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SLOW-REACTING HEMOLYTIC PHOSPHATIDES:

BENZYLATED LYSOLECITHINS*

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SUMMARY

Benzylated lysolecithin derivatives (*rac*-1-alkyl-2-benzylglycero-3-phosphorylcholine) have been found to be useful probes in studying the mechanism of lysolecithin-induced hemolysis on a molecular level. It has been demonstrated that with these lysolecithin analogs the lag time between addition of the lysin and the start of hemolysis can be prolonged to as much as 30 min as compared to 2–20 s with "normal" lysolecithin, *i.e.* 1-palmitoyl- or stearoylglycerophosphorylcholine. Furthermore, the actual leakage of hemoglobin proceeds at a significantly slower rate when benzylated instead of unaltered lysolecithin is used for hemolysis.

While the reasons for the latter phenomenon are not yet known, experiments with radioactively labeled compounds clearly proved that the lag time of the hemolytic reaction is determined by the rate of phosphatide adsorption to the cell surface.

INTRODUCTION

Lysolecithin and other surface-active phosphatides have been found to be useful tools in a number of biological systems. Thus it has been demonstrated that fusion of various cell types, as well as cell junctions, can be facilitated by external addition of lysolecithin[1–4]; and that intraperitoneal injection of lysophosphatides into mice prior to the injection of antigen can lead to a significant enhancement of the immune response[5,6]. It is further known that lysolecithin is a highly membrane-active material in that it induces permeability changes and, at sufficiently high concentrations, leads to cell lysis. Hence it seems conceivable that the biological activities of lysolecithin, such as fusion enhancement or adjuvant action, are related to its interaction with cellular membranes. The mechanism of this interaction, however, is still poorly understood.

Most investigations dealing with the mode of action of lysolecithin on cellular membranes have, for reasons of simplicity, been undertaken with erythrocytes of various mammalian species. Relying on the studies of the hemolytic activities of choline phosphatides, Reman *et al.*[7] recently postulated five consecutive steps in lysolecithin-induced hemolysis: (1) adsorption of the phosphatide to the cellular surface; (2) penetration into the lipoprotein complex; (3) induction of changes in the

* Dedicated to Professor Dr Otto Westphal on occasion of his 60th birthday.

molecular organization of the membrane; (4) permeability changes and disturbance of the osmotic equilibrium; and (5) leakage of macromolecules such as hemoglobin. A detailed study to test this theory, however, has proven to be a difficult task since lysolecithin-induced hemolysis is a very rapid reaction, lasting but a few seconds from the addition of the lysin to completion of lysis. Especially the adsorption step and the final leakage of hemoglobin are considered to be very fast reactions[7]. A slow-reacting derivative of lysolecithin should therefore be of some help in the elucidation of the complex lysis reaction.

As we and others have shown before[7,8], a slight retardation of the hemolysis kinetics can be achieved using synthetic lysolecithin analogs with increased chain length of the fatty acid residue or with a blocked β-hydroxyl group (such as methylated or acetylated lysolecithin[9]). A dramatic increase of the lag phase in the hemolytic reaction, on the other hand, is observed with a group of synthetic intermediates obtained during the chemical synthetis of lysolecithin[10], namely monoalkyl or monoacyl glycerophosphorylcholine with a benzylated hydroxyl group (see Fig. 1).

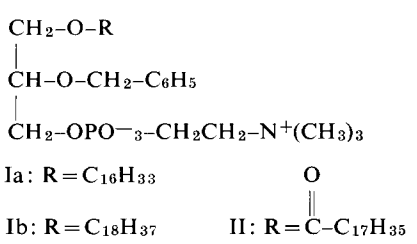

Fig. 1. Chemical structure of *rac*-1-alkyl-2-benzylglycero-3-phosphorylcholine (Ia and Ib) and of *rac*-1-stearoyl-2-benzylglycero-3-phosphorylcholine (II).

