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ASYMMETRY OF THE SITE OF CHOLINE INCORPORATION INTO PHOSPHATIDYLCHOLINE OF RAT LIVER MICROSOMES

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Summary

[^{14}C]Choline was incorporated into microsomal membranes in vivo, and from CDP-[^{14}C]choline in vitro, and the site of incorporation determined by hydrolysis of the outer leaflet of the membrane bilayer using phospholipase C from *Clostridium welchii*. Labelled phosphatidylcholine was found to be concentrated in the outer leaflet of the membrane bilayer with a specific activity approximately three times that of the inner leaflet. During incorporation of CDP-choline and treatment with phospholipase C the vesicles retained labelled-protein contents indicating that they remained intact. When the microsomes were opened with taurocholate after incorporation of [^{14}C]choline in vivo, the labelled phosphatidylcholine behaved as a single pool. Selective hydrolysis of labelled phosphatidylcholine in intact vesicles is not, therefore, a consequence of specificity of phospholipase C. These results indicate that the phosphatidylcholine of the outer leaflet of the microsomal membrane bilayer is preferentially labelled by the choline-phosphotransferase pathway and that this pool of phospholipid does not equilibrate with that of the inner leaflet.

Introduction

During homogenization, the endoplasmic reticulum of rat hepatocytes fragments to form small vesicles having the same orientation with the cisternae side of the membrane inward and cytoplasmic side outward [1,2]. These vesicles are isolated by differential centrifugation in the microsomal fraction. We have recently demonstrated, using phospholipase C as a membrane probe, that there is an asymmetric distribution of phospholipids across the bilayer of microsomes prepared from rat liver [3]. These membranes have the enzymes necessary for the synthesis of phospholipids and, therefore, provide a unique system in which to study the mechanism by which this asymmetry is produced. Phosphatidyl-

choline accounts for approximately half of the membrane phospholipid and 70—75% of this lipid is in the outer leaflet of the membrane bilayer accounting for 85% of this leaflet [3]. The main pathway for synthesis of phosphatidylcholine in rat liver involves a final step, catalysed by choline-phosphotransferase (EC 2.7.2.8), in which phosphorylcholine is transferred from CDP-choline to diglyceride [4,5]. It is possible, therefore, using CDP-[*methyl*-^{14}C]-choline in vitro, or [*methyl*-^{14}C]choline in vivo, to label the membrane phosphatidylcholine, and, using phospholipase C to hydrolyse the phospholipid of the outer leaflet, and thus determine the site of incorporation of the labelled precursor.

Methods

Preparation of microsomes. Livers of male Sprague-Dawley rats (150—200 g) were removed and homogenized in 0.25 M sucrose to yield 20% homogenates. These were centrifuged at 12 500 rev./min (10 000 $\times g_{av}$) for 20 min in the 40 rotor of the Beckman L65 centrifuge. The supernatant was then centrifuged at 40 000 rev./min (105 000 $\times g_{av}$) for 45 min. The pellet was resuspended in the appropriate buffer and used without storage. Microsomes prepared in this way have very low contamination with either plasma membrane or Golgi membranes [6].

Labelling of phosphatidylcholine of microsomes. In vivo [^{14}C]choline-[*methyl*-^{14}C]choline chloride, (spec. act. 40 μCi/μM), The Radiochemical Centre, Amersham, (5 μCi/100 g body weight), was injected intraperitoneally 3 h prior to sacrifice and preparation of microsomes.

In vitro microsomes (approx. 10 mg protein) were incubated with CDP [^{14}C]choline (cytidine-5′-diphospho[*methyl*-^{14}C]choline, The Radiochemical Centre, Amersham) (0.8 mM, spec. act. 1 μCi/μM) in 0.05 M phosphosphate buffer pH 7.4 containing 0.87% NaCl, 1.0 mM EGTA and 15 mM $MgCl_2$.

