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THE ROLE OF NONBILAYER LIPID STRUCTURES IN THE FUSION OF HUMAN ERYTHROCYTES INDUCED BY LIPID FUSOGENS

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Summary

(1) A comparative study of the polymorphic phase behaviour of reconstituted erythrocyte phospholipids with 'fusogens' and chemically related 'non-fusogens' using ^{31}P -NMR techniques has been made. We demonstrate that only fusogens can induce a conformational change in the membrane lipids from the bilayer to the hexagonal (H_{II}) phase.

(2) The tendency of erythrocyte (ghost) membranes to adopt nonbilayer structures is correlated with the extent of fusion in erythrocytes at various membrane concentrations of glycerol monooleate. Above a certain critical membrane concentration of glycerol monooleate, there is an increase in the tendency of ghost membranes to adopt the H_{II} phase, which is paralleled by a marked increase in fusion processes in the intact erythrocytes.

(3) These observations are consistent with a common mechanism of action for fusogenic lipids whereby inverted lipid structures such as long cylinders (H_{II} configuration) or inverted micelles are induced, which serve as intermediates in the fusion event.

Introduction

A model for intercellular fusion of erythrocytes induced by the fusogenic lipid, oleic acid, has been proposed previously which involves the formation of 'inverted' phospholipid structures as intermediates in the fusion event [1]. The model was based on the observation that phospholipids in the membrane of human erythrocyte ghosts undergo a conformational change from the bilayer to the hexagonal (H_{II}) phase in the presence of oleic acid, as detected

by ^{31}P -NMR techniques (for a review of lipid polymorphism as detected by ^{31}P -NMR see Ref. 2).

Several common carboxylic acids, their esters and retinol are able to induce fusion of erythrocytes [3]. In order to understand their mechanism of action, a number of studies have compared the properties of fusogens with chemically related analogues which are unable to induce fusion under the same conditions (for a review see Ref. 4). Differences are found in the melting points of the two types of lipid. In particular, fusogens tend to have a lower melting temperature than nonfusogens and this has led to the proposal that lipids which can induce fusion increase the 'fluidity' of the plasma membrane. Fusogenic lipids also induce structural changes in liposomes of erythrocyte lipids as viewed by negative staining electron microscopy, whereas nonfusogens do not. Differences are also found in the interaction of nonfusogens and fusogens with phospholipids in monomolecular films. Lipids able to induce fusion exhibit deviations from the ideality rule when mixed with monolayers of phospholipids containing a choline headgroup and such deviations are not observed for nonfusogens.

In the present paper, the initial observations made with oleic acid have been extended to include a variety of fusogens and nonfusogens. It is shown that fusogenic lipids are able to induce a conformational change from the bilayer to the hexagonal (H_{II}) phase in the hydrated lipids extracted from human erythrocytes, whereas similar concentrations of nonfusogens have little effect on the bilayer structure normally adopted by hydrated erythrocyte phospholipids. By measuring the extent of erythrocyte fusion induced by different membrane concentrations of glycerol monooleate and comparing this to the amount of nonbilayer phase induced by similar concentrations of fusogen in ghost membranes, a strong correlation is shown to exist between cell fusion and the ability of the membrane phospholipids to adopt nonbilayer phases.

Methods

Isolation of phospholipids from erythrocytes

10 l of packed erythrocytes (obtained from the Red Cross Blood Bank, Vancouver, B.C.) were washed in 150 mM NaCl and haemolysed in 10 vol. of 20 mM Tris at pH 7.4 and 4°C. After 30 min constant stirring, the ghost membranes were centrifuged at $16\,000 \times g$ for 15 min, and the pellet washed with 10 vol. of haemolysing buffer. The resulting pellets of 'red' ghosts were pooled and freeze-dried followed by rehydration in 100 ml of water. Lipids were extracted from the membrane suspension by using the procedure of Rose and Oklander [5]. The total lipid extract was dried by rotary evaporation and suspended in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2 : 1, v/v), which was washed with 0.2 vol. of water and the bottom phase dried by rotary evaporation. The residue was dissolved in 20 ml of CHCl_3 and the lipids separated using a Prep. LC-500 fitted with a Pre-Pack 500 cartridge containing silica gel (Waters Assoc., Milford, MA). Procedures for equilibration of the column and elution of the lipids were similar to those published for the purification of egg phospholipids [6]. The erythrocyte lipids were eluted at 150 ml/min using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (60 : 30 : 1, v/v) and fractions were detected according to their refractive index. Four fractions were collected, with the major lipid being phosphatidylcholine,

