Increased intracellular calcium mobilization in platelets from patients with Type 2 (non-insulin-dependent) diabetes mellitus

H. Ishii, F. Umeda, T. Hashimoto and H. Nawata

3rd Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan

Summary. Enhanced platelet functions have been reported in patients with diabetes mellitus. Our recent study demonstrated that phosphoinositide turnover is increased in platelets from diabetic patients. In the present study, we evaluated the abnormality in platelet intracellular calcium mobilization in patients with Type 2 (non-insulin-dependent) diabetes mellitus using fura-2, a fluorescent calcium indicator. Washed platelets were prepared from six diabetic patients with increased platelet aggregation rates (DM-A group), seven diabetic patients with normal platelet aggregation rates (DM-B group), and eight age-matched healthy control subjects. The basal intracellular free calcium concentrations in platelets were similar among the three groups. Thrombin (0.025–0.1 U/ml) induced a dose-dependent increase in intracellular calcium in both the presence and the absence of extracellular calcium. This increase in the presence of extracellular calcium, which depends on calcium influx and release, was significantly higher in the DM-A group than in the DM-B and control groups. However, there

was no significant difference between the control group and the DM-B group. In the absence of extracellular calcium, thrombin-induced calcium increase, which depends only on calcium release, was also significantly enhanced in the DM-A group. Furthermore, the calcium increase stimulated by platelet-activating factor (10 nmol/l) with and without extracellular calcium was significantly higher in the DM-A group than in the other groups. Additionally, calcium ionophore A23187 (100 nmol/l) caused a significantly higher calcium increase in the DM-A group with extracellular calcium, while the calcium increase without extracellular calcium showed no significant difference among the three groups. These observations suggest that enhanced intracellular calcium mobilization due to increased calcium influx and release may be closely related to platelet hyperfunctions in diabetes mellitus.

Key words: Calcium, platelet, diabetes mellitus, thrombin, platelet-activating factor, A23187.

Enhanced platelet functions have been demonstrated in subjects with diabetes mellitus. These platelet hyperfunctions involve increased platelet adhesiveness and aggregation, serotonin secretion, and prostaglandin synthesis in response to adenosine diphosphate, collagen, thrombin, and platelet-activating factor (PAF) in both platelet-rich plasma and washed platelet suspension [1–5]. However, there have been few studies on the abnormalities in platelet intracellular signal transduction in patients with diabetes mellitus.

Intracellular free calcium plays a major role in platelet signal transduction and causes platelet shape change [6, 7], aggregation [8], release of intracellular granules [9, 10], and prostaglandin synthesis [11]. Phosphoinositide breakdown generates inositol 1,4,5-trisphosphate [12] which induces calcium release from intracellular calcium pools [13]. Our recent study [14] demonstrated that phosphoinositide turnover is increased in platelets obtained from diabetic

patients. Therefore, the possibility that platelet intracellular calcium mobilization is altered in diabetes mellitus may arise. In the present study, we measured platelet intracellular free calcium concentrations ([Ca^{2+}]i) in patients with Type 2 (non-insulin-dependent) diabetes mellitus under basal conditions and following stimulation by thrombin, PAF and calcium ionophore, A23187 using a fluorescent calcium indicator fura-2.

Subjects and methods

Subjects

Thirteen patients with Type 2 diabetes and eight age- and sex-matched healthy control subjects were selected for the present study. Neither the control subjects nor the Type 2 diabetes patients had taken any antiplatelet agents such as aspirin for at least two weeks

Table 1. Clinical characteristics of the subjects

	Control subjects (n = 8)	DM-A (n = 6)	DM-B (n = 7)
Numbers			
Sex (M/F)	4/4	3/3	4/3
Therapy (insulin/drug[a]/diet)		1/2/3	2/2/3
Age (year)	51.3 ± 3.5	53.2 ± 3.1	50.9 ± 7.9
BMI (kg/m^2)	21.9 ± 1.5	22.6 ± 2.1	23.1 ± 2.2
Duration of diabetes (year)		9.0 ± 3.5	8.1 ± 1.9
Fasting blood glucose (mmol/l)	4.9 ± 1.0	13.7 ± 1.4[c]	11.7 ± 1.5[c]
HbA$_{1c}$ (%)		11.2 ± 0.8	9.5 ± 0.4
Platelet aggregation rate (%)[b]	38.5 ± 2.2	63.9 ± 3.7[c,d]	35.0 ± 4.5

