THE EFFECT OF PHOSPHOLIPASE C TREATMENT OF MICROSOMES ON CYTOCHROMES *P*-450 AND b_5

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1. Introduction

Like other phospholipases, phospholipase C is a popular tool for investigating the relationship between membrane phospholipids and proteins. We were interested in investigating the possible involvement of polar headgroups of microsomal phospholipids in maintaining the native conformations of cytochromes *P*–450 and b_5 and in binding these proteins to the membrane of the endoplasmic reticulum. We are also interested in the possibility of solubilizing cytochrome *P*–450 by treating microsomes first with phospholipase C and then with a lipase, a procedure which would hydrolyze membrane phospholipids without at any time producing lysolecithin, a powerful detergent which denatures cytochrome *P*–450 [1] (J. DePierré, unpublished observations).

2. Materials and methods

Microsomes were prepared from the livers of 180–200 g male Sprague-Dawley rats by the method of Ernster et al. [2]. In tne experiments shown these rats received an intraperitoneal injection of 80 mg/kg phenobarbital once a day for three days before sacrifice; similar but less pronounced effects were seen with uninduced animals. Rough and smooth microsomes were separated according to Dallner [3]. When total, rough, or smooth microsomes were treated with phospholipase C (*Cl. welchii* Sigma), the medium contained 50 mM Tris, pH 7.5, 2.0 mM $CaCl_2$. 3–6 mg protein/ml and 100–150 μg phospholipase/mg microsomal protein. Treatment with crude pancreatic lipase (Sigma) was carried out under the same conditions except that in addition to 1 mg lipase/mg microsomal protein, the medium also contained 1 mg trypsin inhibitor/mg lipase in order to inhibit the trypsin which contaminates the lipase preparation. Incubations were carried out at 23°C and stopped by adding EDTA to give a final concentration of 10 mM and placing the tubes in ice.

Cytochromes *P*–450, *P*–420, and b_5 were measured by the method of Omura and Sato [4]. The hydrolysis of phospholipids was determined by extracting the remaining phospholipids into chloroform–methanol [5] and determining the remaining lipid phosphorus according to Bartlett [6]. The control incubations (zero-time incubation, incubation for 30 min without phospholipase C, incubation for 30 min without phospholipase C in the presence of 20 mg/ml fatty acid-free bovine serum albumin (BSA) (Sigma) and incubation for 30 min with phospholipase C and 10 mM EDTA) all gave values of 2.8 nmol cytochrome *P*–450, 1.1 nmol cytochrome b_5, and 600 nmol lipid phosphorus per mg microsomal protein. Solubilized was defined as remaining in the supernatant after centrifugation at 100 000 *g* for 1 hr.

3. Results and discussion

Fig.1 shows that treatment of microsomes with phospholipase C has no effect on the content of cytochrome b_5. On the other hand, fig.2 shows that such treatment causes a loss of some 55% of the microsomal cytochrome *P*–450. The amount of cytochrome *P*–450 that was converted to cytochrome

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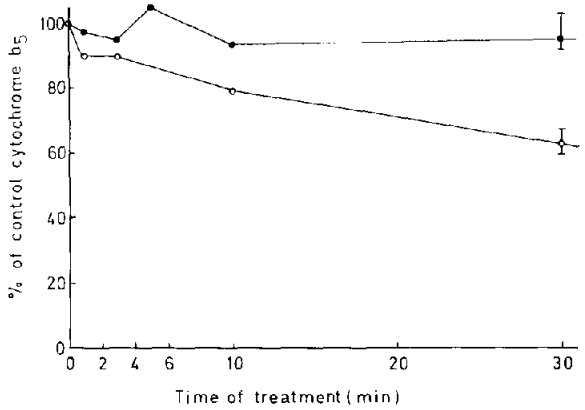

Fig.1. The effect of phospholipase C treatment on cytochrome b_5. Microsomes were treated with phospholipase C and cytochrome b_5 was measured as described in the Methods. All points are from a single or the average of two experiments, except for the points at 30 min, which represent the average and range of 5 determinations. (•) Treatment in the absence of BSA. (○) Treatment in the presence of 20 mg/ml fatty acid-free BSA.

