Increased Intracellular Calcium and Altered Phorbol Dibutyrate Binding to Intact Platelets in Young Subjects With Insulin-Dependent and Non–Insulin-Dependent Diabetes Mellitus

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Intracellular calcium ($[Ca^{2+}]i$) and phorbol ester binding were studied in intact platelets of young patients with insulin-dependent (IDDM) and non–insulin-dependent (NIDDM) diabetes mellitus. Our objective was to evaluate disturbances in calcium regulation and signal transduction in platelets of diabetics. $[Ca^{2+}]i$ in platelets of the IDDM group (135 ± 20 nmol/L) under basal conditions was significantly higher than that of the control group (81 ± 8 nmol/L, $P = .019$), whereas at 60 seconds after stimulation with 0.1 National Institutes of Health (NIH) U/mL thrombin, $[Ca^{2+}]i$ in the NIDDM group (484 ± 36 nmol/L) was significantly higher than that of the controls (347 ± 22 nmol/L, $P = .003$) and IDDM group (360 ± 45 nmol/L, $P = .04$), respectively. Phorbol 12,13-dibutyrate (PdBu) maximal binding capacity (Bmax) in the IDDM group was significantly lower than that in the control group either under basal conditions or after stimulation with thrombin ($P = .0034$ and $P = .015$, respectively). Bmax in the NIDDM group was significantly lower than that in the controls only after stimulation with thrombin ($P = .047$). The K_d for PdBu of the IDDM group was lower than that of the control group under basal conditions ($P = .017$). When analyzing the pooled data of all subjects, a significant correlation was observed between Bmax and K_d (under basal conditions, $r = .544$, $P < .0001$; after stimulation, $r = .601$, $P < .0001$). Our results support the idea that the increased affinity for PdBu may compensate for the decreased binding capacity. We interpret the data as indicating that the change in the binding of phorbol ester to protein kinase C (PKC) units may result in an altered PKC/calcium interaction in the pathogenesis of diabetes mellitus. Our study indicates that such metabolic derangements of $[Ca^{2+}]i$ have already been developing in young diabetic patients.

THE PATHOPHYSIOLOGY OF DIABETES mellitus is characterized by insufficient insulin action, either as an absolute deficiency as in insulin-dependent diabetes mellitus (IDDM) or as a relative deficiency and insulin resistance as in non–insulin-dependent diabetes mellitus (NIDDM). Some groups reported defects of ion transport or of the signal transduction pathway in IDDM or NIDDM.[1,2] Regulation and dysregulation can occur at different levels of the insulin signal transduction cascade.[3] At the receptor level, serine/threonine phosphorylation of the β-subunit appears to reduce tyrosine kinase activity.[4] Although the insulin receptor has been extensively studied, the signal transduction at the postkinase level is not yet understood in detail. There is evidence that protein kinase C (PKC) is involved in this modulation of signal transduction.[5,6] PKC, the calcium/phospholipid-dependent protein kinase, plays a key role in mediating signals generated by hormones, growth factors, and neurotransmitters.[7] An increase in intracellular calcium ($[Ca^{2+}]i$) is important for the activation of PKC.[7,8] $[Ca^{2+}]i$ plays a vital role in the regulation of many physiological events.[9,10] Herein, we intend to verify the hypothesis that an altered $[Ca^{2+}]i$ metabolism plays an essential role in the pathophysiology of diabetes and its complications.[11] Both IDDM and NIDDM induce similar complications, including angiopathy, neuropathy, cataracts, and nephropathy.[11-13] These complications are associated with metabolic derangements common to both diabetic conditions. Activated platelets may play an important role in the increased vascular and thrombotic risks of diabetic patients.[14,15] We focus on diabetic platelet $[Ca^{2+}]i$ and phorbol ester binding, which have been commonly used to study PKC.[16] Phorbol esters, potent activators of PKC, presumably bind to the same site in the regulatory domain as diacylglycerol (DAG).[17] Moreover, [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PdBu)-binding sites are present in platelets.[16,18]

Our study aims to evaluate disturbances of calcium regulation and signal transduction in platelets of diabetics. In parallel, we observed alterations in the binding of phorbol esters in diabetic children and adolescents whose major metabolic derangements have not yet taken place.

