Plasma Membrane Ca\(^{2+}\)-ATPase Overexpression Reduces Ca\(^{2+}\) Oscillations and Increases Insulin Release Induced by Glucose in Insulin-Secreting BRIN-BD11 Cells

Adama Kamagate, André Herchuelz, and Françoise Van Eylen

In the mouse β-cell, glucose generates large amplitude oscillations of the cytosolic-free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that are synchronous to insulin release oscillations. To examine the role played by [Ca\(^{2+}\)]\(_i\) oscillations in the process of insulin release, we examined the effect of plasma membrane Ca\(^{2+}\)-ATPase (PMCA) overexpression on glucose-induced Ca\(^{2+}\) oscillations and insulin release in BRIN-BD11 cells. BRIN-BD11 cells were stably transected with PMCA2wb. Overexpression could be assessed at the mRNA and protein level, with appropriate targeting to the plasma membrane assessed by immunofluorescence and the increase in PMCA activity. In response to K\(^+\), overexpressing cells showed a markedly reduced rise in [Ca\(^{2+}\)]\(_i\). In response to glucose, control cells showed large amplitude [Ca\(^{2+}\)]\(_i\) oscillations, whereas overexpressing cells showed markedly reduced increases in [Ca\(^{2+}\)]\(_i\), without such large oscillations. Suppression of [Ca\(^{2+}\)]\(_i\) oscillations was accompanied by an increase in glucose metabolism and insulin release that remained oscillatory despite having a lower periodicity. Hence, [Ca\(^{2+}\)]\(_i\) oscillations appear unnecessary for glucose-induced insulin release and may even be less favorable than a stable increase in [Ca\(^{2+}\)]\(_i\), for optimal hormone secretion. [Ca\(^{2+}\)]\(_i\) oscillations do not directly drive insulin release oscillations but may nevertheless intervene in the fine regulation of such oscillations. *Diabetes* 51:2773–2788, 2002

**Effects in insulin secretion by endocrine pancreatic β-cells may result in type 2 diabetes. Therefore, the understanding of the fundamental mechanisms of insulin secretion by the β-cell is required to identify the disturbed steps in the disease and to proceed to their correction. One particular feature of insulin secretion is its oscillatory behavior, the importance of which is strengthened by the fact that the loss of insulin oscillations is regarded as an early event in type 1 and type 2 diabetes (1,2).

Glucose, the major physiological stimulator of insulin release, induces the release of the hormone from the pancreatic β-cell by generating both triggering and amplifying signals through distinct pathways (3). According to the first pathway, the metabolism of glucose by the β-cell leads to an increase in intracellular ATP or ATP/ADP ratio that closes the ATP-dependent K\(^+\) (K\(_{\text{ATP}}\)) channels. This closure depolarizes the plasma membrane, leading to the opening of voltage-sensitive Ca\(^{2+}\) channels, with a subsequent increase in Ca\(^{2+}\) inflow and a rise in cytosolic-free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), which triggers the process of exocytotic release of insulin. This corresponds to the K\(_{\text{ATP}}\) channel–dependent or triggering pathway (3,4). In addition to this action, glucose may further increase insulin release, a phenomenon that can be evidenced by blocking K\(_{\text{ATP}}\) channels or preventing the action of glucose on it while maintaining an elevated [Ca\(^{2+}\)]\(_i\). This corresponds to the K\(_{\text{ATP}}\) channel–independent or amplifying pathway (3,4). Another amplifying pathway acts in the absence of a rise in [Ca\(^{2+}\)]\(_i\), but requires the simultaneous and exogenous activation of protein kinases A and C in the β-cell (4). Therefore, a rise in [Ca\(^{2+}\)]\(_i\) is absolutely required for the physiological triggering and amplifying pathways activated by glucose.

Glucose generates large amplitude β-cell [Ca\(^{2+}\)]\(_i\) oscillations that are synchronous to insulin release oscillations. Therefore, it has been suggested that oscillations of insulin secretion could be driven by such [Ca\(^{2+}\)]\(_i\) oscillations (5,6). The rise in β-cell [Ca\(^{2+}\)]\(_i\) induced by glucose is thought to result from the opening of voltage-sensitive Ca\(^{2+}\) channels, the opening of which is intermittent and leads to oscillations of [Ca\(^{2+}\)]\(_i\) (3,7). These oscillations appear to result from oscillations of β-cell glucose metabolism (8,9). Recently, it has been suggested that oscillations of the β-cell [Ca\(^{2+}\)]\(_i\) may have several advantages compared with a sustained elevation (10). Indeed, an oscillatory signal may have the advantage to prevent desensitization of the secretory machinery of the β-cell (10). Likewise, [Ca\(^{2+}\)]\(_i\) oscillations, by driving pulsatile insulin secretion from the β-cell, may prevent the downregulation of peripheral insulin receptors. Such downregulation would in turn increase the amount of insulin to be released, with a subsequent rise in workload for the β-cell and systemic complications due to excessive insulin re-
lease. Last, an oscillatory behavior may avoid cellular toxic effects that are known to be mediated by excessive and sustained [Ca\(^{2+}\)] elevations (10,11).

Recently, oscillatory insulin secretion has been observed in the presence of a stable (nonoscillating) [Ca\(^{2+}\)]\(_{\text{c}}\), under various conditions, e.g., at low (3 mmol/l) and high glucose concentrations (20 mmol/l), or when the plasma membrane is depolarized by extracellular K\(^{+}\) or the hypoglycemic sulfonylurea tolbutamide (12–15). Under the latter conditions, it has been proposed that [Ca\(^{2+}\)] plays a permissive role and that the oscillations in insulin release are directly driven by oscillations in glucose metabolism (12–15). Nevertheless, at intermediate glucose concentration, in which synchronous oscillations of [Ca\(^{2+}\)]\(_{\text{i}}\) and insulin secretion are observed, it is considered that parallel oscillations of glucose metabolism and [Ca\(^{2+}\)] act synergistically to stimulate pulsatile insulin release (15).

Because glucose may stimulate pulsatile insulin release in the absence of [Ca\(^{2+}\)] oscillations at low or high concentrations, one may wonder whether, at intermediate glucose concentrations, the evoked [Ca\(^{2+}\)] oscillations are also necessary for insulin release. Indeed, the burst pattern of electrical activity of mouse islets with concomitant slow [Ca\(^{2+}\)] oscillations is seen only at an extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{o}}\) > 1 mmol/l (1.5 mmol/l for the bursts of electrical activity [16] and 1.28 mmol/l for the oscillations in [Ca\(^{2+}\)]\(_{\text{i}}\) [7]) and disappears at a [Ca\(^{2+}\)]\(_{\text{o}}\) of 1 mmol/l (16) and 0.8 mmol/l (7). Second, such oscillations are not seen in rat islets, even at high [Ca\(^{2+}\)]\(_{\text{o}}\) (17). Therefore, one may even wonder whether [Ca\(^{2+}\)] oscillations, as seen in mouse, may not be detrimental to insulin release as compared with a stable increase in [Ca\(^{2+}\)]\(_{\text{i}}\). Indeed, at variance with the latter pattern, an oscillatory phenomenon that is accompanied by an increase in glucose metabolism and insulin release and that remained oscillatory despite having a lower periodicity. Hence, [Ca\(^{2+}\)] oscillations appear unnecessary for glucose-induced insulin release and may even be less favorable than a stable increase in [Ca\(^{2+}\)] for optimal hormone secretion. [Ca\(^{2+}\)] oscillations do not directly drive insulin release oscillations but may nevertheless intervene in the fine regulation of such oscillations.

