Glycated Calmodulin from Platelets as an Index of Glycemic Control

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In an effort to test whether a significant fraction of calmodulin would become glycated within the lifetime span of the platelet (10–14 days), we monitored the kinetics of calmodulin glycation in vitro. Under the conditions we used, the fraction of glycated calmodulin reached a maximum (~21%) within 10 days. We then extended the studies to human subjects. The intraplatelet concentrations of calmodulin and glycated calmodulin from age-matched type I diabetic subjects were monitored by a combination of m-aminophenylboronate affinity chromatography and enzyme-linked immunosorbent assay. The results indicate that the concentrations of total intraplatelet calmodulin (nonglycated plus glycated) were not dependent on the glycemic state of the subjects. Data from control and diabetic subjects showed a poor correlation between the concentrations of glycohemoglobin and of glycated calmodulin. However, a better correlation was obtained when glycated calmodulin concentrations were compared with those of serum fructosamine. The fraction of glycated calmodulin in the control population (7.71% ± 0.75%) was significantly (P < 0.05) different from that of the diabetic population (21.6% ± 1.26%). Given that the clinical role of the fructosamine assay remains controversial, estimation of glycated calmodulin in platelets might be useful as a short time-window index of glycemic control.

Indexing Terms: diabetes · glycohemoglobin · fructosamine · enzyme-linked immunosorbent assay · chromatography, affinity

A major part of diabetic therapy is centered around the prevention or delay of secondary complications by maintaining a strict control on blood glucose concentrations (1, 2). The effectiveness of glycemic control is monitored by determination of the nonenzymatic glycation end-products in blood proteins. The most routinely assayed glycation product is hemoglobin. However, this protein is useful only as an index of long-term glycemic history, but gives its lifetime span of ~120 days. In many clinical situations a shorter-term glycemic index is desirable. To this end, glycated albumin (half-life 25 days), IgG (half-life 15 days), and serum fructosamine (14–21 days) have been employed (3–6).

Platelets, like erythrocytes, are freely permeable to glucose. Consequently, platelet proteins are potentially susceptible to nonenzymatic glycation. In this study we have devised an assay system that uses readily available reagents to detect nanogram amounts of glycated calmodulin (CaM) in washed human platelets from a 5.0-mL blood sample.6 This assay was used to monitor insulin-dependent diabetes mellitus and control subjects. We found that platelet CaM, which regulates key enzymes such as myosin light-chain kinase (7, 8) and nitric oxide synthase (9), is susceptible to glycation in vivo such that glycated CaM statistically correlates with the glycemic state of the individual. We discuss the results in terms of the potential utilization of platelet glycated CaM in the clinical setting as a 10–14-day index of glycemic control.

Materials and Methods

Materials. Bovine brain calmodulin, polyclonal anticalmodulin IgG, anti-IgG-conjugated alkaline phosphatase, bovine serum albumin, Tween-20, and m-aminophenylboronate (MAPB)-agarose were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium phosphate, potassium phosphate, and sodium chloride were from BDH (Toronto, Ontario, Canada). ELISA plates with eight-well removable flat-bottom strips were purchased from Costar (Cambridge, MA).

Subjects. Blood samples (5.0 mL) were obtained by venipuncture from 21 normal and 20 insulin-dependent diabetic volunteers, ages 12–19 years (average 16), who presented themselves at either the Pediatric Diabetes Centre or at the Hematology Clinic at the Hotel Dieu Hospital of Windsor.

Buffers. Phosphate-buffered saline (PBS): NaCl (0.14 mol/L), Na2HPO4 (1.44 g/L), KH2PO4 (0.24 g/L), pH 7.2. Blocking buffer: PBS containing bovine serum albumin, 10 g/L. Wash buffer: PBS containing 0.5 mL of Tween-20 per liter. Secondary wash buffer: Wash buffer containing diethanolamine (10 mmol/L) and MgCl2 (0.5 mmol/L). MAPB buffer: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (0.05 mol/L), containing 10 mmol/L MgCl2 and 0.2 g/L sodium azide, pH 8.5. MAPB elution buffer: MAPB buffer containing sorbitol (100 mmol/L), pH 9.0.

Protein determinations. Intraplatelet protein concentrations were estimated by the method of Bradford (10). With this procedure for estimating CaM concentrations, we used authentic CaM samples to construct the standard curves. The CaM concentrations in the authentic

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6 Nonstandard abbreviations: CaM, calmodulin; PBS, phosphatebuffered saline; PRP, platelet-rich plasma; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; and MAPB, m-aminophenylboronate.
samples were determined from absorbance at 274 nm ($\varepsilon_{274}^{1%} = 2.1$).

