confirm that SERCA activity was not measured with our method, we measured PMCA activity in a set of islets in the presence or absence of 200 nM Thapsigargin, the most widely used SERCA inhibitor [41].

To assess the possible participation of calmodulin in the changes induced by FRD upon PMCA activity, PMCA activity was also measured in the presence of different concentrations (0-300 µM) of calmidazolium, a calmodulin inhibitor [42].

Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Groups of islets islolated from C and FRD rats were transferred to plates containing Krebs-bicarbonate buffer with 3.3 mM glucose and 1% albumin gassed with 95% O_2-5% CO_2 and incubated for 1 h at 37 °C. Thereafter, 5 µM fura2/AM (Invitrogen, CA, USA) and pluronic acid F-127 (Invitrogen, CA, USA) in a 1:1 ratio were added to the islets and incubated for another 2 h. Islets were then transferred to an open chamber thermostatically regulated (37° C), placed on the stage of an inverted microscope (Nikon Eclipse TE 2000-U UK, Kingston, UK) and perifused (flow rate, 1.5 ml/min) with Krebs-bicarbonate buffer without albumin plus diferent glucose concentrations (3.3, 16.7 and 32 mM) and/or 30mM K^+. The excitation wavelength was set at 340/380 nm and the emitted light was collected at 510 nm by a photomultiplier connected to a digital converter and stored in a computer (Ion Optix, Milton, MA USA). Before obtaining the signal, background fluorescence was subtracted [43].

Immunohistochemical analysis

The whole pancreas was removed and weighed, and a piece of the gland was fixed in 10% formaldehyde and embedded in paraffin. Serial sections of fixed pancreases (5 µm) were obtained from three different levels of the blocks and mounted on silanized slides (3-aminopropyltriethoxy-silane; Sigma). Sections were deparaffinized, incubated for 30 min in 3% H_2O_2 in methanol to

block endogenous peroxidase activity, and rehydrated in a descending ethanol series, followed by incubation with 2.5% porcine serum to reduce non-specific binding. The slides were then sequentially incubated for 24 h at 4 °C in a humidified chamber with our own insulin antibody (1:20,000) and a mixture of antibodies against somatostatin (1:6,000; Dako, Glostrup, Denmark), glucagon (1:400; Peninsula Laboratories, San Carlos, USA) and pancreatic-polypeptide (1:10,000; NovoNordisk, Denmark) to identify β- and non-β-cells, respectively. All these cell types were finally stained through a 30 min incubation with the streptavidin-biotin complex appropriately diluted (1:40 and 1:20 respectively; Sigma), and alkaline phosphatase (1:40; Sigma).

PMCA-positive cells were revealed using appropiate dilutions of 5F10 PMCA antibody (1:100) together with the streptavidin-biotin complex, peroxidase and carbazole [19].

Statistical analysis

Data were statistically analyzed using ANOVA and the paired t-test. Results were expressed as means ± SEM. Differences between groups were considered significant when p values <0.05.

Results

C and FRD rats ate an average of 18.26 ± 2.02 and 10.74 ± 0.67 g/animal/day solid food, respectively; the latter group also consumed 5.26 g/day fructose in the drinking water. The intake of the fructose solution modified the food composition consumed by the two groups as follows: 45% carbohydrates, 43% proteins and 12% lipids for C *vs.* 64% carbohydrates, 28% proteins and 8% lipids for FRD rats. Consequently, there was a small but not significant difference in caloric intake between C and FRD rats (C *vs.* FRD, 55.6 ± 4.54 *vs.* 50.8 ± 3.43 kcal/day). Both groups of animals had similar body weight at the beginning (C *vs.* FRD, 192.4 ± 1.66 *vs.* 190.4 ± 1.16 g) and at the end (261.6 ± 2.50 *vs.* 262.7 ± 2.44 g) of the 21 days of treatment.

