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PHOSPHOLIPID DEPENDENCE OF UDP-GLUCURONYLTRANSFERASE

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Summary

Very extensive hydrolysis of phospholipids with pure *Bacillus cereus* phospholipase C at 5°C greatly inhibited the maximum demonstrable rate of glucuronidation of *p*-nitrophenol by UDPglucuronyltransferase in guinea pig liver microsomes. Lysophosphatidylcholine restored much of the inhibited activity but non-phospholipid surfactants or hydrolysis of diglycerides failed to reactivate. Phospholipid depletion likewise inhibited *o*-aminophenol glucuronidation and phospholipids restored activity. It is concluded that glucuronyltransferase specifically requires phospholipids for optimal activity. It seems unlikely that these phospholipids only serve to dissolve aglycones, or that they are direct physiological regulators of the transferase. Instead, a permissive role is ascribed to phosphlipids, allowing glucuronyltransferase to be regulated by other means.

Introduction

Effects of phospholipids upon UDPglucuronyltransferase (EC 2.4.1.17) are controversial (reviewed in ref. 1). Reports from one laboratory (e.g. refs. 2—5) suggest that phospholipids conformationally constrain the transferase in a state of low catalytic activity and that such constraint is essential for its physiological regulation. However, other evidence suggests that glucuronyltransferase is phospholipid dependent (e.g. refs. 6—12), and some attribute its constraint to structural latency, due to location of the transferase behind the permeability barrier of the endoplasmic reticulum membranes (e.g. refs. 8, 13 and 14).

Since glucuronyltransferase is unstable at physiological temperatures when delipidated [5,15], exhaustive treatment at 5°C with *Clostridium perfringens* and with pure *Bacillus cereus* phospholipase C was used to delipidate in the present study. The *B. cereus* enzyme hydrolysed microsomal phospholipids very extensively and the resultant very large inhibition of maximum demonstrable *p*-nitrophenol glucuronidation could only be reversed with phospholipids. Phospholipid hydrolysis also greatly inhibited maximum demonstrable

o-aminophenol glucuronidation, which was also reactivated by phospholipids. Apparent kinetic parameters of the transferase were examined in lipid-replete and in delipidated microsomes, to explore how phospholipids might act. It is concluded that they play additional roles to simply dissolving aglycones for the transferase.

Materials and Methods

Microsomes were prepared in 0.25 M sucrose, from male Duncan-Hartley strain guinea pigs starved overnight [13]. Microsomal pellets were stored for up to 16 days at −10° C. Pure *B. cereus* phospholipase C (activity 1000 I.U./mg protein) was a generous gift from Dr. R. Zwaal, Department of Biochemistry, Univeristy of Utrecht, The Netherlands. Less pure *Cl. perfringens (welchii)* and *B. cereus* phospholipases, the latter reportedly almost protease free, were purchased from Sigma Chemical Co., London, and from Makor Chemical Co., Jerusalem, respectively. Their nominal specific activities were 3 units and 40—100 units/mg protein, respectively.

Microsomal disruption. Glucuronyltransferase latency was removed by intermittent ultrasonication for 4 min with cooling [13], or by lysophosphatidylcholine treatment [16]. These gave good estimates of maximum demonstrable activity [13].

Assessment of phospholipid hydrolysis. Phosphatidylcholine hydrolysis was assessed radiochemically in every experiment and is corrected for 1.6—2.8% of non-lipid labelling by [*Me*-^{3}H]choline [17]. Hydrolysis of total phospholipids was also sometimes assessed by measuring lipid phosphorus in wet-ashed, purified lipid extracts [18].

Reactivation of delipidated transferase. Glucoronyltransferase was reactivated with ultrasonically dispersed guinea pig liver phospholipids [13], or by adding lysophosphatidylcholine in 10 mM maleate (pH 7.1) [16]; the microsomes were then stood for 10 min at 0° C. Lysophosphatidylcholine was generally at 1.16 mg/ml in assays and only slightly increased glucuronidation rates (3.5%) in lipid-replete, disrupted microsomes.

Assays. Glucuronidation of *o*- and *p*-nitrophenol were assayed with 4 mM UDPglucuronic acid and 0.4 mM acceptor as in ref. 13; EDTA was omitted in assays from Table I. The reverse transferase reaction between UDP and *p*-nitrophenylglucosiduronate was measured in 4 mM EDTA and 10 mM glucaro-1,4-lactone [13]. Protein was assayed by the Lowry method [19].

