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Factors regulating the secretion of lysophosphatidylcholine by rat hepatocytes compared with the synthesis and secretion of phosphatidylcholine and triacylglycerol

Effects of albumin, cycloheximide, verapamil, EGTA and chlorpromazine

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1. The synthesis and secretion of glycerolipid by monolayer cultures of rat hepatocytes was measured by determining the incorporations of [^{3}H]glycerol, [^{3}H]oleate and [^{14}C]choline and by the absolute concentration of triacylglycerol. 2. The presence of albumin in the medium stimulated the accumulation of lysophosphatidylcholine in the medium by 11–13-fold. 3. Cycloheximide did not significantly alter the accumulation of lysophosphatidylcholine. 4. This process was particularly sensitive to inhibition by chlorpromazine and verapamil, compared with the secretion of triacylglycerol and phosphatidylcholine. By contrast, it was relatively less sensitive to EGTA. 5. It is suggested that intracellular Ca^{2+} may be important in the production of lysophosphatidylcholine, which then accumulates in the medium by binding to albumin. *In vivo* this lysophosphatidycholine may be a means of delivering choline and polyunsaturated fatty acids to other organs.

INTRODUCTION

It has recently been reported that perfused livers [1] and rat hepatocytes in monolayer cultures [2,3] can synthesize lysophosphatidylcholine, which appears in the perfusion or incubation medium. The liver could be the source of much of the lysophosphatidylcholine found associated with albumin in the blood. Lysophosphatidylcholine is often the second most prevalent phospholipid in plasma [4], and its concentration is relatively high even in patients with lecithin:cholesterol acyltransferase deficiency [5].

Lysophosphatidylcholine is readily taken up by tissues and can be used for the synthesis of phosphatidylcholine, or for the production of acetylcholine in nervous tissue [6–10]. It seems likely that lysophosphatidylcholine could act as an additional vehicle for transporting choline from the liver to the other tissues.

The lysophosphatidylcholine produced by perfusion of rat livers is predominantly unsaturated [1]. We have recently demonstrated that the secretion of lysophosphatidylcholine from monolayer cultures of rat hepatocytes is stimulated preferentially by unsaturated fatty acids, and further, that these acids are incorporated into the lysophosphatidylcholine [3]. The secretion of this lipid by the liver could therefore provide a route for the transport of both choline and unsaturated fatty acids, and could be particularly important in supplying extra-hepatic tissues with essential fatty acids [3]. In the present work we therefore decided to investigate methods of modulating the production of lysophosphatidylcholine, in order to learn more about this process. This involved determining the effects of adding albumin, cycloheximide, EGTA, verapamil or chlorpromazine to the incubation medium. Changes in the appearance of lysophosphatidylcholine in the medium were then compared with any alteration in the synthesis or secretion of phosphatidylcholine or triacylglycerol.

EXPERIMENTAL

Animals and materials

The sources of the rats and most of the materials have been described [2,3,11–14]. Whatman glass microfibre filters were purchased from Whatman International, Maidstone, Kent, U.K. Verapamil hydrochloride and cycloheximide were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Radiochemicals were purchased from Amersham International, Amersham, Bucks., U.K. Chlorpromazine hydrochloride (Largactil) was a gift from May and Baker, Dagenham, Essex, U.K.

Preparation and incubation of hepatocytes

Hepatocytes were prepared, maintained and incubated as previously described [13]. In all experiments the final concentrations of choline chloride and glycerol were 100 μM and 1 mM respectively. Oleate was added at 2 mM except for the experiments described in Fig. 1 and Table 1. Other conditions are shown in the legends to Figures and Tables. Verapamil, EGTA, chlorpromazine and cycloheximide were added in 30 μl to the 3 ml of incubation medium, cycloheximide was dissolved in 0.9% NaCl.

