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Conference Paper

Treatment of Rat Liver Microsomes with Phospholipase C: Effect on Phospholipids and on Cytochromes *P450* and b_5 *

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Treatment of rat liver microsomes with phospholipase C (*Cl. welchii*) revealed the following:

1. The polar headgroups of 70% of the phospholipids can be removed by treatment of microsomes with phospholipase C. When phospholipids that have been extracted from microsomes are treated with phospholipase C, 90% can be hydrolyzed, suggesting that certain phospholipids are protected from the enzyme *in situ*.
2. Neither the native conformations of cytochromes *P450* and b_5 nor their binding to the microsomal membrane are directly affected by phospholipase C treatment.
3. The diglycerides resulting from the action of phospholipase C can be hydrolyzed by an enzyme in the microsomal membrane to yield free fatty acids which partially denature cytochrome *P450*.
4. The pattern of this partial denaturation is a further indication of the existence of a number of cytochrome *P450* species in the microsomal membrane.

INTRODUCTION

Like other phospholipases, phospholipase C is a popular tool for investigating membrane structure, and in particular the relationship between membrane phospholipids and proteins. We were interested in determining what percentage of the phospholipids in microsomes could be hydrolyzed by this enzyme and why the remaining phospholipids remained intact. In addition, we posed the question as to whether the polar headgroups of phospholipids are involved in maintaining the native conformation of cytochrome *P450* and in binding this protein to the membrane of the endoplasmic reticulum. As a sort of control, we also examined the effect of phospholipase C treatment on cytochrome b_5; from what we know about this enzyme[1], the polar headgroups of phospholipids would not be expected to be involved in its catalytic activity or binding to the membrane. Some of the results discussed here have been published previously[2].

EXPERIMENTAL

Microsomes

Total[3] and rough and smooth[4] microsomes were prepared from the livers of 180—200 g male Sprague-Dawley rats by established procedures. In the experiments shown the rats received an intraperitoneal injection of 80 mg/kg phenobarbital once

* Presented by J. W. DeP. at the Scientific Conference »*Cytochrome P450 — Structural Aspects*« (held in Primošten — Yugoslavia, 6—10. October, 1976).

a day for three days before sacrifice; similar but less pronounced effects were seen with uninduced animals.

Treatment with enzymes

The medium contained 50 mM Tris, pH 7.5, 2.0 mM $CaCl_2$, 3—6 mg microsomal protein/ml and 100—150 μg phospholipase C (*Cl. welchii*, Sigma)/mg microsomal protein. In some cases 20 mg/ml fatty acid-free bovine serum albumin (BSA) (Sigma) was also included. In other cases the mixture minus phospholipase C was incubated for 30 min with 50 μg trypsin (Sigma)/mg microsomal protein before the addition of phospholipase C. Incubations were carried out at 23 °C and stopped either by adding EDTA to give a final concentration of 10 mM and placing the tubes on ice (if cytochromes *P450* and b_5 were to be measured) or by the addition of 19 volumes of chloroform : methanol, 2 : 1 (if only phospholipids were to be determined). Control incubations (zero-time incubation, incubation for 30 min without phospholipase C, incubation for 30 mir without phospholipase C in the presence of BSA, and incubation for 30 min with phospholipase C and EDTA) all gave values of 2.8 nmol cytochrome *P450*, 1.1 nmol cytochrome b_5, and 600 nmol lipid phosphorus per mg microsomal protein.

In one experiment phospholipids were extracted from microsomes with chloroform-methanol[5]; the organic solvent removed; the phospholipid residue suspended in 50 mM Tris, pH 7.5, by sonication for 20 min. at a temperature of 4—6 °C; and treatment with phospholipase C carried out in the same manner as for microsomes (using 100—150 μg enzyme per 600 nmol lipid phosphorus). Treatment with crude pancreatic lipase (Sigma) was carried out under the same conditions as with phospholipase C, except that 1 mg lipase/mg microsomal protein was used and the medium also contained 1 mg trypsin inhibitor/mg lipase in order to inhibit the trypsin which contaminated the crude lipase preparation.

Measurements of cytochromes and phospholipids

Cytochromes *P450*, *P420* and b_5 were assayed by the method of Omura and Sato[6]. The hydrolysis of phospholipids was determined by extracting the remaining phospholipids into chloroform-methanol[5] and determining the remaining lipid phosphorus according to Bartlett[7].

