THE PHOSPHOLIPID-DEPENDENCE OF UDP-GLUCURONYLTRANSFERASE

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Summary. Evidence is presented indicating that specific degradation of the microsomal phospholipid membrane by phospholipase A or phospholipase C produces a concomitant inactivation of UDP-glucuronyltransferase. This inactivation can be reversed by adding phospholipid micelles. It is therefore considered that the activity of the enzyme depends on phospholipids and hence, probably, on the structural integrity of the microsomal membrane.

Enzymes involved in the metabolism of many drugs are located in the microsomal fraction of liver, where they are bound to the microsomal membrane (1,2). Failure of several attempts to solubilise such microsomal enzymes suggest that their attachment to the intact membrane is required for full activity (3,4). The finding (4) that aniline hydroxylase activity may be restored to a lipid-depleted solubilised enzyme by incubation with a microsomal lipid emulsion, lends support to this idea.

Attempts to purify microsomal UDP-glucuronyltransferase (EC 2.4.1.17) have frequently resulted in loss of activity and fractions of reduced stability (5-7). Lueders and Kuff (8) who studied the effects of detergents on microsomal activity suggested that membrane structure is an important factor in the activity and stability of the enzyme. Further work on the effects of deoxycholate, digitonin, detergents, crude lipases and ultrasonication (9-11), support the view that disruption of membrane structure has a marked effect on enzyme activity.

In order to study the membrane-dependence of glucuronyltransferase we have investigated the effects of treating microsomal preparations with phospholipase A and phospholipase C, "reagents" whose sites of action on

membrane phospholipids are fairly well defined. The effects of incubating microsomes, treated in this way, with phospholipid dispersions has also been investigated. This approach has recently proved to be successful in demonstrating the membrane-dependence of microsomal ATPase (12,13) and glucose 6-phosphatase (14).

Materials and Methods

Microsomes were prepared from the livers of male guinea pigs (Hartley strain, 250-400 g) essentially by the method of Pogell and Krisman (15). Washed microsomal pellets were suspended in 0.154M KCl and stored at -18°. Over several weeks the preparations suffered only slight loss of glucuronyltransferase activity. These fractions possessed high microsomal marker enzyme activities (glucose 6-phosphatase and nucleoside diphosphatase) as estimated according to Dallner, Siekevitz and Palade (2). Glucuronyltransferase activity was measured with p-nitrophenol as acceptor (16).

Phospholipase A was purified from lyophilised Crotalus adamanteus venom (Koch-Light Laboratories Ltd.) according to Saito and Hanahan (17), to the stage prior to chromatography on DEAE-cellulose and lyophilised. Partially purified phospholipase C from Clostridium welchii was purchased from Sigma London Chemical Co. Ltd. Microsomes were incubated with phospholipases in presence of 12.5mM tris-HCl buffer, pH 8.0, and 2.5mM $CaCl_2$ and the reactions terminated by adding excess EDTA.

A total lipid fraction was prepared from microsomal pellets by homogenising with $CHCl_3$ - CH_3OH solution (2:1, by vol.), filtering off the sediment and evaporating the solution to dryness under reduced pressure. The phospholipids were isolated by chromatography on a silicic acid column (14) and a diglyceride fraction prepared from them by treatment with phospholipase C (14). Phospholipid micelles were prepared in a tris (20mM), EDTA (15mM), pH 8.0 medium by sonic oscillation.

Inorganic P and total acid-soluble P were determined by the methods described by Leloir and Cardini (18). Phospholipid P was estimated by

the method of Chen, Toribara and Warner (19) and microsomal protein by a biuret method (20).

Results and Discussion

Phospholipase A produced a gradual inactivation of the glucuronyltransferase activity of microsomes in the presence of Ca^{2+} (Figure 1).

