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A STUDY ON THE TOPOLOGICAL DISTRIBUTION OF PHOSPHOLIPIDS IN MICROSOMAL MEMBRANES OF CHICK BRAIN USING PHOSPHOLIPASE C AND TRINITROBENZENESULFONIC ACID

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The transbilayer distribution of phospholipids in chicken brain microsomal membranes has been investigated using trinitrobenzenesulfonic acid and phospholipase C from *Clostridium welchii*. The exposure of intact microsomes to trinitrobenzenesulfonic acid showed that the labelling of aminophospholipids followed biphasic kinetics, indicating that these membranes contain a fast- and a slow-reacting pool of aminophospholipids. Use of microsomes radioiodinated on their surface led to the conclusion that the fast-reacting pool may be located on the outer leaflet of the microsomal vesicles. It contains about 35% of the phosphatidylethanolamine, 29% of the ethanolamine plasmalogens and 18% of the phosphatidylserine. The treatment of intact microsomes with the phospholipase C *Cl. welchii* produced the hydrolysis of 50% of the phospholipids without any loss of their permeability properties, indicating that they are not permeable to the hydrolase. Phospholipids extracted from the microsomes were hydrolyzed rapidly by the phospholipase C with the exception of phosphatidylserine and phosphatidylinositol. In intact microsomes about 90% of phosphatidylcholine, 32% of ethanolamine phospholipids and 60% of sphingomyelin were accessible to the phospholipase. These results suggest that the phospholipids have an asymmetric distribution in chicken brain microsomes, the external leaflet containing about 75% of the choline phospholipids and 25% of the aminophospholipids, whereas an opposite distribution is observed in the inner leaflet.

Introduction

The architecture of nerve cell membranes is of crucial importance for the functional activity of neurons and glial cells. Phospholipids are the major class of lipids in these membranes and play an important role in their fluidity [1,2,3], permeability [4], in synaptic transmission [5,6] and enzyme activities [4]. In view of the different functions of the membrane requiring specific phospholipids (i.e., enzyme activities), the intramembrane distribution of the different phospholipids may be important for the control and the integration of any vectorial process occurring in the membrane.

Several studies have demonstrated an asymmetric distribution of phospholipids in cellular membranes [7–11], including synaptosomal plasma membranes [12,13]. The site of synthesis of the major phospholipids (PC and PE) in brain is located mainly in the microsomes; thus, it may be assumed that the enzymes involved in their synthesis may participate in the asymmetric assembly of

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Abbreviations: TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenyl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

phospholipids in membranes. This hypothesis is consistent with the asymmetric distribution of enzymes catalysing the last step of the synthesis of PC and PE (i.e., phosphocholine and phosphoethanolamine transferases) [14]. It is therefore important to elucidate the organization of phospholipids in a membrane responsible for their biogenesis. This prompted us to investigate the intramembrane distribution of phospholipids in the microsomes of chicken brain using phospholipase C and trinitrobenzene sulfonic acid.

Material and Methods

Materials

Phospholipase C of *Clostridium welchii* (EC 3.1.4.3), specific activity: 10 U/mg protein, trinitrobenzene sulfonic acid, bovine serum albumin type V, lactoperoxidase (EC 1.11.1.7) and glucose oxidase (EC 1.1.2.4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium iodide, $Na^{125}I$, carrier free, was obtained from Amersham International, U.K.

Methods

Preparation of microsomes. Microsomes were prepared from fresh adult chick brains by the method of Porcellati et al [15] with slight modifications. The brains were rapidly removed, rinsed with 0.32 M sucrose/2 mM mercaptoethanol and homogenized in a Potter-Elvehjem homogenizer with the same solution (1 g/10 ml). The homogenate was centrifuged for 10 min at $900 \times g$. The supernatant was carefully removed and centrifuged for 10 min at $7000 \times g$ in a Beckman L2 ultracentrifuge. The supernatant was then centrifuged twice for 20 min at $17000 \times g$, and the microsomes pelleted by centrifugation for 60 min at $105000 \times g$. The supernatant was removed and the pellet washed once with the solution used for homogenization. The microsomes were then homogenized in a proper buffer for phospholipid determination and phospholipid asymmetry analysis.