Values are means ± SEM.
[a] Only glibenclamide was used as an oral hypoglycaemic agent;
[b] normal laboratory range: 18.6–46.4%;
[c] $p < 0.01$ vs control group;
[d] $p < 0.01$ vs DM-B group.
DM-A = Diabetic patients with increased platelet aggregation rates;
DM-B = Diabetic patients with normal platelet aggregation rates

before the study. The patients with Type 2 diabetes were divided into two experimental groups, DM-A group and DM-B group, on the basis of platelet aggregation rate induced by 0.3 U/ml of human thrombin. The DM-A group included the diabetic patients with platelet aggregation rates over 46.4%, which is the upper limit of the normal range of the platelet aggregation rate (mean + 2SD) in our laboratory. The DM-B group included the patients with platelet aggregation rates less than 46.4%. The clinical characteristics of the subjects are summarized in Table 1. There were no significant differences in age, BMI, duration of diabetes, fasting blood glucose levels or HbA$_{1c}$ levels between the DM-A group and the DM-B group.

Platelet aggregation rate

The platelet aggregation rate was determined as previously reported [4]. After overnight fasting, blood was drawn by venous puncture with sodium citrate at a final concentration of 12 mmol/l. The blood sample was immediately centrifuged at 160 x g for 10 min to obtain platelet-rich plasma (PRP). The rest of the blood was recentrifuged at 1500 x g for 10 min to obtain platelet-poor plasma (PPP). The platelet count of PRP was adjusted to 2 x 10^8 cells/ml using autologous PPP. Platelet aggregation was achieved according to Born's turbidimetric method [15] with an Auto RAM-21 aggregometer (Rikadenki Kogyo Co. Ltd., Tokyo, Japan) and recorded with an electronic recorder R-12 (Rikadenki Kogyo Co. Ltd.). Briefly, 250 µl of PRP was stirred magnetically at 1,100 rev/min (37°C). After 1 min of pre-incubation, 0.3 U/ml of human thrombin (Sigma, St. Louis, Mo., USA) was added, and the change in light transmission was recorded. Assuming that the PPP represented 100% aggregation and the PRP represented 0% aggregation, the maximal aggregation within 1 min after the addition of thrombin was determined as the platelet aggregation rate.

Measurement of [Ca^{2+}]i

After overnight fasting, blood was drawn from the antecubital vein with acid citrate dextrose (one volume to six volumes of blood). PRP was obtained by centrifuging the blood at 160 x g for 10 min. The platelets in PRP were incubated with 2 µmol/l fura-2 acetoxymethyl ester (Dojin Chemical Laboratory, Kumamoto, Japan), a fluorescent calcium indicator, for 30 min at 37°C. The PRP was then centrifuged at 800 x g for 10 min, and the platelet pellet was suspended at 2 x 10^8 cells/ml in calcium-free buffer (135 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l MgCl$_2$, 5.5 mmol/l glucose, 20 mmol/l HEPES, pH 7.4). The extracellular calcium concentration was adjusted by adding 1 mmol/l CaCl$_2$ for 5 min at 37°C. This incubation time was chosen because calcium mobilization increased following the addition of CaCl$_2$ and

reached a stable level within 2–4 min which persisted for at least 30 min. Fura-2 fluorescence was measured with a spectrofluorophotometer RF-5000 (Shimadzu, Kyoto, Japan) in a thermostated (37°C) quartz cuvette at 340 nm and 380 nm excitation wavelengths and a 500 nm emission wavelength. Platelets were then stimulated by addition of thrombin (0.025–0.1 U/ml), PAF (10 nmol/l; Sigma) or A23187 (100 nmol/l; Sigma), and the changes in fluorescence were continuously recorded. In parallel experiments, 5 mmol/l EGTA was added to platelet suspension containing 1 mmol/l CaCl$_2$ just before addition of each stimulant. The [Ca^{2+}]i was calculated as described previously using a calcium/dye dissociation constant for fura-2 of 224 nmol/l [16].

