Removal of haem from lipids extracted from intact erythrocytes with particular reference to polyphosphoinositides

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With the use of a modified acid version of a current lipid-extraction technique [Bligh & Dyer (1959) *Can. J. Biochem. Physiol.* **37**, 911–917], 92% of phosphatidylinositol 4,5-bisphosphate obtained by means of three sequential extractions from intact human erythrocytes was obtained during the first one. Some 95% of the haem co-extracted with the lipids could then be removed, with a maximal loss of polyphosphoinositides of less than 3%. About 58 nmol of phosphatidylinositol 4,5-bisphosphate was found per ml of erythrocytes.

INTRODUCTION

Cell-surface-membrane lipids are usually not easily obtained. The total cell lipid of the mature mammalian erythrocyte, however, is generally assumed to be surface-membrane lipid and thereby represents a meaningful entity for extraction. The extraction of PtdIns4P, and in particular of PtdIns(4,5)P₂, requires an acid solvent system that, when applied to erythrocytes, causes the extraction of considerable amounts of haem products. These products interfere in the subsequent separation of the polyphosphoinositides by t.l.c. The present work describes a method by which haem products can be essentially removed from the lipid extract before analysis.

EXPERIMENTAL

All chemicals were of analytical grade. Carrier-free [³²P]P_i (Risø, Denmark) was obtained in 1 ml of 10 mM-HCl, to which 1 ml of Krebs–Ringer buffer (18 mM-Hepes), pH 7.4, was added. [U-¹⁴C]myo-Inositol was from The Radiochemical Centre, Amersham, Bucks., U.K., and [4-¹⁴C]cholesterol was from NEN, Boston, MA, U.S.A.

³²P labelling of erythrocytes

Human blood was drawn into heparinized plastic tubes and the cells were collected by centrifugation for 15 min at about 600 g (*r*_{av}, 13 cm). Then the cells were washed twice in 8 mM-phosphate/saline, pH 7.4, and once in saline (0.9%, w/v). The top layer of cells was removed before each resuspension. The cells were finally suspended at a haematocrit of 30 in Krebs–Ringer buffer (18 mM-Hepes), pH 7.4, at 37 °C, containing 11 mM-glucose and 1 mg of purified bovine serum albumin/ml. After 1 h of preincubation at 37 °C, 8–20 μCi of [³²P]P_i was added/ml of suspension. The incorporation of ³²P was allowed to proceed for 4 h in silicone-treated vessels. Thus essentially the procedure of Allan *et al.* (1976) was followed. Less than 0.5% of cells were lysed, as estimated from a haemolysate dilution series.

[U-¹⁴C]myo-Inositol labelling in lymphocytes

As an aid in the identification of inositides in the bidirectional t.l.c. procedure used initially, a label in inositol was also needed. For this purpose a human lymphocyte line (IM-9) was used to incorporate [U-¹⁴C]myo-inositol into inositides by the labelling procedure of Bell & Majerus (1980). A 5 μCi portion of labelled inositol was added to cells multiplying in Medium 199 (Gibco) supplemented with 5% (v/v) foetal-calf serum. After they had been washed with saline, the cells were stored in saline with 5 mM-Na₂EDTA at –70 °C. Before lipid extraction, about 100 μl of packed lymphocytes was mixed with 200 μl of packed unlabelled erythrocytes (*n* = 2).

Erythrocyte lipid extraction

Basic concepts for the extraction method were taken from Bligh & Dyer (1959), Lang *et al.* (1977) and Griffin & Hawthorne (1978). Samples (1.1 ml) of suspensions of ³²P-labelled erythrocytes were transferred into 16 mm × 100 mm screw-cap test tubes containing 2.5 ml of methanol and 1.25 mg of BHT. The tubes were shaken vigorously a few times within a 30 min period before the admixture of 1.25 ml of chloroform. After a 2 h period with occasional shaking the tubes were stored at –20 °C.