Two of these substances (phosphatides Ia and II) had already been included in a previous investigation of the hemolytic activities of structural lysolecithin analogs[8]. We were then able to demonstrate that these substances are different from other hemolytic phosphatides in that the lag phase of their hemolytic reaction with human red blood cells was extended from 2–20 s to 2–3 min at 37 °C. Meanwhile we obtained the homologous 1-octadecyl-2-benzylglycero-3-phosphorylcholine (phosphatide Ib), first synthesized by Kny[11] according to methods published earlier[10]. This compound proved to be even more valuable for the study of lysolecithin interaction with cellular surfaces.

MATERIALS AND METHODS

(*1*) *Buffer*

Phosphate-buffered saline was used throughout this study: 0.85% NaCl–0.01 M sodium phosphate, pH 7.2.

(*2*) *Erythrocytes*

These were prepared by centrifugation (10 min, $2000 \times g$) of citrate-treated human blood (acid–citrate–dextrose, bloodgroup O/Rh+). The buffy coat was removed and the remaining sediment was washed 3 times with 10 vol. of buffer. No differences in the sensitivity of the cells to lysophosphatides were observed when

1–5-days-old blood was used for preparation. Cells obtained from 7 days or older blood, however, showed a decrease in their stability with respect to lysolecithin.

(3) Phosphatides

Phosphatides (all racemates) were synthetized as described elsewhere[10]. ^{14}C-labeled substances were obtained by a modification of the method of Eibl and Westphal[12]. Solutions of the phosphatides were prepared at concentrations of 10^{-6} moles/ml in buffer, then stored in small volumes at -20 °C and heated to 40 °C before use. Each sample was discarded after use and not frozen again. No differences could be observed in the reactivity of fresh or several months old solutions, nor did sonication (instead of heating) after thawing influence the hemolytic activities of the substances.

Radioactive materials were stored at –20 °C in a methanolic solution. For adsorption experiments, appropriate volumes were evaporated under nitrogen and redissolved in buffer.

(4) Determination of hemolytic activities

Cell suspensions in buffered saline had a count of $4.6 \cdot 10^7$ cells/ml ($\pm 8\%$). (In a previous publication[8] this cell count had been erroneously stated too high by a factor of 10 and should be corrected). After complete lysis and centrifugation, the supernatant of this suspension had an absorbance of 1.0 at 546 nm. 1.2 ml of this suspension ($5.5 \cdot 10^7$ cells) were added to 1.5 ml polyethylene centrifuge tubes (Eppendorf), which contained increasing amounts of the lysin to be tested, dissolved at $2.5 \cdot 10^{-7}$ moles/ml in saline. After 3 h at 37 °C, the samples were spun in a table centrifuge (Eppendorf type 3200) for 1 min at 12000 ref./min ($8000 \times g$) and the absorbance of the supernatant was determined at 546 nm. The dosage which led to a 50% hemolysis (Table I) was extrapolated graphically from the hemolysis–concentration curves obtained with erythrocytes collected from three different individuals.

(5) Kinetics of the hemolytic reaction

At time zero, erythrocytes were mixed with 20 nmoles of phosphatide per $5.5 \cdot 10^7$ cells in 1.2 ml of buffer at 37 °C. Samples were centrifuged at different times, as described in the previous section, with the absorbance of the supernatant being determined at 546 nm. The time for complete sedimentation was set at 5 s after the start of the centrifuge. At that time the maximum acceleration of $8000 \times g$ was obtained.

(6) Adsorption of ^{14}C-labeled phosphatides to cells

8.0 ml of the suspension described under Section 4 (in this experiment $4.44 \cdot 10^7$ cells/ml) were kept in a water bath at 37 °C. At time zero, 400 μl (140 nmoles = 140000 cpm) of ^{14}C-labeled phosphatide Ia or Ib were added. 0.6 ml portions of this mixture, containing 10 nmoles (10000 cpm) of either phosphatide, were transferred into plastic tubes (Eppendorf, 1.5 ml) and centrifuged at various times. The radioactivity was then determined separately in 500 μl of the supernatant and in the sediment containing the remaining part of the supernate. Before counting, both samples were oxidized with 0.4 ml H_2O_2 in 1 ml of Soluene (Packard)–isopropanol (1:1, v/v) at 40 °C. The colorless solutions thus obtained were mixed with 10 ml of Instagel (Packard) and the radioactivity determined in a liquid scintillation counter (Tricarb

3000, Packard Instr. Co.). The sum of the cpm of the two fractions amounted to about 85% of the total radioactivity added at time zero, which implies that some 15% of the phosphatides were adsorbed to the tubes.

