Normal Ca\(^{2+}\) extrusion by the Ca\(^{2+}\) pump of intact red blood cells exposed to high glucose concentrations

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Raftos, Julia E., Amanda Edgley, Robert M. Bookchin, Zipora Etzion, Virgilio L. Lew, and Teresa Tiffert. Normal Ca\(^{2+}\) extrusion by the Ca\(^{2+}\) pump of intact red blood cells exposed to high glucose concentrations. Am J Physiol Cell Physiol 280: C1449–C1454, 2001.—The ATPase activity of the plasma membrane Ca\(^{2+}\) pump (PMCA) has been reported to be inhibited by exposure of red blood cell (RBC) PMCA preparations to high glucose concentrations. It has been claimed that this effect could have potential pathophysiological relevance in diabetes. To ascertain whether high glucose levels also affect PMCA transport function in intact RBCs, Ca\(^{2+}\) extrusion by the Ca\(^{2+}\)-saturated pump [PMCA maximal velocity (V\(_{\text{max}}\))] was measured in human and rat RBCs exposed to high glucose in vivo or in vitro. Preincubation of normal human RBCs in 30–100 mM glucose for up to 6 h had no effect on PMCA V\(_{\text{max}}\). The mean V\(_{\text{max}}\) of RBCs from 15 diabetic subjects of 12.9 ± 0.7 mmol·340 g Hb\(^{-1}\)·h\(^{-1}\) was not significantly different from that of controls (14.3 ± 0.5 mmol·340 g Hb\(^{-1}\)·h\(^{-1}\)). Similarly, the PMCA V\(_{\text{max}}\) of RBCs from 11 streptozotocin-diabetic rats was not affected by plasma glucose levels more than three times normal for 6–8 wk. Thus exposure to high glucose concentrations does not affect the ability of intact RBCs to extrude Ca\(^{2+}\).

plasma membrane calcium pump; glycation; diabetes mellitus; streptozotocin

THE LOW CALCIUM CONTENT of normal red blood cells (RBCs) is maintained by the highly regulated plasma membrane Ca\(^{2+}\) pump (PMCA, hPMCA4b) (2, 16, 20, 22, 23, 26). The Ca\(^{2+}\)-ATPase activity of both isolated membranes and purified PMCA enzyme obtained from human RBCs was found to be inhibited by in vitro exposure of the intact RBCs, isolated membranes, or purified enzyme to high glucose concentrations (6–8, 10). Glucose exposure of inside-out RBC membrane vesicles also induced inhibition of their active Ca\(^{2+}\) transport, relative to mannitol-treated vesicles (8).

The inhibitory effect of glucose had also been documented in membranes prepared from RBCs obtained from human subjects after oral or intravenous glucose administration (5), a procedure that allows in vivo RBC exposure to high glucose levels, and also in diabetic patients (7, 10, 18, 19, 21). This indicated that RBC exposure to elevated glucose, both in vivo and in vitro, was effective in reducing the Ca\(^{2+}\)-ATPase activity of the isolated RBC membranes. The effect of glucose was concentration and time dependent; after incubation of RBCs with 100 mM glucose in high-phosphate media for 6–8 h at 37°C, the Ca\(^{2+}\)-ATPase activity was inhibited by up to 45% (7). In an elegant series of studies by González Flecha et al. (7–9), the enzyme inhibition was found to correlate with nonenzymic glycation of a fraction of the PMCA molecules. Random glycation of up to 6.5% of the 80 lysine (Lys) residues accessible outside the transmembrane domains of the PMCA was accompanied by reduction in the maximum velocity of ATP hydrolysis without effect on the apparent affinities for Ca\(^{2+}\), ATP, or calmodulin (9). Analysis of their data suggested that glycation of a single essential Lys residue, probably located near the catalytic site of the PMCA enzyme, caused full inhibition, whereas those molecules without glycation of the vulnerable Lys residue remained functionally normal (9).

