Biochimica et Biophysica Acta, 513 (1978) 321–337

BBA 78183

ACCESSIBILITY OF PHOSPHOLIPIDS IN THE CHROMAFFIN GRANULE MEMBRANE

R. MARTIN BUCKLAND, GEORGE K. RADDA and CHRISTOPHER D. SHENNAN

Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU (U.K.)

(Received March 6th, 1978)

Summary

1. The accessibility of phospholipids in the membrane of the adrenomedullary storage vesicles (chromaffin granules) has been studied.

2. The reaction of 2,4,6-trinitrobenzenesulphonic acid with both intact granules and their ghosts, results in the labelling of 70% of the phosphatidylethanolamine.

3. The action of phospholipase A_2 (from bee venom), phospholipase C (from *Bacillus cereus*) and sphingomyelinase C (from *Staphylococcus aureus*) on granules and their ghosts was followed as a function of time. No significant difference was observed between the intact granules and their ghosts.

4. In the intact granules the various treatments led to varying amounts of lysis although again no evidence was obtained that such lysis in any way increased the amount of accessible phospholipid.

5. Highly purified granule preparations were also compared with the so-called "large granule" fraction and no significant differences were detected.

6. Approx. 67% of phosphatidylethanolamine + phosphatidic acid, 50% of phosphatidylserine + phosphatidylinositol, 65% of phosphatidylcholine and 20% of sphingomyelin is accessible to enzymatic degradation. In total, approx. 50% of all the phospholipids reacted.

7. It is also shown that, unlike in the enzymatic treatment, all the phosphatidylcholine can be exchanged in the presence of a phospholipid exchange protein (prepared from beef liver).

8. It is concluded that transmembrane movement of phosphatidylcholine is slow in isolated membranes of chromaffin granules. The presence of the exchange protein, however, in conjuction with membrane proteins and specific phospholipid arrangements may catalyse this transmembrane movement.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Introduction

The transverse distribution of phospholipids in the erythrocyte membrane has been extensively documented on the basis of chemical and enzymatic studies [1–8]. Subsequently the distribution of phospholipids has also been investigated in both plasma membranes [9–12] and in membranes of subcellular organelles [13–16]. In each system the conclusion has generally been that total phospholipids are distributed equally between the outer and inner membrane monolayers. The distribution of individual phospholipids may, however, be highly asymmetric. Clearly this knowledge is important in relation to the structure and biosynthesis of membranes and also presumably has implications with respect to function. To what extent particular membrane functions depend on the nature of the lipid asymmetry is unknown.

The aim of this work is to study the properties of phospholipids in the adrenomedullary catecholamine storage vesicles (chromaffin granules) in relation to their possible role in the transmitter release mechanism that is believed to involve fusion between the granule and the plasma membrane (exocytosis) [17,18]. Earlier, we reported experiments based on the use of fluorescent probes [19] and spin labels [20] that explored the motional properties of the lipid phase in the granule and discussed the possibility that lateral lipid phase separation could play a role in the fusion process [20].

In this paper we report our studies on the accessibility of different phospholipids to several specific purified phospholipases and to the reagent 2,4,6-trinitrobenzenesulphonic acid both in intact chromaffin granules and in their "ghosts" from the large granule fraction. We have also compared accessibility of the phospholipids in a highly purified preparation. A further study on the accessibility of phosphatidylcholine in the intact chromaffin granule to a phospholipid exchange protein is reported and the results are discussed in relation to the observed accessibility of this phospholipid in the membrane.

Experimental procedure

Materials

2,4,6-Trinitrobenzenesulphonic acid was obtained from the Sigma Chemical Company and was used without further purification. Phosphatidyl[*N-methyl*-^{14}C]choline (specific activity 60 Ci/mol) was obtained from The Radiochemical Centre, Amersham. Highly purified phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) from bee venom was the kind gift of Dr. G.H. de Haas (University of Utrecht) and was stored at −20°C in glycerol/water (1 : 1, v/v). Highly purified sphingomyelinase C (spingomyelin choline phosphohydrolase) from *Staphylococcus aureus* was the kind gift of Professor L.L.M. van Deenen and Dr. R.F.A. Zwaal (University of Utrecht) and was stored at −20°C in 50 mM Tris, 5 mM $CaCl_2$, glycerol/water (pH 7.5) (1 : 1, v/v). Both of these enzymes were extensively dialysed against 10 mM HEPES (pH 7.4) before use. Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* was obtained from the Sigma Chemical Company and was dissolved in 10 mM HEPES (pH 7.4). Phospholipid exchange protein specific for phosphatidylcholine was prepared from beef liver and was the kind gift of

Dr. K.W.A. Wirtz (University of Utrecht). It was stored in glycerol/water (1 : 1, v/v) at −20°C and was extensively dialysed against 300 mM sucrose/10 mM HEPES (pH 7.0) before use.

Silicagel G and HR were obtained from Merck and the plates for thin layer chromatography were prepared by Mr. R. Prior of the Dyson Perrins Laboratory, Oxford.

Standard laboratory chemicals were obtained in the highest available purity. Water was twice distilled from an all-glass apparatus and other solvents were distilled before use.

Methods

Preparation of chromaffin granules and ghosts. Chromaffin granules and their membranes (ghosts) were prepared from the adrenal medullae of freshly slaughtered cattle by differential centrifugation as previously described [21]. The final pellet was used as the source of the large granule fraction. This preparation has a small mitochondrial and lysosomal contamination [22].

Highly purified granules were prepared by layering 2.5 ml of a suspension of the large granule fraction (approx. 10 mg membrane protein) in 300 mM sucrose/10 mM HEPES (pH 7.0) on 10 ml of 1.6 M sucrose/10 mM HEPES (pH 7.0) as described by Smith and Winkler [22]. After centrifugation for 60 min at $80000 \times g_{av}$, the purified granule pellet was washed with 600 mM sucrose/10 mM HEPES (pH 7.0) and was finally resuspended in 300 mM sucrose/10 mM HEPES (pH 7.0). The purified granule preparation is substantially free from contaminating organelles [22,23] but is known to be more susceptible to osmotic shock than granules derived from the large granule fraction [24].

