Hyperglycemia Induces a Loss of Phospholipid Asymmetry in Human Erythrocytes†

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ABSTRACT: Phospholipid asymmetry in biological membranes is maintained by an aminophospholipid-specific Mg^{2+}-ATPase that transports PS and PE from the outer to the inner monolayer. Recent evidence indicates that a loss of phospholipid asymmetry occurs in erythrocytes from diabetic individuals, resulting in the appearance of PS in the membrane outer leaflet. We show that hyperglycemic treatment of normal erythrocytes duplicates this effect. Erythrocytes incubated for 18–24 h in the presence of glucose were assayed for PS transport and transmembrane phospholipid asymmetry. Phospholipid asymmetry in erythrocytes treated with high concentrations of glucose (>5 mM) showed a time-dependent ($t_{1/2} \sim 12$ h) and concentration-dependent (half-maximal concentration ~7.5 mM) increase in the accessibility of PS and PE, and a decrease in the accessibility of SM and PC, to exogenous phospholipases. After an 18 h incubation with 20 mM glucose, 40% of the endogenous PS and PE was found in the outer monolayer concomitant with a decrease in the outer monolayer content of SM (from 80% to 50%) and PC (from 75% to 65%). These values are consistent with an almost complete transbilayer scrambling of erythrocyte phospholipids. The loss of PS asymmetry was verified using an assay based on the activation of the prothrombinase complex. The observed loss of asymmetry is not due to inhibition of PS transport or glucose-induced Ca^{2+} influx. Hyperglycemic buffers (>5 mM glucose) had no effect on aminophospholipid transport; exogenously-added synthetic PS was transported to the inner monolayer at a rate ($t_{1/2} \sim 8$–9 min) identical to that observed in cells incubated with physiological concentrations (5 mM) of glucose. Cells coincubated with glucose and physiological concentrations of Ca^{2+} showed a loss of phospholipid asymmetry, no change in PS transport, and no increase in intracellular Ca^{2+}. Preliminary evidence indicates that the hyperglycemia-induced loss of asymmetry reflects an increased passive phospholipid flip-flop caused by a secondary effect of hyperglycemia such as phospholipid peroxidation or nonenzymatic protein glycosylation.

Transmembrane phospholipid asymmetry is a characteristic feature of biological membranes. The choline-containing phospholipids phosphatidylcholine (PC)[1] and sphingomyelin (SM) are localized primarily to the membrane outer monolayer, and the amine-containing phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are sequestered selectively in the cytofacial monolayer (Bretscher, 1973; Verkleij et al., 1973; Rothman & Lenard, 1977). Several mechanisms may operate to maintain phospholipid asymmetry: (1) slow flip-flop of endogenous phospholipids; (2) sequestration of inner monolayer lipids by binding to cytofacial proteins; and (3) active transmembrane phospholipid transport. Passive movement of diacylphospholipids across biological membranes is slow, occurring usually with half-times of hours to days (van Meer & Op den Kamp, 1982; Middelkoop et al., 1986). However, many cells have a life span sufficient to allow for phospholipid randomization and are subjected to perturbations, such as membrane fusion, which induce phospholipid reorganization (Huang & Hui, 1990; Song et al., 1992). Binding of acidic phospholipids to cytofacial sites on membrane or cytoskeletal proteins may contribute to the maintenance of phosphatidylserine asymmetry (Haest & Deuticke, 1976; Comfurius et al., 1985; Hubbell, 1990). However, several findings have questioned the role of the cytoskeleton: endogenous erythrocyte phospholipid asymmetry is retained after cytoskeletal protein disruption (Gudi et al., 1990), the energy of PS–cytoskeletal protein interactions is insufficient to trap this phospholipid (Morrot et al., 1986; Maksymiw et al., 1987; Bitbol et al., 1989), and phospholipid asymmetry can be generated and maintained in cytoskeleton-free membrane vesicles (Calvez et al., 1988). Although the erythrocyte cytoskeleton may participate in the maintenance of lipid asymmetry, PS–cytoskeletal interactions are insufficient to generate an asymmetric membrane. Other evidence favors an aminophospholipid-specific transporter, or flippase, first discovered in the plasma membrane of human blood cells (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985), which catalyzes the ATP-dependent selective translocation of PS and PE from the outer to the inner monolayer [for recent reviews, see Devaux (1991) and Schroit and Zwaal (1991)]. This transporter maintains phospholipid asymmetry, in part, by selective inward transport of aminophospholipids. However, the limits of the ability of the flippase to maintain asymmetry in the presence of membrane perturbations have not been well characterized, and its physiological role remains unclear.

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[1] Abbreviations: DLPC, dilauroylphosphatidylcholine; DLPS, dilauroylphosphatidylserine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; HbA_{1c}, glycosylated hemoglobin; HBS, Hepes-buffered saline; HCT, hematocrit; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MI, morphological index; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

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PS transport is reduced in a number of normal and pathologic conditions that result in a redistribution of phospholipids, such as platelet activation (Bevers et al., 1989), sickle cell anemia (Blumenfeld et al., 1991; Zachowski et al., 1985), and Ca^{2+}-loading (Bitbol et al., 1987). Alterations in transmembrane PS asymmetry have profound effects on membrane function. Platelet activation results in an increase in spontaneous phospholipid flip-flop (Bevers et al., 1990), appearance of PS on the cell surface (Bevers et al., 1983), and inhibition of the PS transporter (Bevers et al., 1989; Comfurius et al., 1990). The newly exposed PS provides a catalytic surface for the activation of enzymes of the coagulation cascade (Bevers et al., 1982). Similar alterations in PS asymmetry are associated with several pathologic conditions and may contribute to unwanted thromboses. Sickle erythrocytes and membrane vesicles shed from sickled cells exhibit a loss of PS asymmetry and are procoagulant (Chiu et al., 1981; Lubin et al., 1981; Franck et al., 1985). Erythrocytes from diabetic patients have a reduced membrane deformability (McMillan et al., 1978), an increased membrane viscosity (McMillan, 1976), an increase in spontaneous aggregation (Schmid-Schonbein & Volger, 1976), and exhibit abnormal adherence to cultured endothelial cells (Wautier et al., 1981). In addition, diabetic blood cells show an increase in passive transmembrane lipid movement (Wali et al., 1988), and PS appears in the plasma membrane outer monolayer (Wali et al., 1988; Lupu et al., 1988). The loss of transmembrane PS asymmetry may increase procoagulant activity and contribute significantly to vascular occlusion.

Hyperglycemic treatment of normal erythrocytes produces many of the alterations observed in diabetic cells (Travis et al., 1971; Jain, 1989; Rajeswari et al., 1991). Here we demonstrate that *in vitro* hyperglycemic treatment of human erythrocytes induces a loss of phospholipid asymmetry without concomitant inhibition of PS transport. Possible mechanisms for the exposure of PS and implications for the role of the aminophospholipid transporter in the maintenance of transmembrane phospholipid asymmetry are discussed.

