Platelet hyperactivity and abnormal Ca\(^{2+}\) homeostasis in diabetes mellitus

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Platelet hyperactivity and abnormal Ca\(^{2+}\) homeostasis in diabetes mellitus. Am J Physiol Heart Circ Physiol 280: H1480–H1489, 2001.— We sought to determine the mechanisms for hyperactivity and abnormal platelet Ca\(^{2+}\) homeostasis in diabetes. The glycosylated Hb (HbA_1c) level was used as an index of glycemic control. Human platelets were loaded with Ca\(^{2+}\)-green-fura red, and cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and aggregation were simultaneously measured. In the first series of experiments, the platelets from diabetic and normal subjects were compared for the ability to release Ca\(^{2+}\) to or promote Ca\(^{2+}\) influx. A potent and relatively specific inhibitor of Na\(^+/Ca^{2+}\) exchange, 5-(4-chlorobenzyl)-2',4'-dimethylbenzamil (CB-DMB), increased the second phase of thrombin-induced Ca\(^{2+}\) response, suggesting that the Na\(^+/Ca^{2+}\) exchanger works in the reverse mode to mediate Ca\(^{2+}\) influx. In contrast, in the platelets from diabetics, CB-DMB decreased the Ca\(^{2+}\) response, indicating that the Na\(^+/Ca^{2+}\) exchanger works in the forward mode to mediate Ca\(^{2+}\) efflux. In the second series of experiments we evaluated the direct effect of hyperglycemia on platelets in vitro. We found that thrombin- and collagen-induced increases in [Ca\(^{2+}\)]\(_i\) and aggregation were not acutely affected by high glucose concentrations of 45 mM. However, when the platelet-rich plasma was incubated with a high glucose concentration at 37°C for 24 h, the second phase after thrombin activation was inhibited by CB-DMB. In addition, collagen-stimulated [Ca\(^{2+}\)]\(_i\) response and aggregation were also increased. Thus in diabetes the direction and activity of the Na\(^+/Ca^{2+}\) exchanger is changed, which may be one of the mechanisms for the increased platelet [Ca\(^{2+}\)]\(_i\) and hyperactivity. Prolonged hyperglycemia in vitro can induce similar changes, suggesting hyperglycemia per se may be the factor responsible for the platelet hyperactivity in diabetes.

diabetic complications; Na\(^+/Ca^{2+}\) exchanger; 5-(4-chlorobenzyl)-2',4'-dimethylbenzamil; hyperglycemia

CARDIOVASCULAR COMPLICATIONS are the most frequent cause of morbidity and mortality in diabetic patients (30). These complications include microangiopathy, retinopathy, neuropathy, nephropathy, and macroangiopathy, which is an accelerated form of atherosclerosis. Considering the high frequency of cardiovascular diseases in diabetes, it is important to understand the diabetes pathogenesis. One postulated hypothesis for the pathogenesis is abnormal platelet activation that contributes to both diabetic micro- and macroangiopathy (9, 34). There is evidence supporting this hypothesis. For example, it is well known that platelets play an important role in the pathogenesis of atherosclerosis (36, 38); furthermore, it has been demonstrated that platelets from both type I and type II diabetic patients (preclinical diabetes, clinical diabetes, and diabetic patients with complications) exhibit hyperactivity in vitro and in vivo. This hyperactivity includes increased platelet adhesion, aggregation, thromboxane production, increased plasma levels of platelet-specific proteins, and increased platelet turnover (5, 8, 9). In addition, in vivo thrombosis can occur more readily in large vessels in response to injury in diabetes (18). Platelet aggregates (microthrombi) are shown to exist in the small vessels of the retina in diabetic patients and animals (11, 19). Despite all of this evidence supporting the involvement of platelet abnormality in the pathogenesis of diabetic vascular complications, the mechanisms responsible for the platelet hyperactivity are not known.

That platelet hyperactivity can be found in both platelet-rich plasma (PRP) and washed platelet suspensions suggests that some of the mechanisms exist in the platelet itself. Based on observations in diabetes, the platelets are hypersensitive to many agonists [thrombin, collagen, ADP, and platelet-aggregating factor (PAF)], and many reactions (adhesion, release, and aggregation) are involved. It is reasonable to think that the abnormality may exist in a common pathway in platelet activation. The arachidonate pathway is a common amplifying pathway for many agonists during platelet activation and is found to be increased in platelets from diabetics. However, in clinical trials aspirin had only limited efficacy in preventing chronic complications in diabetic patients (32). An in vitro study showed that in diabetes thrombin-induced platelet hyperactivity still persists after the arachidonate pathway is blocked (44). This suggests that in addition to the overactive arachidonate pathway, there must be other mechanisms for inducing platelet hyperactivity.

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in diabetes. The clinical trial by the Diabetes Control and Complications Trials Research Group (10a) concluded in 1993 that hyperglycemia is correlated with the extent of diabetic vascular disease.

