Transbilayer Distribution and Mobility of Phosphatidylinositol in Human Red Blood Cells*

(Received for publication, March 28, 1990)

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The present studies describe the distribution of phosphatidylinositol (PI) within the membrane bilayer of the human red blood cell (RBC) as well as its transbilayer mobility. The membrane bilayer distribution was determined by measuring the hydrolysis of PI in the exterior leaflet of the RBC membrane using a PI-specific phospholipase C and by extraction of PI from the exterior leaflet using bovine serum albumin. The transbilayer mobility of PI was measured by following the fate of radiolabeled PI which was first incorporated into the outer leaflet of the RBC membrane. Our results indicate that PI is asymmetrically distributed in the membrane, with approximately 80% located in the inner and 20% in the outer leaflet of the bilayer. The rate of transbilayer mobility of PI is similar to that for certain molecular species of phosphatidylycholine and much slower than that reported for the aminophospholipids in the RBC membrane.

The major phospholipid classes of the human red blood cell (RBC) membrane, phosphatidylycholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin, account for more than 94% of total membrane phospholipid. These phospholipids are distributed asymmetrically over the two halves of the lipid bilayer. The aminophospholipids PE and PS are largely confined to the inner leaflet of the bilayer, whereas the choline-containing phospholipids PC and sphingomyelin are predominantly localized in the outer leaflet (Roelofsen, 1982). This asymmetry appears to be generated and maintained by an ATP-dependent translocation of aminophospholipids from outer to inner leaflet (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Connor and Schroit, 1988; Middelkoop et al., 1988), and by interaction of

The phosphoinositides, phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinosito...
phorus was determined according to Bartlett (1959).

Extraction of Endogenous PI from RBC with BSA—Washed RBC were incubated at a hematocrit of 20% in incubation buffer in the presence or absence of 3% BSA for 45 min at 37 °C in a shaking waterbath. Preparation of ghost membranes from control and BSA-treated RBC, lipid extraction, and analysis were done as described above. The changes in PI and lysophosphatidylcholine relative to PS or PE between control and BSA-treated RBC were used to quantitate the amounts of PI and lyso-PC extracted by BSA.

Incorporation of [3H]PI into RBC Membranes—Washed RBC, at a hematocrit of 20%, were incubated with PI donor vesicles at 37 °C. At designated timepoints, RBC were pelleted by centrifugation, and the relative amount of radiolabel was determined from the total amount in the suspension and the amount of radiolabel in the supernatant. The amount of donor vesicle PI added to the RBC suspension was approximately 0.3% of total RBC membrane phospholipid.

Back-extraction of 3HPI with BSA—[3H]PI-labeled RBC were incubated at a hematocrit of 20% at 37 °C in incubation buffer containing 3% BSA. At designated timepoints, aliquots were removed from the incubation mixture, RBC were pelleted as described above, and the relative amount of radiolabel removed from RBC by BSA was calculated from the total amount in the suspension and the amount of radiolabel in the supernatant.

Phospholipase C Treatment of [3H]PI-labeled RBC Membranes—[3H]PI-labeled RBC were incubated at a hematocrit of 20% in incubation buffer at 37 °C. At designated timepoints, RBC were pelleted by low speed centrifugation and the incubation buffer was replaced with PLC buffer. Subsequently, PI-PLC was added to the RBC suspension (see above) and hydrolysis of PI was carried out at 37 °C for 15 min. RBC were pelleted and the amount of radiolabel present in the cell-free supernatant was measured and expressed as the percentage of the total amount of [3H]PI incorporated into RBC. In control experiments, vesicles labeled with [3H]glycerol triolein were used to determine the extent of adherence of PI donor vesicles to RBC during incubation. Hemolysis was measured after PI-PLC treatment and was less than 2% for RBC incubated up to 6 h and 3-5% for RBC incubated for 48 h.

