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LOCALIZATION OF ENZYMES INVOLVED IN POLYPHOSPHOINOSITIDE METABOLISM ON THE CYTOPLASMIC SURFACE OF THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

1. Impermeable inside-out and right-side-out vesicles were prepared from membranes of human erythrocytes. During preparation of each kind of impermeable vesicle, permeable vesicles were also obtained.

2. Incubation of vesicles with [γ-^{32}P]ATP at 37 °C for periods of up to 1 h did not change the topography or the permeability of the vesicles.

3. Vesicles incorporated labeled phosphate from [γ-^{32}P]ATP into both diphosphoinositide and triphosphoinositide, but impermeable inside-out vesicles incorporated significantly more nuclide than did right-side-out vesicles.

4. Permeable vesicles derived during the preparation of inside-out vesicles were as active as impermeable inside-out vesicles in the incorporation of labeled phosphate into the polyphosphoinositides. However, permeable vesicles derived during the preparation of right-side-out vesicles were not as active.

5. Impermeable right-side-out vesicles, treated with 0.01% saponin, incorporated labeled phosphate into the polyphosphoinositides at a level comparable to that of impermeable inside-out vesicles.

6. These data show that the enzymes involved in metabolism of diphosphoinositide and triphosphoinositide are located on the cytoplasmic surface of the erythrocyte membrane.

INTRODUCTION

The polyphosphoinositides, in contrast to most other human erythrocyte membrane phospholipids, actively incorporate labeled phosphate from [γ-^{32}P]ATP [1–3]. This incorporation occurs only in the monoesterified phosphates of the polyphosphoinositides, is enhanced in "leaky" as opposed to "resealed" membranes [3], and the labeled polyphosphoinositides are part of a membrane proteolipid [4]. Taking note of the asymetric distribution of proteins and lipids in the erythrocyte membrane [5–7], we report in this study that diphosphoinositide and triphosphoinositide are

labeled with phosphate only when the cytoplasmic face of the erythrocyte membrane is exposed to [γ-^{32}P]ATP. These data indicate that the enzymes involved in metabolism of di- and triphosphoinositide are situated on the inner surface of the erythrocyte membrane.

MATERIALS AND METHODS

Preparation of inside-out and right-side-out vesicles

Inside-out and right-side-out vesicles were prepared from human erythrocyte membranes by the method of Steck and Kant [8]. The red blood cells from expired units of blood were washed 3 times by centrifugation in 150 mM NaCl, 5 mM sodium phosphate, pH 7.9, care being taken to remove all of the white cells. Hemolysis was performed by thoroughly mixing the washed, packed red cells with 20 vols of 5 mM sodium phosphate, pH 7.9, at 0 °C. The resulting membrane preparation was washed 3 times with the hemolyzing solution and used to prepare inside-out or right-side-out vesicles.

Inside-out vesicles were made by diluting each ml of packed membrane with 20 ml of 0.5 mM sodium phosphate, pH 8.6, and incubating at 0 °C for 1–3 h. Right-side-out vesicles were prepared by following the same procedure except that 0.1 mM $MgSO_4$ was added after the incubation. The membranes were then again centrifuged and stored at 0 °C for periods of 8–12 h. Pellets were resuspended in a small volume of 0.5 mM sodium phosphate, pH 8.6, and passed 3 times through a 27 gauge needle.

Permeable and impermeable vesicles in both preparations were separated on a discontinuous Dextran T 110 gradient (8 % and 15 % w/v) by centrifugation for 1 h at 200 000 $\times g$ at 4 °C in a Spinco SW 40 Ti rotor. Vesicles banded at the top of the 8 % shelf and at the 8 %–15 % interface. Permeability and sideness of vesicles were determined as described below. Vesicles harvested from the top of the gradient were impermeable and were either inside-out if prepared in the absence of Mg or right-side-out if prepared in the presence of Mg. Vesicles which banded at the 8 %–15 % interface were permeable and thus sideness could not be determined. Permeable vesicles resulting from preparation of inside-out vesicles were designated "permeable vesicle I" and those from preparation of right-side-out vesicles were designated "permeable vesicle II".

