Factors influencing triacylglycerol synthesis in permeabilized rat hepatocytes

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Rat hepatocytes were treated with *Staphylococcus aureus* α-toxin to permeabilize their plasma membrane for low-molecular-mass compounds. During incubation with 1 mM labelled fatty acid, phosphatidate and, less clearly, lysophosphatidate rapidly reached a steady state, whereas labelled diacylglycerol accumulated to some extent, at least in the absence of exogenous CDP-choline. Esterification and oxidation were linearly related to the fatty acid concentration, and there was no indication for saturation with acyl-CoA. However, when permeabilized cells were incubated with labelled *sn*-glycerol 3-phosphate and 1 mM unlabelled fatty acid, glycerolipid synthesis and the level of esterification intermediates reached a plateau between 0.25 and 0.50 μmol of the triose phosphate/ml. The synthesis of phosphatidylcholine was dependent on addition of CDP-choline. In presence of the latter, diacylglycerol no longer accumulated and triacylglycerol synthesis was suppressed, although the sum of synthesized diacylglycerol, triacylglycerol and phosphatidylcholine remained constant. This indicates that the same pool of diacylglycerol is shared by choline-phosphotransferase and diacylglycerol acyltransferase and that the relative activity of these enzymes depends on the CDP-choline supply. Comparison of the levels of the esterification intermediates with the activity of the respective steps of the pathway reveals that, at a fixed fatty acid concentration, glycerophosphate acyltransferase determines the esterification rate, whereas lysophosphatidate acyltransferase and, at low CDP-choline levels, diacylglycerol acyltransferase approach saturation at elevated *sn*-glycerol 3-phosphate concentration. There is, however, no indication for a regulatory role of phosphatidate phosphohydrolase in this system. The significance of these findings for the regulation of triacylglycerol synthesis under conditions *in vivo* is discussed.

INTRODUCTION

The use of permeabilized cells for the investigation of biochemical phenomena is becoming more and more frequent. Indeed, the system combines the advantages of intact cells, i.e. preserved intracellular architecture and, presumably, unchanged interaction between organelles, with the ease of substrate manipulation that is typical for broken cell preparations. Different agents have been used to permeabilize cells, e.g. lysophosphatidylcholine (Mayorek & Bar-Tana, 1989), detergents such as digitonin and saponin (Boon & Zammit, 1988; Mick *et al.*, 1988), polyene antibiotics such as filipin (Stephens & Harris, 1987), and cytolytic toxins such as streptolysin O and α-toxin (Bernheimer & Rudy, 1986; Bhakdi & Tranum-Jensen, 1987). The last-named compound is an amphiphilic polypeptide of approx. 34 kDa secreted by *Staphylococcus aureus*. It associates with the plasma membrane, forming an annular hexamer that constitutes a transmembrane channel through which small hydrophilic molecules can pass (Füssle *et al.*, 1981; McEwen & Arion, 1985), while leaving the intracellular membrane systems intact (McEwen & Arion, 1985; Bader *et al.*, 1986). The reported exclusion limit varies from 1000 to 5000 Da (Füssle *et al.*, 1981; Cheung *et al.*, 1989; Schulz, 1990). α-Toxin has been used for the study of hormone release (Ahnert-Hilger *et al.*, 1985; Bader *et al.*, 1986), interaction between urea-cycle enzymes (Cheung *et al.*, 1989) and, recently, for the study of lipid metabolism in hepatocytes (Mayorek & Bar-Tana, 1989).

The regulation of hepatic triacylglycerol synthesis is still incompletely understood. This is mainly due to the relative water-insolubility of the intermediates and the tight interaction of most of the enzymes with intracellular membranes. A further complication is the multi-organellar distribution of the enzymes; only diacylglycerol acyltransferase (EC 2.3.1.20) is present exclusively in the endoplasmic reticulum (Bell & Coleman, 1980). Moreover, the substrates of triacylglycerol synthesis, i.e. *sn*-glycerol 3-phosphate (3-GP) and acyl-CoA, cannot cross the plasma membrane, and their intracellular concentrations are therefore not amenable to easy manipulation in experiments *in vitro* with isolated hepatocytes.

As a consequence of these difficulties studies of the control of triacylglycerol synthesis have been mainly indirect, i.e. changes in the activities *in vitro* of certain enzymes of the pathway have been related to changes in the rate of appearance of the end product. But, in spite of much work, the evidence for a regulatory role of one or more of the enzymes is conflicting (Brindley, 1984; Tijburg *et al.*, 1989). The role of substrate supply has also been extensively investigated. Under certain conditions 3-GP seems to limit glycerolipid synthesis (Declercq *et al.*, 1982). The fatty acid supply determines the rate of both esterification and β-oxidation (Ontko, 1972; Debeer *et al.*, 1981), and the activity of the latter pathway, which is subject to short-term control, may limit the acyl-CoA supply of the esterification pathway (Ontko, 1972; Mannaerts & Debeer, 1979; Zammit, 1984). To our knowledge, however, there is no comprehensive study of the fluctuations in concentration of all the intermediates of triacylglycerol synthesis, demonstrating the interaction between the different steps of the pathway.