sphingomyelin, phosphatidylethanolamine or phosphatidylserine. Chromatographically pure phosphatidylcholine and sphingomyelin were obtained by passing each fraction once more through the column as described for the total extract. The phosphatidylethanolamine and phosphatidylserine fractions were purified using CM cellulose (CM52, pre-swollen, Waters Assoc.) according to published procedures [7].

³¹P-NMR of hydrated mixtures of membrane phospholipids and fusogen/non-fusogens

The purified membrane phospholipids were recombined in the mol ratio of 6 : 6 : 5 : 3 (phosphatidylcholine : phosphatidylethanolamine : sphingomyelin : phosphatidylserine) which is representative of the total membrane phospholipid composition for human erythrocytes, and cholesterol was added to a mol ratio of 0.8 with respect to phospholipid. Fusogenic and nonfusogenic lipids (Sigma Chemical Co.) were combined with membrane lipids so that an equimolar mixture, with respect to membrane phospholipid, was obtained. Aliquots of this mixture, corresponding to 30 μ mol of membrane phospholipid, were added in CHCl_3 to a 10 mm NMR tube and the CHCl_3 was evaporated under N_2 followed by exposure to high vacuum for 2 h. The lipid film was hydrated in 0.6 ml of buffer A: 10 mM Tris, 120 mM NaCl, 2 mM EDTA and 10% $^2\text{H}_2\text{O}$ at pH 7.0. When the lipid was dispersed, 20 μ mol of Ca^{2+} were added and the suspension freeze-thawed. In the present study, the Ca^{2+} dependence of the effects we observed was not investigated, consequently all the results presented were obtained in the presence of Ca^{2+} . ³¹P-NMR measurements were performed at 37°C employing a Bruker WP 200 NMR spectrometer.

Incubation of erythrocyte ghosts and glycerol monooleate

Erythrocyte ghosts were prepared as described above, however, additional washes with haemolysing buffer were made until 'white' ghosts were obtained. These were subsequently lyophilized. Samples were prepared from 200 mg of freeze-dried ghosts which were hydrated in 50 ml of buffer B: 20 mM sodium cacodylate, 120 mM NaCl and 2 mM CaCl_2 , pH 7.0. 25–200 μ g/ml dispersions of glycerol monooleate were prepared by sonication of 250 ml of buffer B and fusogen for 1–2 min at room temperature. The dispersion was added to the suspension of ghost membranes and incubated for 30 min at 37°C. After centrifugation at 16 000 $\times g$ for 15 min the ghost pellet was washed with 200 ml of buffer B. The packed membranes were transferred to a 10 mm NMR tube and the volume made up to 1 ml with a solution of buffer B in $^2\text{H}_2\text{O}$, prior to signal accumulation at 37°C. When the spectrum for each sample had been recorded, a lipid extract was made using the method of Bligh and Dyer [8] and the fatty acids analyzed as described below.

Fusion of erythrocytes induced by glycerol monooleate

The standard procedure developed for *in vitro* study of erythrocyte fusion [3] was used in this study. Glycerol monooleate was dispersed at concentrations ranging from 25–300 μ g/ml in 10 ml of buffer B containing 80 mg/ml of clinical grade dextran, average mol. wt. 60 000–90 000 (Sigma Chemical

Co.). The 10 ml of dispersed fusogen were added to 0.5 ml of packed erythrocytes, which had been drawn from a male donor on the day of each experiment. The cell suspensions were incubated at 37°C for 2 h, 0.5 ml of the incubation mixture was removed and added to 1 ml of buffer B containing 2% glutaraldehyde prior to observation using phase contrast microscopy. Fusion was quantified by expressing the number of fusion events as a percentage of the total cell number in the field of view. No attempt was made to estimate the number of cells involved in any particular fusion event, consequently the percentage fusion data are lower than the actual fusion which occurred. Remaining cells were washed three times with buffer B and a lipid extract made [5] which was submitted to fatty acid analysis.