Results

Ability of lipase and of lysophosphatidylcholine to reactivate p-nitrophenol glucuronidation in delipidated microsomes

Prior to delipidation, some microsomal preparations were ultrasonicated under optimal conditions [13]: their maximum demonstrable rate of *p*-nitrophenol glucuronidation was 6.5—7.6 times the latent rate. Treatment of these ultrasonicated microsomes for 120 min at 5° C with phospholipase C from *Cl. perfringens* or with pure *B. cereus* phospholipase C decreased their maximum demonstrable *p*-nitrophenol glucuronidation by 66 and 85%, respectively, with

the hydrolysis of 77 or 94% of the total microsomal phospholipids and 90 or 99% of the phosphatidylcholine, on average (Table I). The *B. cereus* enzyme hydrolysed more phospholipid because of its broader substrate specificity against microsomal phosphatides (e.g. ref. 18). Similar results were obtained in four experiments by delipidating initially intact microsomes, which had not been ultrasonicated (Table I). Their latent activity was 3.0 ± 0.6 nmol *p*-nitrophenol glucuronidated/min per mg protein. After treatment with *Cl. perfringens* phospholipase, subsequent hydrolysis with *Rhizopus arrhizus* lipase of potentially inhibitory diglycerides [16] caused a small but significant reactivation (mean 23%), to 57% of the maximum demonstrable activity. However, lipase failed to significantly reactivate in *B. cereus*-treated microsomes (Table I), though examination of extracted lipids by thin-layer chromatography showed almost complete diglyceride destruction in both cases [16]. By contrast, 1-acylglycerylphosphorycholine (lysophosphatidylcholine), reactivated glucuronidation greatly to 76—90% of its maximum rate after *B.cereus* and 91% after *Cl. perfringens* phospholipase. Lysophosphatidylcholine was used for technical reasons, since it is a very poor substrate for the *B. cereus* enzyme [16]. This made reactivation possible without having to stop phospholipase activity with 1,10-phenanthroline, since this also inhibits glucuronyltransferase (Berry, C., unpublished). A range of concentrations (%, w/v) of digitonin (0.31—1.25), or Triton X-100 (0.05—0.20), of oleic acid (0.01—0.10) or of cetyltrimethylammonium bromide (0.02—0.10) all failed to significantly reactivate the delipidated transferase.

Reversible inhibition of o-aminophenol glucuronidation by phospholipid depletion

Fig. 1 shows the effect of increasing phospholipid hydrolysis on the glucuronidation of *o*-aminophenol in paired samples from three different microsomal preparations. One sample was untreated before exposure to phospholipase C (and its activity was thus highly latent), while the other had been ultrasonicated under optimal conditions to obtain a good estimate of its maximum demonstrable transferase activity [13], which was 4.5—8 times the latent rate. Extensive phospholipid depletion inhibited maximum activity greatly (89—93%), while with initially intact microsomes, it scarcely increased *o*-aminophenol glucuronidation above its very low latent rate. In another paired experiment, ultrasonically dispersed guinea pig liver phospholipids added to initially intact delipidated microsomes reactivated *o*-aminophenol glucuronidation greatly, to 83% of the maximum demonstrable activity (Table II). When the same microsomes were ultrasonicated before delipidation, reactivation was also substantial, though less effective (Table II). Hydrolysis of inhibitory diglycerides with lipase in the presence of albumin, restored only 19% of the maximum *o*-aminophenol glucuronidation in initially intact microsomes delipidated with *Cl. perfringens* phospholipase.

Effect of delipidation upon apparent v, K and V and K′ parameters of UDP-glucuronyltransferase utilizing p-nitrophenol

Table III shows apparent v values in phospholipid-replete and phospholipid-depleted microsomes, and apparent K parameters for UDPglucuronic acid,

TABLE I

REVERSIBLE INHIBITION OF *p*-NITROPHENOL GLUCURONIDATION BY PHOSPHOLIPID-DEPLETION

Phospholipase C treatment was for 120 min at 5°C, generally in the presence of 60 mg/ml of bovine serum albumin, using 3—6 units/ml of *Cl. perfringens* phospholipase C or 5—30 units/ml of pure *B. cereus* phospholipase C. *Rhizopus arrhizus* lipase suspension (Boehringer), when present, was added to glucuronidation assays at 10 μl/ml for 5—10 min at 37°C [16]. Statistical significance was assessed by *t*-tests: P^a is the probability that control glucuronidation rates do not differ from those of delipidated microsomes and P^b is the probability that lysophosphatidylcholine or lipase do not alter glucuronidation rates in delipidated microsomes.

