Decreased Platelet Phosphoinositide Turnover and Enhanced Platelet Activation in IDDM

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Individuals with diabetes mellitus may have increased in vivo platelet activity. Abnormal platelet function could contribute to the increased incidence of vascular disease in diabetes mellitus. The biochemical mechanism(s) for platelet hyperactivation is unknown. We examined the hypothesis that platelet phosphoinositide turnover, a key signal-transducing mechanism involved in platelet activation, was abnormal in diabetic subjects. Platelets were harvested from 16 subjects with insulin-dependent diabetes mellitus (IDDM) and 19 healthy, nondiabetic control subjects of comparable age. Plasma β-thromboglobulin (β-TBG), a specific marker of platelet activity in vivo, was increased in IDDM (67.1 ± 7.3 ng/ml) compared with control (41.0 ± 6.0 ng/ml) subjects ($P < .005$). [^{32}P]orthophosphate ($^{32}P_i$) incorporation into the individual phosphoinositides and phosphatidic acid (PA) reached isotopic equilibrium by 120 min for IDDM and control subjects. Specific activity (dpm ^{32}P/μg phosphorus) of phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) was not different between IDDM and control subjects. Under these conditions, basal $^{32}P_i$ incorporation into PIP_2 and PIP but not phosphatidylinositol (PI) or PA was significantly lower in IDDM subjects. There was significantly decreased [^{32}P]PIP_2 and [^{32}P]PIP hydrolysis and decreased [^{32}P]PA formation in IDDM after platelet stimulation with 4 U/ml human thrombin. There were no differences in [^{32}P]PI hydrolysis between the two groups. The mass of PIP_2 was reduced ($P < .005$) in the platelets from IDDM (0.71 ± 0.23 nmol/10^9 platelets) compared with control (1.65 ± 0.53 nmol/10^9 platelets) subjects. Similarly, PIP was lower ($P < .001$) in IDDM (0.66 ± 0.09 nmol/10^9 platelets) than in control (2.92 ± 0.43 nmol/10^9 platelets) subjects. There were no differences in PI and PA mass between the two groups. We conclude that increased platelet activation in IDDM is associated with a decrease in platelet polyphosphoinositide content and hydrolysis of PIP and PIP_2. These data suggest that altered phosphoinositide turnover may play a role in abnormal platelet function in IDDM. *Diabetes* 38:1097–1102, 1989

Cholesterol	1 mM = 38.7 mg/dl	Phosphate	1 mM = 3.1 mg/dl
Glucose	1 mM = 18 mg/dl		

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Increased in vivo platelet activity has been reported in subjects with diabetes mellitus (1–3). Exaggerated in vitro platelet aggregation and secretion in response to collagen, epinephrine, adenosine diphosphate (ADP), and thrombin have also been shown (4–7). The abnormality in platelet aggregation occurs soon after platelet activation, with a defective change in platelet shape and an enhanced primary wave of aggregation (8). The mechanism(s) responsible for this hyperactivation is unknown. Previous work in spontaneous and streptozocin-induced diabetes (STZ-D) in rats raised the possibility that enhanced ADP-induced hyperaggregation might be a function of high plasma glucose, but the mechanism of ADP and thrombin hypersensitivity has not been defined (9,10).

Platelet activation by thrombin and ADP is coupled to phosphoinositide turnover, with formation of intraplatelet second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) from hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (11–15). Subsequent formation of arachidonic acid from DAG and by the activation of phospholipase A_2 yields proaggregatory prostaglandin metabolites, such as thromboxane A_2 (16–18). Increased activation of the platelet-arachidonic acid pathway has been described in diabetes (19–21). However, thrombin-induced hyperaggregation persists with aspirin treatment, which presumably blocks arachidonic acid metabolism (9,10,22). Furthermore, change in platelet shape induced by thrombin is closely

linked to phosphoinositide hydrolysis and may be independent of the liberation and metabolism of arachidonic acid (23). It appears likely that platelet hypersensitivity in diabetes may not be caused by an abnormality in the formation of arachidonic acid metabolites. We therefore examined the hypothesis that phosphoinositide turnover and hydrolysis is abnormal in the platelets of subjects with diabetes mellitus.