Inhibition of the Ca\(^{2+}\)-ATPase activity in isolated RBC membranes after exposure of the RBCs to a particular agent does not necessarily indicate that the PMCA function in the intact cells was correspondingly inhibited (25). This is important because only effects documented in intact RBCs are likely to be of pathophysiological relevance. Thus we investigated whether inhibition of the Ca\(^{2+}\)-transport function of the PMCA by high glucose was also present in RBCs. The study was carried out by measuring Ca\(^{2+}\) extrusion by the Ca\(^{2+}\)-saturated pump (PMCA V\(_{\text{max}}\)) in RBCs from normal and diabetic human subjects and in RBCs from rats with experimentally induced diabetes.

METHODS

Studies with human subjects. Ethical approval was obtained from the local Research Ethics Committee, Cambridge Health Authority, Addenbrooke’s Hospital, Cambridge, United Kingdom, and from the Standing Committee on Eth-
ics in Research on Humans at Monash University, Victoria, Australia. Three different studies were carried out. In the first study, RBCs from four healthy volunteers were preincubated in the presence or absence of moderately high glucose concentrations (10–30 mM) for up to 3 h and were then tested for PMCA function. In the second study, RBCs from five healthy volunteers were treated with a very high glucose concentration (100 mM) in high-phosphate media for up to 6 h and assayed for PMCA function. In the third study, PMCA function was measured in RBCs from 15 healthy volunteers and in RBCs from 15 diabetic patients attending the International Diabetes Institute, Caulfield, Victoria, Australia, whose glycated hemoglobin levels exceeded 8%. From these, 13 had type 2 diabetes and 2 had type 1 diabetes. In both diabetic and control groups, there were 4 females and 11 males. Informed consent was obtained in writing from all subjects before they entered the study.

Preparation of human RBCs. All experiments were performed on heparinized venous blood on the day the blood was collected. The RBCs were washed three times at 4°C in solution A and twice more in solution B. White cells and platelets were removed by aspiration after each spin. The washed RBCs were suspended at 10% hematocrit (Hct) in solution B supplemented with 10 mM inosine as the glycolytic substrate. Solution A contained (in mM) 80 KCl, 70 NaCl, 0.15 MgCl2, 10 HEPES-sodium (pH 7.55 at 37°C), and 0.1 sodium-EDTA; solution B was the same as solution A but without sodium-EDTA. The high K+ concentration in both solutions ensured the maintenance of normal cell volume and ionic composition except for Ca2+ during PMCA Vmax measurements (4).

Measurement of the PMCA Vmax in human RBCs. According to the Ca2+-exposure method of DAGHER and LEW (4), 2 ml of the cell suspension (10% Hct) were incubated at 37°C under magnetic stirring. 45CaCl2 (Amersham), with specific activity of 400 kBq/μmol, was added to a concentration of 100–150 μM in the suspension. 45Ca2+ loading of the RBCs was then initiated by addition of ionophore A-23187 (Sigma) from a stock solution of 2 mM in ethanol to a final concentration of 10 μM in the cell suspension. Two minutes later, CoCl2 was added to a final concentration of 350 μM. Added in excess of the Ca2+ concentration, Co2+ instantly blocks Ca2+ transport through the ionophore and exposes the active Ca2+ efflux through the PMCA (4). Frequent samples were taken during the Ca2+-extrusion phase to estimate the Vmax of the Ca2+ saturated pump by linear regression analysis, as reported before (4, 14, 24) and as illustrated here in Fig. 1.

Glucose preincubation routines. The glucose preincubation protocols were designed to reproduce the various RBC treatments reported to inhibit the PMCA activity, as measured in membranes isolated from the treated RBCs (5, 7). In the first study, human RBCs were suspended at 10% Hct in solutions A or B, with or without glucose (to a maximum of 30 mM, as indicated), and preincubated for up to 3 h at 37°C. In the second study, the RBCs were suspended at 10% Hct in solution C, a high-phosphate medium found to be optimal for glucose-induced Ca2+-ATPase inhibition (7), with and without 100 mM glucose, and preincubated for 0.5, 3, and 6 h at 37°C. Solution C contained (in mM) 40 NaH2PO4/Na2HPO4 (pH 7.4), 100 NaCl, 10 inosine, and 1 mg/ml each of penicillin and streptomycin. After preincubation, the RBCs were washed twice in solution B and then resuspended in the same solution with 10 mM inosine for PMCA Vmax measurements, as described above. Suspension aliquots were taken for measurements of cell ATP concentrations.