Trinitrophenylation of microsomal phospholipids. The labelling of aminophospholipids in microsomes with TNBS was carried out as described by Rothmann and Kennedy [16]. Microsomes were homogenized in 170 mM $NaHCO_3$ adjusted to pH 8.00 at a concentration of 0.9 mg protein per ml. Unless otherwise stated, 0.25 ml of 15 mM TNBS in 5% $NaHCO_3$ adjusted to pH 9.00 was added to 2 ml of microsomes. The reaction lasted for various periods of time at a temperature of 0°C or 20°C, and was stopped by the addition of 1 ml of ice-cold 2.5 M perchloric acid. The mixture was centrifuged at $2500 \times g$ for 30 min and the supernatant was discarded. The pellet was washed once with 1 ml 0.6 M perchloric acid and the lipids extracted with 2 ml of chloroform/methanol (2 : 1, v/v) as described by Folch et al. [17]. The treatment of microsomes with perchloric acid produces hydrolysis of plasmalogens into lysophospholipids. This allows the determination of ethanolamine plasmalogens which have reacted with TNBS. The lipid extract was washed with 0.4 ml 125 mM NaCl and the organic phase was evaporated under reduced atmospheric pressure using a rotary evaporator. The phospholipids were separated by two-dimensional thin-layer chromatography using a Silica-gel G plate (Merck, Darmstadt) and developed with chloroform/methanol/27% ammonia (65 : 35 : 5, v/v) in the first dimension and chloroform/ methanol/ acetone/ acetic acid/ water (75 : 15 :30 : 15 : 7.5, v/v) in the second. Phospholipids were visualized by exposure to iodine vapour. The spots from duplicate plates were scraped and analyzed for phosphate content [18]. In some experiments, monodimensional thin-layer chromatography was performed using the alkaline solvent.

Kinetics of the labelling of microsomes with TNBS. The study of the asymmetric localization of aminophospholipids using TNBS requires that the reagent does not penetrate the vesicles during the reaction and that it reacts with all the molecules facing the outside. To determine this, surface lipids of the microsomal membranes were labelled by the lactoperoxidase-catalyzed radioiodination [19,20]. Briefly the microsomes were suspended in a Ca^{2+},Mg^{2+}-free phosphate buffer/NaCl solution (isotonic phosphate-buffered saline, pH 7.4). 100 μCi $Na^{125}I$, 10 μg lactoperoxidase and 6 mU glucose oxidase were added. The reaction was initiated by the addition of 25 nmol D-glucose in a final volume of 0.23 ml. The reaction was carried out for 10 min at room temperature and stopped by the addition of 1 μmol of $Na_2S_2O_3$ in 10 μl H_2O in order to reduce the different forms of

iodine to iodide (I^-). The microsomes were then collected by centrifugation at 105 000 × *g* for 60 min and reacted with TNBS for 1–30 min as described above. After lipid extraction, the labelled lipids were separated from the unbound radioactive iodide by Sephadex LH-20 chromatography as described previously [19,20]. The radioactivity present in the NTP-PE and PE which has not reacted with TNBS was determined after thin-layer chromatography (see above).

Phospholipase C treatment of microsomes. Microsomes were homogenized in 20 mM Tris-HCl buffer (pH 7.4)/0.85% NaCl/0.25 mM $CaCl_2$. 2.5 mg of protein were incubated with 1 unit of phospholipase C in a final volume of 0.45 ml as described by Higgins and Dawson [11]. The reaction was stopped by the addition of 9 ml chloroform/methanol (2 : 1, v/v) and the lipids were extracted at 37°C for 30 min. Under these conditions phospholipase C does not further hydrolyse phospholipids, since similar results were obtained for control tubes containing phospholipase C or not when the reaction was stopped at time zero. The water-soluble compounds were extracted by addition of 1.8 ml 125 mM NaCl. The mixture was vigorously homogenized and centrifuged at 2500 × *g* for 5 min. The upper phase was carefully removed with a Pasteur pipette and the lower phase was washed once with the theoretical upper phase. The rate of hydrolysis of total phospholipids was evaluated by the determination of the phosphate content in both the organic and the water phase. The various phospholipids were separated by a two-dimensional thin-layer chromatography using chloroform/methanol/27% ammonia (65 : 35 : 5, v/v) in the first direction and butanol/acetic acid/water (60 : 20 : 20, v/v) in the second direction. Phospholipids were visualized and quantitated as described above.

