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ADP-INDUCED CHANGES IN [^{32}P]PHOSPHATE LABELING OF PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE IN WASHED RABBIT PLATELETS MADE REFRACTORY BY PRIOR ADP STIMULATION

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Changes in ^{32}P labeling of phosphatidylinositol-4,5-bisphosphate (PIP_2) were examined during ADP-induced aggregation of washed rabbit platelets prelabeled with [^{32}P]phosphate. ADP caused a significant decrease in the amount and ^{32}P labeling of PIP_2 at 10 and 60 sec. The decrease in labeling persisted at 2.5 min when the platelets were still aggregated, but disappeared by 10 min. Platelets refractory to ADP showed no further significant change in ^{32}P in PIP_2 when exposed to ADP; a decrease in PIP_2 labeling could be induced, however, after platelets had recovered their disc shape and sensitivity to ADP. These data indicate that PIP_2 may play a role in the response of platelets to ADP.

The inositol phospholipids comprise less than 10% of the total phospholipids in cell membranes and appear to be involved in the response of the cells to stimuli (1). Changes in the inositol phospholipids have been found in platelets stimulated to aggregate (2-5) and to undergo the release of platelet granule constituents (6-12).

Changes in phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) have been described in nerves (13,14), iris muscle (15), brain tissue (16), parotid acinar cells (17) and platelets (2-7,12,18) in response to a variety of stimuli, although the role of these changes is unknown.

Abbreviations used: Phosphatidylinositol-4,5-bisphosphate (PIP_2); phosphatidylinositol-4-phosphate (PIP); prostaglandin E_1 and I_2 (PGE_1, PGI_2).

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We have recently shown that the amount of PIP_2 decreases within 10 sec of stimulation of platelets with ADP (5). We suggested that diversion of ATP required to maintain the synthesis of PIP_2 from PIP was the cause of the decrease in the amount of PIP_2. Support for this proposal was provided by a study of the effect on PIP_2 of PGE_1 or PGI_2 treatment of platelets (18). The treatment caused a transient shape change in the platelets and at the same time decreased the amount of PIP_2 (18), presumably due to diversion of ATP for formation of cAMP. Based on the earlier proposal of Hendrickson and Reinertsen (19) that conversion of PIP_2 to PIP could result in freeing of Ca^{2+}, we further suggested that Ca^{2+}, (mobilized by the decrease in the amount of PIP_2 caused by ADP) initiated Ca^{2+}-dependent reactions involved in the platelet response to ADP.

When washed platelets have been stimulated with ADP, they are refractory for a period of time to further stimulation by a similar concentration of ADP (20). If a decrease in PIP_2 were involved in the platelet response to ADP, the decrease would not be expected to occur in platelets refractory to ADP stimulation.

In this study, changes in PIP_2 in platelets prelabeled with [^{32}P]phosphate were determined up to 30 min after ADP stimulation. Changes in the labeling of PIP_2 were examined 1.5 min after addition of ADP to platelets made refractory to ADP by the addition of ADP 3 min previously, and in platelets which had recovered their responsiveness to ADP 30 min after the first addition of ADP.

METHODS

[^{32}P]Orthophosphate was supplied by New England Nuclear (Mississauga, Ont). ADP was purchased as the sodium salt from the Sigma Chemical Co. (St. Louis, MO). Silica Gel H made by Merck was from Brinkmann Instruments Inc., (Rexdale, Ont). Phospholipid standards were obtained from Supelco Inc. (Bellefonte, PA) and Serdary Research (London, Ont). Solvents for thin layer chromatography were distilled before use.

Aggregation Studies

Suspensions of washed rabbit platelets were prepared by the method of Ardlie et al.(21) with some modifications for labeling with [^{32}P]phosphate as described previously (22). The platelets were finally resuspended in Tyrode solution (containing calcium (2mM), magnesium (1mM), albumin (0.35%) and apyrase).

