

Heterozygous inactivation of plasma membrane Ca^{2+} -ATPase in mice increases glucose-induced insulin release and beta cell proliferation, mass and viability

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Abstract

Aims/hypothesis Calcium plays an important role in the process of glucose-induced insulin release in pancreatic beta cells. These cells are equipped with a double system responsible for Ca^{2+} extrusion—the Na/Ca exchanger (NCX) and the plasma membrane Ca^{2+} -ATPase (PMCA). We have shown that heterozygous inactivation of NCX1 in mice increased glucose-induced insulin release and stimulated beta cell proliferation and mass. In the present study, we examined the effects of heterozygous inactivation of the PMCA on beta cell function.

Methods Biological and morphological methods (Ca^{2+} imaging, Ca^{2+} uptake, glucose metabolism, insulin release and immunohistochemistry) were used to assess beta cell function and proliferation in *Pmca2* (also known as *Atp2b2*) heterozy-

gous mice and control littermates ex vivo. Blood glucose and insulin levels were also measured to assess glucose metabolism in vivo.

Results *Pmca* (isoform 2) heterozygous inactivation increased intracellular Ca^{2+} stores and glucose-induced insulin release. Moreover, increased beta cell proliferation, mass, viability and islet size were observed in *Pmca2* heterozygous mice. However, no differences in beta cell glucose metabolism, proinsulin immunostaining and insulin content were observed.

Conclusions/interpretation The present data indicates that inhibition of Ca^{2+} extrusion from the beta cell and its subsequent intracellular accumulation stimulates beta cell function, proliferation and mass. This is in agreement with our previous results observed in mice displaying heterozygous inactivation of NCX, and indicates that inhibition of Ca^{2+} extrusion mechanisms by small molecules in beta cells may represent a new approach in the treatment of type 1 and type 2 diabetes.

Nathalie Pachera and Julien Papin contributed equally to this work.

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Keywords Beta cell · Calcium · Diabetes · Plasma membrane Ca^{2+} -ATPase · Sodium/calcium exchange

Abbreviations

ER	Endoplasmic reticulum
GLP-1	Glucagon-like peptide-1
IPGTT	Intraperitoneal glucose tolerance test
NCX	Sodium/calcium exchange
NFAT	Nuclear factor of activated T cells
PMCA	Plasma membrane Ca^{2+} -ATPase

Introduction

Diabetes mellitus is characterised by the loss of functional pancreatic beta cells, leading to insufficient insulin release

[1]. Therefore, enhancing beta cell proliferation and survival is a major goal in diabetes mellitus research. Calcium is an important signalling molecule involved in key cellular functions such as hormone secretion and cell metabolism, division and death. When stimulated by glucose, beta cells display a complex series of events including the opening of voltage-sensitive Ca^{2+} channels, leading to Ca^{2+} entry into the cell with a subsequent rise in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) which triggers insulin release [2]. After entering the cell, the ion must be extruded in order to avoid its excessive accumulation, which may be toxic for the cell [3]. Two systems responsible for Ca^{2+} extrusion are present in the beta cell—the Na/Ca exchanger (NCX) and the plasma membrane Ca^{2+} -ATPase (PMCA) [4]. In a previous study in mice [5], we showed that heterozygous inactivation of the Na/Ca exchanger (isoform 1: NCX1), a protein responsible for Ca^{2+} extrusion from cells [6, 7], induced several modifications of the beta cell including an increase in glucose-induced insulin release and beta cell proliferation and mass. *Ncx1*^{+/-} islets also displayed an increased resistance to hypoxia and when transplanted into diabetic animals produced a four to seven times higher rate of diabetes cure than *Ncx1*^{+/+} islets [5].

Another mechanism responsible for Ca^{2+} extrusion from cells is the plasma membrane Ca^{2+} -ATPase (PMCA) [8]. While NCX has a low affinity but high capacity, PMCA has a high affinity but low capacity to extrude Ca^{2+} from cells [9]. The mammalian PMCA is encoded by four different genes and alternative splicing of the primary transcripts leads to at least several dozen proteins [9]. The beta cell expresses four isoforms of *Pmca* (also known as *Atp2b2*), and six alternative splice mRNA variants have been detected [10]. In the present study, we examined to what extent systemic PMCA2 heterozygous inactivation in mice may also affect beta cell function. Mice with homozygous PMCA2 inactivation display deafness and balance defects [11] while heterozygous PMCA2 inactivation does not cause hearing deficits and the mice have a normal phenotype.

Methods

Animals and isolation of pancreatic islets Principles of laboratory animal care (NIH publication no. 85–23, revised 1985; <http://grants1.nih.gov/grants/olaw/references/phspol.htm>) were followed. Animals were treated following the guidelines of the Belgian Regulations for Animal Care and with approval from the local Ethical Committee. For animal experiments, samples were not randomised.

