RESEARCH COMMUNICATION

Novel evidence for an ecto-phospholipid methyltransferase in isolated rat hepatocytes

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Phospholipids of isolated rat hepatocytes were labelled by preincubation with either 2 μ M [*methyl*-¹⁴C]*S*-adenosylmethionine (AdoMet) or 2 μ M [*methyl*-¹⁴C]methionine. Subsequent addition of phospholipase C to the suspension removed 95 % of the radioactivity from phospholipids methylated by [*methyl*-¹⁴C]AdoMet within a few minutes, but was without effect on phospholipids methylated by [*methyl*-¹⁴C]methionine radioactivity from the latter could, nevertheless, be removed by phospholipase C after permeabilization of the cells with digitonin. The results clearly show that the methyl group of exogenous

AdoMet, contrary to that of methionine, is transferred on to phospholipids located on the external face of the plasma membrane. Accordingly, pretreatment of isolated hepatocytes with trypsin prevented the methylation of phospholipids from exogenous AdoMet by 60–80 %, whereas it was almost without effect when exogenous methionine was the methyl donor. Our data corroborate previous work [Bontemps and Van den Berghe (1997) *Biochem. J.* 327, 383–389], which indicated that AdoMet methylates hepatocyte phospholipids without penetrating the cells.

INTRODUCTION

Phosphatidylcholine (PC), the major cellular phospholipid, can be synthesized either by the CDP-choline pathway or by sequential methylation of phosphatidylethanolamine by phosphatidylethanolamine methyltransferase (PEMT), an enzyme which uses *S*-adenosylmethionine (AdoMet) as the methyl donor. Liver is the only tissue in which PEMT has a significant activity [1] and, hence, could contribute to PC synthesis [2].

Phospholipid methylation can be studied in isolated hepatocytes by incubation with [*methyl*-¹⁴C]methionine, which easily penetrates these cells where it is converted into [*methyl*-¹⁴C]AdoMet by AdoMet synthetase. Although penetration of AdoMet into liver cells is considered highly unlikely [3,4], two studies have, nevertheless, shown that incubation of isolated rat hepatocytes with exogenous [*methyl*-¹⁴C]AdoMet results in labelling of cellular phospholipids [5,6]. Both studies have led to the conclusion that these phospholipids are located on the external face of the plasma membrane. This conclusion is based mainly on experiments with subcellular fractions of liver cells [5], and on the observation that methylation of phospholipids from exogenous AdoMet, contrary to that from methionine, can be inhibited by exogenous *S*-adenosylhomocysteine [6], a metabolite which does not cross the plasma membrane [3,7]. Experiments described in this paper present novel, additional data in favour of this conclusion.

EXPERIMENTAL

Materials

[*Methyl*-¹⁴C]AdoMet and [*methyl*-¹⁴C]methionine (each at 55 mCi/mmol) were purchased from Amersham International

(Little Chalfont, Bucks., U.K.). Collagenase A from *Clostridium histolyticum* and phospholipase C (2 mg/ml) from *Bacillus cereus* were from Boehringer (Mannheim, Germany). Trypsin from bovine pancreas, trypsin inhibitor from soybean and serum albumin (fraction V) were from Sigma (St. Louis, MO, U.S.A.). AdoMet, in the stable form of butane-1,4-disulphonate, was from Knoll Farmaceutici (Liscate, Italy). Other compounds were the purest grade commercially available.

Preparation and incubation of isolated hepatocytes

Rat hepatocytes were isolated from fed, male Wistar rats as described previously [8], suspended in Krebs–Ringer bicarbonate buffer supplemented with 1 % (w/v) of dialysed BSA and 10 mM glucose, and gassed with O₂/CO₂ (19:1). Cells were diluted to a final concentration of 100 $\pm$ 10 mg/ml of cells per ml (wet weight) and incubated in 20-ml glass vials in a water-bath at 37 °C with a shaking frequency of 100 min⁻¹. Cells were preincubated for before starting the experiments. [*methyl*-¹⁴C]AdoMet or [*methyl*-¹⁴C]methionine (0.25 μ Ci/ml) was added to a concentration of 2 nmol/ml. Cell integrity was assessed by measuring lactate dehydrogenase (LDH) activity in the extracellular medium by the method of Vassault [9]. Activity reached 5–10 % of the total LDH activity at the beginning of the incubation period, and was 15 % of total LDH activity at the end of the incubation, in control conditions. The release of LDH activity into the extracellular medium was not influenced by the addition of phospholipase C at the concentration tested, but was increased approx. 2-fold in the presence of trypsin (0.2 mg/ml) as described in the Results section.

