Oleic acid promotes the activation and translocation of phosphatidate phosphohydrolase from the cytosol to particulate fractions of isolated rat hepatocytes

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1. The incubation of hepatocytes with 1–4 mM-oleate increased the total activity of phosphatidate phosphohydrolase that was measured in the presence of Mg^{2+} to about 2-fold. This was accompanied by an increase in the proportion of the enzyme that was isolated with the particulate fractions. 2. Conversely, the addition of up to 4 mM-oleate decreased the recovery of phosphatidate phosphohydrolase in the cytosolic fraction from about 70% to 3% when hepatocytes were lysed with digitonin. 3. Most of the increase in the membrane-associated phosphohydrolase activity was isolated after cell fractionation in the microsomal fraction that was enriched with the endoplasmic-reticulum marker arylesterase. 4. It is proposed that the translocation of phosphatidate phosphohydrolase facilitates the increased synthesis of triacylglycerols in the liver when it is presented with an increased supply of fatty acids.

PAP appears to play an important role in regulating the synthesis of phospholipids and triacylglycerols in the liver. This is based on the observations that changes in the soluble and microsomal PAP activities in different physiological and pharmacological conditions are normally much greater than for other enzymes involved in triacylglycerol synthesis (for review see Brindley & Sturton, 1982). These changes generally parallel the capacity or the potential of the liver to synthesize triacylglycerols. Furthermore, the accumulation of phosphatidate bears an inverse relationship to PAP activity in various physiological conditions (Brindley & Sturton, 1982).

The changes in PAP activity are partly mediated through alterations in hormonal balance. Glucocorticoids increase the synthesis of PAP (Lehtonen *et al.*, 1979; Jennings *et al.*, 1981), whereas insulin suppresses this induction (Lawson *et al.*, 1982*a,b*). Vasopressin (and probably other hormones that cause Ca^{2+} mobilization) stimulate the activity within 5 min (Pollard & Brindley, 1984).

Several different PAP activities occur in cells,

Abbreviation used: PAP, phosphatidate phosphohydrolase (EC 3.1.3.4).

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but the one that is thought to be the most important in glycerolipid synthesis is the activity that is stimulated by Mg^{2+} (Brindley & Sturton, 1982). A large proportion of this activity is normally isolated with the soluble proteins after cell fractionation. This is intriguing, since the other enzymes of triacylglycerol synthesis are tightly membrane-bound, as is the phosphatidate that acts as a substrate for PAP. It is possible that the Mg^{2+} -stimulated PAP dissociates from the endoplasmic reticulum during fractionation. This idea is supported by the finding that addition of KCl to the homogenization medium can increase the microsomal PAP activity from adipocytes, while decreasing the activity in the soluble fraction (Moller & Hough, 1982). However, we have not been able to reproduce these results with rat liver (S. C. Butterwith & D. N. Brindley, unpublished work). Alternatively, it has been suggested that a further level of metabolic control could be exerted by cells if they were able to regulate the reversible binding of PAP to the endoplasmic reticulum (Brindley, 1974). Some experimental evidence for this was provided by Moller *et al.* (1980), who demonstrated with adipocytes that lipolytic agents could increase the microsomal PAP activity and decrease the soluble activity.

The purpose of the present work was to investigate whether the translocation of PAP could be

demonstrated with hepatocytes. It has previously been shown that the inhibition of the synthesis of triacylglycerol and phosphatidylcholine that is produced by cyclic AMP analogues in these cells could be reversed by adding fatty acids to the incubation medium (Pelech *et al.*, 1983b). The restoration of the synthesis of phosphatidylcholine was accompanied by a translocation of CTP:phosphocholine cytidyltransferase (EC 2.7.7.15) from the cytosol to the endoplasmic reticulum. It therefore seemed likely that fatty acids might also promote the translocation of PAP. This, in fact, proved to be the case.

Experimental

Materials

Collagenase (from *Clostridium histolyticum*) was obtained from Boehringer Corp. (London); heparin, digitonin, choline chloride, gentamycin, streptomycin and penicillin-G were from Sigma (London) Chemical Co.; collagen (Vitrogen 100™) was from Flow Laboratories, Irvine, Scotland, U.K.; and newborn-calf serum and Leibovitz L-15 medium were from Gibco Bio-Cult, Hounslow, Middx., U.K. The sources of other reagents have been described previously (Jennings *et al.*, 1981; Lawson *et al.*, 1982a,b).

