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LYSIS OF ERYTHROCYTES BY LONG-CHAIN ACYL ESTERS OF CARNITINE

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SUMMARY

1. Lysis of rat and human erythrocytes by synthetic fatty acyl esters of DL-carnitine and choline was compared to hemolysis by synthetic lysolecithin.

2. Stearoyl carnitine was the most effective lysin, whereas 1-palmitoyl-*sn*-glycero-3-phosphoryl choline and palmitoyl carnitine were more potent than either the shorter-chain acyl esters of carnitine or any one ester in the series of choline esters tried.

3. Even with lower concentrations of any one lysin tested, the rate of hemolysis was very rapid initially and proceeded without a lag period to an extent which was dependent on both the concentration of lysin and the amount of red blood cells present.

INTRODUCTION

According to a widely accepted concept, the membrane consists of a bimolecular leaflet of lipid, held within two surface layers of protein[1,2]. Recent evidence in support of other structural models for the plasma membrane has been summarized by KORN[3]. In any event, the lipid regions of the membrane are in a dynamic state[4] and may well undergo changes in their physicochemical organization[5]. Accordingly, it has been suggested that the local accumulation of a potent lysin such as lysolecithin might alter the permeability of the membrane[6]. In this respect, lysophosphoglycerides may not be exclusive and other lytic substances might also be concerned.

A widely accepted role of carnitine palmityltransferase is to mediate the translocation of fatty acyl groups across the mitochondrial membrane to the site of oxidation[7]. This mitochondrial enzyme is distributed among many animal tissues[8] and recently its occurrence has also been shown in Euglena[9]. Its presence in the red blood cell raises the possibility of a more general role for this transferase in that in certain cells it may also act at the plasma membrane site to promote entry of fatty acids[10].

In this transferase reaction, carnitine acquires a paraffinic chain which renders the product amphipathic. It is not known to what extent this property of fatty acyl carnitines might intervene in the translocation process or to what extent these substances might affect the structure of the membrane at the specific sites where they are produced, however, transient swelling of mitochondria has been demonstrated in the presence of palmitoyl carnitine[11,12]. Moreover, the lytic activity of certain paraffinic quaternary ammonium compounds is well known[13,14].

Since carnitine palmityltransferase is present in the erythrocyte membrane[10] and the products of this enzymatic activity likely possess lytic properties, we have undertaken a study of the effects of acyl carnitines on the red blood cell. This investigation was initiated by a comparative study of the hemolytic properties of fatty acyl esters of carnitine and choline and 1-palmitoyl-*sn*-glycero-3-phosphoryl choline.

MATERIALS AND METHODS

(+)-, (−)- or DL-carnitine·HCl was purchased from Light and Koch Co., England. Acyl carnitines were prepared essentially according to the method of BREMER as modified by FRITZ AND NOVAK (see ref. 15).

Hexanoyl carnitine·HCl (mol. wt. 295.7). Found: N, 4.78. $C_{13}H_{26}NO_4Cl$ requires N, 4.73%.

Octanoyl carnitine·HCl (mol. wt. 323.7). Found: C, 55.09; H, 9.38; N, 4.16. $C_{15}H_{30}NO_4Cl$ requires C, 55.61; H, 9.31; N, 4.32%.

Decanoyl carnitine·HCl (mol. wt. 351.7). Found: C, 58.60; H, 9.84; N, 4.01. $C_{17}H_{34}NO_4Cl$ requires C, 58.00; H, 9.67; N, 3.98%.

Myristoyl carnitine·HCl (mol. wt. 407.8). Found: C, 59.79; H, 10.03; N, 3.14. $C_{21}H_{42}NO_4Cl$ requires C, 61.77; H, 10.38; N, 3.43%.

Palmitoyl carnitine·HCl (mol. wt. 435.8). Found: C, 63.48; H, 10.52; N, 4.25. $C_{23}H_{46}NO_4Cl$ requires C, 63.33; H, 10.64; N, 3.21%.

Stearoyl carnitine·HCl (mol. wt. 463.9). Found: C, 64.59; H, 10.60; N, 3.31. $C_{25}H_{50}NO_4Cl$ requires C, 64.65; H, 10.86; N, 3.02%.

Acyl cholines were purchased from Sigma Chemical Co. and were purified by recrystallization from ethanol–water (5:1, v/v) with addition of ether.

1-Palmitoyl-*sn*-glycero-3-phosphoryl choline was obtained by the action of phospholipase A (EC 3.1.1.4) from *Crotalus adamanteus* venom on the synthetic dipalmitoyl analogue purchased from General Biochemicals, Ohio. The product was isolated by preparative thin-layer chromatography[16]. Human or rat erythrocytes were mixed with 0.4 vol. of 0.14 M glucose containing 0.057 M citrate (pH 7.4), and were extensively washed in saline and finally suspended to give a 50% hematocrit. All lysin solutions were dissolved in saline and adjusted to pH 7.4. The lysis test was determined spectrophotometrically. 100% hemolysed blanks were prepared by adding 25 μl of cells and a known amount of lysin to 3 ml of water in each of two cuvettes of a Spectronics 505 recording spectrophotometer and allowing the mixture to stand at least 20 min. The absorbance was adjusted to 0 and one of the blanks was replaced by another cuvette containing 3 ml of saline to which the same amount of cells and of lysin were added. The change in absorbance at 625 mμ was recorded as a function of time. The extent of hemolysis was usually complete within 10–15 min but was measured over a longer period when required. The percent hemolysis was estimated from a standard curve prepared by mixing different proportions of intact and hemolysed cells.