SUBJECTS AND METHODS

Subjects

Eighteen IDDM patients (10 boys and eight girls, aged 16.1 ± 1.1 years; mean years after onset, 6.0 ± 0.9) and 23 NIDDM patients (nine boys and 14 girls, aged 14.2 ± 0.5 years; mean years after onset, 1.3 ± 0.5) were investigated. Diabetes was defined using National Diabetes Data Group criteria,[19] and NIDDM was defined on the basis of the presence of measurable insulin concentrations, negative islet cell antibody titers, and the ability of diabetic subjects to avoid ketoacidosis without insulin treatment. Slowly progressive IDDM patients who had been treated with insulin within the previous 24 months were excluded from the NIDDM group. Islet cell antibody content was measured in 10 of 23 patients with NIDDM, in whom it was negative. Obesity was expressed as a body mass index (BMI), ie, weight in kilograms divided by the square of the height in meters, greater than 25. IDDM patients were on therapy with subcutaneous insulin, the last dose given 10 hours before blood sampling. The control group consisted of 21 subjects (10 boys and 11 girls, aged 15.5 ± 1.4 years) with normal blood pressure and a negative family history of diabetes mellitus. Control and NIDDM subjects were not treated with any medication, including insulin, and did not show any evidence of endocrine malfunction or recent use of drugs that might potentially alter electrolyte balance. Diabetic patients were free from late complications of diabetes as evaluated by means of

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history, clinical examination, funduscopy, electrocardiography, Doppler ultrasonography of large vessels, renal clearance, proteinuria and microalbuminuria, and tests for the detection of autonomic neuropathy. Informed consent was obtained from the subjects or their parents.

Reagents

Fura 2-AM was obtained from Dojin Chemicals (Kumamoto, Japan). Thrombin was obtained from Sigma (St Louis, MO). [^{3}H]PdBu (13.2 Ci/mmol; NET-692) was obtained from NEN Research Products (Boston, MA). The radioactive scintillation liquid used was Biofluor (NEN Research Products). The glass membrane filters were Whatman GF/C filters (no. 1822025; Whatman International, Maidstone, Kent, UK). All other reagents were obtained from commercial sources.

Platelet Preparation

Platelets were isolated as previously described.[20] After a fast of at least 12 hours, 15 mL blood was drawn into 3.8% (wt/vol) acid citrate buffer (10:1 vol/vol). Blood was centrifuged at 200 × *g* for 10 minutes at room temperature. Platelet-rich plasma was decanted and centrifuged at 1,000 × *g* for 10 minutes, and the cells were washed three times in HEPES-buffered solution (HBS) containing (mmol/L) NaCl 140, KCl 5, glucose 10, HEPES 10 (pH 7.4), and EGTA 0.2. EGTA was omitted from the third washing, and 0.1% fatty acid–free bovine serum albumin (BSA) was added. Platelets were counted in a Celltac counter (Nihon Kohden, Tokyo, Japan). Platelets were studied within 4 hours after blood sampling.

[Ca^{2+}]i Measurement

Unless otherwise indicated, platelets were suspended at a concentration of 2 to 3 × 10^7/mL in HBS plus 1 mmol/L $CaCl_2$ and 0.1% BSA. Platelets were incubated with 5 μmol/L fura 2-AM for 30 minutes at 37°C in HBS with 1 mmol/L $CaCl_2$. [Ca^{2+}]i under basal conditions and after stimulation with 0.1 National Institutes of Health (NIH) U/mL thrombin was monitored under constant stirring in a fluorescence spectrophotometer (model F-2000; Hitachi, Tokyo, Japan). Excitation wavelength was set at 340/380 nm and emission at 505 nm. [Ca^{2+}]i was estimated by the calibration procedure of Grynkiewicz et al.[21]

[^{3}H]PdBu Binding to Intact Platelets

Platelets were preincubated for 30 minutes at 37°C with or without thrombin. For determination of [^{3}H]PdBu binding, 230 μL platelet suspension was mixed in an Eppendorf microcentrifuge tube with 20 μL [^{3}H]PdBu and unlabeled PdBu (final concentration, 10 nmol/L [^{3}H]PdBu and up to 640 nmol/L unlabeled PdBu). To minimize the possibility of internalization of PdBu, all binding experiments were performed at 0°C (on ice) for 15 minutes. At termination of the binding experiments, samples were rapidly filtered on Whatman GF/C filters presoaked with modified Tyrode buffer containing (mmol/L) NaCl 130, KCl 5, NaH_2PO_4 1, $NaHCO_3$ 24, glucose 10, sucrose 12.5, and EDTA 4, plus 0.1% BSA. Cells were rapidly washed four times with 1 mL of the same ice-cold buffer. Filters were allowed to dry and were then counted for radioactivity in Biofluor. Nonspecific binding was determined in the presence of 10 μmol/L PdBu, which comprised 7% to 9% of the total binding.