Pmca2-knockout mice were kindly donated by G. Shull Cincinnati, Ohio, USA [11]. The genotype of each mouse was determined pre-weaning, as previously described [12] (see electronic supplementary material [ESM] Fig. 1a). *Pmca2*^{+/-} mice were viable, fertile, had a normal body weight

and showed no macroscopic anomalies at the level of the main organs. All experiments were performed on *Pmca2*^{+/-} and age-matched *Pmca2*^{+/+} wild-type littermates (129/SvJ background). To verify PMCA2 expression, *Pmca2* mRNA levels were measured by quantitative RT-PCR, since in isolated islets PMCA2 protein is generally expressed at levels too low to be detected by western blot (ESM Fig. 1b). In addition, since *Pmca2*^{+/-} is a whole-body transgenic mouse strain, we assessed PMCA2 protein expression in brain tissue (ESM Fig. 1c). For islet isolation, pancreases were perfused via the common bile duct with collagenase-P and islets were purified using a Histopaque gradient (Sigma-Aldrich, Munich, Germany) [12]. Islets were allowed to recover overnight at 37°C and 5% CO_2 in RPMI-1640 GlutaMAX (Life Technologies, Gent, Belgium) supplemented with 10% heat-inactivated fetal bovine serum and 50 U/ml penicillin and 50 µg/ml streptomycin [13].

Morphological studies, intracellular Ca^{2+} concentration measurement, ^{45}Ca uptake, insulin content and insulin release from isolated islets, and measurement of glucose metabolism ex vivo Golgi proinsulin and beta cell area was evaluated on slides stained for proinsulin with immunoperoxidase [14], digitised through a JVC KY-F58 colour digital camera (Japan Victor, Perkin Elmer, Groningen, the Netherlands). Tissues sections (5 µm thick) were prepared as described by Jacoby et al [15] and stained with haematoxylin–eosin. For electron microscopy, groups of 30 islets were fixed, epoxide-embedded and analysed as described previously [16].

The measurement of intracellular Ca^{2+} concentration using fura-2 in isolated islets was done as previously described [17, 18], using a camera-based image analysis system (MetaFluor, Universal-Imaging, Detroit, MI, USA).

The methods used to measure ^{45}Ca uptake in intact islets and insulin release from perfused islets are described elsewhere [17–20]. Insulin was assayed using an ELISA kit (Mercodia, Uppsala, Sweden). For the measurement of insulin content, the islets were homogenised in water in the absence of glucose.

The methods used to measure D-[5- ^3H]glucose metabolism and D-[U- ^{14}C]glucose oxidation in intact islets have been described previously [21, 22].

Measurement of beta cell viability, proliferation, mass and size Cell viability was measured in isolated islets using Hoechst-33342 and propidium iodide [23]. A minimum of ten islets were analysed per condition, all experiments being performed by two independent observers, one of them unaware of sample identity. In *Pmca2*^{+/+} single beta cells and islets, the viability was 65–70% and 85–95%, respectively. In vitro hypoxia studies were as previously described [24].

The duration of hypoxia was 6 h. Viability of beta cells was measured as described above.

To measure beta cell apoptosis, the TUNEL method was used, with the in situ Cell Death Detection Kit (Roche Diagnostics, Vilvorde, Belgium). The method for beta cell labelling and counting was similar to that used in the measurement of beta cell proliferation (please see below).

To measure beta cell proliferation, tissues sections (5 μm thick) were prepared according to the method of Jacoby et al [15] and then processed for immunofluorescence labelling against Ki67 (rabbit monoclonal [SP6] anti-Ki67; Abcam, Cambridge, UK) and insulin (guinea pig polyclonal anti-insulin; Dako, Hamburg, Germany) [24]. Briefly, after rehydration of the sections, antigen retrieval was realised by incubation in 96°C citrate buffer (10 mmol/l, pH 6.0) for 20 min and blocking in 10% donkey serum in 1% BSA (Sigma, Diegem, Belgium). Primary antibodies were incubated overnight at 4°C and detected with dedicated secondary antibodies (donkey anti-guinea pig–Alexa488 and donkey anti-rabbit–Cy3, Jackson ImmunoResearch, Newmarket, UK) diluted in blocking buffer and incubated for 1 h at room temperature.

Quantification of beta cell mass was performed by point-counting morphometry of insulin-immunostained pancreatic sections, as previously described [25]. Sections were monitored using an Axio-Observer-D1 fluorescence microscope (Carl Zeiss, Zaventem, Belgium) and the individual beta cell size was measured using the associated image analysis software. The beta cell area of the pancreatic section was divided by the number of beta cell nuclei identified in the area. All counts were independently performed by two investigators unaware of sample identity (samples were identified only by randomly assigned numbers).