	Activity (nmol *p*-nitrophenol glucuronidated/min per mg of protein)					
	B. cereus phospholipase			*Cl. perfringens* phospholipase		
	Initially disrupted microsomes	*P*	Initially intact microsomes	*P*	Initially disrupted microsomes	*P*
Maximum demonstrable activity (control)	19.6 ± 2.7 (9)		22.8 ± 4.8 (4)		19.9 ± 3.6 (8)	
Activity after delipidation	2.9 ± 1.1 (9)	<0.001 [a]	4.3 ± 0.8 (4)	<0.01 [a]	6.5 ± 1.7 (8)	<0.001 [a]
Delipidated activity as percent of maximum activity	15%		19%		34%	
Phosphatidylcholine hydrolysed (%)	98.8 ± 1.09 (9)		98.6 ± 0.9% (4)		89.7 ± 6.7 (8)	
Total phospholipids hydrolysed (%)	93.2, 94.5		94.5, 93.6		75.6 ± 4.7 (5)	
Delipidated + phospholipid: activity	14.8 ± 3.0 (5)	<0.001 [b]	20.5 ± 4.9 (4)	<0.01 [b]	18.1 ± 3.1 (3)	<0.01 [b]
Percent of maximum activity	76%		90%		91%	
Delipidated + lipase: activity	3.5 ± 1.4 (8)	>0.2 [b]	4.8, 4.9		11.4 ± 4.5 (7)	<0.02 [b]
Percent of maximum activity	18%		21%, 21.5%		57%	

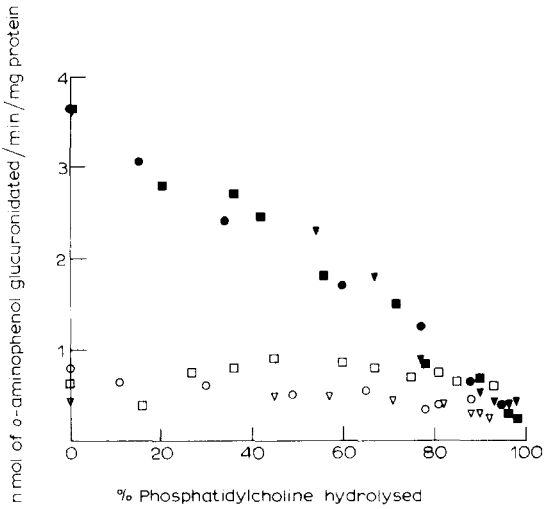

Fig. 1. Effect of progressive delipidation upon *o*-aminophenol glucuronidation in intact and disrupted microsomes. Intact (○, ▽, □) and ultrasonicated microsomes (●, ▼, ■) were delipidated at 5°C with *Cl. perfringens* phospholipase C as in Table II. Samples were withdrawn between 0 and 180 min into 0.004 M EDTA for assaying phosphatidylcholine hydrolysis and *o*-aminophenol glucuronidation activity.

UDP, *p*-nitrophenol and *p*-nitrophenylglucosiduronate, respectively, determined by constructing Lineweaver-Burk plots. These were measured with one substrate concentration held constant (*p*-nitrophenol at 0.4 mM; UDPglucuronic acid at 4 mM; UDP at 4 or 7 mM; *p*-nitrophenylglucosiduronate at 4 mM), and the other varied (*p*-nitrophenol from 0.2 to 0.8 mM; UDPglucuronic acid from 2 to 10 mM; UDP from 0.5 to 4 mM; *p*-nitrophenylglucosiduronate from 0.5 to 10, 1.9 to 10 or 2 to 80 mM). Though parameters differed markedly between separate experiments, phospholipid hydrolysis always impaired the apparent affinity of the transferase for the measured substrate, with 6—8-fold increases in the K for UDPglucuronic acid and 7—15-fold increase in the K for *p*-nitrophenol, which agrees with the findings on rat [8] and mouse liver microsomes [9]. Decreases of lesser magnitude were seen in the apparent affinity for reverse reaction substrates. In paired experiments, the maximum demonstrable apparent v was also generally decreased by delipidation, except for one experiment (Expt. 13).

