Deranged Platelet Calcium Homeostasis in Poorly Controlled IDDM Patients

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OBJECTIVE — Platelet hyperfunction frequently occurs in IDDM. As in many other cellular systems, cytosolic free Ca plays a key role in platelet activation.

RESEARCH DESIGN AND METHODS — We measured cytosolic free Ca concentration ($[Ca^{2+}]i$) by means of the fluorescent probe fura-2 in 60 IDDM patients (mean age 30.8 yr, range 18–50 yr) and in 31 age-matched healthy control subjects. Platelets were studied in both resting conditions and after stimulation with thrombin at 0.05, 0.1, and 0.5 U/ml.

RESULTS — No differences were noted between control subjects and diabetic patients, as a whole. Patients with a poor metabolic control (HbA_{1c} >8%) had significantly ($P < 0.01$ and $P < 0.03$) higher $[Ca^{2+}]i$ in resting platelets. The presence or absence of retinopathy did not modify resting platelet $[Ca^{2+}]i$. After stimulation with thrombin, a significantly ($P < 0.009$) higher rise of platelet $[Ca^{2+}]i$ was observed only in those patients who were both free from complications and had good metabolic control. A highly significant ($P < 0.001$) correlation was found between resting $[Ca^{2+}]i$ and both blood cholesterol and HbA_{1c} in the diabetic patients. Platelets from 10 young healthy subjects also were studied after in vitro incubation with various glucose concentrations (from 1.68 to 56 mM): resting and thrombin-stimulated platelet $[Ca^{2+}]i$ and thrombin-induced aggregation were not modified.

CONCLUSIONS — These data confirm that platelet hyperfunction is present in IDDM patients who have unsatisfactory metabolic control, and give evidence that such an activation involves Ca homeostasis. Acute variations of blood glucose concentration are probably not influent, in this respect.

It is generally agreed that, at some point in the natural history of diabetes mellitus, a derangement of the hemostatic system towards a prothrombotic state takes place (1). Platelets play a central role in this process (2). Intracellular free (ionized) Ca is involved in the mechanisms of signal transduction that lead to platelet activation (3). The same is true for other cell types, such as the myocardial cell (4), the skeletal muscle cell (5), the smooth muscle cell (6), the pancreatic β-cell (7) and the hepatocyte (8). In particular, ionized Ca seems to play a key role in many events that characterize platelet response to stimuli, such as shape change (9), centralization and release of intracellular granules (10), and aggregation (11).

Evaluation of the role of ionized Ca as an intracellular messenger requires quantitative measurements of cytosolic free Ca concentration ($[Ca^{2+}]i$), in both resting and stimulated conditions. At present, one reliable and simple method for measuring $[Ca^{2+}]i$ in mammalian cells is to monitor the fluorescence of indicators such as quin-2 or fura-2 (12). These compounds are loaded into intact cells by incubating them with a membrane-permeant ester derivative. Cytosolic esterases split off the ester groups and leave the membrane-impermeant quin-2 or fura-2 tetra-anions trapped in the cytosol. An increase in quin-2 or fura-2 fluorescence thus signals increased $[Ca^{2+}]i$.

In this study, we evaluated platelet $[Ca^{2+}]i$ in a group of IDDM patients under resting conditions and following stimulation with thrombin, using the fluorescent Ca indicator fura-2.

RESEARCH DESIGN AND METHODS

— Sixty patients with IDDM and 31 age-matched healthy control subjects entered the study after giving informed consent. Their principal clinical characteristics are summarized in Table 1. Criteria for exclusion were: age >50 yr, intercurrent disease, past neoplasia, clinically overt macrovascular disease, arterial hypertension, recent (<10 days) ingestion of platelet-active drugs.

Patients were screened by means of history taking, physical examination, routine blood chemistries, 12-lead ECG, retinography screening, and Doppler sonography of cervical and limb vessels. Two clinical variables were taken into account—the presence or absence of ret-

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IDDM, INSULIN-DEPENDENT DIABETES MELLITUS; CV, COEFFICIENT OF VARIATION; ANOVA, ANALYSIS OF VARIANCE; PRP, PLATELET-RICH PLASMA; NIDDM, NON-INSULIN-DEPENDENT DIABETES MELLITUS.