Animal experiments. The experiments on rats were sanctioned by the Standing Committee on Ethics in Animal Experimentation, Monash University. Eleven male, outbred Wistar rats at 8 wk of age were anesthetized with 1.5–4.5% halothane. Streptozotocin (STZ; Sapphire Bioscience, Alexandria, New South Wales) was dissolved in 100 mM citric acid adjusted to pH 4.5 with NaOH and immediately injected into a tail vein at a dosage of 60 mg/kg. Control rats received the vehicle only. Pairs of control and STZ-treated rats were caged together. After 6–8 wk, the rats were anesthetized with chloroform, decapitated, and bled.

Measurements of PMCA Vmax in rat RBCs. RBCs separated from heparinized blood samples were washed three times at 4°C in solution D, twice more in solution E, and then suspended at 10% Hct in solution E supplemented with 10 mM inosine. Solution D contained (in mM) 40 NaCl, 110 KCl, 0.2 MgCl2, 10 HEPES-sodium (pH 7.55), and 0.1 sodium-EDTA. Solution E was the same as solution D but without sodium-EDTA. The K+ concentrations in these solutions were higher than in those used for human RBCs (80 mM) to maintain the normal K+ content of rat RBCs during Ca2+ loading (5). Because the PMCA rate in rat RBCs is twice that in human RBCs (3), measurements of Ca2+ extrusion were performed at a lower temperature, 25°C rather than 37°C, to permit a sufficient number of timed samples for accurate measurement of PMCA Vmax.

Assays. Hemoglobin (Hb) concentrations were measured spectrophotometrically as cyanmethemoglobin. ATP was estimated using luciferase-luciferin (FLE 50, Sigma) as described previously (4, 14). The PMCA Vmax values measured in human and rat RBCs were standardized to 340 g of Hb, because for both humans and 16-wk-old Wistar rats (17), 1 liter of packed RBCs with normal mean cell volume contains ~340 g of Hb. Glucose concentrations in human and rat

Fig. 1. Comparison of plasma membrane Ca2+ pump (PMCA) maximal velocity (Vmax) measurements in intact human red blood cells (RBCs) preincubated in the presence or absence of 30 mM glucose (37°C) for 2 h. Ca2+ loading of RBCs was initiated by addition of A-23187 (10 μM) to the cell suspension in solution B containing [45Ca]CaCl2 (144 μM). Ca2+ transport by the ionophore was blocked by the addition of CoCl2 (350 μM) to reveal the active efflux of Ca2+. The PMCA Vmax values were determined as the slopes of the regression lines fitted to data points that represent single measurements of total cell Ca2+ during the active Ca2+ efflux, as reported before (4, 14, 24). The Vmax values measured in this experiment were 15.6 and 14.5 mmol·340 g Hb−1·h−1 for controls and glucose-preincubated cells, respectively.
plasma were determined with a standard assay kit (Trinder assay; Sigma). Glycated Hb levels in the human RBCs were estimated as HbA1c using an Ames DCA 2000 automated system, or as total glycated Hb (HbA1a + 1b + 1c), using an affinity resin assay kit (Sigma). HbA1c was measured in blood samples from all 15 diabetic patients and in samples from 11 control subjects. HbA1a + 1b + 1c was measured in samples from the remaining four control subjects. Six control samples were assessed with both methods, and because the difference between the two results proved to be constant, the mean difference was subtracted from the HbA1a + 1b + 1c measurement to estimate HbA1c in the four control samples assayed with the affinity resin method only. All reported glycated Hb levels are HbA1c as a percentage of total Hb.

Statistical analysis. All data are presented as means ± SE. Unless otherwise stated, the Student's t-test performed with Microsoft Excel was used to determine two-tailed P values. Differences were considered significant if P < 0.05. The relationships between age, plasma glucose concentrations, glycated Hb, and the PMCA Vmax were analyzed by linear regression. The effects of age on the PMCA Vmax were also tested using a single-factor analysis of variance (ANOVA) with age as a covariant.