Plasma glucose, triglyceride and insulin levels

There were no significant differences in the plasma glucose levels of both groups; (C *vs.* FRD,

130.1 ± 4.4 *vs.* 131.6 ± 3.2 mg/dl); however, triglyceride and insulin levels were significantly higher in FRD rats (C *vs.* FRD, triglyceride, 98.5 ± 3.5 *vs.* 158.9 ± 5.5 mg/dl; $p < 0.0001$; insulin, 0.77 ± 0.05 *vs.* 1.16 ± 0.07 ng/ml; $p < 0.02$). The higher insulin:glucose ratio (0.017 ± 0.0015 *vs.* 0.027 ± 0.0017; $p < 0.0001$) and HOMA-IR values (9.70 ± 1.42 vs. 22.11 ± 3.2; $p < 0.001$) measured in FRD rats demonstrate that they developed an IR state.

Insulin secretion and islet insulin content

In both experimental groups, insulin secretion in response to different glucose concentrations (2, 4, 8, 12, 16 and 20 mM) showed an increasing dose-response pattern. Islets from FRD rats, however, released more insulin than C in response to high glucose (8, 12, 16 and 20 mM; $p < 0.05$, 0.02, 0.005 and 0.0005, respectively; Fig. 1). Insulin content was higher in FRD islets, but this difference was not statistically significant (C, 69.2 ± 4.4; FRD, 75.0 ± 8.1 ng/ml).

Identification and quantification of PMCA mRNA

Figure 2 shows that none of the PMCA isoforms modified their transcription level with respect to the control (semi quantitative PCR).

Western blot

The different bands of the PMCA isoforms were identified by their molecular weight (~ 134 kDa) using purified enzyme from normal human erythrocytes as standard. The specificity of the reaction was tested by omission of the specific first antibody.

No significant differences between groups were observed when PMCA protein expression was measured using the 5F10 antibody, which reacts with all PMCA isoforms (Fig. 3A). Conversely, using specific antibodies against each PMCA isoform, FRD islets showed a clear decrease in PMCA2 protein expression ($p < 0.001$), an increase in PMCA3 ($p < 0.01$), and no significant changes in the housekeeping isoform 1 (Fig. 3B and C). We detected, however, a small but not significant increase in isoform 4 (Fig. 3B and C).

PMCA activity

No differences were recorded in islet homogenates when PMCA activity was measured in the presence or absence of 200 nM thapsigargin (2.75 ± 0.11 *vs.* 2.82 ± 0.33 μmol Pi/h/mg prot), thus confirming that our method specifically measured only PMCA activity.

Total PMCA activity measured in islet homogenates was significantly lower in FRD islets (C *vs.* FRD, 2.8 ± 0.1 *vs.* 2.2 ± 0.1 μmol Pi/h/mg prot; $p < 0.001$; n=33; Fig. 4).

To assess the possible participation of calmodulin in the changes induced by FRD upon PMCA activity, we measured such activity in islets from both experimental groups incubated with different calmidazolium concentrations. Calmidazolium reduced the activity of the pump at all the concentrations tested (0-300 μM), reaching the greatest inhibition (~ 75%) at 40 μM. Thereafter, we tested the effect of 0 to 40 μM calmidazolium upon isolated islet PMCAs from C and FRD rats (Fig. 4).

Calmidazolium reduced the PMCA activity measured in C and FRD islets in a dose-response manner. While C islets still had higher PMCA activity than FRD ones in the presence of low calmidazolium concentrations (5 and 10 uM), such difference was no longer evident when calmodulin effect was totally inhibited by 40 μM calmidazolium (Fig. 4).

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Islets isolated from both experimental groups incubated in 3.3 mM glucose showed an increase in $[Ca^{2+}]_i$ when perfused with either high glucose (16.7 and 32 mM) or high K^+ (30 mM) medium (Fig. 5A and B), indicating the integrity of the Ca^{2+} handling mechanisms. At every glucose concentrations tested, islets from FRD-treated rats elicited a significantly higher increment in $[Ca^{2+}]_i$ (3.3 mM glucose: 100.0 ± 7.6 *vs.* 159.8 ± 16.4, $p<0.005$; and 16.7 mM glucose: 100.0 ± 3.1 *vs.* 116.6 ± 4.2, $p<0.01$; Fig 5C); this difference in $[Ca^{2+}]_i$ between groups resembled that observed in glucose-induced insulin secretion.