In the present experiments, glycerolipid synthesis was measured in α-toxin-permeabilized rat hepatocytes under near-steady-state conditions. The role of the pathway substrates, acyl-CoA and 3-

Abbreviations used: 3-GP, *sn*-glycerol 3-phosphate.
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GP, and the interactions with the pathways of β-oxidation and phosphatidylcholine synthesis were investigated.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 150–200 g were used in all experiments. The animals had unlimited access to water and were starved for 24 h in order to lower the hepatic rates of fatty acid synthesis *de novo*.

Preparation and permeabilization of hepatocytes

Hepatocytes were isolated by the method described by Zahlten & Stratman (1974), modified by the EGTA-preperfusion technique as described by Seglen (1972). Suspensions of 2.5×10^6 cells/ml were incubated in silicone-treated glass vials in an orbitally shaking water bath at 37 °C with O_2/CO_2 (19:1) as the gas phase (Declercq *et al.*, 1982). The medium routinely consisted of 40 mM-Mops, pH 7.2, 10 mM-$KHCO_3$, 3% (w/v) dextran F70, 2% (w/v) fatty-acid-free BSA, 5 mM-GSH, 140 mM-potassium L-glutamate, 4 mM-ATP and 4 mM-$MgSO_4$. Substrates and other cofactors were added as indicated below. Permeabilization of the hepatocytes was achieved by supplementing the incubation medium with 0.025% (w/v) *Staph. aureus* α-toxin.

Assessing the permeability and intactness of the hepatocyte plasma membrane

The permeability of the hepatocyte plasma membrane was assessed by determining the distribution volume of the cells for 3-*O*-methylglucose and sucrose, as follows. Hepatocytes treated for 5 min with different concentrations of α-toxin were incubated for 1 min with [U-^{14}C]sucrose or [U-^{14}C]3-*O*-methylglucose, each 1 mM and 0.4 Ci/mol. Cells and medium were separated by centrifugation through a 1-bromododecane/dodecane (49:1, w/w) layer, and counted for radioactivity (Cornell, 1980). The distribution volumes for both compounds were corrected for the adhering extracellular fluid, measured by the same procedure but with 1% (w/v) [^{3}H]dextran F70 (0.16 mCi/g) as marker.

Leakage of lactate dehydrogenase (EC 1.1.1.27) was determined by centrifuging the cells at approx. 12000 ***g*** for 15 s and assaying the enzyme in the supernatant and the pellet by a routine kinetic assay after dilution in a Triton X-100-containing buffer.

Esterification and β-oxidation in permeabilized hepatocytes

In the first set of experiments, hepatocytes were incubated with 1-^{14}C-labelled fatty acid (palmitate/oleate, 1:1; 1 Ci/mol; concentrations as indicated in the text or legends) and 1 mM unlabelled 3-GP, together with L-carnitine and CoA at concentrations of 0.5 mM and 0.1 mM respectively. In the other experiments cells were incubated with various concentrations of *sn*-[U-^{14}C]glycerol 3-phosphate (0.8 Ci/mol) and 1 mM unlabelled fatty acid (palmitate/oleate, 1:1), together with carnitine and CoA as above. Incubations were terminated after 6 and 12 min, either with an equal volume of 6 M-$HClO_4$ for the determination of radioactive acid-soluble oxidation products (Mannaerts *et al.*, 1978), or with 20 vol. of chloroform/methanol (2:1, v/v) for the extraction of lipids (Debeer *et al.*, 1977). Neutral lipids were separated into individual classes by t.l.c. as described by Skipski & Barclay (1969). Lysophosphatidate, phosphatidate and phospholipids were chromatographed on oxalic acid-activated silica-gel plates (developed with 0.2 M-oxalic acid, then activated for 1 h at 110 °C) with chloroform/methanol/propan-2-ol/diethyl ether (16:1:1:2, by vol.) as solvent system. After identification with dichlorofluorescein, the spots were scraped off and counted for radioactivity in a liquid-scintillation counter.

Lysophosphatidate and phosphatidate reached steady-state levels after approx. 4 min. The levels indicated in Figs. 3–5 are means of the values measured after 6 and 12 min. As explained below, diacylglycerol accumulated to some extent in the hepatocytes. The diacylglycerol levels indicated in Figs. 3–5 are those measured after 12 min. Phosphatidylcholine and triacylglycerol synthesis is expressed as rates between 6 and 12 min, and β-oxidation as rates between 0 and 6 min. The apparent total flux through the first part of the esterification pathway (glycerophosphate acyltransferase, EC 2.3.1.15; lysophosphatidate acyltransferase, EC 2.3.1.51; phosphatidate phosphohydrolase, EC 3.1.3.4) was calculated as the combined rates of synthesis of triacylglycerol, phosphatidylcholine and diacylglycerol, each measured between 6 and 12 min.