We report here that subjects with insulin-dependent diabetes mellitus (IDDM) and enhanced in vivo platelet activity have a decreased mass of platelet PIP_2 and phosphatidylinositol 4-phosphate (PIP). There is decreased hydrolysis of these polyphosphoinositides, resulting in decreased formation of DAG measured as its phosphorylated product phosphatidic acid (PA). We conclude that there is defective phosphoinositide turnover in the platelets of IDDM subjects, and this may be responsible for the accompanying in vivo hyperactivation.

RESEARCH DESIGN AND METHODS

Subjects. Platelet phosphoinositide turnover and in vivo platelet activation were studied in 16 IDDM subjects and 19 healthy, nondiabetic control subjects. The IDDM subjects were selected from the Diabetes Center Unit at the University of Michigan Medical Center and were free of any clinical evidence of vascular disease. The diagnosis of IDDM had been made previously with the National Diabetes Data Group criteria: corporal weight less than or equal to ideal body weight, spontaneous episodes of diabetic ketoacidosis before the age of 35 yr, and a C-peptide level <2 ng/dl. Control subjects were volunteers who donated blood regularly at the blood bank of the University of Michigan Hospitals. All participants were taking no medicine other than insulin. They were instructed to take no medicine (aspirin or other over-the-counter compounds) for at least 1 wk before the study to avoid affecting platelet function. Blood sampling was performed at the same early-morning hour for each subject after a fast of at least 12 h. Clinical information, including age, duration of diabetes, body mass index, and sex, is given in the patient profile (Table 1). Written, informed consent was obtained from all subjects. The study was approved by the Institutional Review Board and the Committee for Protection of Human Rights at the University of Michigan.

In vivo platelet activity. In vivo platelet activity was assessed by measuring plasma β-thromboglobulin (β-TBG), a platelet-specific protein secreted when platelets are activated (24). Triplicate 2.5-ml samples of whole blood were placed on ice in plastic tubes containing 150 μl 134 mM EDTA and 15 mM theophylline, and β-TBG was assayed by radioimmunoassay (Amersham, Arlington Heights, IL) in the Ligand Laboratory of the Diabetes Research and Training Center at the University of Michigan Medical Center.

Platelet phosphoinositide turnover. Platelet phosphoinositide turnover was carried out by a modification of methods established by Agranoff et al. (25). Two hundred fifty milliliters of platelet-rich plasma was collected and prepared according to blood bank protocol into an acid/citrate/dextrose anticoagulant buffer (85 mM trisodium citrate, 111 mM dextrose, 71 mM citric acid [pH 5.5]). To further ensure that the blood bank product was free from accompanying erythrocyte and leukocyte contaminants, the platelet-rich plasma was centrifuged twice at 200 × *g* for 10 min, followed by centrifugation at 1000 × *g* for 12 min. The platelet pellet was suspended in a small volume of incubating buffer (20 mM Tris-HCl [pH 7.4], 0.025% bovine serum albumin [BSA], 5 mM glucose, 150 mM NaCl) and brought to ~10 ml with autologous plasma. The final volume of plasma added was 7.50 ± 0.04 ml (mean ± SE, range 7.1–7.7 ml), and a final phosphorus concentration of 0.80 ± 0.03 mM (range 0.6–1.0 mM) was reached, with no significant differences in assay conditions between the two groups. The platelet count was adjusted to 2 × 10^9 platelets/ml. For incorporation studies, 500-μl aliquots (1 × 10^9 platelets) of the combined platelet suspension was used for each timed incubation. Aliquots were placed in tubes, and 20 μCi/ml of carrier-free [^{32}P]orthophosphate ($^{32}P_i$), neutralized with 0.02 M NaOH just before use, was added to the separate tubes at 30- or 60-min intervals. At the end of 3 h, all incubations were terminated by addition of 1.0 ml of cold methanol/chloroform (2/1 vol/vol). For the stimulation studies, 500-μl aliquots (1 × 10^9 platelets) were incubated 2 h at 37°C. The incubation was terminated with the addition of phosphate washing buffer (3.2 mM K_2HPO_4, 24 mM NaH_2PO_4, 4.2 mM Na_2HPO_4, 5.5 mM glucose, 150 mM NaCl, 0.025% BSA [pH 7.4]) and then centrifuged at 1000 × *g* for 20 min. The wash was repeated, and the pellet was resuspended in 500 μl of the Tris-HCl pH 7.4 buffer and used for each timed stimulation with 4 U/ml human thrombin (Sigma, St. Louis, MO). At designated times, the incubations were terminated by the addition of 1.0 ml of cold methanol/chloroform (2/1 vol/vol). The denatured preparations were transferred to centrifuge tubes, and 1.5 ml chloroform and 0.5 ml 2.4 M HCl were added. Each tube was vortexed immediately, followed by centrifugation at 150 × *g* for 10 min to separate the aqueous and organic phases. The organic layer was withdrawn, and the aqueous phase was reextracted with 1 ml of chloroform. The combined chloroform extracts were acidified with 2.4 M HCl, followed by addition of 2 ml 50% aqueous methanol. The acidified chloroform extracts were centrifuged at 230 × *g* for 10 min, followed by removal of the aqueous layer. The extract was then dried under N_2 in a water bath at 37°C and resuspended in chloroform/methanol (2/1 vol/vol). Fifty-microliter aliquots were applied to activated oxalate–coated silica thin-layer chromatography (TLC) plates for the identification of the phosphoinositides and PA. After TLC and autoradiography, bands were scraped and counted by liquid scintillation.