In experiments using [^{3}H]leucine, the amount incor-

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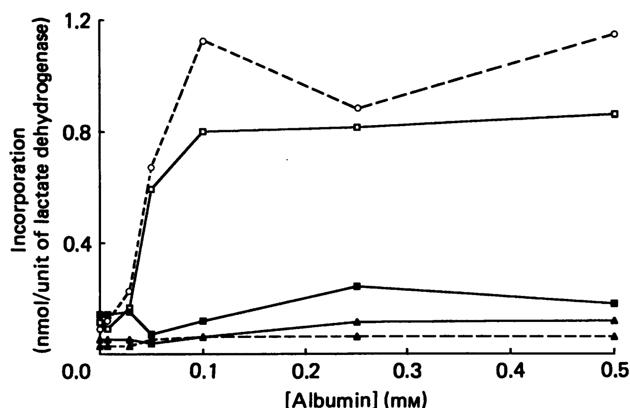

Fig. 1. Effect of the albumin concentration of the medium on the incorporation of glycerol and choline into glycerolipids secreted by rat hepatocytes

Rat hepatocytes were incubated with 1 mM-[^{3}H]glycerol and 100 μM-[^{14}C]choline for 8 h in the presence of various concentrations of albumin (see the Experimental section). No oleate was added. The incorporation of [^{3}H]glycerol into triacylglycerol (■), phosphatidylcholine (▲) and lysophosphatidylcholine (□), and that of [^{14}C]choline into phosphatidylcholine (△) and lysophosphatidylcholine (○) isolated from the medium is shown. Results are from a representative experiment, and the reproducibility of the results is also shown in Table 1.

porated into protein precipitated with trichloroacetic acid was taken as an estimate of the amount of protein synthesized. The protein pellet was solubilized from microfibre filters by eluting overnight in 0.1 M-NaOH and neutralizing with 0.5 M-HCl/0.12 M-Tris. Radioactivity was determined by scintillation counting.

Collection of media and cells and subsequent analysis of radioactive lipids were performed as described elsewhere [2]. The concentrations of triacylglycerol in the incubation medium were determined using essentially the method described previously [3]. To overcome the problem of turbidity encountered because of fatty acid in the lipid extract of the medium, the method was modified to include an alumina adsorption stage. Basic aluminium oxide (1 g) was added to 3 ml of the bottom phase of the lipid extract; the mixture was shaken for 3 min and then centrifuged at full speed in a bench-top centrifuge for 30 min. The supernatant was removed and dried down under N_2, and the residue was assayed for triacylglycerol [3].

RESULTS

Albumin caused a dramatic increase in the incorporation of [^{3}H]glycerol and [^{14}C]choline into lysophosphatidylcholine (Fig. 1). The respective increases were 12.9-fold ($P < 0.05$) and 10.8-fold ($P < 0.02$) when the albumin concentration in the medium was increased from 0 to 0.5 mM. The optimum rate of lysophosphatidylcholine secretion was achieved at 0.1 mM-albumin. No steps were taken to maintain constant osmolarity at different concentrations of albumin in these experiments. However, it appeared that such changes in themselves did not alter the appearance of lysophosphatidylcholine in the medium, at least between 0.1 mM- and 0.5 mM-albumin (Fig. 1).

The secretion of labelled phosphatidylcholine and triacylglycerol was at a low rate because of the absence of added fatty acid in the incubation medium. The addition of albumin to the medium failed to produce significant changes in the synthesis or secretion of triacylglycerol in the three independent experiments that are reported (Table 1).

The incorporation of [^{3}H]glycerol into triacylglycerol in the cells was also low, again due to the absence of any exogeneous fatty acid to stimulate triacylglycerol synthesis, and was not significantly altered by the addition of albumin to the incubation medium (Table 1).

The effects of cycloheximide, chlorpromazine, verapamil and EGTA on the synthesis and secretion of lipids (Table 2) were investigated by using [^{3}H]oleate and [^{14}C]choline under the conditions described.