Reagents

[1-^{14}C]Palmitic acid, [1-^{14}C]oleic acid, [U-^{14}C]sucrose, 3-*O*-methyl-D-[U-^{14}C]glucose and [^{3}H]dextran (70000 Da, average) were from Amersham International, Amersham, Bucks., U.K. *sn*-[U-^{14}C]Glycerol 3-phosphate was from New England Nuclear, Boston, MA, U.S.A. Unlabelled fatty acids and dextran F70 (55000–73000 Da) were from Serva, Heidelberg, Germany. Dextran F70 solutions were dialysed for 48 h against distilled water to remove free glucose. Dodecane, 1-bromododecane, 3-*O*-methylglucose and GSH were from Janssen Chimica, Beerse, Belgium. BSA (fraction V), CoA, collagenase, CDP-choline and *sn*-glycerol 3-phosphate were obtained from Boehringer, Mannheim, Germany. The fraction V BSA was defatted as described by Chen (1967), dialysed in the cold for 48 h against distilled water and freeze-dried. L-Carnitine and standard lipids for t.l.c. were from Sigma Chemical Co., St. Louis, MO, U.S.A., except for phosphatidate, which was purchased from Fluka, Buchs, Switzerland. The α-toxin from *Staph. aureus*, obtained from Calbiochem, San Diego, CA, U.S.A., had an activity of 19 antibody-binding units/mg, as defined by the manufacturer. Soluene-350 and Pico-Fluor were from Packard Instrument Co., Downers Grove, IL, U.S.A. Thin layers of silica gel 60 were from Merck, Darmstadt, Germany. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

Characteristics of the permeabilized cells

α-Toxin was chosen as the permeabilizing agent since it probably does not affect the properties of the intracellular membranes and associated enzymes, whereas its effect on the plasma membrane is discrete and limited, in contrast with detergent-type permeabilizing agents such as digitonin (Mooney, 1988). Moreover, α-toxin is easy to use and, in our hands, gives reproducible results (see below).

Many different incubation conditions were tested before we obtained a medium in which the permeabilized cells were sufficiently stable to carry out the incubations. Our original conditions for permeabilization of hepatocytes were based on the work of McEwen & Arion (1985), but numerous other conditions were tried. The most significant differences with their medium are the following.

(*a*) The addition of a high-molecular-mass compound (BSA and/or dextran of 55000–73000 Da) at a final concentration of 5% (w/v). This proved to be essential for the stability of the permeabilized cells (see below).

(*b*) The substitution of potassium glutamate for mannitol (Schulz, 1990), because with the latter we invariably obtained higher 3-GP levels, apparently owing to limited breakdown of mannitol, as evidenced by the incorporation of radioactivity in

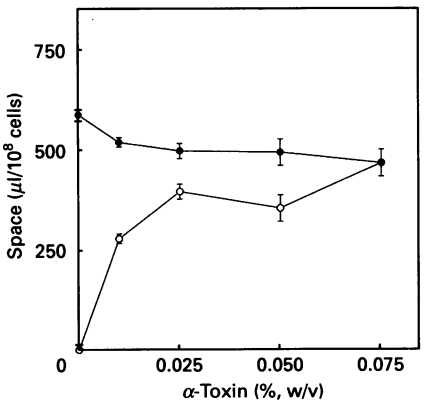

Fig. 1. Effect of α-toxin on the permeability of the hepatocyte plasma membrane

Hepatocytes were incubated as described in the Materials and methods section with increasing concentrations of α-toxin. The Figure shows the net distribution volume ('Space'), determined as described in the Materials and methods section, for [U-^{14}C]sucrose (○) and [U-^{14}C]3-*O*-methylglucose (●). Bars indicate S.E.M.

Table 1. Retention of lactate dehydrogenase in α-toxin-treated versus control cells

Cells were incubated, as described in the Materials and methods section, for different time periods without (control) and with 0.025% α-toxin. BSA was added as indicated; dextran F70 was absent. After incubation, the cells were centrifuged and lactate dehydrogenase was assayed in pellet and supernatant. The results, which are from one representative experiment, show the percentage of total activity present in the pellet.

	BSA	α-Toxin			Control
	(%, w/v)... –	2	4	6	–
Incubation time (min)					
0	74	82	91	88	91
12	73	80	87	85	86
30	28	60	80	80	82

triacylglycerols when labelled mannitol was used (results not shown).

The permeabilization was monitored by measuring the distribution volume of three radioactive markers that are not metabolized by hepatocytes (Craik & Elliott, 1979; Füssle *et al.*, 1981): [^{3}H]dextran F70 (70000 Da, average), which should not penetrate control or permeabilized cells; [U-^{14}C]sucrose, which should penetrate permeabilized cells, but does not enter control cells; [U-^{14}C]3-*O*-methylglucose, which penetrates control as well as permeabilized cells. Fig. 1 shows the effect of increasing concentrations of α-toxin on the net distribution volume of [U-^{14}C]sucrose and [U-^{14}C]3-*O*-methylglucose in isolated hepatocytes. In these experiments, the distribution volume of [^{3}H]dextran F70 remained relatively stable around 400 μl/10^8 cells (range 375–454; results not shown), indicating that the hepatocytes were not permeabilized for this high-molecular-mass compound. The net distribution volume for [U-^{14}C]sucrose increased from approximately zero to 400–450 μl/10^8 cells, which is not different from the net distribution volume of [U-^{14}C]3-*O*-methylglucose. These results show that addition of α-toxin renders hepatocytes