Immunohistochemical studies

Imunocytochemical staining using 5F10 – an antibody that recognizes all the calcium pump

isoforms – showed the presence of PMCA in the plasma membrane of β- (central zone of the islets) and non-β- (islet periphery) cells from both experimental groups. No marked changes were observed in the PMCA distribution pattern between groups (Fig. 6).

Discussion

Our study confirms that FRD administration to normal rats induces a state of IR characterized by normal plasma glucose levels, increased triglyceride and insulin levels, as well as high insulin:glucose molar ratio and HOMA-IR index [22-24]. The combination of high insulin with normal glucose levels displayed by FRD rats can be interpreted as a compensatory response of β cells to cope with the increased demand of insulin induced by the IR state, in order to maintain glucose homeostasis within normal range.

Consistent with the higher serum insulin levels measured in FRD rats (50.6 %), their islets showed a comparable higher $[Ca^{2+}]_i$ (59.8%) at basal glucose and released *in vitro* more insulin in response to glucose than C rat islets. Since PMCA activity decreased in FRD rats by 27%, other mechanisms might contribute to reach the above mentioned increase in $[Ca^{2+}]_i$. The concomitant increase in glucose metabolism measured in islets from rats with FRD-induced IR [44] with the consequent rise in the ATP/ADP ratio and activation of Ca^{2+} channels could be one of those mechanisms. These results would suggest that PMCA exerts *in vivo* a long-term modulation of insulin secretion through changes in cytosolic Ca^{2+} concentration. The fact that increased PMCA2b activity – obtained by its over-expression in β-cell lines – results in a decreased concentration of cytosolic Ca^{2+} (confirming an inverse correlation between expression [and activity] levels of the calcium pump and cytosolic Ca^{2+} concentration; [45,46]) supports this assumption.

The decreased PMCA activity found in FRD islets could be ascribed to significant and uneven changes in the protein expression of PMCA isoforms, namely, a significant decrease of PMCA2 in parallel with an increase of PMCA3, and no significant changes in the expression of housekeeping isoforms 1 and 4. Dissociation between transcription (mRNA levels) and protein expression as the

one currently found is not an unusual finding which has been reported already [47]. The absence of significant changes in the immunocytochemical PMCA distribution pattern of islet cells suggests that such pattern does not play a major role in the mechanism by which IR modulates their activity, at least at this early stage of the process.

The decreased PMCA activity observed in our FRD animals could be ascribed to a direct effect of the increased production of ROS [25,29,30,48]. The decrease in PMCA2 protein level was not apparently completely compensated by the simultaneous increase in the protein level of PMCA3, supporting the concept that the function of PMCA isoforms is not redundant [49]. Since islet PMCA2 (spliced "b" isoform) has the highest affinity for calmodulin, and PMCA3 (spliced "a" and "c" isoforms) has the lowest, the changes recorded would render islet PMCAs less responsive to calmodulin. This fact could explain why calmidazolium inhibition removed the difference in PMCA activity between C and FRD islets. However, we cannot discard the possibility that such difference was due to FRD-induced changes on the stimulatory effectiveness of endogenous calmodulin upon PMCA activity. Our current design cannot define whether one or the sum of all these mechanisms induced the decrease in PMCA activity and the changes in the transcription/expression of its isoforms in FRD animals.

Briefly, our results demonstrate that administration of a FRD to normal rats for 3 weeks induces an IR state and a significant decrease in islet PMCA activity, with a concomitant $[Ca^{2+}]_i$ increase. These functional changes would favor the compensatory higher release of insulin necessary to cope with the increased hormone demand and to maintain glucose homeostasis within normal range. The decreased PMCA activity could be ascribed to a decreased expression of PMCA2 isoform, highly sensitive to calmodulin stimulation, together with a probably lower stimulatory effect of calmodulin upon PMCA activity. These results suggest that changes in islet PMCA activity, due to changes in isoform expression, play a modulatory role upon insulin secretion during both short-acute and long-term adaptation periods.

