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The phosphoinositides exist in multiple metabolic pools in rabbit platelets

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The labelling of the phosphoinositides and phosphatidic acid in washed rabbit platelets incubated with [^{32}P]phosphate or [^{3}H]glycerol was studied in the presence of isotope and after unincorporated isotope had been removed. With both isotopes the increase in the specific radioactivity of phosphatidylinositol 4,5-bisphosphate (PIP_2) lagged behind that of phosphatidylinositol 4-phosphate (PIP) but the specific radioactivity remained higher after unincorporated isotope had been removed. This result was consistent with the presence of a second pool of PIP_2, which interconverted slowly with the pool of PIP_2 which was in direct equilibrium with PIP, proposed to explain the increase in specific radioactivity of PIP_2 which accompanies the decrease in amount of PIP_2 at 10 s in ADP-stimulated platelets. In platelets labelled with [^{3}H]glycerol, the specific radioactivity of PIP_2 became higher than that of PIP and the specific radioactivity of PIP became higher than that of phosphatidylinositol (PI). These results were interpreted to indicate that there were two pools of PIP; of these the pool with the higher specific radioactivity was the precursor of PIP_2. Similarly, two pools of PI were proposed. The presence of pools of the phosphoinositides with different specific radioactivities necessitates the measurement of chemical amount of these compounds when studying the effect of stimulation of the platelets, since changes in labelling may not accurately reflect changes in the amount of the phosphoinositides.

INTRODUCTION

The rapid metabolism of the phosphoinositides in both resting and stimulated platelets was well established by early studies in which the incorporation of [^{32}P]phosphate into the lipids was studied (Cohen *et al.*, 1971; Lloyd *et al.*, 1972; Leung *et al.*, 1977). Little is known, however, about the distribution of phosphoinositides in platelets. The intracellular locations of some of the enzymes of phosphoinositide metabolism have been identified, including phospholipase C (Billah *et al.*, 1980; Rittenhouse-Simmons, 1979) which is primarily cytoplasmic, and phospholipase A_2 and diacylglycerol lipase (Lagarde *et al.*, 1981) which are apparently associated with internal membranes. The asymmetry of phospholipids in the platelet plasma membrane is consistent with that of other cell types, and PI is exposed primarily to the interior of the platelet (Chap *et al.*, 1979; Schick *et al.*, 1975). One study indicates, however, that up to 16% of PI is exposed on the exterior surface of platelets (Perret *et al.*, 1979). The presence of PI on the exterior of the plasma membrane has been shown in other cell types, based on the results of treatment of intact cells with phospholipase C (Low & Finean, 1978; Shukla *et al.*, 1980; Shukla, 1982). Since movement of phospholipids across membranes is considered to be slow, these results raise the possibility that two metabolically separate pools of PI may exist, one inside the platelets and the other on the outside surface. Holmsen *et al.* (1981) and Laffont *et al.* (1981) have concluded that separate metabolic pools of PI may not exist within platelets, but that there is evidence for distinct pools of PA. Based on a comparison of the degradation of PI in thrombin- or ionophore-stimulated platelets, Billah & Lapetina (1982) have suggested that there may be three functional pools of PI, although the apparently separate pools may be the result of different distributions of the enzymes involved rather than physically isolated pools of PI.

We have previously reported that stimulation of rabbit platelets with ADP caused a decrease in the chemical amount of PIP_2, possibly due to reduced synthesis (Vickers *et al.*, 1982, 1983). This decrease in PIP_2 was accompanied by an increase in specific radioactivity of PIP_2 in platelets labelled with [^{32}P]phosphate and [^{3}H]glycerol. We suggested that this might be due to the presence of more than one pool of PIP_2. We have also observed that the specific radioactivity of PIP in platelets labelled with [^{3}H]glycerol did not increase when the amount increased, in spite of the higher specific radioactivities of PI and PIP_2 which were the potential sources of the increased amount of PIP. Again this result indicated that the increase in PIP involved pools of PI or PIP_2 with a specific radioactivity lower than the average specific radioactivity of these phosphoinositides.

Since the interpretation of data from experiments with labelled platelets would be influenced by the presence of pools of the phosphoinositides, we have examined the incorporation of [^{32}P]phosphate and [^{3}H]glycerol into the phospholipids of unstimulated, washed rabbit platelets for up to 3 h, to determine whether multiple pools can be identified on the basis of labelling.