The counts (and thus the number of moles of phosphatide Ia or Ib) adsorbed to the cells were calculated from the total cpm in the sediment fraction by subtracting a correction factor for the cpm of the remaining volume of buffer.

Data for washed sediments (Table II) were obtained from the same reaction mixture. Samples were spun at the same times as in the above-mentioned experiment; however, the supernatant was removed completely (about 97%). The pellet was then resuspended in 0.6 ml of fresh buffer and left at 37 °C for an additonal 20 min. The radioactivity adsorbed to the cells was then determined as described above

RESULTS

Kinetics of the hemolytic reaction

The timecourse of the hemolytic reaction of phosphatide Ib as compared to phosphatide Ia or stearoyllysolecithin (*rac*-1-stearoylglycero-3-phosphorylcholine) is shown in fig. 2. It may be seen that phosphatide Ib reacts by a factor of 5–10 times slower than phosphatide Ia, which in turn shows a 10-fold increase of its lag phase over that of stearoyllysolecithin. The data presented here are given for human erythrocytes at 37 °C. With bovine or sheep red blood cells the hemolytic reactions of all three substances proceed at a significantly higher speed. The relative differences, however, can also be observed with these cell types.

Hemolytic activity

The differences in the lysis kinetics of the three phosphatides are not due to different hemolytic activities, as demonstrated in Table I. Comparing the amounts of phosphatide added in moles per cell necessary for a 50% hemolysis at 37 °C, it is apparent that stearoyllysolecithin and phosphatide Ia exhibit practically identical hemolytic activities, whereas phosphatide Ib is only slightly less active.

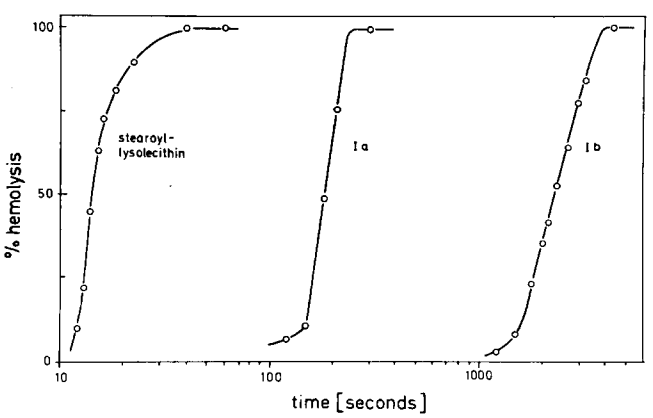

Fig. 2. Hemolysis at 37 °C as a function of time. Data were obtained as described under Materials and Methods, Section 5, using 20 nmoles of lysin per $5.5 \cdot 10^7$ cells (*i.e.* $360 \cdot 10^{-18}$ moles/cell).

TABLE I

HEMOLYTIC ACTIVITIES OF PHOSPHATIDES Ia, Ib AND STEAROYLLYSOLECITHIN AT 37 °C

Data are extrapolated from graphs plotting per cent hemolysis versus concentration of phosphatide in the medium. Incubation: 3 h at 37 °C at a cell count of $4.6 \cdot 10^7$ cells/ml. Values represent the means ± standard deviation of 4 experiments using red blood cells of 3 different individuals (all bloodgroup O).