MATERIALS AND METHODS

Materials. Dilauroylphosphatidylcholine (DLPC), bovine brain sphingomyelin (brain SM), egg phosphatidylcholine (egg PC), and egg phosphatidylethanolamine (egg PE, transphosphatidylated from egg PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Dilauroylphosphatidylserine (DLPS) was obtained from Avanti Polar Lipids or synthesized from DLPC and serine by phospholipase D-catalyzed headgroup exchange (Comfurius & Zwaal, 1977). Cholesterol, phospholipase A_2 (*Apis mellifera*, P-9279; and *Naja naja naja*, P-6139), sphingomyelinase C (*Staphylococcus aureus*, S-8633), phospholipase D (cabbage, P-0282), thrombin (T-7009), and luciferin/luciferase (FL-AAM) were obtained from Sigma (St. Louis, MO). Human prothrombin, factor V/Va, and factor Xa were obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Unless otherwise noted, all chemicals were reagent grade.

Cells. Human erythrocytes were obtained from healthy adult volunteers by venipuncture and collected into EDTA (3.3 mM final concentration). Erythrocytes were pelleted by centrifugation (5 min, 3000*g*), washed 3 times in phosphate-buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, and 1 mM $MgCl_2$, pH 7.4), and stored on ice. To simulate hyperglycemia, erythrocytes were incubated in PBS buffer containing varying amounts of glucose (0–50 mM) at 20% hematocrit (HCT) and 37 °C for 18 h. In order to maintain higher ATP levels, some erythrocytes were incubated at 5% HCT. Buffers for extended incubations (greater than 1 h) contained penicillin (50 IU mL^{-1}) and streptomycin (50 μg mL^{-1}) to retard bacterial growth.

ATP, GSH, and HbA_{1c} Measurements. Aliquots (20 μL) of cell suspensions (50% HCT) were mixed with 1 mL of 0.15 N NaCl, heated at 100 °C for 15 min, and centrifuged to clarity. ATP in the supernatant was measured by the luciferin–luciferase method (Kimmich et al., 1975). Separate aliquots (20 μL) of cell suspensions (50% HCT) were lysed in 10 volumes of water, and reduced glutathione (GSH) was determined (Beutler, 1984). Glycosylated hemoglobin (HbA_{1c}) was measured using a commercial kit (Sigma 441-A or 441-B). ATP and GSH were normalized to hemoglobin quantitated using Drabkin's reagent (Beutler, 1984).

Phospholipase Treatments. Membrane phospholipid asymmetry was determined by phospholipase digestion of outer monolayer phospholipids following a modification of the method of Smith and Daleke (1990). Briefly, 0.2 mL of normal or glucose-treated erythrocytes was suspended in 0.8 mL of Hepes buffer (HBS: 10 mM Hepes/150 mM KCl, pH 7.4) containing $CaCl_2$ (0.5 mM). Phospholipase A_2 (bee venom and *N. naja naja*, 0.02 mL each of a 1000 IU mL^{-1} solution) and sphingomyelinase C (0.02 mL of a 38.5 IU mL^{-1} solution) were added, and the suspension was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.1 mL of HBS containing EDTA (10 mM). Erythrocytes were collected by centrifugation, washed 3 times with 0.8 mL of HBS containing EDTA (1 mM), and stored on ice.

Phospholipid Analysis. Erythrocytes were lysed in Tris buffer (10 mM Tris base/2 mM EDTA, pH 7.4), and the membranes were collected by centrifugation (10 min, 16000*g*). This procedure was repeated until the supernatant was clear (4–6 times). Phospholipids were extracted by a modification of the procedure of Comfurius and Zwaal (1977). Briefly, erythrocyte membranes were mixed for 30 min with 4.3 volumes of chloroform/methanol (5:8). Water (1 volume) and chloroform (3.7 volumes) were added, and the suspension was mixed for 10 min. Following centrifugation to separate the two phases, the organic layer was collected. The aqueous layer was washed 2 times with chloroform, and the washes were combined with the organic layer. The combined phospholipid extract was dried under a stream of nitrogen and reconstituted in 20 μL of chloroform/methanol (4:1). Lipids were separated by two-dimensional thin-layer chromatography on silica gel HL plates (Analtech, Inc., Newark, DE) using chloroform/methanol/water/30% ammonium hydroxide (65:25:2.5:2.5) in the first dimension and chloroform/methanol/formic acid (65:25:5) in the second dimension. Lipids were visualized with iodine vapor, and amine-containing lipids were detected by reaction with fluorescamine (0.05% w/v in acetone). Lipid spots were scraped from the plate and analyzed for phosphate (Bartlett, 1959).

Prothrombinase Assay. Erythrocytes treated with glucose-containing buffers for 24 h were washed by centrifugation, and incubated for 3 min at 37 °C, 0.1% HCT in Tris buffer (50 mM Tris-HCl/120 mM NaCl, pH 7.4) containing $CaCl_2$ (6 mM), factor V/Va (0.33 unit mL^{-1}), factor Xa (0.33 units mL^{-1}), and prothrombin (1.3 units mL^{-1}). EDTA (15 mM final concentration) was added to stop the reaction, the cells were pelleted by centrifugation, and the amount of thrombin in an aliquot of the supernatant (180 μL) was measured using the chromogenic substrate sarcosine-Pro-Arg-*p*-nitroanilide (20 μL of a 500 μM solution; Duncan et al., 1985). Released

thrombin was determined by reference to a standard curve of authentic thrombin. The amount of externalized PS was estimated by incubating the prothrombinase complex (factor V/Va, factor Xa, and prothrombin) with sonicated vesicles composed of a mixture of phospholipids representative of the erythrocyte outer monolayer (SM/PC/PE/cholesterol, 4:4:1:9) containing varying amounts of PS (4 μM total lipid). Similar results were obtained using sonicated liposomes composed of egg PC/cholesterol (1:1) in place of the outer monolayer mixture. Larger multilamellar vesicles of similar composition, extruded 10 times through a 0.2-μm polycarbonate membrane (Poretics Corp., Livermore, CA), yielded results identical to sonicated vesicles after correction for differences in outer monolayer surface area.