MATERIALS AND METHODS

Materials

ADP was purchased from Sigma. Phospholipid standards were from Serdary Research Laboratories

Abbreviations used: PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; IP, IP_2 and IP_3, inositol phosphate, bisphosphate and trisphosphate.

(London, Ont., Canada) except for PIP_2 and PIP which were from Sigma. T.l.c. plates precoated with silica gel H60 (Merck) were from Brinkmann Instruments (Rexdale, Ont., Canada). Solvents for t.l.c. were distilled before use. [^{3}H]Glycerol (10 Ci/mmol) and carrier-free [^{32}P]orthophosphate were purchased from New England Nuclear (Dorval, P. Q., Canada). Aequorin was purchased from Dr. J. Blinks (Mayo Clinic, Rochester, MN, U.S.A.). The platelet ionized calcium aggregometer was generously loaned by Chrono-Log Corp. (Havertown, PA, U.S.A.).

Time course of platelet phospholipid labelling

Suspensions of washed rabbit platelets were prepared according to the method of Ardlie *et al.* (1971). The usual first washing solution (calcium-free Tyrode solution) was replaced with a calcium-free and phosphate-free Tyrode solution containing 5 mM-Hepes, 0.35% albumin and apyrase capable of converting 0.25 μM-ATP to AMP in 120 s. Platelets were washed once in the calcium- and phosphate-free Tyrode solution and adjusted to 10^9 platelets/ml in the same medium before the addition of [^{3}H]glycerol (20 μCi/ml) and [^{32}P]phosphate (20 μCi/ml), the time of addition being taken as 0 time. The 15, 30 and 60 min samples (1 ml) were removed from the incubation mixture and the platelets were freed from unincorporated radioactive precursor by centrifugation in an Eppendorf centrifuge (8000 *g* for 1 min) and resuspension in calcium-free Tyrode solution; this was repeated once. Finally, the platelet pellet was resuspended in Tyrode/albumin solution and lipid extraction started by the addition of chloroform/methanol (1:2, v/v) extraction solvent. The lipids were then extracted with a neutral solvent and then with an acidic solvent system as previously described (Vickers *et al.*, 1982). At 1 h the remaining platelet suspension was freed of unincorporated label by centrifuging and resuspending the platelets twice in calcium-free Tyrode solution. Platelets were finally resuspended in Tyrode/albumin solution containing apyrase and the incubation continued at 37 °C. Samples (1 ml) were removed at 2, 2.5 and 3 h and lipids extracted as described above.

Fractionation of platelet phospholipids and determination of amount and labelling

Platelet phospholipid extracts were fractionated on two-dimensional thin layer chromatograms and the locations of the phospholipids identified by autoradiography and by viewing under u.v. light after spraying with Rhodamine 6G. The silica gel containing the phospholipids was scraped into test tubes for digestion with $HClO_4$ prior to phosphate analysis (Chalvardjian & Rudnicki, 1970; Duck-Chong, 1979) and determination of radioactivity. The methods have been described in detail previously (Vickers *et al.*, 1982).

Analysis of phospholipid data

For all experiments, the values for the platelets resuspended in Tyrode/albumin solution at 2 h were standardized to 10^9 platelets/ml and a total [^{32}P]phosphate incorporation of 2×10^6 d.p.m./10^9 platelets or a total [^{3}H]glycerol incorporation of 4×10^6 d.p.m./10^9 platelets, which were approximately the average values. The factor to standardize the 2 h values in each experiment was used to standardize the results throughout the time course.