	Phosphatide		
	Stearoyl-lysolecithin	*Ia*	*Ib*
Moles × 10^{-18} per cell required for 50% hemolysis	155 ± 15	149 ± 15	185 ± 18

Adsorption kinetics

Consequently, it was concluded that adsorption phenomena must be responsible for the delayed reaction of phosphatides Ia and Ib. To test this possibility we measured the adsorption of radioactively labeled compounds Ia and Ib to human red cells as a function of time. For this purpose ^{14}C-labeled phosphatides Ia and Ib were added to an erythrocyte suspension at concentrations identical to those used for the hemolysis test (see Fig. 2) at 37 °C. At different time intervals radioactivity adsorbed to the cells was determined as described under Materials and Methods. The results of these experiments are shown in Fig. 3. It is quite clear from these curves that the adsorption rates for phosphatides Ia and Ib are indeed markedly different. Moreover, it may be noted that in both cases the amount of phosphatide actually adsorbed to the cells at the time corresponding to the start of hemolysis (3 min for phosphatide Ia, 25 min for phosphatide Ib) is, within the range of error, identical, namely approximately $150 \cdot 10^{-18}$ moles per cell. This amount corresponds to some 40% of the total amounts of phosphatide Ia or Ib added at time zero. Not

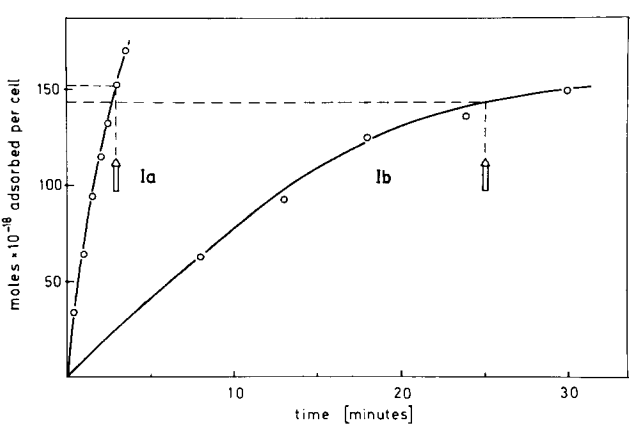

Fig. 3. Adsorption kinetics of ^{14}C-labeled phosphatides Ia and Ib to cells at 37 °C. Measurements were done as described under Materials and Methods, Section 6, with 10 nmoles of lysin per $2.75 \cdot 10^7$ cells ($360 \cdot 10^{-18}$ moles/cell). nmoles adsorbed to cells were calculated from adsorbed cpm, using the known specific activities. Arrows mark the start of hemolysis in the same experiment.

included in Fig. 3 are the adsorption data for stearoyllysolecithin since these are difficult to obtain at lytic concentrations. It is apparent from the above data that there is a direct correlation between the lag phase of a lysolecithin-induced hemolytic reaction and the kinetics of the adsorption of the phosphatide to the cellular surface. Furthermore, the above data reveal that at the beginning of hemolysis the molar amount of phosphatide bound to the cell is approximately identical for different substances.

Delayed hemoglobin release during lysis with phosphatide Ib

We have concluded from the data shown in Fig. 3 that for a given phosphatide the rate of adsorption determines the lag phase of the hemolytic reaction. In addition we found that at least one of the following steps (such as the penetration of lysolecithin into the lipoprotein complex of the membrane, or the rearrangement of the molecular membrane organization, or the final leakage of hemoglobin) must be delayed, when phosphatide Ib is used instead of the "fast-reacting" derivatives. This is demonstrated by the following experiment: Phosphatide Ib at a concentration identical to that used in the previous experiments (Figs 2 and 3) was allowed to adsorb to human red cells at 37 °C. At various times before hemolysis had started, the excess lysin in solution was removed by centrifugation and the pellet was resuspended in fresh buffer at 37 °C. The resuspended sediment was then kept at 37 °C for an additional 3 h, whereupon the samples were spun and hemoglobin in