In vivo glucose metabolism, insulin sensitivity and measurement of serum glucagon, growth hormone and glucagon-like peptide 1 The measurement of glucose metabolism (intraperitoneal glucose tolerance test [IPGTT]) and insulin sensitivity in vivo were done as previously described [26, 27]. Serum glucagon, growth hormone and glucagon-like peptide-1 (GLP-1) were measured using Glucagon Human/Mouse/Rat ELISA (Alpco, Salem, NH, USA), Rat/Mouse Growth Hormone ELISA Kit (Millipore, St Charles, MO, USA) and Mouse GLP-1 ELISA kit (Antibodies-online.com, Aachen, Germany).

mRNA extraction and qualitative RT-PCR Poly(A)+ mRNA was isolated from mouse islets using Dynabeads mRNA Direct kit (Invitrogen, Life Technologies) and reverse transcribed. The real-time PCR amplification reaction was performed using SYBR Green and compared with a standard curve [23]. Expression values were corrected for the housekeeping gene β -actin. The following primers were used: mouse *Pmca2* forward primer 5'-TCATCTGTGTGGTCTGGTCA-

3', mouse *Pmca2* reverse primer 5'-TTCTGCTCCTGCTCA ATTCGG-3'; mouse β -actin forward primer 5'-AGAG GGAAATCGTGCGTGAC-3', mouse β -actin reverse primer 5'-TCTCCTTCTGCATCCTGTCA -3'.

Western blot analysis Mouse brain tissues were lysed in RIPA (Sigma) buffer and the protein content was quantified using Bio-Rad (Marnes-la-Coquette, France) DC Protein-Assay-kit according to the manufacturer protocol. Proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and revealed with antibodies against PMCA2 (Swant, Marly, Switzerland), β -actin (Cell Signaling, BIOKE, Leiden, the Netherlands) and horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich).

Statistics No data were excluded. The results are expressed as means \pm SEM. The statistical significance of differences between data was assessed by using Student's *t* test or ANOVA, followed by the Tukey post test. Statistical significance was accepted at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results

***Pmca2* heterozygous inactivation increases basal $[\text{Ca}^{2+}]_i$, basal ^{45}Ca uptake and glucose-induced insulin release** To evaluate the functional consequences of *Pmca2* heterozygous inactivation on pancreatic beta cells, we measured the effect of glucose (11.1 mmol/l) and KCl (50 mmol/l) on cytosolic $[\text{Ca}^{2+}]_i$ in islets isolated from 12-week-old *Pmca2*^{+/-} and *Pmca2*^{+/+} mice. In both types of islets, glucose induced a biphasic increase in $[\text{Ca}^{2+}]_i$, the increases being of similar magnitude (Fig. 1a). Thus, there was no difference in either the baseline value or in the rise in $[\text{Ca}^{2+}]_i$ elicited by the sugar ($p > 0.05$). KCl also increased $[\text{Ca}^{2+}]_i$ in both types of islets, although the $[\text{Ca}^{2+}]_i$ was slightly higher in *Pmca2*^{+/-} than in *Pmca2*^{+/+} islets during the baseline period ($p < 0.01$, Fig. 1b). We then measured glucose-induced $^{45}\text{Ca}^{2+}$ uptake. In control islets (*Pmca2*^{+/+}), glucose (16.7 mmol/l) significantly increased ^{45}Ca uptake ($p < 0.01$). In *Pmca2*^{+/-} islets, ^{45}Ca uptake at low glucose (2.8 mmol/l) was increased compared with control islets (+70%, $p < 0.05$, Fig. 1c) and 16.7 mmol/l glucose only slightly increased ^{45}Ca uptake (+16%) further, the effect being not statistically significant. Taken as a whole, these data indicate that *Pmca2* heterozygous inactivation slightly affects beta cell $[\text{Ca}^{2+}]_i$ at rest but markedly increases basal beta cell Ca^{2+} content. To further assess this increase, the effect of thapsigargin (an inhibitor of sarcoplasmic/endoplasmic reticulum [ER] Ca^{2+} ATPase) on $[\text{Ca}^{2+}]_i$ under basal condition was examined (Fig. 1d). In *Pmca2*^{+/-} islets, thapsigargin induced an important but transient increase in $[\text{Ca}^{2+}]_i$, a phenomenon that was of larger magnitude in *Pmca2*^{+/-} islets ($p < 0.001$), indicating that ER Ca^{2+} stores