Determination of sideness and permeability of isolated vesicles

Acetylcholinesterase activity was used as a marker for the exterior surface of the erythrocyte membrane [9] and it was assayed by the method of Ellman et al. [10]. Glyceraldehyde-3-phosphate dehydrogenase was chosen as the marker for the cytoplasmic face of the erythrocyte membrane and its activity was determined by the method of Cori et al. [11].

As described by Steck and Kant [8], membrane permeability was estimated by determining the "% accessibility" of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase activity with and without 0.1 % Triton X-100. The rationale for such an estimation is that impermeable vesicles, either inside-out or right-side-out, will have only one marker enzyme exposed to the substrate in the reaction mixture while in permeable vesicles both surfaces of the membrane should be accessible to

substrate. Complete accessibility (100 %) of each vesicle preparation was determined by disrupting the vesicles with 0.1 % Triton X-100.

Incubation of vesicles with [γ-^{32}P]ATP

Vesicles were incubated at 37 °C with 50 mM sodium phosphate, pH 7, 2.5 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (9.6×10^7 cpm/μmole). Incubation was stopped by adding an equal volume of 20 % trichloroacetic acid at 4 °C. The resulting precipitate was washed 3 times with cold 5 % trichloroacetic acid. Phospholipids were extracted from the washed pellet with chloroform/methanol/concentrated hydrochloric acid (200 : 100 : 1, v/v) as described by Folch [12]. The lipid extract was chromatographed on EDTA-treated silicagel impregnated paper by the method of Steiner and Lester [13]. The radioactive phospholipids were localized by autoradiography on Kodak-no screen x-ray film. The appropriate areas on the chromatogram were cut out, placed in 10 ml of toluene phosphor, and counted in a liquid scintillation spectrometer.

Other methods

Protein was determined by the method of Lowry et al. [14], using bovine serum albumin as standard; phosphorus assays were performed as described by Bartlett [15] and [γ-^{32}P]ATP was synthesized according to Glynn and Chappel [16].

RESULTS

Characterization of the erythrocyte membrane vesicles

Data on the accessibility of acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase in typical vesicle preparations are shown in Table I. Acetylcholinesterase, a marker for the exterior surface of the membrane, was highly accessible (115 %) in right-side-out impermeable vesicles and in permeable vesicles I (85 %) and II (132 %). This enzyme activity was only 30 % accessible in inside-out imper-

TABLE I

CHARACTERIZATION OF SIDEDNESS AND PERMEABILITY OF ISOLATED MEMBRANE VESICLES

The % accessibility was calculated as follows: 100 (enzyme activity without triton/enzyme activity with triton). The enzyme assays were performed as described in Materials and Methods. The values given "after incubation" are for vesicles incubated for 1 h at 37 °C under conditions employed for incorporation of [γ-^{32}P]ATP into phospholipids. Inside-out, right-side-out and permeable vesicles I and II were prepared as described in Materials and Methods.

Type of vesicle	% Accessibility			
	Before incubation		After incubation	
	Acetylcholin-esterase	Glyceraldehyde-3-P-dehydrogenase	Acetylcholin-esterase	Glyceraldehyde-3-P-dehydrogenase
Inside-out	31	90	26	87
Right-side-out	115	4	106	5
Permeable I	85	98	65	100
Permeable II	132	89	107	91

meable vesicles. Conversely, glyceraldehyde-3-phosphate dehydrogenase activity, an index of the cytoplasmic face of the membrane, was most evident (90 %) in inside-out impermeable vesicles and, like acetylcholinesterase activity, it was also manifest in permeable vesicles I and II. There was little glyceraldehyde-3-phosphate dehydrogenase activity (4 %) in right-side-out impermeable vesicles. These data, in agreement with those of Steck and Kant [8], indicate that vesicles obtained from 8 % to 15 % Dextran T110 interface, whether prepared in the presence or absence of Mg, are permeable. On the other hand, vesicles obtained at the top of the gradient are impermeable and are either right-side-out (when 0.1 mM $MgSO_4$ was added during vesicle preparation) or inside-out (when $MgSO_4$ was not added). The enzyme assays indicate that the inside-out vesicles preparation may contain a small contamination with right-side-out vesicles, but that the right-side-out vesicle preparation is relatively homogenous. The protein : phospholipid ratio of vesicles prepared in the absence of Mg was 4.54 while that of vesicles prepared in the presence of the divalent cation was 7.15. Yields of 45 % impermeable inside-out vesicles and 85–90 % impermeable right-side-out vesicles were obtained.

