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Solute-induced acceleration of transbilayer movement and its implications on models of blood-brain barrier

Mahendra Kumar Jain [a,*], D.V. Jahagirdar [a], Margreet Van Linde [b], B. Roelofsen [b] and Hansjorg Eibl [c]

[a] *Department of Chemistry, University of Delaware, Newark, DE 19716 (U.S.A.),* [b] *Department of Biochemistry, Central University, 3508 TB Utrecht (The Netherlands) and* [c] *Max Planck Institute for Biophysical Chemistry, Am Fassberg, D-3400 Göttingen (F.R.G.)*

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Hexylglycerol accelerates the transbilayer (flip-flop) movement of phospholipids, lysophospholipids and peptides. For example, lysophosphatidylcholine added to dimyristoylphosphatidylcholine vesicles activates the action of pig pancreatic phospholipase A_2 (Jain and DeHaas (1983) Biochim. Biophys. Acta 736, 157–162) This activating effect is dissipated slowly after mixing, and no activation is observed when the lysophospholipid molecules are equally distributed on both sides of the bilayer. The half time for transbilayer movement of lysophosphatidylcholine is about 7 h, and it is accelerated over 100-fold in the presence of *n*-hexylglycerol, as well as by a variety of other amphipathic solutes including *n*-alkanols, ketamine, and flufenamic acid. Hexylglycerol also accelerates the rate of transbilayer movement of an amphipathic hexapeptide bocLALALW, as well as of the phosphatidylcholine molecules in erythrocyte membrane. These effects are observed without any change in the gross bilayer organization as judged by ^{31}P-NMR. Biophysical significance of such solute induced acceleration of transbilayer movement of amphipathic solutes is discussed to account for the effect of alkylglycerols on blood brain barrier.

Introduction

Incorporation of solutes in phospholipid bilayers induces a variety of structural and functional changes as manifested in altered phase properties [1,2], permeability [3], functions of membrane proteins [4], and induction of anesthesia [5]. These effects could arise from changes in the organizational and motional parameters of phospholipids in the bilayer doped with other solutes. In this paper we show that the transbilayer movement of amphipathic solutes is increased significantly in the presence of hexylglycerol, which is known to promote passage of several amphipathic drugs across blood brain barrier [6]. We have found that the rate of transbilayer movement of lysophosphatidylcholine and of a hydrophobic hexapeptide (bocLALALW) are slow. In both of these cases as well as for phosphatidylcholine in erythrocyte membranes the rates of transbilayer movement are increased up to several hundred-fold in the presence of low concentrations of hexylglycerol which do not disrupt the bilayer organization.

Several methods have been reported to measure

* To whom correspondence should be addressed.

the rates of transbilayer movement of phospholipids [7,8] as well as the permeability of small solutes across bilayer [9]. Both of these processes involve movement of a polar solute or group across the hydrophobic region of a bilayer from one interface to the other. For small solutes like water and urea the transbilayer permeability depends largely upon the membrane/water partition coefficients and the rate of transfer across the bilayer is relatively rapid compared to the rate of the interfacial transfer. However, large amphipathic solutes with large partition coefficients localize in the monolayer half of the bilayer to which they are exposed. The transbilayer movement of amphipathic solutes from one monolayer half of the bilayer to the other monolayer via the intervening hydrophobic barrier is characterized in this paper. Such a process can be very slow even though the lipid solubility or binding constants of these solutes is very large. The amphipathic solutes in a bilayer are localized such that the polar region is in the vicinity of the aqueous phase which makes the transbilayer movement energetically unfavorable since the polar group of the amphipathic molecule will have to interact with the hydrophobic region during the transbilayer transfer. This limitation applies even for the cationic drugs which bind to the anionic surface of a living cell where the driving force for the transbilayer movement could be provided by the inside negative diffusion potential. Thus, acceleration of transbilayer movement by additives has significant physiological and pharmacological implications.

