Interactions of insulin, glucagon and dexamethasone in controlling the activity of glycerol phosphate acyltransferase and the activity and subcellular distribution of phosphatidate phosphohydrolase in cultured rat hepatocytes

Richard A. PITTNER*, Robin FEARS† and David N. BRINDLEY*‡

*Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K., and †Beecham Pharmaceuticals, Biosciences Research Centre, Great Burgh, Yew Tree Bottom Road, Epsom KT18 5XQ, U.K.

(Received 26 March 1985/25 April 1985; accepted 2 May 1985)

1. Rat hepatocytes were incubated in monolayer culture for 8 h. 2. Glucagon (10 nM) increased the total phosphatidate phosphohydrolase activity by 1.7-fold. This effect was abolished by adding cycloheximide, actinomycin D or 500 pM-insulin to the incubations. The glucagon-induced increase was synergistic with that produced by an optimum concentration of 100 nM-dexamethasone. 3. Theophylline (1 mM) potentiated the effect of glucagon, but it did not affect the dexamethasone-induced increase in the phosphohydrolase activity. 4. The relative proportion of the phosphohydrolase activity associated with membranes was decreased by glucagon when 0.15 mM-oleate was added 15 min before the end of the incubations to translocate the phosphohydrolase from the cytosol. This glucagon effect was not seen at 0.5 mM-oleate. Since glucagon also increased the total phosphohydrolase activity, the membrane-associated activity was maintained at 0.15 mM-oleate and was increased at 0.5 mM-oleate. This activity at both oleate concentrations was also increased in incubations that contained dexamethasone, particularly in the presence of glucagon. 5. Insulin increased the relative proportion of phosphatidate phosphohydrolase that was associated with membranes at 0.15 mM-oleate, but not at 0.5 mM-oleate. It also decreased the absolute phosphohydrolase activity on the membranes at both oleate concentrations in incubations that also contained glucagon and dexamethasone. 6. None of the hormonal combinations significantly altered the total glycerol phosphate acyltransferase activity. However, glucagon significantly increased the microsomal activities, and insulin had the opposite effect. Glucagon also decreased the mitochondrial acyltransferase activity. 7. There was a highly significant correlation between the total phosphatidate phosphohydrolase activity and the synthesis of neutral lipids from glycerol phosphate and 0.5 mM-oleate in homogenates of cells from all of the hormonal combinations. 8. Phosphatidate phosphohydrolase activity is increased in the long term by glucocorticoids and also by glucagon through cyclic AMP. In the short term, glucagon increases the concentration of fatty acid required to translocate the cytosolic reservoir of activity to the membranes on which phosphatidate is synthesized. Insulin opposes the combined actions of glucagon and glucocorticoids. The long-term events explain the large increases in the phosphohydrolase activity that occur *in vivo* in a variety of stress conditions. The expression of this activity depends on increases in the net availability of fatty acids and their CoA esters in the liver.

Abbreviations used: GPAT, glycerol phosphate acyltransferase (EC 2.3.1.15); PAP, phosphatidate phosphohydrolase (EC 3.1.3.4).

‡ To whom reprint requests should be addressed.

PAP activity in the liver is subject to both acute and long-term control. Its activity is increased over a period of hours by glucocorticoids (Lehtonen *et al.*, 1979; Jennings *et al.*, 1981) and by cyclic AMP

analogues (Pittner *et al.*, 1985). These two effects are approximately additive (Pittner *et al.*, 1985). They appear to be related to stimulations of PAP synthesis, since the increases can be prevented by inhibition of DNA transcription or protein synthesis (Lehtonen *et al.*, 1979; Jennings *et al.*, 1981; Pittner *et al.*, 1985). The increase in PAP activity produced by glucocorticoids (Lawson *et al.*, 1982a; Pittner *et al.*, 1985) could be antagonized by insulin. However, insulin did not reverse the effects of the cyclic AMP analogues, but this might have resulted from the relative stability of the analogues to degradation by phosphodiesterase, which would have prevented one of the known actions of insulin (Pittner *et al.*, 1985). One reason for the present studies was to determine whether glucagon could increase PAP activity in hepatocytes and whether insulin could overcome this effect. If so, then these combined effects of glucocorticoids, glucagon and insulin could explain why PAP activity in the liver rises in a variety of physiological and pathological conditions in which metabolism is controlled by stress hormones rather than by insulin (Brindley & Sturton, 1982; Brindley, 1985).

The functional expression of the large increases in PAP activity that occur in stress conditions appears to depend on the increased availability of fatty acids and acyl-CoA esters in the liver, which results from the stimulation of lipolysis in adipose tissue. These compounds cause the translocation of PAP from the cytosol to the endoplasmic reticulum, on which glycerolipid synthesis occurs (Cascales *et al.*, 1984; Martin-Sanz *et al.*, 1984). The effectiveness of the fatty acid supply in promoting the translocation of PAP to the endoplasmic reticulum can be modified in hepatocytes by cyclic AMP analogues (Butterwith *et al.*, 1984), and inhibited by chlorpromazine (Brindley, 1985). In cell-free systems, spermine facilitates the action of oleate in promoting the translocation (Martin-Sanz *et al.*, 1985). No information is yet available as to whether hormones that mobilize intracellular Ca^{2+} affect the translocation. However, vasopressin can stimulate PAP activity in hepatocytes within 5 min, and it can stimulate glycerolipid synthesis from added oleate (Pollard & Brindley, 1984). It is believed that the cytosolic PAP constitutes an inactive reservoir of activity that can become metabolically functional when PAP interacts with the endoplasmic reticulum (Brindley, 1985). A further objective of the present work was to examine the interactions of glucocorticoids, glucagon, insulin and fatty acids on the subcellular distribution of PAP in hepatocytes.