Table IV shows the apparent bisubstrate V and K' parameters, in phospho-

TABLE II

PHOSPHOLIPIDS REVERSE THE INHIBITION OF *o*-AMINOPHENOL GLUCURONIDATION, CAUSED BY DELIPIDATION

Microsomes were delipidated at 5°C with *Cl. perfringens* phospholipase C as in Table I, omitting serum albumin and were reactivated with ultrasonicated guinea pig liver phospholipids.

	Activity (nmol *o*-aminophenol glucuronidated/min per mg of protein)			
	Control	Control + phospholipid	Phospholipase treated	Phospholiase treated + phospholipid
Initially intact microsomes	0.55	0.50	0.20	2.50
Ultrasonicated microsomes	3.00	3.20	0.10	1.70

TABLE III

APPARENT MONOSUBSTRATE KINETIC PARAMETERS FOR CONTROL, ULTRASONICATED AND PHOSPHOLIPID-DEPLETED MICROSOMES

Conditions were as in Table II, except that Makor *B. cereus* phospholipase C (1—2.5 units/ml) was used. K is in mmol/l ±S.D. where appropriate; v is in nmol of *p*-nitrophenol glucuronidated/min per mg of protein ±S.D. where appropriate. Abbreviations are UDPGA, UDPglucuronic acid; *p*-NP, *p*-nitrophenol; UDP, uridine diphosphate; *p*-NPGA, *p*-nitrophenyl-β-glucosiduronate.

Expt.	Treatment	Percent phosphatidylcholine hydrolysed	K (mM)		v
1—5	None (intact microsomes)	—	UDPGA	0.7 ± 0.2	2.5 ± 0.3
6—10	*B. cereus*	99.3 ± 2	UDPGA	5.9 ± 2.7	18.2 ± 5.3
11	Ultrasonication		UDPGA	0.71	27
11	Ultrasonication + *Cl. perfringens*	89	UDPGA	4.4	14
12—13	Ultrasonication		*p*-NP	0.14, 0.27	35, 53
12—13	Ultrasonication + *Cl. perfringens*	89, 98.8	*p*-NP	0.95, 4.0	23, 53
14	Ultrasonication		UDP	0.26	16
14	Ultrasonication + *Cl. perfringens*	96.8	UDP	0.69	4.1
15	Ultrasonication		*p*-NPGA	4.4	33
15	Ultrasonication + *Cl. perfringens*	97	*p*-NPGA	8.3	11
16	Ultrasonication + *Cl. perfringens*	98	*p*-NPGA	31	33
16	Above + phospholipid	98	*p*-NPGA	9.6	59

lipid-replete and phospholipid-depleted microsomes glucuronidating *p*-nitrophenol. These parameters were determined from secondary double reciprocal plots of intercepts obtained in primary Lineweaver-Burk plots of rates of glucuronidation at 0.2—0.8 mM *p*-nitrophenol and 1—5 or 2—8 mM UDPglucuronic acid. EDTA was 0.9 or 1.5 mM in assays. In both experiments, large decreases in V accompanied delipidation of ultrasonicated microsomes. Increases in K' were also marked, but ultrasonication, itself, also seemed to lessen bisubstrate affinities.

TABLE IV

EFFECT OF DELIPIDATION UPON THE APPARENT BISUBSTRATE KINETIC PARAMETERS FOR *p*-NITROPHENOL GLUCURONIDATION

Conditions were as in Table II. Phosphatidylcholine hydrolysis was 78% in Expt. 1 and 94% in Expt. 2. V is in nmol *p*-nitrophenol glucuronidated/min per mg of protein; K' is in mmol/l.