Incorporation of H into PIP and PIP2—Phosphorylation of [3H]PI was measured and served as additional evidence that the lipid had translocated from outer to inner leaflet of the RBC membrane bilayer. RBC membranes were labeled with [3H]PI, washed, and incubated as described above. At designated timepoints, aliquots were removed from the incubation mixture and RBC membrane ghosts were prepared from 350 μl of packed cells by hypotonic lysis. Membrane lipids were extracted and separated as described above. All spots visible after staining with iodine vapor were scraped and their radioactivity measured in a liquid scintillation counter.

RBC Morphology—RBC were fixed in 3% glutaraldehyde in 0.9% NaCl and examined by light microscopy using Zeiss-Nomarsky optics.

RESULTS

We determined the bilayer distribution of PI in RBC membranes (which we will refer to as endogenous PI) by incubating RBC with a PI-specific PLC that does not penetrate the RBC membrane and hydrolyzes only PI located in the outer leaflet of the RBC membrane bilayer (Higgins et al., 1989). Treatment of intact RBC with exogenous PI-PLC for 20 min resulted in degradation of 24.3 ± 6.8% of PI (mean of six experiments ± S.D.). In contrast, exposure of (open) ghost membranes to PI-PLC resulted in hydrolysis of 92.4 ± 7.8% of PI (mean of six experiments ± S.D.). Increasing the incubation time to 1 h did not change the amount of PI degraded in intact cells. To confirm these findings using a nonenzymatic approach, we used BSA to extract PI located in the exterior leaflet. When RBC were incubated with BSA for 45 min, 17.6 ± 9.8% of PI was extracted, as well as 38.9 ± 12.9% of lyso-PC (means of three experiments ± S.D.). No differences in the amounts of other phospholipid classes were noted between control and BSA-treated cells. The results of both methods are in agreement and indicate that PI is asymmetrically distributed in the two halves of the membrane bilayer in intact human RBC with approximately 20% located in the outer leaflet.

To determine the transbilayer mobility of exogenous PI added, we incorporated trace amounts of [3H]PI into the RBC and determined its subsequent location in the bilayer using PI-PLC and back-extraction with BSA. When RBC were incubated in the presence of [3H]PI donor vesicles, the exogenous PI spontaneously incorporated into the RBC membrane (Fig. 1A). The rate of incorporation was rapid for the first 60 min and then leveled off, resulting in incorporation of approximately 70% of the PI added to RBC. The amount of PI incorporated into RBC membranes did not cause RBC shape change (results not shown). When RBC were labeled with [3H]PI for a brief (5 min) pulse, washed free of PI donor vesicles and subsequently incubated with BSA, 93.2 ± 0.6% (mean of duplicate determinations from two experiments ± S.D.) of the radiolabel was removed from RBC within 15 min. After RBC were incubated in presence of [3H]PI donor vesicles for 3 h, the amount of [3H]PI removed from RBC by BSA was about 80% during a 15-min extraction (Fig. 1B) and slightly increased if the incubation with BSA was carried out for up to 60 min.