Material and Methods

Pig pancreatic phospholipase A_2 was a gift from Professor G.H. DeHaas (Utrecht) and the hexapeptide bocLALALW (L = leucine, A = alanine, W = tryptophan) was kindly provided by Dr. Lila Geirasch (Newark, DE). All lipids were from Avanti (Birmingham, AL). 1-Hexylglycerol was synthesized as described elsewhere [10]. All other chemicals were best reagent grade available. Phosphatidylcholine-specific phospholipid transfer protein (PC-TP) was purified from bovine liver according to Kamp and Wirtz [11], and prepared for use according to Franck et al. [12]. Rat liver microsomal membranes, containing phosphatidyl[*N-methyl*-^{14}C]choline (37.7 μCi/mmol) were obtained from Wistar rats and prepared by a modification [12] of the procedure of Kamp and Wirtz [11].

Exchangeability of phosphatidylcholine (PC) in human erythrocytes was determined by incubation of the washed cells under gentle shaking at 37°C in the presece of PC-TP (2 nmol/100 μl of packed cells) in a buffer containing 90 mM KCl, 45 mM NaCl. 44 mM sucrose, 10 mM glucose, 10 mM Tris-HCl (pH 7.4), as well as 100 IU/ml penicillin and 100 μg/ml streptomycin. Cell suspensions were preincubated for 5 min in the absence (control) and presence of 1.2 mM hexylglycerol, and the exchange reaction was started by the addition of the [^{14}C]PC-labeled microsomal membranes (120 nmol of microsomal PC/100 μl of packed cells). The final cell concentration was 10%. At various time intervals, aliquots were taken from the incubation mixtures and the exchange reaction terminated by centrifugation for 5 min at 2500 × *g*. Residual microsomal membranes and PC-TP were removed by two additional washes of the cells with buffer. Lipids were extracted from the cells [13] and analyzed for total phosphorus content [14], and specific radioactivity of the PC after two-dimensional thin-layer chromatographic separation of phospholipids [15]. Similarly, specific radioactivity of the PC in the donor microsomal membranes was determined. Calculations of the extent of exchange of PC between microsomes and erythrocytes were carried out as described before [16].

Phospholipase A_2 activity was measured by pH-stat titration on Radiometer titrator [17]. Fluorescence measurements were done with SLM4800S spectrofluorimeter. The rate of transbilayer movement of lysophosphatidylcholine was determined as described elsewhere [18] and the general principle is outlined below. Reaction progress curves for the action of pig pancreatic phospholipase A_2 on sonicated vesicles of dimyristoylphosphatidylcholine exhibits a long latency phase. The latency period (tau) decreases when 1 palmitoyllysophosphatidylcholine in an aqueous solution is added to the preformed vesicles. This decrease in tau depends upon the mole fraction of lysophosphatidylcholine added to the vesicles, as well as on the structure of the lysophospholipid.

This is presumably because under these conditions lysophosphatidylcholine bound to the outer monolayer induces organizational defects to which phospholipase A_2 binds. However, the long latency phase observed with dimyristoylphosphatidylcholine vesicles remains essentially unchanged if lysophospholipid is added to dimyristoylphosphatidylcholine before the preparation of the vesicles, whereby both the components are at equilibrium on both sides of the bilayer. Similarly, low tau seen in the mixture of dimyristoylphosphatidylcholine vesicles and freshly added lysophosphatidylcholine increases when the mixture is allowed to equilibrate, and ultimately the latency period reaches the high value that is seen with unmodified dimyristoylphosphatidylcholine or with the premixed binary vesicles. This is because the lysophosphatidylcholine localized in the outer monolayer distributes in the two monolayers of a bilayer similar to the distribution in the premixed binary vesicles. The halftime for the recovery of the latency period or the halftime for transbilayer movement of 1-palmitoyllysophosphatidylcholine in dimyristoylphosphatidylcholine vesicles is about 7 h [18], which is about the same as the time for transbilayer movement of lysophospholipid determined by other methods in other types of bilayers [19,20].

The transbilayer movement of an amphipathic peptide containing tryptophan was monitored by fluorescence measurements. Tryptophan fluorophore exhibits an increase in the fluorescence intensity when transferred from the aqueous phase to the bilayer. Thus under appropriate conditions (i.e., those corresponding to the linear portion of the binding isotherm) on mixing vesicle with an aqueous solution of a suitable peptide, the overall increase in the fluorescence intensity occurs in two steps: the first 65% of the total change occurs in less than 10 s, and the next 35% of the change occurs more slowly by a first-order process with the halftime of several minutes to hours. We have interpreted the slower change to be due to the transbilayer movement of the peptide initially equilibrated with the outer monolayer to the inner monolayer of the vesicles.

