THE EFFECT OF PHOSPHOLIPASE C TREATMENT ON THE LATENCY AND SEDIMENTABILITY OF RAT LIVER MICROSOMAL NUCLEOSIDE DIPHOSPHATASE

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Received 19 February 1975

1. Introduction

The early studies of Ernster and Jones [1] showed that rat liver microsomes contain an active nucleoside diphosphatase (NDPase–EC 3.6.1.6.) which is activated by detergent (DOC) treatment. These authors [1], and subsequently others found that at least some of this enzyme activity could be easily released from the microsomal vesicles by a variety of treatments including Ultra Turrax blending [1], freeze-thawing [2], low concentration of various surfactants [2,3], high pH [3,4] and conditions favouring lipid peroxidation [2]. Karmanov et al. [2] concluded that, in view of the inverse correlation between vesicle integrity and release of the enzyme, that it is located in the vesicle lumen. In contrast, Kuriyama [3] concluded that NDPase is, in fact, weakly membranebound, primarily because phospholipase A and C treatment of the microsomes resulted in a completely sedimentable though fully activated form of the enzyme which was accessible to proteases and a specific antibody.

In the present investigation, rat liver microsomes were treated with phospholipase C (from *Cl. perfringens*, formely *Cl. welchii*) at 0°C and the reaction was terminated with EGTA. When the resuspended microsomes were post-incubated at 30°C [3], a post-incubation-dependent activation of NDPase occurred. Centrifugation of the suspension suggested that this activation was associated with a release of the enzyme from the microsomal vesicles.

2. Experimental

The livers of 200–250 g male Sprague–Dawley rats that had been fasted for 15 hr were homogenized in 0.25 M sucrose [5] to give a 25% w/v homogenate. Microsomes were prepared by centrifuging 2×10^5 g_{av}. min supernatants (rotor No. 40, Spinco) at 6×10^6 g_{av}. min (50 Ti rotor). In some experiments, these conditions were changed to 10^5 g_{av}. min and 3×10^6 g_{av}. min respectively.

Microsomes (0.25 g liver equiv/ml) were incubated on ice in a medium containing 600–800 μg/ml phospholipase C from *Cl. perfringens* (Sigma), 20 mM sodium maleate buffer pH 7.0 (25°C), 1 mM $CaCl_2$ and 0.188 M sucrose for up to 135 min. Phospholipid hydrolysis was terminated by making the mixture 3.3 mM in EGTA [6] and the suspension was centrifuged (10^7 g_{av}. min; 50 Ti rotor). Controls either contained no phospholipase C (untreated controls), or EGTA and phospholipase C added together (treated controls).

Pellets were resuspended and post-incubated in 0.25 M sucrose or 0.25 M sucrose–20 mM (maleate pH 6.0–7.2 or Tris–HCl pH 7.5 adjusted at either 5 or 30°C) at 30°C for 30 min. The suspensions were cooled in ice and centrifuged (10^7 g_{av}. min).

Free NDPase activity was assayed by adding 0.1 ml fraction to 0.5 ml of a medium which contained 4 mM $MgCl_2$, 50 mM Tris–HCl pH 7.5 (37°C) and 5 mM IDP (Sigma) and which had been preincubated at 37°C. Incubations were for 5–7 min at 37°C. The released phosphate was determined by using either the procedure of Beaufay et al. [7] or the pH4 phosphate assay of Atkinson et al. [8] with Lubrol PX instead of Cirrasol. In the latter case, the phosphatase reaction was stopped by the addition of 0.4 ml ice-cold 0.25 M acetate buffer pH 4.0.

Total NDPase activity was assayed by making the

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microsome suspension 0.1% in DOC and using 0.1 ml of this in the assay.

Phospholipids were extracted with chloroform/methanol [9] and digested with perchloric acid; the phospholipid content was calculated from the phosphorus content [10] by assuming that 1 mg phospholipid contains 40 μg phosphorus.

3. Results

Treatment of rat liver microsomes with phospholipase C (*Cl. perfringens*) at 0°C appeared to result in some activation of sedimentable NDPase when up to 50% of the phospholipid had been hydrolyzed (fig.1). However, post-incubation at 30°C under conditions designed to prevent further phospholipid hydrolysis [6] resulted in a marked elevation of free activity (fig.1). Recentrifugation of phospholipase-treated microsomes showed that this activation was correlated with a loss of NDPase sedimentability, even in the case of microsomes stored at 0°C and not post-incubated (fig. 1). However, little activation and negligible released into the high speed supernatant was observed in the case of treated or untreated controls before or after post-incubation.

Although it is possible that a small amount (no more than 7%) of the phospholipid was hydrolyzed when the microsomes were post-incubated, it can be shown by comparing activation, maximum possible release and phospholipid hydrolysis at 0°C with activation and release occurring on post-incubation that this is not the cause of the above effect.