Samples (2.3 ml) of platelet suspension were stirred in siliconized cuvettes for 1 min at 37°C in Payton aggregometers. A control sample treated with Tyrode solution and a sample treated with ADP dissolved in Tyrode solution were run simultaneously in two aggregometers. The concentrations of ADP given in the Table and Figures are final concentrations in the platelet suspensions. In the case of samples taken 20 and 30 min after stimulation, stirring was continuous for the first 10 min and the last 3 min.

Lipid Extraction and Fractionation

The reactions of the platelets were terminated by addition of chloroform/methanol/10N HCl (1:2:0.12) extraction solvent (a modification of the method of Bligh and Dyer (23) reported by Lloyd et al. (2)) and the lipids recovered as previously described (22). The final extract in chloroform was divided into two portions, half to be fractionated by a thin layer chromatography system (a modification by Lloyd et al. (2) of the method of Gonzalez-Sastre and Folch-Pi (24)) to isolate the polyphosphoinositides and the rest to be fractionated by the method of Rouser et al. (25) which separates phosphatidylinositol and phosphatidylserine. The use of both methods has been described in detail (22).

Analysis of Lipid Phosphate and [^{32}P]Phosphate Incorporation

The phospholipids were identified by comparison with standards after visualization under uv light and autoradiography. For determination of labeling only, the silica gel was scraped directly into scintillation vials and counted (22). For determination of labeling and phosphate, the spots were scraped into pyrex tubes, and the phospholipids digested with perchloric acid in the presence of the silica gel (5). Samples were removed for determination of [^{32}P]phosphate and chemical assay of phosphate as previously described (5). Values were corrected to 100% recovery (5).

Specific Radioactivity of ATP

In experiments to compare the specific radioactivities of PIP_2 and ATP, the lipids were extracted and fractionated as described above. ATP was extracted using 70% perchloric acid and separated from other nucleotides by electrophoresis (26). The amount of ATP was determined in separate samples by the luciferin-luciferase assay (27).

Statistical Analysis

Data were analyzed using a paired t-test. Since the data did not fit a normal distribution as required by the analysis, a logarithmic transformation, which yielded a normal distribution, was used.

RESULTS

Addition of ADP (31 μM) to a suspension of washed platelets prelabeled with [^{32}P]phosphate caused the platelets to change shape and aggregate. The amount of PIP_2 was significantly decreased at 10 sec, although the [^{32}P]phosphate labeling was not (Fig. 1). By 60 sec both the amount and [^{32}P]phosphate labeling of the PIP_2 were significantly decreased (Fig. 1). At the time of maximal platelet aggregation (2.5 min) the ^{32}P labeling of

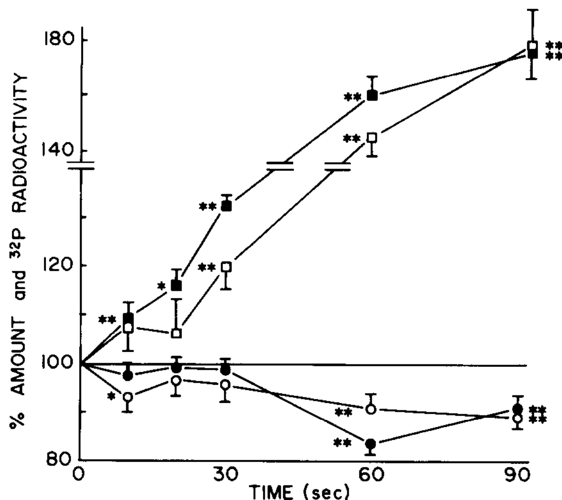

Fig.1 Changes in amount and [^{32}P]phosphate labeling of PIP_2 and PIP during ADP-induced aggregation of washed rabbit platelets. The concentration of ADP was 31 μM. The amount or radioactivity of PIP_2 or PIP in the control samples was taken as 100%. The data were calculated as the mean ± SEM of 9 to 27 pairs of samples from at least 3 experiments at each time. Amount (open symbols) and labeling (solid symbols) of PIP_2 (circles) or PIP (squares). * $p<0.05$; ** $p<0.01$.