RESULTS

As can be seen from Figs. 1A and 1B, acyl carnitines are potent lytic agents when either rat or human red blood cells are used. Stearoyl carnitine was slightly more

effective a lysin than 1-palmitoyl-*sn*-glycero-3-phosphoryl choline or its homologues with shorter acyl chains. In 15 min, little hemolysis was obtained with high concentrations of decanoyl carnitine and none with the octanoyl or hexanoyl derivative. No significant difference could be found between the lytic activity of palmitoyl (+)- or (—)-carnitine and that of the racemic mixture.

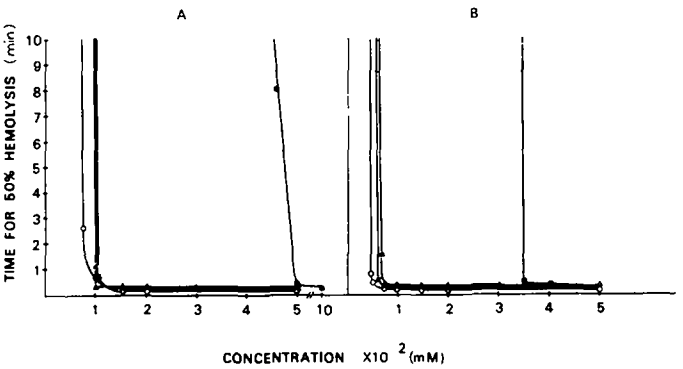

Fig. 1. Hemolysis of (A) rat, (B) human erythrocytes by acyl-DL-carnitines and lysolecithin. O—O, stearoyl carnitine; △—△, 1-palmitoyl-*sn*-glycero-3-phosphoryl choline; ×—×, palmitoyl carnitine; ●—●, myristoyl carnitine.

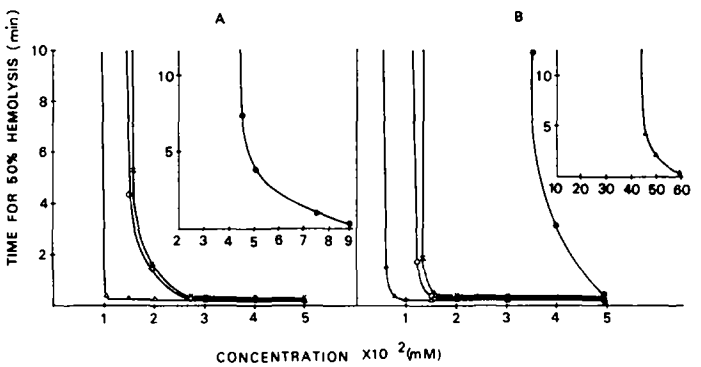

Fig. 2. Hemolysis of (A) rat, (B) human erythrocytes by acyl cholines and lysolecithin. △—△, 1-palmitoyl-*sn*-glycero-3-phosphoryl choline; O—O, stearoyl choline; ×—×, palmitoyl choline; ●—●, myristoyl choline; ▲—▲, lauroyl choline.

Results shown in Figs. 2A and 2B confirm the previous findings of PETHICA AND ANDERSON[13] that acyl cholines are potent lytic agents and the effective minimal concentration decreases with lengthening of the acyl chain. In this respect, acyl carnitines and acyl cholines are very similar.

Figs. 3A–C show that even with lower concentrations of either palmitoyl carnitine, palmitoyl choline, or 1-palmitoyl-*sn*-glycero-3-phosphoryl choline there is no lag period before the onset of hemolysis. This result differs from that obtained with polyene antibiotics on bovine erythrocytes. In the latter case, a lag period which increases as the concentration of lysin decreases was noticed[17]. In the case of the polyene antibiotics, the rate of penetration into the cell membrane is a rate limiting step in the hemolytic process[17]. Such does not appear to be the case with the lysins used in the present study.