Finally, studies were performed to determine how glucocorticoids, glucagon and insulin might interact to control the GPAT activity. Evidence has been provided that mitochondrial GPAT

activity in perfused liver can be increased by insulin, but that the microsomal activity is not significantly affected (Bates & Saggerson, 1977; Bates *et al.*, 1977). There is also indirect evidence from experiments *in vivo* that the mitochondrial and microsomal GPAT activities might be increased by glucocorticoids (Bates & Saggerson, 1979, 1981; Lawson *et al.*, 1981). Other studies with suspension cultures of hepatocytes failed to detect glucocorticoid- or insulin-induced changes in either GPAT activity (Lawson *et al.*, 1982b). The monolayer culture system used for the present work was more stable and responsive to the effects of hormones in controlling PAP activity (Pittner *et al.*, 1985), and therefore it should provide a better model for studying the regulation of GPAT activity.

Experimental

Animals and materials

The sources of the rats and most of the materials have been described (Pollard & Brindley, 1984; Cascales *et al.*, 1984; Pittner *et al.*, 1985). Glucagon and theophylline were from Sigma (London) Chemical Co.

Preparation and incubation of hepatocytes

The preparation of hepatocytes was as described by Cascales *et al.* (1984). The hepatocytes were attached without using collagen to Primaria tissue-culture dishes (Falcon) in a modified Liebowitz L15 medium containing 10% (v/v) newborn-calf serum. They were subsequently maintained under serum-free conditions in media containing 0.2% (w/v) fatty-acid-poor bovine serum albumin (Pittner *et al.*, 1985). The cells were scraped from the plates in 1 ml of ice-cold 0.25 M-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes, adjusted to pH 7.4 with KOH. Homogenates were prepared by sonication (Pittner *et al.*, 1985).

Lysis of hepatocytes with digitonin

The methods used are those described by Cascales *et al.* (1984). Cytosolic enzymes were released from the hepatocytes by a 4 min incubation at 4°C with 0.407 mM-digitonin dispersed in 0.25 M-sucrose containing 0.5 mM-dithiothreitol and 10 mM-Hepes at pH 7.4 (Butterwith *et al.*, 1984). The activities of PAP in the cytosolic and membrane-associated compartments were calculated by correcting for the incomplete release of lactate dehydrogenase in the digitonin fraction. The procedure was based on that originally described by MacKall *et al.* (1979).

Determination of enzyme activities

The activities of lactate dehydrogenase (Saggerson & Greenbaum, 1969), PAP (Pollard & Brind-

ley, 1984), total, microsomal and mitochondrial GPAT (Lawson *et al.*, 1981), arylesterase (Shepherd & Hübscher, 1969) and rotenone-insensitive NADH:cytochrome *c* reductase (Sottocasa *et al.*, 1967) were determined essentially as described previously. Microsomal GPAT was assumed to be the difference between total and *N*-ethylmaleimide-insensitive GPAT. Reaction conditions were optimized in the absence of *N*-ethylmaleimide, and the rates were proportional to the amount of homogenate added whether *N*-ethylmaleimide was present or not.

The total esterification of glycerol 3-phosphate was measured as described by Lawson *et al.* (1982b), except that 0.5 mM-oleate (in a 16% molar excess of KOH) was used instead of potassium palmitate. The distribution of label into various lipid classes was determined by t.l.c. (Brindley & Bowley, 1975), or alternatively phospholipids were removed from the neutral-lipid fraction by adsorption on to alumina (Pollard & Brindley, 1984).

Results

Interactions of glucagon, theophylline, dexamethasone and insulin in controlling the activity of PAP

Incubation of the hepatocytes for 8 h with glucagon increased total PAP activity (Fig. 1; Table 1, column A) and in two out of three experiments this effect was seen with as little as 0.1 nM-glucagon. A concentration of 10 nM-glucagon was chosen for most of the subsequent experiments, since this produces a relatively large and consistent increase in PAP activity (Table 1). When theophylline was added in order to inhibit the breakdown of cyclic AMP, there was a further increase in PAP activity, particularly at low concentrations of glucagon, although theophylline alone had no significant effect (Fig. 1; Table 2).

The addition of 35 nM-insulin to the incubations almost completely overcame the action of glucagon in increasing total PAP activity (Fig. 1a). Theophylline may have slightly protected against the

Fig. 1. Interactions of glucagon, theophylline and insulin in controlling the total activity of PAP in hepatocytes. In (a) hepatocytes were incubated for 8 h in the absence (○) or presence of 35 nM-insulin (△) or 1 mM-theophylline (□), and the homogenates of the cells were assayed for PAP (see the Experimental section); (b) shows the effects of various concentrations of insulin on the PAP activity of cells incubated with 10 nM-glucagon. Results are the means $\pm$ ranges from two dishes in one experiment. Similar results were obtained in three further experiments, and the reproducibility of the results is also shown in Tables 1 and 2.