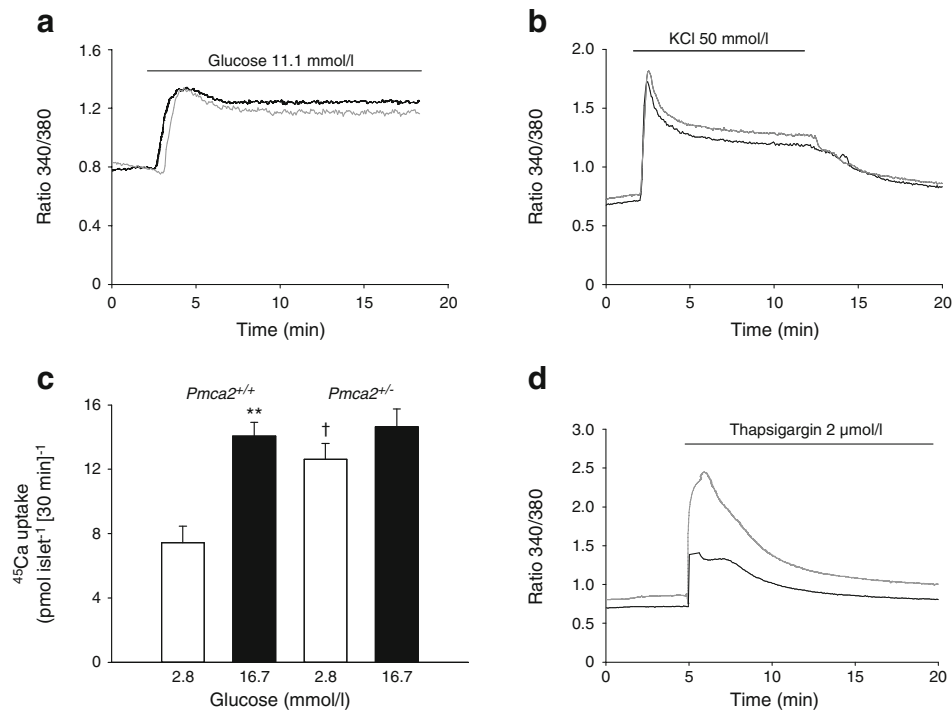


Fig. 1 Effect of *Pmca2* heterozygous inactivation on islet Ca^{2+} movement. **(a, b)** Effect of 11.1 mmol/l glucose **(a)** and 50 mmol/l KCl **(b)** on $[\text{Ca}^{2+}]_i$ in *Pmca2* $^{+/+}$ (black lines) and *Pmca2* $^{+/-}$ (grey lines) islets (groups of 20) measured as the ratio of absorbance at 340 and 380 nm. The period of exposure to glucose or KCl is indicated by a bar above the curves. The mean of five **(a)** and four **(b)** experiments is shown. **(c)** Effect of *Pmca2* heterozygous inactivation on glucose-induced ^{45}Ca uptake in 12-week-

old mouse islets. Mean \pm SEM values are shown for four experiments comprising three to five replicates. $**p < 0.01$ vs control islets at 2.8 mmol/l glucose; $\dagger p < 0.05$ vs *Pmca2* $^{+/+}$ islets at 2.8 mmol/l glucose. **(d)** Effect of *Pmca2* heterozygous inactivation on 2 $\mu\text{mol/l}$ thapsigargin-induced $[\text{Ca}^{2+}]_i$ increase in the presence of 2.8 mmol/l glucose. Black line, *Pmca2* $^{+/+}$ islets; grey line, *Pmca2* $^{+/-}$ islets. The mean of five experiments is shown

were indeed increased in *Pmca2* $^{+/-}$ islets compared with *Pmca2* $^{+/+}$ islets. Thus, the increase in $[\text{Ca}^{2+}]_i$ induced by thapsigargin, measured as the AUC over the baseline value during the 5–20 min period, was approximately doubled in *Pmca2* $^{+/-}$ islets compared with *Pmca2* $^{+/+}$ islets ($p < 0.05$).

We then performed perfusion experiments to measure glucose-induced insulin release in islets isolated from *Pmca2* $^{+/-}$ and *Pmca2* $^{+/+}$ mice. Figure 2 shows the effect of an increase in glucose concentration from 2.8 mmol/l to 11.1 mmol/l on insulin release. In both types of islets, glucose induced a biphasic increase in insulin release characterised by an initial peak followed by a secondary rise. Glucose elicited a larger increase in insulin release in *Pmca2* $^{+/-}$ islets than in *Pmca2* $^{+/+}$ islets. The amount of insulin released during the initial peak (16–20 min) and the whole period of stimulation (16–60 min), measured as the AUC over the baseline value during the 5–15 min period, was about 1.5 and 1.4 times larger in *Pmca2* $^{+/-}$ than in *Pmca2* $^{+/+}$ islets, respectively (Fig. 2, $p < 0.05$). During the pre-stimulatory period there was a tendency for insulin secretion to be higher in *Pmca2* $^{+/-}$ mice than in control mice. However the difference was not statistically significant ($p = 0.18$).