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TABLE 1
Clinical characteristics of insulin-dependent diabetes mellitus (IDDM) and control subjects

	IDDM (*n* = 16)	Control (*n* = 19)
Age (yr)	34.7 ± 2.5	37.1 ± 2.4
Sex (M/F)	12/4	11/8
Body mass index (kg/m²)	23.7 ± 7.1	25.1 ± 1.1
Duration of IDDM (yr)	15.5 ± 1.9	
HbA_{1c}* (%)	11.9 ± 1.0	5.2 ± 0.2†
Fasting blood glucose (mg/dl)	292 ± 49	94 ± 6†
Serum cholesterol (mg/dl)	195 ± 12	142 ± 9†
Serum triglycerides (mg/dl)	137 ± 16	57 ± 8†

Values are means ± SE.
*Normal laboratory range 4–8%.
†$P < .05$.

Phosphoinositide and PA mass. The mass of the individual phospholipids was estimated by a spectrophotometric assay for phosphorus (26). The individual radiolabeled phospholipids were localized and scraped into acid-washed glass tubes. The phospholipids were extracted twice from the silica with 1 ml chloroform/methanol HCl (6/3/1 vol/vol/vol). The extract was neutralized with base, and the remaining silica and salt were removed by centrifugation. Thirty microliters of a 10% $MgNO_3$ solution in methanol/water (1/9 vol/vol) was added, and the solution was dried under N_2. The sample was ashed over a Bunsen burner for at least 30 s and suspended in 1 ml of 2.4 M HCl. The tube was corked and heated for 60 min at 90°C. Five hundred–microliter aliquots were counted for ^{32}P. Thirty microliters of 1% Triton X-100 and 1 ml of dye solution (75% 9.3 mM malachite green HCl/25% 34 mM ammonium molybdate in 4.5 M HCl, which had been mixed and filtered 3 h before) were added to the remainder. After 40 min, absorbance was read at 660 nm in a Hitachi light spectrophotometer (model 100-40, Tokyo). Recoveries from silica gel were typically 79% PA, 39% PIP_2, 54% PIP, and 79% PI. These are similar to recoveries found previously (27).

Additional laboratory measurements. Fasting blood glucose levels were determined by glucose autoanalyzer, and glycosylated hemoglobin (HbA_{1c}) levels were determined by ion-exchange chromatography. Cholesterol and triglycerides were assayed in the Chemical Pathology Laboratory of the University of Michigan Hospital.

Statistical analysis. All statistical evaluations were performed on the University of Michigan IBM 3090-600E computer. Each variable was subjected to a repeated-measures analysis of variance (ANOVA), where time was the repeated measure, and group (diabetic or control) was a grouping factor. The relationship between platelet function and epidemiological variables was evaluated by linear-regression analysis. At all time points (in each subject and epidemiological variable), analysis was performed on the geometric mean of the individual phospholipid concentrations. All data are expressed as means ± SE. Differences were statistically significant at $P < .05$.

RESULTS

In vivo platelet activity. Plasma β-TBG was significantly increased ($P < .005$) in the IDDM (67.1 ± 7.3 ng/ml) compared with the control (41.0 ± 6.0 ng/ml) subjects. Abnormal kidney function can impair β-TBG clearance, but kidney function measured by serum creatinine was normal in all subjects tested.