Table 1. *Effects of glucagon, dexamethasone and insulin on the total activity of PAP in hepatocytes and its presence in the membrane-associated compartment*
Hepatocytes were incubated with the combinations of hormones that are indicated, and after 8 h the cells were harvested and homogenates prepared. In three independent experiments 0.15 mM- or 0.5 mM-oleate was added in fresh medium 15 min before the end of the incubation period (see the Experimental section). This caused PAP to be translocated on to membranes without changing the total activity. The medium was then replaced by 1 ml of an ice-cold suspension of 0.5 mg of digitonin/ml (Cascales *et al.*, 1984). The activities of PAP in column A are expressed relative to that obtained in control incubations, which was 403 ± 152 μ units/unit of lactate dehydrogenase (1 unit = 1 μ mol/min). Columns C and E give the absolute activity in the membrane-associated compartment. Columns B and D show the percentage of the total activity present in this compartment; the remainder was in the cytosol. The results are expressed as means $\pm$ s.d., and for column A the number of independent experiments is given in parentheses. The significance of the difference between the groups, calculated by using a paired *t* test, is indicated by: **P* < 0.05; ***P* < 0.025; ****P* < 0.01; +*P* < 0.005; ++*P* < 0.001.

PAP activity in membrane-associated compartment with:					
Additions	0.15 mM-oleate		0.5 mM-oleate		
	(A) Total activity relative to control (%)	(B) Relative activity (%)	(C) (μ units/unit of lactate dehydrogenase)	(D) Relative activity (%)	(E) (μ units/unit of lactate dehydrogenase)
I None (control)	100 (10)	25 $\pm$ 8	98 $\pm$ 23	41 $\pm$ 4	139 $\pm$ 33
II Glucagon (10nM)	172 $\pm$ 32 (10) I versus II $\dagger\dagger$	15 $\pm$ 6 I versus II*	95 $\pm$ 52	37 $\pm$ 6	199 $\pm$ 52 I versus II*
III Insulin (500pM)	91 $\pm$ 13 (10)	34 $\pm$ 6 I versus III*	119 $\pm$ 18	30 $\pm$ 9	102 $\pm$ 46
IV Glucagon (10nM) + insulin (500pM)	106 $\pm$ 11 (10) II versus IV $\dagger\dagger$	20 $\pm$ 7	86 $\pm$ 33	21 $\pm$ 8	108 $\pm$ 88
V Dexamethasone (100nM)	422 $\pm$ 111 (9) I versus V $\dagger\dagger$	15 $\pm$ 4	273 $\pm$ 37 I versus V*	27 $\pm$ 5	498 $\pm$ 157 I versus V $\dagger$
VI Dexamethasone (100nM) + glucagon (10nM)	830 $\pm$ 263 (9) I and II versus VI $\dagger\dagger$	14 $\pm$ 1	528 $\pm$ 48 I versus VI $\dagger$ V versus VI*	31 $\pm$ 3	1054 $\pm$ 108 I versus VI $\dagger$ V versus VI*
VII Dexamethasone (100nM) + insulin (500pM)	161 $\pm$ 29 (6) V versus VII $\dagger\dagger$	17 $\pm$ 6	150 $\pm$ 28	30 $\pm$ 5	276 $\pm$ 34
VIII Dexamethasone (100nM) + glucagon (10nM) + insulin (500pM)	458 $\pm$ 139 (7) VI versus VIII $\dagger\dagger$	10 $\pm$ 3	192 $\pm$ 25 VI versus VIII*	19 $\pm$ 1	343 $\pm$ 73 VI versus VIII*

Table 2. *Effects of glucagon, dexamethasone and insulin on the activity of PAP in hepatocytes incubated in the presence or absence of theophylline*

Hepatocytes were incubated in the absence of oleate as described in Table 1. The results are expressed as means $\pm$ S.D. for three independent experiments, and means $\pm$ ranges for two independent experiments.

Additions	Relative activity of PAP in homogenate (%)	
	(A) no theophylline	(B) +1 mM-theophylline
I None (control)	100 (5)	109 $\pm$ 9 (5)
II Glucagon (1 nM)	158 $\pm$ 50 (3)	234 $\pm$ 41 (3)
A versus B, $P < 0.05$		
III Glucagon (10 nM)	184 $\pm$ 6 (3)	245 $\pm$ 60 (3)
IV Glucagon (10 nM) + insulin (35 nM)	117 $\pm$ 5 (3)	138 $\pm$ 4 (2)
V Dexamethasone (100 nM)	340 $\pm$ 69 (2)	334 $\pm$ 73 (2)

Table 3. *Effects of cycloheximide and actinomycin D on the increases in total PAP induced by glucagon and dexamethasone*

Hepatocytes were incubated in the absence of oleate as described in Fig. 1. The additions of cycloheximide and actinomycin D are indicated, and the results are expressed as in Fig. 2.

Additions	Relative activity of PAP in homogenate (%)		
	No inhibitor	+ cycloheximide (5 μ g/ml)	+ actinomycin D (1 μ g/ml)
None (control)	100 (5)	92 $\pm$ 22 (3)	102 $\pm$ 18 (4)
Glucagon (10 nM)	173 $\pm$ 31 (3)	104 $\pm$ 25 (3)	80 $\pm$ 1 (2)
Dexamethasone (100 nM)	341 $\pm$ 9 (4)	105 $\pm$ 1 (2)	78 $\pm$ 2 (2)

antagonistic effects of 35 nM-insulin towards the action of 10 nM-glucagon (Table 2). The antagonistic effects of insulin against glucagon occurred when its concentration was decreased to as low as 50 pM. In subsequent experiments, a concentration of 500 pM-insulin was used to antagonize reproducibly the effects of 10 nM-glucagon (Fig. 1b; Table 1, column A). This concentration of insulin had no significant effect on the total PAP activity when it was added alone (Table 1, column A).