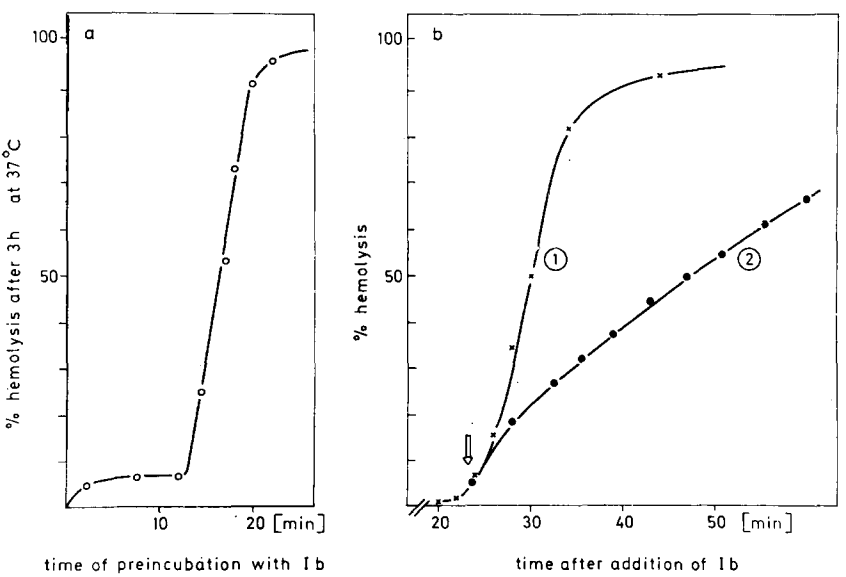

Fig. 4. Delayed hemoglobin release during lysis with phosphatide Ib. All measurements with 20 nmoles phosphatide Ib/$5.5 \cdot 10^7$ cells in 1.2 ml buffered saline at 37 °C. (a) Cells centrifuged at various times after addition of phosphatide Ib and sediment resuspended in fresh buffer. Graph shows the percentage of these cells lysed after an additional 3 h at 37 °C. (b) Curve 1, hemolysis of the complete system without removal of excessive phosphatide Ib as a function of time. Data are taken from the identical erythrocyte–lysin mixture used for data shown in (a). Curve 2, kinetics of the hemolytic reaction when excessive phosphatide Ib is removed by centrifugation after 24 min and the cells are left at 37 °C in fresh buffered saline.

the supernatant was determined. The results are plotted in Fig. 4a. The timecourse of hemolysis of the complete system (without removal of excessive phosphatide Ib) was recorded simultaneously from the same erythrocyte-lysin mixture (see Curve 1 in Fig. 4b). From the two curves it is obvious that after 25 min, when hemoglobin begins to leak out, the cells actually have adsorbed enough of the lysin to undergo complete lysis within the following 3 h. From the adsorption data in Fig. 3 it may further be seen that by this time the cells have adsorbed an amount of approximately $150 \cdot 10^{-18}$ moles of phosphatide Ib per cell. Though this amount is sufficient to cause 100% lysis, the completion of this reaction takes at least an additional 25 min (see Fig. 4, Curve 1) despite a remaining surplus of the lysin in the supernatant. This effect became even more striking when excessive phosphatide Ib was removed by centrifugation after 24 min, as described for Fig. 4a. The sediment (containing enough phosphatide Ib for 100% lysis, see Fig. 4a) was resuspended in fresh buffer and left at 37 °C. At various times, samples of this suspension were spun and hemoglobin was determined in the supernatant. Curve 2 in fig. 4b presents the data obtained in this manner, which clearly demonstrate that lysis with the minimal lytic amount of phosphatide Ib adsorbed to the cells is a very slow process indeed. The reasons causing this slow hemoglobin release following the adsorption of lytic amounts of phosphatide Ib are not yet known.

TABLE II

EFFECT OF WASHING UPON THE AMOUNT OF PHOSPHATIDE Ib ADSORBED TO CELLS

Adsorption experiments were carried out as described under Materials and Methods (Section 6). The total amount of radioactivity added per sample ($2.67 \cdot 10^7$ cells) was 10 000 cpm, corresponding to 10 nmoles of phosphatide Ib.

Adsorption time (min)	*cpm in sediment*	
	Without washing	*After resuspension in fresh buffer**
8	1669	1704
13	2484	2465
18	3326	3089
24	3609	3590
30	3966	3756

* Pellets were resuspended in fresh buffer at 37 °C and centrifuged again after 20 min.

