# EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

# Mobilization of Intracellular $Ca^{2+}$ Induced by ADP and Thrombin in Platelets from Diabetic Patients with Vascular Complications

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It is shown that the baseline level of cytoplasmic $Ca^{2+}$ in platelets from diabetic patients is nearly 1.5 times as high as in healthy donors. The thrombin-induced increase of intracellular $Ca^{2+}$ in patients with angiopathies is reliably lower than in the control, while in patients without angiopathies it is higher than that in donor platelets. The elevation of cytoplasmic $Ca^{2+}$ induced by ADP is greater in both groups of patients. No changes are found in the baseline level of intracellular $Ca^{2+}$ or in the ADP-induced concentration of $Ca^{2+}$ in platelets from diabetic patients during a 12-week course of insulin therapy. The intracellular $Ca^{2+}$ does not rise after 2 weeks of insulin treatment in platelets from diabetic patients.

**Key Words:** *cytoplasmic $Ca^{2+}$; platelet; diabetes mellitus*

Platelet activity has been found to increase in patients with diabetes mellitus. The alterations shown in diabetes by many investigators are as follows: an increased capacity of platelets for aggregation [5,7], a boost of arachidonic acid metabolism by the cyclooxygenase pathway [2,7], a lowering of platelet sensitivity to prostacyclin [5], an increase of the plasma content of markers specific for platelet activation, including B-thromboglobulin and factor 4 [6], and intensification of norepinephrine release from platelets [11]. Disturbances of platelet function may contribute to the development of vascular complications as well as micro- and macroangiopathies in diabetes [5,7,9]. The biochemical mechanisms of platelet hyperactivation in diabetes are obscure; however, it may be assumed, that improved ability of platelets to aggregate is independent of the level of hyperglycemia because there are no documented relationships between glycosylation of platelet proteins and disturbances of their function [12].

Calcium is one of the key regulators of the intracellular processes in many if not all cells of the organism, including platelets. An increase of cytoplasmic $Ca^{2+}$ plays an important role in platelet activation in several ways as follows: by modulation of $Ca^{2+}$-calmodulin-dependent proteinkinases and $Ca^{2+}$-dependent proteases and by activation of phospholipases C and $A_2$ as well as proteinkinase C [8]. Each of these biochemical pathways induces a cell response such as an increase of the adhesion and aggregation of platelets, intensification of the discharge of granule contents, a change of platelet shape, and synthesis of biologically active compounds [8]. Since many are changed in diabetes, the functional parameters of platelets may underlie the alterations in the mechanisms of intracellular signal transmission in platelets from diabetic patients.

The goal of the present investigation was to study the steady-state level of cytoplasmic $Ca^{2+}$ as well

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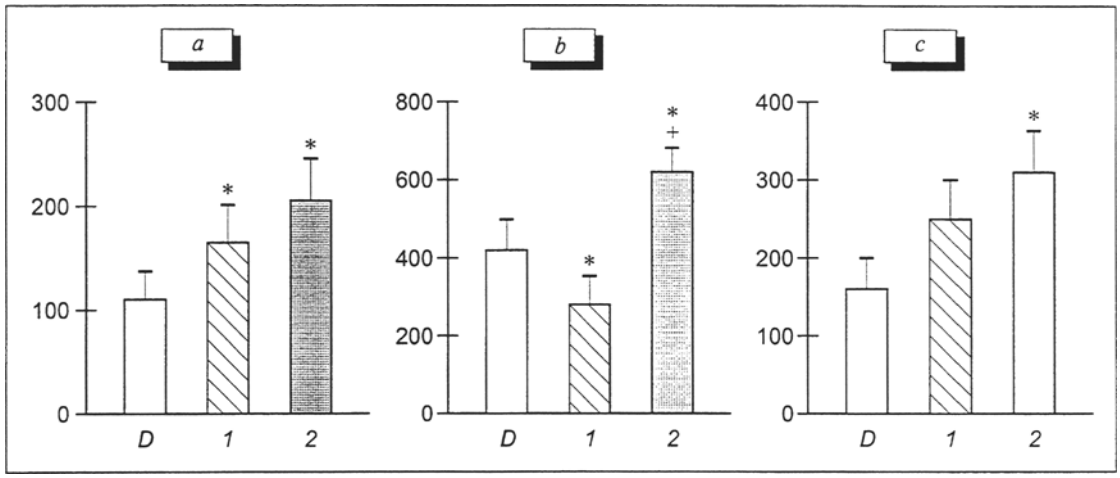


