The erythrocyte calcium pump is inhibited by non-enzymic glycation: studies *in situ* and with the purified enzyme

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In a previous paper we demonstrated that incubation of either intact erythrocytes or erythrocytes membranes with glucose decreases the activity of the membrane Ca^{2+}-ATPase [González Flecha, Bermúdez, Cédola, Gagliardino and Rossi (1990) Diabetes **39**, 707–711]. The aim of the present work was to obtain information about the mechanism of this inhibition. For this purpose, experiments were carried out with purified Ca^{2+}-ATPase, inside-out vesicles and membranes from human erythrocytes. Incubation of the purified Ca^{2+}-ATPase with glucose led to a decay in the enzyme activity of up to 50% of the control activity under the conditions used. The decrease in ATPase activity was concomitant with labelling by [6-^{3}H]glucose of the purified Ca^{2+} pump; the kinetic properties of both processes were almost identical, suggesting that inhibition is a consequence of the incorporation of glucose into the Ca^{2+}-ATPase molecule. In inside-out vesicles, glucose also promoted inhibition of Ca^{2+}-ATPase activity as well as of active Ca^{2+} transport. Arabinose, xylose, mannose, ribose, fructose and glucose 6-phosphate (but not mannitol) were also able to inactive the ATPase. The activation energy for both the decrease in ATPase activity by glucose and the labelling of the pump with [6-^{3}H]glucose was about 65 kJ/mol. Furthermore, inorganic phosphate enhanced the inactivation of the Ca^{2+}-ATPase by glucose. This evidence strongly suggests that inhibition is a non-enzymically catalysed process. Inactivation of the Ca^{2+}-ATPase by glucose was enhanced by reductive alkylation with sodium borohydride. Aminoguanidine, an inhibitor of the formation of the advanced end products of glycosylation, did not prevent the deleterious effect of glucose on the enzyme activity. Therefore it is concluded that inactivation of the Ca^{2+} pump is a consequence of the glycation of this protein.

INTRODUCTION

We have previously reported [1] that erythrocytes from diabetic patients show lower Ca^{2+}-ATPase activity than comparable cells from healthy volunteers. In a subsequent paper we found that preincubation of normal erythrocytes with glucose decreased the Ca^{2+}-ATPase activity, and that this effect was correlated with increased glycosylation of the erythrocyte membrane proteins [2]. In that study we also observed that preincubation of either intact erythrocytes or isolated erythrocyte membranes with glucose caused inactivation of the Ca^{2+}-ATPase, which was enhanced by an increase of the pH of the preincubation medium, a typical characteristic of the non-enzymic glycosylation process.

The reaction of reducing sugars with proteins, which results in non-enzymic 'browning' have been extensively studied [3]. The formation of adducts between sugars and proteins has been reported for several proteins such as haemoglobin [4], RNAase [5] and, more recently, Na^+/K^+-ATPase [6]. Non-enzymic glycosylation of proteins is a reaction which proceeds through several steps; the early ones are reversible and develop in relatively short periods, while the later ones take longer times and become irreversible [7]. Both the initial and end products of the reaction modify the physicochemical properties of the glycosylated protein [8]. Based on such changes, and the permanent high blood glucose levels which characterize diabetes mellitus, glycosylation of proteins has been postulated as the developing mechanism of the chronic complications of this disease.

The experiments in the present paper were designed to: (1) test whether the effect of glucose is exerted directly on the Ca^{2+}-ATPase or whether it involves some other molecules that are present in the erythrocyte membrane; and (2) obtain information about the mechanism of the interaction between glucose and the Ca^{2+} pump.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [9], except that no unlabelled P_i was added to the incubation media. [^{32}P]P_i was provided by the Comisión Nacional de Energía Atómica (Argentina).

Calmodulin–agarose, glucose 6-phosphate, ATP, Mops, phosphatidylcholine, and enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Glucose was from Merck (Darmstadt, Germany), fructose and mannitol from Mallinckrodt Chemical Works (New York, NY, U.S.A.). Arabinose, xylose, mannose and ribose were gifts from Dr. R. Wolosiuk (IIB, Argentina).

$^{45}CaCl_2$ was purchased from du Pont New England Nuclear (Boston, MA, U.S.A.), and D-[6-^{3}H]glucose was a gift from Dr. Mario Parisi (Centre d'études nucléaires de Saclay, France).

All other chemicals were of analytical grade. Solutions were prepared in double-distilled water and the pH was controlled with a Corning 125 pH meter.

Recently drawn human blood for the isolation of Ca^{2+}-ATPase was obtained from the Haematology Section of the Hospital de Clínicas General San Martín (Argentina).

Abbreviation used: IOV, inside-out vesicles.

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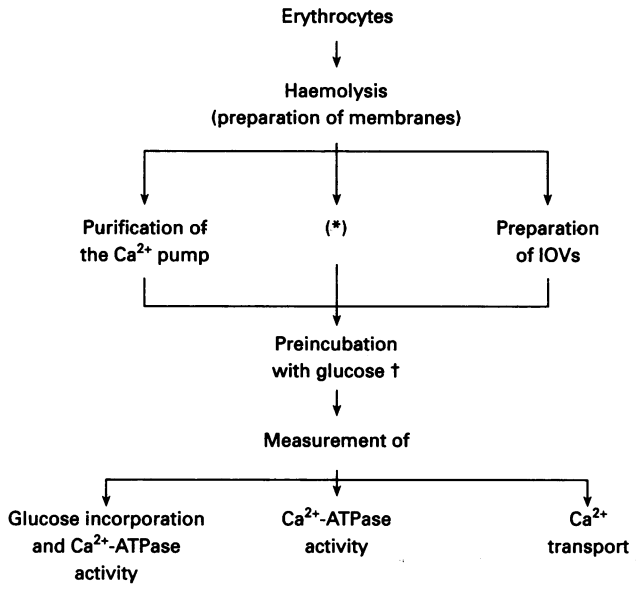

Scheme 1 Steps of experimental design

*Reagents such as $NaBH_4$ and aminoguanidine were included as indicated in the Results section and the Figure legends. †Controls preincubated with equimolar concentrations of mannitol were run in parallel.