Statistical analysis. Results are expressed as the mean ± SEM. Unpaired $t$-test was used to determine the significance of the differences between the control subjects and the diabetic patients. $P < 0.05$ was taken as statistically significant.

Harvesting of platelets. The blood samples (5 mL) were collected in an evacuated tube containing sterile, freeze-dried Na-EDTA and allowed to sit for 10 min. The anticoagulated blood was then gently mixed, transferred to 15-mL centrifuge tubes, and centrifuged at 200 $\times$ g in a Dynac II (Clay Adams Div., Becton Dickinson, Parsippany, NJ) table-top centrifuge for 10 min. The platelet count in the platelet-rich plasma (PRP) was determined with a Coulter counter (Coulter-S-plus; Coulter Electronics, Hialeah, FL). The platelet pellets were washed free of erythrocyte contamination by their repeated (twice) suspension in 0.1 mol/L Tris * HCl containing 5 mmol/L EDTA (pH 7.4) and centrifugation (Model J6B centrifuge; Beckman Instruments, Brea, CA) at 4000 $\times$ g for 20 min. The platelet pellets harvested were examined under phase-contrast microscopy and found to contain <0.1% contamination by other blood cells. The samples (containing $\sim 2 \times 10^{11}$ platelets/mL) were stored at -80°C.

In vitro glycation of bovine brain CaM. Bovine brain CaM (1 mg/mL) was dissolved in HEPES buffer (0.05 mol/L, pH 7.5) containing 0.05 mol of glucose, 0.1 mmol of CaCl$_2$, 10 mmol of EDTA, and 0.2 g of Na$_2$ per liter. The mixture was filter-sterilized (0.25-$\mu$m pore-size filters) and incubated in the dark at 37°C. At the times indicated in Figure 1, 200-$\mu$g aliquots were removed. The glucose--protein conjugates were stabilized by adding 10-fold molar excess of NaBH$_4$ in chilled NaOH (5 mmol/L). The excess NaBH$_4$ was neutralized by acetic acid (0.1 mol/L final concentration). The excess glucose was removed by wash/centrifugation cycles (3x, with MAPB wash buffer) in a Centricon-10 microconcentrator (Amicon, Beverly, MA). The glycated protein was isolated as described below except that more MAPB-agarose (5.0 mL) was used.

Separation of glycated and nonglycated platelet proteins. Platelets were suspended in PBS containing Triton X-100, 1 mL/L. The mixture was homogenized with a hand-held cordless homogenizer (Kontes, Mandel Scientific, Guelph, Ontario, Canada) for 1 min at 5000 rpm and 1 min at 15 000 rpm. The homogenate was then subjected to centrifugation for 5 min at 15 600 $\times$ g (Eppendorf, Fremont, CA, table-top centrifuge). The supernate (containing 100 $\mu$g of total protein) was applied to Spin-x columns (Costar) containing 1.0 mL of MAPB-agarose preequilibrated with MAPB buffer. The nonglycated proteins were eluted by a 10-$\alpha$ centrifugation (Eppendorf microfuge) after suspension of the gel in MAPB buffer (five times, 500 $\mu$L each). The glycated fraction was then eluted in the same manner with MAPB elution buffer. The nonglycated and glycated protein fractions ($\sim 4.0$ mL each) were concentrated by lyophilization and then resolubilized in 400 $\mu$L of PBS.

Estimation of CaM in the glycated and nonglycated protein fractions with the ELISA. We added to each well of the ELISA plates 100 $\mu$L of the nonglycated protein fraction (the fraction that did not bind to MAPB-agarose) or the glycated fraction (the MAPB-agarose-bound fraction). After a 2-h incubation (25°C), the plates were washed with PBS (twice, 200 $\mu$L per well each time). The washed plates were incubated for 2 h (room temperature) with blocking buffer (100 $\mu$L per well). The excess blocking agent was removed by a wash with wash buffer (twice, 200 $\mu$L). The primary antibody (anti-CaM IgG, 125-fold dilution in 100 $\mu$L of wash buffer) was then incubated with the coated, blocked plates for 2 h at room temperature. After washing off the unbound antibody with wash buffer (four times, 200 $\mu$L), we incubated the plates with the secondary antibody (anti-IgG--alkaline phosphatase, 1000-fold dilution) for a further 2 h. The unbound secondary antibody was removed by two 200-$\mu$L washes with secondary wash buffer. The amount of bound antigen was then estimated colorimetrically after the addition of $p$-nitrophenyl phosphate (1 g/L, dissolved in 100 $\mu$L of secondary wash buffer). The enzyme activity was quenched after 30 min with NaOH (2 mol/L, 100 $\mu$L). The color production was quantified at 405 nm with a Micro Reader III (Hyperion, Miami, FL). Standard curves were determined identically, except that 5 to 100 ng of purified bovine brain CaM and glycated CaM were used as the antigen.