Phosphatidylserine Transport Assay. Following incubation in glucose-containing buffers, erythrocytes were pelleted by centrifugation (5 min, 3000*g*) and incubated at 50% HCT with sonicated DLPS vesicles (250 μM). At prescribed time points, erythrocyte suspensions were fixed by adding 5 μL aliquots to 50 μL of glutaraldehyde (1%) in PBS. Samples were examined by light microscopy and their morphology was used as a measure of transbilayer PS distribution (Daleke & Huestis, 1989). Briefly, cells with excess lipid incorporated into the outer monolayer become evaginate echinocytes due to an increase in the outer monolayer surface area, while cells which have transported and accumulated lipid in the inner monolayer become invaginate stomatocytes as a result of inner monolayer expansion (Sheetz & Singer, 1974). Echinocytes are assigned scores of +1 to +5 according to the degree of crenation, discocytes are scored 0, and stomatocytes are given scores of −1 to −4 based on the degree of invagination (Daleke & Huestis, 1989). The average score of a field of 100 cells is defined as the morphological index.

Effects of Glucose on Erythrocyte Ca^{2+}. Erythrocytes were suspended at 20% hematocrit in PBS containing $^{45}CaCl_2$ (2.5 mM, 5 Ci mol^{-1}) and varying concentrations of glucose. Following incubation at 37 °C for 24 h, the erythrocytes were isolated by centrifuging 0.3 mL of the suspension through 0.4 mL of dibutyl phthalate (1.04 g mL^{-1}, 16000*g*, 10 min). The cells were collected and bleached with 1 mL of H_2O_2 (30%) at 60 °C for 24 h. After being cooled to room temperature, the amount of radioactivity was determined by scintillation counting. Hematocrit, against which the results were normalized, was determined using a microhematocrit centrifuge. PS transport and phospholipid asymmetry in the presence of PBS containing $CaCl_2$ (2.5 mM) and varying concentrations of glucose were determined as described above.

Estimation of Inside-to-Outside Flop Rates. An estimate of the outward rate of lipid flip-flop was obtained by analysis of a two-state model for transmembrane PS transport as described by Herrmann and Müller (1986):

$$PS_o \underset{k_o}{\overset{k_i}{\rightleftharpoons}} PS_i \tag{1}$$

where $[PS]_o$ and $[PS]_i$ are the amount of the PS in the outer and inner monolayer, respectively, and k_i and k_o are the respective apparent inward and outward flip rate constants. The equilibrium fraction of PS in the inner monolayer (f_i) is a function of $[PS]_o$ and $[PS]_i$ or k_i and k_o:

$$f_i = \frac{[PS]_i}{[PS]_i + [PS]_o} = \frac{k_i}{k_i + k_o} \tag{2}$$

By rearranging eq 2, the outward rate can be expressed as a fraction of the inward rate and is proportional to the equilibrium fraction of PS in the inner monolayer:

$$\frac{k_o}{k_i} = \frac{1}{f_i} - 1 \tag{3}$$

Williamson et al. (1987) have extended the model of Herrmann and Müller to include the possibility of PS binding to inner monolayer binding sites:

$$PS_o \underset{k_o}{\overset{k_i}{\rightleftharpoons}} PS_i \overset{K_b}{\rightleftharpoons} PS_b \tag{4}$$

where K_b is the inner monolayer site binding constant and $[PS]_b$ is the amount of PS bound to inner monolayer sites. K_b can be written as a function of f_i and the ratio of k_o to k_i:

$$K_b = \frac{k_o}{k_i}\left(\frac{f_i}{1 - f_i}\right) - 1 \tag{5}$$

If k_o and k_i remain unchanged, then K_b will be a function of f_i only.

Miscellaneous Methods. Statistical analysis was performed using a single-tailed Student's *t*-test. Erythrocyte proteins were analyzed by polyacrylamide gel (8%) electrophoresis (Laemmli, 1970).

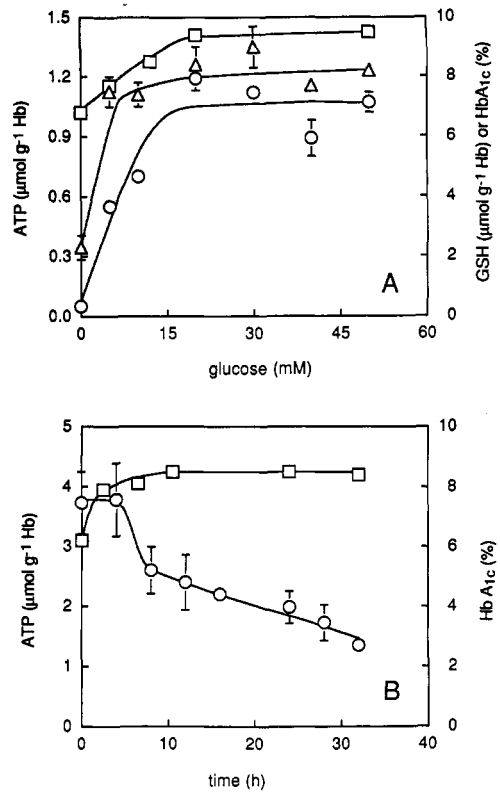

FIGURE 1: Metabolic status of glucose-treated erythrocytes. Washed human erythrocytes were incubated (A) for 18 h in PBS containing the indicated concentration of glucose or (B) for the indicated times in the presence of 20 mM glucose. Erythrocyte lysates were analyzed for ATP (○), GSH (△), and HbA_{1c} (□) as described under Materials and Methods. Values for freshly isolated, untreated erythrocytes were 3.73 ± 0.52 μmol of ATP (g of Hb)$^{-1}$, 7.40 ± 0.25 μmol of GSH (g of Hb)$^{-1}$, and 5.4 ± 0.6% HbA_{1c}.

RESULTS

Metabolic Status of Glucose-Treated Erythrocytes. The ATP, GSH, and HbA_{1c} content of glucose-treated cells was measured as a function of glucose concentration (Figure 1A) and time (Figure 1B) of exposure to hyperglycemic solutions. After an 18-h incubation in the presence of 5–50 mM glucose,

the ATP content of the cells was diminished [Figure 1A; 0.55–1.07 μmol (g of Hb)$^{-1}$ (10–20% of normal)] compared to untreated cells, while GSH levels were within the normal range [7.5–8.2 μmol (g of Hb)$^{-1}$]. The amount of HbA_{1c} was proportional to glucose concentration in the range of 5–20 mM, increasing from 6.8% to 8.5%. At concentrations greater than 20 mM glucose, the fraction of HbA_{1c} remained unchanged. In the absence of glucose, ATP [0.05 μmol (g of Hb)$^{-1}$] and GSH [2.3 μmol (g of Hb)$^{-1}$] were reduced dramatically, while HbA_{1c} levels decreased only slightly. In the presence of 20 mM glucose, intracellular ATP concentration remained unchanged for 4 h [Figure 1B; 3.8 μmol (g of Hb)$^{-1}$; however, ATP concentrations decreased steadily (Figure 1B) to a value of 1.3 μmol (g of Hb)$^{-1}$ after 32 h. During incubation with 20 mM glucose, HbA_{1c} levels increased from approximately 6.2% to 8.5% within 10 h, and remained at this level for up to 32 h. The range of glucose concentrations chosen for these experiments spans the range of plasma glucose levels for nondiabetic (4.5 ± 0.3 mM) and uncontrolled type II diabetic (15.7 ± 3.2 mM) individuals and produces increases in HbA_{1c} levels similar to those observed in diabetics (Wali et al., 1988).