$^{32}P_i$ incorporation into platelet phosphoinositides and PA. Basal incorporation of $^{32}P_i$ into the phosphoinositides and PA was examined over 180 min of incubation. Incorporation of $^{32}P_i$ reached isotopic equilibrium at 120 min for the IDDM and control subjects. Incorporation of $^{32}P_i$ into PIP_2 and PIP was significantly lower in the IDDM platelets (Fig. 1). For PIP_2, the difference was significant as early as 30 min and remained lower at 60, 120, and 180 min. $^{32}P_i$ incorporation into PIP was also significantly lower in platelets of IDDM subjects. In contrast, there were no significant differences in the time course of incorporation of $^{32}P_i$ into phosphatidylinositol (PI) and PA. Variable phosphorus content of the plasma of the subjects tested could not be controlled. Total plasma phosphorus concentrations in the IDDM (3.3 ± 0.5 mg/dl) and control (3.6 ± 0.6 mg/dl) groups did not differ significantly, however.

Phosphoinositide and PA mass. Because the difference in incorporation of $^{32}P_i$ into PIP_2 and PIP could have been due to differences in mass, mass was estimated in IDDM and control subjects. The estimated mass of PIP_2 in the platelets of IDDM subjects (0.71 ± 0.23 nmol/10^9 platelets) was <50% that in the platelets of the control subjects (1.65 ± 0.53 nmol/10^9 platelets; $P < .005$). There was an even greater (~5-fold) decrease in the mass of PIP in platelets of IDDM (0.66 ± 0.09 nmol/10^9 platelets) com-

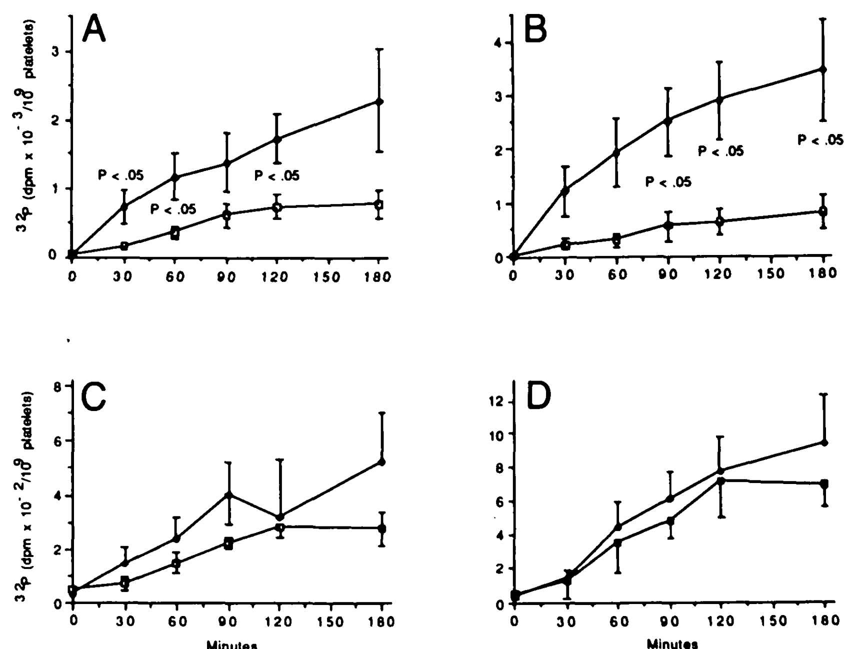

FIG. 1. Incorporation of [^{32}P]orthophosphate into phosphatidylinositol 4,5-bisphosphate (*A*), phosphatidylinositol 4-phosphate (*B*), phosphatidylinositol (*C*), and phosphatidic acid (*D*) in platelets of insulin-dependent diabetic (IDDM [▣]) and nondiabetic (♦) subjects. Each point represents mean of at least 5 subjects; 120-min point represents 16 IDDM and 19 control subjects. Data expressed as means ± SE.

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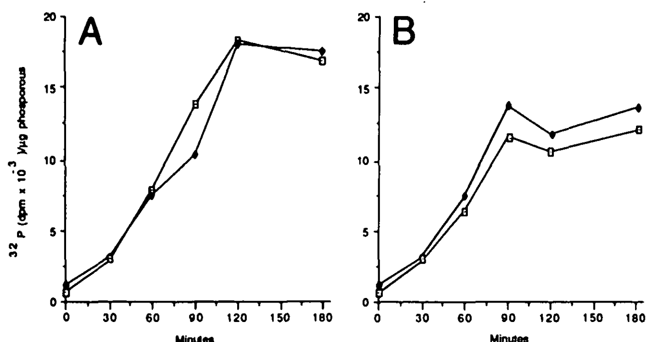