RESULTS

Effects of glucose on the Vmax of the PMCA in intact RBCs from normal human subjects. Figure 1 illustrates a typical experiment from the first study in which PMCA-mediated Ca2+ extrusion by human RBCs was measured after preincubation of the cells (37°C), with and without glucose (up to 30 mM), for various periods of time. In 11 similar experiments with RBCs from 4 different donors, preincubated from 15 min to 3 h, all PMCA Vmax values for both the controls and the glucose-treated RBCs fell within a range of ±7%, the same extent of variation observed between duplicate measurements. Statistical analysis of paired differences (controls minus glucose-treated cells) gave a mean ± SE of 0.18 ± 0.53 (n = 11), which was not significantly different from zero.

Table 1 shows the results of the second study in which PMCA Vmax was measured after preincubation of normal human RBCs, with and without 100 mM glucose, for periods of 0.5, 3, and 6 h, in conditions identical to those in which González Flecha et al. (7, 8) observed up to 45% inhibition of PMCA activity in membranes isolated from the glucose-treated RBCs. There were no significant differences in mean PMCA Vmax values between controls and glucose-treated RBCs.

ATP levels were also measured in the normal RBCs before and after the 6 h of preincubation. After preincubation, the ATP levels were between 95% and 116% of the initial values. There was no difference in ATP levels between RBCs incubated in the presence or absence of glucose (results not shown).

Table 1. PMCA-mediated Ca2+ extrusion following preincubation of RBCs with 100 mM glucose and 40 mM phosphate

<table>
<thead>
<tr>
<th>Preincubation Time, h</th>
<th>PMCA Vmax, %Control Values at Each Preincubation Time</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>97.6 ± 4.2</td>
<td>4</td>
<td>0.80</td>
</tr>
<tr>
<td>3</td>
<td>95.6 ± 2.2</td>
<td>5</td>
<td>0.12</td>
</tr>
<tr>
<td>6</td>
<td>98.3 ± 3.1</td>
<td>4</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, number of experiments. P values are for the differences in plasma membrane Ca2+ pump (PMCA) Vmax values between controls and glucose-preincubated cells at each preincubation time. RBCs, red blood cells.
greater than that of the controls. Similarly, the mean glycated Hb level in the diabetic subjects was twice that of the controls. The elevated glycated Hb levels of the diabetic subjects indicated that their mean glucose levels had been high over the previous 7–8 wk (11).

Nevertheless, there were no significant differences in the mean RBC PMCA \( V_{\text{max}} \) values between controls and diabetic subjects, either before or after age correction (Table 2). Figures 3 and 4 show the relationships between PMCA \( V_{\text{max}} \) values corrected for age and plasma glucose or glycated Hb levels, respectively, in control and diabetic groups. There was no correlation between PMCA function in the intact RBCs and the extent and persistence of hyperglycemia.

**PMCA function in RBCs from STZ-diabetic rats.** After STZ injection, treated rats were kept for 6–8 wk before assaying their RBC PMCA function. At the time of injection, the mean body weight of all the rats was 387 ± 9 g, but by the time of assay, the weight of the STZ-treated rats was 325 ± 23 g, significantly lower than that of the control group (518 ± 54 g, \( P < 0.001 \)). Over the 4 days following STZ injection, the rats showed a 3-fold increase in their plasma glucose levels, from an initial mean value of 6.9 ± 0.6 to 23.4 ± 4.4 mM. After 6–8 wk, the mean plasma glucose in the STZ-treated rats continued to be three to four times higher than in the controls (Table 3, Fig. 5). However, the mean PMCA \( V_{\text{max}} \) did not differ between both groups. Thus sustained, elevated plasma glucose levels in the rats did not reduce the PMCA \( V_{\text{max}} \) in the intact RBCs.

