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Effects of plasma membrane Ca^{2+} -ATPase overexpression upon D-glucose metabolism in insulin-producing BRIN-BD11 cells

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Short title: Overexpression of Ca^{2+} -ATPase isoforms in BRIN-BD11 cells

List of abbreviations: PMCA: plasma membrane Ca^{2+} -ATPase

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Abstract

In order to investigate the possible link between plasma membrane Ca^{2+} -ATPase (PMCA) activity and D-glucose catabolism in insulin-producing cells, BRIN-BD11 cells were transfected with two isoforms of PMCA2. Transfection of insulin-producing BRIN-BD11 cells with plasma membrane Ca^{2+} -ATPase (PMCA2yb and PMCA2wb) was documented by RT-PCR, Western blot analysis, indirect immunofluorescence microscopy and $^{45}\text{Ca}^{2+}$ uptake by microsomes. In the transfected cells, the overexpression of PMCA coincided with three major anomalies of D-glucose metabolism, namely a lower rate of D-[5- ^3H]glucose utilization prevailing at a low extracellular concentration of D-glucose (1.1 mM), a low ratio between D-[U- ^{14}C]oxidation and D-[5- ^3H]glucose utilization prevailing at high extracellular glucose concentration (16.7 mM), and a high ratio between the net generation of ^{14}C -labelled acidic metabolites and amino acids and that of ^3HOH from D-[5- ^3H]glucose. These anomalies resulted in a decreased estimated rate of ATP generation (as linked to the catabolism of the hexose) and a lowered ATP cell content, whether at low or high extracellular D-glucose concentration. The net uptake of $^{45}\text{Ca}^{2+}$ by intact cells was also decreased in the transfected cells, but to a greater extent than apparently attributable to the change in ATP generation rate. These findings document the relevance of plasma membrane Ca^{2+} -ATPase activity to both D-glucose metabolism and Ca^{2+} handling in insulin-producing cells, with emphasis on the key role for both cytosolic and mitochondrial Ca^{2+} concentration in the regulation of D-glucose catabolism. They also reveal that over expression of PMCA leads, in insulin-producing cells, to an imbalance between ATP generation and consumption.

Key words: Ca^{2+} -ATPase, BRIN-BD11 cells, D-glucose metabolism, cell transfection, insulin release, ATP cell content

INTRODUCTION

The plasma membrane Ca^{2+} -ATPase (PMCA) is responsible in part for the ejection of Ca^{2+} from all eukaryotic cells [1]. Glucose, the major physiological stimulus for insulin release, initiates the voltage-dependent entry of Ca^{2+} into pancreatic islet β -cells [2]. This regulatory effect of glucose on insulin secretion involves intracellular metabolism of the sugar by oxidative glycolysis and an increase in the ATP/ADP ratio [3-5]. A rise in intracellular Ca^{2+} is the key trigger of insulin secretion [4-7]. The maintenance of normal Ca^{2+} homeostasis during sustained cellular activity involves *inter alia* plasma membrane Ca^{2+} -ATPase and Na/Ca exchanger [8, 9].

One approach to study the physiological function of plasma membrane Ca^{2+} -ATPase isoforms is to develop systems which would allow modulation of their expression. Successful overexpression of different isoforms has been achieved in different systems [10-12]. Overexpression of PMCA2wb was recently achieved in BRIN-BD11 cells; an insulin-secreting cell line established by electrofusion of RINm5F cells with NEDH rat pancreatic islets cells [13, 14]. BRIN-BD11 cells were selected for such a purpose because they display improved metabolic and secretory response to D-glucose, when considering the difference otherwise found between normal and tumoral islet cells [14, 15]. In response to D-glucose, overexpressing cells showed markedly reduced rise in $[\text{Ca}^{2+}]_i$ and suppression of $[\text{Ca}^{2+}]_i$ oscillations, despite increased D-glucose metabolism and insulin release [13].

The present study extends such investigations to BRIN-BD11 cells transfected with either PMCA2wb or PMCA2yb. It aims mainly at exploring the feedback control of D-glucose catabolism resulting from changes in Ca^{2+} handling, with emphasis on the measurement of ATP cell content, rate of ATP generation attributable to the catabolism of D-glucose, and relationship between the latter rate and the net uptake of ^{45}Ca in control and transfected cells. The results reveal that overexpression of PMCA leads, in insulin-producing cells, to an imbalance between ATP generation and utilization.

MATERIALS AND METHODS

Materials

RPMI, L-glutamine, penicillin, streptomycin, G418, foetal calf serum, Ca^{2+} and Mg^{2+} -free HBSS, cell dissociation buffer, phosphate buffered saline, *NotI* *AlwNI* were from Gibco BRL (Merelbeke, Belgium); *SgrAI* *KpnI* were from Boehringer Mannheim (Brussels, Belgium); oligomycin, thapsigargin and calmodulin were from Calbiochem (Bierges, Belgium); and ouabain from ICN Biomedicals Inc. (Aurora, Ohio).