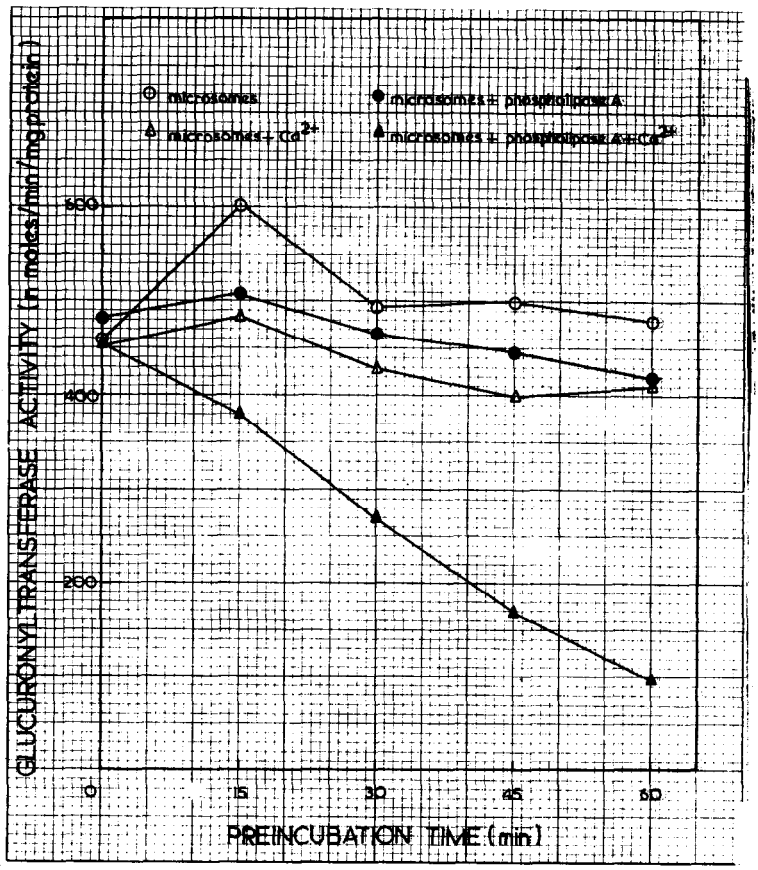

FIG. 1. The effect of phospholipase A on microsomal glucuronyltransferase activity. Microsomes (32 mg protein) were incubated at room temperature (18°) in 12.5mM tris-HCl buffer, pH 8.0, in a total volume 2.4 ml. $CaCl_2$ (2.5mM) or phospholipase A (3.2 mg protein), or both, were added and at the times shown 0.2 ml aliquots of the mixtures withdrawn and assayed for glucuronyltransferase activity in presence of 3.3mM EDTA.

In the absence of Ca^{2+} no significant depression of the enzymic activity was observed, as expected from the known Ca^{2+} -dependence of this enzyme (17). Treatment of microsomes with phospholipase C caused a rapid loss