Measurement of vesicular integrity. The effect of phospholipase C on the microsomal integrity was determined by the sucrose efflux measurements as reported by meissner and Allen [21]. Microsomes were incubated in 20 mM Tris-HCl buffer/0.87% NaCl (pH 7.4)/10 mM [^{14}C]sucrose (spec. act. 1 μCi/mmol). After 1 h, the microsomes were centrifuged at 105 000 × *g* for 1 h and the pellet was homogenized in 20 mM Tris-HCl buffer/0.87% NaCl/0.25 mM $CaCl_2$/10 mM sucrose (pH 7.4). 2 mg protein were incubated for various time periods with or without 1 U phospholipase C in a final volume of 0.45 ml. The efflux of the sucrose was determined by filtration of aliquots of the homogenate (0.2 ml) through 0.45 μm HAWP Millipore filters. The filters were rinsed three times with non-radioactive incubation medium and dried, and the vesicles disrupted by

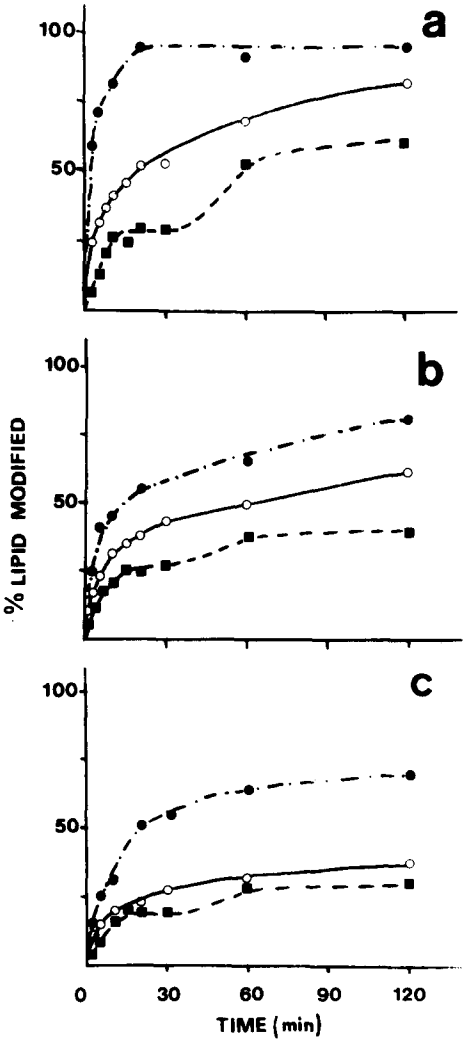

Fig. 1. Time-course for the triphenylation of aminophospholipids of intact chicken brain microsomes as a function of incubation temperature and TNBS concentration. (a) Phosphatidylethanolamine; (b) ethanolamine plasmalogen; (c) phosphatidylserine. ●–·–●, 20°C, 5.00 mM TNBS; ○———○, 20°C, 1.66 mM TNBS; ■-----■, 0°C, 1.66 mM TNBS. Each point represents the average of three separate experiments.

the addition of 10 mM of scintillation fluid (Rotiszint 22, Roth, RFA). The radioactivity was measured in an Intertechnique SL 30 scintillation spectrometer.

Results

Treatment of microsomes with TNBS

When chick brain microsomes were incubated with TNBS, a time-dependent conversion of ethanolamine and serine phospholipids into trinitrophenyl derivatives was observed. The amount of conversion was dependent upon the concentration of TNBS and temperature (Fig. 1). treatment of microsomes with 5 mM TNBS at 20°C yielded more than 90% of triphenylated PE, about 80% of triphenylated ethanolamine plasmalogens and about 70% of triphenylated PS after 2 h. When the reaction was allowed to proceed for 3 h nearly all ethanolamine compounds and more than 80% of the PS had reacted with the probe. With 1.66 mM TNBS, the yield of triphenylated compounds was a little lower after 2 h at 20°C. With both concentrations of TNBS, the reaction kinetic was biphasic, indicating that microsomes contain a fast- and a slow-reacting pool of aminophospholipids, as shown by plotting the date of unreacted phospholipids on a logarithmic scale (Fig. 2). When microsomes were incubated with 1.66 mM TNBS, the fast-reacting pool evaluated by substracting the slow-reacting component from the total, had a half-time reaction of about 2.5–3.0 min for the three aminophospholipids investigated, whereas the half-time reaction of the slow-reacting pool was of about 60 min for PE, 100 min for the corresponding plasmalogens and 300 min for PS. By extrapolation of the slow-reacting pool to time zero, the amount of this pool could be calculated. It is about 65% for PE, 71% for ethanolamine plasmalogens and 82% for PS. Similar results were also obtained with 5 mM TNBS.