RESULTS AND DISCUSSION

Effect on Microsomal Phospholipids

The effect on microsomal phospholipids of treating intact microsomes with phospholipase C is shown in Figure 1. 60% of the phospholipids are hydrolyzed within 3 min, while an additional 10% is hydrolyzed if the incubation period is extended to a half hour. 60 min incubation gives no further hydrolysis.

We were interested in finding out why 30% of the phospholipids in microsomes cannot be attacked by phospholipase C and Table I gives the data from our attempts to answer this question. When microsomal phospholipids are extracted from the membrane, only about 10% are resistant to hydrolysis by phospholipase C; this can presumably be explained by the substrate specificity of the enzyme. Thus, 20% of the phospholipids in microsomes are apparently protected by some aspect of the membrane structure from attack by phospholipase C. Sonication of microsomes, either before treatment with phospholipase C or in the middle of the incubation period, did not render these phospholipids accessible to hydrolysis. The phospholipids in both rough and smooth microsomes were attacked equally by phospholipase C, suggesting that ribosomes are not protecting membrane phospholipids from the enzyme. And finally, treatment of microsomes with trypsin prior to treatment with phospholipase C

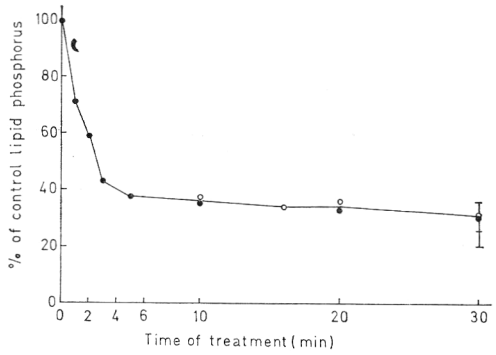

Figure 1. Hydrolysis of phospholipids by treatment of microsomes with phospholipase C. Microsomes were treated with phospholipase C and the remaining lipid phosphorus was determined as described in the Experimental section. All points are from a single or the average of two experiments, except for the 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.

TABLE I

Preparation treated with phospholipase C	% of control lipid phosphorus remaining
phospholipids extracted from microsomes	11.7
sonicated microsomes*	31.5
rough microsomes	33.3
smooth microsomes	32.7
microsomes treated with trypsin	27.3

* The microsomes were either sonicated for 3 min. at 4-6 °C before treatment with phospholipase C or they were first treated for 30 min. with phospholipase C; then sonicated, and subsequently treated for an additional 30 min. with the phospholipase.

resulted in the hydrolysis of a somewhat higher percentage of phospholipids, suggesting that maybe 5% of microsomal phospholipids are shielded from attack by neighboring protein molecules.

Effect on the Binding of the Cytochromes

Using an operational definition of solubilization (*i. e.*, remaining in the supernatant after centrifugation for one hour at 100,000 × g), it was found that approximately 10% of the total microsomal protein but no cytochrome *P450* or b_5 was solubilized by treating microsomes for 30 min with phospholipase C.

On the basis of other studies of these cytochromes[1], this is what would be predicted.

Effect on the Conformation of the Cytochromes

Figure 2 shows that treatment of microsomes with phospholipase C results in denaturation of some 55% of the cytochrome *P450*. The amount of this cytochrome that was converted to cytochrome *P420* was highly variable, ranging between 25 and 50%. Anaerobiosis or the presence of 10 mM aminopyrine (a substrate of the cytochrome *P450* system), 10 mM dithionite (which reduces the cytochrome), or 30% glycerol did not prevent the denaturation of cytochrome *P450* caused by phospholipase C treatment.

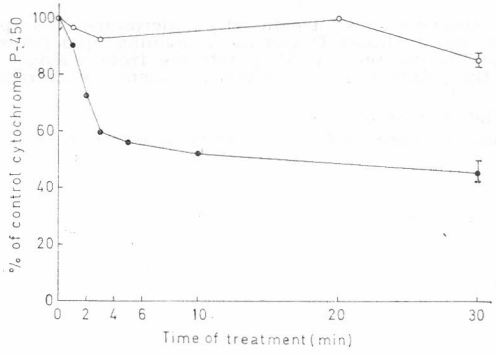

Figure 2. The effect of phospholipase C treatment on cytochrome *P450*. Microsomes were treated with phospholipase C and cytochrome *P450* was measured as described in the Experimental section. All points are from a single or the average of two experiments, except for the points at 30 min, which represent 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.