All experiments were completed within 18 h of the initial preparation of the granules.

Labelling by 2,4,6-trinitrobenzenesulphonic acid. The reaction medium contained 2.4 mM 2,4,6-trinitrobenzenesulphonic acid, 10 mM phosphate (pH 7.75 at 20°C) and approx. 0.1 mg membrane protein/ml. The intact granule medium also contained 400 mM sucrose. The reaction was carried out at 20°C and was stopped by addition of 1 M HCl to give a final pH of approx. 1.2. The membranes were collected by centrifugation for 20 min at $48000 \times g_{av}$. The lipids were extracted according to the method of Bligh and Dyer [25] and were applied as a chloroform solution to silicagel G plates, activated at 100°C for at least 1 h before use. The plates were developed in chloroform/methanol/water (65 : 25 : 4, v/v). The phospholipids were extracted and analysed as described under lipid analysis.

Hydrolysis by phospholipase A. The reaction medium contained 5 international units (I.U.)/ml of purified phospholipase A_2 (from bee venom), 400 mM sucrose, 10 mM HEPES (pH 7.4 at 20°C), 1 mM $CaCl_2$ and 0.5—1.5 mg membrane protein per ml. The reaction was carried out at 20°C and at appropriate times samples were withdrawn. The reaction was stopped by addition of EDTA to give a final concentration of 5 mM.

For lysis determinations, 0.1 ml of the reaction mixture was removed and added to 3.0 ml 40 mM sucrose/10 mM HEPES (pH 7.0). Since the intact granules scatter at 540 nm, the absorbance of the solution at this wavelength

was measured immediately against a water reference. The extent of lysis is proportional to the decrease in absorbance [26].

Hydrolysis by phospholipase C. The reaction medium contained 0.25 I.U./ml of phospholipase C (from *B. cereus*), 400 mM sucrose, 10 mM HEPES (pH 7.4 at 20°C), 2 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.5–1.5 mg membrane protein/ml. The reaction was carried out at 20°C and at appropriate times samples were withdrawn. The reaction was stopped by addition of EDTA and *o*-phenanthroline to give final concentrations of 20 mM and 1 mM, respectively. Lysis was determined as described above.

Hydrolysis by sphingomyelinase C. The reaction medium contained 1.0 I.U./ml of purified sphingomyelinase C (from *S. aureus*), 400 mM sucrose, 10 mM HEPES (pH 7.4 at 20°C), 5 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 0.5–1.5 mg membrane protein/ml. The reaction was carried out at 20°C and at appropriate times samples were withdrawn. The reaction was stopped by addition of EDTA to give a final concentration of 40 mM. Lysis was determined as described above.

Phospholipid exchange. Vesicles were prepared in 300 mM sucrose/10 mM HEPES (pH 7.0) from pure egg phosphatidylcholine (containing phosphatidyl-[*N-methyl*-^{14}C]choline to give a specific activity of 55 μCi/mmol) by sonication on a Dawe sonicator type 7530 (power level 3) until a clear solution was formed. Titanium and liposomes were then removed by filtration through 0.22-μm Millipore filters.

Reaction exchange media were made up in 300 mM sucrose/10 mM HEPES (pH 7.0) to contain vesicles and granules at the concentrations described in Table IV, the reaction being started by addition of an aliquot of purified granules. The exchange protein was added to give a concentration of 6.8 μg/ml. To allow for the possibility that vesicles are absorbed or fused to the granules, parallel reactions were also carried out in the absence of the exchange protein.

Reactions were carried out at 20°C. The time course was monitored by removing aliquots of the solution and collecting the granules by centrifugation for 20 min at $48000 \times g_{av}$. The pellets were resuspended in 1 ml water and transferred to 10.0 ml Triton X-100/toluene (1 : 3, v/v) containing PPO (0.5% w/v) and POPOP (0.1% w/v) scintillation fluid. Samples were counted on an LKB 1210 Ultrobeta scintillation counter for 2 min, the increase in radioactivity representing the extent of reaction.

When no further increase was observed (after approx. 3.5 h) the granules were removed from the reaction exchange media by centrifugation for 20 min at $48000 \times g_{av}$. After removal of the supernatants the pellets were resuspended in water and recentrifuged.

Phospholipids were extracted both from pellets and supernatants and purified by thin-layer chromatography as described below. In both cases, the phosphatidylcholine band was removed and washed by the Skipski procedure for phospholipid elution [27]. The combined washings for each band were then divided to give duplicate samples for both lipid phosphorus analysis (described below) and scintillation counting (removal of solvents followed by addition of 10.0 ml scintillation fluid (as described earlier) and counting for 2 min).

Lipid and protein analysis. The lipids were extracted by the method of Bligh and Dyer [25] and, with the exception of the lipids labelled by 2,4,6-trinitro-

benzenesulphonic acid, were applied in a solution of chloroform as a band to the origin of activated 5 × 20 cm silicagel HR plates. The plates were developed in chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, v/v) [27] and the lipids were visualised by exposure to iodine vapour. Under these conditions excellent separation of the chromaffin granule phospholipids is obtained with the exception of phosphatidylserine from phosphatidylinositol. After removal of the iodine staining, phospholipid spots were removed from the plates and were extracted by the method of Skipski et al. [27]. The lipid extracts were evaporated to dryness and analysed for phosphorus according to the method of McClare [28]. Figures for the percentage hydrolysis of individual phospholipids were calculated as previously described [8].

Protein was estimated by the Biuret method [29].

Results

Analysis of chromaffin granule phospholipids

The phospholipid composition of purified chromaffin granules prepared as described is given in Table I.