Phospholipid Asymmetry in Glucose-Treated Erythrocytes. Phospholipid asymmetry in erythrocytes incubated in hyperglycemic buffers was measured by treating cells with purified phospholipases [phospholipase A_2 (bee venom, 20 IU mL^{-1}, + *N, naja naja*, 20 IU mL^{-1}) and sphingomyelinase C (*S. aureus*, 0.8 IU mL^{-1})]. Incubating erythrocytes for 18 h in buffers of varying glucose content induced a dose-dependent increase in outer monolayer aminophospholipids and a concomitant decrease in outer monolayer choline phospholipids (Figure 2A). The amount of externalized PS increased from 0 to 40% and outer monolayer PE increased from 20 to 40%, coincident with a decrease in outer monolayer SM (from 80 to 50%) and a less pronounced decrease in outer monolayer PC (from 75 to 65%). The effect of glucose on lipid asymmetry was proportional to glucose concentration up to 20 mM glucose. No additional alterations in lipid asymmetry were observed at glucose concentrations higher than 20 mM. The loss of asymmetry was not due to ATP depletion; cells incubated in hyperglycemic conditions under conditions (5% HCT) which maintain higher levels of ATP (50% normal) show a similar loss of asymmetry of all classes of phospholipids (Table I). The amount of phospholipid cleaved ranged from 37.5 ± 6.3% to 55.9 ± 12.1% at the various glucose concentrations tested (Figure 2A, upper panel), consistent with cleavage of outer monolayer phospholipid only. The amount of hemolysis in all cases was less than 2%.

Glucose-induced loss of lipid asymmetry was dependent on the time of exposure to hyperglycemic buffers. Cells treated with 20 mM glucose exhibited a progressive loss of asymmetry up to 12 h; outer monolayer PS increased from 3 to 43%, outer monolayer PE increased from 20 to 46%, outer monolayer PC decreased from 75 to 65%, and outer monolayer SM decreased from 80 to 52%. Incubating cells longer than 12 h produced no further change in lipid asymmetry. The total amount of phospholipid cleaved ranged from 39.1 ± 0.1% to 53.0 ± 4.9% (Figure 2B, upper panel), indicating that only half of the cell phospholipid was cleaved. Cell lysis was less than 3% at each time point.

Prothrombinase Activation. To verify the apparent loss of asymmetry induced by glucose as detected with exogenous phospholipases, an independent assay for phospholipid asymmetry was employed. The prothrombinase complex (prothrombin, factor Va, factor Xa, and Ca^{2+}) is activated by

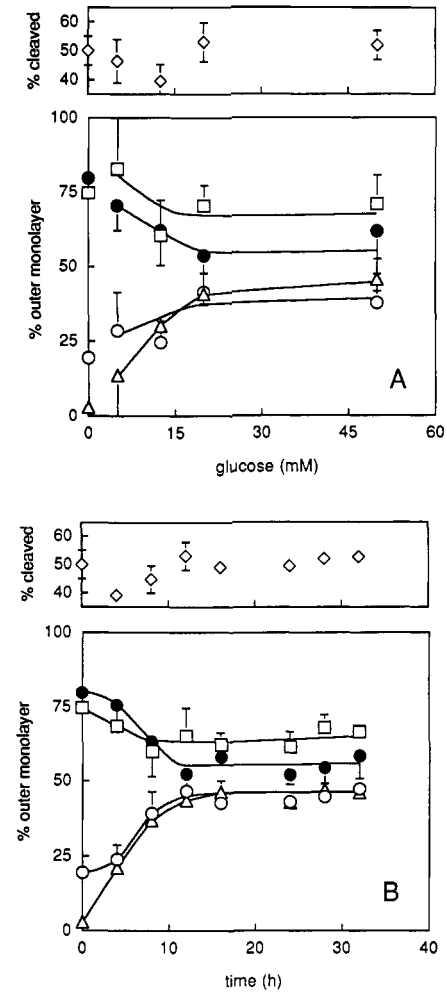

FIGURE 2: Hyperglycemia induces a time- and concentration-dependent loss of phospholipid asymmetry. Erythrocytes were treated (A) for 18 h with the indicated concentrations of glucose or (B) with 20 mM glucose for the indicated times prior to analysis of transmembrane phospholipid asymmetry by accessibility to exogenous phospholipases. Phospholipase treatment and subsequent lipid analysis were performed as described under Materials and Methods: PE (○), PS (△), PC (□), and SM (●) (panel A, $n = 9$; panel B, $n = 3$). The total amount of lipid cleaved at each point is indicated in the upper panels (◇). Values for freshly-isolated cells are indicated on the ordinate. The differences between these values and those after glucose treatment are significant [(A) at 50 mM glucose, $p \leq 0.0005$ (PS and SM) and $p \leq 0.025$ (PE and PC); (B) at 28 h, $p \leq 0.0005$ (PE, PS, and SM) and $p \leq 0.025$ (PC)].

Table I: Outer Monolayer Composition of Erythrocytes Incubated in Hyperglycemic Buffers[a]

phospholipid	5% HCT (3)[b]	20% HCT (9)[b]
PE	44.8 ± 4.9	37.9 ± 14.9
PS	41.7 ± 3.3	45.4 ± 4.0
PC	68.1 ± 6.0	70.8 ± 9.8
SM	51.6 ± 4.2	61.6 ± 14.4

[a] Cells were incubated for 18 h in the presence of 50 mM glucose at the indicated HCT. Phospholipid asymmetry was determined by phospholipase treatment as described under Materials and Methods. ATP concentrations: 20% HCT, 1.07 ± 0.05 μmol (g of Hb)$^{-1}$; 5% HCT, 2.40 ± 0.46 μmol (g of Hb)$^{-1}$. [b] Number of determinations.

PS-containing membranes and has been adapted previously as an assay for transmembrane PS asymmetry (Bevers et al., 1983). Erythrocytes treated with glucose for 24 h were exposed to the prothrombinase complex, and the amount of thrombin produced was measured (Figure 3). Prothrombin converting activity was detected in erythrocytes treated with concen-

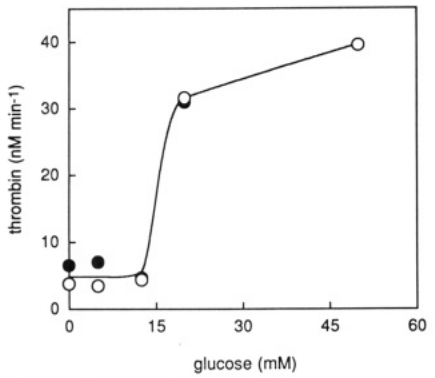

FIGURE 3: Hyperglycemia increases the prothrombin-converting activity of erythrocytes. Erythrocytes were treated with the indicated concentrations of glucose for 24 h. The cells were washed and exposed to a mixture of Ca^{2+}, prothrombin, factor V/Va, and factor Xa. The amount of thrombin released was measured spectrophotometrically using a chromogenic substrate (sarcosine-Pro-Arg-*p*-nitroanilide) by reference to a standard curve. Data are from two separate experiments.