Purification of the Ca^{2+}-ATPase from human erythrocytes

Blood samples were drawn and immediately mixed with an anticoagulant solution containing 1 mM EDTA/Tris (pH 7.4 at 37 °C) and 150 mM NaCl. Afterwards, erythrocytes were washed three times with 10 vol. of cold 150 mM NaCl. Calmodulin-depleted erythrocyte membranes were prepared using a hypotonic solution according to the procedure described by Gietzen et al. [10]. The membrane suspension was frozen at −20 °C. The Ca^{2+}-ATPase was isolated by the calmodulin affinity chromatography procedure and reconstituted in artificial vesicles of phosphatidylcholine as described by Roufogalis et al. [11]. Purified enzyme was stored in liquid nitrogen until use.

Preparation of inside-out erythrocyte membrane vesicles (IOVs)

Calmodulin-deficient IOVs were prepared as described by Sarkadi et al. [12]. After preparation, the IOVs were washed twice with 10 vol. of 10 mM Mops/KOH, pH 7.4, at 37 °C. The final pellet was resuspended to a final concentration of 5 mg of membrane protein/ml in 0.16 M KCl buffered to pH 7.4 at 37 °C with 5 mM Mops/KOH.

Protein concentration

Protein was determined according to the method of Lowry et al. [13], as modified by Peterson [14].

Experimental design

The steps followed to perform the experiments in this paper are summarized in Scheme 1.

Glucose incorporation

Purified enzyme (50 μg/ml) was incubated at 37 °C in 20 mM phosphate buffer (pH 8.4) with 10 mM [6-^{3}H]glucose (specific radioactivity 3000 d.p.m./nmol). At different times, aliquots (200 μl) were removed and mixed with an ice-cold solution of 100 mM glucose. After isotopic dilution, enzyme was precipitated with 7% trichloroacetic acid and filtered through Sartorius membrane filters (0.20 μm pore size). The filters were washed three times with 15 ml of an ice-cold solution of 10 mM glucose and 15 mM Mops/KOH, pH 7.4, dried and transferred to counting vials. Radioactivity was measured by liquid scintillation counting.

Gel electrophoresis

Enzyme was incubated for 1 h at 37 °C with 10 mM [6-^{3}H]glucose and $NaBH_4$, the latter being used as reducing agent as described below. SDS/PAGE and staining of the gels was carried out as described elsewhere [15]. Gels were 5.6% T and 2.5% C [16]. After the destaining procedure the gel was cut into slices and the individual slices were placed into separate vials. Radioactivity was determined by liquid scintillation counting as described (see [17]) and plotted as a function of the distance from the origin. Background corrections were done by subtracting the average radioactivity measured in unstained regions of the gel. The M_r of the glycated fractions was estimated by comparison with the mobilities of standard proteins simultaneously run on the same gel.

Ca^{2+}-ATPase activity

ATPase activity was measured at 37 °C in a medium containing 120 mM KCl, 30 mM Tris/HCl (pH 7.4), 4 mM $MgCl_2$, 1 mM EGTA, 1 mM $CaCl_2$ ($[Ca^{2+}]_{free}$ = 36 μM) and 2 mM [γ-^{32}P]ATP. Ca^{2+}-ATPase activity was taken as the difference between the activity measured in the above medium and that measured in the same medium without calcium. The protein concentration was in the range 50–100 μg/ml (membrane assay) or 2–5 μg/ml (purified enzyme assay). The release of [^{32}P]P_i from the nucleotide was estimated according to the procedure of Richards et al. [18].

Determination of free Ca^{2+} concentrations

The Ca^{2+} concentration in the incubation medium was measured using a selective Ca^{2+} electrode, as described by Kratje et al. [19].

Reducing treatment with sodium borohydride

Reductive alkylation of proteins was performed as described by Means [20], with some modifications. Membranes, or purified enzyme, were incubated at 37 °C in 20 mM phosphate buffer (pH 8.4) and 10 mM glucose. Incubation samples were then cooled in an ice bath, and sodium borohydride was added dropwise under vigorous stirring up to a final concentration of 1–3 mg/ml. The reduction step lasted 30 min, after which the membranes were washed three times with 15 mM Tris/HCl (pH 7.4 at 4 °C) and purified enzyme was mixed with a small amount of HCl, so that excess $NaBH_4$ was eliminated. Alternatively, reduction was performed simultaneously to the incubation with glucose. In this case, $NaBH_4$ and glucose were added in three aliquots at 20 min intervals, reaching the same final concentration as mentioned above.

Ca^{2+} transport measurements

Ca^{2+} influx into IOVs was measured by rapid filtration through Millipore membrane filters (0.45 μm pore size) as described by Rossi and Schatzmann [21].