To localize the two reacting pools on the microsomal vesicle membranes, the labelling of the aminophospholipids with TNBS was performed at 0°C in order to decrease the permeability of the membranes. The results showed that the conversion of the aminophospholipids into TNP derivatives increased for about 15 min, remained constant between 15 and 30 min and increased once more after 30 min. Between 15 and 30 min, the rate of conversion was of about 32% for PE, 27% for plasmalogens and 18% for PS. These values are in good agreement with those obtained for the fast-reacting pools at 20°C. Since in presence of 5 mM TNBS nearly all aminophospholipids reacted with the probe, the results suggest that the fast-reacting pool represents those aminophospholipids located in the outer leaflet and the slow-reacting pool those situated in the inner one.

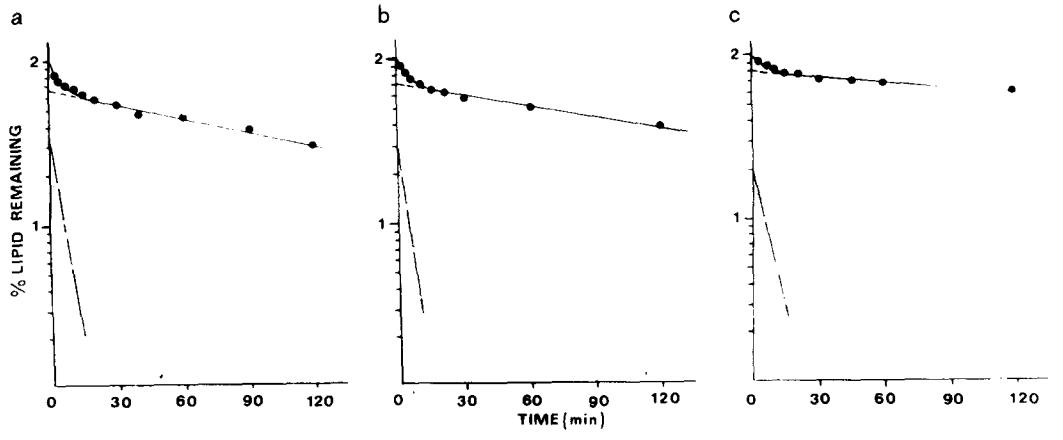

Fig. 2. Time-course for the triphenylation of aminophospholipids of intact chicken-brain microsomes at 20°C with 1.66 mM TNBS. The aminophospholipids remaining are plotted on a logarithmic scale versus time of incubation. Two reacting components are observed. The values of the fast-reacting component was calculated by subtracting the values extrapollated for the slow-reacting component from the total. (a) Phosphatidylethanolamine; (b) ethanolamine plasmalogen; (c) phosphatidylserine. ●——●, slow-reacting component; ○——○, fast-reacting component.

Treatment of iodinated microsomes with TNBS

The enzymatic iodination of microsomes led to the labelling of surface lipids and proteins exclusively [19,20,22,23]. The incubation of these microsomes with TNBS at 20°C showed that the whole iodinated ethanolamine phospholipids has reacted with TNBS in about 7 min, when about 30–35% of the PE have been triphenylated (Fig. 3). Incubation for longer time resulted in an increase of TNP-PE formation. If we assume a random iodination of the ethanolamine phospholipids, these results indicate that (a) TNBS reacted with all molecules of aminophospholipids facing the outer surface, (b) TNBS permeated the microsomal membrane at 20°C and reacted with the aminophospholipids located in the inner leaflet. The permeation is considerably reduced at 0°C and, under the incubation conditions used, more than 30 min are required for the reaction of TNBS with the phospholipids present in the inner leaflet.

Treatment of microsomes with phospholipases

Phospholipases are currently used for the investigation of phospholipid asymmetry in membranes. The choice of the phospholipase is capital, since in such studies the enzymes should hydrolyse the different phospholipids at similar rates, and should digest only the phospholipids located in the outer leaflet of the membrane. Moreover, the hydrolysis should not form holes in the membrane allowing the enzyme to reach the phospholipid present in the inner leaflet. Two different enzymes were assayed, namely *Vipera russeli* phospholipase A_2 and *Cl. welchii* phospholipase C.

(a) Treatment of microsomes with V. russeli phospholipase A_2. The incubation of microsomes with phospholipase A_2 produced a rapid and complete hydrolysis of all phospholipids at similar rates, except for sphingomyelin which was unhydrolysed. Using 0.02 U per incubation, 80%–90% of the different glycerophospholipids were converted into lyso compounds in 30 min, suggesting that the increase of lysophospholipids disrupted the membrane allowing the enzyme to react with the lipids facing the inner side. Therefore this type of phospholipase was not used in further experiments.

