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Effects of okadaic acid on the activities of two distinct phosphatidate phosphohydrolases in rat hepatocytes

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Incubation of hepatocytes with okadaic acid displaced the *N*-ethylmaleimide-sensitive phosphatidate phosphohydrolase from the membrane fraction into the cytosol and partially prevented the oleate-induced movement of phosphohydrolase from cytosol to membranes. However, higher concentrations of oleate still caused translocation and activation of the phosphohydrolase. This enzyme is stimulated by Mg^{2+}, and is probably involved in glycerolipid synthesis. Okadaic acid also decreased the concentration of diacylglycerol within the hepatocytes. Okadaic acid had no observable effect on the activity of an *N*-ethylmaleimide-insensitive phosphatidate phosphohydrolase which remained firmly attached to membranes. This activity is not stimulated by Mg^{2+} and is probably involved in signal transduction by the phospholipase D pathway.

Diacylglycerol; Phosphatidylcholine; Protein phosphatase; Signal transduction; Triacylglycerol

1. INTRODUCTION

At least two different phosphatidate phosphohydrolase (PAP: EC 3.1.3.4) activities exist in rat liver [1]. The first of these enzymes (PAP-1) is inhibited by *N*-ethylmaleimide [1,2] and it has an absolute requirement for Mg^{2+} [2–4]. This enzyme is located in the cytosol and can translocate to the endoplasmic reticulum to become functionally active. The translocation is enhanced by increasing concentrations of fatty acids and acyl CoA esters which may act in their own right or by causing the synthesis of phophatidate which would then act as a specific translocator of PAP-1 [3,4]. Furthermore, the translocation from cytosol to membranes is under hormonal control such that it is decreased by cyclic AMP [5] or glucagon [6], and increased by insulin [6]. This suggests that PAP-1 may undergo phosphorylation and dephosphorylation reactions. However, direct evidence for the existence of a phosphorylated protein has not yet been provided because of the extreme difficulty in purifying PAP-1 and in preparing antibodies. PAP-1 is also under long-term hormonal regulation. Its activity is increased by glucocorticoids, glucagon and growth hormone, whereas insulin antagonizes these effects [3,4,6]. These different levels of regulation indicate that PAP-1 is important in controlling glycerolipid synthesis at the level of diacylglycerol production. Furthermore, the activity of PAP-1 generally parallels the accumulation of triacylglycerols in the liver and in the blood [3,4,7].

PAP-2 is not inhibited by *N*-ethylmaleimide, nor is it stimulated by Mg^{2+} [1]. This newly described PAP activity is located in the plasma membrane where it could function in signal transduction via the phospholipase D pathway and the conversion of phosphatidate to diacylglycerol. The latter compound can activate protein kinase C [8], and phosphatidate is also a potent second messenger [9–13]. PAP-2 could, therefore, regulate the balance between two second messengers.

The objective of the present work was to determine whether further evidence could be gained for the regulation of PAP-1 by a phosphorylation–dephosphorylation cycle, and whether such regulation would also occur with PAP-2. Use was also made of okadaic acid, a polyether fatty acid [14], which is capable of inhibiting protein phosphatases 1 and 2A [15].

2. EXPERIMENTAL

2.1. *Materials*

Okadaic acid was obtained from LC Services Corp., Woburn, MA, USA. Oleic acid, essentially fatty acid-free bovine serum albumin and all other biochemicals were of analytical grade and were obtained either from Sigma Chemical Co., St. Louis, MO, USA or Fisher Scientific, Edmonton, Canada. [^{3}H]Phosphatidate was labelled with [^{3}H]palmitate and diluted with non-radioactive phosphatidate to a specific radioactivity of 0.4 Ci/mol [2]. Tetrahydrolipstatin was a gift from Dr. M.K. Meier, Hoffman-La Roche Ltd., Basel, Switzerland.