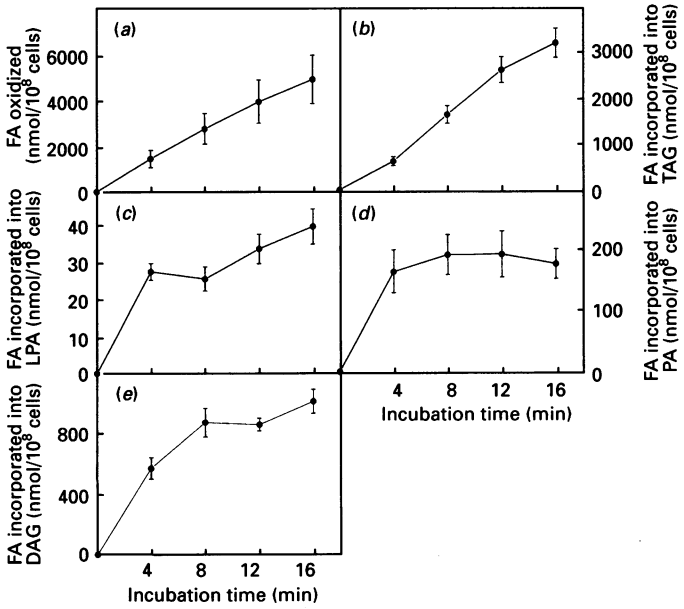

Fig. 2. Time dependence of triacylglycerol synthesis and β-oxidation

Permeabilized cells were incubated with labelled fatty acid and other cofactors as described in the Materials and methods section and in the text. The panels show incorporation of label [nmol of fatty acid (FA)/10^8 cells] into acid-soluble oxidation products (*a*), triacylglycerols (TAG) (*b*), lysophosphatidate (LPA) (*c*), phosphatidate (PA) (*d*) and diacylglycerol (DAG) (*e*), determined as described in the Materials and methods section. The results are means ± S.E.M. for at least three experiments, except for (*a*), which represents means and ranges for two experiments.

permeable to low-molecular-mass compounds, such as sucrose, that thereby acquire a distribution volume equal to that of [U-^{14}C]3-*O*-methylglucose, which penetrates control as well as α-toxin-treated cells. Assuming that the 3-*O*-methylglucose space comes near to the volume of the cytosol and that cytosolic low-molecular-mass solutes can leave the permeabilized cell, it can be calculated that those compounds are diluted roughly 100-fold. Fig. 1 also shows that, under our conditions, near-maximal permeabilization was obtained at 0.025% (w/v) α-toxin. For economical reasons, and because the permeabilized cells were less stable at higher α-toxin concentrations, 0.025% α-toxin was used in all subsequent experiments.

The stability of the permeabilized cells was checked by monitoring the leakage of lactate dehydrogenase, a cytosolic enzyme. During preliminary experiments we found that addition of a macromolecular solute to the incubation medium substantially increased the stability of the permeabilized cells. Table 1 shows lactate dehydrogenase retention as a function of incubation time for hepatocytes treated with 0.025% α-toxin in the absence and presence of different concentrations of BSA; 4–6% (w/v) BSA rendered the α-toxin-treated hepatocytes much more stable. Presumably the presence of the protein in the incubation medium is necessary to compensate for the colloid osmotic pressure of intracellular proteins in the face of the breakdown of the permeability barrier to low-molecular-mass compounds. We also found that dextran F70 (55000–73000 Da) could be substituted for BSA and was equally effective (results not shown). In subsequent incubations a mixture of 2% (w/v) BSA and 3% (w/v) dextran F70 was used; the BSA was added in order to achieve suitable molar ratios of fatty acid to BSA for the study of esterification and oxidation. We then compared leakage of lactate dehydrogenase in permeabilized cells, incubated for

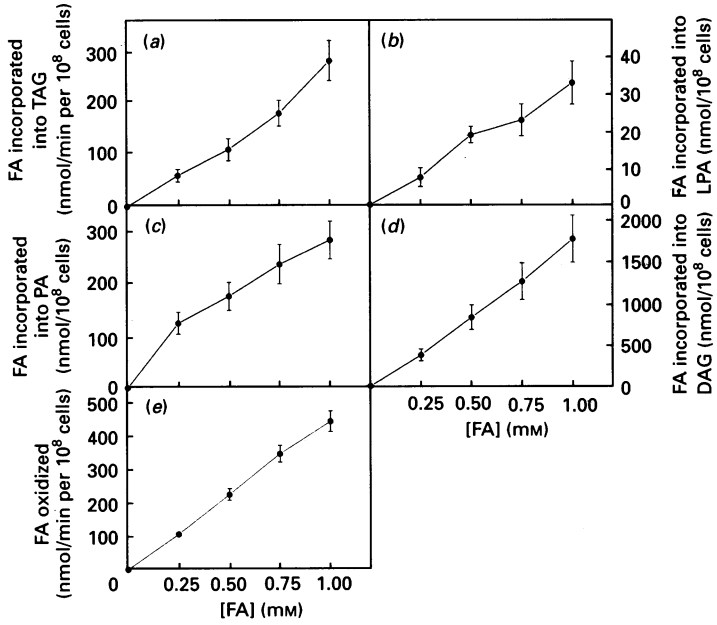

Fig. 3. Fatty acid (FA) dependence of triacylglycerol synthesis and β-oxidation

Permeabilized cells were incubated with increasing concentrations of labelled fatty acid, 1 mM-3-GP and other cofactors as described in the Materials and methods section. Rates of triacylglycerol (TAG) synthesis (*a*) and β-oxidation (*e*), as well as incorporation of label into lysophosphatidate (LPA) (*b*), phosphatidate (PA) (*c*) and diacylglycerol (DAG) (*d*) were determined as described in the Materials and methods section. Results are means ± S.E.M. for five experiments.