When RBC were treated with PI-PLC following 40 min of labeling with [3H]PI, between 90 and 95% of the radiolabeled PI was hydrolyzed (Fig. 2). After incubation for 48 h, the availability of incorporated [3H]PI to hydrolysis by PI-PLC was decreased. The relative amount of radiolabel released from RBC by PI-PLC dropped to approximately 30% of the total after 8 h and subsequently reached a plateau of 20-25% after 48 h of incubation (Fig. 2). If, after prolonged incubation, [3H]PI-labeled RBC were lysed and the (open) ghost membranes incubated with PI-PLC, 70-75% of the total radiolabel was released. Thus, by opening the cells, most of the [3H]PI became accessible to PI-PLC indicating that this lipid was located in the inner leaflet of the RBC membrane. Throughout the incubation, nonspecific adherence of PI vesicles to RBC was determined by measuring the amounts of nonexchangeable [3H]glycerol triolein adhering to RBC. This adhering fraction (i.e. 15.6 ± 3.4%, mean of four determinations ± S.D., after 45 min of incubation of RBC with PI donor vesicles) was used to calculate the actual amount of PI incorporated into RBC. The amount of radiolabel released from RBC during incubation in the absence of enzyme or BSA treatment increased from 1 to 2% at the beginning to approximately 15% of total radiolabel after 48 h. This spontaneous release of radiolabel from RBC was also observed in control experiments, where the [3H]PI had been replaced with [3H]glycerol triolein. Thus, although we did not directly determine the nature of the released radiolabel, it seems likely that it was released as intact [3H]PI which had initially adhered to RBC rather than as free (phosphoryl) [3H]inositol. Even if we assume that all radiolabel was released as free inositol and subsequently entered the cell, it could not have been used for resynthesis of PI at the inner leaflet of the membrane because mature RBC membranes are unable to synthesize PI from its precursors, inositol and CDP-diglyceride or phosphatidate (Percy et al., 1973; Shohet and Nathan, 1970). Therefore, even if spontaneous hydrolysis of [3H]PI had occurred, it would not have affected our results.

The metabolic fate of the [3H]PI introduced into RBC was determined by analyzing the different RBC phosphoinositide classes (PI, PIP, PIP2). At the beginning of incubation, almost all the radiolabel was recovered in the PI fraction (Fig. 3A). After 25 h of incubation, the relative amount of radioactivity in PI decreased to about 85% of label in the phosphoinositides (Fig. 3A) and the radiolabel was detected in PIP (6%) and PIP2 (9%) (Fig. 3B). This process seemed to reverse slightly after prolonged incubation (Fig. 3, A and B), perhaps due to
Membrane Bilayer Organization of Phosphatidylinositol

**Fig. 1.** Incorporation and back-extraction of $[^{3}H]$PI. 

A. RBC were incubated in the presence of $[^{3}H]$PI donor vesicles. At designated timepoints, an aliquot was removed from the incubation mixture and RBC were pelleted. The relative amount of $[^{3}H]$PI in the supernatant was calculated from the total amount of $[^{3}H]$PI in the suspension and the amount of radiolabel in the donor vesicle containing supernatant after centrifugation. The values (■) represent the mean ± S.D. of 10 independent experiments. B. RBC were incubated for 180 min in presence of $[^{3}H]$PI vesicles as described in A. Subsequently, RBC were washed free of unincorporated and nonadherent radiolabel and the $[^{3}H]$PI-labeled RBC were incubated with buffer containing 3% BSA. At designated timepoints, an aliquot was removed from the incubation mixture and RBC were pelleted. The relative amount of radiolabel removed from RBC by BSA was calculated from the total amount of $[^{3}H]$PI in the suspension and the amount of radiolabel in the BSA containing supernatant after centrifugation. The values (○) represent the mean ± S.D. of five independent experiments. The spontaneous release of radiolabel from RBC in the absence of BSA is represented (△).

**Fig. 2.** Release of $[^{3}H]$phosphorylinositol from RBC by PI-PLC treatment. RBC were preincubated with $[^{3}H]$PI for 30-45 min, subsequently washed free of PI donor vesicles, and the $[^{3}H]$PI-labeled RBC were incubated for 48 h. At designated timepoints, an aliquot was removed from the incubation mixture. RBC were pelleted and the incubation buffer was replaced with PLC buffer. PI-PLC was added to the resuspended RBC and hydrolysis of PI was carried out at 37 °C for 15 min. The amount of radiolabel released from RBC by PI-PLC was calculated from the total amount of $[^{3}H]$PI incorporated in RBC and the amount of radiolabel in the supernatant after centrifugation. The data points (■) represent values from three independent experiments. The solid line is the theoretical curve derived from Equation 1.