### RESEARCH DESIGN AND METHODS

**Materials.** RPMI-1640, l-glutamine, penicillin, streptomycin, G418, FCS, Ca\(^{2+}\), and Mg\(^{2+}\)-free Hank’s balanced salt solution (HBSS) as well as cell disassociation buffer, PBS, Nol, and AicoNi were from Life Technologies (Merelbeke, Belgium). Pluronic F-127, furaptra, and fura-2 were from Molecular Probes (Eugene, OR). SprA1 and Kpom1 were from Boehringer Mannheim (Brussels). Oligomycin, thapsigargin, and calmodulin were from Calbiochem (Bieger, Belgium). Digitonin was from Sigma Aldrich (Bornem, Belgium). Ouabain was from ICN Biomedicals (Aurora, OH).

**Construction of the recombinant expression vectors.** Rat pancreatic islets isolation, total RNA preparation, RT, PCR, cloning, and sequencing of PCR products has been described elsewhere (20). To construct the full-length PMCA2wb cDNA, three different fragments of 1,140, 1,669, and 1,562 bp were amplified by PCR using different primer pairs (Table 1) based on rat PMCA2 cDNA sequence (21). The first fragment (I), containing the 135 bp of site A, was amplified using primer pair 1-2. Primer 1 contains the ATG starting codon, which is underlined in Table 1. The second fragment (II) was amplified using primer pair 3-4 and also contains the 135 bp of site A. The third fragment (III) was amplified using primer pair 5-6; primer 6 contains the TAG stop codon, which is underlined in Table 1, and does not contain site C. The PCR fragments I–III were subcloned into the vector pCR-Blunt (Invitrogen, San Diego, CA). DNA sequencing of selected clones was determined using cycle sequencing with AmpliTaq polymerase (Perkin-Elmer). The rat full-length cDNA clone PMCA2wb was constructed by digestion and ligation of fragments I–III. The 5'-end 1,087-bp Nol-AeNi fragment containing the alternatively spliced region site A, the 1,107-bp AecNi-SprA1 central portion fragment, and the 1,542-bp SprA1-Kpom1 3'-end fragment were ligated to each other (Table 2).

The final full-length PMCA2wb was subcloned into the multicloning site of the pcDNA3.1(−) mammalian expression vector (Invitrogen) and digested by Nol and Kpom1. The positive clones were verified by restriction enzyme mapping and sequencing.

**Culture of BRIN-BD11 cells and stable transfection.** Cells were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mmol/l l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin in an incubator with 5% CO\(_2\) and 95% air at 37°C. The glucose concentration in the culture medium was 11.1 mmol/l, except where otherwise stated. Cells were gently washed with Ca\(^{2+}\)- and Mg\(^{2+}\)-free HBSS for 30 s at room temperature, detached from culture flask by incubation with cell dissociation buffer for 1 min, and immediately used for further applications. To establish a cell line that stably expresses rat PMCA2wb, BRIN-BD11 cells were transfected with 5 μg PMCA2wb recombinant pcDNA3.1(−) vector by the LipofectAMINE method (Life Technologies). The pcDNA3.1(−) mammalian vector carrying the neomycin-resistance gene but lacking the PMCA2wb cDNA. The neomycin-resistant colonies were selected with 250 μg/ml G418 in RPMI culture medium. Colonies were then picked and grown as individual clones in the presence of 250 μg/ml G418. The transcription and expression levels of

<table>
<thead>
<tr>
<th>PMCA2 gene fragment</th>
<th>Direction</th>
<th>Sequence</th>
<th>Starting base</th>
<th>Primer no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Forward</td>
<td>5′-CAACATGGGTGATATGACCA-3′</td>
<td>584</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-GTGAGCTTGCCCTGGAAGCA-3′</td>
<td>1,589</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>Forward</td>
<td>5′-AGAGCAGATATGAGCAGGA-3′</td>
<td>1,100</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CCGGGCAGTCTGTTGATCTGT-3′</td>
<td>2,634</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>Forward</td>
<td>5′-GGTATACAGCTCCGATGTT-3′</td>
<td>2,587</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CTAAGCGACGTCTCCCAGGCT-3′</td>
<td>4,149</td>
<td>6</td>
</tr>
</tbody>
</table>

All primers were based on rat PMCA2 cDNA sequences (21). The 5′ position of the primer sequence on the cDNA is indicated. For sake of clarity, the primers and cDNA fragments were numbered 1–6 and I–III, respectively. Reference is made to these numbers in the text.
PMCA2 protein in the transfected cells were determined by RT-PCR, Western blot analysis, immunofluorescence, and PMCA activity. **Quantitative comparison of PCR products.** To determine the transcription pattern of the PMCA2wb isofrom, a quantitative RT-PCR method was used and total RNA was isolated from control and PMCA2wb-transfected BRIN-BD11 cells as previously described (21). The quantitative analysis of PMCA isofroms was performed using 2 μg total RNA in RT reaction. The cDNA was diluted in a final volume of 50 μl, and 3 μl were amplified by PCR using two different primer pairs (Table 3) and the Pwo DNA polymerase kit (Boehringer Mannheim). We used primer pair 7-8 (30 pmol each) for PMCA2 and primer pair 9-10 (10 pmol each) for β-actin (22) as an internal control. After amplification, the samples were analyzed on 1.2% agarose gel stained with ethidium bromide and the cDNA bands were quantified by scanning densitometry.

**Preparation of membrane proteins from cultured BRIN-BD11 cells.** Plasma membranes were prepared from control BRIN-BD11 cells as well as transfected cells as previously described (23), with some modifications. Cells were washed twice with chilled PBS after detachment and suspended in hypotonic 5 mmol/l Tris-HCl buffer (pH 8.0) (5 × 10^6 cell per ml buffer) containing proteinase inhibitors (1 mmol/l PMSF, 1 mmol/l iodoacetate, 1 mmol/l benzamidine, 2 mmol/l DTT, 1 mmol/l EDTA, 0.1 mg/ml soybean trypsin inhibitor, and 10 mmol/l leupeptin), left for 40 min on ice, and then homogenized in a glass homogenizer. The homogenate was centrifuged at 900g for 10 min at 4°C to remove nuclei, and the supernatant was centrifuged for 40 min at 120,000g. The final pellet was resuspended at a concentration of 2–6 mg/ml protein in 20 mmol/l ice-cold Mops buffer (pH 7.4) containing the proteinase inhibitors described above. The membrane preparations were 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.

**Western blot analysis.** Membrane proteins were separated by electrophoresis on 1-mm-thick 7.5% SDS-PAGE according to Laemmli (24). Proteins were transferred electrothermally onto Hybond ECL nitrocellulose membrane (Amersham) using an IM-2 Semi-Dry Blotting Device System (WEP) with a continuous buffer system at 200 mA (10 V) for 2 h at room temperature, as recommended by the manufacturer. Kaleidoscope Prestained Standards (Bio-Rad) were used for molecular mass determination of protein bands. Nitrocellulose membrane (Amersham) was analyzed using a BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Boehringer Mannheim). Membranes were incubated with rabbit polyclonal antibodies raised against PMCA2 (rabbit anti-PMCA2; Swant, Bellinzona, Switzerland) diluted 1:1,000 in blocking solution for 12–16 h at 4°C. The blots were visualized and quantified by scanning densitometry using Chemi Doc (Bio-Rad).