Lipid analyses

Fatty acid profiles were determined for each lipid extract obtained from ghost membranes following accumulation of the ^{31}P -NMR signal and from erythrocytes following incubation with glycerol monooleate for 2 h. Methyl esters were prepared by heating the total lipid extract, dissolved in 2 ml of 5% H_2SO_4 in CH_3OH , at 70°C under N_2 for 2 h [9]. Separation and identification of the esters were achieved employing a Hewlett Packard 7610A high-efficiency gas chromatograph (fitted with a column of ethylene glycol succinate) operated between 170 and 185°C using a 7660A Multilevel temperature programmer. Standards with known concentrations of membrane phospholipid and fusogen were used to determine the mole ratio of fusogen to phospholipid for each sample.

Results

In order to gain definitive insight into the influence of fusogens on the polymorphic preferences of erythrocyte membrane lipids, model membrane systems comprised of appropriate quantities of erythrocyte phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and sphingomyelin to approximate the native lipid composition were employed. There were two reasons for such procedures. First, reconstituted systems were employed in preference to systems comprised of the total extracted lipids of erythrocyte ghosts due to the incomplete nature of the large-scale extraction procedures. This often results in total lipid extracts where phosphatidylserine, for example, is depleted as compared to the intact membrane. The use of reconstituted model systems eliminated such variables. Secondly, the use of pure lipid systems allowed the fusogens to be mixed with the phospholipid in CHCl_3 ensuring that in the derived liposomal system the fusogen was actually incorporated into the phospholipid matrix. This contrasts with the situation where erythrocyte (ghost) membranes are incubated in the presence of fusogen where it is difficult to be sure what fraction of exogenous fusogen actually enters the lipid bilayers.

As we have indicated elsewhere [1], the presence of oleic acid in the erythrocyte (ghost) membrane induces the endogenous phospholipids to assume the hexagonal (H_{II}) phase at equimolar (with respect to phospholipid) or higher membrane concentrations. Glycerol monooleate has the same ability, as indi-

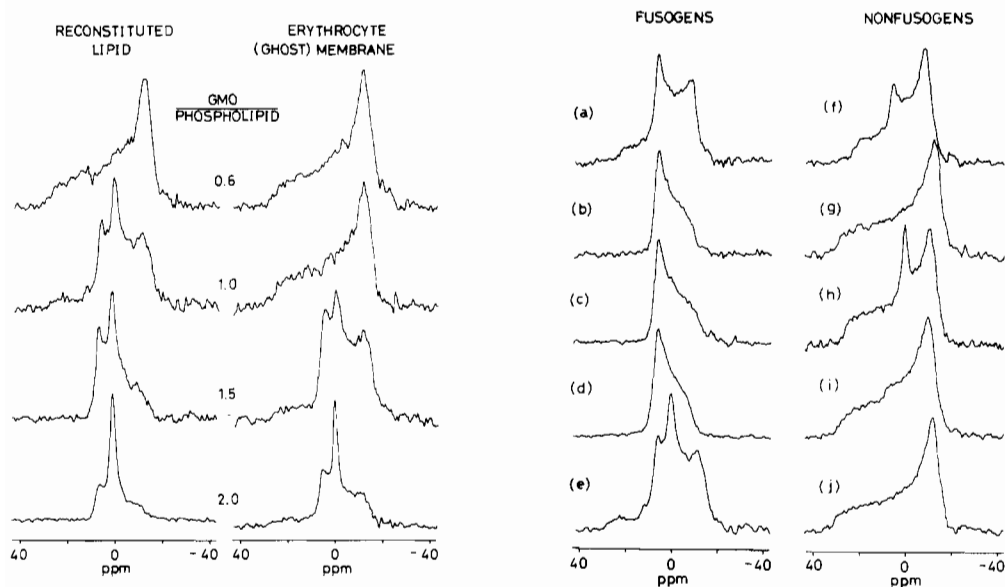


Fig. 1. 81.0 MHz ^{31}P -NMR spectra obtained from samples of reconstituted erythrocyte lipid and erythrocyte (ghost) membrane in the presence of various concentrations of glycerol monooleate (GMO). Samples were prepared as described in Methods. Accumulated free induction decays were obtained at 37°C from up to 4000 transients employing an $11\ \mu\text{s}$ 90° r.f. pulse, a 20 kHz sweep width and gated proton decoupling with an interpulse time of 0.8 s.