^{31}P-NMR spectra were obtained as described elsewhere [21].

Results

Effect of hexylglycerol on the phospholipase A_2 catalyzed hydrolysis

The reaction progress curve for the action of pig pancreatic phospholipase A_2 on ternary codispersions containing premixed dimyristoylphosphatidylcholine + lysophosphatidylcholine + palmitic acid (100 : 22 : 22 mole ratio) has no latency phase, and the initial rate of hydrolysis is zero order at appropriate substrate concentration (> 100 μM). The burst of activity at the end of the latency phase observed in the reaction progress curve for the hydrolysis of dimyristoylphosphatidylcholine vesicles is due to formation of a critical mole fraction of the products [17]. The effect of hexylglycerol on the initial rate of hydrolysis of the ternary codispersions is shown in Fig. 1. The rate of hydrolysis does not change at low concentrations of hexylglycerol, and at higher concentrations the inhibition is apparently noncompetitive. A similar behavior is observed with a large variety of other solutes, and as suggested elsewhere [22] such an inhibition is due to a modification of the substrate interface, rather than due to a direct inhibitory effect of hexylglycerol on the enzyme. For the purpose of further discussion in this paper it should be pointed out that up to about 0.3 mM hexylglycerol has no effect on the rate of phospholipase A_2 catalyzed hydrolysis of the ternary

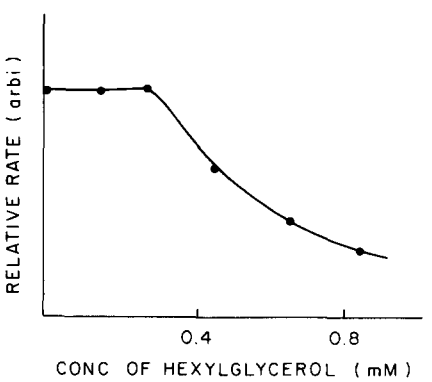

Fig. 1. Effect of *n*-hexylglycerol on the initial rate of hydrolysis of vesicles containing dimyristolphosphatidylcholine + 1-palmitoyllysophosphatidylcholine + palmitic acid (100 : 22 : 11). Phospholipase A_2 (0.4 μg) was added to 5 ml reaction mixture containing 425 μM substrate vesicles at pH 8.0, 35°C.

codispersions. Unless stated otherwise in all the phospholipase A_2 assays the final concentration of hexylglycerol was less than 0.1 mM.

Effect of hexylglycerol on the latency phase

The reaction progress curve for the action of pig pancreatic phospholipase A_2 on dimyristoylphosphatidylcholine vesicles exhibits a long latency phase and the latency period (tau) decreases in the presence of added lysophosphatidylcholine [18]. For most zwitterionic lysophospholipids the rate of transbilayer movement is small, however, it is considerably faster for anionic lysophosphatidic acid (data not shown). This is most probably because the anionic lipid species is translocated as unionized calcium salt.

The latency period (tau) for dimyristoylphosphatidylcholine vesicles under the conditions used in this paper is 30 min. At a constant mole fraction of 1-palmitoyllysophosphatidylcholine (X_p = 0.065) added to dimyristoylphosphatidylcholine vesicles, the time for the recovery of the latency period (tau) to 30 min increases with increasing temperature, that is the recovery is more rapid at higher temperature. The activation energy for this process is 14.4 kcal/mol (60 kjoule/mol), and a small anomalous increase in the recovery rate of the latency period is seen at the phase transition temperature. These and other observations on the transbilayer movement in dimyristoylphosphatidylcholine vesicles [18] are in general agreement with those with intact cells [19,20].