**Indirect immunofluorescence microscopy.** Control and PMCA2wb-transfected BRIN-BD11 cells were plated on coverslips and analyzed by indirect immunofluorescence microscopy 48–60 h after plating. The cells were washed with Tris-buffered saline (TBS; 20 mmol/l Tris and 137 mmol/l NaCl, pH 7.2), fixed for 20 min in 4% formalin at pH 7.4 (4°C), and washed with TBS. The cells were then permeabilized with a solution containing 0.1% Triton X-100, 197 μmol/l MgCl_2, 10.5 μmol/l DTT, and 10% glycerol (pH 7.4, 4°C), washed twice with TBS, and incubated in a blocking buffer containing 1% horse serum (Vector Laboratories, Burlingame, CA) in TBS for 20 min. The coverslips were exposed to the primary antibody (rabbit anti-PMCA2) and diluted 1/1,000 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 then treated with secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG [H + L] conjugate; Molecular Probes), diluted 1/400 in TBS 1% BSA for 45 min, and washed four times with TBS. The cells were incubated with 300 mmol/l 4',6-diamidino-2-phenyldole (DAPI) solution (Molecular Probes) and washed twice with TBS. The coverslips were mounted on slides using Vectashield (Vector Laboratories) and examined using an Axioplan microscope (Zeiss) equipped with an HBO 100 W or XBO 100 W illuminator and a ×100 objective and photographed with a Dual-mode cooled CCD Camera C4880 (Hamamatsu).

**Isolation of microsomes from cultured BRIN-BD11 cells.** Crude microsomal membranes were prepared following a modified version of a procedure previously described (25). Control and PMCA2wb-transfected cells (5–10 × 10^6/ml) were suspended in hypotonic solution containing 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l MgCl_2, 0.1 mmol/l PMSF, 4 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mmol/l DTT. The cells were swelled for 15 min on ice and then homogenized with 40 strokes in a Dounce homogenizer. The homogenate was diluted with an equal volume of a solution containing 0.5 mol/l sucrose, 0.3 mol/l KCl, 2 mmol/l DTT, and 10 mmol/l Tris-HCl (pH 7.5), homogenized again with 20 strokes, and centrifuged at 5,000g for 15 min at 4°C. The supernatant was made in 0.6 mol/l KCl and, to remove calmodulin, an excess of EDTA (1.5 mmol/l) was also added. The suspension was centrifuged at 110,000g for 1 h at 4°C to pellet the microsomal fraction. The final pellet was resuspended in a solution containing 0.25 mol/l sucrose, 0.15 mol/l KCl, 10 mmol/l Tris-HCl (pH 7.5), 2 mmol/l DTT, and 20 mmol/l 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) with BSA as standard.

**Ca^2+ uptake in microsomes.** Ca^2+ influx into microsomal vesicles was measured at 37°C by rapid filtration through filters as previously described, with some modifications (25). The filters were presoaked in a 150 mmol/l KC1/1 mmol/l CaCl_2 solution to reduce background. A total of 20 μg microsomal proteins were resuspended in the uptake buffer containing 100 mmol/l KC1, 25 mmol/l TRIS-triethanolamine (pH 7.2), 5 mmol/l NaNO_3, 4 μg/ml oligomycin, 0.5 mmol/l ouabain, 7 mmol/l MgCl_2, 40 mmol/l KHPO_4, 1 mmol/l EGTA, and sufficient CaCl_2 (including 45Ca*) to obtain the desired free [Ca*] using the Max Chelator program (Stanford University). Thapsigargin (200 mmol/l) was also included to inhibit the activity of the sarco(endoplasmic) reticulum Ca^-2-ATPases (SERCA). Immediately after the addition of 45Ca* (2–3 10^5 cpm/ml), the reaction was started by addition of 6 mmol/l ATP. The incubation time ranged from 0 to 10 min, and the transport activity was determined in the presence or absence of 240 mmol/l calmidoxin. At given times, 100 μl of the mixture were rapidly filtered through 0.45 μm/l

### Table 2

**Summary of the sequences of cDNA fragments used to construct the PMCA2wb expression vector**

<table>
<thead>
<tr>
<th>PMCA2 gene fragment</th>
<th>Position of the 5' restriction site</th>
<th>Restriction enzyme</th>
<th>Position of the 3' restriction site</th>
<th>Restriction enzyme</th>
<th>Type of vector</th>
<th>Fragments length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>*</td>
<td>Not I</td>
<td>1,500</td>
<td>AhwII</td>
<td>pCR-Blunt</td>
<td>~1,087</td>
</tr>
<tr>
<td>II</td>
<td>1,501</td>
<td>AhwII</td>
<td>2,606</td>
<td>SprAI</td>
<td>pCR-Blunt</td>
<td>1,107</td>
</tr>
<tr>
<td>III</td>
<td>2,607</td>
<td>SprAI</td>
<td>*</td>
<td>KpnI</td>
<td>pCR-Blunt</td>
<td>~1,542</td>
</tr>
</tbody>
</table>

The position of the first nucleotide of the palindromic sequence of the corresponding restriction enzyme site is indicated based on rat PMCA2 cDNA sequences (21), except for the 5' and 3' restriction sites of fragments (I) and (III), respectively, which are based on vector sequence (*). Insert in the subcloning vector was in sense orientation.

### Table 3

**Position and sequence of PCR primers used for quantitative PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
<th>Starting base</th>
<th>Primer no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMCA2</td>
<td>Forward</td>
<td>5'-AAGGAGACATAGGGGAC-3'</td>
<td>691</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TTTACCCTTCATCTTGCG-3'</td>
<td>900</td>
<td>8</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>5'-GGTTGAGACCTCACAACCCCA-3'</td>
<td>2,163</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGGATGTCAACGTGACACTCA-3'</td>
<td>2,751</td>
<td>10</td>
</tr>
</tbody>
</table>
nitrocellulose filters (Millipore, Brussels), which were washed three times with a cold 150-mmol/l KCl/3 mmol/l CaCl₂ solution. The filters were then counted with a Beckman scintillation counter.

**Ca**²⁺ uptake in intact BRIN-BD11 cells.** The media used to incubate the BRIN-BD11 cells was a Krebs-Ringer solution (pH 7.4, 37°C) containing 115 mmol/l NaCl, 1 mmol/l CaCl₂, 5 mmol/l KCl, 1 mmol/l MgCl₂, and 10 mmol/l HEPES/NaOH. The media were gassed with ambient air. The different media contained 1.1, 11.1, or 16.7 mmol/l glucose. The method used for the measurements was performed as previously described (26). In brief, BRIN-BD11 cells were preincubated in 1 ml of a nonradioactive solution for 30 min and then incubated for 90 min in 1 ml of the same medium, which also contained 10 μCi/ml ⁴⁴Ca²⁺. At the end of the incubation, the cells were separated from the incubation medium using a combined lanthanum and oil technique (27).

**Intracellular Ca**²⁺ concentration measurements. A drop of the cell suspension containing 50,000 cells was aliquoted onto round glass coverslips placed in Petri dishes and incubated at 37°C in a 5% CO₂/95% O₂ incubator. After 2 h, fresh culture medium was added to the Petri dishes. The cells were further incubated for 24 h at 37°C before use. The medium used to incubate or perfuse the BRIN-BD11 cells consisted of a Krebs-Ringer bicarbonate-buffered solution (pH 7.4, 37°C) containing 115 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, 24 mmol/l NaHCO₃, and 1.1 mmol/l glucose. The medium was equilibrated against a mixture of O₂ (95%) and CO₂ (5%) at pH 7.4. The different media contained 1.1, 11.1, or 16.7 mmol/l glucose, 50 mmol/l fura-2/AM, 10 mmol/l verapamil, and 2 mmol/l thapsigargin. The cells were incubated with fura-2-acetoxymethyl ester (final concentration 2 μmol/l) for 60 min at 37°C in a Krebs-Ringer bicarbonate-buffered solution containing 2.8 mmol/l glucose. The coverslips were then transferred to a tissue chamber mounted on an inverted fluorescence microscope (Diaphot TMD; Nikon, Tokyo) for epifluorescence. Fura-2 fluorescence of single cells was measured by dual-excitation fluorometry using a camera-based image analysis system (Magnetic: Applied Imaging, Sunderland, U.K.). The excitation and emission wavelengths were set at 340/380 and 510 nm, respectively, and a pair of ratiometric images (at the excitations of 340 and 380 nm, 30-ms interval) were taken every 2.5 s. [Ca²⁺] was calculated from the ratios of the 340- and 380-nm signals as previously described (28).