**Fig. 1.** Baseline and stimulated level of $Ca^{2+}$ in platelets from diabetic patients with angiopathy and without vascular complications. Baseline $Ca^{2+}$ level (*a*) and thrombin-induced (*b*) and ADP-stimulated (*c*) elevation of cytoplasmic $Ca^{2+}$ in platelets of donors (*D*), patients with angiopathy (*1*), and patients without angiopathy (*2*), nmol/5×10$^5$ cells. *$p$<0.05 as compared to donor platelets, +$p$<0.001 in comparison between the 1st and 2nd group of patients.

as the increase in the intracellular $Ca^{2+}$ induced by ADP and thrombin in platelets from diabetic patients with angiopathies and without vascular complications under conditions of insulin treatment and carbohydrate metabolism compensation.

## MATERIALS AND METHODS

Seventeen patients (5 women and 12 men) aged from 20-43 years and 15 healthy donors aged 25-46 years were examined. Sixteen patients were in a state of subcompensation (the mean level of glycemia measured on an empty stomach was 9.56±0.27 mmol/liter, glycosylated hemoglobin - $HbA_1c$ - 9.21±0.32%) and one patient was in a compensated state (fasting glycemia was 4.4 mmol/liter, $HbA_1c$ 6.5%) at the time of examination. Diabetic vascular complications, such as nephropathy (transient proteinuria), retinopathy (diabetic angiopathy of the retina), and micro- and macroangiopathies of the lower limbs were observed in 10 patients. The patients were injected insulin (Eli Lilly) twice a day: 11 patients received injections of Humulin S and Humulin lente and 5 patients were treated with Humulin M3. Blood was sampled from the median cubital vein on an empty stomach before the treatment with human insulin preparations as well as after 2, 6, and 12 weeks of therapy. Blood to be tested was mixed with 3.8% sodium citrate solution in the ratio of 9:1 in a plastic test tube. Platelets were obtained by centrifugation at 190 g. FURA 2-AM solution in dimethylsulfoxide was added to the platelet suspension in a final concentration of 10 μM and incubated for 30 min at 37°C with periodic shaking. Thereafter platelets were sedimented at 1200 g for 10 min and resuspended in phosphate buffer, pH 7.4, after which 100 μl of platelet suspension (5×10$^5$ cells/ml) were added to 900 μl of incubation medium. The induced concentration of $Ca^{2+}$ was measured after the addition of 0.5 unit/ml thrombin and 10 μM ADP to the platelet suspension. Fluorescence (FL) was recorded with a Hitachi-2500 spectrofluorimeter using rectangular quartz cuvettes. The wavelengths of excitation and FL were 350 and 500 nm, respectively. The $Ca^{2+}$ concentration in the cytoplasm was computed according to the formula: $Ca^{2+}=224\ nM\times(F-F_{min})/(F_{max}-F)$, where $F$ is the recorded level of FL, $F_{max}$ and $F_{min}$ are the FL intensities when FURA is fully taken up by $Ca^{2+}$ or free of it, respectively, 224 nM is the dissociation constant at pH 7.05 and concentrations of $Mg^{2+}$, $K^+$, and $Na^+$ ions equal to 1, 125, and 20 mM (near-intracellular conditions), respectively. For determination of $F_{max}$ the plasma membrane of platelets contained in medium with a $Ca^{2+}$ concentration that is saturating for FURA was destroyed with 50 μM digitonin. $F_{min}$ was determined by adding $MnCl_2$ (to 0.5 mM), which quenches the fluorescence by eliminating $Ca^{2+}$ from its complex with the dye.

## RESULTS

The baseline level of cytoplasmic $Ca^{2+}$ is increased nearly 1.5-fold in the platelets from diabetic patients as compared with that of healthy donors ($p$<0.05) and does not differ reliably in patients with angiopathies and without vascular complications (Fig. 1, *a*). Thrombin-induced elevation of intracellular $Ca^{2+}$ was reliably lower in patients with angiopathies compared to the control ($p$<0.05), while in patients without them it was higher than in donor platelets ($p$<0.05, Fig. 1, *b*). The increase of cytoplasmic $Ca^{2+}$ induced by ADP was greater in both groups of patients ($p$<0.05, Fig. 1, *c*). The change in the baseline level of intracellular $Ca^{2+}$ was insignificant in platelets from diabetic patients