Control experiments to determine whether the resuspension of the cells in fresh buffered saline removes large amounts of the adsorbed phosphatide from the surface have been carried out with ^{14}C-labeled phosphatide Ib (see Materials and Methods, Section 6). As may be seen from Table II, the amounts of phosphatide Ib bound to the erythrocytes at any time are practically identical, regardless whether the radioactivity of the sediment is measured directly or 20 min after resuspension in fresh buffer. Furthermore, it is obvious from the data presented in Table III that the

TABLE III

ELUTION OF CELL-BOUND PHOSPHATIDE Ib INTO FRESH MEDIUM AS A FUNCTION OF TIME

Same conditions as for data in Table II. Samples were centrifuged after 24 min and the pellets resuspended in fresh buffer at 37 °C. After various time intervals the suspensions were spun again and the radioactivity in the sediment was determined (see Materials and Methods, Section 6).

Time after resuspension in fresh buffer (min)	*cpm in sediment*
2	3684
10	3600
20	3590
60	3391

duration of the incubation of the cells in the fresh medium had no great effect upon the number of counts in the sediment, at least up to 60 min. Thus it appears that phosphatide Ib remains rather strongly adsorbed to the cellular membrane even at a hemolysis rate of more than 50% (see Curve 2, Fig. 4b), and that resuspension of the cells in fresh buffer elutes only small quantities of the phosphatide into the medium. This implies that Curve 2 in Fig. 4b indeed represents the hemolysis kinetics for a constant amount of some $130 \cdot 10^{-18}$– $140 \cdot 10^{-18}$ moles of phosphatide Ib adsorbed per cell, a quantity which leads to 100% lysis within 3 h (see Fig. 4a).

DISCUSSION

The purpose of this paper was to bring attention to a group of hemolytic phosphatides possessing quite unusual properties. These substances, which might be classified as either asymmetrical diether lecithins (*i.e.* alkylbenzylether lecithins) or as benzylated lysolecithins, apparently exhibit one significant difference to "normal" lysocithins: their hemolytic reaction is markedly retarded, due mainly to their delayed adsorption to the cell surface. In all other respects these substances resemble very much the "natural" lysolecithin or other fast-reacting lysosphatides. This is true for their surface activity at air–water interfaces[8], for their quantitative hemolytic activities (see Table I and data published previously[8]), as well as for certain biological properties. Thus it could be demonstrated that phosphatides Ia and Ib, like lysolecithin prepared from egg lecithin, react as immunological adjuvants in mice (Munder, P. G. and Weltzien, H. U., unpublished) and that at least phosphatide Ib is comparable to lysolecithin in its ability to facilitate cell fusion in the system of Croce *et al.*[3] (Shin, S. and Weltzien, H. U., unpublished results). We therefore feel that the use especially of 1-octadecyl-2-benzylglycero-3-phosphorylcholine (phosphatide Ib) might bring certain advantages for the study of lysolecithin–membrane interactions, and that results obtained from such experiments will allow deductive conclusions concerning the reactions of other lysophosphatides which act too fast to be measured directly.

One such reaction is the adsoption of lysophosphatides to the cells. To our knowledge only a few studies have been published which deal with the quantitative correlation between lysolecithin and the cell surface during hemolysis[13–15]. These, however, were done with chemically poorly defined or, by present standards, impure lysosphosphatides. In some early experiments (see Fig. 3) we could demonstrate that with phosphatide Ib as a lysin such measurements become extremely simple and for the first time allow the determination of the molar amount of lysin actually adsorbed to the membrane at the time of lysis with a relatively high degree of accuracy. More detailed studies are being presently carried out in our laboratory to determine the factors controlling this adsorption, such as temperature, chemical structure of the phosphatide, or the nature of the cellular surface (Weltzien, H. U., Arnold, B. and Westphal, O., unpublished).

Another possibility for the use of substances such as phosphatide Ib is indicated by the experiment recorded in Fig. 4. There it has been shown that the actual process of hemolysis, under proper conditions, may be observed at a rate slow enough for detailed morphological as well as chemical or physical investigations. That is to say, it is possible with phosphatide Ib as a lysin first to attach the lytic phosphatide to the cellular surface, to remove excessive lysolecithin from the medium and then study the hemolytic reaction without distortion by further lysin adsorption.

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