**DISCUSSION**

Previous studies by González Flecha et al. (7) have shown that in vitro incubation of human RBCs in concentrations of glucose from 10 to 100 mM progressively inhibits PMCA activity, as measured in isolated membranes. In the current study, we have duplicated their in vitro incubation conditions, but measured instead the \( V_{\text{max}} \) of the active extrusion of Ca\(^{2+}\) from RBCs. In one of the most critical experiments demonstrating the different results obtained with these two types of measurements, we followed the glucose preincubation protocol of González Flecha et al. (7) by suspending RBCs in high-phosphate media, with and without 100 mM glucose, for up to 6 h. Whereas they observed (Ref. 7, see their Fig. 2) a glucose-induced inhibition of 45% in the Ca\(^{2+}\)-ATPase activity of the membranes isolated from these RBCs, we found no significant difference in the Ca\(^{2+}\) extrusion capacity of the PMCA in the RBCs (Table 1).

### Table 2. Plasma glucose, glycated Hb, and PMCA \( V_{\text{max}} \) in human diabetic subjects and nondiabetic controls

<table>
<thead>
<tr>
<th>Subject Groups</th>
<th>n</th>
<th>Plasma Glucose, mM</th>
<th>%Glycated Hb</th>
<th>PMCA ( V_{\text{max}} ) at 37°C, mmol·340 g Hb (^{-1})·h(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>15</td>
<td>4.52 ± 0.24</td>
<td>5.31 ± 0.11</td>
<td>Raw data Age corrected</td>
</tr>
<tr>
<td>Diabetic subjects</td>
<td>15</td>
<td>13.6 ± 1.1*</td>
<td>10.2 ± 0.3*</td>
<td>13.3 ± 0.5</td>
</tr>
</tbody>
</table>

Data are means ± SE. *\( P < 0.001 \) vs. control subjects. **\( P = 0.12 \) vs. age-corrected control subjects.
Furthermore, we found no differences between the PMCA $V_{\text{max}}$ of intact RBCs from diabetic subjects and those from nondiabetic controls and no correlation between PMCA $V_{\text{max}}$ and plasma glucose or glycated Hb (Table 2 and Figs. 3 and 4). By contrast, results from four comparable studies (7, 10, 18, 19) showed Ca$^{2+}$-ATPase activities in RBC membranes from type 1 and type 2 diabetic subjects reduced from those of controls by 42 to 62%, and, in one case (18), a significant correlation between PMCA function and percent of glycated Hb. In all of these studies, the significant reductions of PMCA activity in membrane preparations from RBCs of diabetic patients were observed at levels of increased glycated Hb comparable with those at which we found normal RBC PMCA $V_{\text{max}}$ values.

Similarly, our findings of normal PMCA $V_{\text{max}}$ in RBCs from STZ-diabetic rats with a sustained 3- to 4-fold increase in plasma glucose for 6–8 wk (Table 3 and Fig. 5) contrast with reports that RBC membrane Ca$^{2+}$-ATPase activity from rats that endured 96 h of alloxan-induced type 1 diabetes (1) was inhibited by 55%, and activity from rats 30 wk after STZ injection was 25% less than controls (15).

Another group (13) observed increased RBC membrane PMCA activity 3 wk after type 2 diabetes had been established in rats by injecting the neonates with STZ, whereas adult rats with hyperglycemia for >5 wk after STZ treatment had normal Ca$^{2+}$-ATPase activity. However, PMCA rates in this study were measured as inorganic phosphate production in RBC hemolysates (rather than in washed, isolated membranes), a method which may have inadequate specificity to accurately report changes in PMCA activity when other metabolic factors are varying with the age and diabetic status of the animal.

Taken as a whole, the results presented here show that the $V_{\text{max}}$ of active Ca$^{2+}$ extrusion from RBCs is unaffected by their exposure in vivo or in vitro to high concentrations of glucose, despite abundant documentation that similar glucose exposure resulted in substantial inhibition of the PMCA activity of isolated RBC membranes or purified membrane preparations (5–8, 10, 15, 18, 19). Possible origins for this discrepancy are considered next.