This is in good agreement with data obtained by other workers [30–33]. In contrast to the analysis by Dreyfus et al. [34] we can find no trace of lysophosphatidylethanolamine in untreated granules. They have suggested that separation by thin-layer chromatography of lysophosphatidylethanolamine from lysophosphatidylcholine was not achieved in previous work. In the thin-layer chromatography solvent system used in this work, lysophosphatidylcholine migrates with an R_F value lower than for the other phospholipids of the granule membrane, while lysophosphatidylethanolamine has a greater R_F value, intermediate between that of phosphatidylcholine and of phosphatidylinositol + phosphatidylserine. The two phospholipids are, therefore, clearly separated. After incubation with phospholipase A_2, however, lysophosphatidylethanolamine is formed and a concomitant decrease in the content of phosphatidylethanolamine is observed.

Labelling by 2,4,6-trinitrobenzenesulphonic acid

Phosphatidylethanolamine in the chromaffin granule membrane reacts with

TABLE I

PHOSPHOLIPID COMPOSITION OF CHROMAFFIN GRANULES

Lipids were extracted from purified granules and separated by thin layer chromatograhy. After elution, phospholipids were analysed for phosphorus as described in Experimental procedure. Values are expressed as the means ± S.D. for 5—8 determinations from three preparations.

Phospholipid	Percentage composition
Phosphatidic acid	1.6 ± 0.5
Phosphatidylethanolamine	32.2 ± 0.9
Phosphatidylserine + phosphatidylinositol	10.4 ± 0.7
Phosphatidylcholine	25.4 ± 1.2
Sphingomyelin	13.7 ± 0.8
Lysophosphatidylcholine	16.7 ± 0.6

2,4,6-trinitrobenzenesulphonic acid. The extent of this reaction is identical for both intact granules and their ghosts prepared from the large granule fraction, as shown in Fig. 1. After 7 h approx. 70% of this lipid has been labelled, the remaining 30% being refractory even after 16 h. The extent of labelling cannot be increased by lysing the granules in a hypotonic phosphate solution already containing 2,4,6-trinitrobenzenesulphonic acid, a procedure that should ensure that the reagent is accessible to the inside face of the membrane. No reaction of 2,4,6-trinitrobenzenesulphonic acid with phosphatidylserine is observed, probably because this phospholipid constitutes less than 2.5% of the total phospholipid of the granule membrane [31,33].

Action of phospholipase A_2

The hydrolysis of chromaffin granule phospholipid by purified phospholipase A_2 from bee venom was investigated. Intact granules and their ghosts from the large granule fraction were incubated with this enzyme. Table II shows the percentage of each lipid remaining at 30 and 120 min after the start of the reaction. The small differences observed between the two membrane preparations indicate that lysis has little effect on the extent of reaction. Pretreatment of the ghosts with pronase, trypsin, or neuraminidase did not increase the extent of phospholipid hydrolysis by phospholipase A_2.

The large granule fraction is known to be slightly contaminated by microsomes, lysosomes and mitochondria [23], and there existed the possibility that the refractory fraction of the lipid represented the sum of this contamination. Fig. 2 shows the action of purified phospholipase A_2 on a highly purified preparation of the granules. After 120 min, 57% of the total phospholipids remain unaffected. This consists of 33% phosphatidylethanolamine, 55% phosphatidylserine + phosphatidylinositol and 39% phosphatidylcholine, an essentially identical result to that obtained for the large granule fraction. Fig. 2 also shows the extent of lysis of the granules. Although lysis is complete after approx. 30 min, it is clear that a portion of the phospholipids remain unhydrolysed.

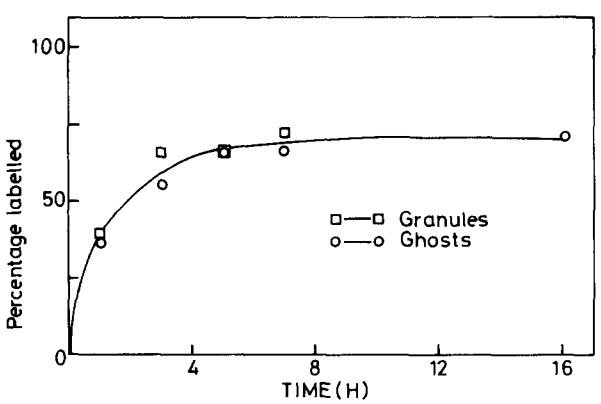

Fig. 1. Labelling of phosphatidylethanolamine by 2,4,6-trinitrobenzenesulphonic acid in chromaffin granule membranes. Intact granules and ghosts (lysed granules) from the large granule fraction (approx. 0.1 mg membrane protein/ml) were incubated in the presence of 2.4 mM 2,4,6-trinitrobenzenesulphonic acid at 20°C, pH 7.75. The extraction and analysis of phospholipids was as described in Experimental procedure. The experiment was repeated three times. Points are the means of 3—6 determinations.

TABLE II

PHOSPHOLIPID HYDROLYSIS BY PHOSPHOLIPASE A_2 (FROM BEE VENOM) IN INTACT GRANULES AND GHOSTS (LYSED GRANULES) FROM THE LARGE GRANULE FRACTION

Experimental details are identical to those under Fig. 2. The values for the times indicated are expressed as percentage of lipid phosphorus remaining (±mean deviation, number of determinations). The experiment was repeated five times for the intact granules and twice for the ghosts. Reactions and phosphorus determinations were carried out as described in Experimental procedure.

	30 min		120 min	
	Ghosts	Intact	Ghosts	Intact
Phosphatidylethanolamine + phosphatidic acid	49.4 (±0.2,2)	40.3 (±0.2,2)	39.3 (±1.2,2)	33.6 (±2.3,3)
Phosphatidylserine + phosphatidylinositol	75.2 (±1.5,2)	68.2 (±0.5,2)	65.7 (±0,2)	58.9 (±1.8,3)
Phosphatidylserine	52.1 (±0.2,2)	46.5 (±0.2,2)	42.6 (±0.4,2)	38.4 (±0.4,3)
Lysophosphatidylcholine	100	100	100	100
Sphingomyelin	100	100	100	100
Total	65.8	60.4	58.6	54.5

Action of phospholipase C

The action of phospholipase C from *B. cereus* on the chromaffin granule phospholipids was investigated.

