MANIPULATION OF PHOSPHOLIPID POLAR HEADGROUP COMPOSITION IN PRIMARY CULTURES OF RAT HEPATOCYTES

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SUMMARY

Isolated hepatocytes in suspension or in primary culture were incubated with different phospholipid bases and the effects on the synthesis and composition of phospholipids were studied. After incubation in the presence of 1 mM diethylethanolamine for three days, an unnatural phospholipid, phosphatidyldiethylethanolamine, constituted more than 20 % of total phospholipids. Its fatty acid composition differed from that of other phospholipids. Incubation of hepatocytes with ethanolamine gave smaller effects and in this case the increased synthesis of phosphatidylethanolamine was compensated for by methylation to phosphatidylcholine. This system can be used for studies on the functional significance of phospholipid polar headgroups in a specialized type of cell.

INTRODUCTION

Analogues to ethanolamine and choline can be incorporated into phospholipids in liver tissue (1, 2). Recently this has been used for modification of phospholipid composition in yeast (3), L-M cells (4) and intact rat liver (5, 6). The hepatocyte has a very low dividing rate under normal conditions, and therefore any manipulation must take place by replacing preexisting molecules rather than by influencing the lipid composition of newly formed membranes in growing cells. In recent years, methods for primary culture of hepatocytes for several days have become available (7, 8). The present report shows that the phospholipid composition of such hepatocytes is considerably changed during incubation in the presence of diethylethanolamine. The accumulation of phosphatidyldiethylethanolamine was more pronounced than that observed for another unnatural phospholipid, phosphatidylisopropylethanolamine, in livers of intact rats (6).

METHODS

Tissue culture media were obtained from Flow Laboratories. Diethylethanolamine was obtained from Aldrich. Phosphatidyldiethylethanolamine was prepared by phospholipase D hydrolysis of soy bean phosphatidylcholine (9) in the presence of diethylethanolamine. Radiochemicals were obtained from the Radiochemical Centre, Amersham, U.K.

Hepatocytes were isolated by collagenase perfusion (10, 11) of livers from male Sprague-Dawley rats fed a balanced diet ad libitum. The perfusate was Hanks' buffer (12) containing 40 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). The isolated hepatocytes were washed twice in this

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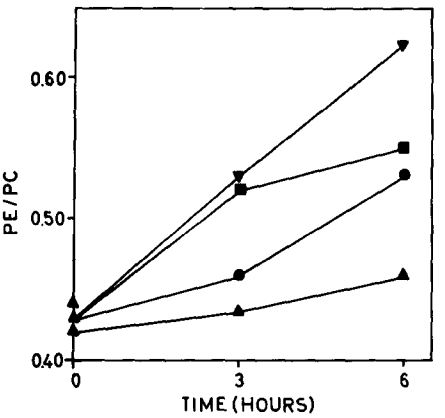

Figure 1. Proportions of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in suspended hepatocytes. Hepatocytes (10.8 mg protein/ml) were incubated in Hanks' buffer containing 20 mM HEPES, 1 % defatted bovine serum albumin and different concentrations of ethanolamine: 0 (▲), 1 (●), 2 (■) and 5 (▼) mM.

medium and once in the respective incubation medium. Suspended hepatocytes were incubated in Erlenmeyer flasks in Hanks' buffer containing 10 mM phosphate and 20 mM HEPES. Primary cultures of hepatocytes were performed in 60 mm Falcon plastic Petri dishes coated with collagen (13). Each dish contained approximately 5 x 10^6 hepatocytes and 2.5 ml of L-15 culture medium, containing 28 mM HEPES (13), 1 mM sodium succinate (7), insulin (0.5 µg/ml), penicillin (100 µg/ml), and streptomycin (100 µg/ml) or gentamycin (50 µg/ml). It also contained 5 % fetal calf serum during the first 4 h and thereafter 2 %. The dishes were kept in a humified incubator at 37°C. The medium was changed after 4 h and then once a day. At different time intervals the medium was removed and 0.2 ml 6 M HCl was added. The cells were removed with a rubber policeman and the dish was rinsed four times with 1 ml methanol:water (2:1). Lipids were then extracted with 4 ml chloroform: methanol (1:1) and the lipid extract was washed (14). Lipids, usually from several pooled extracts, were separated on pre-washed thin-layer silica gel H plates, with chloroform:methanol:conc. ammonia (60:30:5) as developing solvent. The lipids were eluted (15) and quantitated by phosphorous determination (16). When necessary, phosphatidyldiethylethanolamine was further purified with chloroform:methanol:acetic acid:water (65:25:4:4) as developing solvent. Fatty acid composition was determined as described earlier (15) and protein determination was made according to Lowry et al. (17).