2.2. *Isolation, incubation and harvesting of hepatocytes*

Male Sprague–Dawley rats (125–175 g) were fed Purina rat diet at

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22°C and in a room that was lit from 08.00 to 20.00 h. The rats were anesthetized with 2.27 mg of phenobarbital per kg. Hepatocytes were isolated by collagenase perfusion [16] and suspended in Dulbecco's modified Eagle's medium [17]. Between 4–6 × 10^6 hepatocytes were incubated for 30 min in 2 ml medium containing 0.1 mM albumin, and 10% fetal calf serum as described previously [18]. Oleate was prepared in a 20% molar excess of KOH, dissolved by warming and then slowly pipetted with shaking into the albumin-containing medium. Okadaic acid was dissolved in 10% dimethylsulphoxide prior to addition to the medium. The final concentration of dimethylsulphoxide in the medium was 0.25%. The hepatocytes were harvested by centrifugation at 500 × *g* for 2 min at 4°C and the cells washed once with 2 ml of ice-cold phosphate-buffered saline (0.15 M NaCl, 2.68 mM KCl, 8.11 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4). Cytosolic and membrane fractions were obtained by lysing the cells with digitonin [5,19], except that 0.1 mg of digitonin per ml was sonicated in 10 mM HEPES, adjusted to pH 7.4 with HCl, and contained 0.5 mM dithiothreitol in the absence of sucrose. After 1 min the cells were centrifuged for 3 min at 13,000 × *g*, the supernatants removed and the pellets resuspended in 1 ml of the same buffer, but without digitonin. Samples were stored at −70°C until required for analysis.

2.3. *Assays for enzyme activities, diglyceral and protein*

PAP activities were determined by using *N*-ethylmaleimide to distinguish between PAP-1 and PAP-2 [1,2]. Lactate dehydrogenase (LDH: EC 1.1.27) was measured [19], and protein was determined essentially by the method of Bradford [20]. The total LDH activity was used to check that the hepatocytes did not lyse during the pre-incubation period with oleate or okadaic acid [6,19,21]. Expression of the results relative to total LDH also compensates for small differences in the number of viable hepatocytes per incubation flask. However, the activities of PAP-1 and PAP-2 under the different experimental conditions were relatively similar of the results were expressed relative to cell protein. The total content of LDH per flask of cells was 9.39±0.46 μmol of lactate oxidized/min (means ± S.E.M. from 10 independent preparations) and 1 μmol/min is equivalent to 1 unit of activity at 22°C. One flask of cells contained 5.3±0.4 mg of protein (means ± S.E.M. from 4 independent preparations) so that results can be readily calculated relative to protein. Lysis of the hepatocytes with digitonin caused the release of 82–90% of the LDH activity into the cytosolic fraction. Lipids were extracted after incubation of the hepatocytes for 30 min in the absence of oleate, as described previously [22] and diacylglycerol mass was determined [23].

3. RESULTS AND DISCUSSION

Incubation of hepatocytes with 1 μM okadaic acid caused an increased phosphorylation of cytosolic and microsomal proteins (results not shown) as expected [24]. This was accompanied by a decrease of about 50% in the effect of oleate in promoting the translocation of PAP-1 to the membrane compartment (Table I; row III minus I compared with IV minus II). Okadaic acid also decreased the basal activity of PAP-1 in the membranes by 38%.

The concentration of okadaic acid used was 1 μM since that concentration has been shown to give maximum effects in hepatocytes [24]. Furthermore, the intracellular content of protein phosphatases 1 and 2A often reaches concentrations of 1 μM [25] and therefore equimolar concentrations of okadaic acid may be necessary for phosphatase inhibition. Fig. 1 shows the effects of different concentrations of okadaic acid in displacing PAP-1 from the membrane fraction to the cytosol. The results confirm that an optimum effect is obtained between 0.5–1.0 μM okadaic acid.

Oleate alone produced a 42% increase in the activity of PAP-1 that was membrane-associated (Table I). The use of our specific assay [1,2] means that the membrane activity did not contain a contribution from PAP-2. The total activity of PAP-1 was not changed significantly by 1 mM oleate. However, okadaic acid had a small (15–25%) but significant effect in increasing total PAP-1 activity (Table I, Fig. 2). Increasing the concentration of oleate from 1 to 3 mM had two effects as expected from previous work [19]. It increased the proportion of PAP-1 that was associated with membranes and it stimulated the total activity (Fig. 2). The effect of okadaic acid in decreasing the membrane associated PAP and in increasing the cytosolic activity was observed at all

Table I

Effects of okadaic acid and oleate on phosphatidate phosphohydrolase (PAP) activities in rat hepatocytes