Trypsin, when used, was added 15 min before AdoMet or methionine. The reaction was stopped by the addition of soybean trypsin inhibitor (0.5 mg/ml) together with AdoMet or methionine. Controls were run with trypsin inhibitor alone.

Abbreviations used: AdoMet, *S*-adenosylmethionine; LDH, lactate dehydrogenase; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine *N*-methyltransferase.

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Preparation of samples and analyses

In experiments with phospholipase C, analyses were performed on samples of the whole-cell suspension but, where trypsin was used, cells were separated from the extracellular medium to avoid contamination from cells damaged by the addition of trypsin. In the first case, 300 μ l aliquots of the cell suspension were added to 75 μ l of ice-cold 1 M HClO₄; in the second case, 300 μ l aliquots of the cell suspension were transferred to 1.5-ml microcentrifuge tubes containing 500 μ l of silicone AR 200 ($d = 1.04$) layered on to 300 μ l of ice-cold 1 M ClHO₄. The acid-insoluble pellet obtained by centrifugation (10000 g for 30 s) was used to determine the radioactivity incorporated into phospholipids, as described previously [6]. The supernatant was used for the measurement of methylated acid-soluble metabolites by HPLC [10]. [Methyl-¹⁴C]phosphocholine was identified by TLC as described previously [11].

Unless indicated otherwise, the results shown are representative of at least three experiments which gave similar results.

RESULTS AND DISCUSSION

Effect of phospholipase C

The conclusion that the addition of exogenous AdoMet to isolated hepatocyte suspensions resulted in methylation of phospholipids located on the outer surface of the plasma membrane [5,6], led us to investigate the topology of this methylation more thoroughly. For this purpose, phospholipase C was used, as described previously with erythrocyte membranes [12]. Exogenous phospholipase C, because of its inability to penetrate cells, would act only on phospholipids located on the outer side of the cell membrane. Hence, it could decrease the radioactivity of phospholipids in hepatocytes prelabelled by incubation with [methyl-¹⁴C]AdoMet. In accordance with this hypothesis, phospholipase C rapidly removed radioactivity from phospholipids in hepatocytes which had been preincubated for 30 min in the presence of 2 μ M [methyl-¹⁴C]AdoMet (Figure 1A). The effect was dose-dependent and was maximal in the presence of phospholipase C at a concentration of 1–2 μ g/ml, a concentration which removed more than 90 % of phospholipid radioactivity. In parallel, phospholipase C induced the release of an acid-soluble product (Figure 1B), which was detected by radio-HPLC and identified by TLC as phosphocholine. Phosphocholine is the product of the action of phospholipase C on PC, which, as shown previously [6], is the major phospholipid produced during incubation with exogenous AdoMet.

As stressed by Op den Kamp [13], for the localization of an outer membrane lipid it should be shown that the enzyme or other agent used for this purpose does not act on lipids of the inner membrane, and thus, in our experiments, on intracellular phospholipids. Intracellular phospholipids can be methylated by preincubation with methionine, which easily enters cells where it is converted into AdoMet by AdoMet synthetase. The effect of phospholipase C on phospholipids methylated with either [methyl-¹⁴C]methionine or [methyl-¹⁴C]AdoMet are compared in Figure 2. It is clear that phospholipids methylated with methionine, contrary to phospholipids methylated with AdoMet, are resistant to the action of phospholipase C. This result proves that intracellular phospholipids are inaccessible to exogenous phospholipase C. However, they should become accessible to the action of the enzyme after permeabilization of the cells. Accordingly, as also shown in Figure 2, when digitonin was added to the cell suspension as a permeabilizing agent, phospholipase C, which was without effect on phospholipids methylated with methionine in unpermeabilized cells, rapidly removed 90 % of

Figure 1 Dose-effect of phospholipase C on phospholipids methylated from [methyl-¹⁴C]AdoMet

Hepatocytes were incubated for 30 min in the presence of 2 μ M [methyl-¹⁴C]AdoMet before the addition of various concentrations of phospholipase C, as indicated. (A) [methyl-¹⁴C]Phospholipids and (B) acid-soluble [methyl-¹⁴C]products were measured. At zero time, radioactivity incorporated into phospholipids represented 36 % of the initial radioactivity of [methyl-¹⁴C]AdoMet.

the radioactivity. In hepatocytes preincubated with AdoMet, the addition of digitonin resulted in almost complete removal of the residual radioactivity of the phospholipids by phospholipase C. We can therefore conclude that less than 5 % of the phospholipids methylated from exogenous AdoMet are located intracellularly.