Ca\(^{2+}\) is required in platelets for many functions such as shape change, secretion, aggregation, and thromboxane formation. There are several studies in the literature that describe Ca\(^{2+}\) homeostasis in platelets from diabetic patients; however, the reported results are not consistent and the abnormal mechanisms have not been identified. In this report, the first series of experiments confirmed that platelet Ca\(^{2+}\) homeostasis is abnormal in diabetes. We then investigated the mechanisms for abnormal platelet Ca\(^{2+}\) homeostasis. Our focus here was on the Na\(^+/Ca^{2+}\) exchanger and its role in abnormal Ca\(^{2+}\) homeostasis and hyperactivity of platelets in diabetes. We determined the direct in vitro effect of elevated glucose concentration on platelet aggregation, Ca\(^{2+}\) homeostasis, and the Na\(^+/Ca^{2+}\) exchanger. The hypothesis that hyperglycemia is a causative factor for abnormal Ca\(^{2+}\) homeostasis and hyperactivity in platelets was tested.

**MATERIALS AND METHODS**

**Subjects and Platelet Isolation**

The diabetic patients were obtained from the diabetic clinic of the Health Science Center, Winnipeg, Manitoba, Canada. The institutional Human Ethics Committee approved this study. Informed consent was obtained from every study subject. Most of these patients were type II diabetics. The glycosylated Hb levels (HbA\(_{1c}\)) were used as an index of metabolic control. Only the poorly controlled patients (HbA\(_{1c}\) > 9%) were selected. The total serum triglyceride levels, cholesterol levels, and blood pressure values were not significantly different from those of normal subjects. The control subjects were normal healthy people that had glycosylated Hb (HbA\(_{1c}\)) levels in the normal range (3.4–5.4%). No participants had taken any antiplatelet drugs in the previous 2 wk.

Venous blood was drawn into vacutainer tubes. PRP was obtained by centrifugation at 600 rpm for 15 min at 18°C and was then centrifuged at 2,000 rpm for 15 min at 18°C. The supernatant (platelet-poor plasma) was aspirated, leaving the sedimented pellets; these were resuspended in 500 \(\mu\)l of plasma and then loaded with fluorescent dyes.

**Measurement of Platelet Free-Ca\(^{2+}\) Concentration**

The cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) of platelets was determined by Ca\(^{2+}\)-sensitive fluorescent indicators. In most experiments fura 2 was used. In experiments using 5-(4-chlorobenzyl)-2',4'-dimethylbenzamid (CB-DMB), fura 2 could not be used due to quenching (25); instead a combination of long-wavelength dyes, Ca\(^{2+}\)-sensitive fluorescent indicators. The platelet suspension was loaded with the membrane-permeable acetoxymethyl ester (AM) form of fura 2 (10 \(\mu\)M) or a combination of the permeable forms of Ca\(^{2+}\)-green (10 \(\mu\)M) and fura red (20 \(\mu\)M) at 37°C for 1 h. The platelets were separated from plasma and extracellular dye by gel filtration on a Sepharose CL-2B column. The eluted platelets were counted in a Coulter counter and adjusted to 2 \(\times\) 10\(^8\) cells/ml with Ca\(^{2+}\)-free HEPES buffer containing (in mM): 140 NaCl, 4.9 KCl, 1.2 MgCl\(_2\), 1.4 KH\(_2\)PO\(_4\), 11 glucose, and 20 HEPES (pH 7.4).

Fluorescence of fura 2-loaded platelets was measured in the Jasco CAF-102 [Ca\(^{2+}\)]\(_i\) analyzer. [Ca\(^{2+}\)]\(_i\) values were calculated as described by Grynkiewicz and colleagues (14). Autofluorescence was found not to contribute >10% of the fura 2 signal. For Ca-green-1-AM- and fura red-AM-coloaded platelets, an excitation wavelength of 500 nm and dual emission wavelengths of 540 and 680 nm were used. When [Ca\(^{2+}\)]\(_i\) increases, at a 500-nm excitation wavelength the emission intensity of Ca-green at 540 nm increases whereas the emission intensity of fura red at 680 nm decreases. By taking the ratio of emission intensity at 540 nm to 680 nm, [Ca\(^{2+}\)]\(_i\) values can be estimated by the same formula as that for fura 2. The only difference is the dissociation constant \(K_d\), which was taken as 189 nM.

**Measurement of Platelet Aggregation**

Platelet aggregation was simultaneously measured in the Jasco CAF-102 or 110 Ca\(^{2+}\) analyzer. A stirrer speed of 1,000 rpm was used, and the temperature was set at 37°C. Platelet aggregation was measured as a change in optical density. For the initiation of aggregation, thrombin (0.5–1.25 U/ml) and collagen (2–20 \(\mu\)g/ml) were used. The maximum rate of aggregation was determined during the initial 3 min after the addition of agonists.