The extent of phospholipid hydrolysis was compared for granules and ghosts

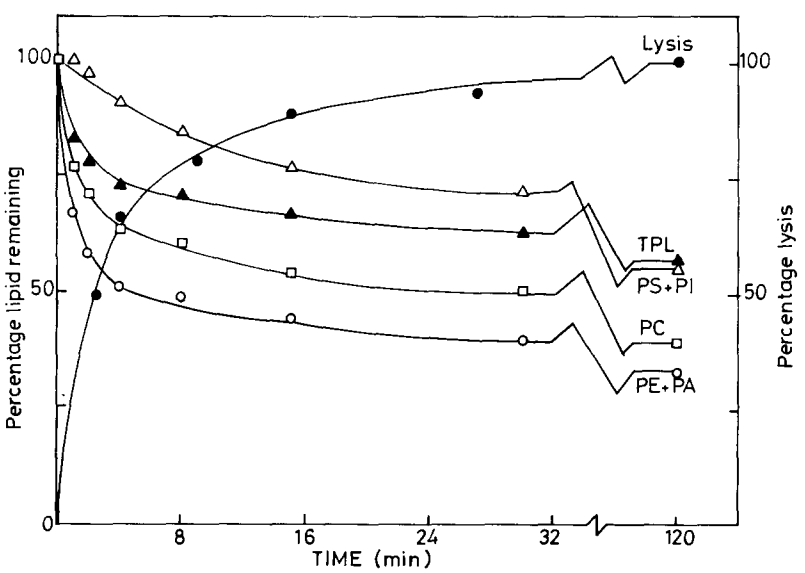

Fig. 2. Phospholipase A_2 (from bee venom) hydrolysis of phospholipids in purified chromaffin granules. Intact granules (1.4 mg membrane protein/ml) were incubated at 20°C with 5 I.U./ml phospholipase A_2 in the presence of 1 mM $CaCl_2$, pH 7.4. Phospholipids were extracted and estimated as described in Experimental procedure. The points are the means of 2—3 determinations. Abbreviations: PE, phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; TPL, total phospholipids.

prepared from the large granule fraction. Table III shows the percentage of each phospholipid remaining at 15, 60 and 120 min of reaction. For the earlier two times some differences exist, reflecting slight changes in the relative kinetics of hydrolysis for the two fractions. After 120 min essentially identical percentages of phosphatidylethanolamine, phosphatidylserine + phosphatidylinositol and phosphatidylcholine remain. However, about 12% more hydrolysis of lysophosphatidylcholine has occured in the ghost fraction.

Subsequently, the action of phospholipase C on a purified granule preparation was investigated. The results are shown in Fig. 3. As found for the large granule fraction, the reaction of each of the lipids except for lysophosphatidylcholine is almost complete after 45 min. After 90 min, 54% of the total phospholipids remain unaffected, the unhydrolysed phospholipids consisting of 92% lysophosphatidylcholine, 46% phosphatidylserine + phosphatidylinositol, 33% phosphatidylethanolamine and 30% phosphatidylcholine.

Action of sphingomyelinase C

The action of purified sphingomyelinase C from *S. aureus* was investigated to gain information on the distribution of sphingomyelin in the chromaffin granule membrane. Fig. 4 shows the time course of the reaction for both the large and purified granule fractions. In contrast to those previously described, the reaction is slow and a slightly increased extent of hydrolysis is found for purified granules. After almost 18 h, 85 and 79% sphingomyelin remains in the large and purified granule fractions, respectively, and in neither case was there any detectable lysis. A comparable reaction carried out on ghosts prepared

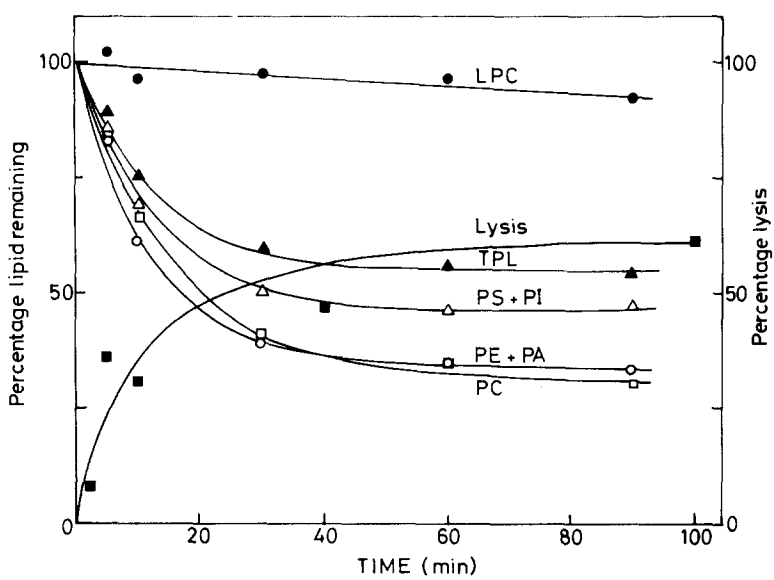

Fig. 3. Phospholipase C (from *B. cereus*) hydrolysis of phospholipids in purified chromaffin granules. Intact granules (1.4 mg membrane protein/ml) were incubated at 20°C with 0.25 I.U./ml phospholipase C in the presence of 2 mM $CaCl_2$ and 1 μM $ZnCl_2$, pH 7.4. Phospholipids were extracted and estimated as described in Experimental procedure. The points are the means of 2—3 determinations. Abbreviations: LPC, lysophosphatidylcholine; for the others, see Fig. 2.

TABLE III

PHOSPHOLIPID HYDROLYSIS BY PHOSPHOLIPASE C (*B. CEREUS*) IN INTACT GRANULES AND GHOSTS (LYSED GRANULES) FROM THE LARGE GRANULE FRACTION

Experimental details are identical to those under Fig. 3. The values for the times indicated are expressed as percentage of lipid phosphorus remaining (±mean deviation, number of determinations). The experiment was repeated twice. Reactions and phosphorus determinations were carried out as described in Experimental procedure.