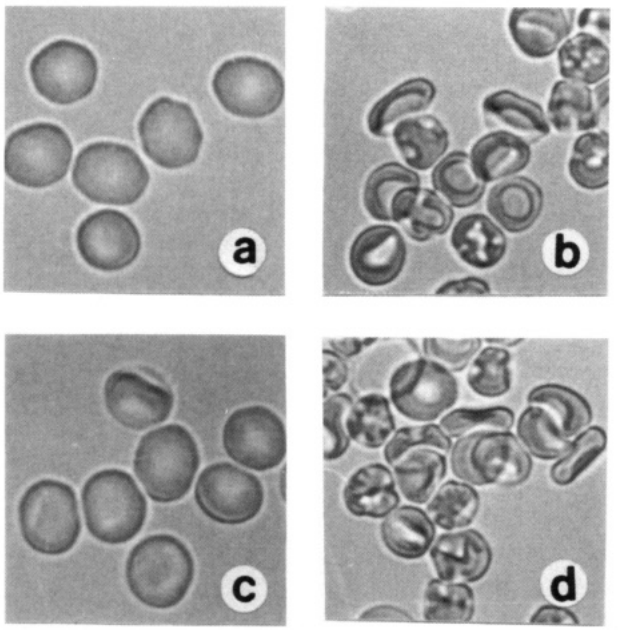

FIGURE 4: Hyperglycemia does not alter aminophospholipid transport. Light micrographs of erythrocytes treated with 5 mM (a, b) or 50 mM (c, d) glucose for 18 h (a, c) followed by treatment with DLPS (250 μM) for 60 min (b, d).

trations of glucose greater than 12.5 mM. Using PS-containing liposomes as standards, the amount of thrombin released in the presence of cells previously incubated with 20 mM glucose corresponds to membranes containing 5.4 mol % PS. The concentration of PS in the outer monolayer of completely scrambled erythrocyte membranes would be 7.5 mol %. Thus, this value corresponds to an outer monolayer concentration of PS in glucose-treated cells of approximately 36%.

Effect of Glucose on PS Transport. PS transport in *in vitro* hyperglycemic erythrocytes was measured using the cell morphology assay described previously (Daleke & Huestis, 1985, 1989). Incubation of erythrocytes in buffers containing normal physiologic concentrations of glucose (5 mM) or hyperglycemic glucose concentrations (50 mM) had no effect on cell shape (Figure 4a,c). Both normal and hyperglycemic buffer-treated cells became echinocytic initially (5–10 min, not shown) when treated with DLPS but reverted to a stomatocytic shape at later time points (60 min, Figure 4b,d).

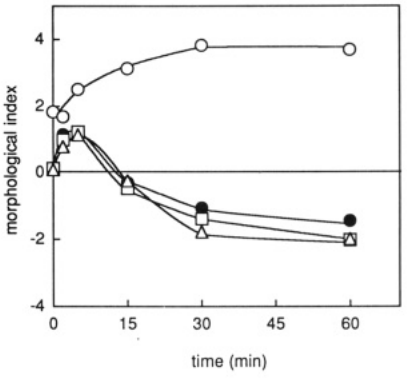

FIGURE 5: Hyperglycemia does not alter the rate or extent of aminophospholipid transport. Time course of erythrocyte shape change (morphological index) of cells treated with DLPS (250 μM) after incubation (18 h) with buffer containing 0 (○), 5 (△), 20 (□), or 50 mM (●) glucose.

This is consistent with initial incorporation of the lipid into, and selective expansion of, the outer monolayer followed by translocation and accumulation of the lipid in the inner monolayer (see Materials and Methods; Daleke & Huestis, 1989).

The time course of shape change in response to DLPS treatment, expressed as the morphological index (MI, Materials and Methods; Daleke & Huestis, 1985, 1989), was similar in cells pretreated with 5–50 mM glucose (Figure 5). The rate of shape change, the extent of shape change, and the half-time for reversion to stomatocytes (10–12 min) were similar in normal, untreated erythrocytes and in cells incubated in the presence of 5–50 mM glucose. In contrast, cells that were incubated in buffer devoid of glucose became echinocytic (MI = +2) and, after addition of exogenous DLPS, became increasingly more crenated (Figure 5). This lack of transport is attributed to low intracellular ATP levels [0.05 μmol (g of Hb)$^{-1}$ (~1% of normal); Figure 1A]. These results indicate that hyperglycemic buffers have no effect on the ability of erythrocytes to transport PS.

Effects of Glucose and Ca^{2+} on Phospholipid Asymmetry and PS Transport. High intracellular Ca^{2+} levels have been shown to induce transmembrane lipid scrambling (Williamson et al., 1992). Therefore, glucose-mediated uptake of Ca^{2+} during both of the asymmetry assays described above may be responsible for the observed loss of phopholipid asymmetry. To test this hypothesis, erythrocytes were incubated in buffers containing physiological concentrations of $^{45}CaCl_2$ (2.5 mM, 5 Ci mol^{-1}) and varying concentrations of glucose. Cells incubated in the absence or presence of low concentrations of glucose exhibit a slightly higher uptake of $^{45}CaCl_2$ [224 ± 34 and 236 ± 20 cpm (μL of cells)$^{-1}$, respectively] than those incubated in the presence of 15–50 mM glucose [169 ± 2 cpm (μL of cells)$^{-1}$]. However, the total amount of intracellular $^{45}CaCl_2$ was less than 0.5% of the extracellular $^{45}CaCl_2$ level, indicating that glucose-treated erythrocytes were effectively excluding Ca^{2+}. Indeed, intracellular [Ca^{2+}] after glucose treatment (estimated from $^{45}CaCl_2$ uptake) were 10–14 μM, within the normal range of total erythrocyte [Ca^{2+}] (10–20 μM; Sarkadi, 1980). Similar results were obtained in glucose-treated erythrocytes that were washed and subsequently incubated in the presence of $^{45}CaCl_2$ (2.5 mM, 5 Ci mol^{-1}) to mimic the exposure of Ca^{2+} during phospholipase or prothrombinase treatments (not shown). Increased [Ca^{2+}] in cells incubated with physiological and lower levels of glucose is likely the result of low intracellular levels of ATP resulting in deficient outward transport by the Ca^{2+}-ATPase. These