Construction of the expression vectors

Pancreatic islet isolation, total RNA preparation, reverse transcription, polymerase chain reaction, cloning and sequencing of PCR products have been described elsewhere [16]. In order to construct full-length PMCA2wb and PMCA2yb cDNA, three different fragments were amplified by polymerase chain reaction, based on rat PMCA2 cDNA sequences [17]. The fragment (I) contains the ATG starting docon and a 93 bp exon (for PMCA2yb) or 135 bp exon (for PMCA2wb) at site A (Figure 1). The fragments (II) and (III) are the same for PMCA2yb and PMCA2wb. The fragment (III) contains TAG stop codon. The fragments I-III were subcloned in the vector pCR®-Blunt (Invitrogen, Leek, The Netherlands), sequenced and cut with *NotI*-*AlwNI* *AlwNI*-*SgrAI* and *SgrAI*-*KpnI* respectively. The complete full-lengthly clone corresponding to the alternatively spliced PMCA isoforms 2yb or 2wb were obtained as follows. The 5'-end 1087 bp *NotI*-*AlwNI* fragment containing the alternatively spliced region site A, the 1107 bp *AlwNI*-*SgrAI* central portion fragment and the 1542 bp *SgrAI*-*KpnI* 3'-end fragment were ligated to each other. The final full-length PMCA2wb was subcloned into the multicloning site of the pcDNA3.1 (-) mammalian expression vector (Invitrogen) digested by *NotI* and *KpnI*. The positive clones were verified by restriction enzyme mapping and sequencing.

Culturing of BRIN-BD11 cells and stable transfection

Cells were grown at 37°C in RPMI-1640 medium supplemented with 10 % foetal calf serum (vol/vol), 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and equilibrated against 5 % CO_2 and 95 % air. The D-glucose concentration of the culture medium was 11.1 mM unless otherwise stated. Cells were gently washed with Ca^{2+} and Mg^{2+} -free HBSS (5 ml per T75 flask) for 30 s at room temperature before detachment from culture flask by incubation with cell dissociation buffer (5 ml per T75 flask) for 1-2 min. They were then immediately used for other application. To establish cell lines that stably express rat PMCA2yb or PMCA2wb, 5 µg/ml PMCA construct was transfected into BRIN-BD11 by Lipofectamin method (Gibco BRL). The neomycin-resistant colonies were selected with 250 µg/ml G418 in RPMI medium containing 10 % foetal calf serum. Colonies were picked and grown as individual cell lines in the presence of 250 µg/ml G418. Each cell line was examined to confirm PMCA expression.

Indirect immunofluorescence microscopy

The cell were plated on coverslips and analysed by indirect immunofluorescence microscopy 48-60 h after plating. The cells were washed with TBS (Tris-buffered saline, 20 mM Tris, 137 mM NaCl, pH 7.2), fixed for 20 min in 4 % formol at pH 7.4 (4°C) and washed with TBS. The cells were permeabilized in a solution containing 0.01 % Triton X-100, 197 µM MgCl_2 , 19.5 µM DTT and 10 % glycerol (pH 7.4, 4°C), washed twice with TBS and incubated in blocking buffer containing 1 % horse serum (Vector Laboratories Inc, Burlingame, CA) in TBS for 20 min. The coverslips were overlaid with the primary antibody (SWant, Rabbit anti- Ca^{2+} -ATPase isoform 2, Bellinzona, Switzerland) diluted 1/1000 in TBS-1 % BSA buffer for 1 h. The control cells were incubated in TBS-1 % BSA buffer without antibody and washed

three times with TBS. The cells were treated with secondary antibody (Alexa Fluor™ 594 goat anti-rabbit IgG (H + L) conjugate, Molecular Probes, Eugene, OR) diluted 1/400 in TBS-1 % BSA for 45 min and washed four times with TBS. The cells were incubated with 300 nM DAPI solution (DAPI: 4', 6-diamino-2-phenylindole, dihydrochloride; Molecular Probes, Eugene, OR) and washed twice with TBS. The coverslips were mounted with Vectashield® (Vector Laboratories Inc, Burlingame, CA) and the cells were observed with Axioplan microscope (Zeiss, Germany) equipped with microscope illuminator with HBO 100 W or XBO 100 W and a x 40 objective, and photographed with Dual-mode cooled CCD Camera C4880 (Hamamatsu, Japan).

Isolation of microsomes from cultured BRIN-BD11 cells

Crude microsomal membranes were prepared by a modified version of a procedure previously described [18]. Cells ($5-10 \cdot 10^6$ cells per ml) were suspended in a hypotonic solution consisting of a 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 4 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 2 mM dithiothreitol. The cells were swollen for 15 min on ice and then homogenized with 40 strokes in Dounce homogeniser. The homogenate was diluted with an equal volume of 0.5 M sucrose, 0.3 M KCl, 2 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, homogenized again with 20 strokes and centrifuged at 5,000 g for 15 min at 4°C. The supernatant was made 0.6 M in KCl and, in order to remove calmodulin, an excess of EDTA (1.5 mM) was also added. The suspension was centrifuged at 110,000 g for 1 h at 4°C to sediment the microsomal fraction. The final pellet was resuspended in a solution containing 0.25 M sucrose, 0.15 M KCl, 10 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol and 20 μ M $CaCl_2$ at a protein concentration of 1-3 mg/ml, aliquoted, frozen immediately in liquid nitrogen and stored at -70°C. Protein concentration was determined using Bio Rad Dc Protein Assay (Bio-Rad, Nazareth, Belgium) with BSA as standard.