**Glucose Transport in Human Platelets**

Glucose transport in platelets was measured by the influx of the \(^{14}\)C-labeled nonmetabolizable glucose analog 3-O-methyl-d-glucose (3OMG). Glucose transport was calculated by measuring the plateau medium distribution of 3OMG. The method was modified from the procedure described by Kim and colleagues (22). Platelet concentrates were obtained from fresh whole blood (random donor) supplied by the Canadian Red Cross Society Blood Services, and pellets were obtained by centrifuging the platelet concentrate at 2,000 rpm for 15 min at 25°C. Pellets were washed with Ca\(^{2+}\)-free and glucose-free buffer and suspended to 2 \(\times\) 10\(^8\) platelets/ml in the same buffer. Influx measurement of radiolabeled 3OMG and unlabeled substrate were carried out at 37°C. Influx was initiated by the addition of 0.4 ml of the platelet suspension to tubes containing 0.05 ml of Ca\(^{2+}\)-free and glucose-free buffer with 1.0 \(\mu\)Ci/ml of \(^{14}\)C\)-3OMG and 0.6 mM of unlabeled 3OMG at 37°C. Influx was terminated by adding 1 ml of ice-cold “stopping” solution (2 mM HgCl\(_2\), and 154 mM NaCl) at different time intervals. Time 0 was determined by adding the stopping solution to the platelets before the addition of labeled substrate. The platelets were quickly centrifuged for 20 s at 25°C, washed twice with 1 ml of cold stopping solution, and lysed with 100 \(\mu\)l of 5% TCA. Radioactivity was then measured. Each point is an average value of four identical measurements. The Ca\(^{2+}\)-free and glucose-free buffer consisted of (in mM): 140 NaCl, 2.5 KCl, 1 KH\(_2\)PO\(_4\), and 5 HEPES (pH 7.2). The effect of insulin on 3OMG flux was determined at two time points. Results were expressed as micromoles glucose per milliliter of platelets.

**Experimental Protocols**

Resting platelet [Ca\(^{2+}\)]\(_i\) and agonist-evoked [Ca\(^{2+}\)]\(_i\) response. We placed 500 \(\mu\)l of dye-loaded platelet suspension (2 \(\times\) 10\(^8\) platelets/ml) in the cuvette and added 1 mM CaCl\(_2\). The platelets were allowed to equilibrate for 5 min and the agonist (such as thrombin) was added. As shown in Fig. 1, several variables were recorded: 1) basal or resting [Ca\(^{2+}\)]\(_i\), (platelet [Ca\(^{2+}\)]\(_i\) before addition of thrombin); 2) thrombin-
evoked peak [Ca\(^{2+}\)], (basal [Ca\(^{2+}\)], was subtracted from peak 1 [Ca\(^{2+}\)], of the thrombin-induced [Ca\(^{2+}\)], response; and 3) thrombin-induced phase 2 response (basal [Ca\(^{2+}\)], was subtracted from the [Ca\(^{2+}\)], at 1 or 3 min after the peak).

**Estimation of the [Ca\(^{2+}\)], store size.** As described earlier, 1 mM CaCl\(_2\) was added and allowed to equilibrate for 5 min, and then 5 mM EGTA was added to chelate extracellular Ca\(^{2+}\) (the external Ca\(^{2+}\) concentration was \(<10^{-8}\) M). Ionomycin (5 \(\mu\)M) was added 1 min later, and the peak [Ca\(^{2+}\)], was used as an estimate of the Ca\(^{2+}\) in the dense tubular system (DTS), which is the intracellular Ca\(^{2+}\) storage site in platelets (31).

**Agonist-releasable Ca\(^{2+}\) and the recovery of cytosolic Ca\(^{2+}\) after release.** Similar to the procedures used for ionomycin, EGTA was used to chelate extracellular Ca\(^{2+}\), and platelets were then stimulated with thrombin. In this condition the peak of the thrombin-induced [Ca\(^{2+}\)], increase indicates thrombin-releasable Ca\(^{2+}\) (Fig. 1). The cytosolic Ca\(^{2+}\) removal rate was determined after the peak [Ca\(^{2+}\)], response.

**Estimation of the Na\(^{+}/Ca\(^{2+}\) exchange in intact platelets.** The direction and activity of the Na\(^{+}/Ca\(^{2+}\) exchange was studied indirectly in intact platelets by comparing the [Ca\(^{2+}\)], before and after the blockade of the exchanger by CB-DMB (2 \(\mu\)M). As shown in the model (Fig. 6), if the function of the exchanger is to mediate Ca\(^{2+}\) efflux (forward mode), then with CB-DMB the [Ca\(^{2+}\)], should increase. If the role of the exchanger is to mediate Ca\(^{2+}\) influx (reverse mode), then CB-DMB should decrease the [Ca\(^{2+}\)]. Specificity of CB-DMB for the Na\(^{+}/Ca\(^{2+}\) exchange has been shown elsewhere (23–25, 28). In intact cells CB-DMB has no effect on the Na\(^{+}/H\) exchanger, the Na\(^{+}\) and Ca\(^{2+}\) pumps, and the Na\(^{+}\) and Ca\(^{2+}\) channels. The effect of CB-DMB was assessed on basal, thrombin-stimulated, and collagen-activated platelet [Ca\(^{2+}\)].