Cycloheximide (5 μg/ml) had no effect on the secretion of lipids into the medium over 8 h (Table 2). Protein synthesis from [^{3}H]leucine was inhibited by 88 % in the cells. The incorporation of [^{3}H]oleate into triacylglycerol in the cells was inhibited by cycloheximide by about 15 % ($P < 0.02$). The synthesis of labelled phosphatidylcholine within the cells did not show any significant change (Table 3).

Table 1. Effect of the albumin concentration of the incubation medium on the incorporation of glycerol and choline into glycerolipids by rat hepatocytes

Rat hepatocytes were incubated in the presence or the absence of albumin, and the incorporation of [^{3}H]glycerol and [^{14}C]choline into glycerolipids was measured over an 8 h period (see the Experimental section). Exogenous fatty acid was not added to these incubations. Results are means ± S.D. for three independent experiments. The significance of the difference between the incorporations in the presence and the absence of albumin was calculated by using a paired t test, and is indicated by: *$P < 0.05$; **$P < 0.02$ (n.d., not detected).

		Substrate incorporated (nmol/unit of lactate dehydrogenase)				
		Lysophosphatidylcholine		Phosphatidylcholine		Triacylglycerol
	Albumin (mM)	Glycerol	Choline	Glycerol	Choline	Glycerol
Medium	0	0.096±0.021	0.13±0.037	0.147±0.103	0.03±0	0.252±0.11
	0.5	1.25±0.34*	1.40±0.22**	0.16±0.046	0.04±0.017	0.153±0.04
Cells	0	n.d.	n.d.	4.96±1.58	8.61±2.09	0.746±0.127
	0.5	n.d.	n.d.	5.99±1.93	14.1±6.42	0.652±0.184

Table 2. Effects of chlorpromazine, verapamil, EGTA and cycloheximide on the incorporation of oleate and choline into glycerolipids of the incubation medium by rat hepatocytes

Rat hepatocytes were incubated with the compounds indicated in the presence of 0.5 mM-albumin, and the incorporation of [^{3}H]oleate and [^{14}C]choline into glycerolipids was measured over an 8 h period (see the Experimental section). The results are means ± S.D. for the numbers of independent experiments shown in parentheses, and the values are expressed relative to the control incubation where no additions were made. This value is taken as 100%, but the absolute incorporation, in nmol of substrate incorporated/unit of lactate dehydrogenase, is also shown (in brackets) in the Table. [Triacylglycerol] is expressed in nmol/unit of lactate dehydrogenase. Means ± ranges are shown where there are only two experiments. The significance of the differences in incorporation relative to the appropriate control value was calculated by using a paired t test, and is indicated by: $^*P < 0.05$; $^{**}P < 0.02$; $^{\dagger}P < 0.001$.

Additions	Relative incorporation (%) into:					[Triacylglycerol] (%)
	Lysophosphatidylcholine		Phosphatidylcholine		Triacylglycerol	
	Oleate	Choline	Oleate	Choline	Oleate	
None	100 [6.23 ± 1.14 (5)]	100 [3.31 ± 1.07 (5)]	100 [1.23 ± 0.54 (5)]	100 [0.105 ± 0.03 (5)]	100 [11.71 ± 3.54 (5)]	100 [5.10 ± 1.08 (3)]
Chlorpromazine (100 µM)	55 ± 19 (4)**	28 ± 9 (4)†	160 ± 136 (4)	53 ± 7 (4)**	75 ± 22 (4)	93 ± 37 (3)
Verapamil (150 µM)	46 ± 24 (4)*	21 ± 8 (4)†	120 ± 67 (4)	54 ± 34 (4)	84 ± 35 (4)	66 ± 5 (2)
EGTA (2 mM)	91 ± 12 (4)	76 ± 17 (4)	57 ± 34 (4)	42 ± 6 (4)†	64 ± 16 (4)**	69 ± 25 (2)
Cycloheximide (5 µg/ml)	106 ± 16 (3)	110 ± 26 (3)	175 ± 68 (3)	92 ± 23 (3)	107 ± 55 (3)	94 (1)