(b) Treatment of microsomal vesicles with Cl. welchii phospholipase C. Incubation of microsomes with 1 U phospholipase C led to the hydrolysis of about 50% of the phospholipids in 30 min (Fig. 4) followed by a slight increase thereafter. When the concentration of phospholipase C was increased up to 3 U, about 50% of the phospholipids were hydrolysed in 5 min. Moreover, a slow but signifi-

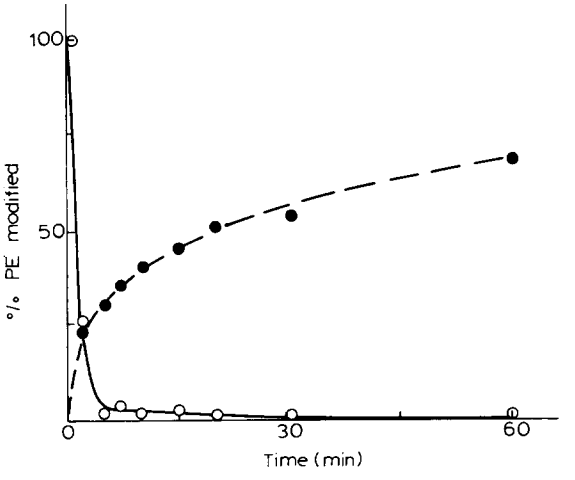

Fig. 3. Time-course for the triphenylation of microsomal PE iodinated with ^{125}I in the external leaflet of intact chicken-brain microsomes. ●-----●, % of PE reacted with TNBS. ○——○, % of ^{125}I-labelled PE remaining. Each point represents the average of two experiments.

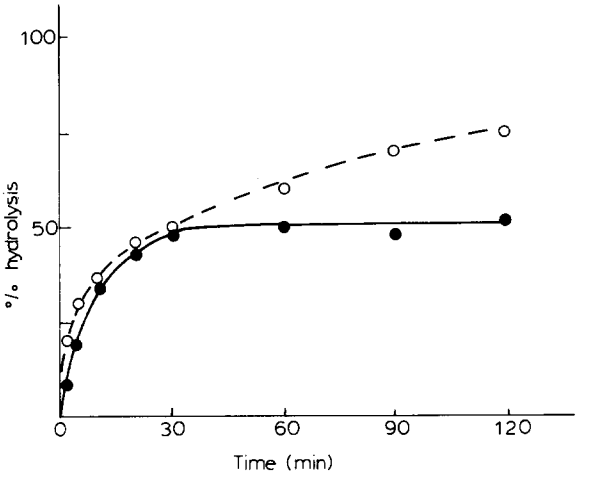

Fig. 4. Hydrolysis of chicken brain microsomal phospholipids with *Cl. welchii* phospholipase C in function of time. Incubation was performed as reported in Material and Methods. Percentage of hydrolysis of total phospholipids in intact microsomes (●——●) and of deoxycholate treated microsomes (○------○). Each point represents an average of three experiments.

cant hydrolysis proceeded at longer time and amounted up to about 70% after 2 h. In the presence of deoxycholate and 1 U of phospholipase C, the hydrolysis proceeded more rapidly, since about 50% of the phospholipids were hydrolysed in 20 min and about 75% after 2 h indicating that when both sides of the membrane were accessible to the phospholipase, the amount of phospholipid hydrolysis is increased (Fig. 4).

Phospholipids extracted from microsomes and dispersed by sonication in the incubation medium were also rapidly hydrolysed. The rate of hydrolysis of total phospholipids reached about 70% after 2 h. Under these conditions, about 75% of PE, 75% SM and 98% of PC were hydrolysed, while PS and PI were not affected (Fig. 5). These observations are in disagreement with those reported in a previous observation [24] and by Higgins and Dawson [11], who observed that phospholipase C hydrolysed PS from liver microsomes. This discrepancy may be due to the physical state of the PS, which may differ in various membrane preparations, depending on their origin and on the procedure of isolation and incubation. Nevertheless, the use of phospholipase C for the study of asymmetry of brain microsomal membranes remains valuable for PC, PE and SM, especially when compared with other methods.