Analysis of the data

All measurements were performed in duplicate or triplicate. Except where indicated, experiments presented in the Results section are representative of at least two independent experiments. The equations were fitted to the experimental data by a non-linear regression procedure based on the Gauss–Newton algorithm [22,23]. The dependent variable was assumed to be homoscedastic (constant variance) and the independent variable to have negligible error. The best-fitting equation was considered to be that which gave the minimal S.D. of the regression and the least biased fit. Parameters were expressed as means ± S.E.M.

RESULTS

[6-^{3}H]Glucose incorporation into purified Ca^{2+}-ATPase and its effects on enzyme activity

Figure 1(a) shows the results of an experiment in which the time course of [6-^{3}H]glucose incorporation into the Ca^{2+}-ATPase was measured. It can be seen that [6-^{3}H]glucose incorporation increased with time along a curve that can be fitted by an exponential function, with a maximal incorporation of 5.4 ± 0.07 mol of [6-^{3}]glucose/mol of ATPase; the apparent rate coefficient ($k_{app.}$) was 0.0223 ± 0.0009 min^{-1}. Incubation with [6-^{3}H]glucose for longer periods of time (results not shown) revealed a very slow phase that can be fitted by a straight line with a slope of 0.016 ± 0.004 mol of [6-^{3}H]glucose/min per mol of Ca^{2+}-ATPase. In a parallel experiment (Figure 1b) we measured the time course of inactivation of the Ca^{2+}-ATPase upon incubation with 10 mM glucose for up to 1 h at 37 °C. It should be pointed out that the enzyme activity decayed during preincubation, even in control tubes, due to thermal inactivation. For this reason, the data are shown as percentages of the activity of the control for each time, and longer times were not tried. The percentage of remnant activity was plotted versus time, and an exponential function was fitted to the data. The apparent rate coefficient for the sugar-induced inactivation was 0.019 ± 0.001 min^{-1}. This value is not significantly different from the $k_{app.}$ for the rapid phase of [6-^{3}H]glucose incorporation ($P > 0.2$), indicating that the combination of glucose with the Ca^{2+}-ATPase and the inactivation of the enzyme are concomitant and cause-related phenomena. These results strongly suggest that inactivation of the Ca^{2+}-ATPase is a consequence of the reaction of glucose with the pump.

To establish whether the glucose binds to the Ca^{2+} pump through covalent or non-covalent interactions, the purified Ca^{2+} pump was incubated with 10 mM [6-^{3}H]glucose and then subjected to electrophoresis in a denaturing SDS/polyacrylamide gel. Only one broad band, of M_r 140000, characteristic of the erythrocyte Ca^{2+} pump, was observed when the gel was stained with Coomassie Blue. Figure 2 shows a pattern of the radioactivity incorporated to the pure Ca^{2+}-ATPase. [6-^{3}H]Glucose appeared mainly as a broad band around the position corresponding to M_r 140000. Some [6-^{3}H]glucose was also detected at the position of the tracking dye, probably due to free [6-^{3}H]glucose and to incorporation of [6-^{3}H]glucose into phospholipids present in the medium. Incorporation of [6-^{3}H]-glucose into Ca^{2+}-ATPase was also observed when the samples were delipidized before running the gel (results not shown). It should be noted that, when the experiment was carried out with trace amounts of [6-^{3}H]glucose, no incorporation over background levels was observed (results not shown). Therefore the results shown in Figures 1 and 2 indicate that when the enzyme is incubated with glucose under the conditions described in the Materials and methods section, the sugar becomes attached covalently to the Ca^{2+}-ATPase and this reaction leads to inactivation of the enzyme.

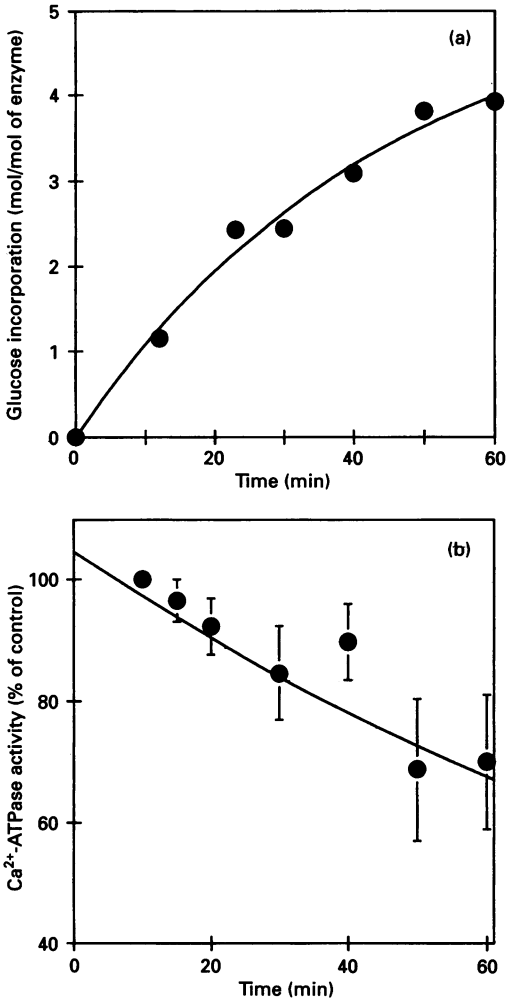

Figure 1 Time courses of (a) glucose incorporation into the Ca^{2+}-ATPase, and (b) inactivation of Ca^{2+}-ATPase by glucose

