The effect of phosphatidyl choline on the degradation of phosphatidyl ethanolamine by the phospholipase of post-heparin plasma or snake venom

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SUMMARY The degradation of phosphatidyl ethanolamine by human post-heparin plasma phospholipase is not significantly affected by phosphatidyl choline added to the assay system as an aqueous emulsion. However, when the two phospholipids are combined in chloroform–methanol and the solvent is removed prior to their joint emulsification, a marked reduction of phosphatidyl ethanolamine degradation is observed. The extent of this reduction is dependent upon the relative amount of phosphatidyl choline added. The results obtained are interpreted on the basis of a hypothetical complex formation between the two phospholipids.

A comparison of the phospholipase activities of post-heparin plasma and *Crotalus adamanteus* venom reveals both similarities and differences in the behavior of the two enzymes.

In a previous study (1) a post-heparin plasma phospholipase was described which readily degrades phosphatidyl ethanolamine (PE). This enzyme had only very slight action on phosphatidyl choline (PC) in incubations of 6–7 hr. Various correlations between a phospholipid particle's charge characteristics and its suitability as a phospholipase substrate (2, 3) or its coagulant action (4, 5) suggested that PC, *when combined* with another phospholipid such as PE, for example, might serve as a suitable substrate for the post-heparin plasma enzyme. All attempts to demonstrate measurable PC degradation to lysophosphatidyl choline (LyPC) and fatty acid (FA) during incubation intervals of 2–3 hr were unsuccessful, regardless of whether aqueous emulsions of PC and PE were added to the plasma phospholipase assay system, or whether they were evaporated together from chloroform–methanol (C–M) prior to emulsification. However, it was observed that as a result of this latter treatment, degradation of PE by the post-heparin plasma enzyme was decreased and that this decrease was related to the relative amount of PC present in the system. A study of this apparently inhibitory effect of PC and some comparisons of the activity of the post-heparin plasma enzyme and that of snake venom are presented in this report.

METHODS AND MATERIALS

Methods for the preparation of PE and PC from egg yolk, for qualitative thin-layer chromatography (TLC), and for the quantitative measurement of post-heparin plasma phospholipase activity have been described previously (1). Amounts of individual phospholipids are reported in terms of their respective phosphorus contents and are abbreviated as follows: PE-P, PC-P, LyPE-P, and LyPC-P for phosphorus of PE, PC, lysophosphatidyl ethanolamine (LyPE), and LyPC respectively.

Preparation of Post-Heparin Plasma

Fifteen minutes after the intravenous administration of 100 mg of heparin to a human donor, approximately 450 ml of blood were collected in a plastic bag without added anticoagulant. Plasma was separated immediately by centrifugation for 30 min at 4° and 1100 × *g*. Aliquots (2 ml) of the plasma were frozen in glass tubes and stored at −40° until used in an experiment as the source of enzyme. Frozen samples were thawed just prior to use and were never refrozen. Except for one instance which is described in the text, all post-heparin plasma used in

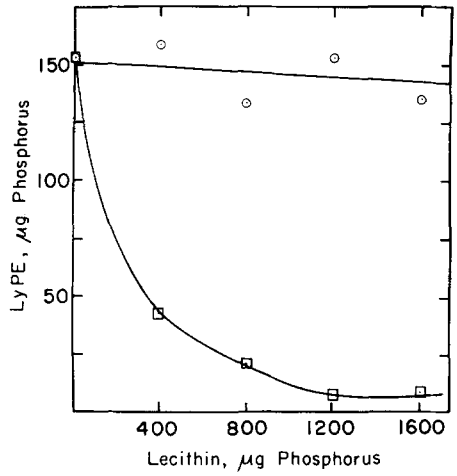

FIG. 1. Effect of PC (lecithin) on PE degradation by post-heparin plasma phospholipase. 1600 μg of PE-P were incubated for 40 min in the standard incubation system alone and together with 400, 800, 1200, and 1600 μg added PC-P. In one experiment, PC was added as an aqueous emulsion ⊙-⊙- . In the other, PE and PC solutions in chloroform–methanol 1:1 were combined prior to evaporation of the organic solvent ⊡-⊡-⊡.

quantitative studies was obtained from this one unit of blood given by one donor.