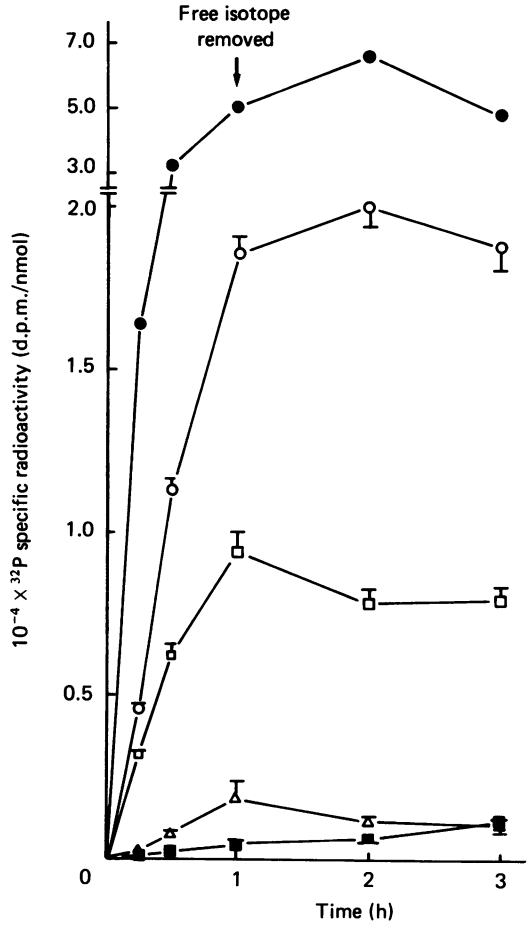

Fig. 1. Time course of changes in the ^{32}P specific radioactivities of ATP (●), PIP_2 (○), PIP (□), PI (■) and PA (△) in washed rabbit platelets incubated for 1 h with 20 μCi of carrier-free [^{32}P]phosphate/ml and then washed free of label as described in the Materials and methods section

The data are from three experiments. The increments in PIP_2 specific radioactivity up to 2 h and the decrease from 2 to 3 h were significant ($P < 0.01$). PIP specific radioactivity increased significantly up to 1 h ($P < 0.001$), decreased significantly from 1 to 2 h ($P < 0.002$) and then did not change significantly. All increments in PI specific radioactivity were significant ($P < 0.01$) and the changes in PA between 0.5 and 1 h and 1 and 2 h were significant ($P < 0.01$). Differences were analysed with a paired *t*-test.

The standardized values were then averaged and the significance of the data was assessed using a paired *t*-test.

Determination of the specific radioactivity of ATP

The specific radioactivity of ATP labelled with [^{32}P]phosphate was determined in samples prepared and incubated with [^{32}P]phosphate as described for the study of the time course of phospholipid labelling. The reactions were terminated by addition of an equal volume of EDTA/ethanol solution (9 vol. of 95% ethanol and 1 vol. of 77 mM-EDTA) at 0 °C. After centrifugation of the mixture, the supernate was fractionated by high voltage paper electrophoresis in a citrate buffer. The spot containing ATP was located by comparison with standards and eluted with water. The amount of ATP was determined by measurement of A_{254}

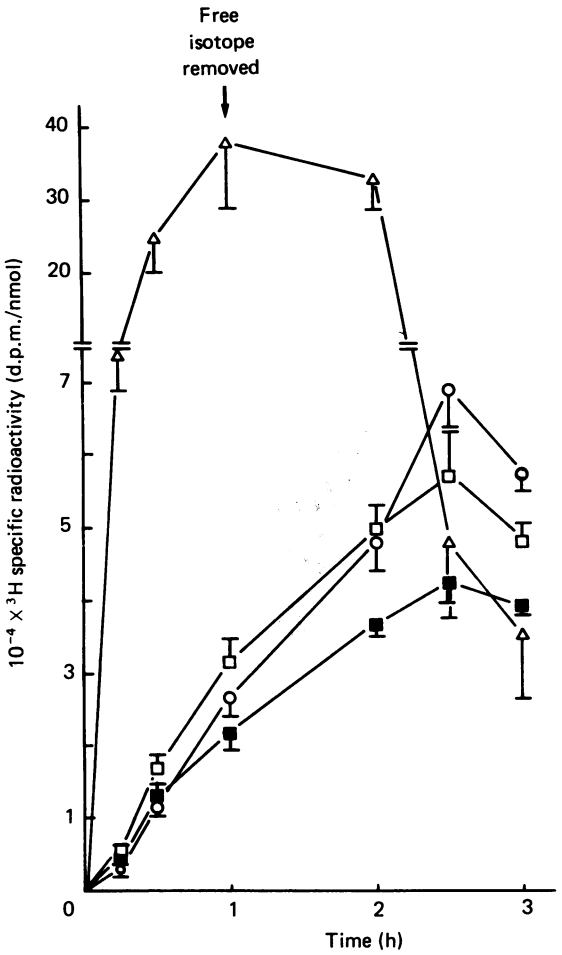

Fig. 2. Time course of the changes in the ^3H specific radioactivities of PIP_2 (○), PIP (□), PI (■) and PA (△) in washed rabbit platelets incubated with [^3H]glycerol as described in Fig. 1