(**a**) Glucose incorporation was measured as described in the Materials and methods section. Unspecific incorporation, determined by incubation of the enzyme with non-radioactive glucose followed by the addition of [6-^{3}H]glucose at the end of the incubation period, was subtracted. The amount of enzyme was calculated assuming that Ca^{2+}-ATPase is the only protein present in the preparation. The continuous line is the graphical representation of the following equation:

$$[EG] = [EG]_{\infty}(1 - e^{-k_{app.}t})$$

where [EG] is the amount of glucose incorporated into the enzyme at time t, $[EG]_{\infty}$ is the maximal incorporation of glucose into the enzyme, and $k_{app.}$ is the apparent rate coefficient of the process. The best-fitting values of these parameters were: $[EG]_{\infty}$, 5.4 ± 0.07 mol of glucose/mol of Ca^{2+}-ATPase; $k_{app.}$, 0.0223 ± 0.0009 min^{-1}. (**b**) Purified Ca^{2+}-ATPase (50 μg/ml) was incubated at 37 °C in 20 mM phosphate buffer (pH 8.4) plus 10 mM glucose. At different times, aliquots (50 μl) were removed and cooled in an ice bath. For each time point, control tubes with 10 mM mannitol were run in parallel. Ca^{2+}-ATPase activity was measured as described in the Materials and methods section and is expressed as a percentage of the activity measured in the corresponding control. The continuous line is the graphical representation of the following equation:

$$A = A_0 e^{-k_{app.}t}$$

where A is the Ca^{2+}-ATPase activity at time t, A_0 is the Ca^{2+}-ATPase activity at zero time, and $k_{app.}$ is the apparent rate coefficient of this process. The best-fitting values of the parameters were: A_0, $109 \pm 8\%$; $k_{app.}$, 0.019 ± 0.001 min^{-1}.

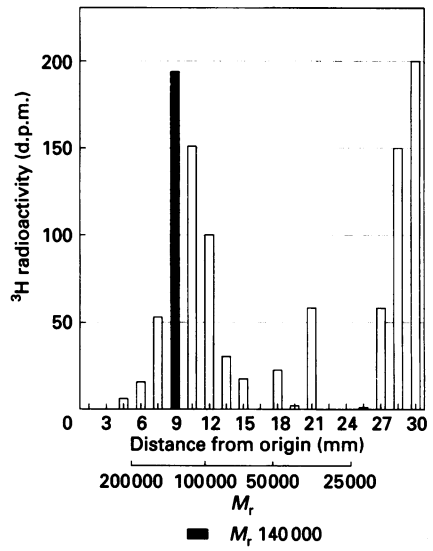

Figure 2 Radioactive pattern after electrophoretic separation (SDS/PAGE) of the Ca^{2+}-ATPase after preincubation with [6-^{3}H]glucose

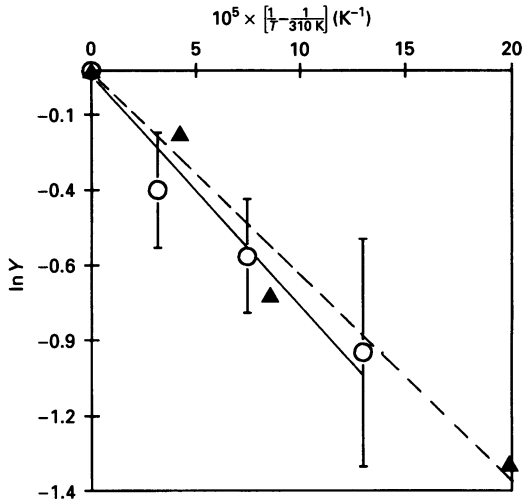

Figure 3 Temperature-dependence of the effect of glucose on Ca^{2+}-ATPase

(i) Ca^{2+}-ATPase activity (○): erythrocyte membranes (1 mg of protein/ml) were incubated at different temperatures for 2 h in media containing 20 mM phosphate buffer (pH 8.4) and 10 mM glucose or mannitol (control). At the end of the incubation period, membranes were washed with 15 mM Mops/KOH; Ca^{2+}-ATPase activity was measured as described in the Materials and methods section, and is expressed as the percentage of control activity. The results shown are the weighed means ± S.E.M. from five independent experiments. In this case, Y = (percentage inhibition at any temperature)/(percentage inhibition at 37 °C). Under the experimental conditions used, $Y = k_{app.}/k_{app.37\,°C}$, where $k_{app.}$ is the rate coefficient of the reaction. The continuous line represents:

$$\ln Y = -E_a(1/T - 1/310\ K)/R$$

where E_a is the activation energy of the reaction, R is the universal gas constant, and T is the thermodynamic temperature of the system. The best-fitting value for E_a was 69 ± 7 kJ/mol. This equation was deduced from the Arrhenius relation for the dependence on temperature of the kinetic coefficient. (ii) Glucose incorporation (▲): purified Ca^{2+}-ATPase was incubated with 10 mM [6-^{3}H]glucose at different temperatures, and glucose incorporation was evaluated as described in the Materials and methods section. In this case, Y = (incorporation of glucose at any temperature)/(incorporation of glucose at 37 °C). It is easy to show that, in this case also, $Y = k_{app.}/k_{app.37\,°C}$. The same equation as given above was used to fit the data (broken line). The best-fitting value for E_a was 57 ± 4 kJ/mol.