**Direct effect of hyperglycemia on platelet [Ca\(^{2+}\)], and aggregation.** Blood was drawn into EDTA-containing vacu- tainer tubes, PRP was isolated by centrifugation, and the glucose concentration was measured by the glucose-oxidase method as described for the YSI 29 glucose analyzer. PRP was divided into three portions: control (no added extra glucose), high glucose concentration (40 mM glucose was added), and high mannitol concentration (40 mM mannitol was added to be used as an osmotic control). The tubes were incubated at 37°C for 24 h. During the incubation glucose concentration was monitored, and extra glucose was added to make up for the consumed glucose. Platelet counts and responses to thrombin and collagen were not altered after incubation in 5–8 mM glucose for 24 h.

**Chemicals**

The pyrazine compound CB-DMB (23) was obtained from Dr. E. J. Cragoe, Jr. It was dissolved in DMSO as stock solutions of 10 or 1 mM. Fura 2-AM, Ca-green-1-AM, and fura red-AM were from Molecular Probes (Eugene, OR). They were dissolved in DMSO and kept as stock solutions of 1 mM. Thrombin (from bovine plasma) was purchased from Sigma Chemical and was dissolved in water as a stock of 50 U/ml. Collagen was from Nycomed Arzneimittel (Munich) and the stock solution was 1 mg/ml. Sepharose 2B-CL was from Pharmacia Biotechnology. All other chemicals were from Sigma.

**Statistical Analysis**

All data are expressed as means ± SE; n is the number of subjects from whom platelets were obtained. The differences between means from nondiabetic subjects and diabetic patients were tested for significance using a two-tailed Student’s t-test for unpaired data. When comparisons were made in the same subject between control and treatment, the paired t-test was used. P < 0.05 was considered to be significant for a difference.

**RESULTS**

**Platelet Ca\(^{2+}\), Homeostasis in Normal and Diabetic Subjects**

**Resting platelet [Ca\(^{2+}\)], level and agonist-stimulated [Ca\(^{2+}\)], response.** The resting platelet [Ca\(^{2+}\)], in a group of poorly controlled diabetic patients was higher (115 ± 7 nM) than in nondiabetic subjects (88 ± 7; Fig. 1B). This difference was statistically significant (P < 0.015) in an unpaired two-sided t-test. In the presence of 1 mM extracellular Ca\(^{2+}\), the addition of thrombin (0.5 and 1.25 U/ml) increased [Ca\(^{2+}\)], in platelets from both study groups. The typical thrombin response was divided into two phases (Fig. 1A): a rapid immediate [Ca\(^{2+}\)], increase after thrombin addition (peak 1) and a sustained [Ca\(^{2+}\)], level (phase 2). There was no signifi-
ionomycin in the absence of extracellular Ca\(^{2+}\) in EGTA-containing medium. Figure 3B shows that the peak [Ca\(^{2+}\)]\(_i\), increase in response to 5 \(\mu\)M ionomycin did not differ between control and diabetic subjects. This suggests that, in platelets from diabetic subjects, the amount of releasable Ca\(^{2+}\) in the intracellular Ca\(^{2+}\) store is not different from that of normal subjects. Figure 3A shows that thrombin (0.5 U/ml) induced the peak Ca\(^{2+}\) increase and the [Ca\(^{2+}\)]\(_i\), at 1 min after the peak in Ca\(^{2+}\)-free medium. The thrombin-induced peak [Ca\(^{2+}\)]\(_i\) increase was not significantly different (\(P = 0.15\)) between these two groups (Fig. 3A, left). This indicates that thrombin-releasable Ca\(^{2+}\) in the DTS is also not changed in diabetes.

In the absence of extracellular Ca\(^{2+}\), the thrombin-stimulated [Ca\(^{2+}\)]\(_i\), at 1 min after the peak was higher (\(P = 0.012\)) in platelets from diabetics compared with controls. At this time point the subsequent fall in the Ca\(^{2+}\) signal indicates either removal of Ca\(^{2+}\) from the cytosol into internal stores or the extrusion of Ca\(^{2+}\) across the plasma membrane. This result thus indi-

![A](image1.png)![B](image2.png)

Fig. 2. Thrombin- and collagen-induced platelet [Ca\(^{2+}\)]\(_i\), responses in the presence of 1 mM extracellular Ca\(^{2+}\) for nondiabetic and diabetic subjects. Values are means ± SE, \(n = 9\). A: thrombin-induced platelet [Ca\(^{2+}\)]\(_i\), at three different time points: peak 1 (\(P = 0.062\)), 1 min (\(P = 0.035\)), and 3 min (\(P = 0.016\)). B: collagen-induced maximal [Ca\(^{2+}\)]\(_i\), for three different concentrations (\(\mu\)g/ml), \(n = 9\); \*\(P < 0.05\).

