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ON THE ACTIVATION OF MICROSOMAL UDPGLUCURONYLTRANSFERASE BY PHOSPHOLIPASE A

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SUMMARY

Activation of rat liver microsomal UDPglucuronyltransferase (EC 2 4 1 17) by phospholipase A (EC 3 1 1 4) was reversed by micellar dispersions of several phospholipids and by serum albumin When microsomal membranes were treated with the phospholipase in presence of albumin, which binds and removes surface-active phospholipid degradation products (lysophosphatides and unsaturated fatty acids), activation was delayed by albumin at a concentration of 1 mg/mg of microsomal protein and completely prevented by higher concentrations of albumin The UDPglucuronyltransferase of intact microsomal membranes was activated by lysophosphatidylcholine and unsaturated fatty acids at concentrations which were produced by phospholipase treatment, and the activation by lysophosphatidylcholine most closely resembled that by phospholipase A

It is concluded that the activation of UDPglucuronyltransferase by phospholipase A is due to the detergent activity of phospholipid hydrolysis products and that the enzyme in intact microsomal membranes probably is not constrained by interaction with a specific phospholipid

INTRODUCTION

Much of our current understandung of the membrane-dependence of the liver microsomal enzyme UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase, acceptor unspecific, EC 2 4 1 17) has been gained by determining its activity before and after perturbing the microsomal membrane with reagents such as phospholipase A (EC 3 1 1 4) [1–6]

UDPglucuronyltransferase of guinea pig liver microsomal fractions prepared in isotonic KCl was inactivated by phospholipase A [1, 2, 6] particularly when activities were measured at a low concentration of UDPglucuronate The enzyme was reactivated specifically by micellar dispersions of phosphatidylcholine [2] We concluded that products from the action of phospholipase A on microsomal phospholipids were not responsible for inactivation since these compounds did not affect significantly the enzyme activity of intact microsomal fractions [1]

Rat liver microsomal fractions prepared in KCl possessed lower UDPglucuro-

nyltransferase activities than similar guinea pig preparations but they were activated by brief treatment with phospholipase A, up to the values of untreated guinea pig liver preparations [6] More prolonged treatment with phospholipase A led to loss of UDPglucuronyltransferase activity, apparently similar to that which phospholipase A induced in the guinea pig microsomal enzyme

Similar differences in the responses of the microsomal enzymes to detergents [6, 7] are also consistent with the view that the influences of membrane phospholipid structure on UDPglucuronyltransferase activity in the two species are not identical

The present paper is concerned with the mechanism of activation of rat liver UDPglucuronyltransferase by phospholipase A and we have addressed ourselves specifically to two questions (1) Is the effect of phospholipase A directly due to degradation of membrane phospholipids which, it has been suggested [8], normally constrain UDPglucuronyltransferase activity, or is it due to the influences of the products of phospholipase A action viz lysophosphatides and/or fatty acids? Lysophosphatidylcholine and several fatty acids are known to enhance the enzyme activity of intact rat liver microsomal preparations [3, 9, 10] (2) Can activation by phospholipase A be reversed by a specific phospholipid as was the inactivation of the guinea pig enzyme (ref 2)?

In this paper, therefore, we have examined the effects, on UDPglucuronyltransferase activity, of micellar dispersions of several phospholipids before and after treating microsomal fractions with phospholipase A, the effects of removing phospholipid degradation products after activation and the effects of these compounds on the enzyme activity of intact microsomal preparations

MATERIALS AND METHODS

Crystalline bovine serum albumin, lysophosphatidylcholine, palmitic, stearic, linoleic and arachidonic acids were purchased from Sigma London Chemical Co Ltd , Kingston-upon-Thames, Surrey, Great Britain Phosphatidylcholine was purified according to Attwood et al [2] The other phospholipids were purchased from Koch-Light Laboratories, Colnbrook, Bucks , Great Britain and had the compositions reported previously [2] Phospholipase A, free from proteolytic activity, was purified from *Crotalus adamanteus* venom [1]