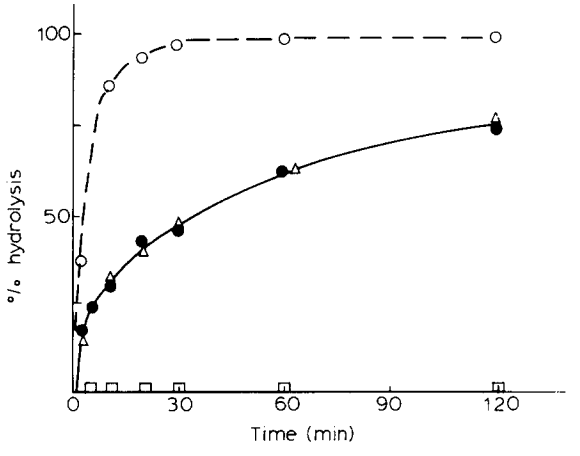

Fig. 5. Hydrolysis of phospholipids extracted from chicken brain microsomes with *Cl. welchii* phospholipase C. Phospholipids were extracted from microsomes and dispersed in the incubation buffer. Aliquots containing 1 μmol phospholipids were incubated with phospholipase as indicated in Material and Methods. Percentage of hydrolysis of phosphatidyl choline (O------O), ethanolamine phospholipids (●———●), sphingomyelin (△———△), phosphatidylserine + phosphatidylinositol (□———□). Each point represents an average of three experiments.

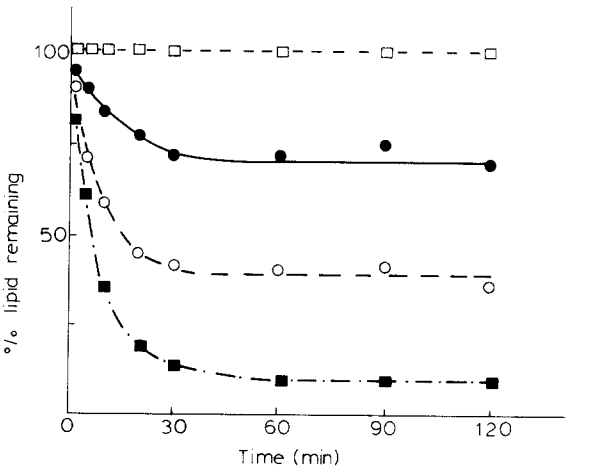

Fig. 6. Hydrolysis of individual phospholipids of intact microsomes with *Cl. welchii* phospholipase C. Intact microsomes were incubated with phospholipase for different times. Lipids were extracted and separated by TLC as reported in material and Methods. Percentage of hydrolysis of phosphatidylcholine (■-·-■), sphingomyelin (O------O), ethanolamine phospholipids (●———●), phosphatidylserine + phosphatidylinositol (□-----□). Each point represents an average of three experiments.

When intact microsomes were incubated with phospholipase C, the loss of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin was rapid for 20–30 min and increased only very slightly thereafter (Fig. 6). Between 30 and 120 min of incubation when about 50% of the total phospholipids were hydrolysed, about 90% of PC, 30% of PE and 60% of SM were digested. The results obtained for PE were in good agreement with those obtained with TNBS.

(c) Vesicular integrity after treatment with phospholipase C. Studies on phospholipid asymmetry of microsomal vesicles using phospholipases are valuable only if during the treatment the microsomes are not disrupted, and therefore the inner side is not accessible to the enzymes. We found, in agreement with the results of Buchanan and Kanfer [25] and Butler and Morell [26], that the mannose-6-phosphatase activity in brain microsomes was too low for the determination of the latency of this enzyme. Therefore the determination of the efflux of [^{14}C]sucrose from microsomal vesicles, which

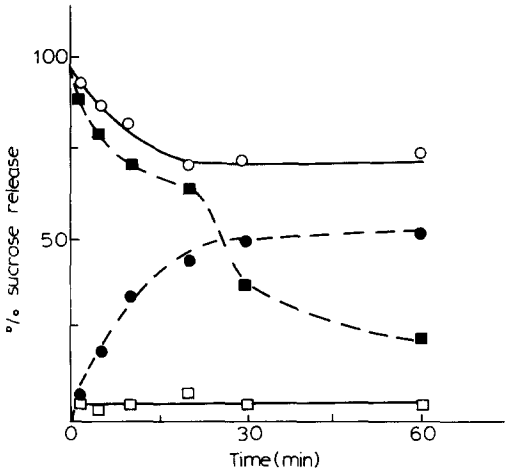

Fig. 7. Effect of *Cl. welchii* phospholipase C on sucrose efflux of chicken-brain microsomes. Microsomes were loaded with [^{14}C]sucrose, treated with phospholipase C as a function of time and the [^{14}C]sucrose released from microsomes was measured as reported in Material and Methods. Percentage of [^{14}C]sucrose release in control microsomes (○——○), in microsomes treated with phospholipase C (■-----■) and in microsomes treated with deoxycholated (□——□). Percentage of hydrolysis of total phospholipids (●-----●).

has been shown to be very slow in liver microsomes [21] was used.