The data were obtained from three experiments. The specific radioactivity of PIP_2 was less than that of PIP at 15, 30 and 60 min ($P < 0.005$) and greater than that of PIP at 2.5 and 3 h ($P < 0.005$). The specific radioactivity of PIP was greater than that of PI at all times ($P < 0.005$). The specific radioactivity of PA was greater than that of PI until 2 h ($P < 0.001$) and not different in the 2.5 and 3 h samples. Differences were analysed with a paired t-test.

after confirmation that the spectrum was consistent with ATP being the major u.v.-absorbing species present.

Incorporation of aequorin into platelets

Rabbit platelets were loaded with aequorin by a modification of the methods of Johnson *et al.* (1985) and Gomperts (1983). Platelets were incubated in calcium-free Tyrode solution with 10 mM-EGTA for 15 min. After centrifugation, they were resuspended at a concentration of 4×10^9 platelets/ml in a medium containing 91 mM-NaCl, 1.8 mM-KCl, 8 mM-$NaHCO_3$, 0.3 mM-NaH_2PO_4, 67 μM-EGTA and 0.67 mg of glucose/ml. The pH was adjusted to 7.35, the osmolarity was approximately 195 mosM and the solution was prewarmed to 37 °C. A 1 ml sample of the suspension was used. To initiate loading, ATP was added to 5 μM and aequorin was added to 200 μg/ml. After a 5 min incubation at 37 °C, Mg^{2+} was added to 2 mM and 16.7 μl of stock solution containing 2.74 M-NaCl, 54 mM-KCl, 238 mM-$NaHCO_3$ and 8.4 mM-NaH_2PO_4 was added to restore the osmolarity to approx. 290 mosM. The platelets were incubated for 15 min in this medium and then centrifuged and resuspended in calcium-free Tyrode solution two times.

For study of the decay of aequorin in the platelets, the final suspension in calcium-free Tyrode solution was divided into two aliquots and centrifuged. One portion was resuspended in calcium-free Tyrode solution and the other in Tyrode/albumin solution with apyrase. In both cases the platelet concentration was adjusted to 10^8 platelets/ml. Samples (1 ml) were removed at 0, 30, 60, 90, 120 and 150 min for determination of total remaining aequorin.

Measurement of total aequorin in platelets

Aequorin-loaded platelets (1 ml) suspended in Tyrode/albumin solution (contains 2 mM-Ca^{2+}) were stirred for 30 s in the platelet ionized calcium aggregometer and then lysed by addition of Triton X-100 to a final concentration of 0.2%. Calcium was added to the platelets suspended in calcium-free Tyrode solution to a final concentration of 2 mM and, after 30 s of stirring, Triton X-100 was added to 0.2%. The peak of the luminescence signal resulting from the addition of Triton X-100 was very narrow and the luminescence was estimated directly from the peak height. Aequorin luminescence in the presence of excess calcium has been shown by Blinks *et al.* (1978) to be proportional to the amount of aequorin over a wide range of aequorin concentrations. The data from two experiments were normalized by expressing peak heights as a percentage of the peak height at time 0 and fitted to the exponential equation:

$$y = C_0 e^{-kt}$$

by using the Expofit program of Barlow (1983).

RESULTS

Time courses of phospholipid and ATP labelling

Incorporation of [^{32}P]phosphate into ATP. Incubation of platelets with 20 μCi of carrier-free [^{32}P]phosphate/ml resulted in rapid incorporation of label into platelet ATP (Fig. 1). When the platelets were centrifuged at 1 h suspension were removed at 15, 30 and 60 min and 2, 2.5 and 3 h for determination of the amount of ATP. The amount of ATP in platelets did not change during this period and the average amount of ATP was determined to be 108.1 ± 3.2 nmol/10^9 platelets. Based on the estimate that 40% of platelet ATP is in the metabolic pool (Reimers *et al.*, 1977), the amount of ATP in the metabolic pool would be 43 nmol/10^9 rabbit platelets. This value was used to express the incorporation of [^{32}P]phosphate into ATP as d.p.m./nmol of metabolic ATP (Fig. 1).