Analytical Methods

Biochemical parameters were measured by standard methods. Plasma insulin level was determined by a commercial double-antibody solid-phase radioimmunoassay (Phadeseph Insulin; Pharmacia Diagnostics, Uppsala, Sweden). Hemoglobin A_{1c} (HbA_{1c}) level was measured by high-performance liquid chromatography according to the method of Akai.[22] 1,5-Anhydroglucitol was determined[23] using high-performance liquid chromatography. Fructosamine content was measured colorimetrically according to the method of Johnson et al.[24]

Data Analysis

Every assay was performed in duplicate. Computation of the binding parameters was performed using the equation,[25] $B = Bmax \times [L/K_d \times [1 + (i/K_d)^h] + L]$, where Bmax is the maximal binding capacity (expressed as molecules or specific binding sites per platelet), B is the binding capacity, L is the concentration of [^{3}H]PdBu, i is the concentration of unlabeled PdBu, and h is the Hill coefficient. The Bmax of PdBu binding reflects the number of PKC receptors. K_d is an inverse measure of binding affinity (low K_d indicates high affinity).

The unpaired *t* test was used to compare mean group values. Regression analysis was performed according to standard methods. Between-group differences in mean [Ca^{2+}]i were assessed with ANOVA. Statistical significance is assumed for *P* less than .05. Data are presented as the mean ± SEM.

RESULTS

Clinical characteristics of the study subjects are shown in Table 1. No statistical differences among the groups were observed for age and gender distribution. BMI was significantly higher in the NIDDM group compared with the control group, but not all NIDDM subjects were obese (15 obese and eight non-obese). As there were no significant gender-related differences among the three groups, data for boys and girls were combined. Systolic and diastolic blood pressure were not significantly different among the three groups. Overnight fasting glucose in the IDDM and NIDDM groups was significantly higher than in the controls. Fasting plasma insulin in the NIDDM group was slightly higher than in the other two groups, although not significantly.

Cytosolic Free Calcium

[Ca^{2+}]i of the IDDM group (135 ± 20 nmol/L) under basal conditions was significantly higher than in the controls (81 ± 8 nmol/L, $P = .012$; Fig 1). [Ca^{2+}]i of the NIDDM group (106 ± 12 nmol/L) under basal conditions was not significantly different. However, in the NIDDM group, [Ca^{2+}]i (484 ± 36 nmol/L) at 60 seconds after stimulation with 0.1 NIH U/mL thrombin was significantly higher than in the control group (347 ± 22 nmol/L, $P = .003$) and IDDM group (360 ± 45

Table 1. Clinical Characteristics of the Subjects

Characteristic	Controls (n = 21)	IDDM (n = 18)	NIDDM (n = 23)
Age (yr)	15.5 ± 1.4	16.1 ± 1.1	14.2 ± 0.5
Male/female	10/11	10/8	9/14
BMI (kg/m^2)	20.9 ± 0.7	20.3 ± 0.6	27.8 ± 1.0†
Systolic blood pressure (mm Hg)	109 ± 3	111 ± 4	115 ± 3
Diastolic blood pressure (mm Hg)	70 ± 3	67 ± 3	73 ± 3
Fasting plasma glucose (mg/dL)	82 ± 2	201 ± 28†	132 ± 17*
Fasting plasma insulin (μU/mL)	12.4 ± 2.7	6.4 ± 0.7	22.1 ± 8.3
Total cholesterol (mg/dL)	131 ± 6	168 ± 11*	164 ± 8*
Triglyceride (mg/dL)	73 ± 8	97 ± 24	83 ± 11
HbA_{1c} (%)	ND	10.1 ± 0.5	8.7 ± 0.6
Fructosamine (μmol/L)	223 ± 14	393 ± 18†	341 ± 21*
1,5-Anhydroglucitol (μg/mL)	16.9 ± 1.8	2.8 ± 0.5†	9.4 ± 3.3

NOTE. Values are the mean ± SEM. Normal range of HbA_{1c} is 4.0% to 6.0%; normal range of plasma insulin is <17 μU/mL.

Abbreviation: ND, not determined.

*$P < .01$, †$P < .005$: *v* control.