Table 1—*Clinical characteristics of the study population*

	Diabetic patients	Control subjects	*P* value
N	60	31	–
Age (yr)	30.8 ± 8.7	28.0 ± 4.6	NS
Sex (men/women)	24/36	11/20	NS
Duration of disease (mo)	141.5 ± 117.0	—	
Mean arterial pressure (mmHg)	102.3 ± 9.6	96.4 ± 10.6	NS
HbA_{1c} (%)	9.1 ± 2.2	5.1 ± 0.2	<0.0001
Cholesterolemia (mM)	5.04 ± 1.15	4.81 ± 0.87	NS
Triglyceridemia (mM)	1.32 ± 0.73	1.28 ± 0.65	NS
Creatininemia (mM)	71.60 ± 26.52	55.69 ± 17.68	NS
Daily insulin requirement (U)	37.05 ± 14.2	—	
Microalbuminuria (*N*)	2/35*	0/31	
Proteinuria (*N*)	6/53†	0/31	

Values are means ± SD.
*Both belonging to subgroup 4.
†Four belonging to subgroup 3, one to subgroup 4, and one to subgroup 1.

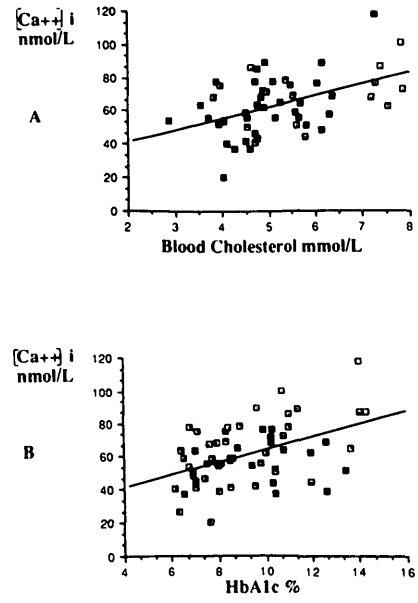

Figure 1—*A: Relationship between resting platelet $[Ca^{2+}]i$ (nmol) and cholesterol (mM) in the diabetic population. (P = 0.002, r = 0.42) B: Relationship between resting platelet $[Ca^{2+}]i$ (nmol) and HbA_{1c} (%) in the diabetic population. (P = 0.001, r = 0.44).*

inopathy, and the degree of metabolic (de-)compensation (arbitrarily defined as HbA_{1c} > or < 8%). Patients were thus placed into four groups: subgroup 1, satisfactorily controlled with complications (n = 10); subgroup 2, satisfactorily controlled without complications (n = 13; subgroup 3, unsatisfactorily controlled with complications (n = 14; subgroup 4, unsatisfactorily controlled without complications (n = 23).

After at least 3 h from the last meal (and regular insulin injection), blood was drawn by clear puncture of an antecubital vein and collected into plastic tubes containing 1 ml of ACD-solution: 0.065 mol citric acid (Riedel, Hannover, Germany), 0.085 mol sodium citrate (Farmitalia, Milano, Italy), 2% glucose monohydrate (Riedel). PRP was obtained by centrifugation at 220 *g* for 15 min at room temperature.

Platelet $[Ca^{2+}]i$ was evaluated according to the method of Rink et al. (9). Briefly, after incubation with fura-2 acetoxymethyl ester (Calbiochem, La Jolla, CA), (3 μl/ml) for 30 min at 37°C, PRP was centrifuged at 550 *g* for 20 min at room temperature, and the supernatant was discarded to yield platelet-poor plasma. The platelet pellet on the bottom was resuspended into a modified Tyrode's solution: 145 mmol NaCl (Baker, Deventer, Holland), 5 mmol KCl (Merck, Darmstadt, Germany), 1 mmol $MgSO_4$ (Farmitalia), 10 mmol HEPES (Sigma, St. Louis, MO), 10 mmol glucose monoidrate (Riedel), sodium hydroxide (Merck), to obtain a pH of 7.38, and kept for 20 min at 37°C. Platelet concentration was adjusted to give a final count of $50 \times 10^3/\mu l$. The fluorescence of the platelet suspension was recorded in a 37°C-termostated cuvette by means of a Perkin-Elmer LS-5B fluorimeter (Perkin-Elmer, Beaconsfield, UK), at 340 and 380 nM excitation and a 495 nM emission wave. Fluorescence values were converted into $[Ca^{2+}]i$ values (nmol) by means of a computer connected to the fluorimeter. The following conversion formula was adopted:

$$[Ca^{2+}]i = Kd \times \frac{(R - F_{min})}{(F_{max} - R)} \times 3.97$$

where F_{min} = 1.03 and F_{max} = 7.17.