Preparation and incubation of hepatocytes

Hepatocytes were prepared by a method based on that of Evans (1981). Male Wistar rats were anaesthetized (Jennings *et al.*, 1981) and injected with 1000 units of heparin in 0.2 ml of 0.9% NaCl via the femoral vein. A catheter was inserted into the superior vena cava, the inferior vena cava was clamped and the hepatic portal vein was cut. The liver was then perfused for about 10 min in a non-circulating manner with Krebs-Henseleit (1932) buffer containing 45 µg of penicillin-G and 50 µg of streptomycin/ml and equilibrated with O₂/CO₂ (19:1). During this process the stomach and intestine were removed and the diaphragm was cut. The liver was lifted clear of the rat and placed on a nylon gauze that was suspended over a plastic funnel. Collagenase (40 mg) was added to give a final concentration of 0.2 mg/ml of medium, and this was recirculated through the liver at a flow rate of 30 ml/min for 30–40 min. The liver was then cut with scissors in about 100 ml of perfusion medium with gentle agitation and the suspension of cells was strained through a coarse nylon gauze. The hepatocytes were then filtered through a 142 µm stainless-steel mesh and centrifuged at 50g for 2 min. They were then washed three times by resuspension in the modified Krebs-Henseleit buffer and re-centrifuged. Finally the hepatocytes were washed once and then suspended in Leibovitz L-15

medium that was supplemented with 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate] adjusted to pH 7.4 with KOH, 8.3 mM-glucose, 50 µg of gentamycin/ml, 50 µg of streptomycin/ml, 100 units of penicillin-G/ml and a final concentration of 28.6 µM-choline chloride. This medium was equilibrated with air. After filtration with a 62 µm stainless-steel mesh, about 4 × 10⁸ hepatocytes were obtained, and 80–88% of the cells excluded Trypan Blue.

About 2 × 10⁶ hepatocytes were pipetted into 60 mm-diam. tissue-culture dishes that were coated with collagen and which contained a final volume of 3 ml of modified Leibovitz medium (as above) to which was added 10% (v/v) of newborn-calf serum. The dishes were incubated for 1–2 h at 37°C in air, and the unattached and non-viable cells were removed together with the medium. The monolayer of cells was then incubated for a further 15–18 h with fresh medium, after which the medium was exchanged. The incubation was continued for 2 h, and the medium was then replaced with 3 ml of new medium that contained the required concentration of oleate. This was achieved by adding a solution of potassium oleate (80 mM in a 20% molar excess of KOH). The hepatocytes were then incubated for a further 1 h at 37°C, after which the incubation medium was removed. The cells were washed twice with the modified Leibovitz medium that did not contain serum, and they were then subjected to cell fractionation, or lysed with digitonin.

Lysis of the hepatocytes with digitonin

Cytosolic enzymes were released from the hepatocytes by a method based on that of Mackall *et al.* (1979). The culture dishes were placed on an ice-cold stainless-steel plate and 1.5 ml of an ice-cold solution of 0.25 M-sucrose containing 0.5 mM-dithiothreitol, 10 mM-Hepes adjusted to pH 7.4 with KOH, and 4.07 mM-digitonin (dispersed by sonication) was added. Three samples of this suspension (each 0.4 ml) were removed at 1 min intervals, and the remainder was collected on the fourth minute. The cell ghosts were then quickly scraped from the plates in 1 ml of an ice-cold solution of 0.25 M-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes, pH 7.4, and the mixture was sonicated at 22 kHz with an amplitude of 8 µm peak-to-peak. The undissolved digitonin was removed from the cytosolic fractions by centrifuging for 10 min at 6000 rev./min and 4°C in a bench centrifuge. Cell fractions were stored at –20°C.