Incubation of vesicles for 1 h at 37 °C under conditions used for the labeling of phospholipid with [γ-^{32}P]ATP did not change the topography or the permeability of the vesicles (Table I).

Phospholipid metabolism in inside-out and right-side-out vesicles

Inside-out and right-side-out impermeable vesicles and permeable vesicles I and II were incubated separately with [γ-^{32}P]ATP and the incorporation of labeled phosphate into phospholipids was determined. Data from a typical experiment is presented in Fig. 1. Inside-out impermeable vesicles avidly incorporated labeled phosphate into phospholipids. Nuclide incorporation into phospholipids of right-side-out impermeable vesicles was 10–20 time less (Fig. 1A). These data indicate that incorporation of phosphate from [γ-^{32}P]ATP into phospholipids occurs on the cytoplasmic surface of the membrane and not on the outer surface and it would be expected that permeable vesicles would incorporate phosphate to the same extent as impermeable inside-out

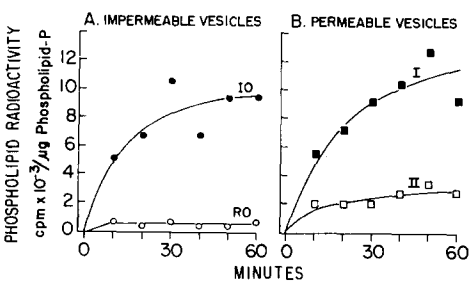

Fig. 1. Time course of incorporation of labeled phosphate from [γ-^{32}P]ATP into phospholipids. The various vesicle preparations were incubated with [γ-^{32}P]ATP and the radioactivity in phospholipids determined, as described in Materials and Methods. The phospholipid radioactivity in each of the vesicle preparations is corrected for the amount of phospholipid-P present in each sample.
Fig. 1A shows the incorporation of labeled phosphate from [γ-^{32}P]ATP into phospholipids of inside out (IO) and right-side-out (RO) vesicles and Fig. 1B shows incorporation into phospholipids of permeable vesicles I and II.

vesicles. As shown in Fig. 1B, permeable vesicles I was as active metabolically in incorporating labeled phosphate into phospholipid as were inside-out impermeable vesicles, but permeable vesicle II was less active. However, nuclide incorporation by permeable vesicle II was greater than that of impermeable right-side-out vesicles. These experiments show that vesicles prepared in the presence of Mg, which is a necessary condition for obtaining right-side-out preparations, are not able to incorporate phosphate into phospholipids as well as vesicles prepared in the absence of Mg. These results suggest that the procedure used to prepare right-side-out vesicles may alter the membrane so that incorporation of labeled phosphate into the phospholipids is reduced. To determine if this is the case, impermeable right-side-out vesicles (prepared in the presence of Mg) were made permeable by the addition of 0.01 % saponin and polyphosphoinositide metabolism was measured (Table II). Right-side-out vesicles, when treated with saponin, actively incorporated labeled phosphate from [γ-^{32}P]ATP into phospholipid and even surpassed the activity of inside-out vesicles

TABLE II

[γ-^{32}P]ATP INCORPORATION INTO VARIOUS VESICLE PREPARATIONS IN THE ABSENCE AND PRESENCE OF SAPONIN

The various vesicle preparations were incubated with and without saponin at 37 °C for the times indicated. The incorporation of [γ-^{32}P]ATP into phospholipid was determined as described in Materials and Methods.