Treatment of Phospholipids

PE and PC were each stored in solution in chloroform–methanol (C–M) 1:1 (v/v) at −40°. Solvent was removed at 38° from an appropriate aliquot added to the tube in which the phospholipid was to be incubated, using a current of nitrogen at first and finally applying a vacuum. For experiments in which varying proportions of PE and PC were evaporated together, suitable volumes of C–M 1:1 were added to the different phospholipid mixtures in order to evaporate each one from an equal volume of solvent.

Standard Incubation System

All incubations with post-heparin plasma were allowed to proceed for 40 min at 38° and in a total volume of 7.0 ml. Incubation intervals with snake venom were 30, 60, and 90 min. The phospholipid was emulsified in a 5.0 ml volume of a solution, adjusted to pH 9.2 with NaOH containing 400 mg of albumin (bovine Fraction V, Armour) and 0.45 ml of 1 M ammonium sulfate. One milliliter of either water or an aqueous PC emulsion was added. The enzyme source was either 1.0 ml of post-heparin plasma or 1.0 ml of an aqueous snake venom solution containing 1 mg of lyophilized venom per milliliter. (The *Crotalus adamanteus* venom was obtained from Ross Allen's Reptile Institute, Inc., Silver Springs, Fla.)

Preparation of Lipid Extracts

For quantification of enzyme activity, lipids were extracted from the incubation systems immediately after the addition of post-heparin plasma (or snake venom) to them and, subsequently, after the selected interval of incubation. A 1.0 ml volume of the standard incubation mixture was added to 18 ml of ethanol–diethyl ether 3:1; the mixture was heated momentarily to boiling, and then diluted at room temperature to a final volume of 25.0 ml with the same solvent. The extract was filtered, 20.0 ml of the filtrate was evaporated to dryness, and the lipid residue redissolved in 3.0 ml of isoamyl alcohol–benzene 1:1. Two aliquots of 200 μl of each solution were then applied to silicic acid-impregnated paper for the duplicate chromatographic separation of the phospholipids and their quantitative measure in terms of phospholipid phosphorus (1). The increase of LyPE-P or LyPC-P during the incubation interval was taken as a measure of enzymatic activity.

RESULTS

Effect of PC on Post-Heparin Plasma Phospholipase Activity

A quantitative evaluation of observations originally made with TLC and two other post-heparin plasmas is presented in Fig. 1, which shows the extent of LyPE formation in the presence of increasing concentrations of PC. It is apparent that addition of an aqueous PC emulsion to the standard incubation system had no significant effect on enzyme activity. Similarly, when post-heparin plasma was incubated with an aqueous PC emulsion for 30 min prior to the addition of PE, identical results were obtained. However, when PC dissolved in C–M 1:1 was combined with PE dissolved in the same solvent mixture prior to removal of the organic solvent, a decrease in LyPE production was observed, the extent of this decrease depending upon the amount of PC present in the assay system. The formation of a small amount of LyPE, even with relatively high concentrations of PC present, was characteristic of all quantitative studies. However, there was no detectable formation of LyPC in either incubation system. There was some formation of suspended flocculent material during the incubations. This was readily dispersed prior to the removal of the aliquots.

Nature of PC Effect on LyPE Production by Post-Heparin Plasma Phospholipase

With 800 μg of PE-P in the standard incubation system, 123 μg of LyPE-P was formed, but greater and equivalent amounts of LyPE-P were formed using amounts of PE-P of 1150 μg or more. Different amounts of PE-P, ranging from 1150 to 2800 μg, were incubated alone as well as following evaporation from C–M 1:1 together with each of three different levels of PC. LyPE-P production was determined in each of these systems and plotted against

substrate concentration (Fig. 2). From the data obtained, it is apparent that for any given amount of PC present, there is greater reduction of LyPE formation at lower PE substrate concentrations than at higher ones. Furthermore, projections of the curves obtained for each of the three PC concentrations suggest that maximum inhibition of LyPE production occurs in those systems in which the PE/PC ratio approximates 2. Although these findings may indicate that PE and PC, when evaporated together from organic solvents, form complexes with a PE/PC ratio of 2, additional considerations make it appear that such 2:1 complexes may not be the only ones formed. Thus in the incubation mixture containing 2800 μg of PE-P and 800 μg of PC-P, the amount of free, unbound PE-P should be 1200 μg if the PE/PC complex ratio were 2 only, and this amount of PE-P had previously been found sufficient to fully saturate the enzyme. Actually, however, the amount of LyPE-P formed under these conditions was only about half that produced in the absence of PC. There would appear to be two possible explanations for this observation. Either PE/PC complexes in ratios of more than 2:1 may be formed, thereby resulting in possible substrate deficiency, or the 2:1 PE/PC complex itself inhibits degradation of free PE by the plasma enzyme.