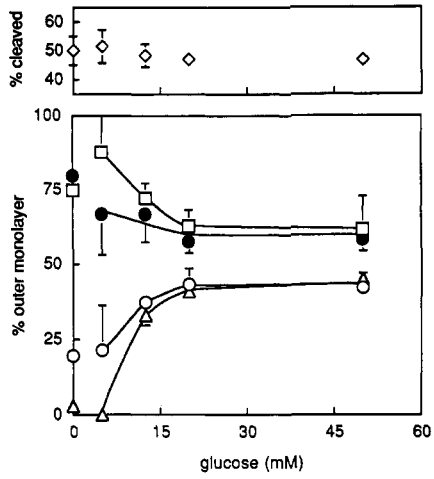

FIGURE 6: External Ca^{2+} does not affect the glucose-mediated loss of phospholipid asymmetry. Erythrocytes were treated for 18 h with the indicated concentrations of glucose in the presence of $CaCl_2$ (2.5 mM) prior to analysis of transmembrane phospholipid asymmetry by accessibility to exogenous phospholipases. Phospholipase treatment and subsequent lipid analysis were performed as described under Materials and Methods: PE (○), PS (△), PC (□), and SM (●) (n = 3). The total amount of lipid cleaved is indicated in the upper panel (◇). Values for freshly isolated cells are indicated on the ordinate.

results indicate that hyperglycemia does not induce an increase in intracellular [Ca^{2+}].

The effect of exogenous Ca^{2+} on the glucose-induced loss of lipid asymmetry was measured using the phospholipase assay described above. Erythrocytes incubated in the presence of Ca^{2+} (2.5 mM) and varying concentrations of glucose for 18 h exhibited a dose-dependent loss of lipid asymmetry (Figure 6), similar to the glucose-induced loss of asymmetry in the absence of Ca^{2+} (Figure 2). The accessibility of PS and PE to phospholipases increased with increasing glucose concentration from 0 to 40% (PS) and from 20 to 41% (PE). Increased PS and PE hydrolysis was coincident with a decrease in the hydrolysis of SM (from 80 to 55%) and a less-pronounced decrease in the hydrolysis of PC (from 75 to 63%). Total lipid hydrolysis varied from 47.4 ± 6.5% to 50.2 ± 5.0%, indicative of cleavage of outer monolayer lipids only. Thus, exogenous Ca^{2+} has no effect on glucose-induced loss of phospholipid asymmetry.

PS transport is inhibited by intracellular Ca^{2+} concentrations in the micromolar range (Bitbol et al., 1987). To determine if glucose treatment alters erythrocyte Ca^{2+} levels sufficiently to affect PS transport, cells were incubated in the presence of Ca^{2+} (2.5 mM) and 5–20 mM glucose for 18 h, and PS transport was measured by the addition of exogenous DLPS. The rate and extent of PS-induced shape changes in glucose- and Ca^{2+}-treated cells were not dependent on the concentration of glucose (Figure 7). Thus, exogenous Ca^{2+} has no effect on the transport of PS in glucose-treated cells.

DISCUSSION

Hyperglycemic treatment of normal erythrocytes induces a time- and concentration-dependent loss of phospholipid asymmetry without concomitant inhibition of the aminophospholipid flippase. An almost complete loss of PS, PE, PC, and SM asymmetry occurs with an identical time course (Figure 2B) and with the same concentration of glucose producing half-maximal loss of asymmetry (Figure 2A). Hyperglycemia may induce lipid scrambling by several potential mechanisms, including alterations in metabolite levels, membrane physical properties, or aminophospholipid transport.

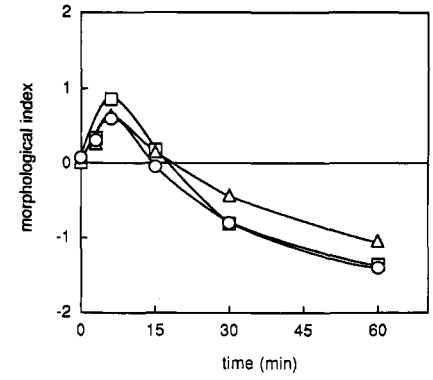

FIGURE 7: Hyperglycemia does not affect aminophospholipid transport in the present of external Ca^{2+}. Erythrocytes were treated with 5 (○), 12.5 (△), or 20 (□) mM glucose in the presence of $CaCl_2$ (2.5 mM) for 18 h, washed, and treated with DLPS (250 μM) for the times indicated. Aliquots of cells were fixed in gutaraldehyde, and cell morphology was analyzed by light microscopy.

Hyperglycemic treatment of erythrocytes produces no gross perturbation to erythrocyte shape, membrane integrity, or levels of essential metabolites. Cell shape is normal after incubation in the presence of high concentrations of glucose (Figure 4), and the relative composition of PC, PS, PE, and SM is identical to that of untreated cells (data not shown). However, under these conditions, glucose alone is incapable of maintaining normal levels of ATP. Incubation in hyperglycemic buffers results in a time-dependent loss of intracellular ATP (Figure 1). However, the decrease of ATP is not sufficient to affect PS transport (Figures 4 and 5) unless cells are incubated in the absence of glucose. Retention of flippase activity in the presence of reduced ATP most likely occurs because ATP levels [0.5–1.0 μmol (g of Hb)$^{-1}$, Figure 1] do not decrease below the apparent K_m [<0.2 mM, ~0.7 μmol (g of Hb)$^{-1}$] of the flippase for ATP (Devaux, 1991). Although the decrease in ATP concentration is coincident with the time-dependent loss of lipid asymmetry (compare Figures 1B and 2B), ATP depletion is not the cause of the loss of phospholipid asymmetry. When erythrocytes are incubated in hyperglycemic buffers under conditions (5% HCT) in which ATP levels remain elevated (50% normal), a loss of phospholipid asymmetry is still observed (Table I). Other metabolites that may affect phospholipid asymmetry or transport, such as GSH, remain unchanged. The maintenance of a high concentration of GSH is essential for the normal functioning of the flippase; transport activity is sensitive to sulfhydryl oxidation (Daleke & Huestis, 1985). *In vitro*, hyperglycemia produces an elevation in HbA_{1c} to levels commonly observed in uncontrolled diabetics (12–20%; Trivelli et al., 1971), coincident with the loss of phospholipid asymmetry (compare Figures 1 and 2). Thus, the hyperglycemia-induced loss of lipid asymmetry likely arises from an effect of glucose treatment other than reduction of ATP or GSH.