**Fig. 3.** Redistribution of radiolabel within the phosphoinositide classes. RBC were labeled with $[^{3}H]$PI as described in Fig. 2 and incubated for 48 h. At designated timepoints, the distribution of $[^{3}H]$ in RBC lipid extracts was determined as described under “Experimental Procedures.” The distribution of radiolabel in PI (A) and PIP and PIP$_2$ (B) is given relative to the total amount of radiolabel in the phosphoinositides. The values represent the mean ± S.D. of four independent experiments.

**Discussion**

Hydrolysis of PI by PI-PLC in the outer leaflet of cell membranes has been found to be a valuable method to study the distribution of PI within the bilayer of membranes (Low and Finean, 1977; Higgins et al., 1989). We used this phospholipase probe to determine the bilayer distribution of PI in human RBC membranes and found that PI is asymmetrically distributed with 24% in the outer leaflet and 76% in the inner leaflet. Our findings are similar to those on RBC from ox, sheep, and pig, where 85–95% of PI was found to be located in the inner leaflet of the membrane (Low and Finean, 1977).

Since PI is slightly water-soluble, it transfers spontaneously from lipid vesicles to membranes (Hohengasser et al., 1986). As with other water soluble lipid probes (Haest et al., 1981;
Mohandas et al., 1982; Morrot et al., 1989). PI can be extracted from membranes with BSA. Thus, the amount of endogenous PI located in the outer leaflet of the RBC membrane detected by PI-PLC was confirmed by BSA extraction of intact RBC (i.e., 18% of PI). The extractability of PI from RBC membranes by BSA suggests that it may be available for exchange between membranes and plasma in vivo.

To determine the transbilayer mobility of PI in human RBC, we incorporated tracers of \(^{3}H\)PI into RBC and followed its transbilayer distribution by measuring its accessibility to hydrolysis by extracellular PI-PLC and to back-extraction by BSA. Between 85 and 95% of incorporated \(^{3}H\)PI could be hydrolyzed from labeled RBC by PI-PLC or was removed by BSA (Figs. 1B and 2). These results indicate that the after the initial labeling procedure most of the exogenously added PI was located in the outer leaflet of the membrane bilayer. The \(^{3}H\)PI not hydrolyzed by PI-PLC likely represents the same pool of radiolabeled PI that was not extracted by BSA which suggests that a small amount of exogenously added PI translocated from outer to inner leaflet of the RBC membrane during the labeling procedure. The slow rate of translocation of PI across the membrane bilayer suggests that, like PC, PI is not actively transported across the RBC membrane bilayer. Therefore, in contrast to the asymmetric distribution of PI can not be explained by a fast and active transport across the membrane bilayer. It has been reported that PI is tightly bound to glycophorin (Yeagle and Kelsey, 1989) and that the state of phosphorylation of PI regulates the affinity of glycophorin for protein A, a membrane skeletal protein (Anderson and Marchesi, 1985). It is possible that such interactions, at the cytoplasmic side of the membrane, prevent the equilibrium of PI between the two leaflets of the membrane bilayer and help maintain the asymmetric distribution of PI in the human RBC.

Our results indicate that the bilayer asymmetry of PI in human RBC is similar to that reported for PE (Roelfsema, 1982). However, PE is actively transported across the membrane bilayer by a putative aminophospholipid translocase (Seigneur et al., 1984; Daleke and Huestis, 1985; Zachowski et al., 1986; Connor and Schroit, 1988) with a halftime for translocation of approximately 50 min. In contrast, the rate of translocation of exogenously added PI is slow, with a halftime in the order of 3 h. This rate is comparable to that for translocation of certain PC molecular species across the RBC membrane bilayer (van Meers and Op den Kamp, 1982; Middelkoop et al., 1986; Morrot et al., 1989).

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**Acknowledgment**—We thank Maggie Yee for excellent technical assistance during part of this study.

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Transbilayer distribution and mobility of phosphatidylinositol in human red blood cells.
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J. Biol. Chem. 1990, 265:16035-16038.

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