Dexamethasone (100 nM) increased the total activity of PAP, and insulin (500 pM) antagonized this effect, as expected (Pittner *et al.*, 1985). The combination of 10 nM-glucagon and 100 nM-dexamethasone produced much greater increases in total PAP activity than when each of the compounds was added alone. The addition of 500 pM-insulin partially overcame these combined effects of glucagon and dexamethasone. Theophylline had no significant effect on the dexamethasone-induced increase in the total PAP activity (Table 2). The incubation of the hepatocytes with cycloheximide or actinomycin D abolished the increases in PAP activity that were produced by glucagon or dexamethasone, although the inhibitors had no significant effect on PAP activity when added alone (Table 3; Pittner *et al.*, 1985).

Effects of glucagon, dexamethasone and insulin on the distribution of PAP between the cytosol and the membrane-associated compartment

In the present experiments, less than 5% of the PAP activity for all of the treatments was recovered in the membrane-associated compartment after the cells were lysed with digitonin when no fatty acid was added to the incubations. This probably reflects the very low fatty acid availability in the medium, which was caused by incubating the hepatocytes with fatty-acid-poor bovine serum albumin. In previous work 10% newborn-calf serum was used in the incubation, and its fatty acid content would have caused some translocation of PAP to the endoplasmic reticulum (Cascales *et al.*, 1984; Butterwith *et al.*, 1984). It was therefore decided to add fatty acids to translocate PAP to membranes in order to determine whether any of the hormones would cause its displacement. This was achieved by adding either 0.15 mM- or 0.5 mM-oleate 15 min before the end of the incubations. This time interval was chosen because the translocation is essentially complete after 15 min (J. E. Morgan & D. N. Brindley, unpublished work). These treatments cause 25% and 41% of the PAP respectively to be associated with the membranes in control incubations (Table 1).

The presence of glucagon in the incubations that were performed after adding 0.15mM-oleate caused less of the PAP to be associated with the membranes in percentage terms. However, because glucagon had caused an increase in the total PAP activity, the activity of the membrane-associated PAP remained constant in absolute terms (Table 1, row II). With 0.5mM-oleate, glucagon did not alter the relative distribution of PAP between the cytosol and the membranes, and therefore the total membrane-associated PAP increased in absolute terms (Table 1).

Dexamethasone appeared to have a similar effect to glucagon. It decreased the percentage of PAP associated with the membranes, particularly at 0.15mM-oleate. However, because of the increase in the total activity, more PAP was attached to the membranes in absolute terms, especially at 0.5mM-oleate. The most pronounced increases in the membrane-associated PAP activities were obtained when glucagon and dexamethasone were added together, and the effect was again higher at 0.5mM-oleate (Table 1, row VI).

Although insulin had no significant effect on the total PAP activity, it did cause a greater percentage of the PAP to become membrane-bound at 0.15mM-oleate. However, it had the opposite effect at 0.5mM-oleate in all of the three independent experiments in Table 1 (row III). In general, insulin decreased the membrane-bound PAP activity when it was added to incubations that contained glucagon or dexamethasone, either alone or in combination. This appeared to result mainly from the changes in the total PAP activity (Table 1).

Effects of glucagon, dexamethasone and insulin on the incorporation of [³H]glycerol 3-phosphate into lipids

Incubating hepatocytes for 8h with glucagon, dexamethasone or insulin had no significant effect on the total rate of incorporation of [³H]glycerol 3-phosphate into lipids. This activity was measured by using exogenous palmitoyl-CoA as an acyl-donor (Table 4), or by generating oleoyl-CoA from oleate (Table 5). However, there was an increase in the microsomal GPAT activity and a decrease in the mitochondrial activity when 10nM-glucagon was added in all of the five independent experiments (Table 4). Dexamethasone decreased the mitochondrial GPAT in four out of five experiments, but overall this effect was not significant ($0.1 > P > 0.05$). Insulin had the opposite effect in decreasing the microsomal GPAT activity. There was no significant effect of dexamethasone, glucagon or insulin on the activities of the endoplasmic-reticulum markers arylesterase and the rotenone-insensitive cytochrome *c* reductase.

The effects of the hormones on the relative rates

Fig. 2. Relationship between the total PAP activity and the rates of incorporation of [³H]glycerol 3-phosphate into neutral lipids by homogenates of rat hepatocytes incubated with various combinations of hormones

The rates of neutral-lipid synthesis for the three experiments described in Table 5 are plotted relative to the total PAP activity for the same experiments. The symbols show the results for hepatocytes incubated with no additions (○), glucagon (□), dexamethasone (△), glucagon + dexamethasone (▲), insulin (▽), glucagon + insulin (■), dexamethasone + insulin (▼), glucagon + dexamethasone + insulin (●).

of synthesis of neutral lipids (di- and tri-acylglycerols) and phospholipids (phosphatidate and lysophosphatidate) were determined by measuring the incorporation of [³H]glycerol 3-phosphate in assays where oleoyl-CoA was generated *in situ*. Glucagon (10nM) in all three experiments increased the relative rate of neutral lipid synthesis, and this was mainly accounted for by the increase in triacylglycerol (Table 5, row II). Dexamethasone (100nM) alone, or in combination with glucagon, also increased the relative amount of radioactivity that was associated with the neutral lipid fractions (Table 5, rows VI and VII). On its own, insulin had no significant effect on the proportion of neutral lipid synthesized, but it did tend to reverse the effects of glucagon and dexamethasone when they were added alone or together.