In order to investigate these two possibilities the following experiment was carried out. PE-P (1200 μg), emulsified in 5.0 ml of the albumin–ammonium sulfate solution described under Methods, was added to each of 4 tubes A, B, C, and D. While tube A served as a control (containing only 1200 μg PE-P), mixtures containing PE and PC in chloroform–methanol had been previously evaporated in tubes B, C, and D so that the dry contents of PE-P and PC-P (in μg) were 400/200, 800/400, and 1600/800 respectively. The 40-min increments of LyPE-P formed in tubes A, B, C, and D were 124, 113, 117, and 71 μg respectively. Thus, it would appear that 2:1 PE/PC complexes do not inhibit post-heparin plasma phospholipase activity unless their concentrations in combined lipids are greater than that of the free PE substrate. (The post-heparin plasma used in this experiment was obtained from a different donor.)

Under the conditions prevailing in tube D, there was a 43% decrease in the amount of LyPE formed. In the previously mentioned mixture of 2800 μg of PE-P and 800 μg of PC-P (Fig. 2) similar amounts of free PE-P and PE/PC complex would be present if a 2:1 ratio were assumed. Since the decrease in LyPE production is 50% under these conditions, it would seem more likely that this decrease is due to enzyme inhibition by PE/PC complex than to limitation of substrate resulting from formation of complexes with greater than 2:1 ratios.

On the other hand, under conditions prevailing in tubes B and C, with relatively smaller concentrations of

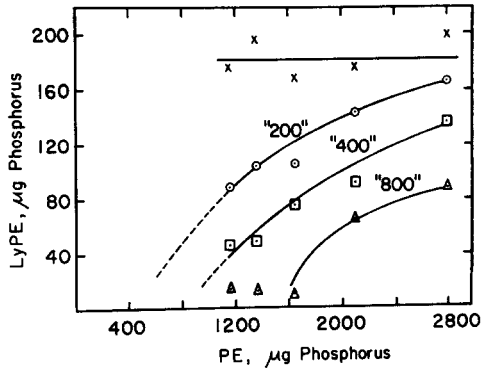

FIG. 2. Effect of varying PC and PE concentrations on PE degradation by post-heparin plasma phospholipase. 1150, 1350, 1650, 2100, and 2800 μg of PE-P were incubated for 40 min in the standard incubation system and the LyPE-P formed was determined ×-×-×. Mixtures of each of these amounts of PE and of varying concentrations of PC in C–M 1:1 were also prepared prior to removal of the organic solvent as described under Methods. The LyPE-P formed in the various systems was determined and plotted as follows: in the presence of 200 μg of PC-P ⊙-⊙-⊙; in the presence of 400 μg of PC-P ⊡-⊡-⊡; and in the presence of 800 μg of PC-P ◬-◬-◬.

the 2:1 PE/PC complex present, there was no significant decrease in LyPE production. It would seem therefore, that under the conditions of the earlier experiment (Fig. 2), where 2800 μg of PE-P were evaporated with 400 and 200 μg of PC-P respectively, the resulting decreases in LyPE production were probably due to PE/PC complex formation in ratios greater than 2:1 which, in turn, resulted in limitation of substrate.

The experiments of Fig. 2 were based on preliminary observations made by TLC with two other post-heparin plasmas. Four additional post-heparin plasmas were incubated with 1200 μg of PE-P alone and also with 600, 400, and 200 μg of PC-P combined in C–M prior to evaporation and emulsification. By TLC all showed nearly complete inhibition of LyPE production in the presence of 600 μg of PC-P, with decreased inhibition with the lesser amounts of PC-P, similar to the results of Fig. 2.