Fig. 2. 81.0 MHz ^{31}P -NMR spectra arising from reconstituted human erythrocyte lipids at 37°C in the presence of equimolar (with respect to phospholipid) fusogens (a–e) and nonfusogens (f–j). (a) Myristic acid, (b) palmitoleic acid, (c) retinol, (d) oleic acid, (e) glycerol monooleate, (f) palmitic acid, (g) triolein, (h) retinol plamitate, (i) stearic acid and (j) glycerol monostearate. Each sample consisted of 30 μmol of phospholipid, dispersed in 0.6 ml of buffer B (see Methods) and 10% $^2\text{H}_2\text{O}$. 20 μmol of Ca^{2+} were added, following lipid dispersion. Accumulated free induction decays were obtained as described in the legend to Fig. 1.

cated in Fig. 1. It is of interest to note that this influence of glycerol monooleate on the intact membrane is paralleled by its action on the total erythrocyte lipid model system, also shown in Fig. 1. This correlation suggests that the glycerol monooleate associated with the ghost membrane is largely contained within the lipid matrix, and that the endogenous proteins do not have a marked influence on the stability of the erythrocyte membrane lipid bilayer.

The influence of equimolar concentrations of assorted lipid fusogens and chemically related nonfusogens on the polymorphic preferences of the reconstituted erythrocyte lipid systems are indicated in Fig. 2. In general, it is clear that all the fusogens have the ability to induce the hexagonal (H_{II}) phase for at least a portion of the phospholipids, whereas the nonfusogens do not. This is with the exception of the saturated fatty acids myristic acid and palmitic acid, for which the distinction is not so clear-cut. Myristic acid is only very weakly fusogenic for hen erythrocytes [3], and in our hands neither palmitic acid nor myristic acid could induce fusion of human erythrocytes. However, both lipids could induce a small fraction of hexagonal (H_{II}) phase in the reconstituted model system (Fig. 2a and f). This led us to suspect that these

lipids are not readily incorporated into the (human) erythrocyte membrane, a possibility which was supported by the observation that neither myristic acid nor palmitic acid were able to induce the H_{II} phase in the ghost membrane system, even at concentrations corresponding to a 4-fold molar excess (with respect to membrane phospholipid).

Glycerol monooleate is one of the most effective of the lipid fusogens and has been used extensively in studies on chemically induced fusion. In Fig. 3, data are presented which show the extent of fusion induced in human erythrocytes by various concentrations of glycerol monooleate, expressed as the mole ratio of fusogen to cell phospholipid, following incubation at 37°C . This is compared to the amount of bilayer remaining in ghost membranes at similar concentrations of fusogen, and is expressed as the percentage of the total ^{31}P -NMR signal which can be attributed to phospholipids in an extended bilayer configuration. Fusion between human erythrocytes is not observed until a critical concentration of fusogen is reached which (in erythrocyte (ghost) membranes) induces the formation of hexagonal (H_{II}) phospholipid structures (Fig. 3). This concentration for glycerol monooleate is approximately equimolar with respect to the erythrocyte phospholipid which is in agreement with previous observations on the concentrations of oleic acid [1] and retinol [10] required to induce significant fusion in human and hen erythrocytes, respectively.

As indicated in Fig. 3, the highest membrane concentrations of glycerol monooleate in the intact erythrocyte membrane (glycerol monooleate-to-phospholipid ratio of approx. 4 : 1) correspond to extensive fusion (60% by our assay procedure). Despite considerable cell damage, examination of these cells under the light microscope reveals that intact membranes are still present, suggesting that a significant proportion of the membranes must be in a bilayer configuration. This is potentially inconsistent with the observation