Table 1 Effect of incubation with different monosaccharides on Ca^{2+}-ATPase activity in erythrocyte membranes

Erythrocyte membranes (1 mg of protein/ml of reaction medium) were incubated for 2 h at 37 °C in the presence of 20 mM phosphate buffer (pH 8.4) and 10 mM of the monosaccharides indicated. After incubation, membranes were washed with 15 mM Mops/KOH (pH 7.7 at 4 °C) and Ca^{2+}-ATPase activity was measured as indicated in the Materials and methods section; values are expressed as percentages of the control activity. Results are means ± S.E.M. from three independent experiments; significant differences from the control are indicated by: $^*P < 0.01$; $^{**}P < 0.001$.

Sugar	Ca^{2+}-ATPase activity (% of control)
Mannitol (control)	100 ± 2.6
Glucose	82.5 ± 3.1*
Glucose 6-phosphate	73.6 ± 1.6**
Fructose	73.6 ± 8.8*
Ribose	92.1 ± 9.6
Mannose	92.6 ± 6.1
Arabinose	88.2 ± 2.2*
Xylose	83.9 ± 5.8*

Effect of temperature on glucose incorporation into purified Ca^{2+}-ATPase

In order to elucidate the characteristics of the reaction between glucose and the Ca^{2+}-ATPase we measured the effect of temperature (from 20 °C to 37 °C) on both the incorporation of glucose into the Ca^{2+}-ATPase and the inactivation of the enzyme by glucose. The data are represented in Figure 3 as an Arrhenius-like plot. Both the incorporation of glucose and the inactivation of the enzyme by glucose could be fitted by approximately the same straight line. From linear regression analysis of the data, the activation energy of glucose incorporation was estimated to be 57 ± 4 kJ/mol, and that for inactivation by glucose of the ATPase was 69 ± 7 kJ/mol. Hence the two phenomena show an almost identical dependence on temperature.

In an attempt to study the characteristics of the reaction of the glucose with the Ca^{2+}-ATPase when the enzyme is in its natural environment, we performed some experiments using whole erythrocyte membranes instead of purified enzyme. Ca^{2+}-ATPase activity was more stable in erythrocyte membranes than in purified enzyme preparations, and consequently we were able to study the effect of glucose using a longer incubation period.

Effect of different reducing sugars on Ca^{2+}-ATPase activity

Table 1 shows the effect on Ca^{2+}-ATPase activity of preincubation of intact erythrocyte membranes for 2 h at 37 °C with 20 mM of several reducing sugars. Incubation of erythrocyte membranes, under identical conditions, with 20 mM sucrose or 20 mM mannitol was used as a control. Ca^{2+}-ATPase was significantly inactivated by glucose, glucose 6-phosphate, fructose, arabinose and xylose. Preincubation with ribose and mannose induced a 8% decrease in the Ca^{2+}-ATPase activity, but this was statistically non-significant under the mild conditions used in these experiments. The results in Table 1 suggest that inactivation of the Ca^{2+}-ATPase by preincubation of isolated erythrocyte membranes with reducing sugars occurs by a non-specific process.

Effect of sodium borohydride

Schiff bases and Amadori products are the compounds resulting from the early steps of the reaction between reducing sugars and

Table 2 Effect of sodium borohydride on the inhibition of Ca^{2+}-ATPase by glucose

Erythrocyte membranes (1 mg of protein/ml of the reaction medium) were incubated for 2 h at 37 °C in 20 mM phosphate buffer (pH 8.4) with 10 mM glucose or mannitol (control). Treatment with sodium borohydride was carried out as described in the Materials and methods section. After incubation, membranes were washed with 15 mM Mops/KOH (pH 7.7 at 4 °C) and Ca^{2+}-ATPase activity was measured; values are expressed as percentages of control activity. Results are means ± S.E.M. from three independent experiments.

Treatment	Ca^{2+}-ATPase activity (% of control)
Mannitol	100 ± 5
Glucose	87 ± 5
Glucose followed by reduction with $NaBH_4$	82 ± 6
Mannitol and $NaBH_4$ added simultaneously	110 ± 4
Glucose and $NaBH_4$ added simultaneously	46 ± 5

Table 3 Effect of different buffers on the inhibition of Ca^{2+}-ATPase by glucose

Erythrocyte membranes (1 mg of protein/ml) or purified enzyme (50 μg/ml) were incubated for 1 h at 37 °C with 10 mM glucose or mannitol in the presence of 20 mM of the buffer indicated in the table. The pH of each buffer was adjusted to 8.4. At the end of the incubation period, the membranes were washed once with 15 mM Mops/KOH (pH 7.4 at 37 °C). The purified enzyme was diluted 10-fold before the assay. Ca^{2+}-ATPase activity was measured as described in the Materials and methods section. The results are means ± S.E.M. of three independent experiments.

Buffer	Ca^{2+}-ATPase activity (% of control)	
	Purified enzyme	Membranes
Tris/HCl	86 ± 5	92 ± 2
Potassium phosphate	68 ± 5	74 ± 4
Bicine/KOH	91 ± 5	100 ± 3
Mops/KOH	100 ± 9	100 ± 7

primary amino groups of proteins. Since formation of these products is a reversible process, it might be possible that the relatively small effects of reducing sugars on Ca^{2+}-ATPase activity were due to the low amounts of Schiff bases or Amadori products formed at the end of the incubation period. To test this hypothesis we performed enzyme incubations with glucose plus $NaBH_4$ as reducing agent, the latter being used to transform both the Schiff bases and the Amadori products to the same stable secondary amine [24]. Table 2 shows that preincubation with 10 mM glucose alone decreased the Ca^{2+}-ATPase activity to 87 % of the control value. Subsequent treatment with $NaBH_4$ did not significantly modify the activity. When glucose and $NaBH_4$ were added simultaneously, Ca^{2+}-ATPase activity was drastically decreased compared with the control. On the other hand, when $NaBH_4$ and 10 mM mannitol were added simultaneously, no significant effect on the Ca^{2+}-ATPase activity was observed.