Ca^{2+} uptake in microsomes

Calcium influx into microsomal vesicles was measured at 37°C by rapid filtration through filters as previously described with some modification [19]. The filters were presoaked in 150 mM KCl, 1 mM $CaCl_2$ to reduce the background. Twenty μ g of microsomal proteins were resuspended in the uptake buffer containing 100 mM KCl, 25 mM TES-triethanolamine (pH 7.2), 5 mM NaN_3 , 4 μ g/ml oligomycin, 0.5 mM ouabain, 7 mM $MgCl_2$, 40 mM KH_2PO_4 , 1 mM EGTA and sufficient $CaCl_2$ (labelled with $^{45}Ca^{2+}$ to obtain the desired free Ca^{2+} using the Max Chelator program (Stanford University). Thapsigargin (200 nM) was also included to inhibit the activity of the SERCA pumps. Immediately after the addition of $^{45}Ca^{2+}$, reaction was started by addition of 6 mM ATP. The incubation time was 10 min, and the transport activity was determined in the absence or presence of 240 nM calmodulin. At given times, 100 μ l of the mixture were rapidly filtered through 0.45 μ M nitrocellulose filters (Millipore, Brussels, Belgium), which were washed three time with cold 150 mM KCl and 1 mM $CaCl_2$. The filters were then counted in Beckman scintillation counter.

Measurement of glucose metabolism

The methods used to measure D-[5- 3H]glucose metabolism, D-[U- ^{14}C]glucose oxidation, and the generation of acidic metabolites (e.g. lactic acid) and amino acids from D-[U- ^{14}C]glucose have been previously described [20-22].

ATP measurement

BRIN-BD11 cells ($4 \cdot 10^6$ cells/ml) were incubated at 37°C for 90 min in bicarbonate- buffered medium [23] containing bovine serum albumin (5 mg/ml) and 1.1 or 16.7 mM D- glucose.

The incubation was halted by addition of PCA (perchloric acid) to a final concentration of 3 %. The tubes were then mixed by sonication, 3 x 10 s, on ice, and centrifuged for 10 min at 15,000 g and 4°C. An aliquot of the supernatant was mixed with an equal volume of 0.5 M KOH-0.2 M imidazole solution and left at -70°C for at least 1 h. Thereafter, the extracts were thawed on ice and centrifuged for 10 min at 15,000 g and 4°C. The supernatants were transferred to new tubes and an aliquot of each tube was diluted 10 times with a buffer containing 5 mM MgCl₂, 50 mM KCl, and 0.1 M imidazole (pH 7.75). ATP content was determined by luciferin-luciferase method using ATP Bioluminescence assay adapted from [24]. Briefly, 0.5 ml of the diluted extract was mixed with 0.5 ml luciferin-luciferase reagent from the ATP Bioluminescence Assay Kit CLSII (Roche Diagnostics GmbH, Mannheim, Germany). Bioluminescence was measured on an LKB Wallace 250 luminometer (LKB-Pharmacia, Uppsala, Sweden). The main characteristics of the method are the following: linearity of ATP concentration between 1 and 9 nM; within-day precision for repeated assay (n = 6) yielding a coefficient of variation ≤ 5.7 %. The recovery of a standard after the whole extraction procedure was 62.9 ± 1.1 % (n = 8).

DNA measurement

DNA content was measured by fluorometric assay according to Labarca & Paigen [25]. Washed cells were ultrasonicated in 950 µl of 50 mM pH phosphate buffer (7.40) containing 2 M NaCl. After addition of 50 µl of the fluorochrome reagent bisbenzimidazole (20 µg/ml in phosphate saline) (Hoechst 33258, Sigma, St Louis, MO), relative fluorescence intensity was measured using a spectrophotofluorometer at excitation and emission wavelengths of 356 and 448 nm respectively. Standard curves were prepared from calf thymus DNA type 1 (Sigma); standard concentration was exactly determined by measurement of the absorbance at 260 nm. Samples and standards were assayed in duplicate.

⁴⁵Ca uptake by intact cells

The media used to incubate the BRIN-BD11 cells consisted in a Krebs-Ringer bicarbonate buffered solution (pH 7.4, 37°C) with the following composition (in mM) : NaCl 115, CaCl₂ 1, KCl 5, MgCl₂ 1, HEPES/NaOH 10. The media were gassed with ambient air. The different media contained 1.1 or 11.1 mM D-glucose. The method used for the measurement of ⁴⁵Ca²⁺ uptake was performed as described previously [26]. In brief, BRIN-BD11 cells were preincubated in 1 ml of a non-radioactive solution during 30 min and then incubated for 90 min in 1 ml of the same medium containing in addition ⁴⁵Ca²⁺ (10 µCi/ml). At the end of the incubation, the cells were separated from the incubation medium using a combined lanthanum and oil technique [27].

Presentation of results

All results, including those already mentioned are presented as mean values (± SEM) together with either the number of individual determinations (n) or degree of freedom (d.f.). The statistical significance of differences between mean values was assed by analysis of variance followed by Tuckey's post test and/or by Student's t-test.

RESULTS

Generation and selection of BRIN-BD11 stable cell line expression the PMCA pump

The full-length clone for PMCA2 pump isoform 2yb and 2wb contains the 93 or 135 bp exon at site A, respectively, without a 227 bp exon at site C. These two isoforms were constructed from polymerase chain reaction amplified cDNA. Stably transfected BRIN-BD11 cell

colonies were picked and grown as individual cell lines in the presence of G418. Quantitative RT-PCR and Western blot analysis confirmed overexpression of PCMA2wb [13] and PCMA2yb (Figure 2).

The overexpression of PMCA2yb and PMCA2wb protein at the level of the plasma membrane of transfected cells was also visualized by immunofluorescence using rabbit anti- Ca^{2+} -ATPase isoform 2 antibody, PMCA2 and nuclei being stained in red and blue, respectively. Results showed that higher levels of Ca^{2+} pump were expressed in the PMCA2yb- and PMCA2wb-transfected cells; as compared with control BRIN-BD11 cells (Figure 3). A pattern of peripheral distribution of stained material was quite obvious in cells overexpressing PMCA2yb and PMCA2wb, suggesting that a major portion of the overexpressed protein was associated with the plasma membrane. In control cells, minimal staining of the endogenous proteins was observed. These experiments support the view that the largest fraction of the overexpressed PMCA2 pump was correctly targeted to the plasma membrane.