12 min in the BSA/dextran-F70-supplemented incubation medium (i.e. the routine incubation medium, as described in the Materials and methods section) with the lactate dehydrogenase leakage in cells incubated in the same medium, except for the omission of α-toxin and Dextran F70 (control cells). Hepatocytes treated with α-toxin retained nearly as much lactate dehydrogenase as control cells: 80.5 ± 1.3% ($n = 21$) versus 90.9 ± 2.1% ($n = 9$). With most of the other permeabilizing agents, a greater leakage of intracellular enzymes occurs (e.g. see Mayorek & Bar-Tana, 1989).

Time dependence of triacylglycerol synthesis and β-oxidation

Fig. 2 shows the time courses of generation of acid-soluble oxidation products (*a*) and of incorporation of labelled fatty acids into triacylglycerols (*b*) and intermediates of the esterification pathway (*c–e*), in permeabilized cells from starved rats, incubated with the necessary cofactors and 1 mM each of 3-GP and labelled fatty acid. Rates of β-oxidation and triacylglycerol synthesis were linear with time for at least 16 min. Although there may have been a slight increase of lysophosphatidate labelling (Fig. 2*c*) in the course of the incubations, phosphatidate labelling (Fig. 2*d*) rapidly reached a steady state. Diacylglycerol labelling (Fig. 2*e*) continued to rise, at least up to 8 min. On the basis of these data, the time points for the calculation of esterification rates and apparent steady-state labelling of intermediates were taken at 6 and 12 min, except for diacylglycerol, for which only the values at 12 min were used (see also the Materials and methods section). The fact that diacylglycerol labelling and, less markedly, lysophosphatidate labelling continued to rise although the rate of triacylglycerol synthesis remained constant suggested to us that diacylglycerol acyltransferase and, possibly, lysophosphatidate acyltransferase might have approached saturation with, respectively, diacylglycerol and lysophosphatidate. More evidence for this is presented below.

Fatty acid dependence of triacylglycerol synthesis and β-oxidation

When α-toxin-treated hepatocytes were incubated with increasing concentrations of labelled fatty acid, in the presence of 1 mM-3-GP and other cofactors, a linear relation was seen between the fatty acid concentration and the rate of triacylglycerol synthesis (Fig. 3*a*). The curve demonstrates the established dependency of esterification on the supply of fatty acid. It is clear that this substrate is a major determinant of the flux through the pathway and it is also obvious that, under our conditions, acyl-CoA synthetase cannot be saturated with fatty acid. The maximum fatty acid concentration that we used was 1 mM, which is in the high physiological range, considering also the rather low albumin concentration in our incubation medium as compared with the normal plasma concentration. Even at 1 mM, there was no indication of saturation of esterification with fatty acid. In fact, incorporation of label into all intermediates continued to rise with increasing fatty acid concentration (Figs. 3*b–3d*). This could indicate that none of the acyl-CoA-utilizing enzymes of triacylglycerol synthesis, i.e. glycerophosphate acyltransferase, lysophosphatidate acyltransferase and diacylglycerol acyltransferase, were saturated with acyl-CoA. However, it has to be taken into account that an increase in fatty acid concentration not only leads to increased acyl-CoA levels but also increases the level of the intermediates of esterification. Thus, for lysophosphatidate acyltransferase as well as for diacylglycerol acyltransferase, both substrates (i.e. respectively, acyl-CoA and lysophosphatidate; acyl-CoA and diacylglycerol) increase in concentration as more fatty acid is supplied. This should lead to an exponential rise in the activity of these enzymes, unless they become saturated with one or other substrate. The latter seems to be the case in our experiments, but it cannot be ascertained from the data of Fig. 3 which of the substrates is at a saturating level.

As expected, the rate of β-oxidation was equally dependent on the fatty acid concentration (Fig. 3*e*). The entry of fatty acids in the permeabilized hepatocytes is presumably unrestricted. Moreover, the rates that we obtained for β-oxidation and triacylglycerol synthesis are very close to what has been seen with intact rat hepatocytes under similar conditions (Debeer *et al.*, 1981). This confirms that the hepatic uptake of long-chain fatty acids does not limit their metabolism (Mayorek & Bar-Tana, 1989).

3-GP dependence of triacylglycerol and phosphatidylcholine synthesis

In the next series of experiments, permeabilized hepatocytes were incubated with increasing concentrations of labelled 3-GP, in the presence of 1 mM unlabelled fatty acid. In some experiments 1 mM-CDP-choline was added to stimulate phosphatidylcholine synthesis maximally. The incorporation of radioactivity was measured in each intermediate of the pathway (lysophosphatidate, phosphatidate and diacylglycerol), as well as in the main end products, triacylglycerol and phosphatidylcholine. The results are shown in Fig. 4. Note that, since radioactive 3-GP was used, only the glycerol esters are labelled and an increasing concentration of the triose phosphate affects only one substrate for each enzyme, in contrast with the experiments with labelled fatty acid. Fig. 4 shows that the synthesis of all intermediates and end products depends on the 3-GP concentration, albeit to varying extents. The 3-GP concentration at which labelling of each intermediate reaches half its maximal value is the result of the kinetic characteristics of the enzymes that synthesize and convert the intermediate. Triacylglycerol synthesis (Fig. 4*a*)