Statistical analysis

To test for differences between the three groups, analysis of variance was used. If significant differences were found, Student's unpaired t-test was used for comparison between the groups. The correlations between changes in [Ca^{2+}]i and epidemiological variables were evaluated by linear-regression analysis. All p values less than 0.05 were accepted as statistically significant.
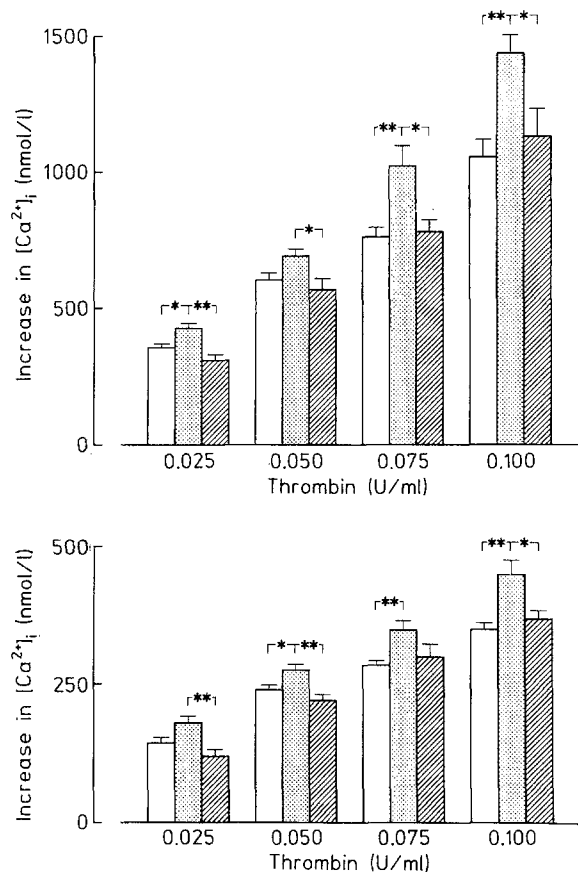
Results

There was no significant difference in platelet [Ca^{2+}]i under basal conditions among the control group (77 ± 2 nmol/l, mean ± SEM), the DM-A group (75 ± 2 nmol/l), and the DM-B group (79 ± 2 nmol/l). These basal [Ca^{2+}]i values were greater than those in our recent report (about 100 nmol/l) [14]. The difference of the [Ca^{2+}]i may have resulted from the difference of platelet count between the studies (2 and 5 x 10^8 cells/ml). Stimulation by thrombin in the presence of extracellular calcium (1 mmol/l CaCl$_2$) induced a rapid and marked increase in platelet [Ca^{2+}]i, which depends on calcium influx from extracellular space and calcium release from intracellular calcium pools. The [Ca^{2+}]i reached a maximal level within 20–30 s after addition of thrombin (Fig. 1). By increasing the thrombin concentrations from 0.025 to 0.1 U/ml, a dose-dependent increase in [Ca^{2+}]i was observed in the control, DM-A, and DM-B groups. This [Ca^{2+}]i increase was significantly higher in the DM-A group than in the DM-B group at all concentrations tested
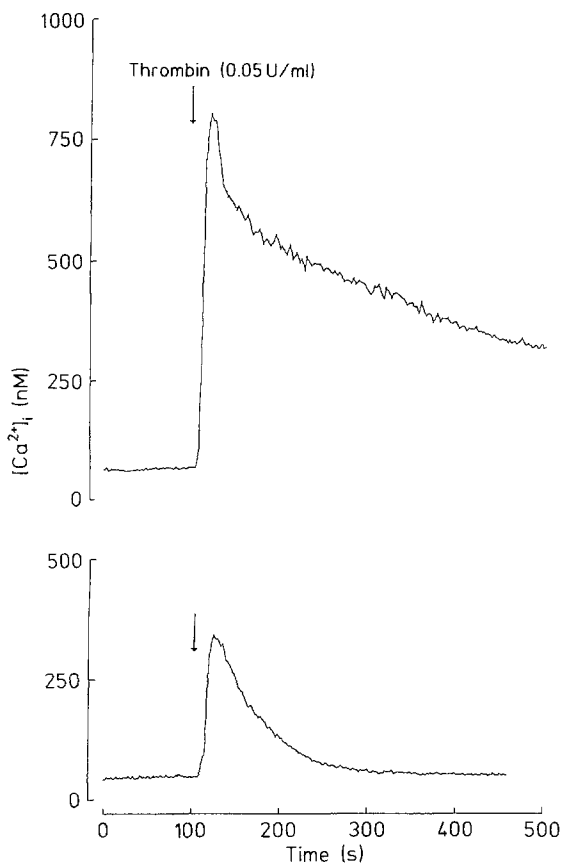
Fig. 1. Representative traces of changes in platelet intracellular free calcium concentrations ([Ca^{2+}]i) induced by thrombin. Fura-2-loaded washed platelets were stimulated with 0.05 U/ml of thrombin in the presence of 1 mmol/l $CaCl_2$ (top panel) or 5 mmol/l EGTA (bottom panel). Changes in fura-2 fluorescence intensity were recorded at 340 nm and 380 nm excitation wavelengths and a 500 nm emission wavelength. The [Ca^{2+}]i was calculated using a calcium/dye dissociation constant for fura-2 of 224 nmol/l [16]. The traces were observed in platelets from one of the diabetic subjects with increased platelet aggregation rate