These results suggest that addition of $NaBH_4$ leads to the irreversible formation of an alkylated product, making the apparent inactivation of Ca^{2+}-ATPase more evident. In keeping with this, the simultaneous addition of glucose and $NaBH_4$ enhanced the efficiency of the irreversible blockage of primary amino groups of the enzyme. Essentially the same results were obtained with $NaCNBH_3$ instead of $NaBH_4$ (not shown). It is worth noting that $NaCNBH_3$ has been used to stabilize the reaction of glucose with Na^+/K^+-ATPase [6].

Effect of aminoguanidine

To evaluate whether the inactivation of Ca^{2+}-ATPase with glucose was due to the formation of advanced glycosylation end products or simply to the formation of a Schiff base or an Amadori product, we tested the effect of the presence of aminoguanidine during preincubation of isolated erythrocyte membranes with glucose [7]. Erythrocyte membranes (1 mg of protein/ml of reaction medium) were preincubated for 2 h at 37 °C in presence of 20 mM phosphate buffer (pH 8.4) and 50 mM glucose with either 0 or 200 mM aminoguanidine. Control tubes were run by replacing glucose with 50 mM mannitol. After preincubation, membranes were washed with 15 mM Mops/KOH (pH 7.7 at 4 °C) and Ca^{2+}-ATPase activity was measured as described in the Materials and methods section. Ca^{2+}-ATPase activities, expressed as the percentages of the respective control, were 82 ± 11 % (mean ± S.E.M.) and 78 ± 10 % when the preincubation medium contained 0 and 200 mM aminoguanidine respectively (three independent experiments). Furthermore, no significant differences were found either between the activities of the controls or between the percentage inhibitions by glucose.

This result supports the assumption that the formation of advanced glycosylation end products is not necessary for the inactivation of Ca^{2+}-ATPase. Hence synthesis of a Schiff base (or even an Amadori product) as a result of enzyme glycation may account for the inactivation of the enzyme.

Effect of buffer composition on Ca^{2+}-ATPase inactivation induced by glycose

Erythrocyte membranes or purified enzyme were incubated with glucose and 20 mM Tris/HCl, Mops/KOH, Bicine/KOH or potassium phosphate, at pH 8.4 and 37 °C in all cases (Table 3). Inactivation of Ca^{2+}-ATPase induced by glucose was greater when phosphate buffer was present in the incubation medium, as expected, since phosphate has been described as the most effective catalytic agent for the Maillard reaction [25]. Inactivation was also significant in the presence of Tris/HCl, but no effect of glucose was observed when Bicine or Mops was used as buffer.

Effect of glucose on active Ca^{2+} transport

Since Ca^{2+}-ATPase is inactivated by glycation, we decided to investigate whether this process also affects active Ca^{2+} transport. For this purpose, ATP-dependent Ca^{2+} uptake was measured into IOVs that had been preincubated for 1 h with either 10 mM glucose or 10 mM mannitol. IOVs were added (final concentration 60 μg of membrane protein/ml) to medium containing 50 mM KCl, 1 mM Mops/KOH (pH 7.4 at 37 °C), 4 mM $MgCl_2$, 1 mM $^{45}CaCl_2$ (specific radioactivity 5×10^5 c.p.m./μmol) and 1 mM EGTA. The reaction was started by the addition of the appropriate amount of ATP so as to obtain a final concentration of 0.9 mM. Passive Ca^{2+} uptake, determined by incubation of IOVs in the same medium without ATP, was subtracted from this total uptake. The rate of ATP-dependent Ca^{2+} uptake was calculated from the slope of the initial part of a plot of $^{45}Ca^{2+}$ trapped by IOVs versus period of incubation. This rate was 3.0 ± 0.13 nmol of Ca^{2+}/min per mg of protein for control vesicles, and 2.37 ± 0.06 nmol of Ca^{2+}/min per mg of protein for

vesicles preincubated with glucose. Thus IOVs preincubated with 10 mM glucose showed a 21% decrease in Ca^{2+} transport compared with those preincubated with 10 mM mannitol ($P < 0.005$). Simultaneous measurements of Ca^{2+}-ATPase activity in the same preparation showed that the reaction of the pump with glucose produces the same degree of inhibition on the two basic functional properties of the Ca^{2+} pump (Ca^{2+}-ATPase activity and active Ca^{2+} transport).

DISCUSSION

Previous results showed that exposure of erythrocyte membranes to a high concentration of glucose, both *in vivo* [1] and *in vitro* [2], decreases the Ca^{2+}-ATPase activity of the membrane. This effect correlates with increased glycosylation of the erythrocyte membrane proteins [2]. These results led us to propose that inactivation of the Ca^{2+}-ATPase could be a consequence of the glycosylation of the ATPase or of another protein able to interact with the pump.