The relationship between the maximum rate of neutral-lipid synthesis from Table 5 and the total PAP activity for the same hormonal conditions, which are included in Table 1, are shown in Fig. 2. There was a significant correlation between these activities ($r = 0.95$; $P < 0.001$).

Discussion

The results establish that glucagon can increase the total PAP activity in hepatocytes over an 8h period. This probably occurs through an increased

Table 4. *Effects of glucagon, dexamethasone and insulin on GPAT activity in cell homogenates*
Hepatocytes were incubated for 8 h with the combinations of hormones indicated, and the GPAT activities were measured by using palmitoyl-CoA as an acyl donor (see the Experimental section). The results are given as means $\pm$ s.d. for the numbers of independent experiments shown in parentheses, and the significances of the differences were calculated and indicated as described in Table 1.

Additions	GPAT activity (nmol of glycerol phosphate incorporated/min per unit of lactate dehydrogenase)				Relative GPAT activity (%)	
	Total	Mitochondrial	In endoplasmic reticulum		Mitochondrial	In endoplasmic reticulum
I None (control)	1.00 $\pm$ 0.181 (5)	0.676 $\pm$ 0.190 (5)	0.324 $\pm$ 0.190 (5)		100	100
II Glucagon (10 nM)	1.07 $\pm$ 0.121 (5)	0.612 $\pm$ 0.196 (5)	0.460 $\pm$ 0.196 (5)		90 $\pm$ 6 (5)	161 $\pm$ 63 (5)
III Insulin (500 pM)	0.956 $\pm$ 0.178 (5)	0.698 $\pm$ 0.166 (5)	0.255 $\pm$ 0.166 (5)	I versus II**	105 $\pm$ 9 (5)	75 $\pm$ 12 (5)
IV Dexamethasone (100 nM)	0.925 $\pm$ 0.162 (5)	0.572 $\pm$ 0.112 (5)	0.354 $\pm$ 0.112 (5)	I versus III***	88 $\pm$ 14 (5)	I versus III*
V Glucagon (10 nM) + insulin (500 pM)	1.07 $\pm$ 0.160 (3)	0.733 $\pm$ 0.111 (3)	0.333 $\pm$ 0.111 (3)		90 $\pm$ 7 (3)	127 $\pm$ 74 (5)
VI Dexamethasone (100 nM) + glucagon (10 nM)	1.13 $\pm$ 0.152 (3)	0.814 $\pm$ 0.100 (3)	0.313 $\pm$ 0.100 (3)		100 $\pm$ 2 (3)	132 $\pm$ 14 (3)
VII Dexamethasone (100 nM) + insulin (500 pM)	0.971 $\pm$ 0.148 (3)	0.787 $\pm$ 0.011 (3)	0.184 $\pm$ 0.011 (3)		98 $\pm$ 10 (3)	141 $\pm$ 46 (3)
VIII Dexamethasone (100 nM) + glucagon (10 nM) + insulin (500 pM)	1.09 $\pm$ 0.203 (3)	0.801 $\pm$ 0.184 (3)	0.287 $\pm$ 0.184 (3)		97 $\pm$ 12 (3)	91 $\pm$ 16 (3)
						127 $\pm$ 30 (3)

Table 5. *Effects of glucagon, dexamethasone and insulin on the incorporation of [³H]glycerol 3-phosphate into lipids*
Hepatocytes were incubated for 8 h with the combinations of hormones indicated and the synthesis of glycerolipids from glycerol phosphate in the homogenate was measured (see the Experimental section). The assays contained optimum concentrations of glycerol phosphate, ATP, CoA, Mg²⁺ and oleate (0.5 mM). Column A shows the incorporation of [³H]glycerol 3-phosphate into total lipids relative to the control, which had an activity of 2.32 $\pm$ 0.39 nmol/min per unit of lactate dehydrogenase. The diacylglycerol and triacylglycerol formed in these assays was separated from phosphatidate and lysophosphatidate by adsorbing the latter phospholipids on to alumina as in the PAP assay. The results in columns A, B and C are means $\pm$ s.d. from three independent experiments, and the significances of the differences are indicated as described in Fig. 1. In two of the experiments the lipids were further characterized by t.l.c. (see the Experimental section), and the results in columns D to G are means $\pm$ ranges.