Additions	PAP-1		PAP-2
	Total activity (mU/U of total LDH)	Membrane-bound activity (%)	Total activity (mU/U of total LDH)
I. None (control)	1.15 ± 0.18	24.7 ± 2.4	0.30 ± 0.06
II. Okadaic acid	1.33 ± 0.18 (I vs. II*)	15.2 ± 1.7 (I vs. II***)	0.28 ± 0.03
III. Oleic acid	1.18 ± 0.16	35.0 ± 3.4 (I vs. III***)	0.30 ± 0.05
IV. Oleic acid + okadaic acid	1.32 ± 0.17	20.3 ± 1.7 (II vs. IV**, III vs. IV***)	0.27 ± 0.05

Rat hepatocytes were incubated for 30 min in the presence of 0.1 mM bovine serum albumin with 1 mM oleate, 1 μM okadaic acid or both as indicated. Results for PAP-1 are means ± S.E.M. of 10 independent experiments and means ± S.E.M. of 4 independent experiments for PAP-2. These activities are expressed relative to the total LDH activity present in the hepatocytes. Membrane-bound PAP-1 activity is defined as the activity not released by digitonin. There was no significant activity of PAP-2 in the cytosolic fraction. Significant differences between groups were calculated by using a paired *t*-test and are indicated by: *$P<0.01$, **$P<0.005$ and ***$P<0.0005$.

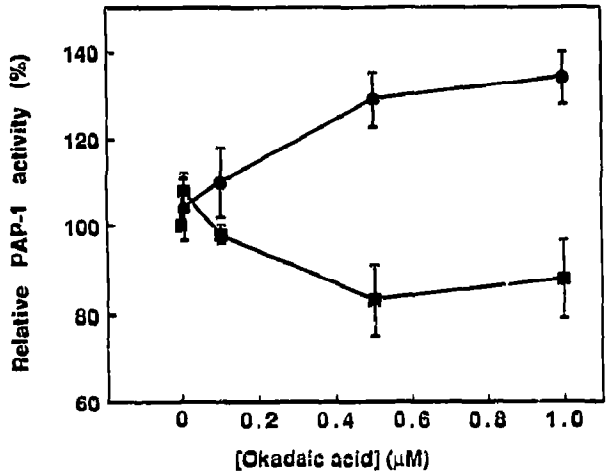

Fig. 1. Effect of okadaic acid on the activity and distribution of PAP-1 in rat hepatocytes. Rat hepatocytes were incubated for 30 min at 37°C with 0.1 mM essentially fatty acid-free BSA and the concentration of okadaic acid indicated. The activity of PAP-1 in the cytosolic fraction (●) and membrane fraction (■) is shown relative to the control values in the absence of okadaic acid. Results are means ± S.E.M. for 3 independent experiments. The absolute values for the cytosolic, membrane and total PAP-1 activities were 1.5 ± 0.16, 0.35 ± 0.06 and 1.40 ± 0.19 nmol of diacylglycerol/min per unit of total LDH, respectively.

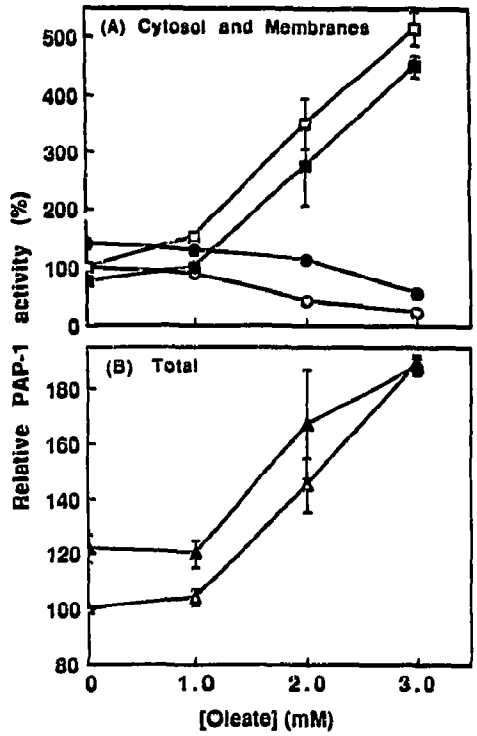

Fig. 2. Effect of oleate on the activity and distribution of PAP-1 in hepatocytes in the presence and absence of okadaic acid. Rat hepatocytes were incubated for 30 min at 37°C with 0.1 mM essentially fatty acid-free BSA and the concentration of oleate indicated in the absence (open symbols) or in the presence (closed symbols) of 1 μM okadaic acid. The activity of PAP-1 in the cytosolic fraction (○, ●) and membrane fraction (□, ■) is shown in (A) and the total PAP-1 activity (△, ▲) is given in (B). Results are expressed relative to the control value of PAP-1 activity in the various fractions in the absence of oleate and okadaic acid. Results are means ± S.E.M. for 10 independent experiments at 1 mM oleate, or means ± range for two independent experiments at 2 and 3 mM oleate. The absolute values for the cytosolic, membrane and total PAP-1 activities in control incubations were 0.88 ± 0.15, 0.27 ± 0.04 and 1.15 ± 0.18 nmol of diacylglycerol/min per unit of total LDH, respectively, in 10 independent determinations.