On rewarming to room temperature the extraction was continued for 1 h. For phase separation, 1.25 ml of chloroform in mixture with 3.75 ml of theoretical neutral lower phase was first added. Theoretical lower phase was obtained when 150 mM-NaCl, methanol, chloroform and water (4:10:10:5, by vol.) were shaken. Disintegration of any lumps was obtained by shaking by hand for few minutes. After a further addition of 1.25 ml of 2.4 M-HCl (Schacht, 1981) the tube was vigorously shaken for 60 s. Phase separation was promoted by centrifugation at 2400 rev./min for 10 min, a procedure that yielded suitably loosely packed protein as a disc at the interface. The yield of PtdIns(4,5)P₂ in this lower phase was 80% of the PtdIns(4,5)P₂ obtained in the entire three-stage extraction (Table 1). Had one used chloroform without the 3.75 ml of the theoretical lower phase, as in the original procedure of Bligh & Dyer (1959), only 53% of

Abbreviations used: PtdIns4P, 1-(3-*sn*-phosphatidyl)-L-myoinositol 4-phosphate; PtdIns(4,5)P₂, 1-(3-*sn*-phosphatidyl)-L-myoinositol 4,5-bisphosphate; BHT, 2,6-di-*t*-butyl-4-methylphenol.

the PtdIns(4,5) P_2 obtained would have been present in the resulting lower phase. The lower phase was pipetted off and 5 ml of chloroform was added to the remainder. The tube was again shaken to disintegrate any lumps for 2 min. The ease with which the dispersion can be achieved is, of course, a function of the degree of packing of the protein disc. After centrifugation the chloroform wash phase was pipetted off. The upper phase was discarded.

For a second extraction the protein-plate material was suspended in 900 μ l of saline, and then 2.5 ml of methanol with BHT was added, followed by 1.25 ml of chloroform. The material dispersed almost to opalescence after shaking for a few minutes. Phase separation followed upon the addition of 1.25 ml each of chloroform and 2.4 M-HCl. The final wash was with 2.5 ml of chloroform. For an occasional third extraction of the plate material (dry wt. $\sim$ 103 mg) the procedure was that used in the second extraction. Each of the lower phases and chloroform wash phases was transferred into an ordinary 10 mm $\times$ 100 mm glass tube. For removal of non-lipidic radioactive contaminants about $\frac{2}{3}$ vol. of methanol/1 M-HCl (1:1, v/v) was added to each tube. The tubes were then capped, shaken for 30 s, and centrifuged. About 99% of the lower phase was pipetted off and saved. Without this rinse the 32 P radioactivity migrating less rapidly than PtdIns(4,5) P_2 (Table 1) was of the same order of magnitude as the sum of the 32 P in phosphatidic acid, PtdIns4P and PtdIns(4,5) P_2 . However, the absolute amounts of 32 P recovered in these three lipids after bidirectional t.l.c. did not depend on this rinse.

Removal of haem

The rinsed lower phase from the first extraction was concentrated, at or below room temperature, in a stream of N_2 . The chloroform wash was gradually added to the evaporating solution. When the volume was reduced to 1–2 ml, haem particles were seen to precipitate. Evaporation was allowed to proceed just to completion with the tube connected in a tilted position to a water pump. A 3 ml portion of chloroform was then added and the tube was swirled. This step took place about 3 h after the beginning of acid treatment. Observation in an ordinary blood-cell-counting chamber revealed a multitude of particles of 1 to a few μ m dimensions suspended in the chloroform/lipid solution. The suspension was stored at -20°C . The next day the particles were sedimented by centrifugation after 1.5 ml of hexane had been added to the chloroform solution in order to reduce its relative density. By means of a stably mounted pipette connected to a syringe, all of the supernatant could be removed, leaving behind the 'dry' black haem precipitate. This precipitate, dissolved in methanol/chloroform (1:1, v/v), had only one definite peak of absorbance, at 400 nm, when scanned in a Unicam SP.800 spectrophotometer in the region 315–700 nm.

The chloroform/hexane supernatant showed a broader and less symmetrical peak, with a maximum at 383 nm. From absorbances and dilution factors it was calculated that less than 5% of the haem material present in the first extract was now left with the lipids. The haem present in subsequent extracts was unimportant in the present context.

Additional methods

T.l.c. Unidirectional t.l.c. was performed as described by Gonzalez-Sastre & Folch-Pi (1968). Bidirectional t.l.c.

followed method no. 4 described by Lloyd *et al.* (1972). For both procedures use was made of plates prepared from a slurry of silica gel 60 HR Merck in 1% (w/v) potassium oxalate. In both types of t.l.c., iodination developed a clearly visible bright-yellow area where the largest amount of lipidic 32 P was found and, therefore, where PtdIns(4,5) P_2 was to be expected. The recovery of 32 P present in chloroform/hexane supernatants was $92 \pm 1\%$ (mean $\pm$ S.E.M.) after unidirectional t.l.c. ($n = 8$). Further, in the bidirectionally obtained spots ascribable to PtdIns(4,5) P_2 and PtdIns4P on account of their prominent 32 P labelling, some of the lipidic [^{14}C]inositol derived from lymphocytes were also found; no ^{14}C was present in the phosphatidic acid spot. Phosphatidyl-inositol, which is not separated from phosphatidylserine (Lloyd *et al.*, 1972), contained 88% of total ^{14}C recovered. T.l.c. spot localization was assisted by autoradiography on Kodak X-Omat film. The haem products still contaminating the lipids when they were applied in t.l.c. migrated faster than PtdIns4P and PtdIns(4,5) P_2 in the unidirectional mode.