Treatment	K' for UDPglucuronic acid (mM)	K' for *p*-nitrophenol (mM)	V
None	0.37	0.15	2.4
Ultrasonication	1.7	0.40	42
Ultrasonication + *Cl. perfringens*	2.5	1.1	22
None	0.71	0.20	3.1
Ultrasonication	3.1	0.55	59
Ultrasonication + *Cl. perfringens*	4.4	0.65	27

Discussion

Extensive loss of glucuronyltransferase activity, specifically reversible by phospholipids, was observed when microsomes were delipidated at 5° C with phospholipase C. However, destruction of potentially inhibitory diglycerides failed to significantly relieve transferase inhibition, after very extensive phospholipid hydrolysis with the pure *B. cereus* enzyme. Therefore we conclude that glucuronyltransferase specifically requires phospholipids to exhibit its maximum demonstrable activity. For reasons discussed elsewhere in respect of glucose-6-phosphatase [18], precisely which phospholipids support glucuronyltransferase activity can best be determined with the completely pure transferase preparation [20], which is now available. Diglycerides are only weak transferase inhibitors [16], while after extensive delipidation residual membrane phospholipids will associate predominantly with those proteins with highest binding affinities [18]. Therefore, the failure of diglycerides to inhibit residual glucuronyltransferase activity, which survives treatment with *B. cereus* phospholipase, may be because this transferase tightly binds some of the small amount of remaining phospholipid. Diglycerides may then be unable to compete effectively for binding.

Others have reported that phospholipids affect glucuronyltransferase in exactly the opposite sense to that discussed above, by constraining the transferase in a conformation with low catalytic activity (e.g. refs. 2–5). However, the claim that glucuronyltransferase can be activated by relieving conformational phospholipid-constraint on it has not been convincingly supported by demonstrating the specific reversibility of this constraint. Reimposition of phospholipid-constraint was sought but not found in microsomes disrupted by three different mechanical methods [13], while inhibition of the transferase by phospholipid dispersion in microsomes briefly pre-treated with phospholipase A_2, is non-specific, since serum albumin also inhibited [21]. Both phospholipids and serum albumin apparently sequester membranolytic lysophosphatides and fatty acids, and thus restore transferase latency [1,21]. Finally, the observation that phospholipids either inhibited (reimposed constraint) or were without effect upon *p*-nitrophenol glucuronidation when added to phospholipase C-treated microsomes [2], is not confirmed here nor in studies from two other laboratories [6,9]. In fact, phospholipids have now been reported to stimulate *p*-nitrophenol glucuronidation in delipidated subfractions prepared from phospholipase A_2-treated microsomes [22].

It has been assumed that conformational phospholipid-constraint of glucuronyltransferase is relieved by treating microsomes with phospholipase A_2 or C or with surfactants or dilute alkali or by ultrasonicating them (e.g. refs. 2, 4 and 5). However, if the transferase is located behind a membrane barrier, these procedures, being membranolytic, would stimulate it by damaging this barrier [13]. A priori, it might also be expected that such severe and diverse membranolytic treatments would each produce quite different changes in the membrane micro-environment of the transferase. This could explain why the transferase, when stimulated by these different treatments, exhibits differences in its kinetic parameters when utilizing *p*-nitrophenol [2,4,5].

As is the case for most, if not all, phospholipid-dependent enzymes [23], it

it still unknown precisely how phospholipids support glucuronyltransferase activity. It seems unlikely that they simply serve to dissolve aglycones, since delipidation should then only decrease aglycone affinity, while the present kinetic studies also generally showed major decreases in v and V on delipidation, and also increases in the K and K' for UDPglucuronic acid.

Though glucuronyltransferase requires phospholipids, these are unlikely to physiologically regulate glucuronidation. Major alterations in phospholipid levels are needed to markedly decrease maximum glucuronidation rates or to stimulate latent activity [11], and the concomitant membrane disruption would be expected to produce a catalytically inefficient enzyme, incapable of functioning well in the presence of likely physiological concentrations of substrates and inhibitors [5,9,24]. Nor can phospholipids be held uniquely responsible for compartmenting the transferase. Though phospholipid hydrolysis is membranolytic, endoplasmic reticulum, like all membranes, is a supramolecular structure and its barrier properties can also be impaired by protein-specific agents such as trypsin and mercurials, both of which can remove the latency of glucuronyltransferase [25—27]. Elsewhere [14], we have very briefly described a model, in which phospholipids are essential for optimal glucuronyltransferase activity but do not directly regulate it. Regulation is instead imposed by other means. The endoplasmic reticular membranes shield the latent transferase from cytosolic inhibitors, and its regulation involves interaction with a postulated UDPglucuronic acid permease and with nucleoside diphosphatase; the effectors, UDP-*N*-acetylglucosamine and divalent cations, would act largely via these two latter components.

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