As shown in Fig. 2, in the presence of hexylglycerol tau increases, and this effect is dependent upon hexylglycerol concentration. The steady-state rate of hydrolysis does not appear to change appreciably even at 1 mM (approx. 20% inhibition), whereas with the ternary codispersion (cf. Fig. 1) the steady-state zero-order rate is inhibited by about 75%. A similar effect is seen with propylglycerol at about 5-fold higher concentration (data not shown). The effect of hexylglycerol on the tau depends on the incubation period over which lysophosphatidylcholine is allowed to equilibrate with the dimyristoylphosphatidylcholine vesicles. In the absence of hexylglycerol the maximum value of tau (about 30 min under the conditions used in this paper) is recovered with a halftime of about 7 h. In the presence of hexylglycerol the halftime for recovery of tau to 30 min depends on the hexylglycerol concentration and it decreases to less than 10 min at 0.84 mM hexylglycerol.

The dependence of the recovery time for tau on the hexylglycerol concentration is also manifested in the plots of tau on the mole fraction of lysophosphatidylcholine in dimyristoylphosphatidylcholine vesicles. As shown in Fig. 3A, the vesicles incubated with lysophosphatidylcholine

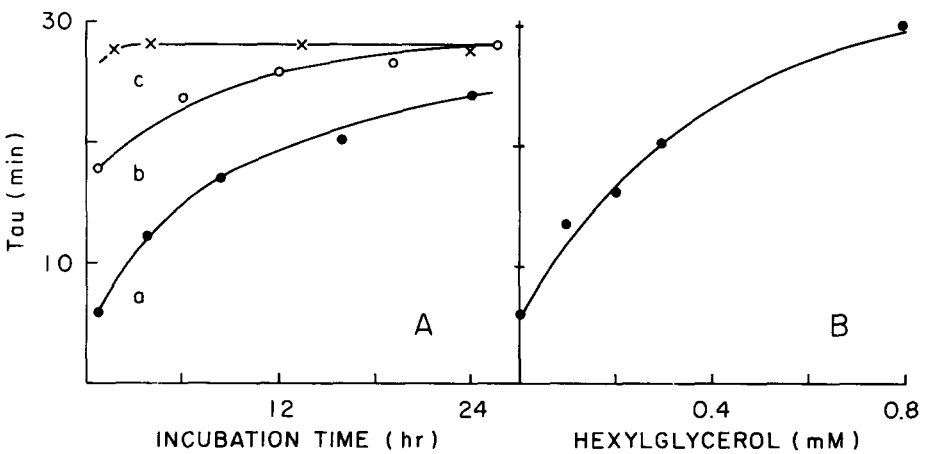

Fig. 2. (A) Variation of the latency period (tau) with the incubation period. Dimyristoylphosphatidylcholine vesicles (425 μM) activated by freshly added lysophosphatidylcholine (28 μM) were incubated at 37°C for the various periods indicated (abscissa). The incubation mixture contained (a) none, (b) 0.2 mM, and (c) 0.84 mM hexylglycerol. (B) Effect of hexylglycerol concentration on the latency period.