$[Ca^{2+}]i$ was evaluated in the following experimental conditions: resting and after the addition of human thrombin at 0.05, 0.1, and 0.5 U/ml, (final concentrations). The three doses of thrombin were added in a randomized fashion. Thrombin was purified according to the method of Miletich et al. (12). The following parameters were chosen to analyze the results: resting $[Ca^{2+}]i$ (nmol); relative increase of $[Ca^{2+}]i$ after stimulation (as ratio stimulated $[Ca^{2+}]i$/resting $[Ca^{2+}]i$).

In a separate set of experiments, the in vitro sensitivity of normal platelets to various concentrations of glucose was tested. Platelets from 10 healthy subjects (5 women, 5 men, mean age 26 yr, range 25–28 yr) were obtained and processed as described previously, but in the last step glucose was added to the modified Tyrode's solution, yielding the following final concentrations: 1.68, 5.6, 14, 28, and 56 mM. After incubation at 37°C for 20 min, platelet $[Ca^{2+}]i$ was evaluated fluorimetrically, both in resting conditions and after stimulation with thrombin 0.1 U/ml (final concentration). The same numerical parameters were

Table 2—*Results of platelet [Ca^{2+}]i in resting condition and its variation (ratio) after thrombin*

	N	Resting (nmol)	After thrombin		
			0.05 U/ml	0.1 U/ml	0.5 U/ml
Control subjects	31	51.35 ± 16.40	3.33 ± 1.69	4.12 ± 1.86	6.29 ± 2.34
Diabetic patients	60	57.99 ± 17.88	2.98 ± 1.17	4.23 ± 1.95	6.63 ± 2.89
Subgroup 1	10	57.10 ± 9.03	3.05 ± 0.99	4.51 ± 1.65	6.96 ± 3.48
Subgroup 2	13	43.82 ± 15.07	3.74 ± 1.21	5.96 ± 2.13*	8.71 ± 3.36†
Subgroup 3	14	65.04 ± 16.25‡	2.62 ± 1.01	3.00 ± 1.19	5.28 ± 1.96
Subgroup 4	23	62.11 ± 19.38§	2.74 ± 1.18	3.87 ± 1.68	6.13 ± 2.22

Values are means ± SD. Subgroup 1, patients satisfactorily controlled with complications; subgroup 2, patients satisfactorily controlled without complications; subgroup 3, patients unsatisfactorily controlled with complications; subgroup 4, patients unsatisfactorily controlled without complications.
*$P < 0.006$ vs. controls.
†$P < 0.009$ vs. controls.
‡$P < 0.01$ vs. controls.
§$P < 0.03$ vs. controls.

adopted as in the in vivo study. In our laboratory, the intra- and interassay CVs for [Ca^{2+}]i are 4 and 7%, respectively.

Similarly processed platelets also were tested aggregometrically (in an Elvi 840 dual-channel aggregometer [Elvi-Logos, Milano, Italy]), after the sequential addition of fibrinogen 100 mg/dl (Boehringer Mannheim, Mannheim, Germany), 1 mmol CaCl (Merck), and thrombin 0.1 U/ml (final concentrations). Aggregability was evaluated in duplicate for each sample, and measured as latency time from stimulus to initial response (s) and as maximal aggregation (%).

HBA_{1c} was determined by HPLC. In our laboratory, the normal reference interval is 4–6%. Microalbuminuria was evaluated using a commercial kit (Albumin RIA kit, Pharmacia, Uppsala, Sweden) and was considered present for albumin escretion rates 20–200 μg/min (13).

Statistical analysis was performed with Student's *t* test and one-way ANOVA, as requested.

RESULTS— As Table 1 shows, the study population was remarkably homogeneous with respect to the principal clinical variables. HbA_{1c} was obviously higher in the diabetic patients as a whole.

Platelet [Ca^{2+}]i was evaluated three times in the resting state (i.e., before the addition of thrombin in predetermined amounts) in each subject. The three sets of values were highly similar ($P > 0.72$), so that each individual value is the mean of three determinations.

Results are summarized in Table 2. Platelet [Ca^{2+}]i in resting conditions was similar in the platelets from control subjects and diabetic patients as a whole group. When the diabetic population was subgrouped with regard to the presence of complications and metabolic status, significantly higher ($P < 0.01$ and $P < 0.03$ vs. control subjects) [Ca^{2+}]i values were observed in those patients with a poor metabolic control independently of the presence of retinopathy (subgroups 3 and 4). In the patients affected by retinopathy, but with a satisfactory degree of metabolic control (subgroup 1), resting platelet [Ca^{2+}]i was not different from that of control subjects.