Ca^{2+} uptake by microsomes

Additional support for correct targeting of PMCA2 came from the assay of PMCA activity in isolated microsomes, as judged from ATP-dependent Ca^{2+} uptake. In these experiments, an excess concentration of thapsigargin was used to ensure complete inhibition of SERCA pump activity, without affecting the calmodulin-dependent Ca^{2+} uptake mediated by the PMCA.

As shown in Table 1, the PMCA activity was higher ($P < 0.05$ or less) in transfected cells than in the control BRIN-BD11 cells, whether in the absence or presence of calmodulin. The enzymic activity in the PMCA2wb-transfected cells exceeded ($P < 0.01$ or less) that in the PMCA2yb-transfected cells both in the absence or presence of calmodulin. Calmodulin increased ($P < 0.05$ or less; paired comparison) the mean value for ^{45}Ca uptake by the microsomes. The relative extent of such an increase was not significantly different in control BRIN-BD11 and transfected cells, with an overall mean value of $84.1 \pm 7.4\%$ ($n = 3$; $P < 0.01$). The expressed isoforms PMCA2yb and PMCA2wb thus displayed characteristic dependence on calmodulin for activation.

D-glucose metabolism

In both the control and transfected BRIN-BD11 cells, the rise in D-glucose concentration augmented significantly ($p < 0.01$ or less) the generation of $^3\text{H}_2\text{O}$ from D-[5- ^3H]glucose and that of ^{14}C -amino acids and ^{14}C -acidic metabolites (e.g. lactic acid) from D-[U- ^{14}C]glucose, whilst failing to affect significantly the production of $^{14}\text{CO}_2$ from D-[U- ^{14}C]glucose (Table 2). Hence, in these tumoral cells, the $^{14}\text{CO}_2/^3\text{H}_2\text{O}$ ratio was always lower ($p < 0.001$) at 16.7 mM than at 1.1 mM D-glucose.

The utilization of D-[5- ^3H]glucose and oxidation of D-[U- ^{14}C]glucose were, on occasion, significantly lower, in transfected cells than in control BRIN-BD11 cells. However, the most substantial difference between control and transfected cells consisted in the higher generation of ^{14}C -acidic metabolites in the latter cells than in the former ones. Whether at 1.1 or 16.7 mM D-glucose, the net production of ^{14}C -acidic metabolites was indeed significantly higher ($p < 0.02$ or less) in BRIN-BD11 cells transfected with PMCA2yb than in control cells. At both 1.1 and 16.7 mM D-glucose, it was further increased ($p < 0.001$) in the PMCA2wb-transfected cells. In this respect, there was thus a close parallelism between the generation of ^{14}C -acidic metabolites (Table 2) and Ca^{2+} -dependent ATPase activity (Table 1).

Three further differences between control and transfected BRIN-BD11 cells merit to be mentioned. First, the rise in D-glucose concentration augmented to a greater relative extent ($p < 0.02$ or less) D-[5- ^3H]glucose utilization in PMCA2yb-transfected cells (multiplication factor : 2.664 ± 0.168 ; d.f. = 79) and PMCA2wb-transfected cells

(multiplication factor : 2.600 ± 0.205 ; d.f. = 80) than in control BRIN-BD11 cells (multiplication factor : 1.998 ± 0.064 ; d.f. = 54). Second, whether at 1.1 or 16.7 mM D-glucose, the mean generation of ^{14}C -labelled acidic metabolites, expressed relative to D-[5- ^3H]glucose utilization, was always higher in the transfected cells than in the control cells, averaging in the former cells $198.3 \pm 10.8\%$ ($p < 0.005$) of the mean corresponding value found at the same D-glucose concentration in the control BRIN-BD11 cells. When the net generation of both ^{14}C -labelled acidic metabolites and amino acids was taken into account, the latter percentage averaged $159.7 \pm 7.9\%$ and, as such, remained significantly higher than unity ($p < 0.005$). Last, the rise in glucose concentration decreased to a greater absolute or relative extent the ratio between D-[U- ^{14}C]glucose oxidation and D-[5- ^3H]glucose utilization in PMCA2-transfected cells than in control BRIN-BD11 cells. Such a difference achieved statistical significance ($p < 0.05$) in the PMCA2wb-transfected cells, but not so in PMCA2yb-transfected cells, once again paralleling the changes in Ca^{2+} -dependent ATPase activity.