![A](image3a.png)![B](image3b.png)

Fig. 3. Thrombin and ionomycin-stimulated [Ca\(^{2+}\)]\(_i\), rise in the absence of external Ca\(^{2+}\) for diabetic and nondiabetic subjects. Platelets were in 1 mM external Ca\(^{2+}\) for 3 min before addition of 5 mM EGTA; 1 min later 5 \(\mu\)M of ionomycin or thrombin (0.5 U/ml) was added. A: peak 1 of thrombin-induced [Ca\(^{2+}\)]\(_i\), (\(n = 9\)), not significantly different; \(P = 0.89\). Phase 2 [Ca\(^{2+}\)]\(_i\), 1 min after peak (\(n = 9\)); \*\(P = 0.012\). B: peak ionomycin-induced [Ca\(^{2+}\)]\(_i\), (\(n = 4\)). Not significantly different; \(P = 1.0\).
data indicate that, in platelets from normal subjects after thrombin stimulation, the Na\(^+\)/Ca\(^{2+}\) exchanger is activated and functions to remove cytosolic Ca\(^{2+}\) out of the cell (forward mode of the Na\(^+\)/Ca\(^{2+}\) exchanger); after the activity of this exchanger was inhibited by CB-DMB, [Ca\(^{2+}\)]\(_i\) increased (see the model in Fig. 6). In contrast, in diabetes the same concentration of CB-DMB significantly decreased both peak 1 (P = 0.016) and phase 2 (P = 0.01) of the thrombin-induced [Ca\(^{2+}\)]\(_i\) response (Fig. 5). This suggests that in platelets from diabetic patients, the Na\(^+\)/Ca\(^{2+}\) exchanger mediates Ca\(^{2+}\) influx (reverse mode), contributing at least in part to the enhancement of thrombin-induced [Ca\(^{2+}\)]\(_i\).

The effect of CB-DMB becomes clear when the data are expressed as a percent change of [Ca\(^{2+}\)]\(_i\) of the control (measured in nanomoles). The different role of the Na\(^+\)/Ca\(^{2+}\) exchanger in platelets from normal and diabetic subjects can be clearly seen in Fig. 6. In nondiabetics the relative change after CB-DMB addition was positive (138%, n = 7), whereas in diabetes the relative change was negative (−63%, n = 14). This figure indicates that after thrombin stimulation, the direction of the Na\(^+\)/Ca\(^{2+}\) exchanger is different in platelets from normal subjects compared with diabetic patients.

The effects of CB-DMB on basal [Ca\(^{2+}\)]\(_i\) and the collagen-stimulated [Ca\(^{2+}\)]\(_i\) response in platelets from the two groups are shown in Fig. 7. In platelets from nondiabetic subjects, addition of 2 μM CB-DMB significantly increased basal [Ca\(^{2+}\)]\(_i\), suggesting that the Na\(^+\)/Ca\(^{2+}\) exchanger works in the forward mode (mediating Ca\(^{2+}\) efflux) under resting conditions. However, in platelets from diabetic patients, addition of the same concentration of CB-DMB did not increase platelet basal [Ca\(^{2+}\)]\(_i\), indicating that in the resting state the activity of the forward mode of the Na\(^+\)/Ca\(^{2+}\) exchanger is decreased in the diabetic state (Fig. 7A, right). Figure 7B shows the role of the Na\(^+\)/Ca\(^{2+}\) exchanger in response to activation by collagen. Platelets from both groups responded to the addition of 2 μM CB-DMB with a significant decrease in collagen-induced [Ca\(^{2+}\)]\(_i\). In nondiabetics [Ca\(^{2+}\)]\(_i\) decreased from 54.69 ± 6.63 to 35.65 ± 7.02 nM, suggesting that the Na\(^+\)/Ca\(^{2+}\) exchanger works in the reverse mode, mediating Ca\(^{2+}\) influx in response to collagen. In diabetes the CB-DMB-sensitive decrease in Ca\(^{2+}\) was greater.

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**Thrombin**

Fig. 4. A: aggregation in platelets from nondiabetics (control). B: typical traces of the potentiating effects of 5-(4-chlorobenzyl)-2’,4’-dimethylbenzamil (CB-DMB) on thrombin-induced [Ca\(^{2+}\)]\(_i\). Thrombin and either 1 μl of DMSO (D) or 1 μl of 1 mM CB-DMB (CB) were added. C: data from seven experiments. NS, nonsignificant \(P = 0.23\) in peak 1 [Ca\(^{2+}\)]\(_i\), control and 2 μM CB-DMB. *Significant effect of CB-DMB on phase 2 [Ca\(^{2+}\)]\(_i\), \(P = 0.021\).


dicates that the rate of Ca\(^{2+}\) removal is slower and the extrusion and/or sequestration mechanisms are impaired. There are two mechanisms for Ca\(^{2+}\) extrusion in platelets: Ca\(^{2+}\)-ATPase and the Na\(^+\)/Ca\(^{2+}\) exchange.