**Measurements of Ca**²⁺ in the intracellular stores. Furaptra loading, cell permeabilization, and measurements of intracellular Ca²⁺ stores were performed as described (26), with some modifications. Briefly, cells were aliquoted onto round glass coverslips as described above and loaded with furaptra acetoxyethyl ester (4 μmol/l) in an extracellular buffer (125 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l MgCl₂, 1.3 mmol/l CaCl₂, and 25 mmol/l HEPES; pH 7.4) containing 0.05% (wt/vol) Pluronic F-127 for 60 min at 37°C. After rinsing, the coverslips were then transferred to a tissue chamber and mounted on an inverted fluorescence microscope. Permeabilization of the plasma membrane was carried out with 4 mmol/l digitonin in intracellular buffer (140 mmol/l KCl, 3 mmol/l NaATP, 2.1 mmol/l MgCl₂, 2 mmol/l EGTA, 10 mmol/l HEPES, and 0.418 mmol/l CaCl₂). Furaptra fluorescence of single-loaded cells was measured by use of dual-excitation microfluorimetry with the Spex photometric system (Optilux, Alphen aan den Rijn, Holland). The excitation wavelengths (340 and 380 nm) were alternated at the frequency of 1 Hz. The emission wavelength was set at 510 nm. After the sudden drop of fluorescence, the detergent was removed and intracellular buffer was added.

**Measurement of insulin release.** Insulin release from BRIN-BD11 cells in tissue culture was determined using cell monolayers. The BRIN-BD11 cells were harvested and seeded into 24-well plates at a density of 5 × 10⁵ per well and allowed to attach overnight at 37°C. Standard RPMI-1640 tissue culture medium containing 11.1 mmol/l glucose, which was routinely used to culture BRIN-BD11 cells, was removed from each well and replaced with culture media containing either 1.1 or 16.7 mmol/l glucose. The cells were incubated for 24 h at 37°C, and aliquots of the culture media were removed from each separate well and stored at −20°C for subsequent insulin measurement by radioimmunoassay (30).

Insulin release from BRIN-BD11 cells monolayers was also measured over 90 min. After 24 h culture at 37°C, the culture medium was removed and replaced by 1 ml of the Krebs-Ringer bicarbonate-buffered solution used for [Ca²⁺]measurement, containing 1.1 mmol/l d-glucose and 0.05% BSA. After 30 min preincubation, the cells were further incubated for 90 min at 37°C in the same medium containing 1.1, 11.1, or 16.7 mmol/l glucose. Aliquots of the culture media were removed from each separate well and stored at −20°C for subsequent insulin measurement by radioimmunoassay (30).

Dynamic insulin release was studied using a multiwell approach as described elsewhere (31). BRIN-BD11 cells were seeded into 48-well plates at a density of 2.5 × 10⁵ per well and allowed to attach overnight at 37°C. The cells were washed and preincubated for 30 min in Krebs-Ringer solution buffered with 10 mmol/l HEPES/NaOH (pH 7.4, 37°C) and supple-

mented with 0.05% BSA. The preincubation medium was then replaced at 30-s intervals with the same medium containing 11.1 or 16.7 mmol/l glucose to produce a time course of insulin secretion. Thus, the first well was exposed to glucose for 24 min and the last for 30 s. Insulin was simultaneously collected from all the wells by inverting the test plate into a second 48-well plate. The plates were stored at −20°C for subsequent insulin measurement by an enzyme immunoassay, Merckodia Rat Insulin ELISA (Merckodia, Uppsala, Sweden), as described by the manufacturer.

Insulin output was expressed in terms of the numbers of cells in each well at the end of the experiment.

**Measurement of glucose metabolism.** The methods used to measure the production of H₂O/H₂O from [D(5-³H)] glucose and the generation of ¹⁴CO₂ from d-[U³H] glucose have been previously described (32–34). Briefly, groups of 10⁶ cells were incubated for 90 min in 80 μl of a bicarbonate-buffered medium. The reaction was stopped by addition of 20 μl of metabolic inhibitors (5 mmol/l KCN, 10 μmol/l Antimycin A, and 10 μmol/l rotenone) in a citrate NaOH buffer (400 mmol/l, pH 4.9).

**Statistics.** The results are expressed as means ± SE. The statistical significance of differences between data were assessed by ANOVA followed by Tukey’s post test.

**RESULTS**

**PMCA2wb expression and activity in stably transfected BRIN-BD11 cells.** The full-length clone for the PMCA2wb isoform, containing the 135-bp exon at splicing site A but without the 227-bp exon at site C, was constructed from PCR cDNA and stably transfected in BRIN-BD11 cells. Two cell clones expressing high levels of PMCA activity (clones 4 and 5) were used for studies described here. However, similar results were observed with a third clone (clone 2) showing a lower level of overexpression (see below). In addition, three clones transfected with the expression vector lacking the PMCA2wb construct were generated.

The transcription and expression levels of PMCA2wb in transfected cells were determined by RT-PCR method, Western blot analysis, immunofluorescence, and measurement of PMCA activity (ATP-dependent ⁴⁴Ca²⁺ uptake) in membrane preparations of control and PMCA2wb-overexpressing cells.

In both control and overexpressing cells, quantitative RT-PCR performed using primer pairs 7–8 and 9–10 for PMCA2 and β-actin (Table 3) yielded two bands of 300 and 589 bp, respectively (Fig. 1A). Quantitative PCR analysis indicated that one to five times higher transcription level of PMCA2wb in clones 4 and 5 than in control (nontransfected and vector-only transfected) cells.

Membrane proteins from BRIN-BD11 cells and positive clones were analyzed using antibodies specific for PMCA2. In control (nontransfected and vector-only transfected) cells, a faint band at the expected molecular mass range (−135 kDa) was observed (Fig. 1B, lanes 1 and 2). Immunoblot of PMCA2wb-overexpressing cells showed a markedly increased amount of the 135-kDa protein, with the amount detected for clones 2, 4, and 5 being about two, four, and seven times higher, respectively, than in control cells (Fig. 1B, lanes 3–5). To test the stability of the expression, the clones were maintained in tissue culture with selection antibiotics for up to 9 months (70 passages) without showing any decrease in PMCA2wb expression level.

The overexpression of the PMCA2wb protein at the level of the plasma membrane of the transfected cells was visualized by immunofluorescence using a rabbit anti-PMCA2 antibody. Overexpressing cells (Fig. 1H–J) showed an intense staining predominantly located along
the cytoplasmic perimeter of the cells, whereas control (nontransfected and vector-only transfected) cells displayed only a faint fluorescence at their periphery (Fig. 2F and G). The results support the conclusion that the largest fraction of the overexpressed PMCA2wb pump was correctly targeted to the plasma membrane.