Comparison of Post-Heparin Plasma and Snake Venom Phospholipases

Snake venom was used as the source of enzyme in place of post-heparin plasma in the standard incubation system (Fig. 3). As in the case of the post-heparin plasma enzyme, LyPE was produced with PE as substrate (curve A), no LyPC formed with PC as substrate (curve B), and LyPE production was reduced when PE and PC were combined prior to removal of the organic solvent (curve F). Contrary to the findings obtained with the post-heparin plasma enzyme were the observations that with PE as substrate and PC added as an aqueous emulsion, LyPE formation was enhanced (curve C) and a small

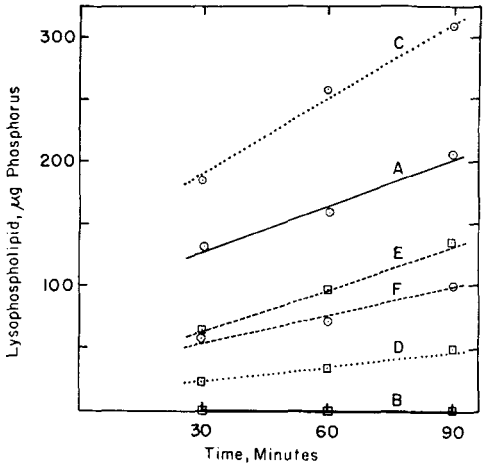

FIG. 3. Effect of PC on PE degradation by snake venom. One milliliter of a freshly prepared 0.1% aqueous snake venom solution was added to various incubation systems. System No. 1, 1200 μg of PE-P; LyPE-P formed is shown by curve A. System No. 2, 1200 μg of PC-P; lack of LyPC-P formed is shown by curve B. System No. 3, 1200 μg of PC-P added as an aqueous emulsion to 1200 μg of PE-P; the resulting LyPE-P and LyPC-P are shown by curves C and D respectively. System No. 4, 1200 μg of PC-P and 1200 μg of PE-P combined in C–M 1:1 prior to removal of solvent and emulsification; the resulting LyPE-P and LyPC-P are shown by curves F and E respectively.

amount of LyPC was formed (curve D), while with the two phospholipids evaporated together, LyPC production was further increased (curve E) and exceeded the LyPE formed (curve F). This latter observation may have been due to the proposed formation of a 2:1 PE/PC complex which would leave some unbound PC. A slow degradation of the complex with the formation of LyPE and LyPC could then cause a more rapid degradation of the free PC.

Demonstration of the activities exhibited by the two phospholipases by means of TLC uncovered additional differences in their behavior. In the previous study of post-heparin plasma phospholipase (1) it had been found that the progressive degradation of PE to LyPE could be studied by the direct application of aliquots of the aqueous assay system to silicic acid plates or to silicic acid-impregnated paper. Such treatment effectively halted enzymatic activity. When the action of snake venom enzyme on phospholipids was studied in the post-heparin plasma assay system, however, it was found that the technique of direct sample application could not be used. Immediately after adding the venom to four incubation systems as described in Fig. 3, 20-μl aliquots of each of the mixtures were applied to a silicic acid plate, and lipid extracts of 1.0 ml volumes of each mixture were prepared as described under Methods. The evaporated lipid aliquots were dissolved in 2.0 ml volumes of C–M 2:1 and 50-μl samples of the resulting solutions applied to the plate. Upon development of the chromatograms there was no degradation of PE or PC in the samples applied from the prepared lipid extracts. However, in those aqueous samples which had been applied directly to and dried on the plate, approximately 15–25% of PC as well as PE were degraded to FA and LyPC and LyPE respectively. This amount of degradation occurred in the presence of either or both of the phospholipids in this system regardless of the method of combination used.

It was repeatedly verified that snake venom did not degrade PC in the standard incubation system. In the familiar diethyl ether system the PC was more rapidly degraded to LyPC and FA when a small amount of $CaCl_2$ was added. TLC of the products of two 2-hr incubations showed about 80–90% degradation of the PC to LyPC and FA in the presence of a small amount of $CaCl_2$, but only about 10% degradation without added $CaCl_2$. A volume of 1.0 ml of 0.035 M $CaCl_2$ was then incorporated in the 7.0 ml standard incubation system, and the incubation of venom and PC was extended to 4 hr. TLC of a lipid extract showed only a trace of LyPC component.