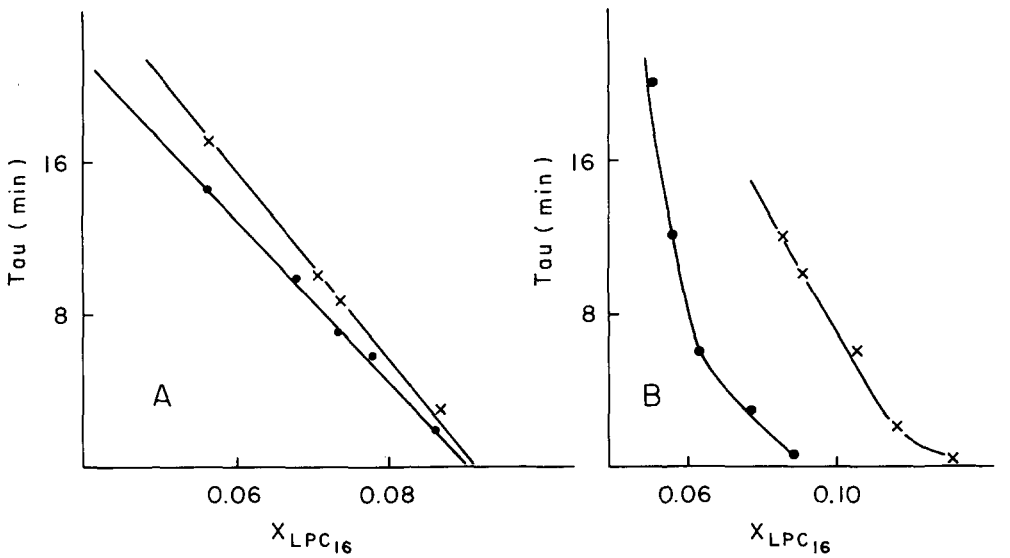

Fig. 3. Dependence of the latency period as a function of the mole fraction of lysophosphatidylcholine in the presence (crosses) and absence (closed circles) of hexylglycerol (0.2 mM) incubated for 1 min (A) or for 1 h (B).

and hexylglycerol for less than one minute exhibit only a small change in tau. In contrast, as shown in Fig. 3B, if the vesicles and lysophosphatidylcholine are incubated for one hour before starting the reaction with the phospholipase A_2 the latency period increases significantly in the presence of hexylglycerol over the whole range of the mole fractions of lysophosphatidylcholine. The results in Figs. 2 and 3 thus show that the recovery time for tau is considerably lowered by hexylglycerol. Since the recovery of tau depends on the rate of transbilayer equilibration of lysophosphatidylcholine, it means that hexylglycerol accelerates the rate of transbilayer movement of lysophosphatidylcholine.

Effect of other solutes on the latency phase

Not only alkylglycerols, but at low concentrations solutes like *n*-hexanol and *n*-heptanol also increase the latency period in the reaction progress curves. The steady-state rate of hydrolysis is not inhibited at these concentrations of solutes. The equipotency concentrations for the homologous alcohols for example suggest that the hydrophobic interaction of alcohols are involved and the effect is not specific for hexylglycerol. Other amphipathic solutes like macrocyclon, flufenamic acid, and ketamine also stimulate the rate of transbilayer movement at < 0.1 mM (data not shown). However, interpretation of these data is somewhat more difficult because the latency phase in the equilibrated bilayers is about 60 min compared to about 30 min in the absence of additives. This is probably because these solutes inhibit the action of phospholipase A_2 at about 0.1 mM, and therefore they could increase the time required for formation of the critical mole fraction of the products in the bilayer that gives a steady-state rate of hydrolysis [17,22]. Lower concentrations of these additives which do not inhibit phospholipase A_2 activity could not be used because of the technical difficulties. Other methods would be required to quantify the effect of these solutes on the transbilayer movement.

Transbilayer movment of the hexapeptide bocLALALW (bhex)

The transfer of a tryptophan containing peptide (bhex) from the aqueous phase to the bilayer of dimyristoylphosphatidylcholine vesicles is accompanied by a large change in the fluorescence emission intensity and a blue shift from 345 to 335 nm (excitation at 290 nm). At low concentrations of the probe and the vesicles the overall change in the fluorescence intensity occurs in two steps. About 65% of the total change occurs in the first step and

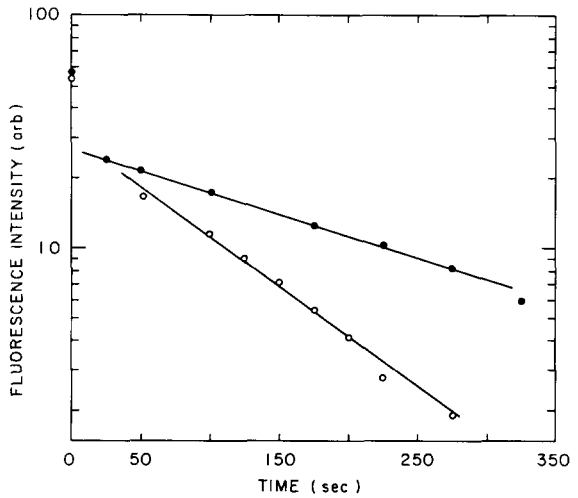

Fig. 4. The first-order plot for the change in the fluorescence intensity of a solution on adding dimyristoylphosphatidylcholine vesicles (0.3 mM) of bocLALALW peptide (4 μM) at 18°C, pH 8.0 in the absence (closed circles) of the presence of 0.2 mM *n*-hexylglycerol (open circles).