After stimulation with thrombin, a highly significant ($P < 0.0001$, $r = 0.54$) dose-response relationship between thrombin concentration and platelet [Ca^{2+}]i relative increase was found both in the control and the patient population.

The percentage changes in platelet [Ca^{2+}]i after thrombin in the various subgroups of the study population are shown in Table 2. Only in the metabolically well-controlled patients free from complications (subgroup 2) was a significantly higher rise of platelet [Ca^{2+}]i observed after stimulation with thrombin ($P < 0.006$ thrombin 0.1 U/ml and $P < 0.009$ thrombin 0.5 U/ml vs. control subjects, respectively).

A highly significant ($P < 0.002$, $r = 0.42$) correlation was found between resting platelet [Ca^{2+}]i and blood cholesterol levels and between resting platelet [Ca^{2+}]i and blood HbA_{1c} ($P < 0.001$, $r = 0.44$) in the diabetic population (Fig. 1). No correlation was found between blood cholesterol or HbA_{1c} and resting platelet [Ca^{2+}]i in the control population ($r = 0.019$ and $r = 0.068$, respectively). No significant correlations were present between resting platelet [Ca^{2+}]i and subject age ($r = 0.21$ and $r = 0.021$), mean arterial pressure ($r = 0.12$ and $r = 0.053$), and triglyceridemia ($r = 0.03$ and $r = 0.057$), both in control subjects and patients, and between resting platelet [Ca^{2+}]i and duration of the diabetes ($r = 0.18$).

The results of the in vitro study are summarized in Table 3. The incubation of normal platelets with a wide array of glucose concentrations did not modify

Table 3—*Results of the in vitro study*

Glucose concentration (mM)	Platelet $[Ca^{2+}]i$ Resting (nmol)	Platelet $[Ca^{2+}]i$ Stimulated (ratio)	Platelet aggregability Latency (s)	Platelet aggregability Maximum response (%)
1.68	74.0 ± 26.1	2.55 ± 1.11	46.8 ± 14.9	47.4 ± 9.4
5.6	67.7 ± 17.5	2.40 ± 1.29	76.0 ± 36.5	48.6 ± 5.2
14	71.1 ± 16.8	2.30 ± 1.21	82.6 ± 28.5	42.7 ± 13.2
28	71.9 ± 16.8	2.11 ± 0.98	76.0 ± 23.7	45.6 ± 12.9
56	78.4 ± 9.5	1.91 ± 0.77	95.0 ± 44.4	43.3 ± 12.2

Values are means ± SD. The agonist used was thrombin 0.1 U/ml. Each value is the mean of five separate duplicate determinations.

either aggregability to thrombin nor calcium homeostasis.

CONCLUSIONS— The influx of ionized Ca through the platelet membranes is one of the basic modes of response of these highly excitable cells to stimulating agents.

The possibility of quantitating platelet $[Ca^{2+}]i$ in resting and stimulated conditions is therefore to be considered a new and potentially sensitive tool in the study of the behavior of platelets both in normal and pathological conditions. Among the fluorescent indicators suitable for this purpose, fura-2 is considered the best for the study of $[Ca^{2+}]i$ in single cells, particularly for its high wavelength sensitivity to Ca (14). Thrombin was chosen because of its recognized role of strong, physiological platelet agonist, the most frequently used in platelet Ca-handling studies (15).

In this study, platelet $[Ca^{2+}]i$ was evaluated three times in the resting state in each subject, and the values obtained were remarkably similar, suggesting a good reproducibility of our method. $[Ca^{2+}]i$ increased in platelets from control subjects stimulated with thrombin proportionately to the thrombin concentration used, and the same behavior was observed in platelets from diabetic patients. In this respect, namely the fact of following the dose-response law, the platelets from diabetic patients behaved as healthy cells. The same was true for resting platelet $[Ca^{2+}]i$ values, which were similar in the control and diabetic population as a whole.