Fig. 2. Thrombin-induced increase in intracellular free calcium concentrations ([Ca^{2+}]i) in the presence of 1 mmol/l $CaCl_2$ (top panel) or 5 mmol/l EGTA (bottom panel) in washed platelets from the control group ($n = 8$; open columns), the diabetic patients with increased platelet aggregation rates (DM-A) group ($n = 6$; dotted columns), and the diabetic patients with normal platelet aggregation rates (DM-B) group ($n = 7$; hatched columns). The increase in [Ca^{2+}]i was calculated by subtracting the basal [Ca^{2+}]i from the maximal [Ca^{2+}]i induced by thrombin. All experiments were performed in duplicate. Values are means ± SEM. * $p < 0.05$; ** $p < 0.01$

and in the control group at all concentrations except for 0.05 U/ml (Fig. 2). However, there were no significant differences between the control group and the DM-B group. When thrombin was added in the presence of 5 mmol/l EGTA in order to reduce extracellular calcium to negligible concentration (approximately 14 nmol/l), the platelet [Ca^{2+}]i increase, which depends only on calcium release from intracellular pools, was rapid but much lower (Fig. 1). The [Ca^{2+}]i increase in the DM-A group was also significantly higher than that in the control group at all concentrations tested except for 0.025 U/ml and that in the DM-B group except for 0.075 U/ml (Fig. 2).

Stimulation by PAF in the range of 2.5 to 200 nmol/l produced a dose-dependent increase in [Ca^{2+}]i with the maximal plateau observed at 10 nmol/l. Therefore, we chose the concentration of 10 nmol/l for the subsequent stimulation studies by PAF. The [Ca^{2+}]i increase induced by PAF was significantly higher in the DM-A group than in the other groups in both the presence and the absence of extracellular calcium (Fig. 3). Finally, A23187 (100 nmol/l) also caused a significantly greater [Ca^{2+}]i increase in the DM-A group than in the other groups in the

presence of extracellular calcium. However, in the absence of extracellular calcium, there was no significant difference among the three groups (Fig. 4).

In addition, correlations were examined between the [Ca^{2+}]i increase stimulated by thrombin, PAF, or A23187 and the epidemiological factors. However, there were no significant correlations between the [Ca^{2+}]i increase and age, BMI, duration of diabetes, fasting blood glucose levels, or HbA_{1c} levels.

Discussion

In the present study, we measured platelet [Ca^{2+}]i in the healthy control subjects, the Type 2 diabetic patients with increased platelet aggregation rates (DM-A group), and the Type 2 diabetic patients with normal platelet aggregation rates (DM-B group). Although basal [Ca^{2+}]i values were similar among the three groups, stimulation by thrombin and PAF induced a significantly greater [Ca^{2+}]i increase in the DM-A group than in the other groups, when the agonists were added in the absence of extracel-
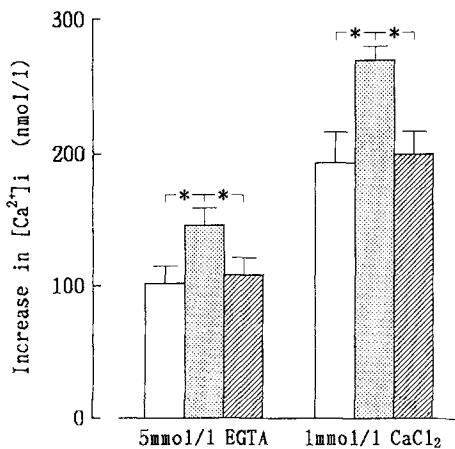
Fig. 3. Platelet activating factor (PAF)-induced increase in intracellular free calcium concentrations ($[Ca^{2+}]i$) in the presence of 1 mmol/l $CaCl_2$ or 5 mmol/l EGTA in washed platelets from the control group ($n = 8$; open columns), the diabetic patients with increased platelet aggregation rates (DM-A) group ($n = 6$; dotted columns), and the diabetic patients with normal platelet aggregation rates (DM-B) group ($n = 7$; hatched columns). Increase in $[Ca^{2+}]i$ was calculated by subtracting the basal $[Ca^{2+}]i$ from the maximal $[Ca^{2+}]i$ induced by PAF. All experiments were performed in duplicate. Values are mean ± SEM. * $p < 0.05$
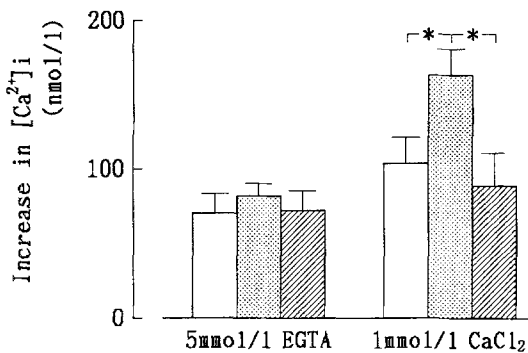
Fig. 4. A23187-induced increase in intracellular free calcium concentrations ($[Ca^{2+}]i$) in the presence of 1 mmol/l $CaCl_2$ or 5 mmol/l EGTA in washed platelets from the control group ($n = 8$; open columns), the diabetic patients with increased platelet aggregation rates (DM-A) group ($n = 6$; dotted columns), and the diabetic patients with normal platelet aggregation rates (DM-B) group ($n = 7$; hatched columns). Increase in $[Ca^{2+}]i$ was calculated by subtracting the basal $[Ca^{2-}]i$ from the maximal $[Ca^{2+}]i$ induced by A23187. All experiments were performed in duplicate. Values are means ± SEM. * $p < 0.05$