Additional support for correct targeting of PMCA2wb came from the assay of PMCA activity using isolated microsomes. An excess concentration of thapsigargin was included in the uptake medium to ensure complete inhibition of SERCA pump activity without affecting the calmodulin-dependent Ca\textsuperscript{2+} uptake mediated by the PMCA (35). Figure 3A illustrates the time courses of ATP-dependent \textsuperscript{45}Ca uptake in control nontransfected and PMCA2wb-overexpressing cells (clone 5). Both cell types displayed a time-dependent increase in \textsuperscript{45}Ca uptake. However, the increase in \textsuperscript{45}Ca uptake was more rapid and
the activity of PMCA2wb in membrane prepared from overexpressing cells (clone 5) was dependent on the Ca\(^{2+}\) concentration in the assay medium within the appropriate range (0.01–10 \(\mu\)mol/l). Addition of calmodulin greatly enhanced the activity without changing the sensitivity to ambient Ca\(^{2+}\) (Fig. 3B). Compared with control cells, the uptake of \(^{45}\)Ca by microsomes from PMCA2wb-overexpressing cells was about four to five times higher in both the presence and absence of calmodulin \((P < 0.001)\).

Taken as a whole, the data presented thus far indicate that PMCA2wb was successfully overexpressed in BRIN-BD11 cells, that the protein was adequately targeted to the plasma membrane, that it was functional, and that its activity showed an appropriate responsiveness to Ca\(^{2+}\) and calmodulin.

**Effect of PMCA2wb overexpression on \([Ca^{2+}]_i\) changes induced by K\(^+\)-mediated membrane depolarization.** To evaluate the role played by PMCA on Ca\(^{2+}\) homeostasis, we examined the effect of PMCA2wb overexpression on changes in \([Ca^{2+}]_i\), induced by membrane depolarization. Figure 4 illustrates the effect of membrane depolarization induced by K\(^+\) (50 mmol/l) on \([Ca^{2+}]_i\). First, in this series of experiments, as in another \((\text{Fig. 5})\), basal \([Ca^{2+}]_i\) did not differ between control and overexpressing cells and averaged 124 \(\pm\) 6 mmol/l \((n = 350)\). Second, whether in control \((\text{Fig. 5A})\) or overexpressing cells \((\text{clone 4, Fig. 5B; clone 5, Fig. 5C})\), K\(^+\) induced a biphasic increase in \([Ca^{2+}]_i\), consisting in an initial peak followed by a plateau phase. The increase in \([Ca^{2+}]_i\) was rapidly reversible upon removal of K\(^+\) from the solution. Cells transfected with the vector only showed a K\(^+\)-induced increase in \([Ca^{2+}]_i\) similar to that of parental nontransfected cells \((P > 0.20)\) \((\text{Fig. 4A})\). Third, in PMCA2wb-overexpressing cells, the magnitude of the increase in \([Ca^{2+}]_i\) was markedly reduced. Thus, a two- and sixfold reduction of the rise in \([Ca^{2+}]_i\) was observed in clones 4 and 5, respectively, compared with vector-only transfected cells. Indeed, the increase in \([Ca^{2+}]_i\), as measured by the area under the curve during the time of K\(^+\) stimulation, averaged 12.3 \(\pm\) 0.5, 6.5 \(\pm\) 0.3, and 2.1 \(\pm\) 0.1 \(\mu\)mol \cdot l\(^{-1}\) \cdot h\(^{-1}\) in vector-only transfected cells, clone 4 cells, and clone 5 cells, respectively \((P < 0.0001)\). In view of the inverse correlation between the level of protein expression and the increase in \([Ca^{2+}]_i\), the effect of K\(^+\) on clone 2, which shows a lower overexpression, was also tested \((\text{Fig. 4D})\). The result obtained was intermediate between control cells and clone 4 and 5 cells (8.7 \(\pm\) 0.5 \(\mu\)mol \cdot l\(^{-1}\) \cdot h\(^{-1}\)), indicating the existence of an inverse correlation between the level of protein expression and the K\(^+\)-induced increase in \([Ca^{2+}]_i\) \((\text{Fig. 6A})\). The reduction in the rise in \([Ca^{2+}]_i\) can be understood as the result of an increased outward Ca\(^{2+}\) transport in PMCA2wb-overexpressing compared with control cells. The results also indicate that there was no difference in PMCA activity between nontransfected and vector-only transfected cells.

**Effect of PMCA2wb overexpression on glucose-induced \([Ca^{2+}]_i\) increases and oscillations.** To further examine the effect of PMCA2wb overexpression on Ca\(^{2+}\) handling in BRIN-BD11 cells, \([Ca^{2+}]_i\) changes evoked by 11.1 mmol/l glucose were investigated. This concentration of glucose elicits characteristic cyclic oscillations of the membrane potential and \([Ca^{2+}]_i\) in pancreatic \(\beta\)-cells and

marked in overexpressing than control cells, and the rate of ATP-dependent Ca\(^{2+}\) uptake by the membrane preparation from the PMCA-transfected cells was about three to four times higher \((\text{clones 4 and 5, respectively})\) than that of control BRIN-BD11 cells \((P < 0.001)\).

A critical parameter for correct functioning of the PMCA is its activation by Ca\(^{2+}\) and calmodulin. Therefore, Ca\(^{2+}\) transport in microsomes isolated from overexpressing cells was tested as a function of the free Ca\(^{2+}\) concentration and the presence of calmodulin. As shown in Fig. 3B,
BRIN-BD11 cells (3,17,36). Figure 5 illustrates typical individual \([\text{Ca}^{2+}]_i\) responses induced by glucose in control and PMCA2wb-overexpressing cells. In response to 11.1 mmol/l glucose, 40 and 50% of control cells (nontransfected and vector-only transfected cells, respectively) displayed large amplitude \([\text{Ca}^{2+}]_i\) oscillations (Fig. 5A). In contrast, overexpressing cells never showed such large \([\text{Ca}^{2+}]_i\) oscillations but instead displayed medium size (clone 4, Fig. 5B) or small size oscillations (clone 5, Fig. 5C). An intermediate situation was observed in clone 2 (data not shown).

A similar picture was observed in response to a higher glucose concentration (16.7 mmol/l). Indeed, in control nontransfected (Fig. 7A) and vector-only transfected cells (Fig. 7B), large amplitude \([\text{Ca}^{2+}]_i\) oscillations were observed in 46 and 44% of the cells, respectively, while transfected cells did not show such large amplitude oscillations (clone 4, Fig. 7C; clone 5, Fig. 7D). The oscillations induced in control cells by 16.7 mmol/l glucose were less individualized than those evoked by 11.1 mmol/l glucose and tended to occur in bursts (compare Figs. 5 and 7).

A similar difference is seen with respect to \(\beta\)-cell electrical activity; an increase in duration of the bursts of electrical activity has been observed when the concentration of glucose is increased from 11.1 to 16.7 mmol/l (3,17). The data obtained in glucose-stimulated cells are consistent with those obtained in response to K\(^+\).

**Effect of PMCA2wb overexpression on glucose-induced \([\text{Ca}^{2+}]_i\) spikes.** In control parental cells (solid line, arrow a) and control cells transfected with the vector only (dashed line, arrow b) are shown. The bar above the curves indicates the period of K\(^+\) exposure. The curves shown are the mean of 104, 36, 61, 85, and 64 traces, respectively.

Effect of PMCA2wb overexpression on the endoplasmic reticulum \([\text{Ca}^{2+}]_i\) stores. To evaluate the depletion of the intracellular \([\text{Ca}^{2+}]_i\) stores, we examined the effect of the SERCA inhibitor thapsigargin on \([\text{Ca}^{2+}]_i\) (Fig. 9). Because thapsigargin releases \([\text{Ca}^{2+}]_i\) from endoplasmic reticulum (ER) stores, the rise in \([\text{Ca}^{2+}]_i\) induced by the drug can be taken as an estimate of ER \([\text{Ca}^{2+}]_i\) content. In control cells (nontransfected or vector-only transfected cells) and at 1.1 mmol/l glucose (Fig. 9A), thapsigargin provoked a transient increase in \([\text{Ca}^{2+}]_i\). In overexpressing cells, the rise in \([\text{Ca}^{2+}]_i\) was reduced by \(~82\%\) (clone 2, Fig. 9B; clone 4, Fig. 9D) and 100% (clone 5, \(P < 0.001\); Fig. 9C) compared with vector-only transfected cells, indicating a depletion of the ER \([\text{Ca}^{2+}]_i\) stores.