Serum fructosamine determinations. Fructosamine was assayed with the Roche (Nutley, NJ) Fructosamine Test Plus kit in a thermostatically controlled Flexigem (Electro-Nucleonics, Inc., Fairfield, NJ) recording analyzer. The fructosamine concentration in the plasma was measured as follows: 25 $\mu$L of EDTA-plasma was reacted with 0.5 mL of 0.2 mol/L carbonate buffer, pH 10.3, containing nitroblue tetrazolium (0.48 mmol/L) and uricase (≥2.5 kU/L) in a disposable rotor cuvette (Electro-Nucleonics, Inc.) for 10 min at 37°C. The absorbance was measured at 550 nm. All measurements were made against the Roche fructosamine calibrator (442 $\mu$mol/L), which had been standardized by using $[^14]$C glycidyl polysine and human serum. The human-based lyophilized controls (Roche) Fructosamine Control N (normal range 278 $\mu$mol/L) and Fructosamine Control P (pathological range 552 $\mu$mol/L) were used as references in the assay procedure.

Amino acid analysis. Aliquots of the glycated CaM and authentic CaM were hydrolyzed with 6 mol/L hydrochloric acid containing 10 g/L phenol at 110°C for 24 h. The resulting free amino acids and the standard were dried, redried, and derivatized with phenylisothiocyanate. The analyses were performed with a Waters 600 multi solvent delivery system and a Waters 441 Detector (Waters, Mississauga, Ontario, Canada). The derivatives of the amino acids were dissolved in sample diluent [4.75 mmol/L disodium hydrogen phosphate/acetonic acid (95/5 by vol), pH 7.4] and applied to a Waters Picotag™ column; the effluent was monitored at 254 nm.
Results and Discussion

The nonenzymatic glycation of proteins is a relatively slow process. The glycation rates for a particular protein depend on several factors, including solvent exposure of the susceptible residues and their proximity to protein side chains such as imidazole, which are thought to enhance rates of glycation (11). In addition, some anionic buffers, e.g., phosphate, have been shown (in vitro studies) to catalyze the glycation reaction (12). Therefore, before undertaking the in vivo study, we wanted to ensure that a significant fraction of purified CaM would become glycated within the life span of the platelet. To this end, purified bovine brain CaM was incubated with 50 mmol/L glucose. Aliquots were withdrawn from the incubation mixture at the times indicated (Figure 1), and the glycated and the nonglycated fractions were separated by MAPB–agarose. The Lys content of the glycated CaM fraction was determined by amino acid analysis. Under these conditions we determined that ~17% of the CaM was glycated within 5 days. There was ~3% increase in the amount of glycated protein upon a further 5-day incubation, suggesting that a near maximal glycation is reached within 10 days. After a 3-day incubation, the mole fraction of all amino acid residues analyzed in the glycated CaM fraction remained constant, except for Lys. The decrease in [Lys]/[CaM] (mol/mol) at the end of 3 and 10 days of incubation was 2.02 ± 0.1 and 3.06 ± 0.19, respectively, indicating that three of the eight free Lys residues of CaM can be glycated within 10 days. In previous in vitro studies, CaM was maximally glycated within 6 days (13). This faster rate of glycation is probably due to the fact that phosphate buffer was used in the in vitro glycation, given reports that anionic buffers enhance in vitro glycation rates (12).

The focus of the present study then turned to the estimation of in vivo glycated CaM from human platelets. The ELISA standard curves obtained with authentic untreated and glycated (from in vitro incubation) CaM samples are presented in Figure 2. The absorbance (405 nm) as a function of either authentic or glycated CaM (5 to 100 ng) could be fitted by an equation for a quadratic polynomial (y = mx + nx^2 + b). To test the efficiency of the separation of authentic from glycated CaM in the MAPB–agarose step, we mixed 15 ng of glycated CaM plus 85 ng of authentic CaM with 100 µg of bovine serum albumin. This mixture was then carried through the affinity chromatography and the ELISA procedures. Recovery of the authentic and glycated CaM under these conditions was 97.8% ± 3% and 96% ± 8%, respectively (n = 12).