it is essentially complete in less than 10 seconds. The remaining 35% of the change occurs slowly by a first-order process as indicated by a linear semi-log plot with a halftime of about 2 min (Fig. 4). The 65 : 35 ratio for the change in the fluorescence intensity in the two steps is seen only in intact sonicated vesicles at peptide concentrations below the apparent dissociation constant. This is because in this linear region of the binding isotherm the proportion of the bound probe is directly proportional to the 'concentration' of the lipid interface available for binding [23]. For example, if the vesicles are sonicated or osmotically shocked after mixing the peptide, the total increase in the fluorescence intensity occurs almost instantaneously. On the other hand in multilamellar vesicles, the increase in fluorescence intensity in the first phase is only about 10% of the total change, and the increase in the intensity during the second phase follows a complex kinetics. Based on these observations we have assumed that the increase in the intensity in the first rapid step is due to the binding of the peptide to the outermost monolayer which constitutes about 65% of the total interface in sonicated vesicles and about 8–13% in multilamellar liposomes [24]; and the second slower step is due to the transbilayer movement of the peptide from the outer monolayer to the inner monolayer of the bilayer of the vesicles.

The proportion of the probe bound to bilayers also depends upon the apparent dissociation constant [23]. The fraction of the total probe bound to the two interfaces would be proportional to the interface concentrations only at probe concentrations below apparent K_d. Under these conditions, the fraction of the probe bound to the bilayer is almost linearly proportional to the concentration of the total probe as well as that of the interface. At the concentration of the hexapeptide used in this paper (4 μM), not only this condition for linearity is satisfied, but also the inner filter effect is negligible and a linearity of fluorescence intensity with the probe concentration is assured.

As shown in Fig. 4, the second phase of the change in the fluorescence intensity is accelerated by hexylglycerol although the proportion of the fluorescence intensity change in the two steps remains the same. The total change in the fluorescence intensity decreases at higher hexylglycerol concentrations, probably because the peptide in the bilayer containing hexylglycerol is in a somewhat less hydrophobic environment. The halftime for transmembrane movement of the peptide decreases with hexylglycerol concentration, and the effect is more pronounced at higher temperature. Similar effects are seen with 1-propylglycerol at about 10-fold higher concentration. The activation energy for the transbilayer movement of the hexapeptide is about 8 kcal/mol (33 kjoule/mol) in the absence as well as in the presence of 0.2 mM hexylglycerol. These results thus show that the rate of transbilayer movement of bhex is accelerated by hexylglycerol.

Exchangeability of phosphatidylcholine in intact human erythrocytes

Incubation of normal human erythrocytes at 37°C, in the presence of [^{14}C]PC containing rat liver microsomal membranes and PC-TP, results in an exchange of over 60% of the erythrocyte PC within 5 h (Fig. 5). During prolonged incubations, up to 76% of the PC can be exchanged in the native cells (results not shown), representing the PC pool in the outer monolayer. This is in full agreement with previous observations [12,16,25]. In contrast, a similar incubation in the presence of

1.2 mM hexylglycerol results in an essentially complete equilibration of the entire PC pool (100% that is present in the inner and outer monolayers) within 4 h, while the rate at which this process proceeds is considerably enhanced when compared to that in the absence of hexylglycerol (Fig. 5). If the partition coefficient of hexylglycerol between erythrocyte lipid and water is assumed to be 80, the amount of hexylglycerol present in the red cell membranes would be 2.5 mol% of the total phospholipid in the outer monolayer, or 1.4 mol% of the total lipid present in those membranes. The fact that the PC-TP mediated exchange of PC in native normal human erythrocytes reaches a plateau value at 76% of all of the PC, representing the fraction of this phospholipid present in the outer monolayer, has been interpreted to be a consequence of the relatively slow transbilayer movement of PC molecules in the red cell membrane [12,16,25,26], preventing the PC present in the inner leaflet to take part in this exchange process. In the presence of hexylglycerol, however, the entire PC complement (100%) appears to be available for such an exchange process, which proceeds at a higher rate (Fig. 5), thus indicating that the transbilayer movement of PC is no longer the rate-limiting step in the exchange of the PC from the inner monolayer.