In 1985, Davis et al. [26] reported that exposure of erythrocyte membranes to high glucose concentrations *in vitro* resulted in inactivation of Ca^{2+}-ATPase. Subsequently the same group suggested that cellular membranes possess an enzyme capable of catalysing a glycosylation that modulates Ca^{2+}-ATPase [27].

The experiments in the present paper were designed to test whether: (1) the effect of glucose is directly exerted on the Ca^{2+}-ATPase or involves some other molecules present in the erythrocyte membrane; and (2) the effect of glucose is a consequence of non-enzymic reaction or is catalysed by a specific enzymic system.

With regard to the first point, we found that inactivation of the Ca^{2+} pump is probably due to the incorporation of sugar into the pump. This conclusion is based in the fact that both phenomena, Ca^{2+}-ATPase inactivation and glucose incorporation, have the same time course and temperature-dependence. Furthermore, Ca^{2+}-ATPase activity and Ca^{2+} uptake showed the same degree of inhibition by glucose. These observations, together with the fact that labelled glucose remains bound to the pump after electrophoresis under denaturing conditions, suggest that inactivation of the Ca^{2+} pump by glucose occurs by the covalent reaction of the sugar with this protein. This does not exclude the possibility of an interaction of reducing sugars with the lipid environment. Indeed, incorporation of glucose takes place in two phases: an early exponential phase and a linear, slower one. The inactivation of the enzyme by glucose presents the same time course as the early phase of the incorporation of glucose into the Ca^{2+}-ATPase. The slow, linear phase of glucose incorporation into the enzyme may be related to the glycation either of amino groups that are only exposed in the inactive state of the Ca^{2+} pump or of primary amino groups with trace amounts of lipids (i.e. phosphatidylethanolamine) present in the reconstitution medium of the enzyme. Furthermore, as we previously reported [2], the presence of 1 mM ATP during preincubation of erythrocyte ghosts with glucose prevents inactivation of Ca^{2+}-ATPase but not the glycation of other membrane proteins. This experimental evidence argues against the participation of phospholipids or other proteins in the mechanism of inactivation of the Ca^{2+} pump by reducing sugars under the conditions used throughout this work.

Regarding the second point, the experiments described in this paper strongly suggest that inhibition of the membrane Ca^{2+} pump was mostly due to the non-enzymic glycation of primary amino groups in the Ca^{2+}-ATPase, for the following reasons. (i) Inactivation of the purified Ca^{2+}-ATPase by preincubation with glucose occurs under identical conditions and with a similar rate as the inactivation of Ca^{2+}-ATPase from isolated erythrocyte membranes (see the Results section and [2]). (ii) Since Ca^{2+}-ATPase was purified by calmodulin affinity chromatography, it is rather improbable that a glycosylating enzyme could co-purify with the Ca^{2+} pump. Even assuming this last possibility, the hypothetical glycosylating enzyme was not detectable on electrophoresis of the purified Ca^{2+}-ATPase. (iii) The only feasible, but very unlikely, hypothesis is that Ca^{2+}-ATPase has an autoglycosylating capability. The hypothetical autoglycosylating activity should be poorly specific, since as shown in Table 1, different sugars can substitute for glucose as inactivators of the Ca^{2+} pump. (iv) The temperature-dependence of both the incorporation of glucose into the Ca^{2+}-ATPase and the inactivation of the enzyme by glucose showed an activation energy of about 65 kJ/mol. This value is between the previously described values for the early steps of non-enzymic glycosylation, i.e. 35 kJ/mol for α-amino acids [28] and 109 kJ/mol for food proteins [29].

On the other hand, the reaction of the Ca^{2+} pump with sugars showed several characteristics of non-enzymic glycation: (i) the reaction showed no remarkable specificity with regard to the reducing sugar used, (ii) inactivation by glucose was greatly enhanced by phosphate anions, and (iii) the inactivation of the Ca^{2+} pump by preincubation with reducing sugars could be enhanced by treatment with $NaBH_4$ or $NaCNBH_3$.

The inactivation of the Ca^{2+} pump could in theory be explained by the formation of a Schiff base, an Amadori product or an advanced glycosylated end product between a reducing sugar and the primary amino groups of the Ca^{2+}-ATPase. It is well known [7] that aminoguanidine prevents the development of advanced glycosylated end molecules through the formation of a dead-end product. As shown in the Results section, aminoguanidine did not block the inactivation of the Ca^{2+} pump during preincubation with glucose, indicating that formation of advanced glycosylated end products is not necessary for inhibition of the Ca^{2+}-ATPase. This behaviour agrees well with the fact that the Ca^{2+}-ATPase activity in erythrocytes from poorly controlled diabetic patients reverted to its normal specific activity after 10 days of euglycaemia [30]. At this point formation of the Schiff base or the Amadori product could explain the results. Due to the short incubation period employed in our experiments, Schiff bases were thought likely to be the main compounds resulting from non-enzymic glycation of the Ca^{2+}-ATPase. However, the fact that the effect of sugars was enhanced by phosphate anions suggests that an Amadori-type rearrangement takes place. Additional studies are required in order to identify the stage of the glycation that is responsible for the inhibition of the Ca^{2+} pump.

A question that is still open is: which are the primary amino groups that are the target for glycation? The N-terminal group is blocked [31], so should be excluded. The other possible targets for glycation are the ϵ-amino groups of Lys residues. A Lys residue is present in the active site of the enzyme [32]. In our laboratory, experiments involving the chemical modification of the pump revealed another Lys residue that is involved in the E_1–E_2 transition [33]. Such chemical modification could also be prevented by ATP. Since inhibition of the pump by glucose is prevented by ATP [2], both of these residues are likely to be involved in the glycation of the pump.