A similar picture was observed when the experiments were carried out in the presence of 16.7 mmol/l glucose and 10 \(\mu\)mol/l verapamil; the latter drug was added to offset the effect of glucose-induced membrane depolarization (Fig. 10).

The low-affinity \([\text{Ca}^{2+}]_i\) indicator furaptra was used to monitor free \([\text{Ca}^{2+}]_i\) in the ER of individual BRIN cells after controlled permeabilization of the plasma membrane, as previously described (29,37).

According to the fluorescence obtained by excitation at 340 and 380 nm, cells were permeabilized in intracellular medium containing 4 \(\mu\)mol/l digitonin. Upon the sudden drop in fluorescence caused by the loss of cytoplasmic furaptra, the detergent was immediately removed but the
measurement of the fluorescence at both wavelengths was continued. The loss of cytoplasmic furaptra was associated with an inversion of the 340/380-nm fluorescence excitation ratio, indicating that the remaining indicator was exposed to higher concentrations of free Ca\textsuperscript{2+}/H\textsubscript{1001}/11001 prevailing in intracellular stores (29,37). In a previous study (37), we observed that thapsigargin, an inhibitor of ER Ca\textsuperscript{2+}/H\textsubscript{1001}/11001-ATPase, released 50\% of the Ca\textsuperscript{2+}/H\textsubscript{1001}/11001 pool sensed by furaptra under the present condition. Therefore, thapsigargin was used to estimate Ca\textsuperscript{2+}/H\textsubscript{1001}/11001 stores in intracellular organelles and more specifically in the ER. In control cells (nontransfected, Fig. 11A and vector-only transfected cells, Fig. 11B), thapsigargin induced a drop of 50 and 43\% in furaptra 340/380-nm fluorescence excitation ratio, respectively, and the effect of thapsigargin was reduced by 58/10067% and 47/10065% in PMCA2wb-overexpressing cells (clone 4, Fig. 11C; clone 5, Fig. 11D; P < 0.001) compared with vector-only transfected cells. This indicates that the Ca\textsuperscript{2+}/H\textsubscript{1001}/11001 concentration and content of ER stores were markedly reduced in PMCA2wb-overexpressing cells. Incidentally, it is conceivable that part of the difference seen in control and overexpressing cells may result from a reduced capacity of the ER to take up Ca\textsuperscript{2+}. Indeed, in view of

FIG. 5. Effect of 11.1 mmol/l glucose on [Ca\textsuperscript{2+}]/1. A: control cells (nontransfected and vector-only transfected cells); B and C: PMCA2wb-overexpressing BRIN-BD11 cells (B: clone 4; C: clone 5). The bar above the curves indicates the period of glucose (11.1 mmol/l) exposure. The curves shown are representative of 115, 56, 49, and 40 traces, respectively.

FIG. 6. Correlation between level of protein expression and K\textsuperscript{+}-induced increase in [Ca\textsuperscript{2+}], and comparison of insulin release and glucose metabolism in control and PMCA2wb-overexpressing cells. A: Protein expression was estimated from densitometric measurement of Western blot analysis of control and overexpressing cells as shown in Fig. 1B. The increase in [Ca\textsuperscript{2+}], induced by K\textsuperscript{+}, was measured by the area under the curve during K\textsuperscript{+} stimulation (Fig. 4). B: Comparison of insulin release between clones 2, 4, and 5 and control cells at 16.7 mmol/l glucose (90 min incubation). C: Comparison of glucose utilization between clones 2, 4, and 5 and control cells at 16.7 mmol/l glucose.
of the presence of ATP in the medium, the ratio reached after permeabilization may indicate ATP-driven uptake by the ER. However, thapsigargin was added immediately after permeabilization; therefore, the difference also results from a decreased accumulation of Ca\(^{2+}\) before permeabilization. In a previous study, PMCA overexpression was also shown to reduce ER Ca\(^{2+}\) concentration (38).

**Effect of PMCA2wb overexpression on glucose-induced insulin secretion.** Insulin release was initially measured over 24 h of static incubation in RPMI-1640 tissue culture medium in the presence of 1.1 and 16.7 mmol/l glucose. The latter concentration of glucose elicits near-maximal stimulation of insulin secretion from BRIN-BD11 cells (18). In control cells (nontransfected or vector-only transfected cells), 16.7 mmol/l glucose induced a two- to threefold increase in insulin release \((P < 0.05)\) (Fig. 12A). In PMCA2wb-overexpressing cells (clones 4 and 5), basal insulin release, measured at 1.1 mmol/l glucose, was increased by \(-80\) and \(-19\%\) in clones 4 and 5, respectively \((P < 0.05)\), and the stimulation in insulin release induced by 16.7 mmol/l glucose was increased by \(-88\) and \(-76\%\) (clones 4 and 5, respectively, compared with vector-only transfected cells; \(P < 0.05)\). Similar data were observed in response to 11.1 mmol/l glucose (data not shown). Because the increase in insulin release was of lower magnitude in clone 5 than in clone 4, insulin secretion was also measured in clone 2. In the latter clone (Fig. 12A), insulin release, whether basal or stimulated, was increased by \(-180\) and \(-260\%\) compared with vector-only transfected cells \((P < 0.01)\). The release of insulin obtained in control and transfected cells in response to 16.7 mmol/l glucose is illustrated in Fig. 6B. Thus, all overexpressing cells (clones 2, 4, and 5) showed a larger increase in glucose-induced insulin release than control cells. However, within the overexpressing cells, there was an inverse relationship between PMCA overexpression and insulin release (Fig. 6B).

To relate insulin release and Ca\(^{2+}\) concentrations, insulin release of 16.7 mmol/l glucose was measured over a shorter period of time (90 min) (Fig. 12B). The data obtained were similar to those obtained over 24 h. Thus, compared with vector-only transfected cells, basal insulin release was increased by \(-36\), \(-15\), and \(-8\%\) in clones 2, 4, and 5, respectively \((P < 0.05)\). Likewise, glucose-stimulated insulin release was increased by \(-60\), \(-23\), and \(-10\%\) (clones 4 and 5, respectively, compared with vector-only transfected cells; \(P < 0.05)\). Again, there was an inverse relationship between PMCA overexpression and insulin release. To understand such a relationship, glucose metabolism was measured.

**Effect of PMCA2wb overexpression on glucose metabolism.** Table 4 illustrates glucose utilization and oxidation in control and overexpressing cells. At 1.1 mmol/l glucose, no difference in glucose utilization or oxidation \((P > 0.05)\) was observed between control and overexpressing cells, except that glucose utilization was lower in vector-only transfected cells than in nontransfected cells \((P < 0.01)\). Glucose (16.7 mmol/l) increased glucose utilization \((P < 0.001)\) but failed to increase glucose oxidation \((P > 0.06)\). In overexpressing cells (clones 2, 4, and 5), at 16.7 mmol/l glucose, there was an inverse relationship between PMCA overexpression and glucose utilization (Fig. 6C) that was parallel to that relating PMCA overexpression and insulin release (Fig. 6B).