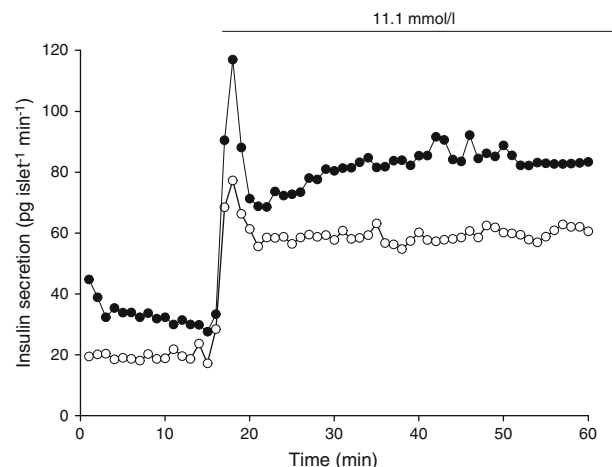


Fig. 2 Effect of 11.1 mmol/l glucose on insulin release from *Pmca2* $^{+/+}$ (white circles) and *Pmca2* $^{+/-}$ (black circles) islets (groups of 20). The period of exposure to glucose is indicated by a bar above the curves. The mean of four experiments is shown for each type of islet. The amount of insulin released during the initial peak (16–20 min) and the whole period of stimulation (16–60 min), measured as the AUC over the baseline value during the 5–15 min period, was about 1.5 and 1.4 times larger in *Pmca2* $^{+/-}$ islets than in *Pmca2* $^{+/+}$ islets, respectively ($p < 0.05$). There was no difference in insulin content between *Pmca2* $^{+/+}$ and *Pmca2* $^{+/-}$ islets (batches of ten islets, four and five individual determinations)

Effect of *Pmca2* heterozygous inactivation on insulin content, glucose metabolism and morphology No difference in insulin content was observed between *Pmca2*^{+/-} islets and *Pmca2*^{+/+} islets (data not shown).

To stimulate insulin release, glucose must be metabolised by the pancreatic beta cell [2, 28]. As seen in ESM Fig. 2a, the utilisation of D-[5-³H]glucose increased in both types of islets when the glucose concentration increased from 2.8 mmol/l to 16.7 mmol/l. At both hexose concentrations, ³H₂O generation from D-[5-³H]glucose tended to be slightly higher in *Pmca2*^{+/-} cells than in *Pmca2*^{+/+} cells, but the difference was not statistically significant. The generation of ¹⁴CO₂ from D-[U-¹⁴C]glucose was also increased in response to the rise in glucose concentration in both types of islets (ESM Fig. 2b). At both concentrations, there was a tendency for the oxidation to be higher in *Pmca2*^{+/-} islets than in *Pmca2*^{+/+} islets, but again the differences were not statistically significant.

There was no difference in morphology between *Pmca2*^{+/-} and *Pmca2*^{+/+} islets. Proinsulin staining was not increased in *Pmca2*^{+/-} islets compared with *Pmca2*^{+/+} islets, a finding in line with the absence of an increase in insulin content. Electron microscopy analysis revealed no difference in the features of pancreatic beta cells of *Pmca2*^{+/+} and *Pmca2*^{+/-} mice (ESM Fig. 2c). The observed ultrastructure perfectly matched the ultrastructure described in the literature [5]. A non-quantitative electron microscopy examination of the samples demonstrated no obvious differences between *Pmca2*^{+/+} and *Pmca2*^{+/-} beta cells. Quantitative analysis of the relative volume of the organelles showed that there was a decrease in mitochondrial volumetric density compensated for by an increased cytoplasmic density in *Pmca2*^{+/-} mouse islets ($p < 0.0001$) (ESM Table 1).

***Pmca2* heterozygous inactivation increases beta cell mass, proliferation and viability** Beta cell mass, size and proliferation rate were measured in adult (aged 12 weeks) *Pmca2*^{+/+} and *Pmca2*^{+/-} mice. Beta cell mass was increased by about 60% in *Pmca2*^{+/-} mice when compared with *Pmca2*^{+/+} mice, averaging 3.22±0.44 mg and 5.21±0.76 mg in *Pmca2*^{+/+} and *Pmca2*^{+/-} mice, respectively ($p < 0.05$, Fig. 3a). This increase was not due to beta cell hypertrophy because no change in beta cell size was observed (Fig. 3b); rather, it was due to an increase in islet size with concomitant beta cell hyperplasia. Indeed, the islet size averaged 20,948±384 μm² and 29,991±458 μm² in *Pmca2*^{+/+} and *Pmca2*^{+/-} mice, respectively ($p < 0.001$, Fig. 3c). The islet number, however, was not increased (Fig. 3d). In agreement with these observations, the beta cell proliferation rate was 4.3 times higher in *Pmca2*^{+/-} mice than in *Pmca2*^{+/+} mice ($p < 0.05$, Fig. 3e).