Lipid	15 min		60 min		120 min	
	Ghosts	Intact	Ghosts	Intact	Ghosts	Intact
Phosphatidylethanolamine + phosphatidic acid	46.8 (1)	63.5 (±1.4, 2)	30.9 (±0.4, 2)	29.8 (±0.2, 2)	25.0 (±0.7, 2)	27.9 (±1.2, 2)
Phosphatidylserine + phosphatidylinositol	57.9 (1)	55.6 (±0.5, 2)	38.0 (±2.0, 2)	27.7 (±0.6, 2)	29.5 (±1.3, 2)	34.4 (±2.5, 2)
Phosphatidylserine	43.3 (1)	62.3 (±0, 2)	22.1 (±0.7, 2)	22.8 (±0.5, 2)	17.3 (±0.6, 2)	17.3 (±0.2, 2)
Lysophosphatidylcholine	88.3 (±1.2, 2)	102.6 (±1.0, 2)	88.3 (±0.1, 2)	96.9 (±0, 2)	81.7 (±2.6, 2)	93.5 (±0.4, 2)
Sphingomyelin	100	100	100	100	100	100
Total	60.7	72.1	42.8	45.9	40.4	44.4

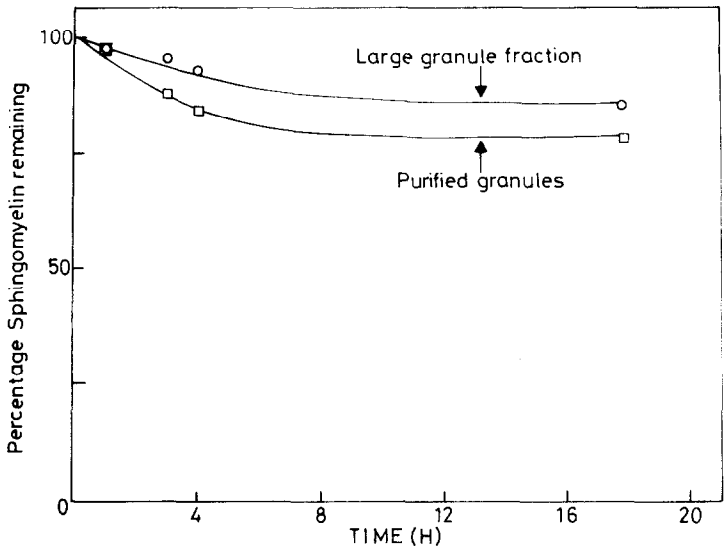

Fig. 4. Sphingomyelinase C (from *S. aureus*) hydrolysis of sphingomyelin in chromaffin granule membranes. Intact granules (1.4 mg membrane protein/ml) were incubated at 20°C with 0.5 I.U./ml sphingomyelinase C in the presence of 5 mM $CaCl_2$ and 0.25 mM $MgCl_2$, pH 7.4. Phospholipids were extracted and estimated as described in Experimental procedure. The experiment was repeated twice. Points are the means of 2—3 determinations.

from the large granule fraction indicated that 82% of the sphingomyelin remained after the same time. Under similar conditions 85% of the sphingomyelin in erythrocytes was hydrolysed by the same enzyme preparation in 1 h (C. Grathwohl, personal communication).

Phosphatidylcholine exchange

The accessibility of the membrane phosphatidylcholine was further investigated by the nonperturbing technique of phospholipid exchange catalysed by a protein from beef liver [35]. In a previous study [36] the protein was used to

TABLE IV

ACCESSIBILITY OF CHROMAFFIN GRANULE PHOSPHATIDYLCHOLINE TO BEEF LIVER EXCHANGE PROTEIN

^{14}C-labelled egg phosphatidylcholine vesicles (specific activity 55 μCi/mmol) and purified chromaffin granules were incubated in 300 mM sucrose/10 mM HEPES (pH 7.0) at the concentrations shown in the table with 6.8 μg/ml beef liver exchange protein. After approx. 3.5 h vesicles and granules were analysed for phosphatidylcholine phosphorus and ^{14}C-labelled lipid as described in Experimental procedure. Figures for the percentage accessible are the means ± S.D. for 14 determinations and the calculation of these is described in the Appendix.

Experiment	Lipid phosphorus concentration (μg/ml)	Membrane concentration (mg/ml)	Protein/lipid ratio (normalised)	Percentage phosphatidylcholine accessible
A	4	0.07	1	103 ± 4
B	2	0.28	8	98 ± 6

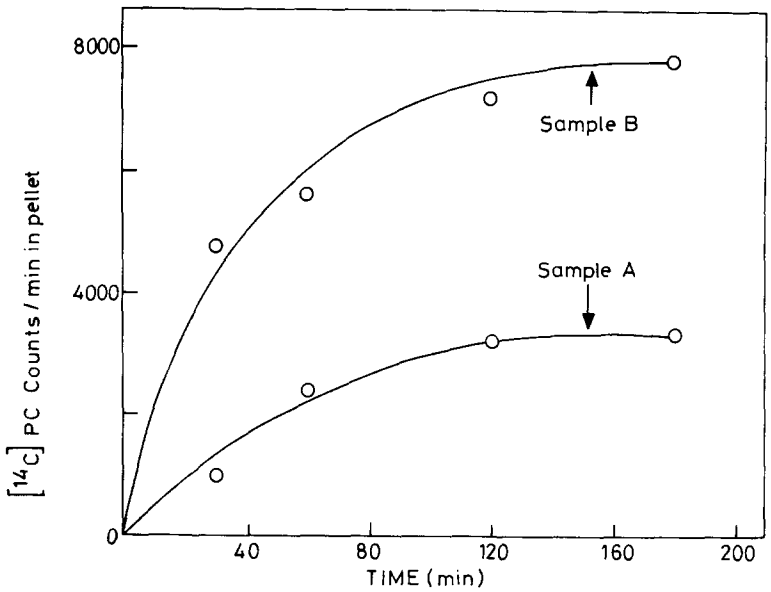

Fig. 5. Uptake of ^{14}C-labelled phosphatidylcholine by purified chromaffin granules. ^{14}C-labelled egg phosphatidylcholine vesicles (specific activity 55 μCi/mmol) and purified chromaffin granules were incubated in 300 mM sucrose/10 mM HEPES (pH 7.0) at the concentrations shown in Table IV with 6.8 μg/ml beef liver exchange protein. At the times indicated aliquots were removed and centrifuged at 48 000 × g_{av} to pellet the granules. The pellets were resuspended in 1 ml water and 10 ml toluene/Triton X-100 scintillation fluid and were counted for 2 min. The abscissa scale is uncorrected for scintillator counting efficiency. Further details are given in Experimental procedure. Abbreviation: PC, phosphatidylcholine.