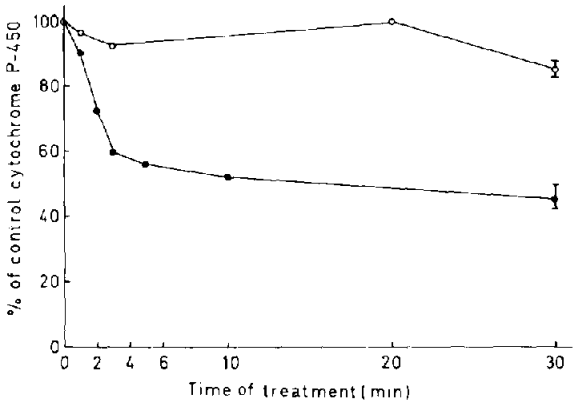

Fig.2. The effect of phospholipase C treatment on cytochrome *P*–450. Microsomes were treated with phospholipase C and cytochrome *P*–450 was measured as described in the Methods. All points are from a single or the average of two experiments, except for the points at 30 min, which represent the average and range of 5 determinations. (•) Treatment in the absence of BSA. (○) Treatment in the presence of 20 mg/ml fatty acid-free BSA.

P–420 was highly variable, ranging between 25 and 50%. Anaerobiosis or the presence of 10 mM aminopyrine (a substrate of the cytochrome *P*–450 system), 10 mM dithionite (which reduces the cytochrome), or 30% glycerol did not prevent the denaturation of cytochrome *P*–450 caused by phospholipase C treatment.

Since it is known that microsomes contain a diglyceride lipase activity that rapidly breaks down the diglycerides that are products of phospholipase C hydrolysis [7], it seemed possible that cytochrome *P*–450 was being denatured by free fatty acids rather than as a direct result of phospholipase C action. Indeed, fig.2 shows that 20 mg/ml fatty acid-free BSA prevented almost all of the denaturation. Half as much BSA afforded significantly less protection, while twice as much did not afford any more protection. Addition of BSA after phospholipase C treatment did not reverse the denaturation of cytochrome *P*–450. Fig.1 shows that the presence of BSA in the medium during phospholipase C treatment resulted in denaturation of about 30% of cytochrome b_5, a finding for which we have no ready explanation.

It is, of course, possible that BSA prevents denaturation of cytochrome *P*–450 by inhibiting breakdown of the microsomal membrane by phospholipase C. That this is not the case is illustrated in fig.3: 70% of the microsomal phospholipids are hydrolyzed by phospholipase C both in the absence and presence

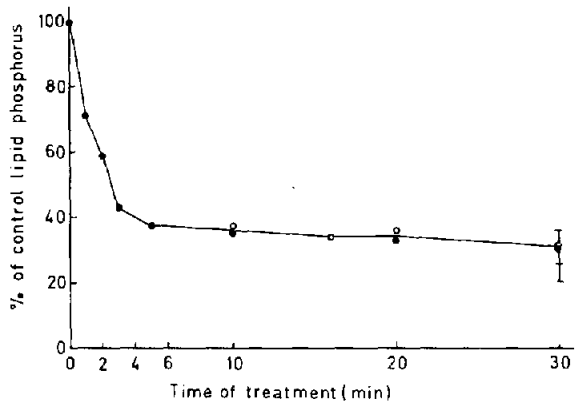

Fig.3. Hydrolysis of microsomal phospholipids by phospholipase C. Microsomes were treated with phospholipase C and the remaining lipid phosphorus was determined as described in the Methods. All points are from a single or the average of two experiments, except for the points at 30 min, which represent the average and range of 5 determinations. (•) Treatment in the absence of BSA. (○) Treatment in the presence of 20 mg/ml fatty acid-free BSA.

of BSA. In order to further test the explanation that BSA protects cytochrome *P*–450 by binding free fatty acids, microsomes were treated with pancreatic lipase to hydrolyze the membrane glycerides [8] and produce large amounts of free fatty acids. As depicted in table 1, this procedure denatured 92% of the cytochrome P–450; and 80% of this could be measured as cytochrome *P*–420. BSA was able to prevent more than one-half of this denaturation. Pancreatic lipase treatment had no effect on cytochrome b_5.