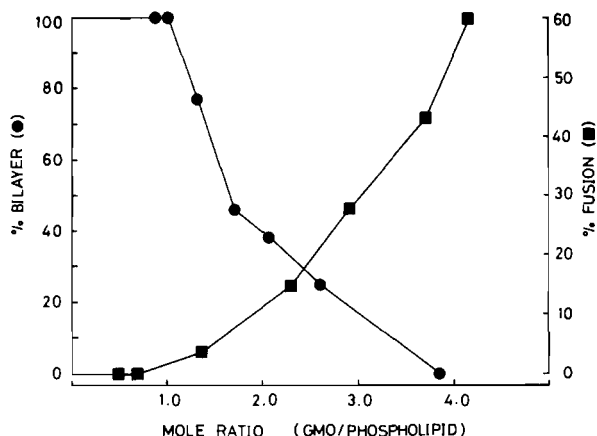


Fig. 3. A comparison of the extent of fusion between erythrocytes and the amount of bilayer phase remaining in erythrocyte (ghost) membranes at various membrane concentrations of glycerol monooleate (GMO). The % of ^{31}P -NMR signal which can be attributed to membrane phospholipids in an extended bilayer configuration (●), and % fusion of erythrocytes following incubation for 2 h with fusogen (■). Incubations and lipid analyses were performed as described in Methods.

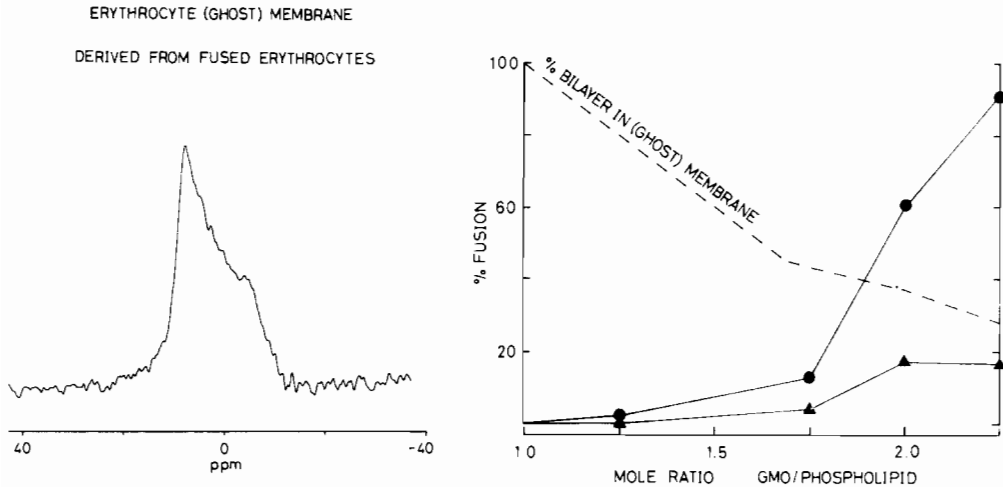


Fig. 4. 81.0 MHz ^{31}P -NMR spectrum of erythrocyte (ghost) membrane in the presence of fusogen at 37°C . The sample was prepared as follows. 10 ml of packed erythrocytes were suspended in 50 ml of buffer B containing dextran, and mixed with 200 ml of the same buffer in which 33 mg of glycerol monooleate had been dispersed by sonication. This concentration of fusogen was sufficient to yield a mol ratio of glycerol monooleate : phospholipid of approx. 4.0 : 1, which would be expected to induce greater than 50% fusion after 2 h (Fig. 3). However, the incubation was stopped after 20 min when fusion was less than 10%. The cells were haemolysed and white ghosts prepared as described in Methods. The ^{31}P -NMR spectrum was obtained as described in the legend to Fig. 1.

Fig. 5. Erythrocytes were incubated at 37°C in the presence of glycerol monooleate (GMO) for 1 h. Half the samples were allowed to stand for 20 min at room temperature whilst the remaining cells were centrifuged at $2000 \times g$ for 20 min, also at room temperature. Following centrifugation, the erythrocytes were resuspended and all the samples incubated for a further 15 min at 37°C prior to fixation. The extent of fusion for cells which were subjected to centrifugation at $2000 \times g$ (\bullet) and for the control cells (\blacktriangle). The discontinuous line represents the amount of extended bilayer remaining in the erythrocyte (ghost) membrane at the concentration of fusogen used for this experiment.