exchange ^{32}P-labelled lipid (prepared in vivo) from rat liver microsomes to beef heart mitochondria. We introduce here a new method for investigating phosphatidylcholine accessibility by following the catalysed uptake by chromaffin granules of ^{14}C-labelled lipid from vesicles. The description of the theory is given in the Appendix, and experimental details are given in the Methods section.

Table IV gives details of a typical experiment which was carried out at two different granule:vesicle concentrations, Fig. 5 showing the progress of these reactions with time. In contrast to the conclusions reached for phospholipases A_2 and C catalysed hydrolysis, after approx. 3.5 h 100% of the phosphatidylcholine in the granule membrane is accessible in the presence of the exchange protein.

Discussion

The comparison here of intact and lysed granules indicates that the same constituent phospholipids may be hydrolysed by either phospholipase A_2 or C at different rates for the two membranes. The different kinetics for each lipid hydrolysed indicates that lysis may have caused some membrane reorganization. However, the extent of reorganization must be minimal since on completion of each reaction the lipids of each preparation have been affected to the same extent. The exception is lysophosphatidylcholine which is hydrolysed to

a greater extent by phospholipase C in granule ghosts, an effect which is discussed later, Similarly, no significant difference has been observed between the two preparations in the labelling reaction of 2,4,6-trinitrobenzenesulphonic acid or in the hydrolysis catalysed by sphingomyelinase C.

In studies on the membranes of erythrocytes [2,3,6,8], rat liver organelles [16] and blood platelets [11] it was shown that the extent of reaction using either covalent labelling reagents or hydrolytic enzymes was greater once the membranes had ruptured. The extent of enzyme action, for instance, increased to hydrolyse 100% of the available lipids [16]. The absence of any difference observed with the chromaffin granule membrane may be explained in two ways.

Firstly, the membrane of a lysed granule in hypotonic solution may act as a barrier, preventing access to the extracytoplasmic side. The ghost formed would appear to an extrinsic reagent or enzyme as an intact granule. Large molecules such as proteins may be excluded but for smaller molecules such as 2,4,6-trinitrobenzenesulphonic acid, this effect would seem unlikely. Even granules lysed directly in hypotonic phosphate solution containing 2,4,6-trinitrobenzenesulphonic acid show no difference in the extent of phosphatidylethanolamine labelling from preformed ghosts subsequently incubated with 2,4,6-trinitrobenzenesulphonic acid in solution.

Secondly, the extracytoplasmic side of the membrane might be shielded by tightly bound species such as proteins originating either from the membrane itself or from the contents of the granule usually referred to as soluble. Strong adhesion between the contents and the membrane has been observed within intact granules by electron microscopy [37]. In granule ghosts tight binding of proteins to the inner monolayer may help to explain the presence of two components also found in the soluble contents. Thus, even the most thorough procedures for ghost preparation have shown that small quantities of chromogranin A, a strongly hydrophilic and acidic protein, are present with the membrane [38]. In a similar way, dopamine β-hydroxylase activity has been found associated with both the soluble and insoluble fractions of lysed chromaffin granules [39]. No difference appears to exist between these forms of the enzyme [39]. Since the insoluble form is thought to be situated on the extracytoplasmic side of the membrane [39] it may simply represent a strongly bound form rather than a true membrane component.

Shielding of the inner membrane phospholipids by tight binding of hydrophilic proteins would prevent an attack to that surface by phospholipid-directed extrinsic reagents and enzymes. However, pretreatment of ghosts with pronase, trypsin, or neuraminidase did not increase the accessibility of the phospholipids to phospholipase A_2. There is no evidence, though, that these proteins were able to reach the inner membrane monolayer as discussed earlier.

A conspicuous feature of the reactions studied is that the rate and limiting extent of lysis varies greatly. The products of each reaction are likely to affect membrane stability to different extents. More importantly the method used for the preparation of chromaffin granules produces a population of organelles having a heterogeneous distribution of sizes. Membrane stability will almost certainly change with size, and changes in the rate and extent of lysis are to be expected. Nevertheless, it is important to emphasize that there is no relation

between extent of lysis and lipid accessibility.

Table V summarises the percentage of each lipid remaining in purified granules after the reactions with each enzyme had apparently reached the limit. The action of phospholipase A_2 results in hydrolysis of 43% of the total lipid. Sphingomyelinase C removes a further 3%, corresponding to 21% of the total sphingomyelin content. About 46% of the total phospholipids of the chromaffin granule membrane can thus be hydrolysed by these two enzymes. The hydrolysed phospholipids consist of approximately 67% phosphatidylethanolamine, 45% phosphatidylserine + phosphatidylinositol, 61% phosphatidylcholine and 21% sphingomyelin. The accessibility of the diacyl phosphatides is supported by the results obtained for phospholipase C (Table V) by which a further 3% of the total phospholipid may be hydrolysed. The accessibility of phosphatidylethanolamine is further supported by 2,4,6-trinitrobenzenesulphonic acid labelling of the large granule fraction in which 70% of the total phosphatidylethanolamine reacted.