Cell ATP content

Table 3 provides the absolute values for the ATP content of control BRIN-BD11 cells and PMCA2yb- or PMCA2wb-transfected cells as measured after 90 min incubation in the presence of either 1.1 or 16.7 mM D-glucose. In the three cell types, the ATP content was lower ($P < 0.02$ or less) at 16.7 mM D-glucose than at 1.1 mM D-glucose. Thus, at 16.7 mM D-glucose, the values averaged, in control BRIN-BD11 cells and PMCA2yb- or PMCA2wb-transfected cells, respectively $87.6 \pm 2.7\%$ ($n = 9$), $90.2 \pm 2.3\%$ ($n = 27$) and $87.8 \pm 1.8\%$ ($n = 27$) of the corresponding mean values found within the same experiment(s) and same cell type at 1.1 mM D-glucose (i.e. $100.0 \pm 1.2\%$, $n = 8$; $100.0 \pm 2.9\%$, $n = 27$; and $100.0 \pm 2.3\%$, $n = 27$). Whether at 1.1 or 16.7 mM D-glucose, the ATP content of the PMCA2yb and PMCA2wb-transfected cells was significantly lower ($P < 0.005$ or less) than that recorded at the same hexose concentration and within the same experiment(s) in control BRIN-BD11 cells. Relative to the latter control values, the readings made in the transfected cells were not significantly different at 1.1 or 16.7 mM D-glucose and in PMCA2yb- or PMCA2wb-transfected cells, with an overall mean value of $81.3 \pm 1.5\%$ ($n = 108$).

^{45}Ca uptake by intact cells

The measurements of ^{45}Ca net uptake made at low and high D-glucose concentration are summarized in Table 4. In all cell types, the net uptake of ^{45}Ca was significantly higher ($P < 0.005$ or less) at high, rather than low, hexose concentration. At the two concentrations of D-glucose, the net uptake of ^{45}Ca was lower ($P < 0.001$) in transfected cells than in the control BRIN-BD11 cells.

Figure 4 illustrates the relationship between ^{45}Ca net uptake and the estimated rate of ATP generation. The latter rate was calculated from the data listed in Table 2 as follows. The ATP generation rate was taken as the sum of (i) the rate of D-[U- ^{14}C]glucose oxidation (multiplied by 36) and (ii) the differences between the rate of D-[5- ^3H]glucose utilization and that of D-[U- ^{14}C]glucose oxidation (multiplied by 2). Figure 4 documents that, in all three cell types, a given increase in ATP generation (as resulting from a rise in D-glucose concentration) coincided with a comparable increase in ^{45}Ca net uptake. However, for any given ATP generation rate, the net uptake of ^{45}Ca was lower in transfected cells than in the control BRIN-BD11 cells. Such a difference appeared most pronounced in the PMCA2wb-transfected cells, in which the activity of PMCA2 was also the highest (see Table 1).

DISCUSSION

The present data document that, in BRIN-BD11 cells transfected with either PMCA2yb or PMCA2wb, the estimated ATP yield resulting from the catabolism of D-glucose is decreased, this coinciding with a lower ATP content and lower ^{45}Ca net uptake in intact transfected cells. The latter cationic anomaly is likely to be directly caused by overexpression of PMCA2 in the transfected cells. It coincides with a lesser increase in cytosolic Ca^{2+} concentration in these cells, when exposed to insulin secretagogues [13].

The alteration of the ATP content in the transfected cells is likely to be attributable mainly to the increased consumption of ATP caused by enhanced activity of PMCA2, rather than to any major change in D-glucose catabolism, at least in the PMCA 2wb-transfected cells.

The metabolism of D-glucose in both the control and transfected BRIN-BD11 cells displayed the typical features of a Crabtree effect, namely inhibition of cellular respiration at high concentration of D-glucose, as previously documented in tumoral insulin-producing cells of the RINm5F line [28].

In the BRIN-BD11 cells overexpressing PMCA2 activity, changes in D-glucose metabolism, when present, are probably attributable, in part at least, to the modulation by mitochondrial Ca^{2+} of the activity of FAD-linked glycerophosphate dehydrogenase [29, 30]. Such a proposal is supported by three sets of findings. First, the rise in glucose concentration indeed decreased to a greater absolute or relative extent the ratio between D-[U- ^{14}C]glucose oxidation and D-[5- ^3H]glucose utilization in PMCA2-transfected cells than in control BRIN-BD11 cells. Second, the anaerobic modality of glycolysis as judged by the generation of ^{14}C -acidic metabolites from D-[U- ^{14}C]glucose, was indeed higher in PMCA2-transfected cells than in control BRIN-BD11 cells. In these two cases, the hierarchy in mean values paralleled that of microsomal Ca^{2+} -dependent ATPase activity. Last, the generation of ^{14}C -acidic metabolites, considered alone or together with that of ^{14}C -amino acids, represented a greater fraction of total glycolytic flux, as judged from the production of $^3\text{H}_2\text{O}$ from D-[5- ^3H]glucose, in the PMCA2-transfected cells than in the control BRIN-BD11 cells.

In conclusion, the present work extends to changes in PMCA activity the concept of a feedback control by cationic variables of D-glucose catabolism in insulin-producing cells [20]. It indeed documents alterations of both glycolysis and mitochondrial oxidative events in cells overexpressing PMCA, such alterations impeding the rate of ATP generation attributable to the catabolism of the hexose to match the accelerated consumption of ATP caused by increased activity of this Ca^{2+} pump.