lular calcium to exclude calcium influx from the extracellular space. These observations suggest that calcium release from intracellular calcium pools may be enhanced in the DM-A group. Both thrombin and PAF induce phosphoinositide breakdown to produce inositol 1, 4, 5-trisphosphate [12, 17], which causes calcium release from intracellular pools [13]. Our recent study [14] indicated that thrombin-induced phosphoinositide turnover is increased in platelets from Type 2 diabetic patients associated with enhanced platelet aggregation rates. From this evidence, the enhanced $[Ca^{2+}]i$ increase in the absence of extracellular calcium may be attributed to the increased phosphoinositide breakdown. An alternative mechanism is that calcium contents in intra-platelet calcium pools may be increased in the hyperfunctioning platelets in diabetes mel-

litus. However, this seems unlikely, since, in the absence of extracellular calcium, there was no significant difference among the three groups in the $[Ca^{2+}]i$ increase induced by calcium ionophore A23187, which directly mobilizes calcium from intracellular pools [18] without provoking phosphoinositide breakdown [19].

Thrombin and PAF are known to cause calcium influx from the extracellular space [20–22]. In the presence of extracellular calcium, we demonstrated that thrombin and PAF induced a 2- to 3-fold increase in $[Ca^{2+}]i$ compared with those in the absence of extracellular calcium and that these $[Ca^{2+}]i$ increases were significantly enhanced in the DM-A group, suggesting that calcium influx may also be increased in platelets from the DM-A group. The detailed mechanisms behind calcium influx stimulated by thrombin or PAF are not fully understood. Recently, Zschauer et al. [23] reported the platelet calcium channel activated by thrombin stimulation. The increased calcium influx in the DM-A group may be due to increased activation of the calcium channel. We also showed that A23187 induced significantly greater $[Ca^{2+}]i$ increase in the presence of extracellular calcium in the DM-A group. However, it is not likely that A23187-induced calcium influx was enhanced, because A23187 causes calcium influx through permeabilizing platelets, not by opening the calcium channel. The mechanism of the enhanced $[Ca^{2+}]i$ increase evoked by A23187 in the presence of extracellular calcium remains to be determined.

Previous studies [2, 24] have shown that platelet aggregability is positively correlated with fasting blood glucose levels. We have also reported a significant correlation between platelet phosphoinositide breakdown induced by thrombin and fasting blood glucose levels [14]. However, in the present study, we could not find any significant differences between the $[Ca^{2+}]i$ increase and the fasting blood glucose levels or other epidemiological factors. The enhanced platelet $[Ca^{2+}]i$ increase in the DM-A group may be due to the original abnormalities in platelets themselves rather than to the effect of epidemiological factors. Further studies are needed to clarify the mechanisms of the abnormal platelet calcium mobilization in patients with diabetes mellitus.

In conclusion, thrombin and PAF caused significantly enhanced $[Ca^{2+}]i$ increase in platelets from Type 2 diabetic patients with increased platelet aggregation rates in both the presence and the absence of extracellular calcium. A23187-induced $[Ca^{2+}]i$ increase was also enhanced in these patients in the presence, but not the absence, of extracellular calcium. These results suggest that the increased intracellular calcium mobilization may be closely related to the enhanced platelet functions in diabetes mellitus and that this increased calcium mobilization may be due to the increased calcium release and influx.

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Dr. H. Ishii
3rd Department of Internal Medicine
Faculty of Medicine
Kyushu University
3-1-1 Maidashi, Higashi-ku, Fukuoka 812
Japan