Fig. 3 shows that lysophosphatidylcholine in chromaffin granules may be hydrolysed at a slow rate by phospholipase C from *B. cereus*. Hydrolysis of lysophosphatidylcholine by phospholipase C from *Clostridium perfringens* has been observed at a rate ten times slower than that for phosphatidylcholine [40]. If these slow rates are of a comparable magnitude, the data in Fig. 3 may be extrapolated to the limit of reaction. After 100 min, 8% of the lysophosphatidylcholine in chromaffin granules is accessible to the enzyme. On the basis of this extrapolation, hydrolysis ought to be complete after approx. 200 min when approx. 16% of the lysophosphatidylcholine would have reacted. An upper limit of approx. 20% may therefore be set as the percentage of lysophosphatidylcholine in chromaffin granules that would have been accessible to phospholipase C (from *B. cereus*) after complete reaction.

It was shown recently that lysophosphatidylcholine is not acylated in intact chromaffin granules by a microsomal acyl transferase, but in broken vesicles up to one-third of lysophosphatidylcholine can be acylated [41]. These observations strengthen our tentative conclusions about this phospholipid component.

It has been shown that up to 49% of the total phospholipids in the chro-

TABLE V

SUMMARY OF THE EFFECTS OF HYDROLYTIC ENZYMES ON PHOSPHOLIPIDS OF PURIFIED CHROMAFFIN GRANULES

Values are percentages of original lipid phosphorus remaining after completion of enzyme hydrolysis. The data are taken from Figs. 2, 3 and 4.

	Phospholipase A_2	Phospholipase C	Sphingomyelinase C
Phosphatidylethanolamine + phosphatidic acid	33	33	100
Phosphatidylserine + phosphatidylinositol	55	46	100
Phosphatidylcholine	39	30	100
Sphingomyelin	100	100	79
Lysophosphatidylcholine	100	(92) *	100
Total	57	54	97

* Reaction not completed.

maffin granule membrane can be hydrolysed by different phospholipid hydrolytic enzymes. It has also been shown that lysis of the membrane produces little, if any, effect on lipid accessibility and this observation differs from those seen in other membranes. The remaining 51% of the total phospholipids are inaccessible to the reagents used. These phospholipids must therefore be shielded in some way. The methods used here do not allow us to distinguish shielded from asymmetrically distributed phospholipids.

In complete contrast to the findings with the hydrolytic enzymes, about 100% of the total phosphatidylcholine is accessible to the exchange protein used. A similar apparent paradox has been observed for the phosphatidylcholine in rat liver microsomes in which 54% was hydrolysed at 0°C by phospholipase A_2 [14] while up to 95% was accessible for exchange at 30°C [36]. A temperature increase of 30°C may be expected to change the motional properties of phospholipids and their accessibility as a result. However, the reactions described here were all carried out at 20°C. There are clearly at least three possible explanations for this observation. (1) 100% of the phosphatidylcholine may be situated in the cytoplasmic monolayer. Absolute asymmetry of phosphatidylcholine has never previously been observed and from steric considerations the accessibility of the lipid would probably be similar to proteins of similar size (such as phospholipase A_2 and phosphatidylcholine exchange protein). (2) Phosphatidylcholine may be distributed between the two sides of the membrane and "flip-flop" may be rapid, allowing 100% accessibility to the exchange protein. Reaction (e.g., hydrolysis) of the outer phospholipids must then produce products that inhibit further "flip-flop" from taking place and prevent complete reaction. The excellent agreement between the results of phospholipases A_2 and C and 2,4,6-trinitrobenzenesulphonic acid labelling treatments, each of which produces quite different products, makes this very unlikely. (3) Phosphatidylcholine may be distributed between the two sides of the membrane and "flip-flop" may be slow. Reaction only takes place with outer phospholipids. In this case 100% accessibility of the membrane phosphatidylcholine to the exchange protein implies that the protein is catalysing exchange both between and across the granule membrane. Increased rates of "flip-flop" have not been seen in vesicles under similar conditions [42,43]. It is therefore concluded that in conjuction with membrane proteins or particular phospholipid arrangements the exchange protein can catalyse transmembrane movement of phosphatidylcholine to take place at rates considerably greater than those found in vivo.

These studies have shown that only one half of the chromaffin granule membrane phospholipids are accessible to extrinsic reagents. It is unclear why the remaining phospholipids are inaccessible. This unusual feature may confer some advantage to the functioning of the membrane and, in particular, its role in exocytosis with the chromaffin cell plasma membrane.

Appendix

Use of the exchange protein to determine the fraction of accessible membrane phosphatidylcholine

It is assumed that the exchange protein catalyses the exchange of phospha-

tidylcholine molecules only between all membrane surfaces present in the system. Freshly prepared chromaffin granules are incubated in the presence of the exchange protein with phosphatidylcholine vesicles containing a small fraction (approx. 0.1%) of ^{14}C-labelled phospholipid molecules. It is also assumed that labelled and unlabelled molecules exchange at identical rates such that the increase in radioactivity in the granule fraction is proportional to both the decrease in radioactivity of the vesicle fraction and to the extent of reaction.

Equilibrium will be reached when there is no further net exchange between vesicles and granules. For the vesicles, let the number of radioactive molecules initially present be N. If the ratio of the number of molecules in the vesicle outer monolayer to those in the inner monolayer is X then the fraction of molecules present in the outer monolayer equals $X/(X + 1)$, and $N \cdot X/(X + 1)$ are accessible to the exchange protein provided that "flip-flop" in the vesicle is slow in relation to the time taken to attain equilibrium [35]. Let the number of radioactive molecules present in the vesicles at equilibrium be N^*, in which case $(N - N^*)$ molecules have been transferred.