Time courses of ^{32}P labelling of the phospholipids. Incubation of platelets with [^{32}P]phosphate resulted in incorporation of ^{32}P into the phosphoinositides and PA (Fig. 1). when the platelets were centrifuged at 1 h and resuspended in a medium containing unlabelled phosphate (these procedures were complete at about 1.5 h), the specific radioactivities of PIP_2, PIP and PA increased to a peak between 1 and 2 h and then

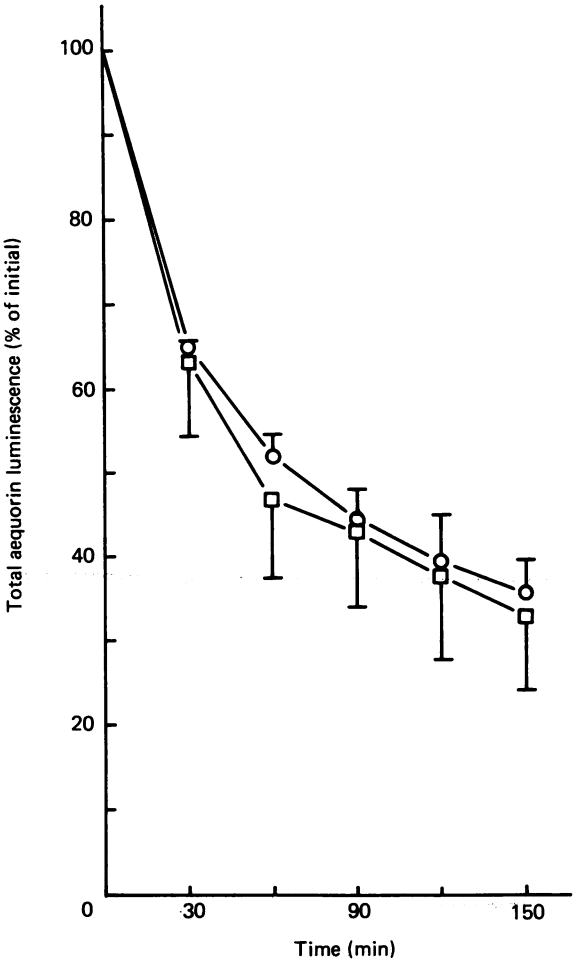

Fig. 3. Time course of the decrease in total aequorin luminescence in rabbit platelets incubated in calcium-free Tyrode/albumin solution (□) or Tyrode/albumin solution (containing 2 mM-calcium) (○) at 37 °C

Platelets were loaded with aequorin and total luminescence was determined by lysis of the platelets in a medium containing 2 mM-calcium with 0.2% Triton X-100 in a platelet ionized calcium aggregometer as described in the Materials and methods section. The results, which are expressed as a percentage of initial aequorin luminescence which was similar in the two media, are the means of two experiments.

decreased slowly, while the specific radioactivity of PI was still increasing at 3 h.

Time course of labelling of the phospholipids with [^{3}H]glycerol. The incorporation of [^{3}H]glycerol into the phospholipids followed a different pattern (Fig. 2). Of the phospholipids studied, the specific radioactivity of PA increased most rapidly while the [^{3}H]glycerol was present and decreased most rapidly after removal of [^{3}H]glycerol. The specific radioactivities of PIP_2, PIP and PI reached a maximum at 2.5 h. By 3 h their specific radioactivities had decreased, but the specific radioactivities of PIP_2 and PIP were still greater than the specific radioactivities of PA and PI. PI showed only a slow decrease in specific radioactivity after 2.5 h. Although the specific radioactivity of PIP was initially significantly greater than that of PIP_2, after 2 h the specific radioactivity of PIP_2 was greater than that of PIP. Throughout the period up to 3 h the specific radioactivity of PIP was greater than that of PI.