Fig. 2(*a*) is a typical experiment showing the effect of increasing concentrations of chlorpromazine on the incorporation of [^{3}H]oleate and [^{14}C]choline into lysophosphatidylcholine in the medium. Subsequent experiments utilized 100 µM-chlorpromazine (Tables 2 and 3). At this concentration of chlorpromazine, the incorporation of [^{3}H]oleate and of [^{14}C]choline into secreted lysophosphatidylcholine was decreased by 45% and by 72% respectively, compared with the control incubation. Incorporation of [^{3}H]oleate into secreted phosphatidylcholine was not significantly altered, but incorporation of [^{14}C]choline was decreased by 47%. The incorporation of [^{3}H]oleate into secreted triacylglycerol and the concentration of triacylglycerol in the medium were not significantly affected by 100 µM-chlorpromazine (Table 2).

Chlorpromazine again did not affect [^{3}H]oleate incorporation into phosphatidylcholine in the cells, but did decrease the incorporation of [^{14}C]choline into phosphatidylcholine by about 39% (Table 3). This inhibition did not become more pronounced at higher concentrations of chlorpromazine (results not shown). There was no significant effect on [^{3}H]oleate incorporation into triacylglycerol in the cells (Table 2).

Fig. 2(*b*) is a typical experiment illustrating the effect of increasing concentrations of verapamil incorporation of [^{3}H]oleate and [^{14}C]choline into lysophosphatidylcholine. At 150 µM, verapamil decreased the incorporation of [^{3}H]oleate and [^{14}C]choline into lysophosphatidylcholine by 54% and 79% respectively (Table 2). There was no effect on the incorporation of [^{3}H]oleate into secreted phosphatidylcholine, but the incorporation of [^{14}C]choline was decreased by 46%. There appeared to be a decrease of around 15% in [^{3}H]oleate incorporation into secreted triacylglycerol, and, although this was not significant, the inhibition became more pronounced at 350 µM-verapamil (results not shown). There was no effect on [^{3}H]oleate incorporation into triacylglycerol in the cells (Table 3), but 150 µM-verapamil caused a decrease of 50% on the incorporation of [^{14}C]choline into cell phosphatidylcholine ($P < 0.01$).

Fig. 2(*c*) is a typical experiment showing the secretion of labelled lysophosphatidylcholine at increasing concentrations of EGTA. At 2 mM, EGTA did not significantly alter the incorporation of either label into lysophosphatidycholine. However, it did significantly decrease the amount of [^{14}C]choline incorporated into secreted phosphatidylcholine and the incorporation of [^{3}H]oleate into secreted triacylglycerol (Table 2). EGTA (2 mM) also decreased the incorporation of [^{14}C]-choline into phosphatidylcholine in the cells by around 32% but did not significantly alter the incorporation of [^{3}H]oleate into phosphatidylcholine or triacylglycerol in the cells (Table 3).

DISCUSSION

The main objective of this work was to investigate factors which modulate the appearance of lysophosphatidylcholine in the medium of cultured rat hepatocytes and to compare these changes with the secretion of phosphatidylcholine and triacylglycerol. These latter lipids are mainly associated with very-low-density lipoproteins [2]. The action of such factors on synthesis within the cells was also examined to see whether a

Table 3. Effects of chlorpromazine, verapamil, EGTA and cycloheximide on the incorporation of oleate and choline into glycerolipids of rat hepatocytes

The incorporations of [^{3}H]oleate and [^{14}C]choline into the cell lipids of the experiments described in Table 2 are shown in the same way as described for that Table.

	Relative incorporation (%) into:			
	Phosphatidylcholine		Triacylglycerol	[Triacylglycerol] (%)
	Oleate	Choline	Oleate	
None	100 [68 ± 14 (5)]	100 [23 ± 5.9 (5)]	100 [413 ± 195 (5)]	100 [371 ± 242 (3)]
Chlorpromazine (100 μM)	99 ± 31 (4)	62 ± 27 (4)	94 ± 28 (4)	112 ± 21 (3)
Verapamil (150 μM)	79 ± 14 (4)	51 ± 16 (4)**	100 ± 24 (4)	108 ± 4.9 (2)
EGTA (2 mM)	79 ± 19 (4)	68 ± 20 (4)†	116 ± 23 (4)	124 ± 7.8 (2)
Cycloheximide (5 μg/ml)	102 ± 7.8 (3)	90 ± 15 (3)	84 ± 3.6 (3)**	113 ± 3.2 (2)

specific effect on secretion was being observed or whether this resulted from a more general effect on glycerolipid synthesis.