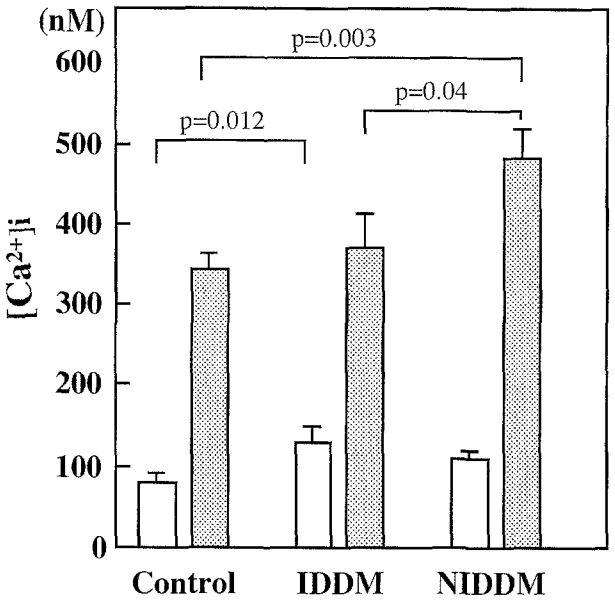

Fig 1. Platelet [Ca2+]i of control, IDDM, and NIDDM patients basally (□) and 60 seconds after stimulation with 0.1 NIH U/mL thrombin (▨).

nmol/L, $P = .04$). The percent increase of [Ca2+]i in the IDDM group (308% ± 37%) was lower than in the control group (538% ± 85%, $P = .024$). The percent increase of [Ca2+]i in the NIDDM group (452% ± 53%) showed no significant difference.

PdBu Binding

Table 2 shows the PdBu binding parameters of platelets under basal conditions and after stimulation with thrombin. PdBu Bmax in the IDDM group was significantly lower than in the controls either under basal conditions or after stimulation. Bmax in the NIDDM group was significantly lower than in the controls only after stimulation. The K_d for PdBu of the IDDM group was lower than for the controls under basal conditions ($P = .017$). No significant difference was observed for the Hill coefficient (h) among the three groups under either basal or stimulated conditions.

In analyzing the pooled data for all subjects, a significant correlation was observed between Bmax and K_d under basal conditions and after stimulation ($r = .544$ and $r = .601$, respectively, in each state, $P < .0001$; Fig 2A and B). The percent

Table 2. Binding Parameters of PdBu to Platelets

Group	$B_{max} \times 10^{-4}$ (molecules per platelet)	K_d (nmol/L)	Hill Coefficient
Control			
Basal	3.07 ± 0.50	45.5 ± 8.6	1.05 ± 0.08
+Thrombin	2.59 ± 0.43	46.9 ± 8.3	1.10 ± 0.09
IDDM			
Basal	1.26 ± 0.23†	18.8 ± 5.4*	1.00 ± 0.10
+Thrombin	1.27 ± 0.21*	36.4 ± 11.3	1.00 ± 0.09
NIDDM			
Basal	2.15 ± 0.33	43.3 ± 7.3	0.99 ± 0.07
+Thrombin	1.51 ± 0.31*	33.9 ± 6.8	0.99 ± 0.09

*$P < .05$, †$P < .005$: *v* control.

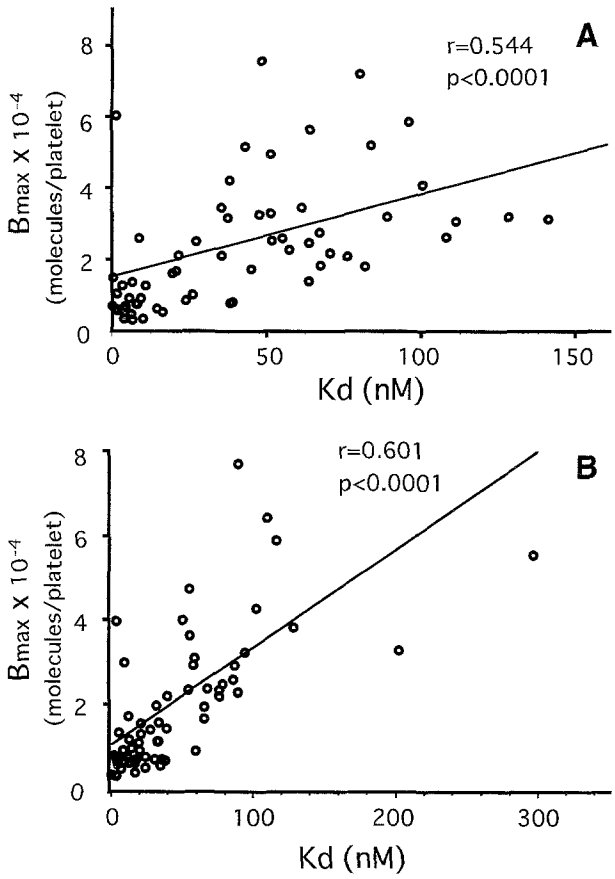

Fig 2. Correlation between Bmax and K_d of PdBu binding to platelets basally (A) and after stimulation with 0.1 NIH U/mL thrombin (B).

increase of [Ca2+]i was correlated with the K_d of the basal state ($P = .0036$). There was no sex difference for [Ca2+]i or PdBu binding in all the groups together or in the subgroup.