Na\(^+\)/Ca\(^{2+}\) exchanger in platelets from normal subjects and diabetic patients. The Na\(^+\)/Ca\(^{2+}\) exchanger in intact platelets was studied by comparing changes in [Ca\(^{2+}\)]\(_i\) in response to the blocker CB-DMB. The effect of CB-DMB on 0.5 U/ml thrombin-induced [Ca\(^{2+}\)]\(_i\) response in platelets from normal subjects is shown in Fig. 4. After the platelets were pretreated with 2 μM CB-DMB, the thrombin-stimulated peak 1 [Ca\(^{2+}\)]\(_i\) response was not significantly changed, but the phase 2 response (3 min) was increased significantly. These

Fig. 5. A: typical traces of inhibitory effects of 2 μM CB-DMB on thrombin-induced [Ca\(^{2+}\)]\(_i\) in platelet aggregation (agg) of diabetic patients. Experimental conditions were same as in Fig. 4. B: summarized data from 14 patients. CB-DMB decreased both thrombin-induced peak 1 and phase 2 [Ca\(^{2+}\)]\(_i\), compared with DMSO control. *\(P = 0.041\) and **\(P = 0.001\).
These data suggest that after activation with collagen, the platelet Na\(^+\)/Ca\(^{2+}\) exchanger functions in the reverse mode. This activity was further increased in the diabetic state.

**Effect of high glucose concentration on Ca\(^{2+}\) homeostasis in platelets from normal subjects.**

High concentrations of glucose in vivo in diabetic patients may have direct or indirect effects on platelets. Hyperglycemia may produce effects from the outside or inside of platelets. To investigate the direct intracellular effects of high glucose concentration, we needed to know whether the glucose transport in platelets was insulin sensitive. The influx rate of the nonmetabolizable glucose analog 3OMG was very rapid in the first 10 s of incubation in 0.6 mM 3OMG and reached steady state at 10 min (data not shown). This time course was consistent with the results of Kim and colleagues (22). Based on this time course we chose 10-s and 60-min time frames to study the effects of insulin on glucose transport. As shown in Fig. 8, there was no significant difference in the 3OMG influx in the control and in the presence of insulin (10 \(\mu\)U/ml).

To determine the acute effects of hyperglycemia, the dye-loaded platelets from normal subjects were suspended in medium with 5 or 45 mM glucose. Both the thrombin- and collagen-induced \([\text{Ca}^{2+}]_i\) responses of platelets in 5 and 45 mM glucose media were not significantly different (Fig. 9).

The prolonged effect of high glucose concentration was studied by incubating PRP for 24 h at 37°C in different glucose concentration. As shown in Figs. 10 and 11, prolonged exposure of platelets to high glucose concentration (45 mM) markedly enhanced the \([\text{Ca}^{2+}]_i\) response to thrombin and collagen compared with both controls (5 mM glucose or isosmolar mannitol). There were no significant differences in platelet \([\text{Ca}^{2+}]_i\) and aggregation between control and isosmolar control with high mannitol concentration. The platelet aggregation was increased after 24 h in high glucose concentration. After 24 h of hyperglycemia, the thrombin-induced aggregation rate was increased by 125 ± 14%. Collagen stimulation increased the rate of aggregation by 164 ± 17% in high glucose concentration compared with controls with isosmolar mannitol, and this in-
crease was found to be significantly different \((P = 0.014, n = 6)\).

Because we found that the platelet \(\text{Na}^+/\text{Ca}^{2+}\) exchanger is different in diabetes, it was thus important to assess whether the exchanger was also altered by hyperglycemia in vitro. Figure 12 shows the effects of CB-DMB in platelets from nondiabetics exposed to hyperglycemia for 24 h. CB-DMB significantly \((P = 0.04)\) inhibited the thrombin-induced \([\text{Ca}^{2+}]_{i}\) response, which was similar to the results in platelets from diabetic patients. These data suggest that the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger contributes in part to the enhanced thrombin-induced \([\text{Ca}^{2+}]_{i}\) response in platelets by hyperglycemia.

**DISCUSSION**

This study confirmed that in poorly controlled diabetic patients, platelets have increased basal \([\text{Ca}^{2+}]_{i}\) and larger agonist-stimulated \([\text{Ca}^{2+}]_{i}\) response compared with those in normal subjects. Furthermore, it was found that in diabetes, the direction and activity of the platelet \(\text{Na}^+/\text{Ca}^{2+}\) exchanger was altered. In addition, we report that prolonged hyperglycemia in vitro can induce platelet \(\text{Ca}^{2+}\) abnormality and hyperactivity similar to those seen in diabetic patients.

The results of studies reported in the literature that deal with platelet basal \([\text{Ca}^{2+}]_{i}\) in diabetes are conflicting. Our data are in agreement with the findings of Yamaguchi and colleagues (45), who found that platelet basal \([\text{Ca}^{2+}]_{i}\) was greater in diabetes than in con-
trols. Tschöpe and co-workers (43) reported that platelet [Ca\(^{2+}\)]\(_i\) in the basal condition and after stimulation with collagen (2 \(\mu\)g/ml) was increased in type II diabetics. Mazzanti and colleagues (29) demonstrated that platelet basal [Ca\(^{2+}\)]\(_i\) was significantly higher in diabetic patients with poor metabolic control (15 type I and 22 type II), and there was no difference between types I and II diabetic patients. In contrast to these studies, there are reports that have shown no difference in basal platelet [Ca\(^{2+}\)]\(_i\) between type II diabetes and controls (20, 21, 39). There are also reports that are in between these two types of conclusions: e.g., Pellegratta and co-workers (33) measured [Ca\(^{2+}\)]\(_i\) in 60 non-insulin-dependent diabetes mellitus patients, and as a whole group they found no difference between control subjects and diabetic patients. However, when they divided the diabetic patients into subgroups according to metabolic control and presence of complications, they found that the resting platelet [Ca\(^{2+}\)]\(_i\) was higher in patients with poor metabolic control (HbA1c > 8%). The inference from these published data and our results would be that metabolic control is an important factor that determines the platelet basal [Ca\(^{2+}\)]\(_i\) levels and there is no difference in this regard between the different types of diabetes.