Treatment of microsomes with phospholipase C. Incorporation of [^{14}C]-phosphorylcholine into phosphatidylcholine of microsomes in vitro was stopped by addition of $CaCl_2$ to give a final concentration of 4 mM. Phospholipase C (Type 1 from *Clostridium welchii*, Sigma Chemical Co.) was added to give a final concentration of 10 U/ml, and the microsomes incubated for 15 min. A higher phospholipase C concentration compared with our previous investigations [3] was used to hydrolyse microsomal phospholipid more rapidly and minimise potential movement of labelled phospholipids. Hydrolysis reached a final level within 5 min. The longer incubation was used to ensure that equilibrium was achieved.

Microsomes labelled in vivo were suspended in the same buffer, or in 0.87% NaCl 4 mM $CaCl_2$ adjusted to pH 7.4 with sodium bicarbonate [3], and treated with phospholipase C as above. Similar results were obtained with either buffer.

Extraction and separation of phospholipids. After incubation, with and without phospholipase C, the microsomal lipids were extracted with chloroform/methanol, 2/1 (20 vols.); the phases were separated by addition of 0.05 M CaCl (5 vols.) and aliquots of the lower phase were taken to determine hydrolysis of labelled phosphatidylcholine and hydrolysis of total phospholipid. The solvent was removed, the lipids dissolved in chloroform methanol

(1/1) and the phospholipids separated on thin layers of silica gel (Merck 60F 254) using a one dimensional system, chloroform/methanol/glacial acetic acid/water (60/50/1/4). In some experiments the phosphatidylcholine-containing spots were removed, extracted with chloroform/methanol (1/1) and the specific activity of the phospholipid determined. In other experiments, each phospholipid-containing spot was removed and the extent of hydrolysis of each determined.

Lipid phosphorus was determined by the method of Bartlett [7] after digestion of the phospholipid with 1.5 ml of 72% perchloric acid. Protein was determined by the method of Lowry et al. [8].

Results

Incorporation of [^{14}C]phosphorylcholine into microsomal phospholipid

When microsomes were incubated with CDP-[^{14}C]choline, the phosphoryl choline moiety was incorporated rapidly into the microsomal phospholipid and reached a final level within fifteen minutes of incubation. If the microsomes were initially passed through a French pressure cell at 20 000 lb/inch^2, to open the vesicles [3] neither the rate nor the final level of incorporation of choline was increased indicating that CDP-choline can gain access to the enzyme and the membrane-bound diglyceride of intact microsomes. The use of detergents to open vesicles was avoided, as these may potentially affect the enzyme activity. However, Coleman and Bell [9] investigated the effect of taurocholate, deoxycholate and Tween 20, at concentrations which open the microsomal vesicles, on the enzymes involved in phospholipid synthesis. They also concluded that choline-phosphotransferase was not latent in microsomal vesicles.

Site of incorporation of [^{14}C]phosphorylcholine into microsomal phosphatidylcholine in vitro

After incorporation of [^{14}C]phosphorylcholine, from CDP-choline, into microsomal phospholipids for 2 min hydrolysis of total phospholipids was approx. 50%, as in previous experiments (Table I). However, hydrolysis of labelled phosphatidylcholine was almost 90%, suggesting that most of the newly incorporated choline was in the outer leaflet of the membrane bilayer. The specific activity of the total membrane phosphatidylcholine was 2.5 times that of inner leaflet of the bilayer and, by calculation, the specific activity of the outer leaflet was more than three times that of the inner leaflet.

A similar labelling pattern was found at different times when incorporation of choline was allowed to proceed up to 120 min. When unlabelled CDP-choline at ten times the concentration of the labelled CDP-choline was added to the incubation medium after 2 min, and incubation continued for a further 120 min, the hydrolysed and relative specific activities of the phosphatidylcholine pools were unchanged (Table II). It is not possible therefore to chase the labelled phosphatidylcholine from the outer to the inner leaflet, although synthesis of phosphatidylcholine continues after addition of unlabelled CDP-choline.