The total CaM in the soluble fraction of platelet extracts from control and diabetic subjects was 1130 ± 0.06 and 1097 ± 0.1 ng/mg of soluble platelet protein, respectively (n = 41). There was no correlation between [CaM]_total and [serum fructosamine] (Figure 3). In addition, the average CaM concentration in the control subjects was not statistically different from that of the diabetic subjects (Figure 3, inset). This result is contrary to earlier reports that concentrations of platelet CaM are nearly doubled in diabetic subjects (14). We consider

Fig. 1. Effect of incubation time on the percentage of glycated CaM (%) and the decrease in its Lys content (V). Bovine brain CaM (1 g/L) was incubated with 0.05 mol/L glucose. At the times indicated the glycated and nonglycated CaM were separated by MAPB–agarose. Protein concentration in either fraction was determined by the Coomassie Blue dye-binding assay (10). The Lys content of the glycated CaM fraction was determined by amino acid analysis. Bars indicate SEM (n = 3, % glycated CaM determinations; n = 2, amino acid analysis)

Fig. 2. ELISA standard curves of authentic untreated (O) or glycated (V) CaM

Antibody-linked phosphatase-catalyzed p-nitrophenyl phosphate hydrolysis (detected at 405 nm) as a function of the presence of untreated or glycated calmodulin. The best-fit lines are described by the equations y = 0.30 ± 0.0008x + 0.0006; r = 0.9988 (glycated CaM); and y = 0.123x + 0.0004 + 0.054; r = 0.9988 (untreated CaM). The bars indicate SEM (n = 4)

Fig. 3. Serum fructosamine vs [CaM]_total as determined by ELISA

The best-fit line is described by the equation y = 1224 + 0.00057x (r = 0.00218). Inset: mean [serum fructosamine] vs mean [CaM]_total for normal subjects (n = 21) and diabetic subjects (n = 20); units are identical to those of main plot. Bars indicate SEM

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our results more reliable because we included Triton X-100 in the buffers at the protein extraction step, whereas detergents were not used in the earlier study (14). Given that ~20% of total CaM in platelets reportedly is membrane associated (15), the omission of detergent would result in lower yields of CaM from platelets. In addition, with the present extraction procedures, nearly 98% of the platelets are broken down, compared with 90% in the earlier report (14). Finally, the sample size we used is nearly double that of the earlier study. It is therefore possible that their reported increases in CaM concentrations (14) actually reflect diabetes mellitus-dependent mobilization of membrane-associated CaM.

We then attempted to correlate the fraction of glycated CaM measured with currently accepted methods for monitoring the glycation status of blood (i.e., glycohemoglobin and serum fructosamine). A poor correlation ($r = 0.43$) was obtained between the glycohemoglobin content of the diabetic subjects ($n = 28$) and their concentrations of glycated CaM (Figure 4). This is not unexpected, because glycohemoglobin is a long-term index of glycemic status, whereas intraplatelet glycated CaM should report 10–14-day glycemic history. The correlation was better ($r = 0.71$) between the glycated CaM concentrations and serum fructosamine, an established index (4, 5) for 14–21-day glycemic control (Figure 5). The results indicate that intraplatelet CaM is susceptible to glycation both in control subjects and in the diabetic population. The mean proportions of glycated CaM in the normal subjects and the diabetic subjects were $7.71\% \pm 0.75\%$ ($n = 21$) and $21.6\% \pm 1.26\%$ ($n = 20$), respectively (Figure 5, inset), significantly different by $t$-test analysis ($P < 0.05$).

In the present study we have presented a simple procedure for the detection of glycated CaM at the nanogram level. This procedure has been used to obtain the first indirect evidence for the susceptibility of platelet CaM to glycation in vivo. Our data also indicate that the fraction of glycated CaM in diabetic subjects is 2.8-fold greater than that of control subjects. Given that

![Figure 4](image1.png)  
**Fig. 4.** % glycated hemoglobin vs % glycated CaM as determined by ELISA

$\%$ glycated CaM = 100 x glycated CaM/(nonglycated CaM + glycated CaM).

The best-fit regression line was described by the equation $y = 0.76x + 3.98$; $r = 0.43$ ($n = 29$)

![Figure 5](image2.png)  
**Fig. 5.** [Serum fructosamine] vs % glycated CaM as determined by ELISA

The best-fit regression line was described by the equation $y = 0.57x - 3.95$; $r = 0.71$. Inset: mean [serum fructosamine] vs mean % glycated CaM for normal subjects ($n = 21$) and diabetic subjects ($n = 20$); units are identical to those of main plot. Bars indicate SEM

the clinical role of fructosamine assay (which has been well evaluated) remains controversial, a potential role of platelet glycated CaM remains to be determined in the clinical setting as a short time-window index of glycemic control.

A more intriguing question that comes to mind concerns the central role played by CaM in platelet physiology. CaM has been implicated in platelet aggregation through its regulation of platelet myosin light-chain kinase (7, 8). More recently, CaM also has been shown to regulate platelet deactivation through platelet nitric oxide synthase, which is Ca$^{2+}$-CaM dependent (9). The results presented here indicate that in diabetic subjects about one-fifth of the platelet CaM was glycated. It is as yet unclear how these high proportions of glycated CaM affect platelet function. To this end, we have initiated several studies to examine the role of CaM glycation in diabetic complications.

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