It was found that approximately 10% of the microsomal protein but no cytochrome *P*–450 or b_5 was solubilized by phospholipase C treatment. And finally, it seemed possible that phospholipase C might have different effects on the phospholipids and cytochrome *P*–450 of rough and smooth microsomes, due to a protection of the membrane surface by ribosomes, to different levels of diglyceride lipase activity, or to some other difference between these two microsomal subfractions. Table 1 demonstrates that this was not the case.

The conclusions which can be drawn from this study are as follows: Neither the conformations of cytochrome *P*–450 and b_5 in situ nor the binding of these proteins to the microsomal membrane is critically dependent on the polar headgroups of the 70% of microsomal phospholipids that can be hydrolyzed by phospholipase C (*Cl. welchii*). This 70% includes 80% of the phosphatidylcholine and sphingomyelin, 50% of the phosphatidylethanolamine, and 40% of the phosphatidylserine and phosphatidylinositol [9,10].

Cytochrome *P*–450 can be denatured by free fatty acids produced in situ. Glucose 6-phosphatase is another microsomal enzyme that can be denatured in the same manner [7]. The erythrocyte membrane, liver plasma membrane, muscle microsomes, and myelin of rats are all known to contain diglyceride lipase activity [11]; and investigators should in general be aware of the danger of denaturing membrane enzymes by providing substrate for this activity, e.g., by phospholipase C treatment of the membrane. It has also been observed that high levels of laurate added to the medium denature cytochrome *P*–450 to cytochrome *P*–420 [12].

It has been reported that phospholipase C treatment of microsomes strongly inhibits type I metabolism catalyzed by the cytochrome *P*–450 system (e.g., aminopyrine demethylation) but does not affect or only slightly inhibits type II metabolism (e.g., aniline hydroxylation) [10,13,14]. These findings may be partially explained by the results shown in fig.2, which suggest that about one-half of the cytochrome *P*–450 pool is more sensitive to free fatty acids than the other half. It is becoming more and more clear that cytochrome *P*–450 is a family of proteins; and it is possible that the cytochrome *P*–450 species involved in type I metabolism is more sensitive to denaturation by free fatty acids produced in situ than is the species involved in type II metabolism. Alternatively, it has also been shown that laurate [12] and unsaturated long chain fatty acids [15] can inhibit type I metabolism in microsomes. The inhibition by laurate was competitive with and therefore probably selective for type I

Table 1

Conditions	% of control cytochrome *P*-450 remaining	% of control lipid phosphorus remaining
Microsomes incubated with pancreatic lipase	8.0	
Microsomes incubated with pancreatic lipase and 20 mg/ml fatty acid-free BSA	70.2	
Rough microsomes incubated with phospholipase C	46.7	33.3
Smooth microsomes incubated with phospholipase C	45.2	32.7

metabolites and occurred at low fatty acid levels where there was no apparent denaturation of cytochrome *P*–450; whereas the inhibition with unsaturated long chain fatty acids was shown to occur at concentrations where type II metabolism is unaffected. Thus, it seems likely that the inhibition of type I metabolism caused by phospholipase C treatment results from a combination of direct inhibition and denaturation of cytochrome *P*–450 by free fatty acids arising from diglyceride lipase activity.

Finally, it would seem feasible to attempt the solubilization of cytochrome *P*–450 and other proteins by treating microsomes first with phospholipase C and then with a lipase, provided BSA is always present in the medium.

Acknowledgements

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