The appearance of lysophosphatidylcholine in the medium was highly dependent on the presence of albumin in the medium (Fig. 1; Table 1; ref. [15]). In our system, the maximum stimulation was obtained at 0.1–0.5 mM-albumin. The normal physiological concentration of albumin in the blood is in the region of 0.5 mM. The effect of the albumin was unlikely to have been caused by the presence of a contaminating phospholipase, since heat treatment of the albumin at 60 °C before use did not significantly affect the production of lysophosphatidylcholine. We had thought that lysophosphatidylcholine might have been co-secreted with albumin by the hepatocytes. However, cycloheximide did not significantly alter the appearance of lysophosphatidylcholine in the medium (Table 2). This indicates that the synthesis of new protein is not needed for lysophosphatidylcholine production. Furthermore, colchicine does not significantly inhibit lysophosphatidylcholine production, which shows that it is not released from the cell by a process which involves the production of microtubules [2,16]. It therefore seems likely that the albumin is acting as a binding site for the lysophosphatidylcholine, thus enabling it to accumulate in the medium. This conclusion is compatible with the observation that an α-cyclodextrin which binds lysophosphatidylcholine is also able to stimulate the appearance of lysophosphatidylcholine in the medium [15].

The second part of our work investigated the effects of compounds that are known to interfere with Ca^{2+} metabolism. This seemed to be relevant since Ca^{2+} antagonists are known to inhibit the secretion of very-low-density lipoproteins [17–19]. Furthermore, Ca^{2+} is a known cofactor for the action of many phospholipases, and it seemed likely that the production of lysophosphatidylcholine might involve such Ca^{2+}-dependent enzymes.

The first of these compounds was the amphiphilic amine, chlorpromazine, which can displace Ca^{2+} from various intracellular binding sites [20]. Chlorpromazine inhibited the appearance of lysophosphatidylcholine in the medium at concentrations of 25–200 μM (Fig. 2*a*; Table 2). Lysophosphatidylcholine production therefore appeared to be particularly sensitive in this respect relative to the secretion of very-low-density lipoprotein, since there was no significant inhibition of the secretion of triacylglycerol (Table 2). There was also no significant effect on the synthesis and accumulation of triacylglycerol within the cells (Table 3) at 100 μM-chlorpromazine. This concentration of chlorpromazine did, however, produce a significant decrease in the secretion of phosphatidylcholine as measured with [^{14}C]choline, but not with [^{3}H]oleate (Table 2). This latter discrepancy could have resulted partly from the fact that methylation of phosphatidylethanolamine can be a significant source of the phosphatidylcholine that is secreted from hepatocytes [21–23].

Chlorpromazine can also modify glycerolipid synthesis by its interaction with membranes, which makes them more positive. This inhibits phosphatidate phosphohydrolase and results in an inhibition of the synthesis of phosphatidylcholine and triacylglycerol and an accumulation of phosphatidate [20,24]. Such an effect was not observed in the present work, probably because of the much longer period of incubation, which was 8 h rather than 15 min. Futhermore, the use of a high (2 mM) oleate concentration in the present work would counteract the effect of chlorpromazine on membrane charge [20,24].

A further effect of chlorpromazine is its action in increasing lysosomal pH, which can contribute to an inhibition of lysosomal phospholipases [25]. Such an effect could theoretically be involved in the inhibition of the production of lysophosphatidylcholine in the incubation medium.