The total agonist-stimulated [Ca\(^{2+}\)]\(_i\) response in platelets from diabetes was larger in the presence of external Ca\(^{2+}\). Phase 2 of the thrombin-stimulated [Ca\(^{2+}\)]\(_i\) response was greater in diabetes; however, the peak 1 response was not different. The thrombin-induced initial and transient [Ca\(^{2+}\)]\(_i\) spike (peak 1) reflects the discharge of Ca\(^{2+}\) from intracellular stores such as the DTS, whereas the sustained plateau (phase 2) reflects Ca\(^{2+}\) transport across the plasma membrane. Collagen has an activating mechanism that is different from thrombin and mainly depends on extracellular Ca\(^{2+}\). We have demonstrated here that the Na\(^+\)/Ca\(^{2+}\) exchanger contributes to the influx of Ca\(^{2+}\) during collagen activation of platelets. Our data with collagen also indicate that the abnormality mainly exists in the Ca\(^{2+}\) flux across the plasma membrane. Furthermore, the data in the absence of external Ca\(^{2+}\) clearly suggest that in platelets from diabetics, the release mechanism from the intracellular store is not altered. The intracellular store size was not different as estimated by the peak response to the maximum dose of ionomycin in the absence of extracellular Ca\(^{2+}\). Involvement of extracellular Ca\(^{2+}\) but not the intracellular stores has been documented by Bergh and colleagues (4), Mazzanti and co-workers (29), Tschöpe and colleagues (43), and Levy (27). In the DTS, Ca\(^{2+}\) pumps move Ca\(^{2+}\) against its concentration gradient, and saturation may limit the uptake by this compartment despite increased [Ca\(^{2+}\)]\(_i\) in diabetes. Cytosolic Ca\(^{2+}\) extrusion by the plasma membrane Na\(^+\)/Ca\(^{2+}\)-ATPase and the Na\(^+\)/Ca\(^{2+}\) exchanger is necessary for maintaining a low [Ca\(^{2+}\)]\(_i\) in nondiabetics; hence, in diabetes, a problem with one of these pathways will result in increased [Ca\(^{2+}\)]\(_i\). It has been shown by Mazzanti and colleagues (29) that in both type I and type II diabetic patients the platelet plasma membrane Na\(^+\)/Ca\(^{2+}\)-ATPase activity is increased compared with control subjects. These workers also demonstrated that there is a positive correlation between the platelet Ca\(^{2+}\) and the increase in Ca\(^{2+}\)-ATPase, which may be a compensatory mechanism. All of this led us to consider the Na\(^+\)/Ca\(^{2+}\) exchanger as the possible candidate contributing to abnormal Ca\(^{2+}\) homeostasis.