In the pooled data for all subjects, BMI was correlated with both systolic and diastolic blood pressure ($P < .005$). No significant correlation was found between BMI and [Ca2+]i with or without stimulation or between BMI and any PdBu binding parameters.

DISCUSSION

In this study, we evaluated [Ca2+]i and derangements in phorbol ester binding of intact platelets in young diabetic patients. [Ca2+]i was high both in the basal state and after stimulation with thrombin in IDDM patients, whereas it was high only after stimulation with thrombin in NIDDM patients. The high [Ca2+]i might account for the hyperreactivity to stimuli and hypercoagulability observed in diabetic platelets.[14,15] Homeostasis of [Ca2+]i appears to be impaired in both types of diabetes, and this impairment may be differently expressed. In the IDDM group, a percent increase over the resting level in the presence of thrombin was lower than in the other two groups. The higher [Ca2+]i in the NIDDM group after stimulation may be correlated with insulin resistance.[26] The biochemical defects causing changes in [Ca2+]i metabolism in the platelets of diabetics are not clear.

Reportedly, the activity of Ca2+-adenosine triphosphatase

was increased in diabetic patients.[27] Several reports have shown that $[Ca^{2+}]i$ in both types of diabetes was higher than in control subjects either under basal conditions or after stimulation.[27,28] $[Ca^{2+}]i$ was not high under basal conditions in the younger group in our study. This is because the disease process in our NIDDM group may not have reached the complete state.[29,30] Therefore, residual cells could still maintain $[Ca^{2+}]i$ regulation. The discrepancy in $[Ca^{2+}]i$ between NIDDM and IDDM groups in our study may also be due to the degree of dysregulation. The duration of the disease after the onset of IDDM is longer than for NIDDM. Therefore, the disease process might have been more advanced.

A number of reports showed that obesity induces hypertension[31] and that blood pressure is related to $[Ca^{2+}]i$.[32] In the pooled data for all subjects, BMI was correlated with both systolic and diastolic blood pressure but not with $[Ca^{2+}]i$ with or without stimulation. We consider that hypertension is advanced in obese subjects. Augmented PKC activity and calcium dysregulation could underlie the diabetic state.[33]

Binding of PdBu to intact cells depends on several factors, ie, induction of condensation of PKC units, $[Ca^{2+}]i$, and DAG in the plasma membranes, as well as the affinity of phorbol ester for the DAG binding site. Previously, we reported that platelet membranes have Ca^{2+}-sensitive and Ca^{2+}-insensitive populations of PdBu binding sites and that an increase in $[Ca^{2+}]i$ induces PKC affinity for PdBu.[20] The results in the IDDM group are consistent with our previous report, ie, $[Ca^{2+}]i$ under basal conditions was significantly higher and K_d was decreased. These observations in platelets of subjects with both types of diabetes suggest that increased $[Ca^{2+}]i$ downregulates PKC receptors and increases the binding affinity of the remaining receptors for phorbol esters in diabetic patients. There are several PKC isoforms,[7] and these isoforms may have different binding affinity for phorbol esters.[34] Among the pooled data for all subjects, a significant correlation was observed between Bmax and K_d both under basal conditions and after stimulation. The correlation between Bmax and K_d suggests that the downregulated Bmax was compensated for by the increased affinity for PdBu. In the NIDDM group, no significant differences in binding parameters were observed in the basal state. After stimulation that induces elevation of $[Ca^{2+}]i$, these parameters in NIDDM were amplified. This finding suggests that NIDDM is a hyperreactive state concerning the regulation of $[Ca^{2+}]i$. The increased affinity of the binding sites, presumably PKC units, for PdBu in both types of diabetes may play an essential role in the pathophysiology of diabetes and its complications.

In conclusion, the present study of platelet $[Ca^{2+}]i$ and PdBu binding shows that the increased $[Ca^{2+}]i$ and elevation in affinity for PdBu may compensate for the decreased density of membrane-bound PKC receptors. The defects of $[Ca^{2+}]i$ metabolism are ubiquitous in both types of diabetes and are responsible for the development of diabetic complications. It is therefore possible that these derangements have already been developing in young diabetic patients.

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