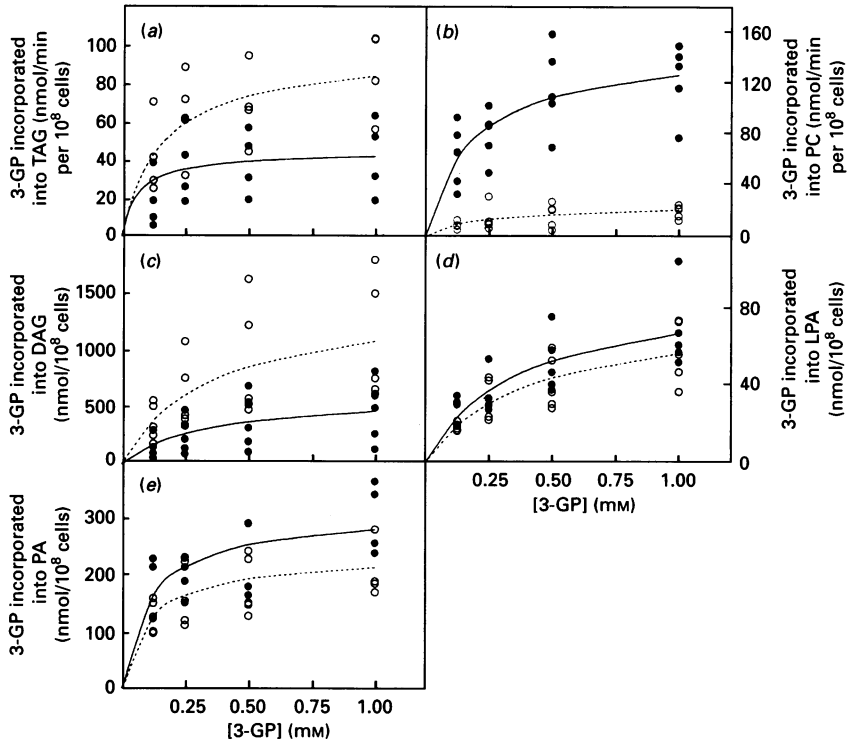

Fig. 4. *sn*-Glycerol 3-phosphate dependence of triacylglycerol and phosphatidylcholine synthesis

Permeabilized cells were incubated with increasing concentrations of labelled 3-GP, 1 mM fatty acid and other cofactors, as described in the Materials and methods section. White symbols (broken lines) and black symbols (continuous lines) indicate the absence and presence, respectively, of 1 mM-CDP-choline in the incubation medium. Rates of triacylglycerol (TAG) synthesis (*a*) and phosphatidylcholine (PC) synthesis (*b*), as well as incorporation of label into diacylglycerol (DAG) (*c*), lysophosphatidate (LPA) (*d*) and phosphatidate (PA) (*e*) were determined as described in the Materials and methods section. The results are individual data points and non-linear regression curves, calculated by assuming Michaelis–Menten kinetics. The curves with and without CDP-choline in (*a*) and (*b*) were significantly different at the 95% level by using an F-test on the residual sums of squares (Alvord *et al.*, 1990).

reached a plateau between 0.25 and 0.50 μmol of 3-GP/ml of incubation medium. Thus, in our system and at 1 mM fatty acid, triacylglycerol synthesis is limited by a 3-GP concentration below approx. 0.25 mM. From previous results (Declercq *et al.*, 1982) it can be calculated that the cytosolic 3-GP concentration in rat hepatocytes *in vivo*, in both nutritional states, is approx. 1 mM, which is sufficiently high to saturate the esterification pathway.

As expected, addition of 1 mM-CDP-choline markedly stimulated phosphatidylcholine synthesis (Fig. 4*b*). On the other hand triacylglycerol synthesis was clearly suppressed, presumably because a significant fraction of the diacylglycerol was diverted to phosphatidylcholine synthesis. This is apparent from the level of labelled diacylglycerol, which fell substantially when CDP-choline was added (Fig. 4*c*). These data agree with the results obtained by Mayorek & Bar-Tana (1989). Interestingly, diacylglycerol levels at 6 and 12 min were not significantly different in the presence of CDP-choline (results not shown), whereas, in its absence, diacylglycerol accumulated between those two time points (see also Fig. 2). These experiments indicate that diacylglycerol acyltransferase and cholinephosphotransferase (EC 2.7.8.2) share a common pool of diacylglycerol and that the availability of this intermediate can limit triacylglycerol synthesis. The labelling of lysophosphatidate (Fig. 4*d*) and phosphatidate (Fig. 4*e*) was not altered significantly by CDP-choline.