Male Wistar rats (150–200 g), purchased from Fisons Pharmaceuticals, Loughborough, Leicestershire, Great Britain, were starved overnight and liver microsomal fractions prepared in 0 154 M KCl [6] The twice-washed microsomal pellets were suspended in sufficient 0 154 M KCl to yield a protein concentration of 20 mg/ml, measured with a biuret reagent [11] standardised with bovine serum albumin The microsomal suspensions were stored at 0 °C and used within 1 h of preparation

Microsomal suspensions (5 or 10 mg of protein per ml, final concentration) were digested with phospholipase A (10 μg/mg of microsomal protein except where otherwise stated) by shaking at 20 °C in a medium containing 12 5 mM Tris–HCl buffer, pH 8 0 and 2 5 mM $CaCl_2$ The reaction was terminated by adding an excess of EDTA The time-course of the phospholipase A-catalysed hydrolysis of microsomal phospholipid was followed by measuring the release of lysophosphatides EDTA (10 mM, final concentration) was added to portions (2 ml) of a digest and the mixtures freeze-dried The phospholipids were extracted with four 5-ml volumes of

chloroform–methanol (2 1, v/v) The solutions were washed and the phospholipids separated by two-dimensional thin-layer chromatography and the proportions of individual phospholipids determined as described previously [10] The quantities of the lysophophatides released were then calculated, using the known phospholipid content and composition of native microsomal preparations

Other microsomal fractions (5 mg of protein per ml) were treated with lysophosphatidylcholine or fatty acids in the concentration range 0–0 3 μmole/mg of microsomal protein for 10 min at 20 °C in the presence of 25 mM Tris–HCl buffer, pH 8 0 Lysophosphatidylcholine was added as a 3 mM solution in water, the fatty acids were added as fine dispersions in water (3 μmole/ml) produced by sonication

Phospholipid micellar dispersions were prepared in 20 mM Tris–HCl buffer, pH 8 0, containing 1 mM EDTA [2] The phosphorus contents of phospholipid micellar dispersions and microsomal suspensions were measured after drying by the method of Chen et al [12] The phospholipid content of microsomal fractions was calculated as described previously [10] after subtracting from the phosphorus content 1 7 μg P/mg of microsomal protein which could not be extracted from microsomal membranes by extensive washing with chloroform–methanol

UDPglucuronyltransferase activity was measured with *p*-nitrophenol as acceptor at a UDPglucuronate concentration of 0 5 mM (ref 7) Activities of the various microsomal preparations were determined with portions of suspensions containing 0 5–1 0 mg of microsomal protein In this series of experiments the UDPglucuronyltransferase activities of unactivated microsomal fractions were in the range 0 74–1 19 nmole of *p*-nitrophenol glucuronidated per min per mg of microsomal protein

RESULTS

Activation of UDPglucuronyltransferase by phospholipase A

Activation of rat liver UDPglucuronyltransferase by phospholipase A is now well documented [3, 5, 6, 10] Confirming these reports, Fig 1 shows the marked increase in enzyme activity which occurred on treating a freshly prepared microsomal suspension with a low concentration of phospholipase Since activation occurred only in presence of added Ca^{2+} (Fig 1) which are required for phospholipase activity [13] and no activation was observed after microsomal suspensions had been incubated alone even for 4 5 h at 30 °C we concluded that the effect was entirely dependent on the catalytic action of phospholipase A

The extent of activation depended on the time of treatment (Fig 1) and on the concentration of phospholipase A In a separate experiment employing a fixed 15-min digestion period, maximum activation of UDPglucuronyltransferase was observed at a phospholipase concentration of 10 μg/mg of microsomal protein

Reversal of phospholipase A activation by phospholipid micelles

Treatment of guinea pig liver microsomal fractions prepared in 0 154 M KCl with phospholipase A inactivated UDPglucuronyltransferase [1, 2] While full activity was restored to the treated preparations by phosphatidylcholine micelles, none of several other phospholipids reactivated significantly and we concluded that phosphatidylcholine in intact microsomal preparations was specifically required for the enzyme's activity In the light of those results it is reasonable to speculate that UDP-