The effects of verapamil on the secretion of phosphatidylcholine and triacylglycerol differed from those observed by others [17,26]. These authors observed optimal inhibition at 200 μM-verapamil, and they demonstrated that the inhibition of secretion took place in the Golgi complex [26]. In our experiments the inhibition of triacylglycerol secretion was not significant at 150 μM-verapamil, and the concentration had to be increased to 350 μM before inhibition was more marked. The incorporation of [^{14}C]choline into secreted phosphatidylcholine was inhibited by 50% at about 150 μM-verapamil (Table 2). The concentrations of verapamil required to elicit these changes are rather greater than those which produce effects on secretory or excitable cells [27–30]. However, a major regulator of very-low-density-

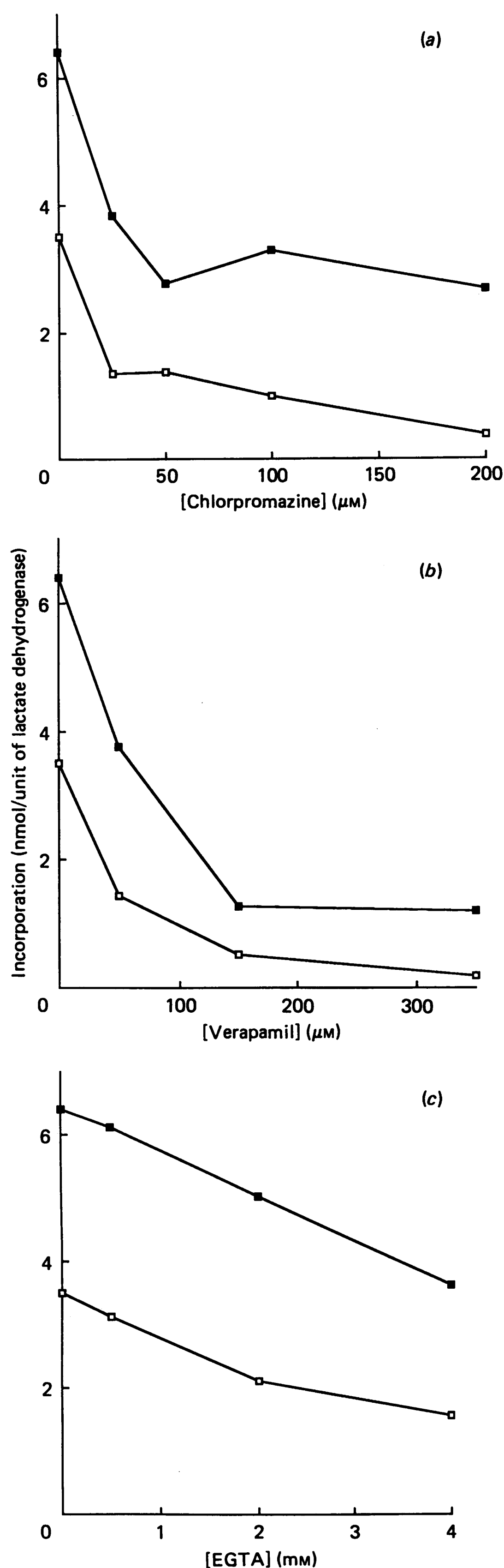

Fig. 2. Effects of chlorpromazine, verapamil and EGTA on the incorporation of oleate and choline into the lysophosphatidylcholine secreted by rat hepatocytes

Rat hepatocytes were incubated with 0.5 mM-albumin, 2 mM-[^{3}H]oleate and 100 μM-[^{14}C]choline for 8 h (see the Experimental section). The effects of (*a*) chlorpromazine, (*b*) verapamil and (*c*) EGTA on the incorporation of [^{3}H]oleate (■) and [^{14}C]choline (□) are shown for one representative experiment. The reproducibility of the results is also shown in Table 2.

lipoprotein secretion is the availability of fatty acids for triacylglycerol synthesis and secretion [31,32]. In our experiments 2 mM-oleate was used, whereas Nossen *et al.* [17,26] used 1 mM-oleate. Therefore the availability of fatty acid was much greater, and this may have overridden the effects of the verapamil. This appears to indicate, in agreement with Nossen *et al.* [17], that Ca^{2+} fluxes through voltage-dependent Ca^{2+} channels in the plasma membrane may be of relatively minor importance for the secretion of very-low-density lipoproteins.