The loss of asymmetry induced by glucose was verified by two independent methods: phospholipase digestion and prothrombinase activation. In order for phospholipase digestion to provide an accurate indication of outer monolayer phospholipid content, conditions must be chosen such that (1) all of the phospholipid in the outer monolayer is digested (~50% of the total phospholipid) and (2) the exogenous phospholipases must not gain access to the inner monolayer. In each of the measurements illustrated in Figures 2 and 7, cellular lysis did not exceed 3%, and the total amount of phospholipid hydrolyzed ranged from 38 to 50% indicating that these conditions have been met. A second phospholipid asymmetry assay, based on the activation of the prothrom-

binase complex, verified the presence of PS on the cell surface (Figure 3). However, qualitatively different results are obtained with these assays. Glucose-treated erythrocytes demonstrate prothrombin converting activity only at glucose concentrations greater than 12.5 mM (Figure 3). In contrast, phospholipase digestion detects an increase in outer monolayer PS (25%) in cells incubated in the presence of relatively low (5–10 mM) concentrations of glucose (Figures 2 and 6). One possible explanation is that prothrombin activation requires a threshold concentration of PS. However, the prothrombinase converting ability in model membranes composed of PS and a mixture of lipids representative of the erythrocyte outer monolayer is linearly proportional to the concentration of PS (data not shown). More likely, the discrepancy reflects the rate of outward movement of PS and the time scale of the two assays; phospholipase digestion requires 1 h to completely digest outer monolayer phospholipid, while the prothrombinase assay samples the PS content of the membrane in 3 min. These data are consistent with a glucose-induced dymamic loss of asymmetry at low concentrations that it partly corrected by inward active transport and, at higher concentrations of glucose, an apparent further increased rate of outward "flop" that overwhelms the ability of the flippase to restore PS asymmetry.

The loss of asymmetry induced by glucose can be analyzed using two kinetic models for the maintenance of PS asymmetry: a two-state model (eq 1; Herrmann & Müller, 1986) and a modification of this model to include binding to inner monolayer sites (three-state model, eq 4; Williamson et al., 1987). Separate assumptions are required for analysis by these models. For the two-state model, the binding of PS to inner monolayer sites is assumed to be an insignificant regulator of PS asymmetry, and the effects of hyperglycemia are on k_o alone. For the three-state model, the assumption is made that hyperglycemia has no effect on k_i ork_o, but exerts its effects through a reduction in K_b, perhaps through selective damage to cytoskeletal proteins. When the present data are fit to the two-state model, the outward flop rate of PS (k_o) can be expressed as a fraction of the rate of inward PS transport (k_i) and the equilibrium fraction of PS on the inner monolayer, f_i (eq 3, Figure 8). Using the three-state model, K_b can be expressed as a function of k_o/k_i and f_i (eq 5, Figure 9). The increase in k_o predicted by the two-state model closely mimics the rate and extent of the concentration- and time-dependent loss of PS asymmetry (compare Figures 2, 6, and 8). However, the three-state model predicts that K_b decreases to less than 10% of its original value at concentrations of glucose (5–12.5 mM) and times of incubation (5–10 h) which produce little change in phospholipid reorientation (compare Figures 2, 6, and 9). Although absolute values of K_b cannot be determined from these data, direct measurements of PS binding to cytoskeletal components indicate that PS–cytoskeletal interactions are weak (Morrot et al., 1986; Maksymiw et al., 1987; Bitbol et al., 1989). Thus, using the attendant simplifying assumptions presented here, the data fit the two-state model well and argue for an increase in outward flop as the major contributor to asymmetry loss in glucose-treated cells.

An estimate of the absolute value for the rate of PS flop (k_o) in normal and hyperglycemic cells can be determined from these data. During exposure to hyperglycemic solutions, k_o increases from 3% to a maximum of 85% of k_i (Figure 8). Using a value of k_i determined for spin-PS (0.21 min^{-1}; Bitbol & Devaux, 1988), k_o increases from 6.3×10^{-3} to 0.18 min^{-1} upon exposure to glucose. This increase in k_o should prevent the stable accumulation of exogenously added DLPS in the

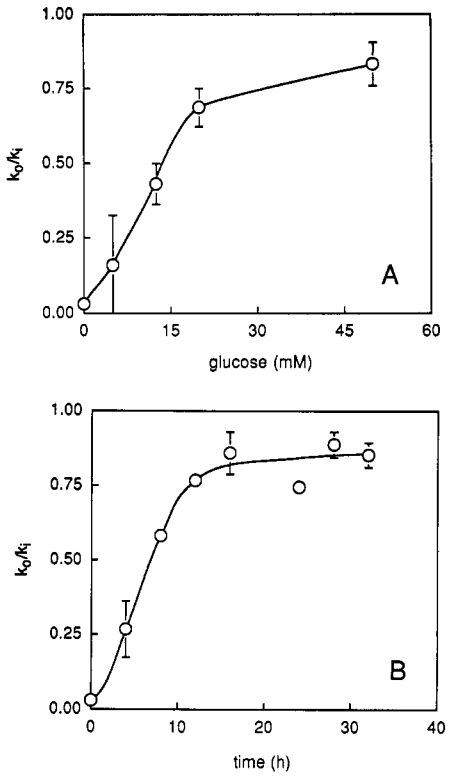

FIGURE 8: Hyperglycemia induces an increase in outward PS translocation. Data from Figure 2A,B were used to estimate the rate of outward PS translocation (k_o) as a fraction of the inward transport rate (k_i) employing the two-state model as described under Materials and Methods.

inner monolayer, contrary to the results obtained here (Figures 4 and 5). However, PC flip-flop in erythrocytes is dependent on acyl chain length (van Meer & Op den Kamp, 1982; Bergmann et al., 1984; Middelkoop et al., 1986), and the half-time for inward flip of DLPC in erythrocytes (8 h; Daleke, 1986) is significantly slower than that of lipids with an acyl chain composition similar to endogenous PCs [16:0,18:2-PC (2.9 ± 1.7 h); Middelkoop et al., 1986]. Similarly, the half-times for PS flop measured using synthetic DLPS (~15 h; Daleke & Huestis, 1989), C_6-NBD-PS (~1.5 h; Connor et al., 1992), and spin-PS (0.96 h; Bitbol & Devaux, 1988) differ significantly. Although the outward flop rate of endogenous PS in normal cells determined here (~0.01 min^{-1}) is similar to that obtained with spin-PS (0.012 min^{-1}; Bitbol & Devaux, 1988), a slower flop rate of DLPS compared with endogenous PS would allow this lipid to accumulate in the inner monolayer of glucose-treated cells.