Measurement of radioisotopes. The entire lanes from unidirectional t.l.c. plates were divided into about 20 bands. The silica-gel material from each band was scraped into a suitable flat circular container for counting for radioactivity in a Berthold low-level proportional argon/methane flow counter system. The background measure was 0.7 c.p.m. Radioactivity in extracts was measured by applying portions on to 17 mm $\times$ 17 mm filter-paper squares or on to silica-gel plates for imitation of the conditions for bands. Corrections were applied for background, physical decay and differences in efficiency. For ^{14}C this implied the construction of an experimental self-absorption curve.

Estimation of phosphate. Assay of phosphate was performed after transfer of the silica gel from the flow-counter system to glass tubes with conical bottoms and digestion at 170°C . The ultramicro method of Bartlett (1959) was used with, however, the H_2SO_4 replaced by 350 μ l of 70% (w/v) HClO_4 . The use of HClO_4 in the presence of silica gel was discussed by Rhee & Dugan (1967). As shown by titration, almost no HClO_4 was lost by evaporation during digestion. The PtdIns(4,5) P_2 revealed in t.l.c. lanes containing the lipids from the first extraction was located within one band typically containing 38 nmol of phosphate. The radioactivities in the two bordering bands were divided by the specific radioactivity for this phosphate. Values of about 4 nmol of phosphate thus obtained were added to those from above, as was also a few nanomoles similarly arrived at for PtdIns(4,5) P_2 phosphate from lanes containing lipids from subsequent extractions. The 45.9 nmol in Table 1 was obtained this way.

RESULTS AND DISCUSSION

Values for the sequential extraction of those three lipids which incorporate significant radioactivity during incubation of mammalian mature erythrocytes with [^{32}P]P $_i$ (Allan & Michell, 1978) are given in Table 1. PtdIns(4,5) P_2 is the least extractable of the three phospholipids dealt with. However, 92% of the PtdIns(4,5) P_2 that could be obtained by three extractions was obtained by the first one. For all the three

Table 1. Radioactivity (c.p.m.) values obtained sequentially in a three-stage extraction of ^{32}P -labelled lipids from intact erythrocytes using an acid version of the method of Bligh & Dyer (1959)

Data are from a single representative experiment that confirmed the results obtained during work with about 180 erythrocyte samples. The cells had been incubated for 4 h in Krebs-Ringer buffer containing $^{32}\text{P}[\text{PP}_1]$ (Allan & Michell, 1978) before the addition of methanol to samples each containing $330\ \mu\text{l}$ of cells. See the Experimental section for further details. Haem was removed in particulate form from the lipids of the first extract. This haem contained $485 (\pm 22)$ c.p.m. ($n = 8$). The lipids were fractionated by unidirectional t.l.c. (Gonzalez-Sastre & Folch-Pi, 1968). The four fractions studied together accounted for 98–99% of radioactivity in the entire t.l.c. lanes. The c.p.m. values are means $\pm$ S.E.M. for the numbers of samples indicated in parentheses. The $\text{PtdIns}(4,5)\text{P}_2$ from the first extraction contained 42.5 ± 0.5 nmol of phosphate ($n = 8$). Assuming identical specific radioactivities for $\text{PtdIns}(4,5)\text{P}_2$ in the first and second extracts, the accumulated 14819 c.p.m. would indicate a total of 45.9 nmol of phosphate or 15.3 nmol of $\text{PtdIns}(4,5)\text{P}_2$ from $330\ \mu\text{l}$ of cells. Assuming that 20% of extracted material is not represented, owing to losses during t.l.c., haem-separation and volume-transfer procedures, plus limited hydrolytic cleavage, 58 nmol of $\text{PtdIns}(4,5)\text{P}_2$ should have been extracted from 1 ml of packed erythrocytes. Total lipid phosphate was close to 4000 nmol/ml of cells. Thus, as a molar percentage of total phospholipid, the value obtained for $\text{PtdIns}(4,5)\text{P}_2$ becomes 1.5 .