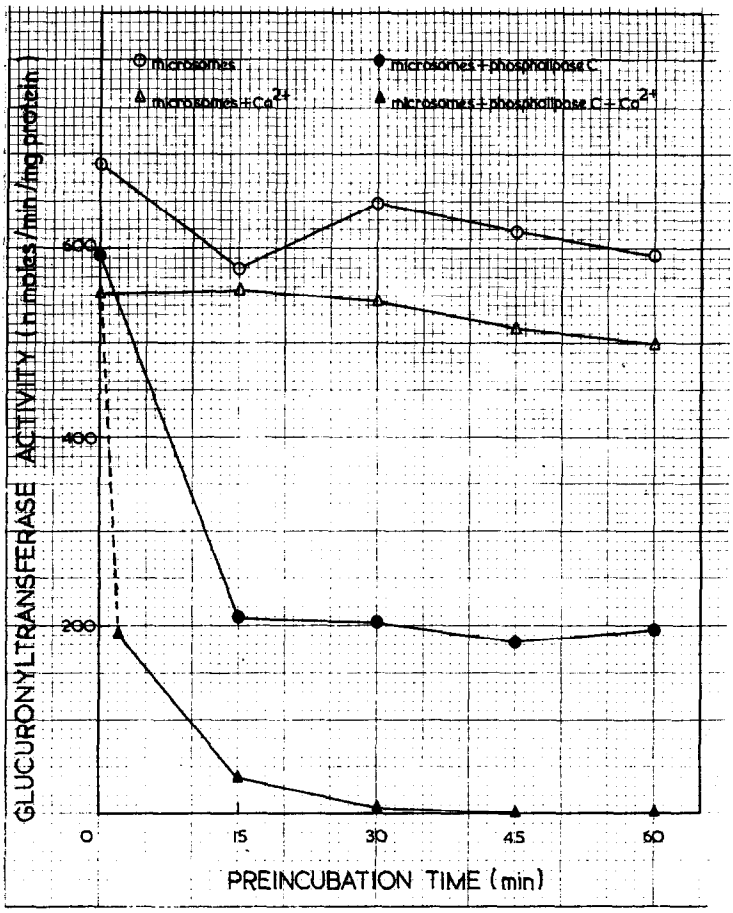

FIG. 2. The effect of phospholipase C on microsomal glucuronyltransferase activity. Microsomes (30.6 mg protein) were incubated at room temperature (19°) in 12.5mM tris-HCl buffer, pH 8.0, in a total volume of 2.4 ml. $CaCl_2$ (2.5mM) or phospholipase C (3.06 mg), or both, were added and at the times shown 0.2 ml aliquots of the mixtures withdrawn and assayed for glucuronyltransferase activity in presence of 3.3mM EDTA.

of glucuronyltransferase activity which was accelerated by Ca^{2+} (Figure 2). The rapid inactivation by phospholipase C in presence of Ca^{2+} was, however, totally inhibited by EDTA (10mM). In separate similar experiments, the release of phospholipase A and phospholipase C hydrolysis products (fatty acids, i.e. titrable acidity, and total acid-soluble P respectively) was found to parallel the diminution of glucuronyltransferase activity. These results suggest that the phospholipase-catalysed degradation of the membrane phospholipids is accompanied by inactivation of glucuronyltransferase and

that the enzyme is dependent on phospholipids for activity.

It could be argued that products of phospholipase hydrolysis might have inhibited glucuronyltransferase activity in the above experiments, and that degradation of the microsomal membrane phospholipid is not primarily responsible for inactivation of the enzyme. Crystalline albumin was used to remove any inhibitory activity due to fatty acids released by phospholipase A. However, microsomal glucuronyltransferase activity was rapidly inactivated by phospholipase A both in presence and absence of albumin, while no significant loss of activity was noted in the controls (Table 1). Since, under these conditions the fatty acids released are almost totally bound by albumin it is concluded that they are not responsible for inactivation of glucuronyltransferase. Phospholipase A also liberates lysolecithin from phospholipids; phospholipase C liberates diglycerides and phosphomonoesters. Lysolecithin, a diglyceride fraction and phosphorylcholine were incubated with microsomes and the glucuronyltransferase activities assayed. The results (Table 2) indicate that none of these hydrolysis products inhibited enzymic activity to any significant extent even at concentrations three times those produced when phospholipase A or C was incubated with microsomes for 60 min. It is concluded, therefore, that release of products during phospholipase-treatment of microsomes does not account for the inactivation of glucuronyltransferase.

Crude phospholipase preparations often contain considerable quantities of proteolytic activity. Proteolytic contamination of the partially purified preparations used in these experiments might degrade the glucuronyltransferase protein and inhibit its activity. Trypsin-treatment is known to abolish the Ca^{2+} transport activity associated with microsomal ATPase (13). However, Saito and Hanahan (17) report that the heat-treatment stage in the purification of phospholipase A removes proteolytic activity. In any case, such activity could not be detected in either phospholipase fraction, using serum albumin as substrate.

Phospholipase A	Albumin	Activity (n moles/min/mg protein) Initial	After preincubation	Inactivation (% initial activity)
-	-	587	420	28.5
-	+	729	610	16.3
+	-	582	65	88.8
+	+	596	134	77.6

TABLE 1. The effect of bovine serum albumin on inactivation of glucuronyltransferase activity by phospholipase A. Microsomes (23.4 mg protein), tris-HCl buffer, pH 8.0, (12.5 mM) and $CaCl_2$ (2.5 mM) were incubated at room temperature (20°) in a final volume of 2.4 ml. Crystalline bovine serum albumin (10 mg/ml) or phospholipase A (2.34 mg protein), or both, were added. Samples (0.2 ml) of the mixtures were withdrawn immediately and after 60 min, and assayed for glucuronyltransferase activity in presence of 3.3mM EDTA.

