Biochemical Pharmacology, Vol. 42, No. 10, pp. 1947–1951, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

PERTURBATION OF THE FLUIDITY OF THE ERYTHROCYTE MEMBRANE WITH IONOPHORIC ANTIBIOTICS AND LIPOPHILIC ANAESTHETICS

KHALID M. ABU-SALAH

Department of Biochemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

(*Received* 21 *March* 1991; *accepted* 4 *July* 1991)

Abstract—The fluidity of the rat erythrocyte membrane was evaluated by measurement of excimer fluorescence of an intra-molecular forming fluorophore, 1,3-di(1-pyrenyl)propane. The polyene ionophoric antibiotics, amphotericin B and nystatin, were found to fluidize the erythrocyte membrane, as assessed by the increase in the excimer/monomer fluorescence intensity ratio, by 42 and 13%, respectively, compared with control samples. In contrast, of the peptide ionophoric antibiotics, valinomycin demonstrated about twice the effect which gramicidin A had on depressing the fluidity of the erythrocyte membrane. On the other hand, the general lipophilic anaesthetics, propanidid and althesin, led to an increase, by 70 and 32%, respectively, while the local anaesthetic, procaine, led to a decrease by 20%, in the fluidity of the erythrocyte membrane. These results were explained in the light of the partition coefficients determined for these drugs in decane and native membranes, their affinities for specific membrane components and the changes which they induce in the permeability properties of erythrocyte and other biological membranes.

Ionophores are lipid-soluble substances which can carry specific ions across model and biological membranes by forming moderately stable complexes with the transported species.

Amphotericin B and nystatin are polyene macrolide antibiotics used in the treatment of a wide range of fungal infections [1]. They achieve their fungicidal and lytic activities by complexing with the sterols available in plasma membranes [2, 3], thus forming transmembrane channels which allow the efflux of vital cations such as potassium [2, 4]. Amphotericin B and nystatin, however, inhibit the exchange of some metabolically important anions such as phosphate and sulphate [5, 6].

Gramicidin A and valinomycin are peptide antibiotics which act, respectively, as a channel-forming ionophore [7] and a carrier [8, 9], across biological [10, 11] and model membranes [11, 12]. Gramicidin A increases the rate of uptake of alkali metals and H^+ [8] and valinomycin, used as a pesticide, is specific for the translocation of H^+, Na^+ and Rb^+ [8, 9].

Althesin and propanidid are general lipophilic anaesthetics which have been found to inhibit glucose and phosphate transport across the human erythrocyte membrane [13]. Procaine, on the other hand, is a local anaesthetic which has a perturbing effect on the conductance of nerve membranes for Ca^{2+} [14]. Although the effects of these ionophoric antibiotics and lipophilic general anaesthetics on the exchange of cations has been investigated, their effects on the fluidity of synthetic and natural membranes have not been reported. This study investigated the effect of ionophoric polyene and peptide antibiotics and the general anaesthetics propanidid and althesin on the fluidity of the rat erythrocyte membrane, in order to shed more light on their ionophoric and anaesthetic properties, their functions and their modes of action.

MATERIALS AND METHODS

Amphotericin B was purchased from the Sigma Chemical Co. (Poole, U.K.). Nystatin, gramicidin A and ouabain were supplied by Windsor Laboratories Ltd (Slough, U.K.). L-α-Phosphatidyl choline from egg yolk was obtained from Calbiochem Corporation (San Diego, CA, U.S.A.). Valinomycin was provided by Fluka AG, Chemical Factory (Buchs, Switzerland). Procaine hydrochloride was purchased from BDH Chemicals (Poole, U.K.). Althesin and propanidid were obtained from Glaxo Ltd and Bayer U.K. Ltd, respectively. 1,3-Di(1-pyrenyl)propane (DPP) was supplied by Molecular Probes Inc. (Eugene, OR, U.S.A.). All other reagents were analytical grade or better, and were obtained from BDH Chemicals. Amphotericin B, nystatin, valinomycin and ouabain were solubilized with dimethyl sulphoxide. Gramicidin A, propanidid and the commercial formulations of althesin for clinical usage were dissolved in ethanol. Procaine hydrochloride was dissolved in water.

Preparation of erythrocyte membranes. In a typical experiment blood was withdrawn, into syringes containing EDTA, directly from the hearts of rats, under ether anaesthesia, by a cardiac puncture after thoraco-abdominal incision. Erythrocytes were prepared by washing the normal blood with isotonic 0.17 M Tris–HCl buffer, pH 7.6, and centrifuging at 1000 *g* for 20 min at 4°. The erythrocytes were washed twice more after removal of the buffy coats, as described by Hanahan and Ekholm [15]. Protein content of prepared membranes was measured after solubilizing the membranes in 2.0% sodium dodecyl sulphate using the manual ninhydrin method [16].

Determination of intramolecular excimer fluorescence. The effect of various drugs on membrane fluidity was monitored with the intramolecular excimer forming fluorophore 1,3-di(1-pyrenyl)-propane. The method was essentially as modified for rat hepatocyte plasma membrane [17]. After loading erythrocyte membranes with DPP, they were washed twice with Tris-buffered saline (TBS, 5 mM Tris, 146 mM NaCl, 4 mM KCl, pH 7.4) by centrifuging at 45,000 *g* for 25 min at 20°. The washed pellet was resuspended in 4 mL of deoxygenated TBS containing the drug concerned and then incubated at 37° for 30 min in a shaking water bath to allow the drug to partition into the lipid bilayer of the membrane. Fluorescence emission spectra were recorded at 20° with a Perkin–Elmer 650-15 fluorescence spectrophotometer at a time constant of 0.3 and sensitivity of 0.1. An excitation wavelength of 345 nm was employed. Excimer and monomer fluorescence peak intensities were monitored at 485 and 396 nm, respectively. Sample intensities were corrected for membranes carried through the procedure with drug but without DPP and for the probe added to TBS containing the drug only. All drugs used, along with a control, were examined in duplicate in one experiment.