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Table 1. Ca^{2+} -dependent ATPase activity in microsomes from control and PMCA2 - transfected cells

Calmodulin	Ca^{2+} uptake (nmol/mg protein per 10 min)	
	Nil	240 nM
BRIN-BD 11	0.513 ± 0.143 (4)	0.885 ± 0.130 (4)
BRIN-BD 11 transfected with PMCA2yb	1.537 ± 0.166 (4)	3.041 ± 0.276 (4)
BRIN-BD11 transfected with PMCA2wb	2.585 ± 0.213 (8)	4.705 ± 0.147 (4)

Table 2 . D-[U-¹⁴C]glucose and D-[5-³H]glucose metabolism in control and PMCA2 gene transfected BRIN-BD11 cells

Cells	BRIN-BD11		BRIN-BD11 transfected with PMCA2yb		BRIN-BD11 transfected with PMCA2wb	
	1.1 mM	16.7 mM	1.1 mM	16.7 mM	1.1 mM	16.7 mM
D-glucose						
D-[U- ¹⁴ C]glucose oxydation	1.54 ± 0.07 (27) ^a	1.82 ± 0.12 (28)	1.11 ± 0.08 mM (41)	1.35 ± 0.10 (39)	1.37 ± 0.09 (37)	1.46 ± 0.10 (38)
D-[5- ³ H]glucose utilization	9.66 ± 0.11 (28)	19.30 ± 0.58 (28)	6.23 ± 0.27 (42)	16.47 ± 0.77 (39)	8.74 ± 0.51 (41)	22.72 ± 1.21 (41)
¹⁴ CO ₂ / ³ H ₂ O ratio (%)	15.92 ± 0.73 (27)	9.48 ± 0.60 (28)	17.43 ± 0.91 (41)	8.89 ± 0.66 (34)	15.13 ± 0.43 (36)	6.47 ± 0.23 (39)
¹⁴ C-amino acids	0.95 ± 0.04 (28)	1.33 ± 0.03 (21)	0.81 ± 0.02 (41)	1.33 ± 0.04 (21)	0.8 ± 0.02 (42)	1.44 ± 0.03 (40)
¹⁴ C-acidic metabolites	1.04 ± 0.08 (27)	1.51 ± 0.14 (28)	1.25 ± 0.04 (41)	2.44 ± 0.09 (42)	1.7 ± 0.03 (41)	3.32 ± 0.08 (39)

^aAll results are expressed as pmol of D-glucose equivalent per 90 mg and ng DNA

Table 3. ATP content in BRIN-BDII non-transfected or transfected cells

	ATP content	
	1.1 mM D-Glucose	16.7 mM D-Glucose
BRIN-BD11	606 ± 67 (8) ^a	541 ± 49 (9)
BRIN-BD11 transfected with PMCA2yb	489 ± 27 (27)	432 ± 13 (27)
BRIN-BD11 transfected with PMCA2wb	500 ± 29 (27)	438 ± 25 (27)

^aAll results are expressed as pmol of ATP per µg DNA

Table 4. Effect of D-glucose on ^{45}Ca net uptake by BRIN-BD11 non-transfected or transfected cells

D-glucose	1.1 mM	16.7 mM
BRIN-BD11	696 ± 23 (12) ^a	814 ± 24 (12)
BRIN-BD11 transfected with PMCA2yb	495 ± 19 (30)	591 ± 24 (30)
BRIN-BD11 transfected with PMCA2wb	487 ± 11 (30)	563 ± 11 (30)

*All results are expressed as fmol per 10^3 cells at the 90th min of incubation

Legend of figures

Figure 1. Scheme summarizing the splice variants of the plasma membrane Ca^{2+} -ATPases isoform 2 found in insulin-producing cells. cDNA organization of PMCA2 is shown on top. Putative transmembrane segments 1-10 are indicated by black bars. Exons involved in the alternative splicing at sites A, B and C are represented by grey boxes. Flanking sequences that are conserved are represented by white boxes.

Figure 2. RT-PCR amplification and Western blot analysis of PMCA2yb in control and PMCA2yb transfected BRIN-BD11 cells.

(A) RT-PCR amplification of PMCA2yb at cycles 32 yielded a bands of 300 and 589 for PMCA2 and β -actin, respectively. Lines 1-4 : control RT-PCR (BRIN-BD11 non transfected), BRIN transfected with PMCA2yb clone 1, clone 2 and clone 5, respectively. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. MW : 1 kb DNA ladder molecular weight marker (sizes in bases). (B) Membrane proteins (100 μg) were separated by SDS-PAGE on 7.5 % gels. The gel was electroblotted onto nitrocellulose membrane which was then cut and incubated with primary antibodies (Swant, rabbit anti- Ca^{2+} -ATPase isoform 2 raised against PMCA2, diluted 1:1000). The bound antibody was detected with a POD-labelled secondary antibody as described by the manufacturer. Lines 1-4 : control BRIN-BD11 cells, the stable transfected cells clones 1, 2 and 5, respectively. MW : Kaleidoscope Prestained Standard (Bio-Rad), molecular mass.

Figure 3. Immunological localization of the overexpressed pump protein.

Immunofluorescence was performed as outlined in Materials and Methods using rabbit anti- Ca^{2+} -ATPase isoform 2 raised against PMCA2. Ctrl BRIN refers to experiments without the first antibody on BRIN-BD11 cells. NT BRIN, PMCA2yb and PMCA2wb refer to experiments on BRIN-BD11 cells, BRIN-BD11 transfected with PMCA2yb and BRIN-BD11 transfected with PMCA2wb, respectively, using the first antibody. PMCA2 protein and nuclei were staining by immunofluorescence in red and blue, respectively.

Figure 4. Mean values for ^{45}Ca net uptake and the estimated ATP generation rate in control BRIN-BD11 cells and in either PMCA2yb- or PMCA2wb-transfected cells incubated in the presence of 1.1 mM D-glucose (open circles) or 16.7 mM D-glucose (closed circles).