Concentration of hydrolysis product	Glucuronyltransferase activity (% control) Lysolecithin	Diglyceride	Phosphorylcholine
0	100	100	100
x1	107	84	115
x2	92	83	113
x3	92	118	116

TABLE 2. The effect of phospholipid hydrolysis products on glucuronyltransferase activity. Microsomes (7.74 mg protein) were incubated with and without lysolecithin, a diglyceride fraction or phosphorylcholine at room temperature (21°) in a total volume of 0.6 ml. (Lysolecithin and diglyceride were added in ethanolic solution). After 20 min samples (0.2 ml) were withdrawn and assayed for glucuronyltransferase activity.

In the first column x1 represents the amount of phospholipid hydrolysis product liberated from microsomes in 60 min by phospholipase A or phospholipase C; x2 and x3 represent 2 and 3 times these concentrations. The calculation assumes M.W. for lysolecithin and diglyceride of 500 and 560 respectively, and is based on that given by Duttera, Byrne and Ganoza (14).

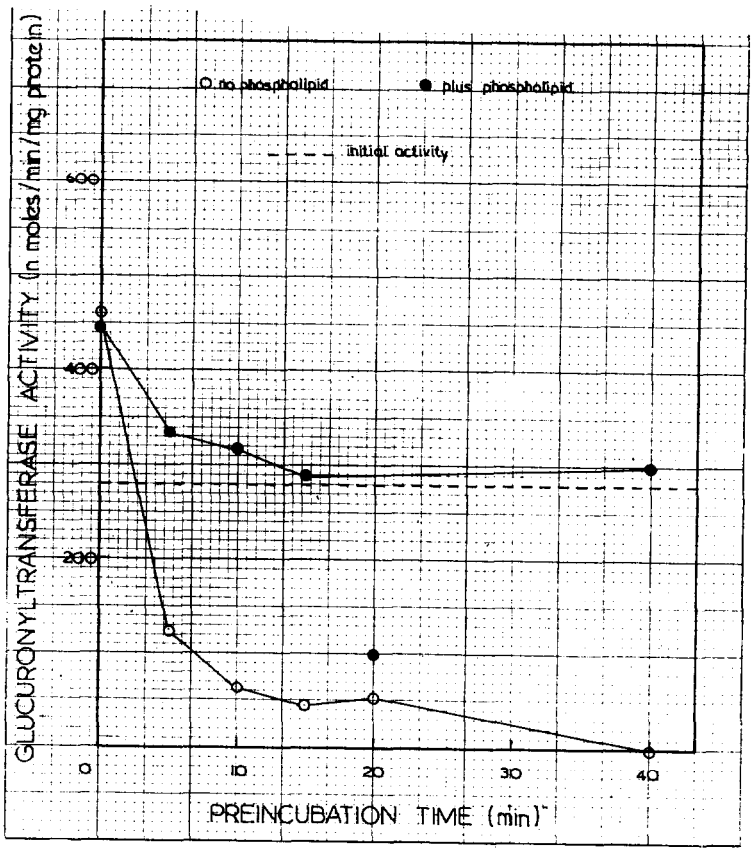

FIG. 3. Reactivation of glucuronyltransferase activity by phospholipid micelles after pretreatment with phospholipase A. Microsomes (74.4 mg protein) were incubated at room temperature (22°) with tris-HCl buffer, pH 8.0 (12.5mM), $CaCl_2$ (2.5mM) and phospholipase A (3.72 mg protein), in a total volume of 5.0 ml. At the time shown samples (0.3 ml) of the mixture were withdrawn and added to 0.6 ml of tris (20mM), EDTA (15mM), pH 8.0 and to 0.6 ml of phospholipid dispersion (238 µg P). After 5 min at room temperature 0.2 ml aliquots of the treated microsomes, with and without phospholipid, were assayed for glucuronyltransferase activity. Initial activity represents the activity of untreated microsomes.