RESULTS

Earlier studies showed that ethanolamine and its N-methylated analogues markedly affected phospholipid biosynthesis during short-term incubation of suspended hepatocytes (18). To evaluate the effects on phospholipid composition suspended hepatocytes were incubated for up to 6 h with increasing amounts of ethanolamine (Fig. 1). The proportion of phosphatidylethanolamine increased with time in the presence of ethanolamine and the change was more

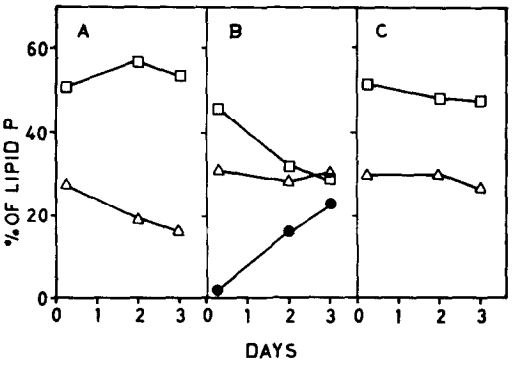

Figure 2. Phospholipid composition in primary cultures of hepatocytes. A, control; B, 1 mM diethylethanolamine; C, 1 mM ethanolamine. □, phosphatidylcholine; △, phosphatidylethanolamine; ●, phosphatidyldiethylethanolamine.

marked with increasing concentration of ethanolamine. Since the hepatocytes are viable only for limited periods of time under these experimental conditions, long-term effects of phospholipid bases were studied in primary cultures of hepatocytes in Petri dishes. In addition to ethanolamine and choline, several of their analogues were tested.

The most dramatic changes in phospholipid composition were observed when hepatocytes were incubated with diethylethanolamine (Fig. 2). The proportion of phosphatidyldiethylethanolamine progressively increased during incubation for three days. This phospholipid appeared as a distinct band between neutral lipids and phosphatidylethanolamine upon thin-layer chromatography. It cochromatographed in several systems with an authentic standard prepared enzymatically. The rise in phosphatidyldiethylethanolamine was mainly compensated for by a decrease in phosphatidylcholine. Interestingly, the proportion of phosphatidylethanolamine was higher in the incubations with diethylethanolamine than in the controls. Whether this reflects utilization of the ethanolamine moiety after dealkylation, for phosphatidylethanolamine synthesis remains to be elucidated. In any case, no accumulation of phosphatidylmonoethylethanolamine took place. In this experiment [^{3}H]glycerol was added to the incubation 1 h before harvesting to obtain information on the spectrum of glycerolipids synthetized at the different time intervals. As expected the addition of bases caused even more marked changes in [^{3}H]glycerol distribution than in phosphorous distribution (Fig. 3). After three days' incubation in the presence of diethylethanolamine, phosphatidyldiethylethanolamine constituted 35 % of lipid ^{3}H and 60 % of phospho-

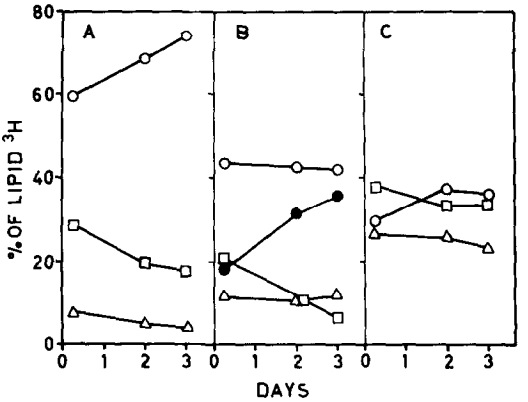

Figure 3. Glycerolipid synthesis from 1mM [^{3}H]glycerol added 1 hour before interruption of hepatocyte cultures. A, control; B, 1 mM diethylethanolamine; C, 1 mM ethanolamine. ○, neutral lipids; other symbols as in Figure 2.

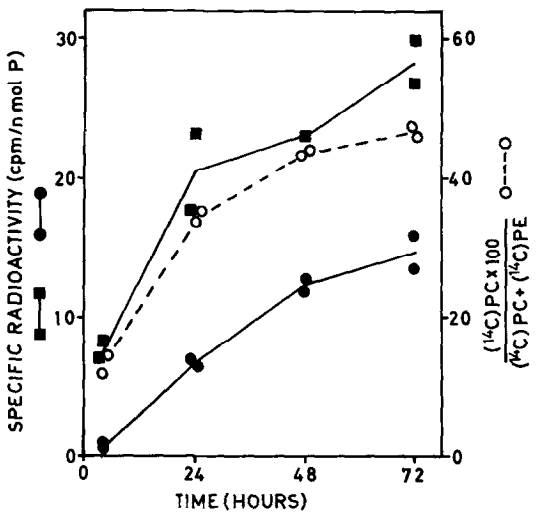

Figure 4. Methylation of phosphatidylethanolamine in primary cultures of hepatocytes. [^{14}C]Ethanolamine (0.4 mM) was added at the beginning of the culture and the specific radioactivity of phosphatidylethanolamine (■) and phosphatidylcholine (●) was followed. Also the total ^{14}C in phosphatidylcholine (PC) expressed as percent of the total radioactivity in phosphatidylcholine plus phosphatidylethanolamine (PE) is shown.

lipid ^{3}H. The compensating decrease occurred mainly in neutral lipids and also in phosphatidylcholine.