The experiment reported in fig.1 has been modified by using different media for both the initial phospholipase treatment and the post-incubation. In all cases, little activation occurred after 120–135 min phospholipase treatment at 0°C (never more and usually less than that shown in fig.1) whereas a further activation to at least 70% of the total activity always occurred on post-incubation. In some cases, (0.25 M sucrose or 0.25 M sucrose–Tris–HCl as the post-incubation medium) essentially maximum NDPase activation occurred when less than 50% of the phospholipid had been hydrolyzed. Maximum activation was associated with the release of a large part of the NDPase (table1).

Direct incubation [3] of microsomes with phospholipase C (*Cl. perfringens*) also resulted in activation and release (table 2). However, significantly more latent activity remained in the pellet and, therefore as expected, less supernatant activity was obtained despite the fact that phospholipid hydrolysis was

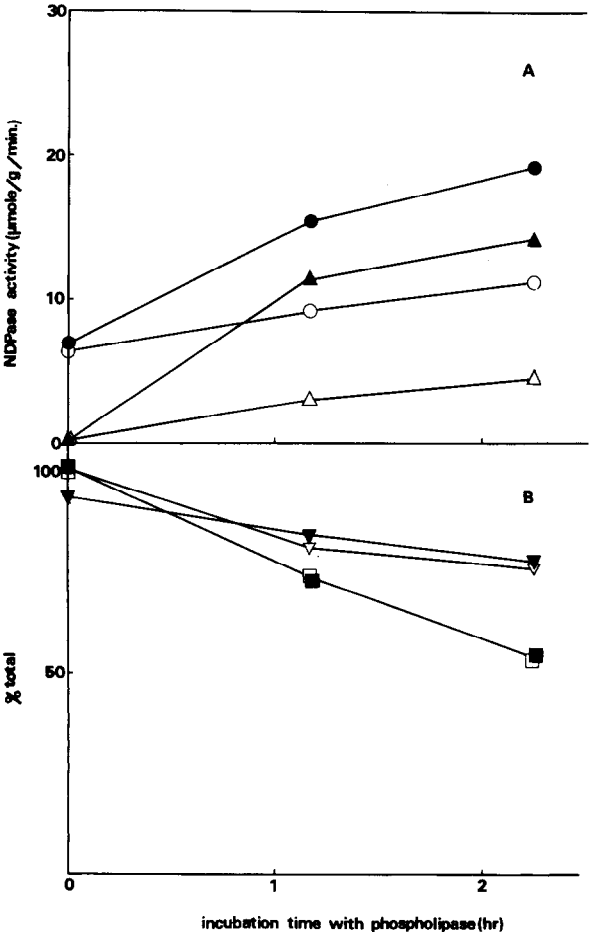

Fig.1. Effect of phospholipase treatment and post-incubation on the latency and sedimentability of NDPase. For details of procedures, see Experimental. A and B refer to the same experiment (post-incubation medium: 0.25 M sucrose–20 mM Tris–HCl pH 7.5 at 30°C). Free activity in resuspended microsomes before (–○–) and after (–●–) post-incubation; activity in 10^7 g_{av}. min supernatant before (–△–) and after (–▲–) post-incubation; total NDPase activity before (–▽–) and after (–▼–) post-incubation; phospholipid content before (–□–) and after (–■–) post-incubation. The absolute phospholipid content of control microsomes which had not been post-incubated was 8.0 mg/g. '% total' (fig.1B) means: percentage of value obtained for untreated control microsomes which had not been post-incubated.

Table 1
Release of NDPase into the high speed supernatant fluid (indirect incubation)

Expt No.	% phospholipid remaining	Free activity as % total activity	NDPase activity in 10^7 g_{av}. min supernatant fluid		
			in μmol/g/min	as % total NDPase in uncentrifuged post-incubated fraction	as % total non-post-incubated control NDPase
1	61	95	18.4	52 (3)	42
2	59	96	12.2	54 (2)	44
3	54	92	14.0	68 (1)	52

Microsomes were treated with phospholipase C (*Cl. perfringens*) at 0°C for 120–135 min and sedimented as described in Experimental except in expt 2 where the initial phospholipase digestion was carried out in 0.25 M sucrose–20 mM Tris–HCl buffer pH 7.5 (adjusted at 5°C) containing 1 mM $CaCl_2$. Post-incubation at 30°C was performed in 0.25 M sucrose (expt 1), 0.25 M sucrose–20 mM Tris–HCl pH 7.5 (5°C) (expt 2) and 0.25 M sucrose–20 mM Tris–HCl pH 7.5 (30°C) (expt 3). The NDPase activity in the high speed supernatant for untreated control microsomes is given in brackets. The recovery of free NDPase in the supernatant plus pellet compared to that of the uncentrifuged whole mixture for expt 1 and 2 was 91% and 89% respectively. 'Total non-post-incubated control NDPase' (extreme right column) refers to microsomes incubated without phospholipase at 0°C, sedimented, resuspended and then assayed for total activity.

more extensive than in the indirect experiments. This residual latency was apparently not affected by using increasing concentrations of phospholipase (table 2), suggesting that the cause was not that phospholipid hydrolysis was rate limiting. One possibility is that the phospholipase preparation contains an inhibitor of the activation phenomenon.