FIG. 2. Specific activity of phosphatidylinositol 4-phosphate (PIP, *A*) and phosphatidylinositol 4,5-bisphosphate (PIP_2, *B*). Each point represents mean of 5 subjects. SEs not given to maintain clarity of data points. No significant differences were noted between insulin-dependent diabetic (▣) and control (♦) subjects for PIP or PIP_2.

pared with the control (2.92 ± 0.43 nmol/10^9 platelets; $P <$ .001) subjects. However, there was no difference between IDDM and control subjects in the mass of PI (16.0 ± 1.4 vs. 13.7 ± 1.5 nmol/10^9 platelets) and PA (3.5 ± 0.6 vs. 3.8 ± 0.9 nmol/10^9 platelets).

Specific activity of phosphoinositides and PA. With time, there was an increase in $^{32}P_i$ incorporation per microgram of phosphorus. Equilibrium for PIP_2 and PIP was reached by 120 min and was not different between platelets of IDDM and control subjects (Fig. 2). Specific activity in PA and PI did not increase after 60 min (data not shown). Differential labeling with $^{32}P_i$ between IDDM and control subjects thus appears to be a function of mass and not a result of differences in $^{32}P_i$ uptake.

Stimulated phosphoinositide turnover. Because isotopic equilibrium incorporation of $^{32}P_i$ into the phosphoinositides was reached by 120 min, this was selected as optimal labeling for subsequent stimulation experiments. After stimulation with human thrombin, platelets of IDDM subjects showed decreased phosphoinositide turnover measured by hydrolysis of [^{32}P]PIP and [^{32}P]PIP_2 (Fig. 3). Whereas the expected fall in [^{32}P]PIP and [^{32}P]PIP_2 was found in control subjects, there was a small rise in [^{32}P]PIP and [^{32}P]PIP_2 in the platelets of IDDM subjects. [^{32}P]PA formation was decreased in IDDM compared with control subjects (Fig. 4).

Correlations. No relationship was found between any of the indices of platelet phosphoinositide turnover and plasma concentrations of β-TBG, body mass index, duration of diabetes, insulin dosage, total cholesterol, triglycerides, fasting glucose, and HbA_{1c}.

DISCUSSION

Platelet phosphoinositide turnover and formation of second messengers are key signal-transducing mechanisms linked to platelet activation (28–31). We examined the hypothesis that abnormalities in this mechanism accompany abnormal platelet function in IDDM. We found a reduction in mass of individual polyphosphoinositides and decreased turnover and formation of PA in platelets of IDDM subjects compared with platelets from healthy nondiabetic subjects of comparable age.

The cause of decreased PIP_2 and PIP mass in IDDM is not readily apparent. It does not appear to be related to methodological details. The levels of these polyphosphoinositides in the platelets of our control subjects are similar to those previously reported (27). Previous studies showed that advancing age and increasing cholesterol content in the platelet membrane have been associated with a concomitant decrease in phospholipid content (32–34). However, no correlation was found between total serum cholesterol level, triglycerides, or age and $^{32}P_i$ incorporation or mass of the individual phosphoinositides and PA. Interestingly, decreased phosphoinositide (total PI and PIP_2) content has been variably reported to occur in the sciatic nerve of STZ-D rats (35). Consistent with these findings, cytidine 5-diphosphate–DAG transferase and PIP kinase activities were decreased (36). Thus, our findings of decreased polyphosphoinositide content in the platelet may be evidence for a more ubiquitous phenomenon in diabetes.

Although decreased mass of the polyphosphoinositides in platelets of IDDM subjects might account for the decreased formation of PA when stimulated with thrombin, the decrease in hydrolysis of PIP and PIP_2 remains unexplained. Furthermore, we and others have demonstrated that platelets from patients with diabetes mellitus have exaggerated in vitro aggregatory responses to various stimuli, including thrombin (4–8). Therefore, there is also an apparent paradox between our findings of decreased polyphosphoinositide content and hydrolysis and increased in vivo platelet activity. Several explanations may account for the decreased phosphoinositide hydrolysis accompanying platelet hyperactivity in IDDM.