Incubation of microsomes loaded with [^{14}C]sucrose produced a slow efflux of [^{14}C]sucrose as a function of time (Fig. 7). About 25% of the radioactivity present in microsomes was lost in 30 min. Treatment of microsomes with deoxycholate led to a complete loss of [^{14}C]sucrose, indicating complete release of the [^{14}C]sucrose when the vesicles are disrupted. The treatment of microsomes with *Cl. welchii* phospholipase C produced a release of [^{14}C]sucrose at a rate similar to that obtained for control microsomes when less than 50% of the phospholipids are hydrolysed (Fig. 7). A rapid and nearly complete release was observed when more than 50% of the phospholipids were hydrolysed. These results indicated that the microsomal vesicles remained sealed when less than 50% of the phospholipids were hydrolysed. It seems, therefore, likely that the 50% of the microsomal phospholipids hydrolysed by the phospholipase C are present on the external leaflet.

Phospholipid distribution in the two leaflets of the microsomes

Assuming that all external phospholipids of the microsomes were accessible and thus likely to react with TNBS or with *Cl. welchii* phospholipase C, the distribution of the different phospholipids in each leaflet of the bilayer was evaluated (Table I). About 90% of the PC and 60% of SM were located in the outer leaflet and only about 32% of the PE, 29% of the plasmalogen PE and 18% of the PS. Phosphatidylinositol was not hydrolysed with *Cl. welchii* phospholipase C, nor did it react with TNBS. However, since about 50% of the total phospholipids were hydrolysed by phospholipase C, phosphatidylinositol can be tentatively assigned to the inner leaflet of the bilayer.

Discussion

The microsomal membranes used in this study derived predominantly from the endoplasmic reticulum. This fraction was enriched in NADPH cytochrome *c* oxidoreductase [15,27]. Moreover, the activities of CDPcholine: 1,2-diacylglycerol

TABLE I

DISTRIBUTION OF PHOSPHOLIPIDS IN THE INNER AND OUTER LEAFLET OF CHICKEN BRAIN MICROSOMES

Results are expressed as nmol phospholipids per mg microsomal protein. In brackets, the percentage of each phospholipid in the inner and outer leaflet.

Phospholipids	Whole microsomes	Inner leaflet	Outer leaflet
Phosphatidylcholine	241.8	27.1 (11.2)	214.7 (88.8)
Sphingomyelin	40.2	16.1 (40.0)	24.1 (60.0)
Phosphatidylethanolamine	102.0	69.4 (68.0)	32.6 (32.0)
Ethanolamine plasmalogens	120.0	85.2 (71.0)	34.8 (29.0)
Phosphatidylserine	60.0	49.2 (82.0)	10.8 (18.0)
Phosphatidylinositol	36.0	?	?

cholinephosphotransferase, CDPethanolamine: 1,2- diacylglycerol ethanolaminephosphotransferase and the enzymes catalyzing the base exchange reaction (enzymes known to be localized essentially in the endoplasmic reticulum [28]) were present to more than 80% in this fraction [16,29–31]. The microsomal fraction was almost devoid of cytochrome *c* oxidase or succinate oxidoreductase [15,27], indicating that the mitochondrial contamination was very low or nil. The main problem concerned the possible contamination of the endoplasmic reticulum, with plasma membranes. Determination of 5′-nucleotidase and $(Na^+ + K^+)$-ATPase showed a small decrease in their specific activities relative to homogenate [26,27]. These results may indicate the presence of plasma membrane in the microsomal fraction. Subfractionation of the microsomes by centrifugation on a discontinuous gradient has shown that the contamination with plasma membranes did not exceed 20% [26]. However, if the distribution of the phospholipids in the two leaflets of the plasma membranes is different from those in endoplasmic reticulum, then the asymmetric distribution in the microsomal fraction will be underestimated.

Electron microscopy also showed that ribosomes were localized on the outer surface of the vesicles originating from rough endoplasmic reticulum (unreported observations), suggesting that in the microsomal vesicles the outer leaflet corresponds to the cytoplasmic one in situ.