**Effect of PMCA2wb overexpression on insulin oscillations.** Last, to better relate cytosolic Ca\(^{2+}\) and insulin release oscillations, a multiwell approach was used to measure the time course of insulin release (31). Nontransfected cells were grown in 48-well plates and stimulated by 11.1 and 16.7 mmol/l glucose one well at a time in 30-s intervals (Fig. 13A and C, respectively). The secretion
patterns from the wells were linearized by smoothing the data with a three-point moving average, fitting it to a straight line (31), and then determining the positive or negative differences between the data and the straight line (residuals). Plotting the secretion as residuals more clearly shows the oscillatory pattern of insulin release (Figs. 13B and D).

Figure 14 shows insulin release oscillations in response to 11.1 mmol/l glucose (plotted as residuals) in vector-only transfected cells and in the three clones overexpressing PMCA. Vector-only transfected cells showed clear oscillations over 4-min periods (Fig. 14A). Clones 4 and 5 also showed oscillations, but the period of the oscillations was increased to 6 min (P < 0.0001). Surprisingly, clone 2 did not show such oscillations but instead showed an initial peak followed by a plateau phase. Exactly the same picture was observed when the cells were stimulated by 16.7 mmol/l glucose. Thus, vector-only transfected cells showed oscillations over 4-min periods, clones 4 and 5 oscillated over 6-min periods (P < 0.05), and clone 2 showed an initial peak followed by a plateau phase (Fig. 15).

DISCUSSION

The PMCA belongs to the P-type family of transport ATPases that form a phosphorylated intermediate during the reaction cycle. Four different genes corresponding to four isoforms PMCA1, -2, -3, and -4 have been evidenced. Genes 1 and 4 are transcribed in most tissues, whereas genes 2 and 3 are transcribed in specialized tissues. PMCA1 and -4 may thus represent housekeeping isoforms, while PMCA2 and -3 may be required for specialized functions in a limited number of tissues. Diversity among the ATPases is also generated by alternative splicing of the primary transcripts that may involve two major sites, termed A and C (for review see 19,39,40). In a previous work, we identified the PMCA transcripts expressed in the β-cell and characterized them at alternative splicing sites A and C (20). The identified isoforms were PMCA1x, -2yb, -2wb, -3za, -3zc, and -4xb. Interestingly, our work was the first to demonstrate the presence, at the protein level, of substantial amounts of PMCA2 and -A3 in non-neuronal tissue (20), a finding that attests the special needs of β-cells in terms of Ca\(^{2+}\) homeostasis. Therefore, PMCA2(wb) was chosen to be cloned and overexpressed in BRIN-BD11 cells. The overexpression could be assessed at both the mRNA and protein level. The overexpressed protein was appropriately targeted to the plasma membrane, as shown by immunofluorescence and the increase in ATP-dependent \(^{45}\)Ca uptake observed in plasma membrane microsomes. As expected for PMCA-mediated Ca\(^{2+}\) transport, \(^{45}\)Ca uptake was regulated by the Ca\(^{2+}\) concentration in the assay medium within the submicromolar range and by calmodulin without changing the sensitivity to ambient Ca\(^{2+}\) (41).

Overexpression of PMCA2wb had substantial effects on [Ca\(^{2+}\)]\(_{i}\) and Ca\(^{2+}\) homeostasis in BRIN cells. Indeed, the rise in [Ca\(^{2+}\)]\(_{i}\) induced by extracellular K\(^+\) was markedly blunted in overexpressing cells, and a positive and inverse correlation was observed between the level of PMCA expression and the area under the curve of [Ca\(^{2+}\)]\(_{i}\) during K\(^+\)-induced release in fura-2 or furaptra-loaded cells. In addition, control cells transfected with the vector alone (without the PMCA2wb construct) did not show any difference in K\(^+\)-induced [Ca\(^{2+}\)]\(_{i}\) changes compared with nontransfected parental cells (Fig. 4A). Despite such major changes in cell Ca\(^{2+}\) homeostasis recorded in PMCA2wb-overexpressing cells, basal [Ca\(^{2+}\)]\(_{i}\) was not reduced. In two other studies of PMCA1a and -4 overexpression, no change in basal [Ca\(^{2+}\)]\(_{i}\) was observed (38,41), although a third study evidenced a 20–30% drop in basal [Ca\(^{2+}\)]\(_{i}\) in PMCA4b-overexpressing myogenic cells (42). Taken as a whole, the
present data indicate that PMCA2wb operates within the range of the changes in $[\text{Ca}^{2+}]_i$ induced by $K^+$ (100–1,200 nmol/l) but that although being overexpressed, the PMCA remained regulated and did not decrease $[\text{Ca}^{2+}]_i$ below basal levels. Incidentally, the effect of PMCA overexpression on $K^+$-induced $[\text{Ca}^{2+}]_i$ increase was strikingly different from that of Na/Ca exchanger overexpression, although this protein also contributes to $\text{Ca}^{2+}$ outflow from the β-cell (43). Indeed, in Na/Ca exchanger–overexpressing cells, the increase in $[\text{Ca}^{2+}]_i$ induced by $K^+$ was accelerated, as was its decrease on $K^+$ removal, but the global rise in $[\text{Ca}^{2+}]_i$ was rather comparable or even slightly higher than that in control cells (43). This points to the more complex actions exerted by the Na/Ca exchanger, which participates in both $\text{Ca}^{2+}$ inflow and outflow and also generates a current due to the stoichiometry.

FIG. 9. Effect of thapsigargin on $[\text{Ca}^{2+}]_i$ at 1.1 mmol/l glucose. A: Control cells; B–D: PMCA2wb-overexpressing BRIN-BD11 cells (B: clone 4; C: clone 5; D: clone 2). A: Effect of 2 μmol/l thapsigargin on $[\text{Ca}^{2+}]_i$ in both control parental cells (solid line, arrow a) and control cells transfected with the vector only (dashed line, arrow b) are shown. The bar above the curves indicates the period of thapsigargin exposure. The curves shown are the mean of 46, 47, 66, 47, and 26 traces, respectively.

FIG. 10. Effect of thapsigargin on $[\text{Ca}^{2+}]_i$ at 16.7 mmol/l glucose. Same presentation as in Fig. 9 except that the experiments were carried out at 16.7 mmol/l glucose plus 10 μmol/l verapamil. The curves shown are the mean of 46, 46, 40, 49, and 45 traces, respectively.
The major observation of the present study was that PMCA overexpression markedly damped glucose-induced Ca\(^{2+}\) oscillations while simultaneously increasing the effect of glucose on insulin release. In PMCA-overexpressing cells, as in control cells, stimulation by glucose leads to a progressive increase in basal Ca\(^{2+}\), but without the development of large and distinct oscillations seen in control cells. Again, there was a positive and inverse correlation between the level of PMCA expression seen in the different clones and the amplitude of the [Ca\(^{2+}\)]\(_i\) oscillations induced by glucose. As in the case of K\(^+\), the decrease in glucose-induced [Ca\(^{2+}\)]\(_i\) oscillations can best be understood as resulting from an increased Ca\(^{2+}\) outflow from PMCA-overexpressing cells. Despite this reduction in glucose-induced [Ca\(^{2+}\)]\(_i\) oscillations, insulin release was not reduced but instead was increased. Such a phenomenon was observed whether the cells were exposed to 11.1 or 16.7 mmol/l glucose during short (90 min) or long periods of time (24 h). Our data indicate that [Ca\(^{2+}\)]\(_i\) oscillations are unnecessary for insulin release, even at an intermediate glucose concentration (11.1 or 16.7 mmol/l), and that their presence may even lead to a less efficient insulin secretory process compared with that induced by a stable increase in [Ca\(^{2+}\)]\(_i\).