Additions	Relative distribution of ³ H in:						
	(A) Relative incorporation into total lipids	(B) Phospho-lipids	(C) Neutral lipids	(D) Lysophos-phatidate	(E) Phos-phatidate	(F) Diacyl-glycerol	(G) Triacyl-glycerol
I None (control)	100	79 $\pm$ 2	21 $\pm$ 2	11 $\pm$ 2	49 $\pm$ 7	16 $\pm$ 4	6 $\pm$ 1
II Glucagon (10 nM)	94 $\pm$ 23	71 $\pm$ 10	29 $\pm$ 10	13 $\pm$ 2	43 $\pm$ 2	18 $\pm$ 2	19 $\pm$ 2
III Insulin (500 pM)	94 $\pm$ 18	81 $\pm$ 2	19 $\pm$ 2	12 $\pm$ 2	57 $\pm$ 1	15 $\pm$ 5	5 $\pm$ 1
IV Glucagon (10 nM) + insulin (500 pM)	95 $\pm$ 18	79 $\pm$ 5	21 $\pm$ 5	11 $\pm$ 1	52 $\pm$ 6	18 $\pm$ 2	6 $\pm$ 1
V Dexamethasone (100 nM)	88 $\pm$ 21	53 $\pm$ 9	47 $\pm$ 9	11 $\pm$ 1	47 $\pm$ 1	27 $\pm$ 6	8 $\pm$ 2
VI Dexamethasone (100 nM) + glucagon (10 nM)	88 $\pm$ 29	32 $\pm$ 17	68 $\pm$ 17	10 $\pm$ 2	36 $\pm$ 1	35 $\pm$ 8	14 $\pm$ 1
VII Dexamethasone (100 nM) + insulin (500 pM)	89 $\pm$ 19	70 $\pm$ 1	30 $\pm$ 1	11 $\pm$ 4	51 $\pm$ 3	22 $\pm$ 6	9 $\pm$ 2
VIII Dexamethasone (100 nM) + glucagon (10 nM) + insulin (500 pM)	98 $\pm$ 28	54 $\pm$ 6	46 $\pm$ 6	11 $\pm$ 3	48 $\pm$ 4	22 $\pm$ 7	11 $\pm$ 4

rate of PAP synthesis, since the effect can be blocked with cycloheximide and actinomycin D (Table 3). The glucagon effect is likely to be mediated by its ability to raise the concentrations of intracellular cyclic AMP. This conclusion is compatible with the increases in PAP activity demonstrated with cyclic AMP analogues (Pittner *et al.*, 1985) and with the action of theophylline in potentiating the glucagon-induced stimulation (Table 2). By contrast, theophylline did not significantly alter the dexamethasone-induced increase in PAP activity, and this provides a further indication that the dexamethasone effect is partly independent of cyclic AMP. Also, glucagon (Table 1) and cyclic AMP analogue (Pittner *et al.*, 1985) can produce a marked increase in PAP activity in the presence of an optimum concentration of 100 nM-dexamethasone.

The action of insulin in blocking the glucagon-induced increase in PAP activity can probably be explained by its stimulation of phosphodiesterase activity, thus decreasing cyclic AMP concentrations (Houslay & Marchmont, 1981). The cyclic AMP analogues used previously (Pittner *et al.*, 1985) would have been fairly resistant to this degradation. Thus insulin can oppose the increases in PAP activity that occur in response to hormonally induced increases in cyclic AMP (Table 1; Figs. 1 and 2), and by glucocorticoids (Table 1; Lawson *et al.*, 1982a,b; Pittner *et al.*, 1985). These interactions can be observed at the concentrations of the hormones that are expected *in vivo*.

The long-term control of PAP activity in the liver by glucagon and glucocorticoids demonstrated in the present work is compatible with the relatively large increases in this activity that are seen in a variety of stress conditions. These include starvation, surgical stress, subtotal hepatectomy, diabetes, hypoxia and toxic fatty liver. Similar increases in PAP activity are also seen when the control of metabolism by stress hormones relative to insulin is increased by feeding fructose, sorbitol, glycerol or ethanol, or after injecting glucocorticoids or corticotropin (Brindley & Sturton, 1982; Brindley, 1985). The effect of insulin in suppressing these actions of glucagon and glucocorticoids is particularly well demonstrated *in vivo* when insulin is injected into diabetic rats (Murthy & Shipp, 1979; Woods *et al.*, 1982). Insulin can also operate in controlling the diurnal variation of hepatic PAP activity (Knox *et al.*, 1979), and in preventing the rise in PAP activity when rats are fed with a test load of glucose rather than fructose (Brindley *et al.*, 1979).

The present results, particularly with glucagon and insulin, confirm the similarities that exist between the long-term control of PAP activity and that of the regulatory enzymes involved in gluco-

neogenesis and the associated degradation of amino acids. This hormonal co-ordination facilitates the hepatic secretion of energy in the form of glucose, ketones and triacylglycerols in stress conditions (for further details, see Pittner *et al.*, 1985).

The metabolic expression of the PAP activity depends on its translocation from the cytosol to the membranes of the endoplasmic reticulum. This is promoted by long-chain fatty acids, acyl-CoA esters (Cascales *et al.*, 1984; Martin-Sanz *et al.*, 1985), phosphatidate (R. Hopewell & D. N. Brindley, unpublished work) and possibly by polyamines (Moller & Hough, 1982; Jamdar & Osborne, 1983; Martin-Sanz *et al.*, 1985). The effectiveness of fatty acids in this respect appears to be under hormonal control. Cyclic AMP analogues displace PAP from the membranes at low fatty acid concentrations, but the effect was reversed when these were high (Butterwith *et al.*, 1984). These changes paralleled the ability of the hepatocytes to synthesize triacylglycerol and phosphatidylcholine (Pelech *et al.*, 1983). The results in Table 1 indicate that glucagon has a similar effect to cyclic AMP on the translocation. However, because it increases the total PAP activity in the long term, the PAP activity on the membranes remained relatively constant at 0.15 mM-oleate, and at 0.5 mM-oleate there was an increase. Similarly, dexamethasone and dexamethasone plus glucagon decreased the proportion of the total PAP that was isolated with the membranes. However, the increases in total PAP activity that were obtained with these treatments meant that more PAP in absolute terms associated with the membranes, especially at the higher oleate concentration (Table 1). Thus cyclic AMP (Butterwith *et al.*, 1984), and presumably glucagon through cyclic AMP, increases the concentration of fatty acid required to promote the association of PAP with the endoplasmic reticulum. However, this effect would normally be compensated for in stress conditions *in vivo* by the immediate increased flux of fatty acids from adipose tissue, and in the long term by the action of glucocorticoids and cyclic AMP in stimulating PAP synthesis.