Since it is known that microsomes contain a diglyceride lipase activity that rapidly breaks down the diglyceride products of phospholipase C action[8], it seemed possible that cytochrome *P450* was being denatured by free fatty acids rather than as a direct result of phospholipase C hydrolysis. Indeed, Figure 2 also shows that the presence of 20 mg/ml 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 citochrome *P450*.

One simple explanation of these findings is that BSA prevents denaturation of cytochrome *P450* by inhibiting breakdown of the microsomal membrane by phospholipase C. However, Figure 1 illustrates that phospholipase C is equally effective in hydrolyzing microsomal phospholipids in the presence as in the absence of BSA. In order to further test the explanation that BSA protects cytochrome *P450* by binding free fatty acids, microsomes were treated with pancreatic lipase to hydrolyze the membrane glycerides[9] and produce large amounts of free fatty acids. This procedure denatured 92% of the cytochrome *P450,* and 80% of this could be measured as cytochrome *P420*. However, when lipase treatment was carried out in the presence of BSA, only 30% of the cytochrome was denatured.

Figure 3 shows that treatment of microsomes with phospholipase C has no effect on the content of cytochrome b_5, as expected. However, 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 explanation.

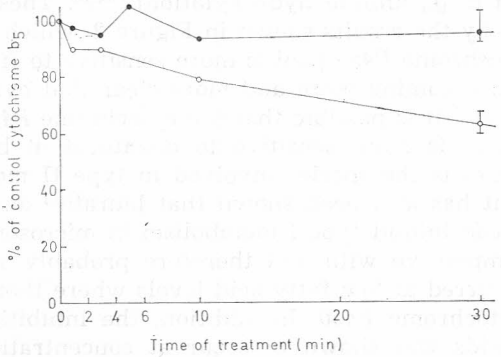

Figure 3. The effect of phospholipase C treatment on cytochrome b_5. Microsomes were treated with phospholipase C and cytochrome b_5 was measured as described in the Experimental section. All points are from a single or the average of two experiments, except for the points at 30 min, which represent 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.

CONCLUSIONS

1. The polar headgroups of 70% of the phospholipids can be removed by treatment of microsomes with phospholipase C (*Cl. welchii*). This 70% includes 80% of the phosphatidylcholine and sphingomyelin, 50% of the phosphatidylethanolamine, and 40% of the phosphatidylserine and phosphatidylinositol[10,11]. However, when phospholipids are extracted from microsomes, 90% of them can then be hydrolyzed with phospholipase C. This suggests that some aspect of the membrane structure is shielding 20% of the microsomal phospholipids from attack *in situ*. Treatment with trypsin suggests that 5% of the phospholipids are shielded by neighboring proteins. Some or all of the remaining 15% may be so-called »boundary lipids«, which are more highly organized than the bulk membrane phospholipid and may consequently be more resistant to enzymatic attack. It will be of interest to investigate the chemical nature of the 20% of microsomal phospholipids which can be attacked by phospholipase C after extraction but not *in situ*.

2. Neither the conformations of cytochromes *P450* and b_5 nor the binding of these proteins to the microsomal membrane is critically dependent on the polar headgroups of the 70% of the phospholipids of microsomes that can be hydrolyzed by phospholipase C (*Cl. welchii*).

3. Cytochrome *P450* 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[8]. The erythrocyte membrane, liver plasma membrane, muscle microsomes, myelin, and liver microsomes of rats are all known to contain diglyceride lipase activity[12]; 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 *P450* to cytochrome *P420*[13].

4. It has been reported that phospholipase C treatment of microsomes strongly inhibits type I metabolism catalyzed by the cytochrome *P450* system (*e. g.*, aminopyrine demethylation) but does not affect or only slightly inhibits type II metabolism (*e. g.*, aniline hydroxylation)[11,14,15]. These findings may be partially explained by the results shown in Figure 2, which suggest that about one-half of the cytochrome *P450* pool is more sensitive to free fatty acids than the other half. It is becoming more and more clear that cytochrome *P450* is a family of proteins; and it is possible that the cytochrome *P450* 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[13] and unsaturated long chain fatty acids[16] can inhibit type I metabolism in microsomes. The inhibition by laurate was competitive with and therefore probably selective for type I metabolites and occurred at low fatty acid levels where there was no apparent denaturation of cytochrome *P450*. In addition, the inhibition by 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 *P450* by free acids arising from diglyceride lipase activity.