Fractionation of the hepatocytes

The hepatocytes were scraped from each plate in 1 ml of ice-cold 0.25 M-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes, pH 7.4, and the contents of six dishes incubated in parallel were

combined in duplicate. The suspensions were homogenized with five strokes up and down in a Teflon/glass homogenizer, followed by sonication for 10 s at 22 kHz with an amplitude of 4 μ m peak-to-peak. A portion (80%) of these homogenates was centrifuged for 10 min at 20 000 g ($r_{av.} = 6.44$ cm) at 4°C. The pellets were resuspended in 2 ml of the same medium by homogenization and sonicated for 5 s at 22 kHz, and the suspensions were centrifuged as above. The pellets were again suspended in 2 ml of sucrose medium, and the combined supernatants were centrifuged for 1 h at 180 000 g ($r_{av.} = 6.44$ cm) at 4°C. The microsomal pellets were resuspended in 1 ml portions of the sucrose medium. Cell fractions were stored at -20°C.

Assessment of cell viability

This method of culturing hepatocytes was adopted, since it is known to provide an experimental model that is metabolically stable for several days (Evans, 1981; Evans & Mayer, 1982, 1983). This is indicated by the high percentage retention of lactate dehydrogenase activity, and the maintenance of stable rates for protein turnover. We can also routinely induce the synthesis of tyrosine aminotransferase and PAP by adding 0.1 μ M-dexamethasone, and obtain 3–6-fold increases in these activities in 6–8 h (E. H. Mangiapane & R. A. Pittner, unpublished work).

Measurement of enzyme activities

The activities of lactate dehydrogenase (Saggerson & Greenbaum, 1969), arylesterase (Shephard

& Hübscher, 1969) and PAP (Pollard & Brindley, 1984) were determined as described.

Results

Mackall *et al.* (1979) showed that cytosolic enzymes can be released rapidly from cells with digitonin. There is minimum disruption of the organelles, and membrane-bound enzymes are retained. This technique was therefore chosen in order to investigate the relationship between cytosolic and membrane-associated PAP activity. Fig. 1(a) confirms the work of Mackall *et al.* (1979), in that digitonin produces a rapid release of lactate dehydrogenase activity from the cells. This enzyme was used as a marker for the cytosolic compartment. After 4 min 61–69% of the lactate dehydrogenase was released, and the addition of oleate did not significantly alter this process.

By contrast, only about 43% of the PAP was released after 4 min in the absence of oleate. The addition of oleate progressively decreased the amount of PAP that was found in the digitonin extract (Fig. 1b). The only apparent anomaly was the higher release of the PAP in the presence of 0.5 mM-oleate at shorter times. This effect was also seen in three out of four independent experiments. The remaining PAP activity was recovered in the hepatocyte ghosts. The addition of oleate promoted the retention of this activity in the cells at the expense of that which was released, and it also increased the total PAP activity (Fig. 2). This increase was $198 \pm 74\%$ (mean $\pm$ s.d. for four inde-

Fig. 1. Effect of oleate on the digitonin-mediated release of lactate dehydrogenase (a) and PAP (b) from rat hepatocytes. The hepatocytes were incubated for 1 h in the absence of oleate (○), or in the presence of (Δ) 0.5 mM-, (□) 1.0 mM-, (●) 2.0 mM- or (▲) 4.0 mM-oleate. The incubation medium was replaced with 1.5 ml of an ice-cold solution containing 0.5 mg of digitonin/ml (see the Experimental section). Samples of the digitonin extract were removed at time intervals up to 4 min and assayed for enzyme activity. The values are the means for three dishes, and similar results were obtained in three further independent experiments.

pendent experiments) when the oleate concentration was 4 mM.

Fig. 3 shows the percentage distribution of PAP that was estimated to be cytosolic or membrane-associated. These values were calculated by assuming that all of the lactate dehydrogenase was cytosolic. The PAP activities at 4 min (Fig. 1) were corrected by factors that compensated for the incomplete release of lactate dehydrogenase in order to give the total cytosolic PAP. The membrane-associated PAP was determined by subtracting the cytosolic PAP activity that was estimated to have been retained in the cell ghosts from that depicted in Fig. 2. It was estimated that 70% of the PAP was cytosolic when no oleate was added to the medium used for incubating the hepatocytes. This value fell to about 3% when the concentration of oleate was raised to 4 mM.