Beta cell viability was measured in isolated islets from adult mice (12 weeks old). Figure 3f shows that cell viability after 24 h of culture was higher in *Pmca2*^{+/-} than in *Pmca2*^{+/+} islets ($p < 0.01$), the percentage of apoptotic and necrotic cells

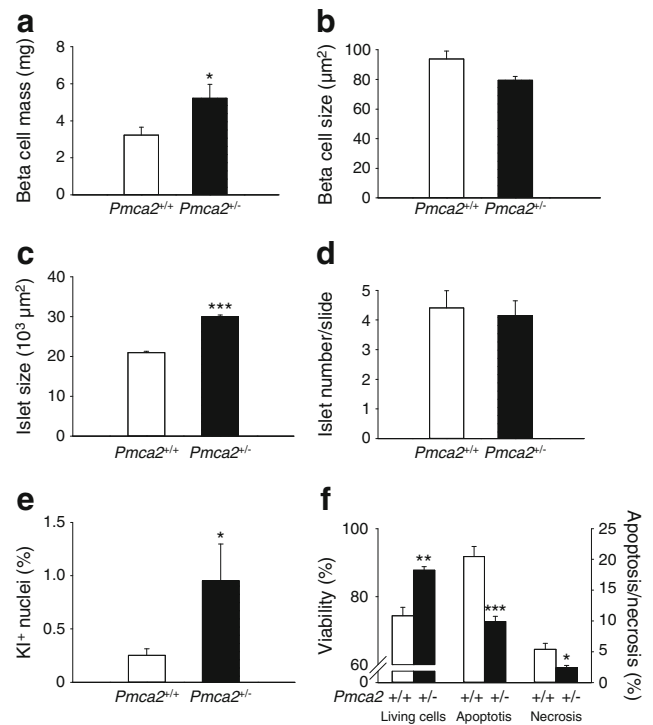


Fig. 3 The effect of *Pmca2* heterozygous inactivation is shown on beta cell mass (a) and size (b), islet size (c) and number (d) and beta cell proliferation rate (e) and viability (f) in 12-week-old *Pmca2*^{+/+} (white bars) and *Pmca2*^{+/-} (black bars) mice. The means±SEM from four pancreas specimens are shown. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, *Pmca2*^{+/-} vs *Pmca2*^{+/+}

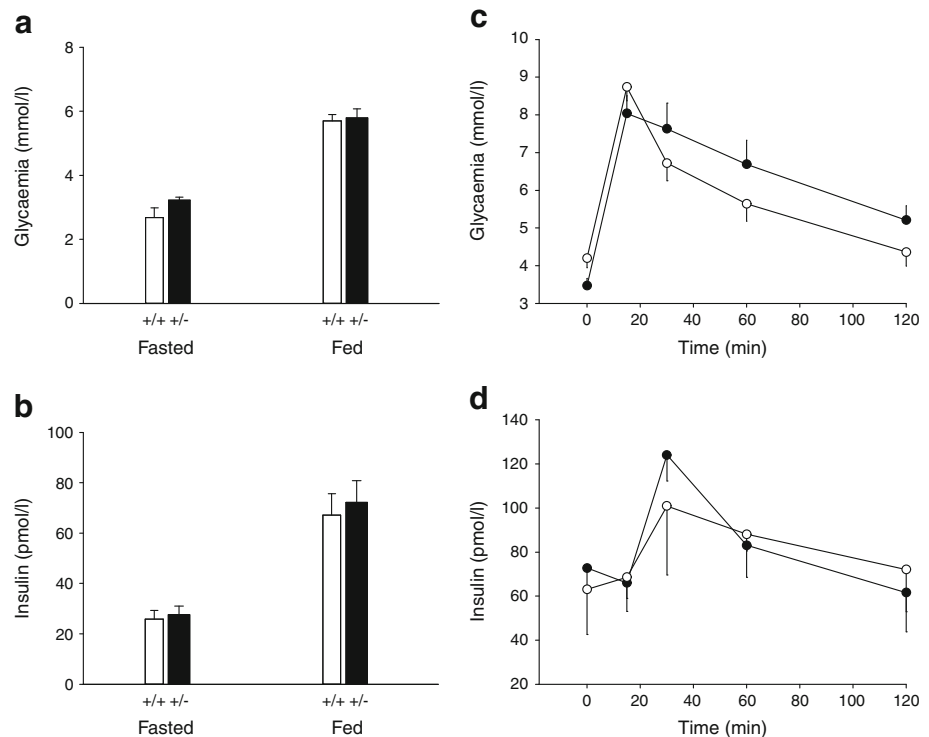
being significantly lower ($p < 0.05$ and $p < 0.001$, respectively). Similar data were observed after 48 h and 72 h of culture (data not shown). To test their resistance to hypoxia, the islets were then subjected to hypoxia for 6 h. ESM Fig. 2d shows that there was no difference in viability between *Pmca2*^{+/-} and *Pmca2*^{+/+} islets.