More information on the kinetic characteristics of the separate enzymes of glycerolipid synthesis can be obtained by plotting the flux through each enzymic step as a function of the corresponding substrate level. This alternative representation of the data is given in Fig. 5. In Fig. 5(*a*) the combined rates of synthesis of triacylglycerol and phosphatidylcholine, measured in the absence and presence of CDP-choline, are shown as a function of 3-GP concentration. Although omitting CDP-choline appeared to have a suppressive effect, this was compensated for by the accumulation of diacylglycerol under these circumstances (see above). The 'flux' through the pathway was therefore calculated as the combined rates of synthesis, between 6 and 12 min, of triacylglycerol, phosphatidylcholine and diacylglycerol (Fig. 5*b*). This was unaffected by the presence of CDP-choline and actually represents the activity of glycerophosphate acyltransferase. As shown in Fig. 5(*b*), this enzyme becomes saturated at 3-GP concentrations of 0.25–0.50 mM, which is compatible with earlier work (Declercq *et al.*, 1982) showing a K_m of glycerophosphate acyltransferase for 3-GP of 0.16 mM, as measured in homogenates from hepatocytes of starved rats at a molar palmitoyl-CoA/BSA ratio of 0.65. Mayorek & Bar-Tana (1989), working with cultured hepatocytes permeabilized with lysophosphatidylcholine, obtained an apparent K_m of neutral lipid synthesis for 3-GP ranging from 0.8 to 3.0 mM. The reason for the discrepancy is not clear, but it may be related to the use of a different permeabilizing agent.

The lysophosphatidate–flux relationship (Fig. 5*c*) is also curvilinear, reflecting accumulation of lysophosphatidate. We consider this as evidence that lysophosphatidate acyltransferase approaches saturation with lysophosphatidate at saturating 3-GP concentrations. Although the curves were obtained by non-linear regression analysis, the wide scatter of the data, which is inherent in this type of experiment, and the possible accumulation

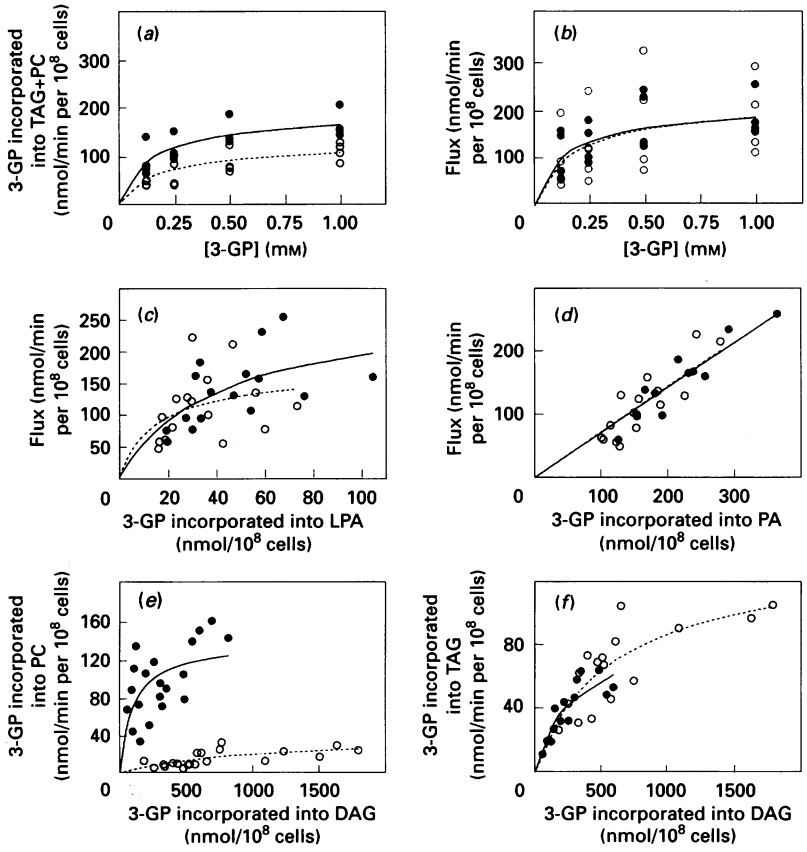

Fig. 5. Activity of the individual steps of esterification as a function of the respective substrates

The data are taken from Fig. 4 with the following re-arrangements. The ordinates show the rate of incorporation of labelled 3-GP (nmol/min per 10^8 cells) into triacylglycerol (TAG) + phosphatidylcholine (PC) (*a*), triacylglycerol + phosphatidylcholine + diacylglycerol ('flux': *b*, *c* and *d*), into phosphatidylcholine (*e*) and into triacylglycerol (*f*). The abscissae show the 3-GP concentration (*a*, *b*), and the incorporation of labelled 3-GP (nmol/10^8 cells) into lysophosphatidate (LPA) (*c*), phosphatidate (*d*) and diacylglycerol (*e*, *f*). Panels (*a*), (*b*), (*c*), (*e*) and (*f*) represent non-linear-regression curves, calculated by assuming Michaelis–Menten kinetics. The plot in panel (*d*) was obtained by linear regression.

of lysophosphatidate in the lipid phase of membranes make calculation and comparison of apparent K_m values pointless.

The phosphatidate–flux relationship (Fig. 5*d*) has a very different appearance. There is no indication that phosphatidate phosphohydrolase can be saturated with its substrate under our conditions. The linear relationship suggests that phosphatidate phosphohydrolase merely propagates the flux, without limiting it.