Changes in aequorin luminescence in unstimulated platelets

Incubation of platelets in media containing different concentrations of Ca^{2+} might influence the internal Ca^{2+} concentration of the platelets and thus phosphoinositide metabolism. To examine this possibility, the rate of disappearance of aequorin, due to the basal, internal Ca^{2+} concentration of platelets, was examined in the different media. When platelets loaded with aequorin were incubated at 37 °C, the amount of aequorin (as indicated by total luminescence when platelets were lysed with Triton-X100 in the presence of 2 mM-Ca^{2+}) decreased with time (Fig. 3). The decrease was similar whether the platelets were incubated in calcium-free Tyrode solution (no added Ca^{2+}) or in Tyrode/albumin solution (2 mM-Ca^{2+}). The initial amount of aequorin in the two samples was comparable. The data fitted an exponential equation with correlation coefficients of 0.99 (platelets in calcium-free Tyrode solution) and 1.0 (platelets in Tyrode/albumin solution). The rate constants (k) calculated from the equation were not significantly different (0.00767 ± 0.00077 for platelets in calcium-free Tyrode solution and 0.00844 ± 0.00128 for platelets in Tyrode/albumin solution) indicating that the rates of decrease in the amount of aequorin in the two samples of platelets were not significantly different. Thus the internal Ca^{2+} concentration of platelets suspended in the absence or presence of 2 mM-Ca^{2+} is not significantly different for up to 2.5 h.

DISCUSSION

The primary purpose of the experiments reported in this paper was to test the hypothesis that there may be more than one metabolic pool of each of the phosphoinositides in platelets. The results are consistent with this hypothesis.

In previous studies using rabbit platelets prelabelled with [^{3}H]glycerol or [^{32}P]phosphate and stimulated with ADP (Vickers *et al.*, 1982, 1983; Leung *et al.*, 1983), we observed that while the amount of PIP_2 decreased significantly at 10 s, the specific radioactivity with either isotope increased (Fig. 4). The increase in specific radioactivity at 10 s could be explained by two pools of PIP_2 which interconvert slowly (Fig. 5). One pool of PIP_2 (pool 1) is envisioned to interconvert rapidly with PIP. The second pool of PIP_2 is proposed to interconvert slowly with the first and in prelabelled platelets to have a higher specific radioactivity. The observed increase in specific radioactivity would be due to stimulation by ADP causing a shift in the equilibrium between PIP and the PIP_2 in pool 1. The net effect of this would be to decrease the amount of PIP_2 while increasing the average specific radioactivity, since the decrease in amount is in the pool of lower specific radioactivity.

To test the hypothesis that there were multiple pools of PIP_2 and to determine how pool 2 of PIP_2 came to have a higher specific radioactivity, the time courses of labelling of the phosphoinositides with [^{32}P]phosphate and [^{3}H]glycerol were studied. When platelets were

Table 1. Ratios of the specific radioactivities of PIP_2 and PIP in platelets incubated with [^{32}P]phosphate for 60 min after which the radioactive phosphate was replaced with unlabelled phosphate

Values were calculated from the time course shown in Fig. 1 (three experiments).

	Time (min)	Specific radioactivity ratio PIP_2/PIP
^{32}P present	15	1.46
	30	1.81
	60	1.94
^{32}P removed	120	2.57
	150	2.37
	180	2.35

labelled with ^{32}P, PIP and PIP_2 became labelled more rapidly than PI and PA (Fig. 1). This is consistent with the reports (Cohen *et al.*, 1971; Lloyd *et al.*, 1972; Leung *et al.*, 1977) which emphasized the very rapid labelling of the monoesterified phosphates in the polyphosphoinositides (which are turning over because of the futile phosphorylation–dephosphorylation cycles) compared with the slower labelling of the diesterified phosphate of PI and monoesterified phosphate of PA, which are incorporated in synthesis *de novo*. In these circumstances, the diesterified phosphates in PI, PIP and PIP_2 would have a similar specific radioactivity which would be much lower than that of the monoesterified phosphates in PIP and PIP_2 which are turning over as a result of the futile cycles. On this basis the diesterified phosphate of PIP and PIP_2 would be expected to contribute little to the specific radioactivity of PIP and PIP_2. Thus the ratio of the specific radioactivities of PIP_2, which has two monoesterified phosphates and PIP, which has one monoesterified phosphate, would be approx. 2:1 if there were only one pool of each. While ^{32}P was present in the suspending medium the ratio (Table 1) was less than 2:1 (ranging from 1.46 to 1.94) and then after removal of ^{32}P from the suspending medium the ratio became greater than 2:1 (ranging from 2.57 to 2.35). These observations are compatible with there being at least two pools of PIP_2. The specific radioactivity of the monoesterified phosphates in PIP_2 in pool 1 and PIP, which are rapidly turning over, would increase in parallel with the specific radioactivity of ATP when ^{32}P was present in the medium. However, the increase in specific radioactivity of the monoesterified phosphates in PIP_2 in pool 2 would lag behind the increase in specific radioactivity of the monoesterified phosphates in PIP_2 in pool 1 and PIP (Fig. 4), leaving the average specific radioactivity of the monoesterified phosphates in PIP_2 less than that of the monoesterified phosphate in PIP and thus the ratio less than the 2:1 ratio that would have been expected if there were only one pool of PIP_2. When the ^{32}P in the suspending medium is removed, there would be a decrease in the specific radioactivity of the monoesterified phosphates of PIP_2 and PIP in parallel with the decrease in specific radioactivity of ATP. However, due to the slow interchange between PIP_2 in pool 1 and pool 2, the decrease in specific radioactivity of the monoesterified phosphates of PIP_2 in pool 2 would lag behind that in