PIP_2 was still significantly decreased (Fig. 2). When the platelets had deaggregated after exposure to ADP, the labeling of PIP_2 had returned to values which were not significantly different from those of control platelets (Fig. 2).

During the first 90 sec following stimulation with ADP there were significant increases in both the amount and labeling of PIP (Fig. 1). Following the point of maximum aggregation the labeling of PIP gradually declined toward the prestimulation level (Fig. 2).

No change was observed in phosphatidylinositol (PI) until 30 sec when the amount was decreased (-12%, $p<0.01$, n=17).

The increase in the amount of ^{32}P label in PIP_2 after aggregation reached maximum (Fig. 2) could be due to an increase in the amount of PIP_2 or incorporation of higher specific radioactivity [^{32}P]phosphate into the PIP_2. For the specific radioactivity of the PIP_2 to increase, the specific radioactivity of the [^{32}P]ATP in the platelets would have to be higher than that of PIP_2. As shown in Fig. 3 the specific radioactivity of ATP in unstimulated platelets was more than 10 times that of PIP_2 3 hrs after label-

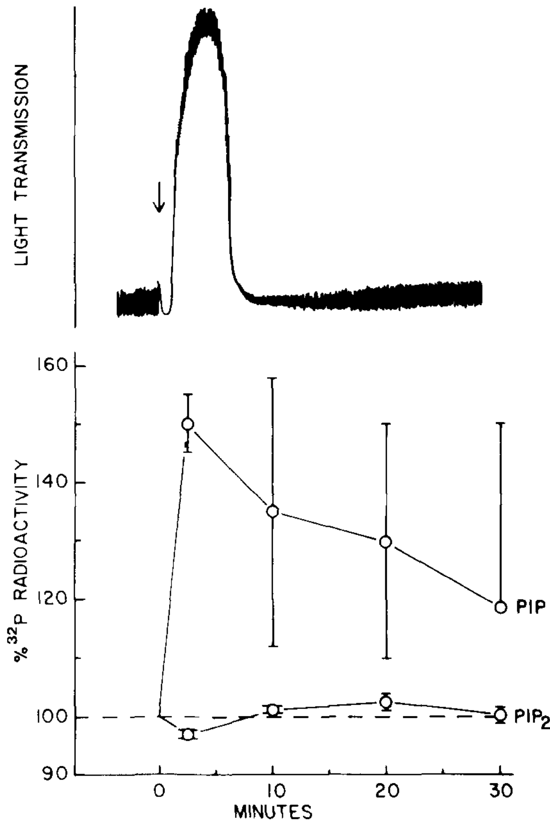

Fig.2 Changes in light transmission and in the [^{32}P]phosphate labeling of PIP_2 and PIP during platelet aggregation and deaggregation. The concentration of ADP added at the point indicated by the arrow was 10 μM The data, which represented 10 to 20 pairs of samples from at least 3 experiments, were expressed relative to control samples which were taken as 100%. The labeling of PIP_2 was significantly reduced at 2.5 min ($p<0.05$). The labeling of PIP was significantly increased only at 2.5 min ($p<0.001$).

ing. The experiments in Fig. 1 and 2 were conducted during the period between 3 and 4 hrs of the time course shown in Fig. 3.

A second addition of ADP 3 min after the first did not cause aggregation or a significant decrease in the ^{32}P labeling of PIP_2 (Table 1, group I). In contrast, a second addition of ADP 30 min after the first, did cause aggregation and a significant decrease in PIP_2 labeling (Table 1, group II). In the sample to which Tyrode solution was added 30 min after ADP stimulation, the ^{32}P labeling of PIP_2 was similar to that of the samples to which both additions had been Tyrode's solution (Table 1, group II), whereas at 3 min the labeling was significantly less (Table 1, group I).