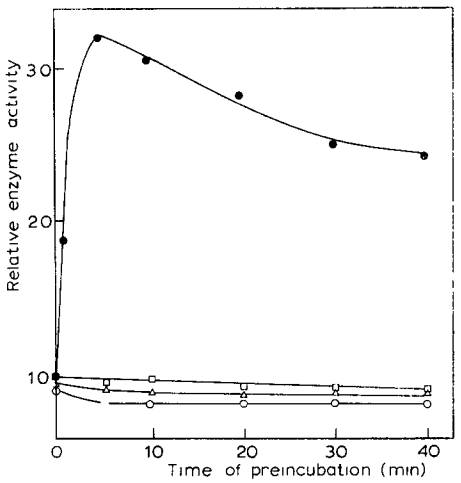

Fig 1 Activation of UDPglucuronyltransferase by phospholipase A Microsomal suspensions (5 mg of protein per ml) were preincubated without phospholipase A or Ca^{2+} (□), without phospholipase A but with Ca^{2+} (△), with phospholipase A but without Ca^{2+} (○), and with both phospholipase A and Ca^{2+} (●) Portions of the mixtures were withdrawn after various times and enzyme activity determined in presence of 3 3 mM EDTA Enzyme activity is expressed relative to that of the mixture without phospholipase A and Ca^{2+} at zero time

glucuronyltransferase in intact rat liver microsomal membranes might be restrained from expressing its maximum activity through interaction with a specific phospholipid To investigate this, rat enzyme was activated to near its maximum level by a brief treatment with phospholipase A and portions of the microsomal suspensions added to various concentrations of micelles of the phospholipids found in microsomal membranes [10] Activation by phospholipase A was reversed by low concentrations of all the phospholipids (Fig 2) Phosphatidylserine, however, at concentrations greater than 10 μg of phosphorus per mg of microsomal protein reduced the activity of phospholipase-treated enzyme to less than unactivated values None of the phospholipids except phosphatidylserine had any significant effect on the UDPglucuronyltransferase activity of intact microsomal preparations (Fig 2) Phosphatidylserine was strongly inhibitory In our earlier work [2] phosphatidylserine and phosphatidylinositol inhibited the enzyme activity of intact guinea pig microsomal preparations

The results of Fig 2 indicate that abolition by phospholipid micelles of the increased UDPglucuronyltransferase activities of phospholipase A-treated microsomal preparations is an unspecific process and do not support the view that in intact rat liver microsomal membranes the enzyme might be constrained by interaction with a specific phospholipid species An alternative, non-specific, mode of action of the phospholipids might be the removal from the microsomal membranes, probably as mixed micelles, of materials that stimulate UDPglucuronyltransferase activity which are produced by the catalytic action of phospholipase A

Reversal of phospholipase A activation by albumin

Crystalline serum albumin has a considerable affinity for lysophosphatides and fatty acids and has been used to bind and remove these products of phospholipase A