Several potential mechanisms exist for the glucose-induced loss of phospholipid asymmetry, some of which have been shown to produce lipid scrambling in erythrocytes and platelets. These include an increase in intracellular [Ca^{2+}], membrane vesiculation, and lipid oxidation. Ca^{2+}-loading of erythrocytes by ionophore treatment in the presence of exogenous Ca^{2+} potently inhibits flippase activity at intracellular concentrations of free Ca^{2+} greater than 1 μM (Bitbol et al., 1987) and, at higher concentrations (50 μM), induces a rapid reorientation of all major classes of phospholipids (Williamson et al., 1992). Platelet activation is accompanied by a loss of lipid asymmetry, increased cytoplasmic [Ca^{2+}] (Wiedmer et al., 1990), and calpain-mediated proteolysis of cytoskeletal proteins (Comfurius et al., 1985; Verhallen et al., 1988). However, hyperglycemia-induced lipid scrambling differs from Ca^{2+}-induced lipid reorientation in several aspects. Ca^{2+} levels in

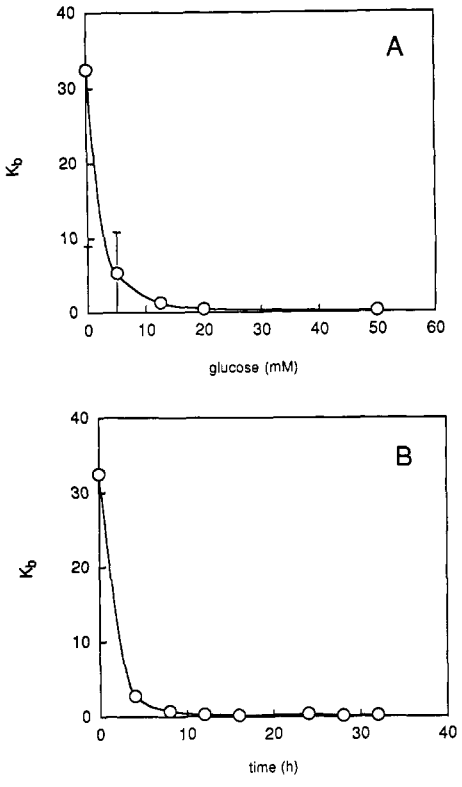

FIGURE 9: Hyperglycemia induces an apparent decrease in the binding of PS to inner monolayer binding sites. Data from Figure 2A,B were used to estimate the effect of glucose concentration on the binding constant (K_b) of PS to potential inner monolayer binding sites employing the three-state model as described under Materials and Methods and assuming the inward (k_i) and outward (k_o) rates of PS translocation are unaffected by glucose treatment.

erythrocytes are unchanged by hyperglycemic treatment. Flippase activity is normal (Figure 7), confirming that internal free Ca^{2+} levels have not risen above 1 μM (Bitbol et al., 1987). Calpain activation inhibits platelet flippase activity (Comfurius et al., 1990), but erythrocyte flippase activity is not altered by glucose treatment (Figures 4, 5, and 7) and proteins from erythrocytes treated for 18 h with 0–50 mM glucose show no evidence of proteolysis by polyacrylamide gel electrophoretic analysis (data not shown). Finally, the relative rates of transmembrane lipid movement induced by Ca^{2+} and glucose differ. Ca^{2+}-loading of erythrocytes results in a similar rate of PS, PE, and PC transmembrane movement (3–4 min^{-1}) that is 2–3-fold faster than SM reorientation (~0.9 min^{-1}; Williamson et al., 1992). In contrast, the effects of glucose on the transmembrane redistribution rate of PS, PE, PC, and SM are of similar magnitude (Figure 2). Thus, hyperglycemia does not induce lipid scrambling through an increase in intracellular [Ca^{2+}] or calpain activation, and the effects of Ca^{2+}-loading on lipid reorientation are qualitatively different from those of glucose treatment.

Another potential mechanism for transmembrane lipid scrambling is membrane fusion. Fusion of model membranes induces lipid randomization (Huang & Hui, 1990; Song et al., 1992), and platelet activation-induced membrane scrambling has been linked to membrane vesiculation (Sims et al., 1989; Fox et al., 1990). If glucose were to induce membrane vesiculation in erythrocytes, echinocytes would be observed after hyperglycemic treatment. However, erythrocyte morphology is unchanged by incubation in hyperglycmeic buffers, indicating that membrane vesiculation does not occur (Figure 4a,c). Thus, another mechanism must be driving the loss of PS asymmetry.

Hyperglycemia may cause lipid reorientation by direct alteration of proteins or lipids. Glucose modifies proteins via Schiff base formation with amine residues. Glycated proteins often exhibit altered enzymatic (Garner et al., 1990; Rajeswari et al., 1991) or structural properties (Kowluru et al., 1989; McMillan & Brooks, 1982; Miller et al., 1980). Furthermore, glucose promotes the accumulation of sorbitol by activation of aldose reductase (Travis et al., 1971) and sorbitol dehydrogenase (Gabbay, 1975). Altered osmotic properties of the sorbitol-enriched cells may result in a loss of phospholipid asymmetry (Baldwin et al., 1990). Finally, glucose promotes the metal-catalyzed oxidation of lipids (Jain, 1989) and proteins (Wolff et al., 1991), which in turn may alter protein structure and function (Rajeswari et al., 1991) or lipid asymmetry (Jain, 1984). Phospholipid oxidation results in the cleavage of unsaturated fatty acyl chains and releases short-chain aldehydes and an abbreviated phospholipid. These oxidized lipid byproducts may perturb membrane asymmetry. Phospholipids with short acyl chains flip faster than lipids with longer acyl chains (van Meer & Op den Kamp, 1982; Middelkoop et al., 1986) and may alter the transmembrane mobility of other phospholipids. Indeed, amphipathic compounds, such as chlorpromazine, induce a temporary loss of asymmetry in erythrocytes (Rosso et al., 1988; Schrier et al., 1992). In addition, oxidation of polyunsaturated fatty acids releases malondialdehyde [which alone is capable of altering phospholipid asymmetry (Jain, 1984)] and reactive short-chain aldehydes. Although extensive modification of phospholipids was not observed by two-dimensional thin-layer chromatographic analysis (data not shown), preliminary evidence indicates that erythrocytes coincubated with glucose and antioxidants display a reduced scrambling of phospholipids (M. Wilson and D. Daleke, unpublished results). Thus, minor products of lipid oxidation may produce a loss of asymmetry through direct or indirect modification of lipids and proteins.

SUMMARY

Hyperglycemia induces a unique and dramatic loss of transmembrane phospholipid asymmetry in erythrocytes without gross changes in membrane integrity, aminophospholipid transport, or cellular Ca^{2+} content. Randomization of PS, PE, PC and SM asymmetry is likely a result of increased passive transmembrane flip-flop. The appearance of PS (and PE) on the membrane surface indicates that the flippase is incapable of regulating aminophospholipid asymmetry in the face of a large increase in lipid flip-flop. Externalized erythrocyte PS may contribute to thromboses in diabetes by stimulating blood clotting enzymes. These findings define a limit to the ability of the flippase to regulate aminophospholipid asymmetry and suggest that hyperglycemic damage to blood cell membranes may be a significant contributor to vascular complications in diabetes.

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