On the other hand, in poor metabolic control, platelets from diabetic patients had significantly higher $[Ca^{2+}]i$ than those of both normal subjects and well-controlled diabetic patients, independently of the presence or absence of retinopathy. The pathophysiological meaning of a platelet $[Ca^{2+}]i$ higher than normal has not been fully elucidated. In the normal platelet, a sharp rise of $[Ca^{2+}]i$ is a precocious event in the mechanism of platelet activation, being induced by agonist-receptor interaction on the cell surface and subsequent transduction of the signal by the system of G-proteins (16). Whatever its origin, either from the extracellular medium through non–voltage-dependent ionic channels (17) or from the dense tubular system by the action of inositol 1,4,5,trisphosphate (18), the increment of $[Ca^{2+}]i$ plays a pivotal role in many steps of the platelet response, such as protein kinase C activation (19), myosin light-chain phosphorilation (20), and phospholipase A2 activation (21).

It therefore seems reasonable to postulate that a rise of resting $[Ca^{2+}]i$ could lower the threshold for platelet activation. Such an interpretation has been proposed previously (22).

A condition of chronic activation of the circulating platelets has been recognized in diabetic patients from many years (23). The aspects of platelet function found to be deranged include adhesion (24), in vitro aggregability (25) and sensitivity to antiaggregating agents (26), α-granule content discharge (27,28), and thromboxane production (29). Such an activation was initially considered secondary to the presence of micro- and macroangiopathic alterations and/or to a condition of hyperglycemia (30).

Although studies in vitro (31) and in vivo (32) had subsequently excluded hyperglycemia per se to be a cause of platelet activation, debate continues about the possibility that long-term metabolic control could normalize platelet hyperfunction in diabetic patients.

We found no alterations in $[Ca^{2+}]i$ either in resting conditions or after thrombin challenge of platelets taken from normal subjects and incubated with medium containing glucose in a wide range of concentrations. Our finding thus confirms, from a different point of view, that acute derangements of glucose homeostasis are probably harmless to the human platelet.

The presence of endothelial discontinuations or atherosclerotic lesions undoubtedly represents a strong stimulus for platelet activation. However, the finding of signs of platelet activation in animal models (33) and in young patients with uncomplicated IDDM of very recent onset (34) suggests an alternative, non–complication-dependent pathogenesis. The results of this study indicate that an unsatisfying metabolic control determines an increase of $[Ca^{2+}]i$ in the platelets of IDDM patients. The close correlation between $[Ca^{2+}]i$ and HbA_{1c} further strenghtens the hypothesis of a causative role of the midterm glycemic status.

The behavior of ionized Ca in the platelets of diabetic patients has been seldom studied. Bergh et al. (35) studied the exchange of $45Ca^{2+}$ in and out the cytosol of platelets from 8 insulin-treated diabetic patients apparently free from complications but with suboptimal met-

abolic control. Influx rate was significantly higher in the patients' platelets, while efflux rate was similar to that of control platelets.

Our results agree with those of Mazzanti et al. (36), who observed in 37 IDDM patients (with mean HbA_{1c} >8.5%) that resting platelet $[Ca^{2+}]i$ was significantly higher than in control subjects. In the absence of extracellular Ca (a condition quite similar to ours), the addition of thrombin or ADP induced a rise of $[Ca^{2+}]i$ of the same magnitude in both diabetic patients and control subjects. Fura-2–loaded platelets from NIDDM patients have been shown to have basal $[Ca^{2+}]i$ similar to that of control subjects. After stimulation with thrombin, a significant increment of $[Ca^{2+}]i$ was found in a subgroup of patients whose platelets manifested an in vitro hyperaggregability (37).

Two other findings of our study require a comment. The first is the highly significant linear correlation between basal platelet $[Ca^{2+}]i$ and blood cholesterol levels observed in the diabetic population but not in the control group. The subgroup of diabetic patients with poor metabolic control had a mean ± SD blood cholesterol of 5.31 ± 1.2 mM, which is significantly ($P = 0.02$) higher than that of the subgroup of well-controlled diabetic patients (blood cholesterol 4.59 ± 0.92 mM). Moreover, it has been demostrated that hypercholesterolemia is both epidemiologically (38) and causatively (39) associated with platelet hyperfunction. These data, therefore, give further evidence to the role of the metabolic control in the pathogenesis of the platelet hyperfunction observed in this study. It has been demonstrated by means of quin-2 that $[Ca^{2+}]i$ in unstimulated platelets from patients with essential hypertension is significantly higher than in control subjects (40), possibly because of a transferable plasma factor (41). A significant correlation between resting platelet $[Ca^{2+}]i$ and mean arterial pressure also was found in normotensive subjects (40). The results of our study are not in agreement with those cited before, and we cannot offer any explanation for this discrepancy at present.

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