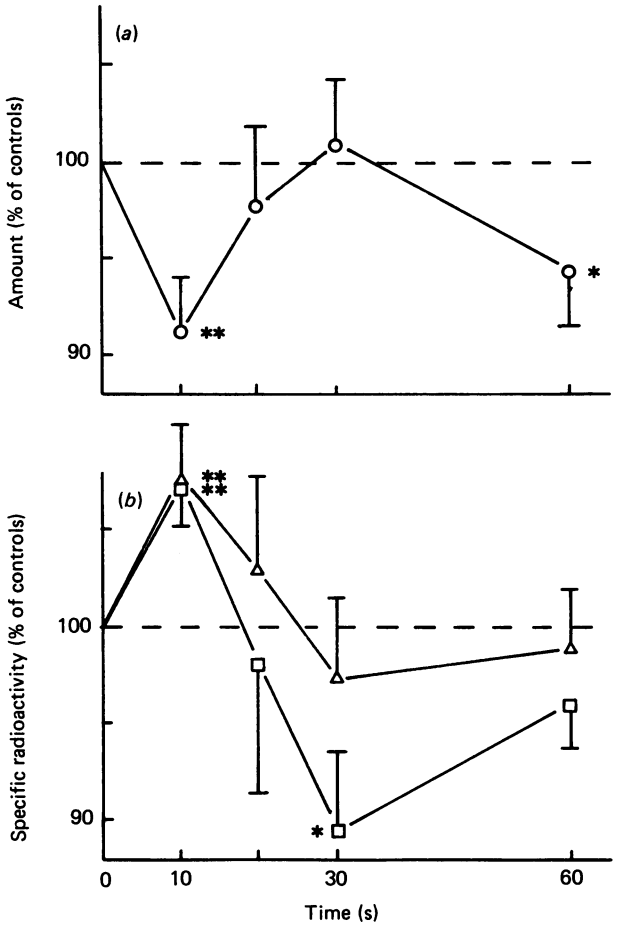

Fig. 4. Time courses of the changes in the (*a*) amount and (*b*) specific radioactivity of PIP_2 in rabbit platelets prelabelled with [^{32}P]phosphate or [^{3}H]glycerol and stimulated with ADP

Platelets were prelabelled with the isotopes for 1 h, transferred to medium containing unlabelled phosphate and incubated for an additional 1 h. After stimulation with 10 μM-ADP, samples were removed at the indicated times. The phospholipids were extracted, fractionated by t.l.c. and quantified as described in the Materials and methods section for samples obtained from the time course study. Amount (○) and ^{3}H (□) and ^{32}P (△) specific radioactivity of PIP_2 are shown. Parts of these data have been presented previously (Vickers *et al.*, 1982, 1983). $*P < 0.05$, $**P < 0.01$.