Addition of 1 mM ethanolamine also decreased neutral lipid ^{3}H and markedly stimulated phosphatidylethanolamine synthesis (Fig. 3), as already noted in

Table 1. Fatty acid composition of hepatocyte phospholipids after culture for two days in the presence and absence of diethylethanolamine (1 mM).
Abbreviations: PDEE, phosphatidyldiethylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

Fatty acid	+ diethylethanolamine			- diethylethanolamine		freshly isolated cells	
	PDEE	PE	PC	PE	PC	PE	PC
16:0	28.2	17.9	20.7	19.2	22.6	21.8	22.5
16:1	1.0		0.4		0.3	0.4	1.2
18:0	17.6	29.2	25.5	33.5	25.3	21.2	22.3
18:1	8.9	4.3	5.5	3.4	5.9	3.7	6.3
18:2	29.8	11.2	15.4	9.0	15.3	11.2	19.8
20:4	13.3	25.6	25.3	23.3	24.3	26.9	19.0
22:5	0.2	1.2	1.4			2.3	0.8
22:6	1.0	10.1	5.9	8.0	4.5	11.7	6.2
others		0.5		0.6	1.9	0.9	2.2

short-term incubations of suspended hepatocytes (18). The changes in phospholipid composition were much smaller and in several experiments the main observation was that the decrease in phosphatidylethanolamine with time occurring in control cultures was prevented by ethanolamine (Fig. 2). Neither the proportion of lipid ^{3}H nor lipid phosphorous in phosphatidylcholine did change with time. Although the culture medium L-15 contained some choline (0.007 mM), this may indicate that ethanolamine after methylation in the form of phosphatidylethanolamine increased the supply of choline in the cell. This point was further substantiated by studies on phosphatidylethanolamine methylation (Fig. 4). The proportion of lipid ^{14}C in phosphatidylcholine after addition of [^{14}C]ethanolamine at the start of the culture, steadily increased with time and was almost 50 % after three days. The specific radioactivity rose during the whole experiment indicating that an increasing proportion of both polar headgroups was substituted by exogenous [^{14}C]ethanolamine.

Since rat liver phosphatidylethanolamine and phosphatidylcholine differ specifically in fatty acid composition, it was of interest to determine this parameter for the newly synthetized phosphatidyldiethylethanolamine (Table 1).

This phospholipid had a fatty acid composition considerably different from those of the two normal phospholipids. Palmitate, oleate and linoleate were more abundant whereas stearate, arachidonate and docosahexaenoate had lower proportions in phosphatidyldiethylethanolamine. Some acyl changes also occurred in phosphatidylethanolamine and phosphatidylcholine during incubation (Table 1). Stearate and in the latter phospholipid also arachidonate increased at the expense of palmitate and linoleate.

DISCUSSION

This study shows that the system of isolated hepatocytes is feasible for manipulation of the polar headgroups of phospholipids. The extent of substitution by diethylethanolamine was higher than reported for another analogue, isopropylethanolamine, in L-M cells (19) and intact rat liver (6). It may be further increased using techniques permitting still longer periods of culture (20). Other groups obtained high proportions of phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine after supplementation with corresponding bases (4, 5). These bases and also ethanolamine (Fig. 2) and choline gave less dramatic changes in the present system. This reflects, at least in part, the effective phospholipid methylation (Fig. 4). The concentration of L-methionine in L-15 is 0.5 mM, which is optimal at least in suspended hepatocytes (18). The pronounced accumulation of phosphatidyldiethylethanolamine may therefore be due to the fact that it is not efficiently methylated. It or its parent base may, however, undergo dealkylation since the proportion of phosphatidylethanolamine increased in the presence of diethylethanolamine. Such a conversion was also suggested for isopropylethanolamine (6).

Most of the diethylethanolamine was probably incorporated by de novo synthesis via phosphatidate, as indicated by the high labeling of phosphatidyldiethylethanolamine by [^{3}H]glycerol (Fig. 3). In addition, its fatty acid composition with high proportions of palmitate, oleate and linoleate is similar to the fatty acids incorporated into glycerolipids via phosphatidate. The low proportions of stearate and arachidonate may also indicate that this phospholipid is less prone to undergo deacylation - reacylation cycles than other phospholipids. This agrees with the observation that 1-acyl-glycerophosphodiethylethanolamine is not very active as acyl acceptor (21). Incorporation by base exchange preferentially occurs for highly unsaturated molecular species (22) and has therefore low quantitative importance for the incorporation of diethylethanolamine.

Further studies are necessary to elucidate the functional significance of

the reported changes in phospholipid composition. Of special interest is the fact that several phospholipid bases have hypolipidemic effects when administered to rats (23).

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