Insulin has the opposite effect to glucagon in that it increases the relative amount of PAP associated with membranes, but only at the lower fatty acid concentration (Table 1). It also antagonizes the effects of glucagon and dexamethasone in stimulating the synthesis of PAP. *In vivo*, fatty acid and fatty acyl-CoA concentrations are lower in the liver when insulin predominates over glucagon in controlling metabolism than vice versa, e.g. in the fed compared with the starved state (Zammit, 1981). The changes that are brought about by glucagon and insulin in the ability of PAP to translocate to the endoplasmic reticulum therefore

appear to anticipate to some extent the changes in fatty acid availability. How these changes in the translocation are brought about is not yet established, but they may involve alterations in the phosphorylation state of PAP (Butterwith *et al.*, 1984).

The hormonal combinations used in the present work produced no marked changes in the total GPAT activity as measured with palmitoyl-CoA (Table 4), or with the oleoyl-CoA-generating system (Table 5). *In vivo*, changes in GPAT activity are also relatively small by comparison with those of PAP (Brindley & Sturton, 1982), but we had expected from previous work (Bates & Saggerson, 1977; Bates *et al.*, 1977) that mitochondrial GPAT might have increased with insulin. In fact, we did detect a decrease in the mitochondrial GPAT with glucagon (Table 4). GPAT activity in the endoplasmic reticulum was increased by glucagon and decreased by insulin, with no significant changes in the activities of arylesterase and NADH:cytochrome *c* reductase. However, it must be emphasized that the microsomal GPAT activity was calculated by difference, rather than being measured directly.

The maximum capacity of the hepatocytes to synthesize di- and tri-acylglycerol from glycerol phosphate was studied in Table 5 by using an optimum concentration of 0.5mM-oleate in the assay. Fig. 2 shows that there was a highly significant correlation ($P < 0.001$) between this and the total PAP activity in the cells. In other words, glucagon and dexamethasone increase the maximum capacity for neutral-lipid synthesis, provided that this can be expressed by high substrate availability. The use of an optimum concentration of oleate in the experiments in Table 5 would have translocated PAP to the membranes in this cell-free system (Martin-Sanz *et al.*, 1984; 1985; Brindley, 1985). We also established a similarly high correlation between the rates of neutral-lipid synthesis from glycerol phosphate (Table 5) and the PAP activity that was associated with the membranes in parallel incubations of hepatocytes to which 0.5mM-oleate was added.

It should be noted that the concentrations of glycerol phosphate, fatty acids and acyl-CoA esters in the liver can increase in conditions such as starvation and diabetes (Debeer *et al.*, 1981; Zammit, 1981) because of the increased mobilization of glycerol and fatty acids from adipose tissue. The increased capacity for triacylglycerol synthesis in starvation may not be expressed because the increased supply of fatty acids may be diverted to β -oxidation. However, when this latter pathway is blocked by octanoyl-(+)-carnitine, the fatty acids are rapidly converted into triacylglycerols if glycerol phosphate concentrations are optimum (Debeer

et al., 1981; McGarry & Foster, 1980). Table 5 also demonstrates that an increased proportion of triacylglycerol is synthesized from glycerol phosphate in the homogenates of cells that have been incubated with glucagon. Therefore, even if a decreased activity of diacylglycerol acyltransferase is caused by a cyclic AMP dependent phosphorylation (Haagsman *et al.*, 1981, 1982), it is not evident in this system. Such an inhibition is also likely to be overcome by fatty acid, and in fact Haagsman & van Golde (1981) have shown that fatty acid stimulates diacylglycerol acyltransferase activity. It is also unlikely that the liver should limit its ability to respond to an increased fatty acid supply by stimulating triacylglycerol synthesis, since this performs a protective function.

The ability of PAP to translocate to the endoplasmic reticulum and to promote triacylglycerol synthesis helps to control the excessive accumulation of fatty acids and acyl-CoA esters that might otherwise be toxic. Triacylglycerol synthesis also regenerates CoA. It can result in the fatty liver that is often observed in diabetes, in stress and in toxic conditions. Alternatively, the triacylglycerol can be secreted provided that the necessary apoproteins and phosphatidylcholine can be synthesized. It is therefore significant that CTP:cholinephosphate cytidyltransferase (EC 2.7.7.15), which regulates the synthesis of phosphatidylcholine by controlling the availability of CDP-choline, is also activated by a fatty-acid-induced translocation to the endoplasmic reticulum. This is modulated by cyclic AMP in the same way as for PAP (Pelech *et al.*, 1983; Vance & Pelech, 1984; Pelech & Vance, 1984). Furthermore, diacylglycerol (Pelech & Vance, 1984) and phorbol esters (Pelech *et al.*, 1984) also promote the translocation. Therefore PAP, producing more diacylglycerol in response to an increased fatty acid load, could provide a further feed-forward signal for phosphatidylcholine synthesis. It is considered that further studies on the role of substrate supply and the mechanisms of hormone action on enzyme translocation could improve our knowledge of how glycerolipid metabolism is co-ordinated.