Prolonged exposure of RBCs to elevated glucose concentrations causes substantial glycation of membrane proteins; exposure of purified PMCA preparations suggests a stoichiometry of 5 mol of glucose-bound Lys residues per mole of PMCA enzyme (9). The presence of 5 mM ATP during glycation of RBC membranes by incubation in 10 mM glucose prevented inhibition of the PMCA but had no detectable effect on the overall level of membrane glycation (7). From these results, the authors concluded that only one Lys residue was involved in the inhibition of the PMCA by glycation and that the vulnerable Lys was probably located near the ATP catalytic site (9). They further suggested that glycation of the amino group on this Lys residue resulted in total loss of ATP binding capacity of the affected enzyme molecule, with consequent reduction in the measured $V_{\text{max}}$ of the PMCA.

Within the above framework, our present observations of a normal PMCA $V_{\text{max}}$ in RBCs exposed to high glucose suggest that the vulnerable Lys residue was not glycated, perhaps because of a protective effect of cytoplasmic ATP. But if so, why should membranes prepared from the glucose-treated RBCs show marked inhibition of their Ca$^{2+}$-ATPase activity? One possible explanation is that glycation of the vulnerable Lys residue occurred during the hemolysis and membrane isolation procedures, in the absence of extracellular glucose. For this to be feasible, it would be necessary to postulate the transfer of trapped glucose molecules between neighboring Lys residues within a diffusion-restricted domain at the ATP catalytic site.

Alternatively, an apparent inhibition of the Ca$^{2+}$-ATPase might result from an increased amount of membrane-associated protein obtained from glucose-treated RBCs, resulting in a decreased ratio of PMCA enzyme to membrane protein, despite normal enzyme activity per RBC. Also, glucose-induced functional denaturation (other than through direct glycation) of the enzyme may also contribute to the observed decrease of

### Table 3. Plasma glucose and the PMCA $V_{\text{max}}$ in RBCs from STZ-treated rats and normal controls

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>n</th>
<th>Plasma Glucose, mM</th>
<th>PMCA $V_{\text{max}}$ at 25°C, mmol·340 g Hb$^{-1}$·h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>11</td>
<td>7.2 ± 0.5</td>
<td>17.5 ± 1.2</td>
</tr>
<tr>
<td>STZ-treated rats</td>
<td>11</td>
<td>27.3 ± 0.9</td>
<td>16.7 ± 0.9</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.001 vs. control rats. STZ, streptozotocin.

Fig. 5. RBC PMCA $V_{\text{max}}$ as a function of plasma glucose concentration in streptozotocin (STZ)-diabetic and control rats. For each rat, the $V_{\text{max}}$ was measured twice, and their average was plotted as a function of plasma glucose concentration. Regression lines fitted to the plots showed a positive correlation between the plasma glucose concentration and PMCA $V_{\text{max}}$ for the STZ-diabetic rats ($r = 0.65$, $P = 0.03$). There was no significant correlation for control rats ($r = 0.18$, $P = 0.59$).
activity in in vitro conditions. Similar mechanisms might account for the discrepancies between measurements of PMCA-mediated Ca\(^{2+}\) fluxes in RBCs and of ATPase activities in isolated RBC membranes from diabetic patients or animals.

The present results indicate that PMCA-mediated Ca\(^{2+}\) transport in RBCs is not affected by type 2 diabetes in humans, by STZ-induced (type 1) diabetes in rats, or by any of the experimental incubations at high glucose levels found to inhibit both the ATPase activity in isolated RBC membranes and the Ca\(^{2+}\) uptake into inside-out vesicles prepared from RBC membranes. The results expose a real difference, not a contradiction: the functional state of the pump in the intact RBC is not affected by glucose, whereas in the disrupted cell, it apparently is. This is important with regard to claims, based on studies with isolated membranes and purified enzyme, that the Ca\(^{2+}\) homeostasis of cells in vivo may be affected directly by high glucose inhibition of PMCA activity (7, 12, 18, 19, 21). Our results show that there is no experimental basis for such an effect on RBCs. Thus PMCA function of RBCs in vivo should not be affected by short- or long-term exposure to elevated glucose concentrations. Understanding the basis of the observed differences of PMCA responses to high glucose may affect directly by high glucose inhibition of the pump in the intact RBC.

REFERENCES