At equilibrium, the number of radioactive molecules in the outer monolayer of the vesicles per mole of phosphatidylcholine in that monolayer must equal the equivalent ratio for the accessible pool (or pools) of phosphatidylcholine molecules in the granules. Let the number of moles of vesicle phosphatidylcholine be P. Then $P \cdot X/(X + 1)$ is both accessible and in the outer monolayer. The specific activity of that lipid (number of counts per mol) is initially

$$\frac{N \cdot X/(X + 1)}{P \cdot X/(X + 1)}$$

and at equilibrium

$$\frac{N \cdot X/(X + 1) - (N - N^*)}{P \cdot X/(X + 1)}$$

Let the number of moles of granule phosphatidylcholine be P_{CG} and the ratio of accessible to unaccessible phosphatidylcholine molecules be Y. The fraction of accessible granule phosphatidylcholine is therefore $Y/(Y + 1)$ and the number of moles of accessible lipid is $P_{CG} \cdot Y/(Y + 1)$. If the number of radioactive molecules in the granules at equilibrium is N_{CG}, then the specific activity of the granule phosphatidylcholine becomes

$$\frac{N_{CG}}{P_{CG} \cdot Y/(Y + 1)}$$

The equality that was defined earlier is the condition for equilibrium and may now be written as

$$\frac{N_{CG}}{P_{CG} \cdot Y/(Y + 1)} = \frac{N \cdot X/(X + 1) - (N - N^*)}{P \cdot X/(X + 1)}$$

The calculation of $Y/(Y + 1)$ therefore involves the determination of N/P, N^*/P, N_{CG}/P_{CG} and assumes that $X = 1.85$ [44] (in fact, values in the range 1.7—2.0 make changes of less than 3% in the calculated value of $Y/(Y + 1)$). Since only the relative values of N, N^* and N_{CG} are required, it is unnecessary

to correct for scintillation counter efficiency. The experimental procedure used for the determinations is described under Methods.

In the absence of the exchange protein, a small fraction of labelled lipid is inevitably trapped in the pelleted granule fraction. The values quoted for the percentage accessible allow for a correction of this.

Acknowledgements

R.M.B. and C.D.S. thank the Medical Research Council for research studentships. This work was supported by the Science Research Council.

References

1 Bretscher, M.S. (1972) Nat. New Biol. 236, 11—12
2 Gordesky, S.E., Marinetti, G.V. and Segel, G.B. (1972) Biochem. Biophys. Res. Commun. 47, 1004—1009
3 Gordesky, S.E. and Marinetti, G.V. (1973) Biochem. Biophys. Res. Commun. 50, 1027—1031
4 Colley, C.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 307, 74—82
5 Whiteley, N.M. and Berg, H.C. (1974) J. Mol. Biol. 87, 541—561
6 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83—96
7 Renooij, W., van Golde, L.M.G., Zwaal, R.F.A. and van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53—58
8 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178—193
9 Tsai, K.H. and Lenard, J. (1975) Nature 253, 554—5
10 Fong, B.S., Hunt, R.C. and Brown, J.C. (1976) J. Virol. 20, 658—663
11 Chap, H.J., Zwaal, R.F.A. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146—164
12 Rothman, J.E. and Kennedy, E.P. (1977) J. Mol. Biol. 110, 603—618
13 Nilsson, O.S. and Dallner, G. (1975) FEBS Lett. 58, 190—193
14 Nilsson, O.S. and Dallner, G. (1977) Biochim. Biophys. Acta 464, 453—458
15 Vale, M.G.P. (1977) Biochim. Biophys. Acta 471, 39—48
16 Higgins, J.A. and Dawson, R.M.C. (1977) Biochim. Biophys. Acta 470, 342—356
17 Winkler, H. (1971) Philos. Trans. R. Soc. Lond. B 261, 293—303
18 Grynszpan-Winograd, O. (1971) Philos. Trans. R. Soc. Lond. B 261, 291—292
19 Bashford, C.L., Morgan, C.G. and Radda, G.K. (1976) Biochim. Biophys. Acta 426, 157—172
20 Marsh, D., Radda, G.K. and Ritchie, G.A. (1976) Eur. J. Biochim. 71, 53—61
21 Bashford, C.L., Casey, R.P., Radda, G.K. and Ritchie, G.A. (1975) Biochem. J. 148, 153—155
22 Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 480—482
23 Helle, K.B., Flatmark, T., Serck-Hanssen, G. and Lönning, S. (1971) Biochim. Biophys. Acta 226, 1—8
24 Edwards, W., Phillips, J.H. and Morris, S.J. (1974) Biochim. Biophys. Acta 356, 164—173
25 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
26 Casey, R.P., Njus, D., Radda, G.K. and Sehr, P.A. (1976) Biochem. J. 158, 583—588
27 Shipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374—378
28 McClare, C.W.F. (1971) Anal. Biochem. 39, 527—530
29 Dawson, R.M.C., Elliot, W.H. and Jones, K.M. (1969) Data for Biochemical Research, 2nd edn. p. 618, Clarendon Press, Oxford
30 Blaschko, H., Firemark, H., Smith, A.D. and Winkler, H. (1967) Biochem. J. 104, 545—549
31 Winkler, H., Strieder, N. and Ziegler, E. (1967) Naunyn-Schmiedeberg's Arch. Pharmakol. Exp. Pathol. 256, 407—415
32 Da Prada, M., Pletscher, A. and Tranzer, J.P. (1972) Biochem. J. 127, 681—683
33 Phillips, J.H. (1973) Biochem. J. 136, 579—587
34 Dreyfus, H., Aunis, D., Harth, S. and Mandel, P. (1977) Biochim. Biophys. Acta 489, 89—97
35 Zilversmit, D.B. and Hughes, M.E. (1976) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 7, pp. 211—259, Plenum Press, New York
36 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110
37 Phillips, J.H. (1974) Biochem. J. 144, 311—318
38 Winkler, H., Hörtnagl, H. and Smith, A.D. (1970) Biochem. J. 118, 309—310

39 Winkler, H. and Smith, A.D. (1975) in Handbook of Physiology, Section 7, Endocrinology, Vol. VI, Adrenal Gland (Blaschko, H., Sayers, G., Smith, A.D., eds.), pp. 321—339, American Physiological Society, Washington, D.C.
40 Stahl, W.L. (1973) Arch. Biochem. Biophys. 154, 47—55
41 Voyta, J.C., Slakey, L.L. and Westhead, E.W. (1978) Biochem. Biophys. Res. Commun. 80, 413—417
42 Johnson, L.W., Hughes, M.E. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176—185
43 Rothman, J.E. and Dawidowicz, E.A. (1975) Biochemistry 14, 2809—2816
44 Berden, J.A., Barker, R.W. and Radda, G.K. (1975) Biochim. Biophys. Acta 375, 186—208