If the concentration of lysin is kept constant and the amount of red blood cells is increased (Figs. 4A–C), there is a corresponding decrease in the percent of hemolysis. The extent of hemolysis by lysolecithin, acyl carnitines or acyl cholines varies not only with their concentration but also with the ratio of lysin concentration to the amount of cells present. A similar effect is found with polyene antibiotics and this

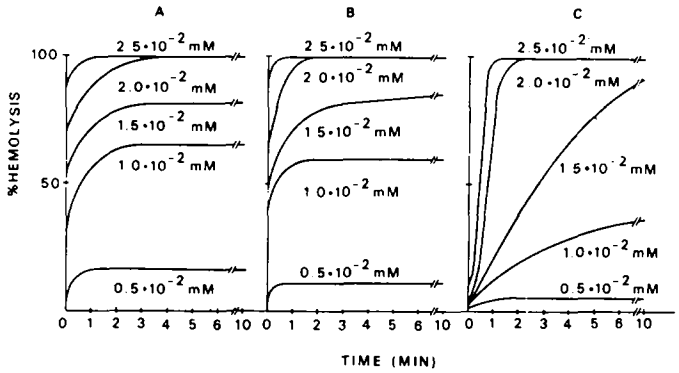

Fig. 3. The effect of decreasing concentrations of lytic palmitoyl esters on hemolysis of rat erythrocytes. (A) 1-Palmitoyl-*sn*-glycero-3-phosphoryl choline; (B) palmitoyl-DL-carnitine; (C) palmitoyl choline.

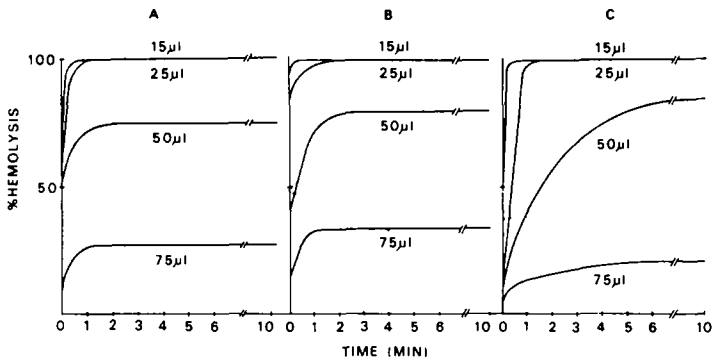

Fig. 4. The effect of increasing the amount of rat erythrocytes on hemolysis by $2.5 \cdot 10^{-2}$ mM concentrations of (A) 1-palmitoyl-*sn*-glycero-3-phosphoryl choline, (B) palmitoyl-DL-carnitine, (C) palmitoyl choline.

has been explained on the basis that the lysin combines with a definite proportion of lipid of the cell suspension. In this case complete hemolysis was obtained only when the number of filipen molecules added per molecule of lipid in the erythrocyte membrane was approximately 0.10–0.21 under the conditions used[17].

DISCUSSION

The red blood cell has served as a very useful model for studying the properties of biomembranes and the mechanisms involved with lysis. The hemolytic activity of paraffinic–ionic, surface-active compounds has been related to their ability to penetrate monolayers of cholesterol[17]. Such a relationship also exists between the lytic

activity of polyene antibiotics, the presence of sterol in the membrane and the ability of these lysins to interact with monolayers containing cholesterol[18,19]. On the other hand, certain non-ionic surfactants such as the copolymer of heptyl alcohol-11 ethylene oxide, probably exert their lytic activity by lowering the surface tension[20]. Yet other lysins exemplified by a number of organic solvents miscible with water, cause rupture of the membrane possibly by raising the effective value of the dielectric constant within the membrane thereby reducing the stability of the ionic bonds constituting the structural network[21].

Since acyl carnitine and acyl cholines are close analogues, they probably both interact in a similar manner with membrane constituents. The higher lytic potency in both these series of compounds with an increase in chain length suggests some degree of Van der Waals interaction between the agent and the apolar entities in the membrane. Although cholesterol is likely to be involved in such an interaction, other hydrophobic segments either from phospholipid or protein might also be implicated. In a preliminary investigation we were able to show that not only cholesterol but also serum albumin is very effective in protecting rat red blood cells from lysis by acyl carnitines.

Aside from non-ionic interactions, perturbations in the metal–ligand arrangements or in the ionic bonds involving protein and lipid might also intervene in the lytic process caused by the substances studied. In the case of acyl cholines which possess a net positive charge this seems more likely since relevant studies by BLAUSTEIN[22] and by FEINSTEIN[23] have shown that a number of cationic drugs can displace Ca^{2+} from a binding to acidic phospholipids. In the case of acyl carnitines which possess little or no net charge at physiological pH a displacement of Ca^{2+} from acid phospholipid is not as likely, although lipids such as lecithin, uncharged at physiological pH are capable of binding Ca^{2+} (refs. 24, 25).

The aforementioned discussion has dealt with mechanisms involved with a gross phenomenon resulting in the death of the cell. Nevertheless, similar mechanisms implicating small concentrations of naturally occurring lysins might be concerned in a more refined phenomenon such as the permeability of the cell. In this regard a study of sublytic concentrations of acyl carnitines and other natural amphipaths on the permeability of the red blood cell may be of considerable value. Furthermore, studies possibly implicating the lytic properties of such compounds with the release of substances from cells or from subcellular organelles, *e.g.* the release of bound acetylcholine or adrenalin, might well define in broader terms the physiological role of acyl carnitine and the enzyme responsible for its formation.

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