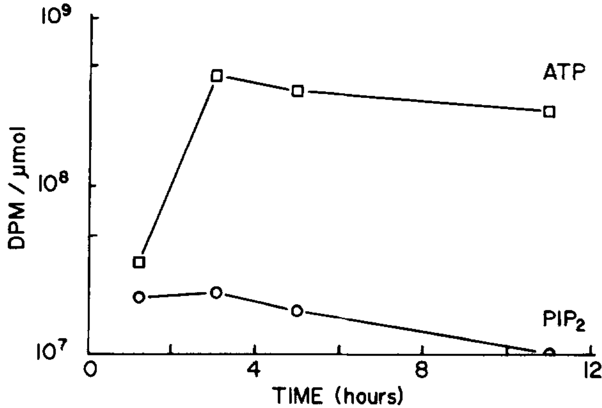

Fig.3 Incorporation of [^{32}P]phosphate into ATP and PIP_2 of rabbit platelets. Platelets were washed and resuspended in a medium containing unlabeled phosphate after 2 hr of incubation with label. The data are the means of three samples.

DISCUSSION

In this study we have confirmed our previous reports (2-5) that aggregation of prelabeled, washed rabbit platelets in response to ADP stimulation is accompanied by changes in both the amount and labeling of PIP_2. Furthermore, the decrease in PIP_2 labeling with [^{32}P]phosphate, 90 sec after ADP treatment of platelets, does not occur if the platelets are refractory to

Table 1.

^{32}P Labeling of PIP_2 90 sec after Addition of Tyrode Solution or ADP to Platelets Treated 3 or 30 min Previously with Tyrode Solution or ADP.

Time Between First and Second Additions	First Addition	Second Addition	^{32}P Labeling of PIP_2 After Second Addition ($dpm/10^6$ platelets) Mean ± SEM	(n)	
Group I	(A) Tyrode	Tyrode	14.5 ± 0.43	(11)	
					$p<0.05$
(3 min)	(B) ADP	Tyrode	14.0 ± 0.48	(11)	
					NS
	(C) ADP	ADP	13.7 ± 0.45	(11)	
Group II	(A) Tyrode	Tyrode	16.8 ± 0.49	(12)	
					NS
(30 min)	(B) ADP	Tyrode	17.1 ± 0.38	(12)	
					$p<0.001$
	(C) ADP	ADP	15.8 ± 0.55	(12)	

ADP was added initially to give a final concentration of 4 µM. Three min (Group 1) or 30 min (Study II) after the first ADP addition an equal amount of ADP was added. The platelet suspensions (10^9 platelets in 1 ml) were subjected to lipid extraction 90 sec after the second addition. The higher ^{32}P-labeling in PIP_2 at 30 min is likely due to incorporation of higher specific radioactivity phosphate from [^{32}P]ATP.

aggregation due to previous stimulation with ADP. The initial, transient decrease in PIP_2 in response to ADP stimulation has been previously reported (5) and was suggested to be due to diversion of ATP required to maintain the level of PIP_2.

In the series of experiments to study platelets refractory to ADP, the samples in which the second addition (at 3 min after the initial ADP stimulation) was Tyrode solution, showed a lower PIP_2 labeling (Table 1) which was consistent with the time course of PIP_2 labeling changes after ADP stimulation (Fig. 2). The small increase in the labeling of PIP_2 in the 30 min ADP-Tyrode sample, compared to the Tyrode-Tyrode control, may be due to a return of the amount of PIP_2 to the prestimulation level, accompanied by incorporation of higher specific radioactivity [^{32}P] phosphate.

The absence of a significant further decrease in the labeling of PIP_2 in platelets upon the second stimulation with ADP after 3 min, is consistent with the lack of aggregation in response to ADP. The significant decrease in PIP_2 labeling upon the second stimulation with ADP after 30 min, indicates that the concentration of PIP_2 may have to return to normal before its concentration can decrease again in response to a second addition of ADP.

The results of this study support the hypothesis that PIP_2 may play a role in the response of platelets to agents that exert some or all of their effects through ADP.

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