It has been proposed that the rapidly descending phases of mouse β-cell [Ca\(^{2+}\)]\(_i\) oscillations result from the outward transport of Ca\(^{2+}\) from the cell, a finding that is compatible with the view that glucose stimulates such an outward transport (7). Consequently, it can be expected that enhanced removal of Ca\(^{2+}\) from the cytoplasm would prevent the establishment of the critical [Ca\(^{2+}\)]\(_i\) required for a feedback inhibition of glucose-stimulated entry of the ion (7). Such a view is consistent with the observation that the acceleration of the outward transport of Ca\(^{2+}\) by protein kinase activation suppresses the slow [Ca\(^{2+}\)]\(_i\) oscillations seen in mice (7). Thus, overexpression of PMCA, by preventing the establishment of the critical [Ca\(^{2+}\)]\(_i\) required for a feedback inhibition of glucose-stimulated entry of the ion, would suppress periods during which [Ca\(^{2+}\)]\(_i\) drops to or below the basal level and would hence lead to an improved insulin release. In fact, PMCA overexpression may simply offset part of the consequences resulting from the use of a high extracellular Ca\(^{2+}\) concentration. Therefore, the oscillations in electrical activity and [Ca\(^{2+}\)]\(_i\) seen in mouse at high [Ca\(^{2+}\)]\(_i\) may not represent a physiological model for the study of the process of insulin release. In this respect, it is interesting to notice that Gilon et al. (5), in order to study the synchronicity between [Ca\(^{2+}\)]\(_i\) and insulin release oscillations, had to use an extremely elevated concentration of extracellular Ca\(^{2+}\) (10 mmol/l). Recently, Ravier et al. (44) studied the effect of “imposed” or “forced” oscillations of cytoplasmic Ca\(^{2+}\) or metabolism on insulin secretion in mouse islets. However, the protocol used in the latter study corresponds to intermittent stimulations of Ca\(^{2+}\) rises and not to real or spontaneous Ca\(^{2+}\) oscillations, as could be explored in the present study using PMCA2wb-overexpressing cells.

Insulin release was increased in all three clones overexpressing PMCA compared with control cells. However, within the three clones, there was an inverse relationship between insulin release and PMCA level of expression.
This inverse relationship was paralleled by an inverse relationship between PMCA expression and glucose utilization at 16.7 mmol/l, indicating that the difference in insulin release resulted from a difference in glucose utilization among the three clones. In a recent study, Jung et al. (45) correlated the temporal changes of glucose level, oxygen level, and $[\text{Ca}^{2+}]_{i}$ in single islets. They observed that although rises in $[\text{Ca}^{2+}]_{i}$ preceded pulses in oxygen consumption during oscillations, $[\text{Ca}^{2+}]_{i}$ increase was associated with decreases in glucose consumption through glycolysis (45). Our present data showing that PMCA-overexpressing cells, which display inhibited rises in $[\text{Ca}^{2+}]_{i}$, and a higher rate of glucose utilization, are in agreement with the latter data. They are also in agreement with those of Detimary et al. (46), who showed that a rise in $[\text{Ca}^{2+}]_{i}$ causes a decrease in ATP/ADP ratio, a phenomenon that probably results from the activation of ATPases involved in the maintenance of $[\text{Ca}^{2+}]_{i}$ and $[\text{Na}^{+}]_{i}$ homeostasis in $\beta$-cells or insulin exocytosis. Incidentally, in contrast with the situation found in normal islets (47), a rise in glucose concentration did not cause any increase in glucose oxidation in BRIN-BD11 cells, a situation reminiscent of that found in RINm5F, where glucose even causes, through a Crabtree effect, inhibition of glucose oxidation (48). BRIN-BD11 cells result from the fusion of normal $\beta$-cells with RINm5F cells, and the metabolic situation found in BRIN-BD11 cells, which show no change in glucose oxidation, is intermediate between that of normal $\beta$-cells and RINm5F cells (49).

What is not clear from the present study, and warrants further study, is the reason why there was an inverse relationship between PMCA expression level and glucose utilization in overexpressing cells. Although increases in $[\text{Ca}^{2+}]_{i}$ may be associated with decreases in glucose consumption, it is conceivable that a decrease in $[\text{Ca}^{2+}]_{i}$ that is too large may have the opposite effect, perhaps through direct $\text{Ca}^{2+}$ effects on glycolytic enzymes (50,51).

The present data confirm the observation of Deeney et al. (31) showing that clonal $\beta$-cells grown in separate wells display oscillations in insulin release in response to glucose. This indicates that the synchrony that must exist between $\beta$-cells to lead to oscillations in insulin release is apparently not mediated, or not exclusively mediated, by either electrical coupling or a putative soluble synchronizing factor that is possibly secreted by some or all cells of the population (31). It is conceivable that the synchrony of the cells in separate wells results from the similar metabolic effects of glucose on the different groups of cells (31). Interestingly, oscillations were in 4-min periods, which is similar to the findings of Deeney et al. (31) in HIT cells.

Another major observation of the present study was that insulin release oscillations induced by glucose (11.1 and 16.7 mmol/l) persisted in PMCA-overexpressing cells (clones 4 and 5) despite the absence, in the latter cells, of $[\text{Ca}^{2+}]_{i}$ oscillations. This indicates that even at an intermediate glucose concentration, $[\text{Ca}^{2+}]_{i}$ oscillations are not necessary for insulin release oscillations, which are apparently directly driven by oscillations of glucose metabolism (12–15). Interestingly, the periodicity of the oscillations was changed from 4 to 6 min in overexpressing cells,
which may indicate that $[\text{Ca}^{2+}]_i$ oscillations and/or intracellular Ca$^{2+}$, although not directly driving insulin release oscillations, play a regulatory role of such oscillations. Interestingly, no oscillations in insulin release was observed in the clone showing the largest insulin response to both 11.1 and 16.7 mmol/l glucose (clone 2). It is conceivable that a change in intracellular Ca$^{2+}$ homeostasis, as induced by PMCA overexpression, may not only regulate the periodicity of insulin release oscillations but may also lead to their disappearance, with a subsequent increase in total insulin release.

In conclusion, our data show that overexpression of PMCA2wb inhibits glucose-induced oscillations of $[\text{Ca}^{2+}]_i$, a phenomenon that is accompanied by an increase in

FIG. 13. Insulin release oscillations in response to glucose. Time course of insulin release from adherent control (nontransfected) BRIN-BD11 cells grown in a 48-well plate in response to 11.1 mmol/l (A and B) and 16.7 mmol/l glucose (C and D). Data smoothed with a three-point moving average are shown in A and C. To illustrate these oscillations more clearly, a best-fit line was calculated for the data, after which the points above and below the line were plotted as residuals (B and D).

FIG. 14. Effect of PMCA overexpression on insulin release oscillations in response to 11.1 mmol/l glucose. Multiwell measurement of insulin release oscillations. Data obtained in vector-only transfected cells (A) together with those obtained in clone 4 (B), clone 5 (C), and clone 2 (D) are presented as residuals.
glucose-induced insulin release. Hence, [Ca\(^{2+}\)]\(_i\) oscillations appear unnecessary for glucose-induced insulin release and may be less favorable than a stable increase in [Ca\(^{2+}\)]\(_i\) for optimal hormone secretion, and large increases in [Ca\(^{2+}\)]\(_i\) occurring during [Ca\(^{2+}\)] oscillations, lead to inhibition of both \(\beta\)-cell glucose metabolism and insulin release. In addition, oscillations of [Ca\(^{2+}\)]\(_i\) do not directly drive insulin release oscillations but may nevertheless intervene in the fine regulation of such oscillations. It would be of interest to confirm such observations in normal pancreatic \(\beta\)-cells.

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