The investigation of the transverse asymmetry of the lipid bilayer of microsomes using chemical and enzymatic reagents requires that: (a) the probe should not permeate through the membrane and should not lyse the cell; (b) the probe should have accessibility to the lipids facing the outer surface and should react quantitatively with them; (c) the distribution of the lipids in both leaflets should not change during the reaction through, for instance, a transbilayer movement. Such transbilayer movements have been reported to occur in biological membranes and can be induced by localization techniques, which may explain the contradictory observations about phospholipids localization in liver microsomes [8]. In brain microsomes, this movement seems to be slow under our experimental conditions, since the study on the distribution of newly synthesized phosphatidylethanolamine in both leaflets after in vitro incorporation of radioactive ethanolamine showed that the transfer of this lipid from the outer to the inner leaflet represented less than 1% per h [32,33]. The difficulty in obtaining a probe which may fulfill all of these prerequisities led us to the use of two different probes to approach the phospholipid distribution in brain microsomes.

The use of TNBS for the investigation of aminophospholipids in chicken brain microsomes showed that at 20°C the reaction with phosphatidylethanolamine, ethanolamine plasmalogens, and phosphatidylserine proceeded following two kinetics, a fast and a slow one. The quantitation of the two pools by the semilogarithmic evaluation of the time-course reaction showed that the fast-reacting pool contains about 35% of the phosphatidylethanolamine, 29% of the ethanolamine plasmalogens and 18% of the phosphatidylserine. When triphenylation was performed at 0°C, a plateau was obtained between 15 and 30 min, when about similar amounts of the different aminophospholipids have reacted. Moreover, the labelling of the external lipids with ^{125}I revealed that all the radioactive ethanolamine phospholipids were trinitrophenylated when about 30–35% of these compounds were converted to trinitrophenyl derivatives. The results suggest that the TNBS permeates the microsomal membrane and that the fast-reacting pool of aminophospholipids is located in the outer leaflet.

The phospholipase C of *Cl. welchii* was used previously by Higgins and Dawson to investigate the distribution of the phospholipids in liver microsomes [11]. The results suggest that this enzyme is a valuable probe for the study of the distribution of PC, PE and SM in brain microsomal-membrane. The investigation of the effect of phospholipase C on the efflux of sucrose showed a nearly complete release of the sugar when 50% of the phospholipids are hydrolysed, indicating that the hydrolysis of 50% of microsomal phospholipids produced a disruption of microsomal vesicles, allowing the phospholipase C to react with phospholipids facing the inner surface and suggest that at least the 50% of the phospholipids which react with the hydrolase are located in the outer leaflet. The analysis of the different phospholipids revealed that about 30% of PE 90% of PC and 60%

of SM were hydrolysed. These results are in agreement with those reported by Higgins and Dawson [11] for liver microsomes. Moreover, the amount of ethanolamine phospholipids accessible to phospholipase C is of the same order of magnitude as that reacting with TNBS according to fast kinetics.

The results of the present work suggest an asymmetric distribution of the phospholipids in chicken brain microsomes. The outer leaflet of the microsomal vesicles contains about 75% of choline phospholipids and about 25% of aminophospholipids, whereas an opposite distribution is observed in the inner leaflet (Table I). Similar results were also reported for synaptosomal plasma membranes by Fontaine et al. [9]. This asymmetric distribution raises the question of which mechanisms set up and maintain the asymmetry in microsomal membranes. The investigation on the intramembrane localization of enzymes involved in the synthesis of ethanolamine and choline phosphoacylglycerols have shown that CDPcholine:1,2-diacylglycerol cholinephosphotransferase, and CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase are differently embedded in the microsomal membrane [14]. Similar results have also been obtained for choline and ethanolamine base-exchange enzymes [25], suggesting that these enzymes may participate in the asymmetric distribution of the phospholipids in brain microsomes. However, the study on the side of synthesis of the different phospholipids revealed that the ethanolamine and choline phospholipids are synthesized on the cytoplasmic side, either through the Kennedy's pathway or by base exchange [26,32,33]. Similar observations have also been reported by Higgins [34], Huston and Higgins [35] and Bell et al. [36] for liver microsomes. It seems, therefore, likely that if the phospholipid synthesizing enzymes may participate in part in the setting up of phospholipid asymmetry in membranes, other mechanisms should be taken into account. Recent in vitro studies on the transbilayer movements of phospholipids have shown that chelation of divalent cations with EDTA facilitates the distribution of the newly synthesized ethanolamine phospholipids in the two microsomal leaflets [33].

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