that similar membrane concentrations of glycerol monooleate in ghost membranes induce the hexagonal (H_{II}) configuration for the vast majority of the endogenous phospholipids (Fig. 3). A possible source of this discrepancy concerns the centrifugation step ($16\,000 \times g$, 15 min) employed in the preparation of the ghosts. This results in a tightly packed pellet in which intermembrane fusion events would be expected to be greatly facilitated, and where formation of the H_{II} phase (which appears to take place between closely opposed bilayers [11]) would be favoured. Results supporting this possibility are presented in Figs. 4 and 5. Fig. 4 shows the ^{31}P -NMR spectrum obtained from ghosts prepared from erythrocytes incubated in the presence of glycerol monooleate sufficient to induce greater than 50% fusion within 2 h. However, the incubation was stopped after only 20 min when approx. 10% fusion had occurred. Clearly, the large majority of the endogenous phospholipid in this ghost system adopts the hexagonal (H_{II}) phase. This would be consistent with the possibility that membranes rich in fusogen are able to enter the H_{II} phase more easily when in close proximity. Such a proposal would predict that erythrocyte fusion would be enhanced if cells were packed by centrifugation following incubation in the presence of fusogen. This was found to be the

case. In Fig. 5, the extent of fusion in human erythrocytes incubated with various concentrations of glycerol monooleate is greatly enhanced following centrifugation at approx. $2000 \times g$ for 20 min when compared to cells which were not subjected to centrifugation. It is interesting to note that even when centrifugation is included in the procedure, fusion of erythrocytes is not observed until sufficient fusogen is present to induce the formation of (H_{II}) structures in the erythrocyte (ghost) membrane.

Discussion

Earlier comparative studies of fusogenic and nonfusogenic lipids suggested that lipids capable of inducing fusion increase the fluidity in the hydrocarbon region of membranes [4]. The results presented here, on the other hand, clearly suggest that an additional distinguishing feature between fusogenic and nonfusogenic lipids is that fusogens have the ability to induce the hexagonal (H_{II}) phase for a portion of the phospholipids in the biological membrane.

In a previous work [1], we demonstrated that membrane concentrations of oleic acid which were sufficient to induce appreciable H_{II} phase structures in erythrocyte (ghost) membranes were similar to the membrane concentrations of oleic acid required to induce fusion between erythrocytes. This observation led to the proposal that fusion induced by oleic acid proceeds via intermediate nonbilayer lipid structures such as long inverted channels (H_{II} configuration) or inverted micelles (see Fig. 4 of Ref. 1) which is facilitated by the presence of fusogen. The results of this study suggest that this may be a common mechanism of action of fusogens, as the more active lipid fusogens induce the H_{II} configuration in erythrocyte phospholipids. Furthermore, our basic proposal is supported by the detailed analysis of glycerol monooleate-induced fusion, which shows a direct correlation between erythrocyte fusion and the tendency of the (ghost) membrane to adopt the H_{II} phase.

The mechanism of action whereby lysophosphatidylcholine induces cell fusion would appear to constitute an exception to the behaviour described above. Although this lipid was one of the first shown to have fusogenic properties towards erythrocytes, it is extremely haemolytic and fusion can only be observed if cells are fixed within 30 s of adding lysophosphatidylcholine [4]. In situations where lysophosphatidylcholine does induce fusion, micelles bridging two membranes [1] would appear to be most likely to correspond to the intermediate situation.

In summary, previous studies on fusogenic lipids established correlations between fusogenic capacity and factors such as fluidity changes and ideality of mixing in monolayers of phospholipid [4]. These effects do not, however, suggest an obvious intermediate structure which the fusion process requires. The results presented here, on the other hand, are consistent with a common mechanism of action for the more effective lipid fusogens, which appear able to generate non-bilayer inverted lipid configurations which can be logically proposed to constitute an intermediary structure in the fusion event. Furthermore, the ability of naturally occurring membrane lipids to adopt the hexagonal (H_{II}) phase [2] or other inverted structures such as inverted micelles

(lipidic particles) [13], and the ability of Ca^{2+} to generate such structures [14,15] strongly support our contention that membrane fusion in vivo proceeds via a similar mechanism.

Acknowledgements

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