oleate concentrations (Table I, Fig. 2). The oleate-induced increase in total PAP activity was also observed in the presence of okadaic acid. These results imply a control of the subcellular distribution of PAP-1 by a phosphorylation–dephosphorylation cycle that involves protein phosphatases.

This displacement of PAP-1 from the membranes is normally related to a decrease in functional enzyme activity [3,4,26]. Such an effect was also found for okadaic acid since only 2.26 ± 0.24 (mean ± S.E.M. for 3 independent experiments) nmol of diacylglycerol were isolated per flask in three independent experiments. This represented a decrease of about 45% ($P<0.05$) compared to the 4.11 ± 0.08 nmol of diacylglycerol per flask isolated from the equivalent control cells. This compares well with the 48% decrease PAP-1 activity bound to the membranes and represents a substantial change in diacylglycerol metabolism. Diacylglycerol production is also required for vital functions of phospholipid synthesis such as bile secretion and membrane turnover. Consequently, a basal level of diacylglycerol synthesis needs to be maintained. The proposed phosphorylation of PAP-1 is thought to be a modulating event which modifies the effects of fatty acids as feed-forward activators [3,4].

Diacylglycerol is a major signal in the activation of CTP: phosphocholine cytidylyltransferase [27,28]. This enzyme, like PAP-1, is also translocated to the endoplasmic reticulum during its activation by fatty acids [29]. By contrast, cAMP analogues [30], and okadaic acid [18] promote translocation from endoplasmic reticulum to cytosol. The fatty acid induced translocation of the cytidylyltransferase may be an indirect effect in cells mediated through the enhanced formation of diacylglycerol, which is facilitated by the activity of PAP-1 on the endoplasmic reticulum. Therefore, there is a close coordination of PAP-1 and the cytidylyltransferase activity. The present work demonstrates that this is probably effected by phosphorylation reactions. Previous work implicated the involvement of kinase A in the regulation of PAP-1 [5,6] and the present results with okadaic acid are compatible with a phosphorylation mechanism. The reasons for the coordinate regulation of the synthesis of triacylglycerols and phosphatidylcholine may relate to the formation of very low density lipoproteins which contain about 60% by weight of triacylglycerol and 20% of phosphatidylcholine [4].

The other important part of the work was to determine whether PAP-2 activity is controlled by phosphorylation. Neither oleate (1–3 mM), nor okadaic acid (0.001–1 μM) were able to produce any significant

changes in the activity of PAP-2, which was completely recovered in the membrane fraction (Table I). We also tested to see whether there might have been a change in the apparent K_m by measuring PAP-2 activity with 0.05–1 mM phosphatidate. However, no change in reaction rate was observed at suboptimum or optimum substrate concentrations. This failure to observe an effect of okadaic acid should not have been caused by toxic effects of decreased ATP concentrations in the hepatocytes in these short-term incubations [24]. Furthermore, we observed a simultaneous effect of okadaic acid on the subcellular distribution of PAP-1 (Table I). The involvement of phosphatases in the regulation of PAP-2 cannot be excluded, since this might involve protein phosphatases 2B and/or 2C, or tyrosine phosphate phosphatases. However, we were previously unable to modify PAP-2 activity by incubating hepatocytes with vasopressin, glucagon, insulin, triiodothyronine or dexamethasone [1]. The other possibility is that PAP-2 may be phosphorylated, but that the effect of the phosphorylation may not be detected in the assay system that we used in vitro. PAP-2 activity is known to be regulated indirectly by inhibition with sphingosine [1,31–33]. A final decision on whether PAP-2 is regulated by phosphorylation in vivo must await its purification and characterization. This is important in fully understanding the role of PAP-2 in regulating signal transduction via the phospholipase D pathway. Furthermore, PAP-1 might also translocate from the cytosol to plasma membranes and participate in signal transduction.

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