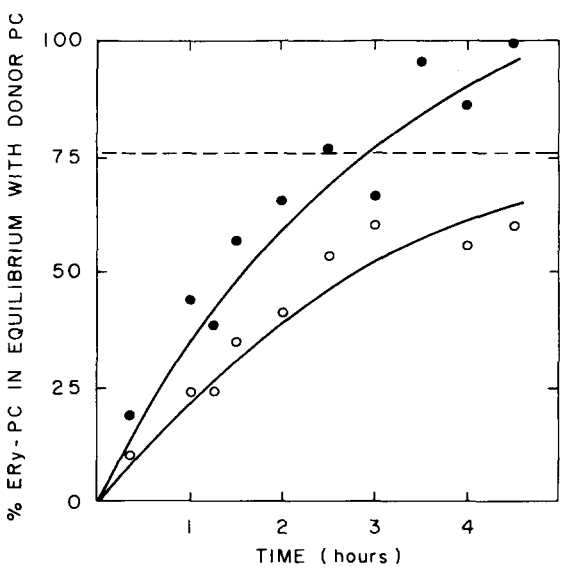

Fig. 5. Kinetics of exchange of erythrocyte phosphatidylcholine in the absence (open circles) and in the presence (closed circles) of hexylglycerol. Cells were incubated with phosphatidylcholine-specific transfer protein and microsomal membranes as the [^{14}C]phosphatidylcholine donor. The extent of exchange, expressed as per cent of the total phosphatidylcholine present in the erythrocyte, was determined at various time points for control cells (open circles) and cells incubated in the presence of 1.2 mM hexylglycerol (closed circles). During these incubations, cell lysis was always less than 4%. The horizontal broken line indicates the amount (76%) of phosphatidylcholine which is present in the outer layer of the erythrocyte membrane.

Effect of hexylglycerol on the bilayer organization

Hexylglycerol at the concentrations used in the preceding studies does not noticeably modify the bilayer organization. For example, the fluorescence studies and the phosphatidylcholine transfer studies show that the lysis/leakage of the bilayer bound structures is very low, if any. Similarly, differential scanning calorimetric profiles of dimyristoylphosphatidylcholine with or without lysophosphatidylcholine is not significantly altered (date not shown) even at the highest mole fractions used. As shown in Fig. 6, the ^{31}P-NMR line shape of egg phosphatidylcholine dispersions is not altered in the presence of equimolar con-

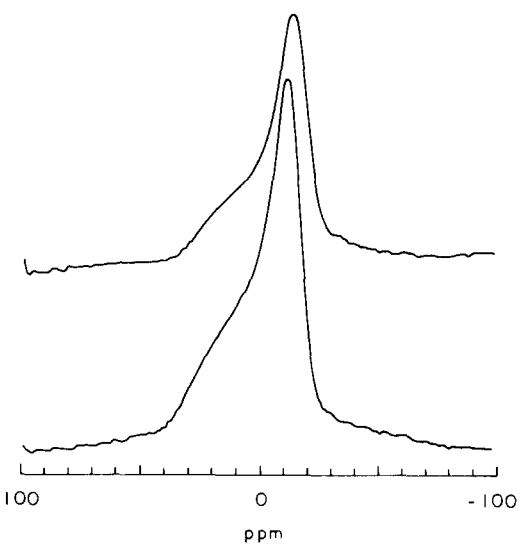

Fig. 6. ^{31}P-NMR spectrum (high power proton noise-decoupled measurements at 101.27 MHZ on Brucker WM-250 instrument) of egg phosphatidylcholine (approx. 100 mg/ml in $^{2}H_2O$, however, the exact concentrations are somewhat different in different samples) in the absence (top) and presence (bottom) of hexylglycerol (1 : 1 mole ratio) at 30°C.

centrations of hexylglycerol. All these observations suggest that hexylglycerol does not modify the gross bilayer organization.