***Pmca2* heterozygous inactivation fails to affect glucose metabolism in vivo** Plasma glucose and insulin levels were comparable in *Pmca2*^{+/+} and *Pmca2*^{+/-} mice both in the fasted and fed state (Fig. 4 a, b). Likewise, the IPGTT did not show any difference between the two types of islets, except that the decrease in glucose concentration after the initial peak (15 min) was more rapid in *Pmca2*^{+/-} mice than in *Pmca2*^{+/+} mice (Fig. 4c). Thus, the decrease in glucose concentration between 15 and 60 min and 15 and 120 min averaged 1.3±0.3 and 2.8±0.3 mmol/l and 2.8±0.3 and 4.5±0.4 mmol/l in *Pmca2*^{+/+} and *Pmca2*^{+/-} mice, respectively ($p < 0.05$ and $p < 0.01$). No difference in insulin levels were observed between the two types of mice (Fig. 4d).

The intraperitoneal insulin sensitivity test showed no difference between *Pmca2*^{+/-} and *Pmca2*^{+/+} mice (data not shown).

There were no differences in serum levels of insulin, glucagon or growth hormone in the fasting state and GLP-1 in the

Fig. 4 (a, b) Glycaemia and insulin levels measured in the fasting and the fed state of *Pmca2*^{+/+} and *Pmca2*^{+/-} mice ($n=9-13$). (c, d) Glycaemia and insulin levels during IPGTT in *Pmca2*^{+/+} (black circles) and *Pmca2*^{+/-} (white circles) mice ($n=13$ in each case, $*p<0.05$). The decrease in glucose concentration between 15 and 60 min and 15 and 120 min during the IPGTT averaged 1.3 ± 0.3 and 2.8 ± 0.3 mmol/l and 2.8 ± 0.3 and 4.5 ± 0.4 mmol/l in *Pmca2*^{+/+} and *Pmca2*^{+/-} mice, respectively ($p<0.05$ and $p<0.01$)



fed state when comparing 12-week-old *Pmca2*^{+/-} mice with *Pmca2*^{+/+} mice ($p>0.05$, ESM, Table 2).

Discussion

The aim of the present study was to examine the effects of heterozygous inactivation of the PMCA on beta cell function in comparison with those induced by NCX heterozygous inactivation [5]. The data obtained are compatible with the characteristics of these two Ca^{2+} -extruding mechanisms, namely that while NCX has a low affinity but high capacity, PMCA has a high affinity but low capacity to extrude Ca^{2+} from cells [9]. In other words, while NCX takes care of large amounts of Ca^{2+} when the ion is entering the cell in the stimulated state, the PMCA controls $[\text{Ca}^{2+}]_i$ under basal condition. Indeed, PMCA2 inactivation tended to increase basal $[\text{Ca}^{2+}]_i$ but failed to increase $[\text{Ca}^{2+}]_i$ in glucose- and K^+ -stimulated islets, while NCX1 inactivation produced the opposite effects. Likewise, PMCA2 inactivation increased basal ^{45}Ca uptake and failed to affect glucose-stimulated ^{45}Ca uptake, in contrast to NCX1 inactivation. As a consequence, the effects induced by PMCA2 inactivation compared with NCX1 inactivation were of lower magnitude and sometimes absent. For instance, there was a 1.5- and 2.4-fold increase in glucose-induced insulin release after PMCA2 and NCX1 inactivation, respectively. Insulin content and proinsulin staining were unaffected by PMCA2 inactivation but were increased by NCX1 inactivation and glucose metabolism and glucose-induced $[\text{Ca}^{2+}]_i$

oscillations were unaffected by PMCA2 inactivation but were reduced or altered by NCX1 inactivation, respectively.

Nevertheless, PMCA2 inactivation induced interesting changes in beta cell function, including an increase in beta cell proliferation ($\times 4.3$ vs 5.3 in the NCX1 model) with a resulting increase in beta cell mass ($\times 1.6$ vs 2.3) and islet size ($\times 1.4$ vs 1.0) but not in islet number, indicating beta cell hyperplasia. Interestingly, PMCA2 inactivation induced a larger increase in ER Ca^{2+} stores (twofold) when compared with NCX1 inactivation (1.6-fold). Taken as a whole, the present data confirm the view arising from the NCX1 experiments [5], that inhibition of Ca^{2+} extrusion from the beta cell with its subsequent intracellular accumulation may stimulate beta cell function, proliferation and mass. The inhibition of such a process in the beta cell by small molecules may represent a new approach in the treatment of diabetes that could prevent the development of both type 1 and type 2 diabetes in at-risk patients. It could also preserve residual beta cell function and mass in recent-onset diabetes and activate endogenous beta cell regeneration by stimulation of beta cell proliferation. Indeed, both types of diabetes, although differing in aetiology and physiopathological features, have in common a decrease in beta cell mass that contributes to insufficient insulin release [1]. Hence, a means of increasing beta cell proliferation would be invaluable in both types of diabetes. One potential danger of molecules that stimulate cell proliferation, though, is their potential tumorigenic effect. This could be overcome by an intermittent or temporary treatment using drugs specifically targeting beta cells. In this respect, benzyoxyphenyl NCX