The Na\(^+\)/Ca\(^{2+}\) exchanger is a reversible carrier that can mediate the transport of Ca\(^{2+}\) across the plasma membrane in both directions. In most cells and situations the role of the Na\(^+\)/Ca\(^{2+}\) exchanger is to remove Ca\(^{2+}\) from the cell (forward mode); however, under some conditions the exchanger can mediate the net influx of Ca\(^{2+}\) (reverse mode). The net driving force for the exchange is the difference between the membrane potential and the reversal potential of the exchanger. Thus the activity of the Na\(^+\)/Ca\(^{2+}\) exchanger is determined by the Na\(^+\) gradient, the Ca\(^{2+}\) gradient, and the membrane potential. In the diabetic state, any of these factors could be abnormal. It is possible that an increase in platelet cytosolic Na\(^+\) occurs. It has been shown that platelet Na\(^+\)-K\(^-\)-ATPase activity is smaller in both type I and type II diabetes compared with control subjects, and Na\(^+\)-K\(^-\)-ATPase activity is inversely related to platelet [Ca\(^{2+}\)]\(_i\) (29). This supports our results, because a decrease in Na\(^+\)-K\(^-\)-ATPase activity can produce an increase in cytosolic Na\(^+\). This would decrease the activity of the forward mode (as in the resting state) and depolarize the platelet membranes. Because the membrane potential from the platelets of diabetics is yet to be determined, any theoretical calculations related to the exchanger are largely speculative. The reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger has been reported in other disease states. For example, in central nervous system anoxia/ischemia, most of the Ca\(^{2+}\) influx in the white matter is mediated by a reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (40). Even in certain normal physiological conditions, the Na\(^+\)/Ca\(^{2+}\) exchanger has been described as mediating Ca\(^{2+}\) entry, e.g., in cardiac cells (26) and lymphocytes (1).
The Diabetes Control and Complications Trials Research Group (10a) found that in insulin-dependent diabetes mellitus patients with retinopathy or nephropathy, there was a correlation between glycemic control and the development of chronic vascular complications. The mechanism underlying this observation is not known. Our data suggest that glucose per se can affect platelet Ca\(^{2+}\) homeostasis and behavior and thus could contribute to diabetic complications. We found that acute exposure of normal platelets to a pathological glucose concentration had no effect on the platelets, which is in agreement with the results of Pellegatta and colleagues (33). Therefore, acute hyperglycemia is probably not harmful to platelet behavior. In contrast, we found that when the platelets were exposed to a pathological glucose concentration for longer periods (24 h, 37°C), the thrombin- and collagen-induced [Ca\(^{2+}\)]i response and aggregation were enhanced. The effect of hyperglycemia was time dependent and specific for glucose. An isomolar manitol concentration did not mimic the effect of the high glucose concentration, indicating that the changes observed were not due to an osmotic effect of glucose. Cohen (7) has shown that in vitro exposure of the arteries to glucose concentrations of 400–800 mg/dl (22.2–44.4 mM) for a period of 3–6 h induced changes in arteries that were similar to those observed in arteries from diabetic rabbits, which had plasma glucose concentration of ~300 mg/dl (16.7 mM) for 6 wk. It has been shown that 45 mM glucose could affect the function of cultured endothelial cells (12) and aortic strips (41) within a few hours. Our data for platelets are consistent with reports for other cells. It has been found that high glucose concentration can increase [Ca\(^{2+}\)]i in normal vascular smooth muscle cells (2), human erythrocytes (35), pancreatic β-cells (3), and insulinoma cells (17). Despite all these observations, which suggest that glucose itself can alter [Ca\(^{2+}\)]i, the mechanisms by which glucose modulates Ca\(^{2+}\) homeostasis remain unclear. Our study shows that in the hyperglycemic condition, the Na\(^{+}\)/Ca\(^{2+}\) exchanger may mediate Ca\(^{2+}\) influx and be involved in the enhanced [Ca\(^{2+}\)]i in hyperglycemia. There is evidence for high glucose concentration-mediated inhibition of Na\(^{+}\)/K\(^{+}\)-ATPase (15) in the aorta. Similar to what occurs in diabetic patients, in vivo chronic hyperglycemia could decrease Na\(^{+}\)/K\(^{+}\)-ATPase and produce an increase in Na\(^{+}\) concentration, which in combination with a possible decrease in membrane potential may shift the Na\(^{+}\)/Ca\(^{2+}\) exchanger to the reverse mode, resulting in Ca\(^{2+}\) entry and elevation of [Ca\(^{2+}\)]. How hyperglycemia produces an inhibition of Na\(^{+}\)/K\(^{+}\)-ATPase is not known.

We found that the glucose transport in platelets is insulin insensitive. This finding was not surprising because Craik and co-workers (10) found that platelet glucose transport is the GLUT-3 (brain type), which is not insulin sensitive. If glucose transport is insulin insensitive, then intracellular glucose will accumulate inside platelets in the presence of a high extracellular glucose concentration. Intracellular glucose could affect platelets by the sorbitol-polyol pathway. The Michaelis-Menten constant of aldose reductase for glucose is high. When the intracellular glucose concentration is increased, the intracellular level of sorbitol could increase in the platelets due to the very slow degradation process (13) and produce adverse effects. For example, Na\(^{+}\)-K\(^{+}\)-ATPase inhibition in nerve fibers in hyperglycemic diabetic patients has been related to this mechanism, which can be prevented by aldose reductase inhibitors and by raising plasma myoinositol (37). In platelets the aldose reductase inhibitor 5-(3-thienyl)tetrazol-1-yl acetic acid monohydrate has been shown to reduce ADP-induced platelet hyperaggregation in streptozotocin-induced diabetic rats with neuropathy, suggesting that increased polyol pathway activity plays an important role in platelet aggregation in the development of diabetic neuropathy (16). Other possible mechanisms cannot be ruled out but await further evaluation.

Oral and intravenous glucose administration or in vitro glucose addition has been shown to increase platelet adhesion (6). There is evidence showing that platelet hyperactivity in diabetic patients is correlated with metabolic control in vivo. Some studies have shown that, after control of blood glucose in non-insulin-dependent diabetes mellitus patients, ADP and arachidonic acid-stimulated platelet hyperaggregation and other biochemical parameters can be reversed to normal (39). These in vitro effects of high glucose concentration and the corresponding studies in diabetic patients suggest that blood glucose level is an important factor in determining platelet activity.

In conclusion, this study shows that Ca\(^{2+}\) homeostasis is deranged in platelets from uncontrolled diabetic patients. This abnormality can be at least partly explained by alteration of the plasma membrane Na\(^{+}\)/Ca\(^{2+}\) exchanger activity. Prolonged hyperglycemia in vitro affects the Na\(^{+}\)/Ca\(^{2+}\) exchanger, alters Ca\(^{2+}\) homeostasis, and induces hyperactivity, suggesting that hyperglycemia per se is a plausible factor that can induce the platelet abnormalities observed in patients with diabetes.

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