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Figure legends

Fig. 1. *In vitro* insulin secretion. Insulin released by islets isolated from C (white circles) and FRD (black circles) rats in response to different glucose concentrations (2, 4, 8, 12, 16 and 20 mM). Both experimental groups showed an increasing dose-response pattern although FRD islets released more insulin than C in response to high glucose Results represent the means ± SEM of three independent experiments; *p* values between groups *<0.05; **<0.02; ***<0.005.

Fig. 2. Semi-quantitative RT-PCR. Analysis of the expression levels of the different PMCA isoforms in islets from C (white bars) and FRD (black bars) rats. Each panel shows a representative picture of the agarose gel together with the quantification chart (panel A: PMCA1, panel B: PMCA2, panel C: PMCA3, panel D: PMCA4). None of the PMCA isoforms modified their transcription level with respect to the control. The ratio between the gene of interest (GOI) and the internal standard is shown in relative units with respect to the ratio measured in C islets. Results represent the means ± SEM of three different independent experiments.

Fig. 3. **Panel A**: PMCAs Western blot from C and FRD isolated islets performed with 5F10 antibody, which reacts with all PMCA isoforms. Lane 1: Relative molecular weight marker. Lanes 2 and 3: Islets isolated from C rats (300 and 400 islets, respectively). Lanes 4 and 5: Islets isolated from FRD animals (300 and 400 islets, respectively). Arrows show the position and size (KDa) of the relative standard molecular weight. **Panel B**: Western blot of PMCA isoforms identified in islets from C and FRD rats using specific antibodies (NR1, NR2, NR3 and JA9). First lane, relative molecular weight markers; lanes 2-9, each pair represents islets from C (left) and FRD (right) animals; lanes 2 and 3, isoform 1; lanes 4 and 5, isoform 2; lanes 6 and 7, isoform 3; lanes 8 and 9, isoform 4. The arrow indicates the position and size (KDa) of the relative standard molecular weight. The figure is a representative picture from three independent experiments. **Panel C**: Quantitative expression of band intensity from C (white bars) and FRD (black bars) rat islets; FRD islets showed a decrease in PMCA2 and an increase in PMCA3 protein expression, with no significant changes in PMCA1 and 4. Results represent the means ± SEM of three independent

experiments expressed as relative values with respect to the intensity found in C islets. In every case, we considered the main (most intense) band for quantitation. FRD *vs*.C; **p*<0.001; ***p*<0.01

Fig. 4. Total PMCA activity measured in homogenates of islets isolated from C (white circles) and FRD (black circles) rats in the presence of 1.77 μM Ca^{2+} and in the absence or presence of different calmidazolium concentrations (0 to 40 μM). Calmidazolium reduced the PMCA activity measured in C and FRD islets in a dose-response manner. C islets showed higher PMCA activity than FRD ones in the absence of calmidazolium, and this diference was no longer evident when calmidazolium was present at a high concentration (40 μM). Results represent the means ± SEM of four independent experiments, where each determination was performed in triplicate. FRD *vs.* C, *p* * <0.05; **<0.02; ***<0.001.

Fig. 5. Measurement of $[Ca^{2+}]_i$ in islets isolated from C and FRD rats. **Panel A:** Islet $[Ca^{2+}]_i$ increased when glucose concentration in the perifusion medium shifted from 3.3 mM to either 16.6 mM or 32.0 mM. **Panel B:** The same effect was observed in the presence of 30 mM K^+ in the medium. Charts are representative of 6 individual experiments. **Panel C:** FRD islets (black bars) showed significantly higher $[Ca^{2+}]_i$ than *C* islets (white bars) at both glucose concentrations tested (3.3 mM, **p*<0.005; and 16.6 mM ***p* <0.01). Units are expressed as relative values with respect to the levels measured in C islets and represent the means ± SEM of 6 independent experiments.

Fig. 6. Pancreatic islets stained with PMCA 5F10 antibody. PMCA immunopositive cells were found in the periphery (non-β cells) and in the central zone (β cells) of the islets in C (left panel) and FRD (right panel) rats. No marked changes were observed in the PMCA distribution pattern between groups (× 40) .