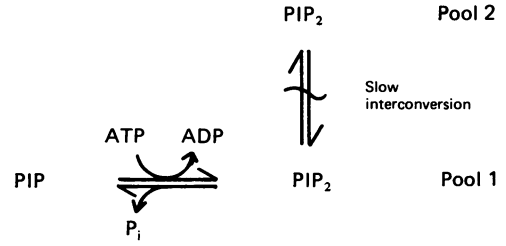

Fig. 5. Proposed relationship of two pools of PIP_2 to PIP in rabbit platelets

pool 1 and thus remain higher. This would lead to the specific radioactivity of the monoesterified phosphates in PIP_2 being higher than that of the monoesterified phosphate in PIP, giving a ratio that is greater than 2:1. The observation that the specific radioactivity of PIP_2 began to decrease later (decrease apparent at 3 h) than that of PIP (decrease apparent at 2.5 h) following removal of ^{32}P is consistent with this interpretation.

The time courses of labelling of PIP and PIP_2 in platelets incubated with [^{3}H]glycerol are also compatible with the proposed pools of PIP_2 (Fig. 4). During the labelling with [^{3}H]glycerol, the specific radioactivity of PIP_2 lagged significantly behind that of PIP. Following removal of [^{3}H]glycerol from the suspending medium, the specific radioactivity of PIP_2 became greater than that of PIP. The lag in the movement of label into and then out of pool 2 of PIP_2 would explain these changes in specific radioactivity. With [^{3}H]glycerol, the lag in increases in specific radioactivity of PIP_2 compared with PIP could also be explained by the delay in the synthetic pathway to PIP_2, although the rapid interconversion of PIP and PIP_2 indicated by the rate of labelling with ^{32}P makes this seem unlikely. However, when taken together with the observed changes in ADP-stimulated platelets, which are best explained by the presence of the two pools of PIP_2, the lag in the specific radioactivity of PIP_2 is consistent with the proposal of two pools of PIP_2.

At 1.5 h after washing out unincorporated isotope, the specific radioactivity of PIP_2 in platelets labelled with [^{3}H]glycerol became higher than the specific radioactivity reached by PIP at any time. One hypothesis to account for this observation is that there could be two pools of PIP with different specific radioactivities, of which the one with the higher specific radioactivity acts as a direct precursor of PIP_2. Similarly, at least two pools of PI have to be postulated to explain the observation that the specific radioactivity of PIP is higher than that of PI. An inactive pool of PI may be similar to that demonstrated for PI on the external surface of the plasma membrane of a number of cells (Low & Finean, 1978; Shukla *et al.*, 1980). The low specific radioactivity of PI labelled with [^{3}H]glycerol compared with PA is consistent with the existence of a pool of PI which is not labelled. The data give no information on the possibility of pools of PA in platelets.

Since in these experiments the platelets were incubated without added calcium in the suspending medium (concentration about 10^{-5} M) for the first 1 h and, after centrifugation, resuspended in a medium containing 2 mM-calcium, it is of concern whether or not the shift from a medium containing low calcium to one with 2 mM-calcium affected the results. The study with aequorin demonstrates that the internal calcium concentration of the platelets over a period of 2.5 h is not affected by the low concentration of calcium in the medium compared with the platelets incubated in the presence of 2 mM-calcium. Therefore, it seems unlikely that the changes in the labelling and amount of the phosphoinositides reported in this study could be attributed to changes in the internal calcium content of the platelets. As well, the changes in [^{3}H]glycerol specific radioactivity upon which the proposals of pools are based occur well after the platelets are resuspended in the medium containing 2 mM-calcium. Finally, the platelets were suspended in a medium containing 2 mM-calcium throughout the study of phosphoinositide changes in ADP-stimulated platelets, upon which the proposal of the pools of PIP_2 was based. Thus, although the shift from low to 2 mM-calcium in the suspending medium may have some influence, the overall data indicate that the changes that are being observed in the phosphoinositides are related to the existence of pools of the phosphoinositides rather than the shift in external calcium.

The results of this study indicate that there is more than one pool of each of the phosphoinositides and provide an explanation for the increase in specific radioactivity of PIP_2 when prelabelled platelets are stimulated with ADP, which causes a decrease in the amount of PIP_2. The results also demonstrate that caution must be exercised in the interpretation of labelling data in such studies; for example, because the specific radioactivity of PIP_2 increased as a result of the change in the proportions of the two pools of PIP_2, while the amount decreased, the labelling was not significantly changed (Vickers *et al.*, 1982). If only labelling were studied in this case, the effect of ADP stimulation on PIP_2 would not be obvious.

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