The diacylglycerol–phosphatidylcholine relationship (Fig. 5*e*) suggests a very strong dependence of phosphatidylcholine synthesis on the diacylglycerol level, at least in the presence of 1 mM-CDP-choline, which is almost certainly saturating for cholinephosphotransferase (Kanoh & Ohno, 1976). This is in agreement with the results obtained by Lim *et al.* (1986). Groener *et al.* (1979) concluded that the microsomal level of diacylglycerol is not important for the rate of synthesis of phosphatidylcholine, although their data do not necessarily contradict our conclusions.

Fig. 5(*f*) illustrates that diacylglycerol acyltransferase reaches saturation with diacylglycerol at high 3-GP levels and without addition of exogenous CDP-choline. However, when phosphatidylcholine synthesis is maximally stimulated by a high concentration of CDP-choline, diacylglycerol decreases to non-saturating levels.

General discussion

Regulation of triacylglycerol synthesis, as it emerges from our experiments and those of other investigators, is clearly multifactorial. Our main focus has been on the role of the different substrates and on the interaction with pathways that compete for common substrates or intermediates. 3-GP determines glycerolipid synthesis below 0.25 mM, but the physiological role of this substrate appears to be limited when it is taken into account that the level *in vivo* is sufficiently high to saturate the pathway (Declercq *et al.*, 1982).

The data suggest that glycerophosphate acyltransferase itself sets the maximal activity or flux of total glycerolipid synthesis, since none of the intermediate glycerol esters accumulate in sufficiently large amounts to be indicative of complete saturation of any of the subsequent enzymes. Evidently, a weak point in this analysis is that we do not know how far hydrolases, which are ubiquitously present in the cell, may limit accumulation of such intermediates. We also appreciate the fact that, since we studied incorporation of label into the lipids, we did not measure their true concentrations if there was significant dilution of radioactivity by endogenous material. However, we do not believe the latter was the case, for the following reasons. First, since we used starved rats, the endogenous lipogenesis was low. Second, the endogenous 3-GP was diluted in the incubation medium (see above). Third, the linearity of the time curves and concentration curves argues against significant dilution of radioactive fatty acid. Lastly, the rate of triacylglycerol synthesis measured with 1 mM labelled fatty acid, in the presence of 1 mM unlabelled 3-GP, equalled the rate measured with 1 mM labelled 3-GP, in the presence of 1 mM unlabelled fatty acid, taking into account that 3 mol of fatty acid are incorporated per mol of 3-GP. At saturating 3-GP levels, and when the fatty acid concentration is

fixed, lysophosphatidate acyltransferase approaches its maximal activity, as does diacylglycerol acyltransferase in the presence of low CDP-choline concentrations. Under these conditions triacylglycerol synthesis reaches its maximal rate. The CDP-choline level appears to be of importance: it limits the rate of phosphatidylcholine synthesis and, because diacylglycerol acyltransferase and cholinephosphotransferase draw from the same pool of diacylglycerol, it indirectly determines the rate of triacylglycerol synthesis as well. In the presence of saturating concentrations of CDP-choline, diacylglycerol no longer accumulates, but rather decreases to levels that are not saturating for diacylglycerol acyltransferase and thus rate-limiting for that last enzyme of the triacylglycerol synthesis pathway. These data also suggest that the fat accumulation in livers of choline-deficient rats (Haines & Rose, 1970) may partly be caused by increased triacylglycerol synthesis and not only by suppression of lipid export owing to phosphatidylcholine deficiency (Pelech & Vance, 1984). Interestingly, in the presence of sufficient CDP-choline, the affinity of cholinephosphotransferase for diacylglycerol seems to be several-fold higher than that of diacylglycerol acyltransferase (compare Figs. 5*e* and 5*f*), so that at low diacylglycerol levels the synthesis of phosphatidylcholine is favoured. This conclusion has been reached by others through separate lines of evidence (Groener & Van Golde, 1977; Groener *et al.*, 1979). Under our conditions phosphatidate phosphohydrolase, which has often been considered as the rate-limiting enzyme of triacylglycerol synthesis (Brindley, 1984; Björkhem *et al.*, 1984; Pittner *et al.*, 1985), appears to have no regulatory impact.

The substrate the supply of which is most susceptible to fluctuation under conditions *in vivo* is fatty acid. At saturating 3-GP concentrations, there was no saturation of the esterification pathway with fatty acid at a concentration as high as 1 mM. Since, under these conditions, glycerophosphate acyltransferase, lysophosphatidate acyltransferase and diacylglycerol acyltransferase (low CDP-choline concentrations) were saturated or nearly saturated with 3-GP, lysophosphatidate and diacylglycerol respectively, it is hard to conceive that these enzymes were already saturated with acyl-CoA. Thus fatty acids, and as a consequence the intracellular acyl-CoA levels, appear to determine the rate of esterification over a wide concentration range. The fatty acid dependence of triacylglycerol synthesis is presumably governed by the acyltransferase which has the highest K_m for acyl-CoA. However, our experiments do not allow us to identify this enzyme. In conclusion, some new insights on the regulation of triacylglycerol synthesis have been obtained from our experiments, in which we have presented an integrated view of the pathway and its interactions with other metabolic pathways that compete for a common substrate or intermediate. This would not have been feasible without the use of permeabilized hepatocytes which, at present, constitute the only experimental system to study this complex pathway *in situ*, with very little restriction for substrates and cofactors.

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