The oleate-induced translocation of PAP could also be observed when the hepatocytes were subjected to cell fractionation. In the absence of oleate, about 70% of the PAP activity was in the cytosolic fraction (Figs. 4a and 4c), and about 3% of the activity was in the microsomal fraction, which also contained about 35% of the arylesterase activity (Fig. 4b). This latter enzyme was used as a marker for the endoplasmic reticulum. Most of the remaining arylesterase activity (52%) was in the low-speed pellet, which contained about 26% of the

PAP activity. This is more than would be expected from contamination by the endoplasmic reticulum. However, PAP is known to occur in mitochondria, plasma membranes and lysosomes (see Brindley & Sturton, 1982).

Most of the increase in PAP activity that was induced by oleate occurred in the microsomal fraction. This activity rose from 3% in the absence of oleate to 36% in the presence of 4 mM-oleate. A parallel increase in the proportion of PAP activity should have occurred in the low-speed pellet, since this also contained about 40–50% of the arylesterase activity (Fig. 4). However, there was no increase in the PAP activity in this fraction, except at 4 mM-oleate. Concentrations of oleate greater than 3 mM caused some release of the arylesterase from the endoplasmic reticulum into the cytosolic fraction (Fig. 4b). A similar effect was seen with the same preparation of hepatocytes in the experiments described in Figs. 1–3, where 21% of the arylesterase activity was released by digitonin at 4 mM-oleate. The equivalent value for incubations that did not contain oleate was 7%.

The assays for PAP were also performed in the absence of Mg^{2+} , but in the presence of 1 mM-EDTA. For the experiment shown in Fig. 4, no significant PAP activity was observed in the absence of oleate. With 3 mM- and 4 mM-oleate the PAP activity in the total homogenate was 7 and 23% respectively of the Mg^{2+} -stimulated activity. This, and the similar effects in other experiments,

Fig. 2. Effect of oleate on the activity and distribution of PAP activity in rat hepatocytes

Hepatocytes were incubated as described in Fig. 1, and the activity of PAP in the digitonin extract at 4 min (■), in the cell ghosts (▲) and the total activity (●) is shown. The values are the means for three dishes, and similar results were obtained in three further independent experiments.

Fig. 3. Effect of incubating hepatocytes with oleate on the percentage distribution of PAP between the cytosolic and membrane-associated compartments

The distribution of PAP in the cytosolic (■) and membrane-associated (▲) compartments was calculated from the results shown in Figs. 1 and 2 as described in the Results section.

Fig. 4. Effect of oleate on the subcellular distribution of (a) lactate dehydrogenase, (b) arylesterase and (c) PAP from isolated hepatocytes

Hepatocytes were incubated for 1 h in the presence of various concentrations of oleate, and the percentage distributions of the enzymes in the low-speed pellet (Δ), the microsomal fraction ($\circ$), the low-speed pellet plus the microsomal fraction ($\blacktriangle$) and in the soluble fraction ($\blacksquare$) were determined. The average recoveries relative to activities in the homogenate for lactate dehydrogenase, arylesterase and PAP were 96%, 98% and 130% respectively. The values are the means for duplicate fractionations for each concentration of oleate. Similar results were obtained in two further independent experiments.

demonstrates that the activities quoted in Figs. 1–4 are predominantly those of the Mg^{2+} -stimulated PAP.

Discussion

This paper provides some potential answers to the problems involved in understanding the role of PAP in regulating the synthesis of glycerolipids in the liver. It provides evidence that the Mg^{2+} -stimulated PAP activity in the cytosol of hepatocytes is able to associate with the membrane fractions, where glycerolipid synthesis occurs. The magnitude of this translocation was greater when this was estimated after lysing the cells with digitonin (Figs. 1–3) rather than after a more conventional cell fractionation (Fig. 4). The vigorous homogenization and the greater time taken for the latter separation probably leads to some loss of PAP from the particulate fractions. However, both of these techniques can be used to demonstrate that adding oleate to the incubation medium promotes the translocation of the cytosolic PAP to the membrane-associated compartment. This was accompanied by an increase in the total PAP activity (Fig. 2).