First, platelet activation can occur through mechanisms involving Ca mobilization and/or protein phosphorylation (37–39). The hydrolysis of PIP_2 results in the formation of IP_3 and DAG. IP_3 mobilizes Ca, and DAG activates protein kinase C (PKC). Subsequent phosphorylation of DAG yields PA. Ca mobilization and protein phosphorylation via PKC appear to be synergistic in platelet activation (39). Therefore, an elevated stimulus-coupled influx of intracellular Ca in di-

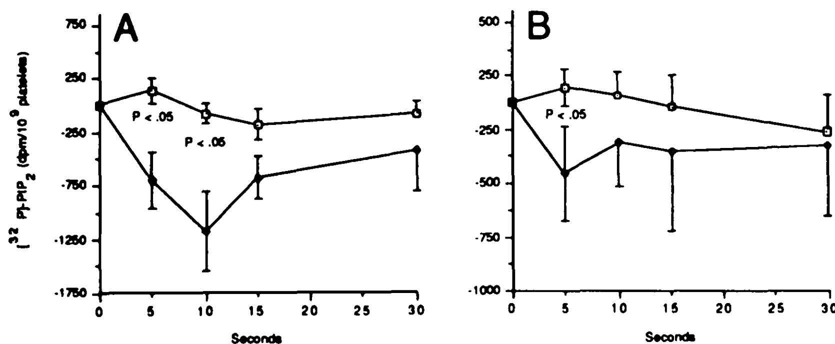

FIG. 3. Phosphatidylinositol 4,5-bisphosphate (PIP_2, *A*) and phosphatidylinositol 4-phosphate (PIP, *B*) hydrolysis after stimulation with thrombin. Data are means ± SE and are expressed as change in disintegrations per minute from basal. n = 14 insulin-dependent diabetic (▣) and 14 control (♦) subjects.

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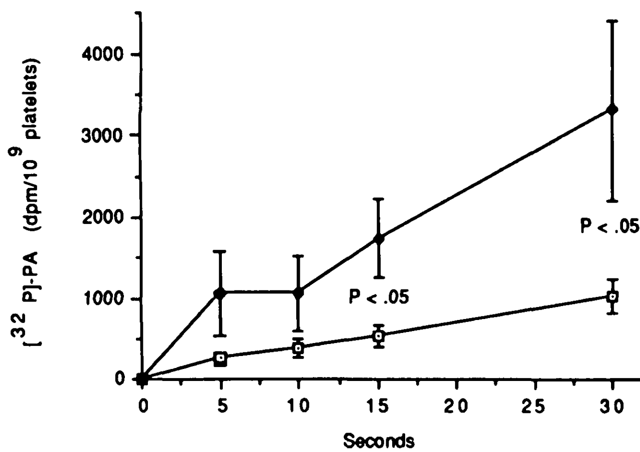

FIG. 4. Phosphatidic acid (PA) formation after stimulation with thrombin. Data are means ± SE and are expressed as change in disintegrations per minute from basal. n = 14 insulin-dependent diabetic (▣) and 14 control (♦) subjects.

abetic platelets, as has been seen (40), might require minimal IP_3 generation from PIP_2 hydrolysis to cause platelet activation. Alternatively, a decrease in the threshold for PKC activation or an increase in the amount of PKC in the platelet might make an apparent small amount of DAG that is sufficient for platelet activation. Both of these possibilities are being investigated in our laboratories.

Finally, a plasma factor could be responsible for the differences seen in phosphoinositide turnover. Because each incorporation and stimulation profile was undertaken in a medium containing ~75% autologous plasma, a factor present in the plasma could have contributed to the differences in phosphoinositide turnover. Insulin excess might be implicated as a factor responsible for alterations in platelet function, and ultimately atheromatous changes in the vascular bed (41,42). Insulin has been shown to activate phospholipase C and hydrolysis of a unique phosphoinositide glycan in adipocytes and myocytes (43). However, insulin has not been found to stimulate the hydrolysis of PI or the polyphosphoinositides (44,45). In those cell systems, insulin actually increased labeling of phospholipids (46–48). Although our laboratory has been unable to demonstrate an effect of insulin on in vitro aggregation (unpublished observations), another study has shown that insulin decreases rather than increases in vitro platelet sensitivity to various stimuli (49).

In conclusion, IDDM subjects with enhanced in vivo platelet activation have decreased platelet polyphosphoinositide content. When platelets of IDDM subjects are stimulated with human thrombin, there is decreased hydrolysis of PIP and PIP_2 and decreased formation of PA. Aberrant phosphoinositide turnover may be linked to increased in vivo platelet activity in IDDM.

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