The work was supported by a Project Grant from the Medical Research Council and an Equipment Grant from the Humane Research Trust. We also thank the Science and Engineering Research Council and Beecham Pharmaceuticals for providing a CASE award to R. A. P., and the Boots Co. for its gift of insulin.

References

- Bates, E. J. & Saggerson, E. D. (1977) *FEBS Lett.* **84**, 229–232
- Bates, E. J. & Saggerson, E. D. (1979) *Biochem. J.* **182**, 751–762

- Bates, E. J. & Saggerson, E. D. (1981) *FEBS Lett.* **128**, 230–232
- Bates, E. J., Topping, D. L., Soorana, S. R. & Saggerson, E. D. (1977) *FEBS Lett.* **84**, 225–228
- Brindley, D. N. (1985) *Prog. Lipid Res.* **23**, 115–133
- Brindley, D. N. & Bowley, M. (1975) *Biochem. J.* **148**, 461–469
- Brindley, D. N. & Sturton, R. G. (1982) *New Compr. Biochem. J.* **4**, 179–213
- Brindley, D. N., Cooling, J., Burditt, S. L., Pritchard, P. H., Pawson, S. & Sturton, R. G. (1979) *Biochem. J.* **180**, 195–199
- Butterwith, S. C., Martin, A. & Brindley, D. N. (1984) *Biochem. J.* **222**, 487–493
- Cascales, C., Mangiapane, E. H. & Brindley, D. N. (1984) *Biochem. J.* **219**, 911–916
- Debeer, L. J., Declercq, P. E. & Mannaerts, G. P. (1981) *FEBS Lett.* **124**, 31–34
- Haagsman, H. P. & van Golde, L. M. G. (1981) *Arch. Biochem. Biophys.* **208**, 395–402
- Haagsman, H. P., de Haas, C. G. M., Geelen, M. J. H. & van Golde, L. M. G. (1981) *Biochim. Biophys. Acta* **664**, 74–81
- Haagsman, H. P., de Haas, C. G. M., Geelen, M. J. H. & van Golde, L. M. G. (1982) *J. Biol. Chem.* **257**, 10593–10598
- Houslay, M. D. & Marchmont, R. J. (1981) *Biochem. J.* **198**, 703–706
- Jamdar, S. C. & Osborne, L. J. (1983) *Biochim. Biophys. Acta* **752**, 79–88
- Jennings, R. J., Lawson, N., Fears, R. & Brindley, D. N. (1981) *FEBS Lett.* **113**, 119–122
- Knox, A. M., Sturton, R. G., Cooling, J. & Brindley, D. N. (1979) *Biochem. J.* **180**, 441–443
- Lawson, N., Jennings, R. J., Pollard, A. D., Sturton, R. G., Ralph, S. J., Marsden, C. A., Fears, R. & Brindley, D. N. (1981) *Biochem. J.* **200**, 265–273
- Lawson, N., Jennings, R. J., Fears, R. & Brindley, D. N. (1982a) *FEBS Lett.* **143**, 9–12
- Lawson, N., Pollard, A. D., Jennings, R. J. & Brindley, D. N. (1982b) *FEBS Lett.* **146**, 204–208
- Lehtonen, M. A., Savolainen, M. J. & Hassinen, I. E. (1979) *FEBS Lett.* **99**, 162–166
- MacKall, J., Meridith, M. & Lane, M. D. (1979) *Anal. Biochem.* **95**, 270–274
- Martin-Sanz, P., Hopewell, R. & Brindley, D. N. (1984) *FEBS Lett.* **175**, 284–288
- Martin-Sanz, P., Hopewell, R. & Brindley, D. N. (1985) *FEBS Lett.* **179**, 262–266
- McGarry, J. D. & Foster, D. W. (1980) *Annu. Rev. Biochem.* **49**, 395–420
- Moller, F. & Hough, M. R. (1982) *Biochim. Biophys. Acta* **711**, 521–531
- Murthy, V. K. & Shipp, J. C. (1979) *Diabetes* **28**, 472–478
- Pelech, S. L. & Vance, D. E. (1984) *Biochim. Biophys. Acta* **779**, 217–251
- Pelech, S. L., Pritchard, P. H., Brindley, D. N. & Vance, D. E. (1983) *Biochem. J.* **216**, 129–136
- Pelech, S. L., Paddon, H. B. & Vance, D. E. (1984) *Biochim. Biophys. Acta* **795**, 447–451
- Pittner, R. A., Fears, R. & Brindley, D. N. (1985) *Biochem. J.* **225**, 455–462
- Pollard, A. D. & Brindley, D. N. (1984) *Biochem. J.* **217**, 461–469
- Saggerson, E. D. & Greenbaum, A. L. (1969) *Biochem. J.* **115**, 405–417
- Shephard, E. H. & Hübscher, G. (1969) *Biochem. J.* **115**, 405–417
- Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Berstrand, A. (1967) *J. Cell Biol.* **32**, 415–438
- Vance, D. E. & Pelech, S. L. (1984) *Trends Biochem. Sci.* **9**, 17–20
- Woods, J. A., Knauer, T. E. & Lamb, R. G. (1982) *Biochim. Biophys. Acta* **666**, 482–492
- Zammit, V. A. (1981) *Biochem. J.* **198**, 75–83