At present, we do not know the mechanisms that cause these changes to take place. It is also difficult to be entirely certain as to which membranes the PAP attaches. The endoplasmic reticulum is likely to be involved (Fig. 4), but there was not a complete parallel with the distribution of arylesterase (Fig. 4). Oleate could also cause a redistribution of PAP activity between mitochondria and the endo-

plasmic reticulum, since both of these are potential sites of glycerolipid synthesis in the liver (Brindley & Sturton, 1982). A more extensive cell fractionation is required before these questions can be fully answered. However, it is probable that PAP can be included in the list of enzymes that have collectively been termed 'ambiquitous' (Wilson, 1980), i.e. that it can exist in different subcellular compartments, and its translocation is involved in the regulation of its activity.

The addition of fatty acids to the medium in which hepatocytes are incubated has been widely reported to stimulate glycerolipid synthesis (see Pelech *et al.*, 1983b). The present studies demonstrate that this is accompanied by the loss of PAP activity from the cytosol and its association with particulate fractions. Thus, in assessing the role of PAP in controlling glycerolipid synthesis, it is necessary to take this into account rather than simply considering the total activity, which only indicates the maximum potential.

The results in Figs. 1–4 were obtained in the presence of 10% newborn-calf serum, and so the albumin concentrations were correspondingly lower than *in vivo*. Consequently the concentrations of free oleate in some incubations were very high relative to those encountered physiologically. Therefore, the physiological significance of the effects that are demonstrated in this paper still needs to be established.

In vivo, fatty acid concentrations in the blood increase markedly in stress and in ketotic diabetics. When fatty acids are taken up by the liver in these conditions they are preferentially oxidized, es-

pecially when their concentrations are relatively low. These changes are partly brought about by the high concentrations of cyclic AMP, which decrease fatty acid synthesis and increase β -oxidation, and which can decrease the synthesis of triacylglycerol and phosphatidylcholine (Pelech *et al.*, 1983b). However, high concentrations of fatty acids reverse the cyclic AMP-induced inhibition in the synthesis of triacylglycerol and phosphatidylcholine (Pelech *et al.*, 1983b). Part of this latter effect results from the stimulation and translocation of the regulatory enzyme CTP:phosphocholine cytidyltransferase from the cytosol to the endoplasmic reticulum (Pelech *et al.*, 1983a,b). We have now shown that a fatty acid can also cause a similar translocation of PAP. This indicates that there is a co-ordinated control in the synthesis of triacylglycerol and phosphatidylcholine in this respect.

The synthesis of PAP in the liver is increased in stress and diabetes, and this is caused by the increased ratio of glucocorticoids (and possibly other stress hormones) relative to insulin (Lawson *et al.*, 1982a,b). These conditions also cause the mobilization of fatty acids from adipose tissue. The increased availability of fatty acids could activate PAP in the liver and cause its translocation from the cytosol to the membranes on which glycerolipids are synthesized. These events could provide the liver with the ability to match its capacity to synthesize triacylglycerols to the fatty acid load that it is receiving. Thus the stimulated release of corticosterone *in vivo* is accompanied by increases in PAP activity in both microsomal and soluble fractions of the liver (Sturton *et al.*, 1978; Brindley *et al.*, 1979). High rates of fatty acid supply cause the liver either to become fatty, or to increase its secretion of very-low-density lipoprotein (Brindley & Sturton, 1982; Brindley & Lawson, 1983). The latter event also requires a co-ordinated increase in the synthesis of phosphatidylcholine, which the stimulated activity of CTP:phosphocholine cytidyltransferase could provide.

PAP activity is also known to reach a peak in its diurnal cycle at the time when the rats begin to feed and when their serum insulin concentrations rise (Knox *et al.*, 1979). In this situation fatty acid synthesis in the liver increases and the endogenous acids could activate PAP and the cytidyltransferase. This could also facilitate the synthesis of triacylglycerol and phosphatidylcholine and the subsequent release of very-low-density lipoproteins.

PAP is known to exist in the cytosolic fraction of a variety of tissues, including liver, small intestine, lung and adipose (Brindley & Sturton, 1982). It is proposed that the activation and the translocation of PAP from the cytosol to membranes that are res-

ponsible for glycerolipid synthesis could provide an additional level of metabolic regulation.

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