If the enzyme is membrane-dependent it should be possible to restore the activity to preparations which have been inactivated by modification of the membrane structure, by replacing the phospholipid. The inactivation of microsomal glucuronyltransferase activity by phospholipase A was reversed by phospholipid micelles to values slightly greater than that of untreated microsomes (Figure 3 shows typical results). In similar

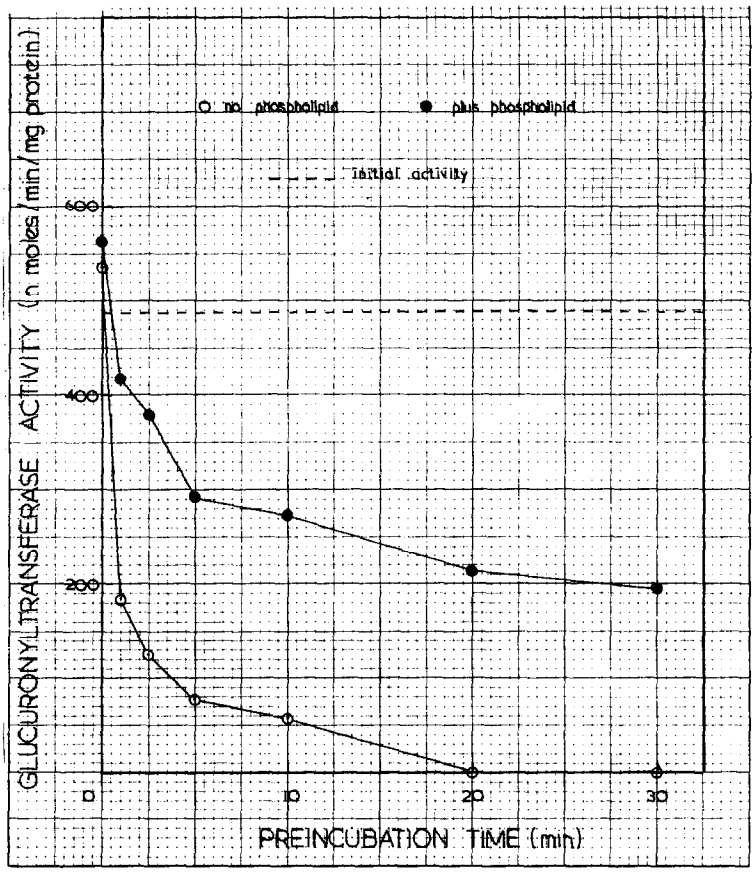

FIG. 4. Reactivation of glucuronyltransferase activity by phospholipid micelles after pretreatment with phospholipase C. Microsomes (69.3 mg protein) were incubated at room temperature (22°) with tris-HCl buffer, pH 8.0 (12.5mM), $CaCl_2$ (2.5mM) and phospholipase C (6.93 mg), in a total volume of 5.0 ml. At the times shown samples (0.3 ml) of the mixture were withdrawn and added to 0.6 ml of tris (20mM), EDTA (15mM), pH 8.0 and to 0.6 ml of phospholipid dispersion (232 µg P). After 5 min at room temperature 0.2 ml aliquots of the treated microsomes, with and without phospholipid, were assayed for glucuronyltransferase activity. Initial activity represents the activity of untreated microsomes.

experiments using phospholipase C (Figure 4 shows typical results) the diminished activity of all enzyme-treated samples was partially restored by phospholipid micelles. Although phospholipid reactivation after phospholipase C treatment decayed markedly with time, when no glucuronyl-transferase activity could be detected in the absence of phospholipid, its presence activated the enzyme to 40 - 44% of the initial activity. The initial enhancement of activity at zero time in Figures 3 and 4 is

presumably a result of the additional dilution and incubation stages not involved in the experiments of Figures 1 and 2. It was also observed in a separate control experiment, in which microsomes were preincubated in the absence of phospholipases. The inclusion of phospholipid micelles in this control experiment resulted in activities at approximately the initial activity level. It is concluded that the data of Figures 3 and 4 represent genuine reactivations by phospholipid micelles.

These results support the view that the activity of the enzyme depends on phospholipids and hence, probably, on the structural integrity of the microsomal membrane.

Acknowledgement

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