inhibitors [29] could be of interest because they are about 20 times more active in inhibiting the NCX1 isoforms that are expressed in the beta cell than those expressed in the heart and other tissues [30]. As suggested in the case of the *Ncx1*^{+/-} model, the increase in beta cell function, proliferation and mass seen in *Pmca2*^{+/-} islets is thought to result from the activation of the calcineurin–nuclear factor of activated T cells (NFAT) signalling pathway by raised cellular Ca²⁺ [5]. Calcineurin is a calmodulin-dependent serine/threonine phosphatase, which upon activation by Ca²⁺ dephosphorylates the cytoplasmic subunits of NFAT (NFATc). Dephosphorylation of NFATc allows its rapid translocation to the nucleus where it interacts with NFAT nuclear proteins (NFATn); subsequent activation of insulin transcription and promotion of beta cell proliferation occurs through increased expression of cell cycle promoters such as cyclin D1, cyclin D2 and cyclin-dependent kinase 4 [31]. Although we did not observe a significant increase in basal [Ca²⁺]_i in *Pmca2*^{+/-} mice, we observed an increased basal Ca²⁺ uptake and ER Ca²⁺ stores. Since elevated basal [Ca²⁺]_i can induce cell death [32, 33], the ion is stored in the ER and released into the cytosol when necessary [32]. Therefore, we hypothesise that the elevated ER Ca²⁺ concentrations observed in *Pmca2*^{+/-} mice are sufficient to activate the calcineurin–NFAT signalling pathway. A hypothesis needs to be confirmed.

We observed only a slight improvement in the IPGTT results between control and *Pmca2*^{+/-} mice, despite increased insulin secretion and beta cell mass in the latter. However, the beta cell population is heterogeneous, so while some beta cells are more sensitive or responsive to low glucose concentrations, others only respond to higher levels of glucose [34]. Therefore, there is a concerted response of the beta cells to maintain stable glucose levels in the blood. This plasticity in beta cell response can be observed in impaired glucose tolerance or obesity, where these cells are able to compensate for the increase in insulin resistance and/or decrease in beta cell mass [35, 36]. Since our studies were performed in ‘healthy’ mice, the increased beta cell mass observed in *Pmca2*^{+/-} mice may be still at rest and an effect on IPGTT is not clearly observed. However, it is plausible that these mice would respond better than control mice when used in diabetes-inducing models such as high-fat diet or multiple-low-dose-streptozotocin treatment.

One may wonder whether the increase in beta cell mass seen in *Pmca2*^{+/-} islets is of developmental origin. For *Ncx1*^{+/+} islets this is not the case. Indeed, in the latter model [5], beta cell mass was not different in 4-week-old *Ncx1*^{+/+} and *Ncx1*^{+/-} mice. In 12-week-old mice, beta cell mass was much more important in *Ncx1*^{+/-} than in *Ncx1*^{+/+} islets [5]. Therefore, for the *Ncx1*^{+/-} mice, the increase in beta cell mass is acquired and we imagine that the situation is the same in the *Pmca2*^{+/-} model. This, however, remains to be demonstrated.

Another interesting finding was the observation that PMCA2 inactivation increased beta cell viability. This was unexpected because NCX1 heterozygous inactivation does not affect cell viability. The reason for this difference is unclear. The calcineurin–NFAT pathway may also affect apoptosis. According to immunologists’ ‘two-signal hypothesis’ for T cell activation, calcineurin–NFATc signalling in the absence of a second activated signalling pathway (e.g. one that activates NFATn proteins) may induce cell death and dysfunction rather than cell growth and proliferation [37]. The exact mechanism involved in PMCA inactivation-induced increase in beta cell viability remains to be further examined. Incidentally, because cell viability was evaluated in isolated islets, the increase in viability cannot be taken as a mechanism by which *Pmca2* heterozygous inactivation increases beta cell mass.

Quantitative analysis of the relative volume of organelles showed that there was a decrease in mitochondrial volumetric density compensated for by an increased cytoplasmic density. Because glucose oxidation, which occurs in the mitochondria, was not affected in *Pmca2*^{+/-} islets compared with *Pmca2*^{+/+} islets, the meaning of this observation is unclear.

In conclusion, inhibition of Ca²⁺ extrusion from the beta cell, either by inhibiting NCX or the PMCA, may represent a novel approach for the prevention and treatment of both types of diabetes.

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