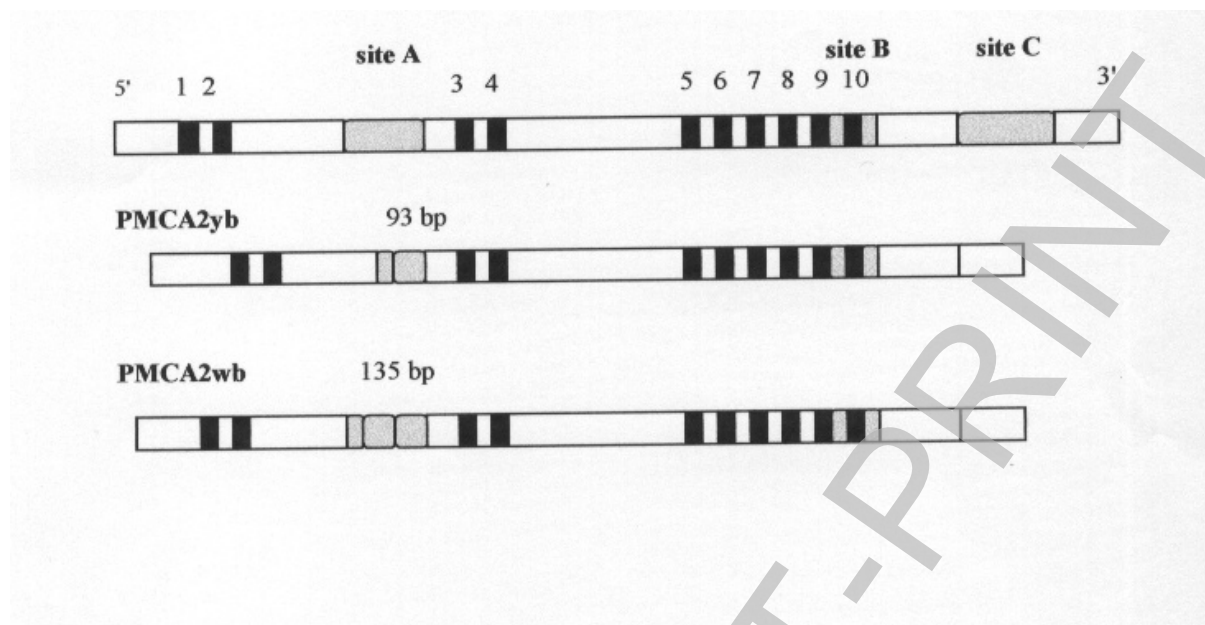


Figure 1

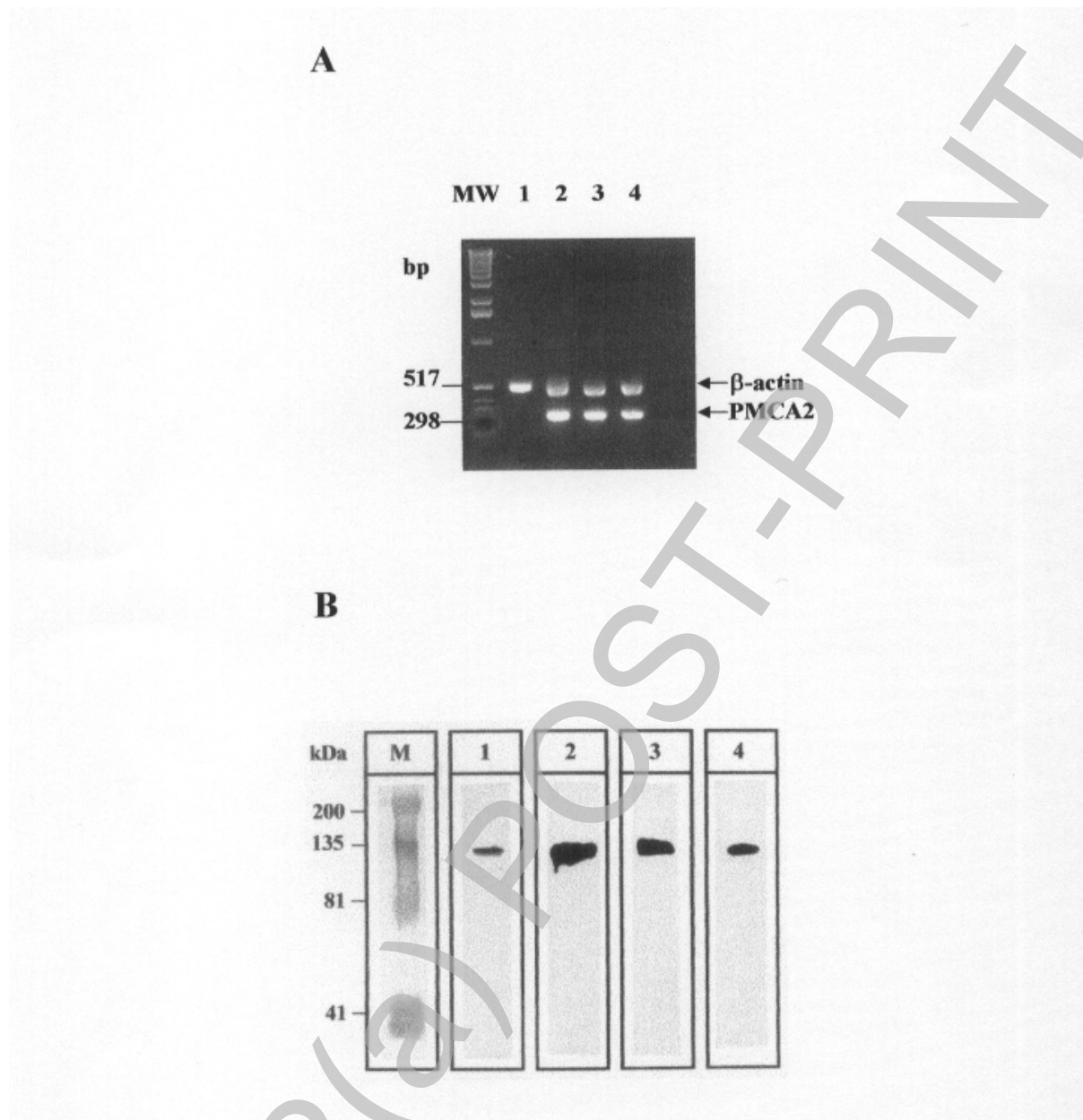


Figure 2