Lipid	Radioactivity (c.p.m.)					
	Accumulated in to extractions	From first extraction	From second extraction	From third extraction	From first extraction	
					In lower phase	In chloroform wash
Phosphatidic acid	4801 ± 82 (8)	4698 ± 84 (8)	103 ± 8 (8)	7 ± 2 (3)	4207 ± 52 (4)	353 ± 30 (4)
$\text{PtdIns}4\text{P}$	2475 ± 47 (8)	2340 ± 41 (8)	135 ± 20 (8)	19 ± 6 (3)	1976 ± 44 (4)	327 ± 9 (4)
$\text{PtdIns}(4,5)\text{P}_2$	14819 ± 138 (8)	13751 ± 118 (8)	1068 ± 53 (8)	92 ± 8 (3)	11794 ± 59 (4)	1743 ± 60 (4)
Migrating more slowly than $\text{PtdIns}(4,5)\text{P}_2$ or not migrating	1238 ± 106 (4)	1047 ± 85 (4)	191 ± 16 (8)	28 ± 6 (3)		

phospholipids, 99% of the total extractable was obtained after two extractions.

The acidity of the extraction system probably causes the appearance of lyso compounds owing to breakage of vinyl ether bonds. The bottom-row values in Table 1 for compounds migrating slower than PtdIns(4,5) P_2 probably include the lyso products of this lipid. To obtain a rough estimate of the generation of lyso compounds, portions of the lower phase from a first extraction were processed without delay, after 3.5 h and after 16 h delay, at room temperature. For the two last portions the c.p.m. values for compounds migrating slower than PtdIns(4,5) P_2 were twice the corresponding c.p.m. value for the portion processed without delay. This doubling corresponded to concomitant 7% decreases in c.p.m. values for PtdIns(4,5) P_2 ; no changes were found for PtdIns4P and phosphatidic acid. Further, no significant loss of any labelled lipid was observed from a 6.5 h delay in the separation of phases in the second extraction ($n = 2$).

The haem particles: haem was removed from the first extract only. For assay of ^{32}P the haem particles were dissolved in 2 ml of methanol/chloroform/water (25:75:2, by vol). Erythrocyte samples incubated with [^{32}P]P_i for less than 1 min gave haem particles containing 32 c.p.m. ($n = 2$). The particles otherwise contained 485 (± 22) c.p.m. as compared with 20 789 (± 200) c.p.m. for all ^{32}P in the three major labelled lipids of the first extract (Table 1). In some cases the particles were applied in a 12–15 cm-long zone for t.l.c. ^{32}P -labelled compounds with the same relative distribution as shown in Table 1 were obtained, except that the most slowly migrating labelled material accounted for about 25% rather than 5% of the total label applied. The loss of labelled lipids during removal of haem thus seems to be of little qualitative or quantitative significance. Also, less than 1% of added [4- ^{14}C]cholesterol was lost during that procedure. Some samples extracted in the complete absence of BHT showed blackening at various steps, but gave rise to results identical with those of Table 1.

Two neutral systems for the extraction of lipids were found less satisfactory for the present purpose. In the first system, 1.25 ml of 2 M-KCl in 0.5 M-phosphate buffer, pH 7.4, substituted for HCl (Garbus *et al.*, 1963).

Although phosphatidic acid was well extracted, virtually no PtdIns(4,5) P_2 or PtdIns4P radioactivity was obtained. With erythrocyte samples identical with those mentioned in Table 1, the method of Cohen *et al.* (1971) was also tried. In this method, 0.1 M- Na_2EDTA in 2 M-KCl is added before the methanol and chloroform, and, once again, at phase separation. The results were: 4599 ± 445 c.p.m. for phosphatidic acid, 1278 ± 90 c.p.m. for PtdIns4P and 1938 ± 495 c.p.m. for PtdIns(4,5) P_2 ($n = 3$). Thereafter 160 (± 15) c.p.m., 420 (± 229) c.p.m. and 9066 (± 464) c.p.m. respectively were obtained during the acid extraction described in the Experimental section for the second extraction.

The valuable assistance of Mrs. K. Kandborg is acknowledged. Cand. Scient. Dr. H. Nielsen, Institute of Medical Biochemistry, University of Aarhus, is thanked for helpful discussions. The P. Carl Petersen Foundation is thanked for financial support.

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