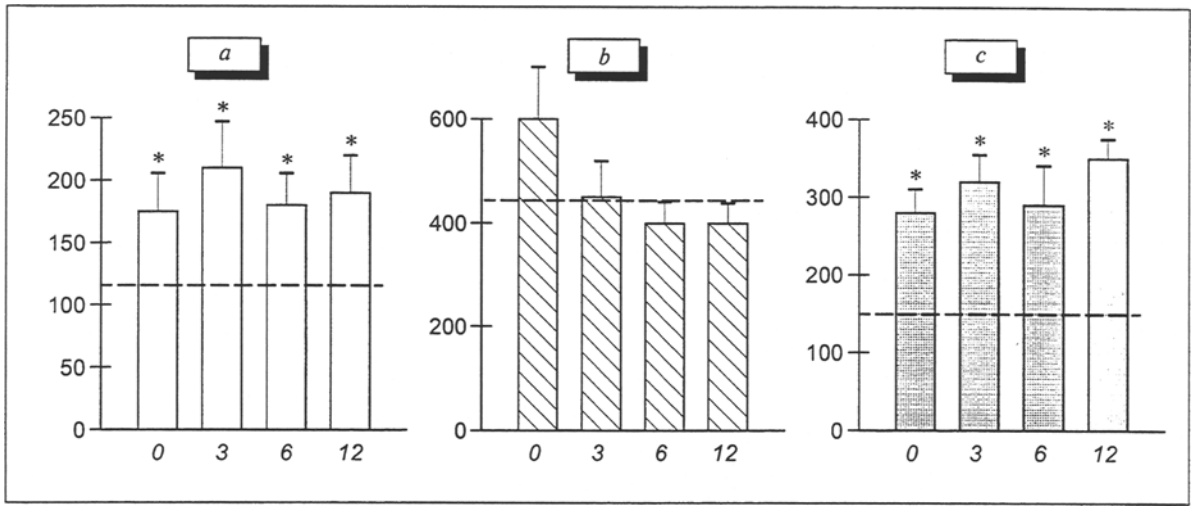


**Fig. 2.** Change of baseline and stimulated level of $Ca^{2+}$ in platelets from diabetic patients during a course of insulin therapy. Baseline $Ca^{2+}$level (*a*) and thrombin-induced (*b*) and ADP-stimulated (*c*) elevation of cytoplasmic $Ca^{2+}$ (nmol/5×$10^5$ cells) in platelets of patients before the start of insulin treatment (*0*) and during the course of treatment after 2, 6, and 12 weeks. *$p<0.05$ compared with donor platelets. Dotted line shows $Ca^{2+}$ level in donor platelets.

during the 12-week course of insulin therapy (Fig. 2, *a*). There were no alterations in the $Ca^{2+}$ concentration in the ADP-stimulated platelets from diabetic patients during the period of insulin treatment and compensation of carbohydrate metabolism. The thrombin-induced elevation of intracellular $Ca^{2+}$ in the platelets from diabetic patients was just leveled to the control value after 2 weeks of insulin treatment (Fig. 2, *b*). Several conclusions can be drawn from these results.

1. The rise of the intracellular $Ca^{2+}$ baseline level (as well as the induced one) in the platelets from diabetic patient attests to an elevation of platelet activity *in vivo* whether or not vascular complications have developed.