	Phospholipid radioactivity (cpm/μg Phospholipid P)			
	Control		0.01 % Saponin	
	30 min	50 min	30 min	50 min
Inside-Out	1115	1115	1031	1330
Right-Side-Out	236	311	2219	2088
Permeable I	1346	1289	914	823
Permeable II	360	450	182	124

in this respect. The explanation for an activity greater than that of inside-out vesicles is not clear, but it may be that inside-out vesicles have lost some enzymes located on the cytoplasmic face during vesiculation and subsequent washing of the membranes, as evidenced by the lower protein : phospholipid ratio of inside-out vesicles as compared with that of right-side-out vesicles. Saponin had no effect on the phospholipid metabolism of inside-out vesicles, as would be expected if the relevant enzymes are located on the cytoplasmic face of the membrane, and also had no effect on permeable vesicles I and II (Table II).

These data show that impermeable right-side-out vesicles are capable of phospholipid metabolism but that the activity is occluded when [γ-^{32}P]ATP is not accessible to the cytoplasmic face of the membrane. These data further suggest that permeable vesicle II is damaged, insofar as being able to incorporate labeled phosphate into phospholipids, since they are not as active as the other preparations either in the presence of absence of saponin.

Separation of the phospholipids on paper chromatography revealed that labeled phosphate from [γ-^{32}P]ATP was incorporated into diphosphoinositide and triphosphoinositide but not into any other phospholipid (Fig. 2). Inside-out and permeable vesicle I incorporated labeled phosphate into di- and triphosphoinositide

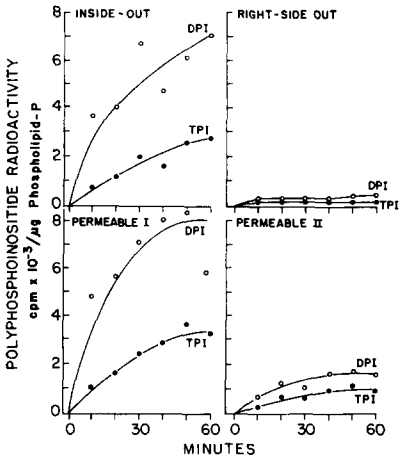

Fig. 2. Incorporation of labeled phosphate from [γ-^{32}P]ATP into the polyphosphoinositides of isolated vesicles. Inside-out, right-side-out, permeable I and permeable II vesicles were incubated with [γ-^{32}P]ATP as in Fig. 1. The radioactivity in the phospholipids was determined after the lipids were separated by paper chromatography as described in Materials and Methods. Diphosphoinositide (DPI) and triphosphoinositides (TPI) were the only radioactive phospholipids detected.

to approximately the same extent, with labeling of diphosphoinositide being about 2.5 that of triphosphoinositide after 60 min of incubation. Right-side-out vesicles showed negligible incorporation into the polyphosphoinositides. Permeable vesicle II incorporated labeled phosphate into both di- and triphosphoinositide but at a much reduced level as compared with inside-out and permeable vesicle I, and the ratio of di- to triphosphoinositide was reduced to approximately 1.5 after 60 min incubation.

DISCUSSION

The phospholipid distribution in the human erythrocyte membrane is highly asymmetric. The outer surface of the membrane consists of the choline-containing lipids, sphyngomyelin and phosphatidylcholine [5] and the glycolipids [7] while the interior or cytoplasmic face of the membrane is high in phosphatidylethanolamine and phosphatidylserine [5, 6]. Previously it was shown that human erythrocyte membranes contain a proteolipid which does not contain sphyngomyelin and phosphatidylcholine but which is enriched in phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine and which contains 100 % of the polyphosphoinositides which can be labeled with phosphate from [γ-^{32}P]ATP. We show now that di- and triphosphoinositides are labeled with [γ-^{32}P]ATP only when the substrate is accessible to the cytoplasmic surface of the membrane, indicating that this is where the enzymes are localized and possibly that this is also the location of these radiolabeled phospholipids. This

high asymmetric distribution may indicate that these enzymes are regulated by intracellular factors. Although the cellular functions of the metabolically active di- and triphosphoinositides have not yet been defined, several possibilities such as the regulation of calcium binding [17], involvement in the active transport of cations [18, 19] and in lysis and resealing of membranes [3, 4], have been postulated. The feasibility of these postulated functions should be evaluated in light of the asymmetric distribution of the enzyme involved in polyphosphoinositide metabolism.

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