TABLE I

AVAILABILITY OF MICROSOMAL PHOSPHATIDYLCHOLINE FOR HYDROLYSIS BY PHOSPHOLIPASE C

Microsomes were labelled with CDP-[^{14}C]choline or [^{14}C]choline in vitro or in vivo as described in Methods. These were incubated with phospholipase C, the phospholipids extracted and the % hydrolysis of total phospholipid, labelled phospholipid and the specific activity of the phosphatidylcholine determined as described. The results tabulated are averages of two determinations on one preparation of microsomes. The experiments were repreated three times with the same results; however, as the specific activity of the phosphatidylcholine varied between experiments only data from one experiment is given.

	Label incorporated	
	in vivo	in vitro
Hydrolysis (%)		
Total phospholipid	51.2	53.1
Labelled phospholipid	87.0	89.3
Spec. act. (cpm/μmol)		
Total phosphatidylcholine	6646	6992
Unhydrolysed phosphatidylcholine	3621	2705
Hydrolysed phosphatidylcholine *	7654	8421

* Calculated from hydrolysis of total phosphatidylcholine.

Site of incorporation of [^{14}C]choline into microsomal phosphatidylcholine in vivo

It is possible that mechanisms for transfer of phospholipid across the microsomal membrane bilayer are disrupted in isolated microsomes, or that the results in vitro are due to restriction of diglyceride, one substrate for cholinephosphotransferase, to the outer leaflet of the membrane bilayer. The site of incorporation of choline in vivo, when physiological mechanisms of transfer or provision of substrates must be operative, was therefore investigated. When choline was injected intraperitoneally and microsomes isolated and treated with phospholipase C, the labelling pattern was the same as that in vitro. 87% of the

TABLE II

EFFECT OF ADDITION OF UNLABELLED CDP-CHOLINE ON THE SITE OF INCORPORATION OF CDP-[^{14}C]CHOLINE

Microsomes were incubated with CDP-[^{14}C]choline as described in Methods. Incorporation into one group of samples was stopped after 2 min and unlabelled CDP-choline was added to a second group, which was incubated for a further 120 min. Both groups of sample were treated with phospholipase C and the hydrolysis of total phospholipid, labelled phospholipid and the specific activity of phosphatidylcholine were determined as described. Results given are the average of two determinations.

	Time of incubation	
	2 min	120 min
Hydrolysis (%)		
Total phospholipid	49	52
Labelled phospholipid	86	84
Spec. act. (cpm/μmol)		
Total phosphatidylcholine	911	1526
Unhydrolysed phosphatidylcholine	457	780

labelled phosphatidylcholine was hydrolysed by phospholipase C and the specific activity of the total phosphatidylcholine was twice that of the unhydrolysed pool (Table I). Choline is therefore incorporated preferentially into the phosphatidylcholine of the outer leaflet of the bilayer in vivo and in vitro and does not cross the bilayers to equilibrate with the phosphatidylcholine of the inner leaflet.

Integrity of microsomal vesicles

The secretory protein contents of both rough and smooth microsomes are labelled by injection of [^{3}H]leucine into rats 30′ prior to sacrifice [10]. The labelled contents can be released by opening the vesicles either mechanically or with low concentrations of detergents [3,10]. Loss of labelled contents from microsomes incubated without phospholipase C was 9.9% of the total label (Table III). This loss was similar to that from microsomes washed with ice-cold 0.25 M sucrose, and may represent loss of adsorbed cytoplasmic protein and/or leakage from a small proportion of the vesicles. On treatment of the same preparations with phospholipase C, 11.3% of the labelled contents were lost (Table III). The vesicles therefore retain their contents both during incubation with CDP-choline and after treatment with phospholipase C.

An alternative method of measuring the integrity of microsomal vesicles is to demonstrate that mannose-6-phosphatase is latent throughout experimental manipulation [9,11]. Over 90% of the mannose-6-phosphatase of our microsomal preparations was only expressed when the vesicles were opened with 0.4% taurocholate. Phospholipase C treated microsomes exhibited the same activity as unopened vesicles, suggesting that the enzyme remains latent. However, treatment of microsomal vesicles with phospholipase C followed by taurocholate resulted in complete inhibition of mannose-6-phosphatase. Latency of this enzyme is not therefore a completely satisfactory method for the demonstration of membrane integrity in phospholipase treated microsomes.