Discussion

The results described in the preceding section show that the rate of transbilayer movement of three very different amphipathic molecules across phospholipid bilayers is accelerated several-fold in the presence of hexylglycerol, and under these conditions the gross bilayer organization is not altered by hexylglycerol. Until now, considerably enhanced transbilayer movements of PC in the human red cell membrane, as detected by a complete exchangeability of this lipid, have been ascribed to either a modification of the membrane skeleton [25,29] and thereby an uncoupling of this protein network from the lipid bilayer [12], or the absence of the antigens of particular blood group systems [26,30]. The present study shows that the rate of PC flip-flop in erythrocyte membrane can also be increased by a compound which, as demonstrated above, may have a direct effect on the lipid-lipid interactions in the lipid bilayer. However, it cannot yet be excluded that hexylglycerol in some cases interferes in lipid-protein interactions, and particularly those that stabilize the lipid bilayer of the red cell membrane by maintaining the characteristic asymmetric distribution and slow transbilayer movement of its phospholipids.

The mechanism of acceleration of transbilayer movement can only be speculated at this stage by comparing transbilayer movement with solute-induced permeability change. Several interesting features of the phenomenology of acceleration of transbilayer movement may be noted. Amphipathic solutes like alkanol [4,27] and local anesthetics [3,4] are known to increase the permeability of bilayers for small solutes. The alkanol induced increase in permeability of small solutes like water is seen at 20–50 mM concentration of hexanol. On the other hand, the effect of hexylglycerol on the transbilayer movement of larger amphipaths is seen at less than 1 mM. The bilayer/water partition coefficients for heptanol and hexylglycerol are expected to be approximately the same. We suggest that the difference in the effect of activators on the transbilayer movement is primarily due to a difference in the mechanism of transbilayer movement of the various solutes. Transfer of small solutes by solubility-diffusion mechanism apparently depends upon the mobility of kinks arising from the thermal disorder in the packing of the acyl chains [28]. Enhancement of such a solute-induced disorder probably requires higher concentration of additives like alkanols, and their effect would be essentially independent of the structure of the activating solute. On the other hand transbilayer movement of larger amphipathic molecules like lysophospholipids could occur by a mechanism involving sites of local disorder formed by the phase separation induced by lysophosphatidylcholine added to preformed dimyristoylphosphatidylcholine vesicles. The ^{31}P-NMR data (Fig. 6) suggest that such a local disorder is not due to the hexagonal phase. The most significant implication of the observations reported here is on the molecular characteristics of blood-brain barrier. As early as 1944 [31] Krogh suggested that the transendothelial passage which regulates the blood-brain barrier has characteristics of plasma membrane. Recently, Oldendorf [32] has pointed out that the permeability of blood-brain barrier is largely based on specific molecular conformation and hardly at all on molecular size or the lipid solubility. Both of these suggestions, as well as a variety of other observations [32], are consistent with the hypothesis that for the medium-sized amphipathic molecules the rate of transbilayer movement is slow and therefore these solutes can not cross the blood-brain barrier. Thus along with the carrier-mediated and the solubility diffusion mechanisms for the passage of solutes across membranes, the transbilayer movement could be yet another mechanism that is activated in the presence of additives like hexylglycerol. This interpretation of our observations provides a molecular explanation for the results of Eibl and co-workers [6] who showed that hexylglycerol indeed promotes passage of several amphipathic drugs across the blood-brain barrier. It should be pointed out that in these studies a transiently higher concentration of hexylglycerol is used because the activator does not attain an equilibrium distribution within the capillary where the transit time is less than one second.

The phenomenon of solute induced change in

the rate of transbilayer movement also raises other interesting possibilities. Hexylglycerol can be used for loading solutes into vesicles and cells. Some endogenous molecules (proteins or monoglycerides) could serve as accelerators of not only the transfer of solutes and membrane lipids but also for the transbilayer transfer of signal peptides and proteins during their biosynthesis. It may also be noted that the rate of transbilayer movement of mono- and diacylphospholipids is significantly accelerated in glycophorin containing vesicles [33], which implies that the local organizational defects induced by glycophorin and hexylglycerol may be similar or they may have the same precursor state.

Acknowledgments

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