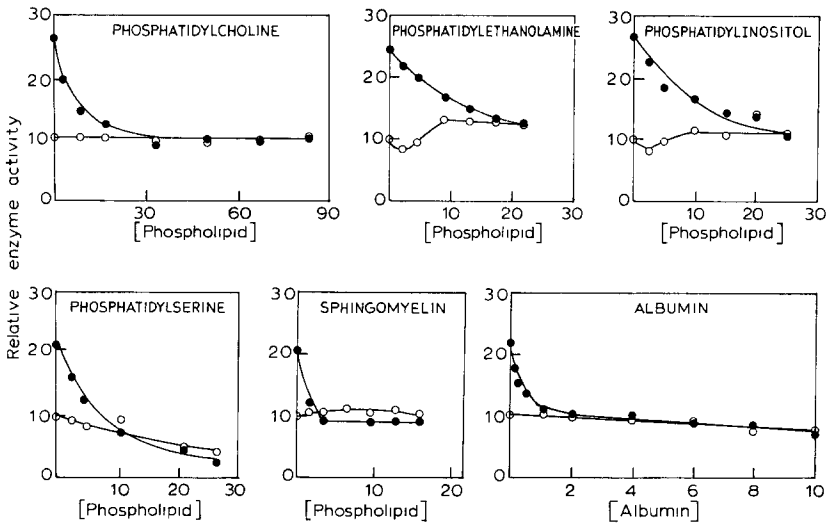

Fig 2 Effects of phospholipid micelles and albumin on UDPglucuronyltransferase activity in untreated (○) and phospholipase A-treated (●) microsomal fractions Microsomal fractions (10 mg of protein per ml) were preincubated with and without phospholipase A for 5 min EDTA was added (final concentration, 10 mM) and portions of the mixtures added to various concentrations of the different phospholipids or albumin in Tris (20 mM)–EDTA (1 mM), pH 8 0 After 5 min, portions of these mixtures were assayed for enzyme activity which is expressed relative to that of the untreated microsomal suspension added to Tris–EDTA, pH 8 0 Phospholipid concentrations are quoted as μg of phospholipid phosphorus per mg of microsomal protein and those of albumin as mg/mg of microsomal protein

action from treated microsomal membranes [1, 14–16] Albumin, at concentrations equal to or greater than 1 mg/mg of microsomal protein, completely restored the high activities of phospholipase-treated microsomal membranes to unactivated values and had no effect on UDPglucuronyltransferase activity of intact microsomal fractions even at a concentration of 10 mg/mg of microsomal protein (Fig 2) These observations strongly support the idea that it is the products from phospholipid hydrolysis which activate UDPglucuronyltransferase

As a further test, a microsomal fraction was treated with phospholipase A in the presence and absence of different concentrations of albumin The usual activation of UDPglucuronyltransferase was observed in the absence of albumin while increasing concentrations of albumin in the digests resulted in increasing delays in activation (Fig 3) Indeed, at the highest concentration of albumin (3 mg/mg of microsomal protein) enzyme activation was completely prevented during the entire duration (30 min) of phospholipase A treatment It is unlikely that this effect is due to inhibition of phospholipase A by albumin, since albumin is known to stimulate phospholipase A activity [17]

In a similar experiment with guinea pig microsomal fraction, albumin (1 mg/mg of microsomal protein) in the digest did not affect the time course of UDPglucuronyltransferase inactivation and we concluded that phospholipase A reaction products had not inhibited UDP glucuronyltransferase activity (ref 1, Graham, A B and Wood, G C, unpublished)

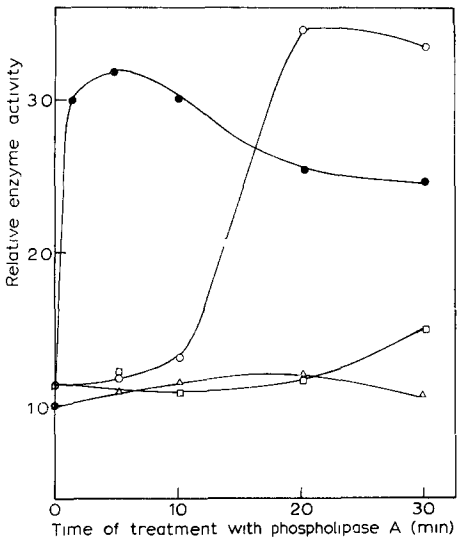

Fig 3 Effect of albumin on the activation of UDPglucuronyltransferase by phospholipase A Microsomal suspensions (5 mg of protein per ml) were treated with phospholipase A in the absence (●) and presence of albumin at concentrations of 1 (○), 2 (□) and 3 (△) mg/mg of microsomal protein Portions of the digests were removed at various times and assayed for enzyme activity in presence of 3 3 mM EDTA Enzyme activity is expressed relative to that of an untreated microsomal suspension

Activation by lysophosphatidylcholine and fatty acids

If fatty acids and/or lysophosphatides produced by phospholipase A are responsible for the activation of rat liver UDPglucuronyltransferase, these compounds should stimulate the enzyme activity of intact microsomal fractions at concentrations produced by the hydrolase acting on the phospholipids of microsomal membranes Fig 4 shows the time-course of microsomal phospholipid hydrolysis by phospholipase A under the conditions of our experiments After 30 min, 0 37 μmole of lysophosphatide per mg of microsomal protein was released From the stoichiometry of the reaction, release of an equal amount of fatty acid is expected In a previous experiment [10] in which phospholipid hydrolysis had been followed by titration, an approximately equivalent amount of NaOH was consumed