Hydrolysis of phosphatidylcholine of microsomal vesicles opened with taurocholate

In order to open the vesicles, microsomes, labelled in vivo, were treated with

TABLE III

LOSS OF LABELLED CONTENTS OF MICROSOMAL VESICLES TREATED WITH PHOSPHOLIPASE C OR EDTA

[^{3}H]LEUCINE (5 μCi/100 g) was injected intraperitoneally into rats 30 min prior to killing and preparation of total liver microsomes. The microsomes were suspended in the buffer for phospholipase treatment. To one group of samples were added 10 vols. of 10 mM EDTA. A second group of samples was treated with phospholipase C as described in Methods. All samples were centrifuged 40 000 rev./min for 45 min and aliquots of the supernatant taken and counted. Loss of contents is expressed as counts appearing in the medium as a % of the counts in the original sample. Results are means of four determinations ± standard deviation.

	Loss of contents (%)	
	Phospholipase C	EDTA
Control	9.9 ± 0.5	9.61 ± 0.9
Experimental	11.3 ± 0.3	15.23 ± 2.1

0.4% taurocholate followed by phospholipase C. Hydrolysis of total phospholipid was increased and the specific activity of the phosphatidylcholine remaining unhydrolysed was similar to that of the total phosphatidylcholine (Table IV). Selective hydrolysis of labelled phosphatidylcholine in unopened vesicles is not therefore a consequence of specificity of phospholipase C. In intact vesicles, there are two pools of phosphatidylcholine one of which is preferentially labelled by the CDP-choline pathway and is also preferentially hydrolysed by phospholipase C.

Taurocholate may open microsomal vesicles and allow access of phospholipase C to both sides of the membrane, and/or, may disrupt lipid-protein or lipid-lipid interactions so that phospholipids normally sequestered become available for hydrolysis. It is difficult to distinguish unequivocally between these two possibilities, as any experiment designed to make the membrane permeable to phospholipase C may also release sequestered phospholipids. However, it is well established that taurocholate opens microsomal vesicles [11]. It is probable, therefore, that it also allows phospholipase C to gain access to phospholipids of the inner leaflet of the membrane bilayer. This is also suggested by increased hydrolysis of phospholipid of taurocholate treated microsomes (Table IV). Thus, labelled phosphatidylcholine of the outer leaflet of the bilayer and unlabelled phosphatidylcholine of the inner leaflet of the bilayer are not distinguished by phospholipase C, when the vesicles are opened by taurocholate.

Effect of EDTA on the distribution of labelled phosphatidylcholine

In early experiments, 10 mM EDTA was used to halt incorporation of choline in vitro. The labelled phosphatidylcholine was found to be concentrated in the inner leaflet of the bilayer, in contrast to the opposite finding when incorporation was stopped by calcium ions and phospholipase C. When choline was incorporated in vivo, and the microsomes resuspended in 10 mM EDTA and centrifuged prior to treatment with phospholipase C, a similar high level of labelling of the inner leaflet phosphatidylcholine was found, although without EDTA treatment the label was concentrated in the outer leaflet of the bilayer of the same microsomes (Table V). EDTA treatment therefore appears to shift the labelled phosphatidylcholine across the microsomal membrane. The

TABLE IV

AVAILABILITY OF PHOSPHATIDYLCHOLINE OF TAUROCHOLATE-TREATED MICROSOMES TO PHOSPHOLIPASE C

Microsomes labelled with [^{14}C]choline in vivo were suspended in 0.25 M sucrose containing 0.4% taurocholate for 30 min at 0°C. The microsomes were isolated by centrifugation and treated with phospholipase C as described in Methods. Results are means of four determinations ± S.D. standard deviations.