2. The dissimilar nature of the alterations in intracellular $Ca^{2+}$ in response to thrombin and ADP stimulation presumably testifies to different mechanisms of disturbance in the mobilization of intracellular $Ca^{2+}$ in platelets from diabetic patients with vascular complications. Judging from this the decrease of the thrombin-induced cytoplasmic $Ca^{2+}$ concentration in platelets from patients with angiopathies may be related to attenuation of the mechanism of platelet activation. Probably *in vivo* platelet activity is induced by thrombin to a greater extent than by ADP. Low thrombin concentrations produce an initial activation of platelets, a "priming" effect, which leads to a more intensive cell response to the subsequent stimulus. Such a phenomenon may occur in diabetic patients without vascular complications. High doses of thrombin and prolonged cell stimulation dampen the activation mechanism and lower the cell response to additional stimulation *in vitro*. Thrombin-induced elevation of the intracellular concentration of $Ca^{2+}$ is realized in at least two ways, namely, due to $Ca^{2+}$ discharge from the intracellular reservoir and as a result of activated $Ca^{2+}$ entry from outside [10]. Inositol-1,4,5-triphosphate ($IP_3$), which is generated from thrombin-dependent splitting of the membrane phospholipid phosphatidylinositol-4,5-diphosphate by phospholipase C, plays a key role in the release of $Ca^{2+}$ from the intracellular reservoir [8,10]. Inflow of $Ca^{2+}$ from the environment is accomplished via receptor-dependent selective calcium channels [1,10], which are thought to be responsible for most of the intracellular $Ca^{2+}$ increase in response to agonists [1]. It has been shown that in platelets of diabetic patients the rate of labeled $Ca^{2+}$ flow into the cell is significantly higher than in healthy donors, while the rate of outflow is lowered [4]. However, the release of $Ca^{2+}$ from the compact tubular system in response to $IP_3$ in platelets from diabetic patients is below the level of that in healthy donors [4]. In addition, in the platelet membrane from patients the content of phosphatidylinositol-4,5-diphosphate and phosphatidylinositol-4-phosphate is also lowered and hydrolysis is reduced in thrombin-stimulated platelets [3]. Thus, there is no question but that the phosphatidylinositol pathway of intracellular $Ca^{2+}$ mobilization is disturbed in response to thrombin. Unfortunately, there is no analysis of the changes found in relation to angiopathy in the literature cited. Our data suggest that a drop in the level of intracellular $Ca^{2+}$ induced by thrombin is observed only in the group of patients with angiopathies, whereas in patients without these complications the level is higher than in donor platelets. It may be assumed that in patients with angiopathies the mechanism of $Ca^{2+}$ mobilization from the reservoir in platelets is dampened, while in patients without angiopathies either this mechanism is preserved

or else its deficiency is compensated by the boosted $Ca^{2+}$ influx from outside. The data on a marked increase of thromboxane synthesis in platelets from diabetic patients without vascular complications and a slight decrease in platelets from patients with vascular complications compared to that of healthy donors may indirectly confirm our hypothesis [9]. Synthesis of thromboxane in activated platelets is also initiated by receptor-dependent splitting of phosphatidylinositol-4,5-diphosphate by phospholipase C with the formation of diacylglycerol, which affects the release of endogenous arachidonic acid and its metabolism in the cyclooxygenase pathway via the activation of proteinkinase C [8]. On the basis of our own and published data it may be presumed that the phosphoinositol chain of receptor signal transmission is disrupted in platelets from diabetic patients with vascular complications. The mechanisms of platelet activation in response to strong agonists such as thrombin and weak ones such as ADP are different and involve different metabolic pathways [8]. Unlike thrombin, ADP is not responsible for receptor-dependent $IP_3$ formation in platelets but increases the intracellular $Ca^{2+}$ concentration by opening the ADP-dependent nonselective cation channels [10]. The increased concentration of cytoplasmic $Ca^{2+}$ induced by ADP in both groups of patients is reportedly due to the elevated extracellular $Ca^{2+}$ inflow into platelets found in diabetes [4].

3. Insulin therapy and normoglycemia do not diminish platelet hyperactivity because the baseline level of the intracellular $Ca^{2+}$ remains elevated. Compensation of carbohydrate metabolism does not affect the ADP-induced rise of cytoplasmic $Ca^{2+}$ either, probably due to the boosted $Ca^{2+}$ influx into platelets from the environment. Normalization of the thrombin-stimulated $Ca^{2+}$ concentration in platelets of diabetic patients after just 2 weeks of insulin treatment may result from the direct or indirect effect of insulin on $Ca^{2+}$ mobilization from the reservoir and its inflow via the thrombin-dependent $Ca^{2+}$ channels. The data on the restoration of the rate of $Ca^{2+}$ outflow from the depot in patients' platelets in response to $IP_3$ after insulin therapy [4] are interpreted as indicating that disturbances in intracellular calcium metabolism can be corrected by insulin.

Thus, the intracellular $Ca^{2+}$ increase in platelets from diabetic patients is one of the causes of elevated platelet activity, which is preserved under conditions of compensated carbohydrate metabolism. The increase of cytoplasmic $Ca^{2+}$ in patients' platelets is above all due to enhanced entry of extracellular $Ca^{2+}$ into the platelets. Probably, $Ca^{2+}$ mobilization from the intracellular reservoir is disrupted in platelets from diabetic patients with vascular complications.

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