Phospholipase A catalyses the hydrolysis of phospholipids by specifically attacking the 2-ester position, thus releasing fatty acids which are predominantly unsaturated [18] The major unsaturates of rat liver microsomal phospholipids are linoleic and arachidonic acids, the major saturated species, palmitic and stearic acids [19], and lysophosphatidylcholine was the major lysophosphatide released by phospholipase A from our microsomal preparations (Fig 4) The saturated fatty acids (0–0 3 μmole/mg of microsomal protein) did not affect the UDPglucuronyltransferase activity of intact microsomal fraction while the unsaturates, especially linoleic acid stimulated significantly at concentrations greater than 0 2 μmole/mg of microsomal protein (Fig 5) Very similar results were obtained when dispersions of the unsaturated fatty acids were prepared under N_2 to avoid peroxidation of the lipid The most powerful stimulation of activity was observed with lysophosphatidylcholine, while an

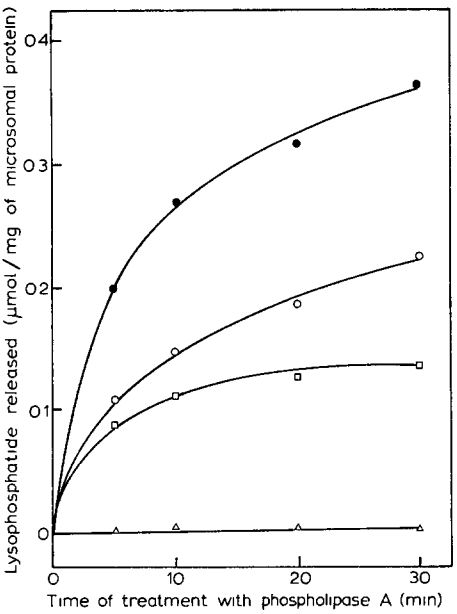

Fig 4 Release of lysophosphatides from microsomal phospholipid catalysed by phospholipase A See Materials and Methods Lysophosphatidylcholine (○), lysophosphatidylethanolamine (□), lysophosphatidylinositol plus lysophosphatidylserine (△), and total lysophosphatide (●) The phospholipid content of the microsomal suspension at zero time was 555 μg/mg of microsomal protein and its phospholipid composition, 59 4% phosphatidylcholine, 25 6% phosphatidylethanolamine, 11 8% phosphatidylinositol plus phosphatidylserine, and 3 2% sphingomyelin To calculate the quantities of individual lysophosphatides released the following molecular weights were assumed, for phosphatidylcholine, 810, for phosphatidylethanolamine, 750, and for phosphatidylinositol plus phosphatidylserine, an average of 830

equimolar mixture of linoleic acid and the lysophosphatide produced a stimulation approximately equivalent to that of linoleic acid alone

The activation by lysophosphatidylcholine most closely resembles that produced by phospholipase A although the latter occurred at lower product concentrations Perhaps this is not surprising since in the experiment of Fig 5 lysophosphatide and fatty acids were added to the membranes' normal complement of intact phospholipid so that the activations by phospholipase A and added hydrolysis products occurred under conditions which were not identical

The results of Fig 5 are in reasonable agreement with those of Hanninen and Puukka [3, 9] However, those authors reported [3] that phosphatidylcholine at a concentration of 40 μg P per mg of microsomal protein stimulated the UDPglucuronyltransferase activity of an intact microsomal preparation while we found (Fig 2) no activation of the enzyme by the phospholipid at any concentration up to 80 μg P per mg of microsomal protein To explain this discrepancy we suggest that the commercial preparation of phosphatidylcholine used by Hanninen and Puukka might have contained lysophosphatidylcholine In addition, we observed no activation of UDPglucuronyltransferase by the saturated fatty acids at concentrations below 0 3 μmole/mg of microsomal protein, while they recorded 2–3-fold increases in activity at this concentration of both palmitic and stearic acids [9]