	Hydrolysis (%)	Spec. act. (cpm/μmol)
Phospholipid	78.0 ± 3.7%	—
Labelled phospholipid	88.7 ± 1.6%	—
Total phosphatidylcholine	—	24 461 ± 5315
Unhydrolysed phosphatidylcholine	—	22 882 ± 2487

TABLE V

EFFECT OF EDTA TREATMENT ON THE HYDROLYSIS OF PHOSPHOLIPIDS OF MICROSOMES BY PHOSPHOLIPASE C

Microsomes, labelled with [^{14}C]choline in vivo, were treated with phospholipase C. Hydrolysis of total phospholipids, labelled phospholipids and individual phospholipids were determined as described in Methods. In A, microsomes were suspended in the buffer used for phospholipase treatment, 10 vols. of 10 mM EDTA in the same buffer were added and the microsomes isolated by centrifugation. The pellets were washed and resuspended in the same buffer for phospholipase C treatment. In B, total microsomes were prepared as described by Sundler et al [13] using 0.25 M sucrose containing 1 mM EDTA as a homogenizing medium. The pellets were rinsed and resuspended in buffer for phospholipase C treatment.

Hydrolysis (%)	A	B
Total phospholipid	49.8 ± 2.5	54.7 ± 7.6
Labelled phospholipid	65.6 ± 0.2	87.2 ± 1.7
Phosphatidylcholine	49.2 ± 1.7	72.8 ± 3.7
Phosphatidylethanolamine	44.0 ± 15	37.9 ± 5.9
Sphingomyelin	35.0 ± 18	70.3 ± 56
Phosphatidylserine	18 ± 2	35.7 ± 10

distribution of the individual phospholipids on either side of the bilayer was also markedly altered in EDTA treated microsomes (Table V). The phospholipids were more evenly distributed and showed more variation than in untreated microsomes with large standard deviations about the mean. The EDTA treatment does not cause a large increase in loss of vesicular contents (Table III) therefore the change in distribution of phospholipid occurs without major loss of microsomal integrity. These observations emphasise that care should be taken in treatment of microsomes in studies on the distribution of phospholipids. Discrepancies between results of different investigators may possibly be due to such treatment.

Sundler et al., [13] have also investigated the distribution of phospholipids across microsomal membranes, using phospholipase A as a probe. They concluded that there was no asymmetry of phospholipids in these membranes. As these investigators used 1.0 mM EDTA in 0.25 M sucrose as a homogenizing medium, we also prepared microsomes using their methods. The site of incorporation of [^{14}C]choline in vivo and the distribution of phospholipids across the bilayer was similar to those found above and in our previous investigations [3] (Table V). The lower concentration of EDTA does not shift the labelled phosphatidylcholine, therefore; and, in our hands, microsomes prepared as described by Sundler et al. exhibit the same asymmetry as microsomes prepared by our methods.

Discussion

The present results indicate that both in vivo and in vitro choline is incorporated preferentially into phosphatidylcholine of the outer leaflet of the microsomal membrane bilayer. This conclusion is based on the assumption that labelled phosphatidylcholine hydrolysed by phospholipase C is in the outer leaflet of the bilayer. Several places of evidence support this assumption. As in our previous investigations [3] hydrolysis of membrane phospholipids reached equilibrium at approximately 50%, although the phospholipase C treated

microsomes remain intact. Approx. 90% of the labelled phosphatidylcholine was hydrolysed and the specific activity of this pool was two and a half to three times that remaining unhydrolysed. When the vesicles were opened with taurocholate, however, the phosphatidylcholine of the microsomes behaved as a single pool.

After labelling in vitro the specific activity of phosphatidylcholine of the outer leaflet of the microsome bilayer was greater than that of the inner leaflet at all times investigated. Nor was it possible to chase the label from the outer leaflet by addition of excess unlabelled CDP-choline. After labelling in vivo, when any physiological mechanism for transfer of phospholipids across the bilayer would be operative, the outer leaflet was still preferentially labelled. Phosphatidylcholine synthesised by the CDP-choline pathway appears therefore to be formed and remain on the outside of the bilayer. Approximately a quarter of the phosphatidylcholine of microsomes is in the inner leaflet of the bilayer [3]. An alternative pathway must therefore give rise to the inner pool of this phospholipid. The second major pathway for synthesis of phosphatidylcholine in rat liver is by methylation of phosphatidylethanothamine by *S*-adenosyl methionine [14]. The site of this activity is presently under investigation in this laboratory.