In a preliminary experiment, it was found that a

Table 2
Recovery of NDPase in the high speed supernatant fluid (direct incubation)

Expt No.	Source of phospholipase C	Phospholipase concentration (units/ml) *	Phospholipid remaining (%)	NDPase (as % total recovered NDPase **) in		
				Pellet		Supernatant
				−DOC	+DOC	
1a	*Cl. perfringens*	2.7	27	46	64	36 (2)
1b	*B. cereus*	5.0	7	10	11	89 (3)
2a	*Cl. perfringens*	2.7	30	39	61	39 (1)
2b	*Cl. perfringens*	6.4	29	34	57	43 (1)
2c	*Cl. perfringens*	9.5	28	40	58	42 (1)

Microsomes were incubated with various concentrations of phospholipase and centrifuged as described in [3]. One control (expt 1) and the incubation mixture containing *B. cereus* phospholipase also contained 20 mM $(NH_4)_2SO_4$. The recovery of NDPase relative to that of an incubated untreated control was 87, 92, 80, 81 and 80% for expts 1a, 1b, 2a, 2b and 2c. The NDPase activity in the high speed supernatant for (incubated) control microsomes is given in brackets.

* The units referred to here are defined in the Sigma catalogue and are different to the egg units of [3].

** Total recovered NDPase: total activity in pellet plus activity in supernatant.

purified preparation of *B. cereus* phospholipase C (Boehringer-Mannheim) also activated and released NDPase (table 2). In this case, both phospholipid hydrolysis and NDPase release were more extensive than for the *Cl. perfringens* enzyme.

4. Discussion

The simplest explanation for the post-incubation effects (fig.1) is that the diglyceride product of phospholipase C action, which sediments with the microsomes [11,12], is hydrolyzed by the very active endogenous microsomal lipase [13] during post-incubation; the fatty acids generated, being surfactants, then damage the membrane locally and cause release of NDPase. In fact, surfactants are known to release NDPase [1–3]. However, preliminary attempts to inhibit activation with defatted bovine serum albumin have been inconclusive. The latter inhibited post-incubation-dependent activation and release by 50–100% but had essentially no inhibitory effect when microsomes were incubated directly with phospholipase. Experiments are now in progress to elucidate the cause of this discrepancy and determine whether the above explanation is correct.

The finding that activation of NDPase following phospholipase treatment at 0°C and post-incubation was always associated with release into the high speed supernatant is consistent with the suggestion [2] that NDPase is at least partly located in the microsomal vesicle lumen. In constrast, Kuriyama [3] observed full activation but essentially no release of NDPase when microsomes were directly incubated with phospholipase C (*Cl. perfringens*). In an attempt to clarify the matter, microsomes were incubated with phospholipase C under conditions similar to those described by Kuriyama [3] (table 2). However, the relationship between activation and release was still apparent.

A completely satisfactory explanation for this discrepancy has not been found. The NDPase release reported here does not appear to be due to the presence of proteolytic or other contaminants in the *Cl. perfringens* phospholipase C preparation because little activation or release ($\leqslant 5\%$) was observed for treated controls. Moreover, in many experiments, phospholipase treatment at 0°C resulted in only 15–20% of the activation occurring on post-incubation, yet many contaminating activities should have been removed by the centrifugation prior to post-incubation. Finally, a purified *B. cereus* phospholipase C was also found to activate and release NDPase (table 2).

It should be pointed out that the evidence supporting the view that NDPase is really membrane-bound in vivo [1,3] is largely circumstantial and rests mainly on the previous [3] phospholipase results. Incomplete release following the mechanical treatment of microsomes [1] does not necessarily indicate that NDPase is membrane-bound since other factors, e.g. non-specific absorption effects, the entrapment of NDPase by ruptured and subsequently resealed vesicles and/or the formation of damaged vesicles permeable to IDP but not to NDPase, may be responsible. The fact that the distribution of NDPase on sucrose gradients and induction by phenobarbital parallels the distribution and induction of a number of other (membrane-bound) enzymes [3] seems to have little bearing on the question but also does not require that NDPase be membrane-bound.

Acknowledgements

I would like to thank Dr D. B. Roodyn (University College London) and Dr T. Hallinan (Royal Free Hospital Medical School) for helpful discussions and Dr D. Saggerson (University College London) for the gift of defatted bovine serum albumin. The author is a post-doctoral overseas scholar of the Royal Commission for the Exhibition of 1851.

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