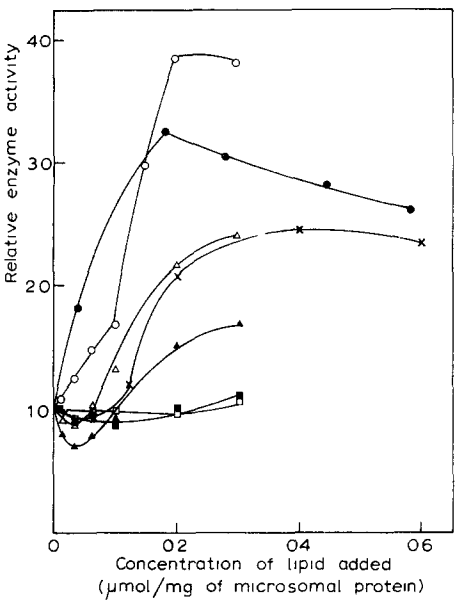

Fig 5 Effects of phospholipase A hydrolysis products on UDPglucuronyltransferase activity Microsomal suspensions were treated with various concentrations of palmitic acid (□), stearic acid (■), linoleic acid (△), arachidonic acid (▲), lysophosphatidylcholine (○), and an equimolar mixture of linoleic acid and lysophosphatidylcholine (×) as described in Materials and Methods Enzyme activity is expressed relative to that of an untreated microsomal suspension For comparison is included the effect of phospholipase A (●, data calculated from those of Figs 1 and 4)

The results of Fig 5 indicate that activation of UDPglucuronyltransferase can be simulated by adding the products of the phospholipase A reaction to intact microsomal membranes Moreover, activation of the enzyme by products was reversed by albumin as was activation by phospholipase A (Fig 2) When UDPglucuronyltransferase was activated by an equimolar mixture of linoleic acid and lysophosphatidylcholine (0 2 μmole of each lipid per mg of microsomal protein) the effect was fully reversed by albumin at concentrations in excess of 4 mg/mg of microsomal protein

Since the activation by lysophosphatidylcholine most closely resembled that by phospholipase A (Fig 5) we suggest that lysophosphatides are probably responsible for activation of UDPglucuronyltransferase by phospholipase A

DISCUSSION

To account for activation of microsomal UDPglucuronyltransferases by phospholipase A it has been proposed [8] that an intact membrane phospholipid environment constrains the enzyme in a conformation of low activity and that perturbation of phospholipids releases it to express full activity If this theory is correct, our data (Fig 2) indicate that UDPglucuronyltransferase is not specifically constrained by any particular phospholipid since all the phospholipids tested reversed the activation by phospholipase A Indeed, activation does not appear even to be due directly to degradation of membrane phospholipids The results of Figs 2, 3 and 5 establish that it is

the surface-active products (especially lysophosphatides) of the catalytic action of the phospholipase which are responsible Thus, activation of rat liver microsomal UDP-glucuronyltransferase by phospholipase A closely resembles that by detergents [6, 7, 22, 23] and by the lysophosphatides in microsomal membranes isolated from protein-deficient rats [10]

The reported [3] activation of UDPglucuronyltransferase by phospholipase C (EC 3 1 4 3) also might be due to surface-active compounds, even though the immediate products of phospholipase C action (diglycerides and phosphomonoesters) are not surface-active, rat liver microsomal fractions contain an acylhydrolase which liberates fatty acids from the diglycerides produced by phospholipase C [20, 21] Moreover, it has been recorded [3] that phospholipase D (EC 3 1 4 4), which does not liberate surface-active products, did not activate UDPglucuronyltransferase Therefore, we propose that activation or UDPglucuronyltransferase by phospholipid hydrolases is due to the release (directly or indirectly) of stimulatory surface-active compounds Recently, Mookerjea and Yung [24] concluded that the activation of rat liver microsomal UDPgalactose glycoprotein galactosyltransferase by phospholipase A is due to the detergent properties of the lysophopshatidylcholine produced, suggesting that our hypothesis may be of more widespread application

Release of UDPglucuronyltransferase from a conformational constraint, to which it is subject in intact microsomal membranes [8], is only one plausible explanation for the enzyme's activation Winsnes [22] and Mulder [23] proposed that detergents unmask enzyme molecules or active sites on the enzyme which are normally inaccessible to substrates due to the location of the protein within the membrane The data presented in this paper do not allow us to distinguish between these two theories but in view of the findings that detergents [25], phospholipase A, unsaturated fatty acids and lysophosphatidylcholine [14] increase the permeability of microsomal membranes they provide some support for the second

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