Effect of trypsin

The transfer of the methyl group of exogenous AdoMet to phospholipids located on the external face of the plasma membrane implies the existence of an ecto-phospholipid methyltransferase. Incubation of isolated hepatocytes with a proteolytic should destroy this activity and prevent phospholipid methylation from exogenous AdoMet. The effect of preincubation of hepatocytes for 15 min with various concentrations of trypsin on phospholipid methylation from exogenous AdoMet, when compared with methionine, is depicted in Figure 3. Phospholipid methylation with exogenous AdoMet appeared to be much more sensitive to the action of trypsin. Methylation was decreased by more than 60 % at a trypsin concentration of 0.05 mg/ml, when compared with less than 10 % for phospholipid methylation from methionine. The latter, as well as a minor part of the inhibitory effect of trypsin observed on phospholipid methylation

Figure 2 Comparison of the effect of phospholipase C on phospholipids methylated from AdoMet or methionine

Hepatocytes were incubated for 30 min in the presence of 2 μ M [$methyl-^{14}C$]AdoMet (circles) or 2 μ M [$methyl-^{14}C$]methionine (Met) (squares) before the addition of phospholipase C (2 μ g/ml). Digitonin (200 μ g/ml) was added (closed symbols) 15 min after phospholipase C. At zero time, radioactivity incorporated into phospholipids represented 29% of the initial radioactivity of [$methyl-^{14}C$]AdoMet and 24% of initial radioactivity of [$methyl-^{14}C$]methionine.

Figure 3 Effect of preincubation with trypsin on phospholipid methylation from AdoMet or methionine

Hepatocytes were incubated for 15 min in the presence of the indicated concentrations of trypsin before the addition of 2 μ M [$methyl-^{14}C$]methionine (Met) or 2 μ M [$methyl-^{14}C$]AdoMet. Radioactivity was measured 30 min after the addition of the methylated substrates. Radioactivity incorporated in phospholipids in the absence of trypsin represented $26 \pm 4\%$ of the initial radioactivity of [$methyl-^{14}C$]AdoMet or $28 \pm 4\%$ of the initial radioactivity of [$methyl-^{14}C$]methionine. Results shown are the means $\pm$ S.E.M. ($n = 3$).

in the presence of AdoMet, could be explained by a damaging effect of the enzyme on cell viability. Extracellular LDH activity, measured at the end of the incubation, was $21.5 \pm 2.8\%$ (means $\pm$ S.E.M.) of total LDH in the presence of trypsin (0.2 mg/ml), versus $9.9 \pm 1.6\%$ in control conditions ($n = 6$). Nevertheless, the results presented in Figure 3 provide convincing evidence that the active site of the methyltransferase, which uses exogenous AdoMet, is located on the external side of the plasma

membrane. Similar experiments, performed with erythrocyte ghosts [12] and with microsomal vesicles of rat liver [14,15], to determine the orientation of phospholipid methyltransferase have shown that trypsin completely destroyed the activity of the exposed enzyme. It should be mentioned that the results of the present study were obtained with 5–10-fold lower concentrations of trypsin than those used in the studies cited above. The concentrations used by the latter were very toxic to hepatocytes, shown by the extensive ($> 50\%$) release of total LDH activity in to the extracellular medium, and therefore unusable in experiments with intact cells.

Conclusion

Taken together, our results demonstrate that, in isolated rat hepatocytes, the phospholipid methyltransferase(s) that use exogenous AdoMet and those that utilize intracellular AdoMet produced from methionine and ATP by AdoMet synthetase, have different locations. Two immunologically distinct PEMTs have been identified in rat liver; PEMT1, mainly located on the endoplasmic reticulum and PEMT2, found exclusively on a mitochondrial-associated membrane fraction [16,17]. To our knowledge, no PEMT activity has ever been purified from rat liver plasma membranes. The results presented here should stimulate a search for this ectoactivity. It is worth mentioning that our experiments were performed with micromolar concentrations of exogenous AdoMet, which are more than one order of magnitude lower than those measured inside hepatocytes [6] and comparable with those achieved in plasma after its therapeutical administration as an hepatoprotective agent [18–20]. The presence of a PEMT activity on the outer surface of liver cells could thus result in the utilization by the hepatocyte membrane of the methyl group of AdoMet administered as an hepatoprotective agent, but also, more generally, in the salvage of the methyl group of AdoMet released from damaged cells. As discussed by Hirata and Axelrod [21], methylation of the phospholipids of the plasma membranes could contribute to the preservation of their fluidity.

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