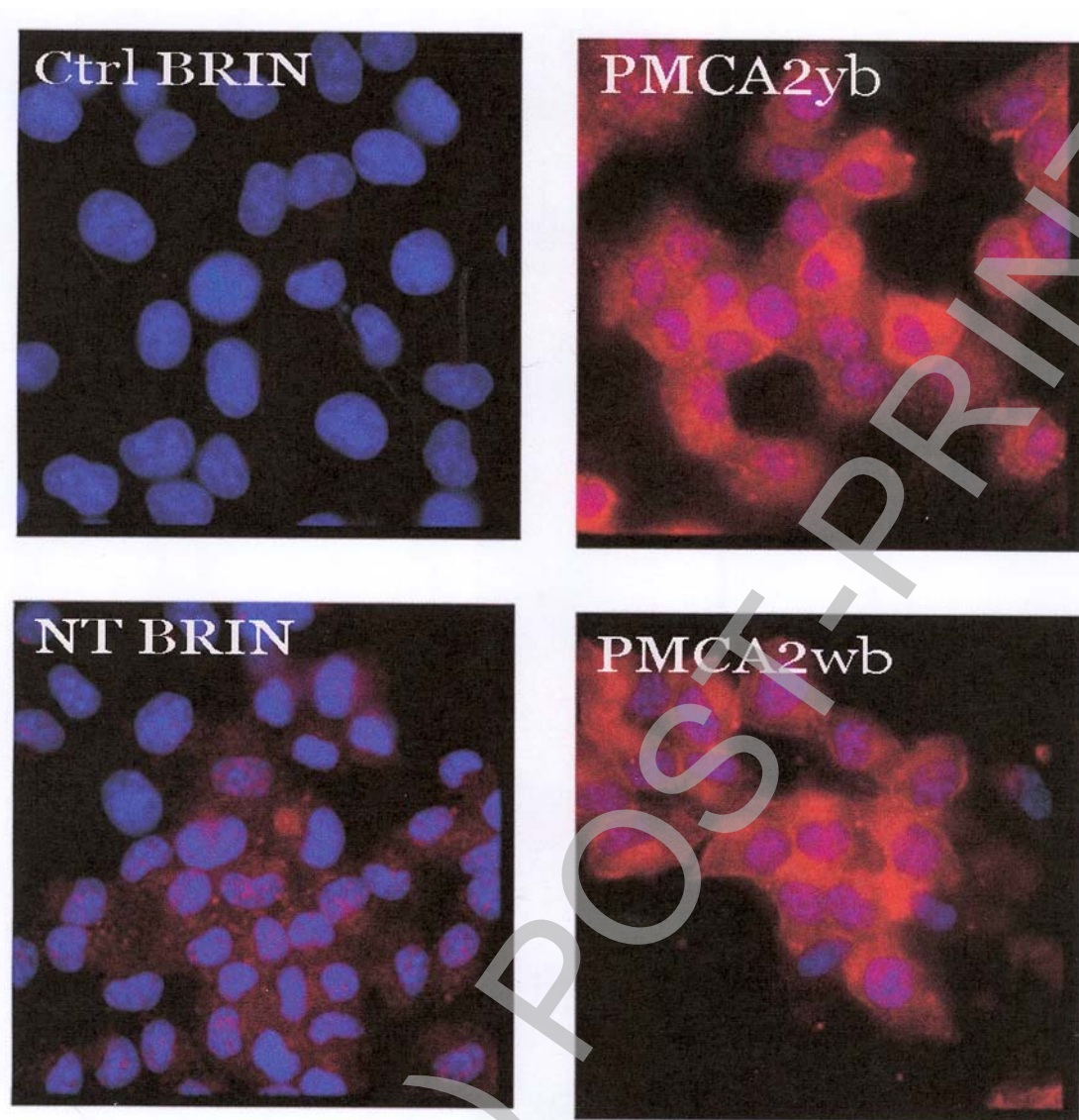


Figure 3

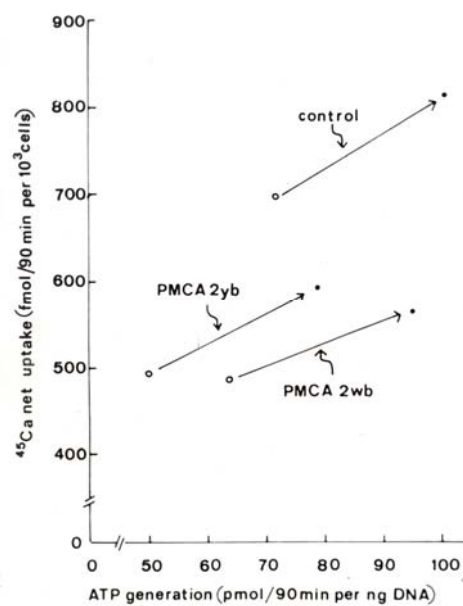


Figure 4