The degradation of PC to LyPC and FA by snake venom phospholipase in the aqueous aliquots applied directly to chromatoplates was found to be largely dependent upon the $CaSO_4$ used as binder of the silicic acid. In addition to the usual chromatoplates prepared with Mallinckrodt silicic acid containing 4% $CaSO_4$, plates were prepared without $CaSO_4$. The standard incubation and also one modified with added $CaCl_2$, as described above, were used. Immediately after adding the venom, aliquots were applied to and dried on the two types of chromatoplates before TLC. Aliquots from both incubation systems showed a similar 15–25% conversion on the plates containing $CaSO_4$. On the plates without $CaSO_4$ the aliquot from the standard incubation system showed a 3–5% conversion, while the aliquot from the incubation system with added $CaCl_2$ showed the same 15–25% conversion as on the plate containing $CaSO_4$. The possible presence of some calcium in the Mallinckrodt silicic acid itself could account for the 3–5% conversion described above. The involvement of calcium in the reaction occurring while these particular aqueous samples are drying on the chromatoplate seems established. Its possible involvement with the similar degradation of PE or mixtures of PE and PC was not explored.

DISCUSSION

A decrease in the degradation of PE by post-heparin plasma phospholipase was observed only when PC was mixed with PE prior to removal of organic solvent. Little or no effect on PE breakdown was noted when aqueous emulsions of the two phospholipids were combined. Experiments carried out following evaporation of PE–PC mixtures of varying proportions have led to the conclusion that the respective effects observed did not involve

direct inhibition of enzyme activity by free PC. Rather the results suggest the formation of complexes between PE and PC, such complexes having (depending upon the particular conditions) ratios of 2 or more moles of PE to 1 mole of PC. Whereas PE–PC complexes having a 2:1 ratio did not seem to inhibit enzyme activity as long as the level of free PE was greater than that of the sum of the two phospholipids combined as complex, complexes with PE/PC ratios of greater than 2:1 resulted in decreased LyPE production, presumably because of substrate limitation. The fact that formation of the hypothetical PE–PC complex takes place only during concentration and evaporation from organic solvents may indicate that the spatial configurations and charge characteristics of PE and PC micelles in an aqueous emulsion do not favor complex formation. The proposed complexing of PE and PC is supported by findings that phospholipid mixtures evaporated from a combination of organic solvents and water migrate as "a single band of electrophoretically homogeneous material" (5).

The repeated formation of small amounts of LyPE, even in the presence of relatively high levels of PC, may be due to some limited degree of dissociation of the complex when emulsified in an aqueous system, to a trace of water in the organic solvents used, or to the possibility that a small portion of the egg PE (which is composed of phosphatidyl ethanolamines of varying FA composition) may not complex with PC at all.

When the action of *Crotalus adamanteus* venom on PE, PC, and mixtures of the two phospholipids was examined in the post-heparin plasma phospholipase assay system, some of the effects observed were similar to and others different from those noted with post-heparin plasma. Of particular interest here are the findings that LyPE production was enhanced by PC added to the assay system as an aqueous emulsion while, at the same time, there was some LyPC formation which could not be observed in the absence of PE. There is no information at this time as to whether LyPC formation by snake venom is due to the same enzyme which is involved in PE degradation or whether there are, in fact, two different phospholipases in snake venom, one attacking PE and another specific for PC. It is of interest in this connection that two different proteins with phospholipase A activity have recently been isolated from the venom of *Crotalus adamanteus* (6).

From observations made on the behavior of the post-heparin plasma and snake venom phospholipases in an aqueous assay medium and on chromatoplates it would seem that the two enzymes are similar in some of their properties while differing in others. A general conclusion from these results is to re-emphasize that lack of a demonstrable phospholipase activity in a given system does not necessarily imply absence of the enzyme per se, nor does the presence of a given phospholipid substrate guarantee action on it by a particular phospholipase. The effects of the physicochemical nature of both substrate and enzyme on phospholipase activity have been described recently (7).

The observed effects of two phospholipids in combination, presumably bound together as a complex, on post-heparin plasma phospholipase activity call to mind observations made by different investigators of an enhanced coagulant or "thromboplastic" activity of similarly combined phospholipid preparations (8, 9, 4, 5). Interestingly enough, these same lipids by themselves usually have either negligible or low coagulant activity. The fact that no increased coagulant activity is obtained when the two phospholipids in question are emulsified in water without having been mixed previously in chloroform suggests that the observed effect is due to an interaction which took place between them while in chloroform solution.

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