Coleman and Bell [9] have investigated the site of enzymes involved in synthesis of phospholipids in rat liver microsomes using proteases to hydrolyse enzymes accessible from the outer surface of the membrane. More than 90% of the choline-phosphotransferase activity of microsomes was hydrolysed by chymotrypsin in vesicles, which remained intact, judged by latency of mannose-6-phosphatase. These results are consistent with ours and indicate that incorporation of choline is into the outer leaflet of the microsomal bilayer.

There have been two other studies of the distribution of phospholipids in the microsomal membrane. Nillson and Dallner [12] used phospholipase A_2 (*Naja naja* or *Viperai russelli*) and concluded that phosphatidylethanolamine is located in the outer leaflet of the bilayer and phosphatidylcholine is evenly distributed. Sundler et al. [13] used phospholipase A_2 (*Naja naja* or *Crotalus*) and concluded that this enzyme opens microsomal vesicles, but that before these open the hydrolysis is not selective, indicating that the membrane is not asymmetric with respect to phospholipids. The reasons for the discrepancies between these observations and ours are not clear. We found that phospholipase A_2 (bee venom or *Naja naja*) hydrolysed microsomal phospholipids to completion, and, immediately caused leakage of contents so that it was not possible to allow hydrolysis to proceed and be sure that the vesicles were intact [3]. We have prepared microsomes by Sundler's method and have obtained similar results, with phospholipase C, to those reported previously. We have also used phospholipase D as a probe for microsomal asymmetry and found the same distribution of phospholipids to that reported here and previously (Higgins, J.A., unpublished observations). Sundler et al. [15] have also investigated the use of phospholipases as probes for asymmetry, using model phospholipid vesicles. They concluded that phospholipase C caused extensive hydrolysis of both sides of the bilayer. This is in conflict with our observations. However, model membranes without protein and biological membranes are sufficiently different that the same results might not necessarily be expected with both.

Zilversmit and Hughes have demonstrated that 82—85% of the phosphatidylcholine labelled in vivo with [*methyl*-^{14}C]choline is exchangable by phospholipid-exchange protein [16]. This observation is consistent with our results in which 84—89% of the phosphatidylcholine, labelled by choline in vivo, is available for hydrolysis by phospholipase C. This however, is due to selective hydrolysis of the labelled phospholipid and not to hydrolysis of 85% of the total phosphatidylcholine. Thus, the extensive exchangability of choline-labelled phosphatidylcholine may be a consequence of asymmetry labelling by this pathway. Zilversmit and Hughes also labelled microsomes with ^{32}P in vivo in normal rats and hepatectomized rats in which rapid growth of liver occurs. 85—95% of the phospholipids, including phosphatidylcholine, were exchangable in these microsomes. Similarly, van den Besselaar et al. [17] demonstrated that 90—95% of the phosphatidylcholine of rat liver microsomes labelled in vivo with ^{32}P was exchangable by phospholipid exchange protein. As the experimental conditions of both groups were consistent with uniform labelling of phospholipids, these results suggest that a high proportion of the phospholipids, including phosphatidylcholine, of the inner leaflet can traverse the membrane bilayer, and presumably are replaced by phospholipids from the outer leaflet. We have no explanation for the fact that randomization of phosphatidylcholine labelled by choline, does not occur in our studies. It may be that this pool of phospholipid, as it is already on the outer leaflet of the membrane, differs from the phospholipids of the inner leaflet. Further investigations of the site of synthesis of all the membrane phospholipids are necessary before these discrepancies can be resolved.

Asymmetry of the phospholipid bilayer of microsomal membranes may be produced by two mechanisms, (a) the phospholipid may be synthesised in situ by asymmetrically located enzymes, or, (b) the phospholipids may be synthesized on one side of the membrane and traverse the membrane by some controlled mechanism. The present results suggest that the former mechanism is operative for phosphatidylcholine of the outer leaflet of the membrane and that this is synthesized by the CDP-choline diglyceride-choline-phosphotransferase pathway.

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