Inactivation of Ca^{2+}-ATPase by non-enzymic glycation might produce an imbalance in the transmembrane Ca^{2+} flux. Indeed, the results in this paper showed that active Ca^{2+} transport is blocked to the same extent as the Ca^{2+}-ATPase activity. As a consequence, changes in the cytosolic Ca^{2+} concentration would be expected. Whether or not these changes are significant *in vivo*, and the importance of such changes in the pathogenesis of some

chronic complications of diabetes, are an interesting field for future study.

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REFERENCES

1 Gronda, C., Rossi, J. P. F. C., Cédola, N. V. and Gagliardino, J. J. (1986) IRCS Med. Sci. **14**, 476
2 González Flecha, F. L., Bermúdez, M. C., Cédola, N. V., Gagliardino, J. J. and Rossi, J. P. F. C. (1990) Diabetes **39**, 707–711
3 Ellis, G. P. (1959) Adv. Carbohydr. Chem. **14**, 63–134
4 Bunn, H. F., Shapiro, R., McManus, M. J., Garrick, L., McDonald, M. J., Gallop, P. J. and Gabbay, K. H. (1979) J. Biol. Chem. **254**, 3892–3898
5 Watkins, N. G., Thorpe, S. R. and Baynes, J. W. (1985) J. Biol. Chem. **260**, 10629–10636
6 Garner, M. H., Bahador, A. and Sachs, G. (1990) J. Biol. Chem. **265**, 15058–15066
7 Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P. and Cerami, A. (1986) Science **232**, 1629–1632
8 Baynes, J. W. and Monnier, V. M. (eds.) (1989) The Maillard Reaction in Aging, Diabetes and Nutrition, Alan R. Liss, New York
9 Glynn, I. M. and Chappell, J. B. (1964) Biochem. J. **90**, 147–149
10 Gietzen, K., Wüthrich, A. and Bader, H. (1981) Biochem. Biophys. Res. Commun. **101**, 418–425
11 Roufogalis, B. D. and Villalobo, A. (1989) in The Red Cell Membrane: A Model for Solute Transport (Raess, B. U. and Tunnicliff, G., eds.), pp. 76–83, Humana Press, Clifton, NJ
12 Sarkadi, B., Szász, I. and Gárdos, G. (1980) Biochim. Biophys. Acta **598**, 326–338
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
14 Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
15 Rossi, J. P. F. C., Gronda, C. M., Fernández, H. N. and Gagliardino, J. J. (1988) Biochim. Biophys. Acta **943**, 175–182
16 Hjertén, S. and Mosbach, R. (1962) Anal. Biochem. **3**, 109–118
17 Andrews, A. T. (1986) Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications, 2nd edn., pp. 52–54, Oxford University Press, New York
18 Richards, D. E., Rega, A. F. and Garrahan, P. J. (1978) Biochim. Biophys. Acta **511**, 194–201
19 Kratje, R. B., Garrahan, P. J. and Rega, A. F. (1983) Biochim. Biophys. Acta **731**, 40–46
20 Means, G. E. (1977) Methods Enzymol. **47**, 469–478
21 Rossi, J. P. F. C. and Schatzmann, H. J. (1982) J. Physiol. (London) **327**, 1–15
22 Fraser, R. D. B. and Suzuki, E. (1973) in Physical Principles and Techniques of Protein Chemistry, Part C (Leach, S. J., ed.), pp. 301–355, Academic Press, New York
23 Rossi, R. C. (1987) Doctoral Thesis, University of Buenos Aires, pp. 38–56
24 Lundblad, R. L. and Noyes, C. M. (1984) Chemical Reagents for Protein Modification, vol. 1, pp. 127–170, CRC Press, Boca Raton, Florida
25 Watkins, N. G., Neglia-Fisher, C. I., Dyer, D. G., Thorpe, S. R. and Baynes, J. W. (1987) J. Biol. Chem. **262**, 7207–7212
26 Davis, F. B., Davis, P. J., Nat, G., Blas, S. D., MacGillivray, M., Gutman, S. and Feldman, M. J. (1985) Diabetes **34**, 639–646
27 Deziel, M. R., Safeer, R. S., Blas, S. D., Davis, F. B. and Davis, P. J. (1992) Biochim. Biophys. Acta **1110**, 119–122
28 Katchalsky, A. and Sharon, N. (1953) Biochim. Biophys. Acta **10**, 290–301
29 Cheftel, J. C. and Cheftel, H. (1978) Introduction a la Biochimie et la Technologie des Aliments, p. 346, Enterprise Moderne d'Edition, Paris
30 Gronda, C., Rossi, J. P. F. C., Cédola, N. V. and Gagliardino, J. J. (1987) I Simposio de la Sociedad Platense de Endocrinología, La Plata
31 Zvaritch, E., James, P., Vorerr, T., Falchetto, R., Modyanov, N. and Carafoli, E. (1990) Biochemistry **29**, 8070–8076
32 Filoteo, A. G., Gorski, J. P. and Penniston, J. T. (1987) J. Biol. Chem. **262**, 6526–6530
33 Donnet, C., Caride, A. J., Fernández, H. N. and Rossi, J. P. F. C. (1991) Biochem. J. **279**, 121–127

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