In contrast, the effects of verapamil on secretion of lysophosphatidylcholine were more marked, inhibitory effects being observed at 50 μM-verapamil (Fig. 2*b*), a concentration analogous to those eliciting effects in secretory and excitable cells [27–30]. This appears to indicate a role for Ca^{2+} fluxes through voltage-dependent channels in the production of lysophosphatidylcholine. The inhibition observed of 50% in [^{14}C]choline incorporation into cell phosphatidylcholine (Table 3) is mirrored by the decrease in the secretion of phosphatidylcholine (Table 2). However, the decrease in lysophosphatidylcholine secretion is about 80% for [^{14}C]-choline incorporation.

EGTA, a fairly specific Ca^{2+} chelator, caused an inhibition of secretion of both phosphatidylcholine and triacylglycerol from the hepatocytes (Table 2). The latter is in agreement with Nossen *et al.* [17]. It has also been shown that EDTA causes inhibition of secretion of triacylglycerol [33] and cholesterol ester [34] from isolated rat hepatocytes. It therefore appears that bivalent-cation chelators remove Ca^{2+} from the medium and decrease the secretion of very-low-density lipoproteins.

EGTA did not decrease the synthesis of triacylglycerol from [^{3}H]oleate in the cells, but the synthesis of phosphatidylcholine, measured with [^{14}C]choline, was decreased (Table 3). This relative decrease, however, appeared to be smaller than that observed for secreted phosphatidylcholine (Table 2). The decreased concentrations of Ca^{2+} in the medium did not significantly decrease the secretion of lysophosphatidylcholine (Table 2) indicating a different mechanism in the secretion.

Mangiapane & Brindley [2] showed that the appearance of lysophosphatidylcholine in the medium of cultured rat hepatocytes could be dissociated from the secretion of very-low-density lipoproteins [2]. The present work extends and confirms this observation by studying the effects of albumin, chlorpromazine, verapamil and EGTA. So far, little is known about the mechanism by which lysophosphatidylcholine accumulates in the medium. The process is specifically stimulated by unsaturated rather than by saturated fatty acids [3], and the lysophosphatidylcholine contains a relatively high proportion of polyunsaturated fatty acids (refs. [1] and [15]; A. Graham, V. A. Zammit, W. W. Christie & D. N. Brindley, unpublished work). The present work and that of Baisted *et al.* [15] demonstrate that the accumulation is very dependent on the presence of albumin in the medium, and that this probably results from the binding of lysophosphatidylcholine to the

albumin. The accumulation of this lipid in the medium is not dependent on the coincident synthesis of protein. It is very sensitive to inhibition by chlorpromazine and verapamil (Figs. 2*a* and 2*b*), which probably indicates a requirement for intracellular Ca^{2+}. By contrast, the concentration of free Ca^{2+} in the medium appears to be less important, since EGTA had relatively little effect (Fig 2*c*).

The results indicate that a Ca^{2+}-dependent phospholipase may be involved in producing the lysophosphatidylcholine that appears in the medium. However, this is inferred from indirect evidence. The site at which the lysophosphatidylcholine is produced is also obscure. There are indications that intracellular Ca^{2+} may be more important than extracellular Ca^{2+}, since chlorpromazine and verapamil were more effective at inhibiting lysophosphatidylcholine formation than was EGTA (Table 2). However, there was no significant accumulation of lysophosphatidylcholine in the cells in any of the experimental conditions (ref. [2]; Table 3). The appearance of lysophosphatidylcholine in the medium depends on its being bound to albumin. It has been proposed [3] that *in vivo* the lysophosphatidylcholine is then carried to other organs, to supply them with choline and polyunsaturated fatty acids.

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