REFERENCES

1. J. W. DePierre and G. Dallner, *Biochim. Biophys. Acta* **415** (1975) 411.
2. J. W. DePierre and L. Ernster, *FEBS Lett.* **55** (1975) 18.
3. L. Ernster, P. Siekevitz, and G. E. Palade, *J. Cell. Biol.* **15** (1962) 541.
4. G. Dallner, *Acta Pathol. Microbiol. Scand. Suppl.* **166** (1963).
5. J. Folch, M. Lees, and G. H. S. Stanley, *J. Biol. Chem.* **226** (1957) 497.
6. T. Omura and R. Sato, *J. Biol. Chem.* **239** (1964) 2379.
7. G. R. Bartlett, *J. Biol. Chem.* **234** (1959) 466.
8. B. R. Cater and T. Hallinan, *FEBS Lett.* **16** (1971) 137.
9. H. Glaumann and G. Dallner, *J. Lipid Res.* **9** (1968) 720.
10. S. Jothy, S. Tay, and H. Simpkins, *Biochem. J.* **132** (1973) 637.
11. M. D. Chaplin and G. J. Mannering, *Mol. Pharmacol.* **6** (1970) 631.
12. R. H. Michell, R. Coleman, and J. B. Finean, *Biochim. Biophys. Acta* **318** (1973) 306.
13. S. Orrenius and H. Thor, *Eur. J. Biochem.* **9** (1969) 415.
14. T. E. Eling and R. P. DiAugustine, *Biochem. J.* **123** (1971) 539.
15. B. R. Cater, V. Walkden, T. Hallinan, *Biochem. J.* **127** (1972) 37.
16. R. P. DiAugustine and T. Fouts, *Biochem. J.* **115** (1969) 547.

DISCUSSION

H. Rein:
Do you think there are species differencies?

J. W. DePierre:
My studies have only been carried out on rat liver microsomes, and there may well be species differences.

J. I. Mason:
(*a*) Is information available on processes of this kind in induced microsomes, *e. g.* by phenobarbital or 3-methylcholanthrene? (*b*) Do other *P450* inactivating agents, *e. g.* mesalyl, inactivate similar species of cyt *P450*? (*c*) Trypsin treatment of microsomes should result in removal of cytochrome. Are there studies available on phospholipase C treatment of preparations of this kind?

J. W. DePierre:

(*a*) Most of the experiments which I have reported were done with microsomes from animals treated with phenobarbital. Control microsomes give the same results, though the effects are less pronounced. The experiment has not yet been done with methylcholanthrene-treated animals. (*b*) I don't know, the comparative experiments have not been done. (*c*) To my knowledge, no one has done such an experiment. I agree that the results would be quite interesting.

F. Jung:

(*a*) Does the added bovine serum albumin enter into the vesicles (or into the membranes)? (*b*) Did you follow the modification of the vesicles to micelles by electron microscopy?

J. W. DePierre:

(*a*) Added BSA does not enter into microsomal vesicles as long as their permeability barrier is intact. However, no one has investigated the possibility that BSA may be incorporated into the microsomal membrane or into the micellar structures formed by phospholipase C treatment. This seems unlikely to me. (*b*) Yes, the various stages of transformation of microsomal vesicles to micellar structures during treatment with phospholipase C were followed with the electron microscope.

P. Debey:

Was there any solubilization of cytochrome *P450* or b_5 after the treatment by phospholipase C, and did you do the measurements after centrifugation?

J. W. DePierre:

Neither treatment of microsomes with phospholipase C, in the presence or in the absence of bovine serum albumin, solubilized any cytochrome *P450* or b_5. This was determined by centrifuging the treated microsomes at 100,000 x g for one hour and then assaying for these cytochromes in the supernatant and in the pellet.

K. Ruckpaul:

With regard to Dr Debey's question: what was the molecular weight of your lipase treated microsomes?

J. W. DePierre:

I do not know the molecular weight of the »micelles« formed upon treatment of microsomes with phospholipase C. I think what you and Dr. Debey are getting at is that this treatment may give rise to small membrane pieces containing cytochrome *P450* or cytochrome b_5, so that these cytochromes can be said to have been solubilized. This depends on what one means by »solubilized«. I have used an empirical criterion — that, which remains in the supernatant after centrifugation at 100,00 x g for one hour, is solubilized.

L. K. Hanson:

You mentioned in the summer school (»*Membrane Bound Enzymes*«, Primošten, 2—6. October, 1976) that there are only ~25—40 phospholipid molecules per microsomal protein molecule. Is 30% of the phospholipid sufficient to account for boundary lipid? What happens to the 90% protein which is not solubilized upon hydrolysis of the microsomes by phospholipase C?

J. W. DePierre:

One can calculate roughly that there are approximately 27—42 molecules of phospholipid per polypeptide chain in the membrane of the endoplasmic reticulum. After treatment with phospholipase C, 8—13 molecules of phospholipid per polypeptide chain remain. It's not possible to say, in our present ignorance, whether this is enough phospholipid to provide all the membrane protein molecules with their normal complement of »boundary lipid«. The 90% of microsomal protein, which is not solubilized by treatment with phospholipase C, seems to be incorporated into micellar structures which also contain the remaining phospholipid and the product diglyceride.

H. Schleyer:

What might be the ratio phospholipid/*P450* when you reach the »magic number« of ~30% phospholipid left, after the phospholipase C treatment? (Incidentally it is a figure which is also observed with other preparations of mammalian origin.)

J. W. DePierre:

About 8—13 molecules of phospholipid per polypeptide chain remain after phospholipase C treatment. It is impossible to say at this point whether these phospholipid molecules are evenly distributed among the different proteins, or whether cytochrome *P450* is not changed into *P420*. Can b_5 be separated from *P450* after this treatment?

K. Ruckpaul:

You said that after treatment with phospholipase in the presence of serum albumin, *P450* is not changed into *P420*. Can b_5 be separated from *P450* after this treatment?

J. W. DePierre:

No, or rather I should say, probably not. Neither of these cytochromes is solubilized by treatment of microsomes with phospholipase C. Presumably, they are both bound to mixed micelles containing diglyceride, the remaining phospholipids, and various microsomal proteins. If *P450* and b_5 are not bound to the same micelles, if they are segregated into different particles after the enzyme treatment, it might be possible to separate them. However, I think this is very unlikely. What I would really like to know is the nature of the remaining 30% of the phospholipids which cannot be hydrolized by treatment of microsomes with phospholipase C. Firstly, do these phospholipids have a special functional relationship to the cytochrome *P450* system and secondly, do they have any special structural relationship to this system? For instance, one might compare the fatty acid composition of phospholipids which can be hydrolyzed by treatment of microsomes with phospholipase C and of those which cannot. If anyone has any other suggestions, I would be happy to hear them.
Another possibility is to treat microsomes with phospholipase C from other sources (I used the enzyme from *Cl. welchii*). It has been shown very nicely, in van Deenen's laboratory, that the action of different phospholipases A on membranes is affected by the surface tension of the membrane, that is to say, that some phospholipases A will hydrolyze membranes with high surface tension while other will not. So there are differences in the ability of phospholipases to penetrate into membranes and hydrolyze their components. If I could find a phospholipase C which would attack 100% of microsomal phospholipids, it would be interesting to see if cytochrome *P450* is still structurally and functionally intact after such a treatment.

SAŽETAK

Djelovanje fosfolipaze C na mikrosome jetre štakora: utjecaj na fosfolipide i na citokrome *P450* i b_5

J. W. De Pierre i L. Ernster

Djelovanje fosfolipaze C *(Cl. welchii)* na mikrosome jetre štakora pokazalo je: Polarni završeci od 70% fosfolipida mogu se ukloniti iz mikrosoma hidrolizom, ali kad se prethodno ekstrahiraju iz mikrosoma onda ih se 90% hidrolizira fosfolipazom C, što ukazuje na to da je dio fosfolipida *in situ* zaštićen od enzima.

Fosfolipaza C ne djeluje direktno niti na nativne konformacije niti na vezivanje citokroma *P450* i b_5 u membrani mikrozoma.

Digliceridi koji nastaju djelovanjem fosfolipaze C hidroliziraju se drugim enzimom, iz membrane mikrosoma, pa tako nastaju slobodne masne kiseline koje djelomice denaturiraju citokrom *P450*. Način te denaturacije još je jedan znak postojanja grupe citokroma *P450* u membrani mikrosoma.

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