Determination of partition coefficients. Drug partitioning, at the concentrations indicated in Tables 1 and 2, was studied either between erythrocyte membrane and TBS, at 3.25 mg membrane protein/mL of TBS, or between decane and TBS (1:1 v/v). In each case the media were mixed using a vortex mixer and then incubated for 20 min at room temperature to allow the drug to partition between the phases used. In all the experiments, the concentration of solubilizing reagent did not exceed 1.5%. The drug was initially solubilized in either TBS alone or decane alone, depending on drug polarity. The complete separation of the two phases or precipitation of the membranes was achieved by centrifugation at 1500 rpm using MSE Coolspin 2 centrifuge. The amounts of amphotericin B, nystatin, gramicidin A, althesin, propanidid and procaine in the aqueous phases were determined by measuring their absorbances at the wavelengths of 405, 303, 280, 268, 274 and 284 nm, respectively. Correction for the residual membrane proteins in the supernatants was applied where necessary. A pure sample of each drug was used as a standard. The partition coefficients were calculated by dividing the proportion of the drug in decane or in erythrocyte membranes by that in the aqueous phase.

Measurement of potassium exchange. Erythrocytes were prepared by washing whole blood in Tris buffered saline (TBS, 5 mM Tris, 146 mM NaCl, 4 mM KCl, pH 7.4) containing 5 mM glucose and centrifuging at 1000 *g* for 10 min at 4°. The erythrocytes were washed twice more after removal of the buffy coats.

Washed erythrocytes were preincubated for 15 min at a haematocrit value of 45% and 37° with TBS. Antibiotics and anaesthetics were subsequently added in the proper solvent. Samples were removed from the reaction medium at various timed intervals and centrifuged for 4 min at 2000 *g*. The amount of

Table 1. Fluidity parameters and partition coefficients of different ionophoric antibiotics in decane and erythrocyte membranes

Ionophore	Concentration (μM)	Excimer/monomer fluorescence intensity ratio*		P value	Partition coefficients in decane†	Partition coefficients in membrane†
		Control	Treated			
Amphotericin B	10	0.252 ± 0.003	0.358 ± 0.003	<0.001	1.20 ± 0.004	0.40 ± 0.69
Nystatin	10	0.252 ± 0.003	0.286 ± 0.001	<0.001	0.077 ± 0.005	0.026 ± 0.004
Gramicidin A	15	0.252 ± 0.003	0.200 ± 0.001	<0.002	2.06 ± 0.070	0.898 ± 0.079
Valinomycin	15	0.252 ± 0.003	0.145 ± 0.005	<0.001	ND	ND

* Values of fluidity parameters represent the mean ± SEM, N = 12. P values were determined by Student's *t*-test.
† Values of the partition coefficients represent the mean ± SD of six different determinations.
ND, not determined.

Table 2. Fluidity parameters and partition coefficients of different anaesthetics in decane and erythrocyte membranes

Anaesthetic	Concentration (μM)	Excimer/monomer fluorescence intensity ratio* Control	Treated	P value	Partition coefficients in decane†	Partition coefficients in membrane†
Althesin	350	0.250 ± 0.003	0.330 ± 0.004	<0.001	0.162 ± 0.006	0.049 ± 0.004
Propanidid	350	0.250 ± 0.003	0.425 ± 0.013	<0.001	0.620 ± 0.045	0.200 ± 0.022
Procaine	350	0.250 ± 0.003	0.199 ± 0.004	<0.001	0.050 ± 0.004	0.900 ± 0.060

* Values of fluidity parameters represent the mean ± SEM, N = 12. P values were determined by Student's *t*-test.
† Values of the partition coefficients represent the mean ± SD of five different determinations.

K^+ in the supernatants was determined using a Pye-Unicam 5P9 atomic absorption spectrophotometer.

RESULTS

The dynamic component of erythrocyte membrane fluidity was assessed by measurements of excimer fluorescence of the intramolecular excimer forming fluorophore, DPP. DPP has been used to monitor short-range lateral diffusion of pyrene substituents in biological membranes [18]. The short range lateral diffusion in plasma membranes was evaluated by use of the excimer/monomer fluorescence intensity ratio of DPP. The increase in this ratio observed in erythrocyte membranes treated with amphotericin B and nystatin, by 42 and 13% respectively, indicated an increase in their fluidity (Table 1). The fluidizing effect of amphotericin B was about three times more than that of nystatin. Similarly, the proportions of amphotercin B partitioned in decane and in the erythrocyte membrane were higher than those of nystatin by 15.6 and 15.4 times, respectively (Table 1). The values of partition coefficients obtained for amphotericin B and nystatin are in accordance with their hydrophobic nature. In contrast, the ionophoric peptide antibiotics, gramicidin A and valinomycin, which are also specific for cation transport [7, 12], were found to reduce the fluidity of the erythrocyte membrane, as evident from the decrease observed in their excimer/monomer fluorescence intensity ratio (Table 1). Valinomycin demonstrated about twice the effect which gramicidin A had on depressing the fluidity of the erythrocyte membrane (Table 1). The effect might be explained, at least in part, in the light of the characteristic "doughnut shape" conformation, which valinomycin assumes in lipid bilayers.

The partition of the general anaesthetics, althesin and propanidid, in the non-polar phase of the erythrocyte membrane led to a significant increase in the short range lateral diffusion of DPP in the hydrophobic milieu of the lipid bilayer (Table 2). The values obtained for the excimer/monomer intensity ratio of DPP increased by 32 and 70% in the presence of althesin and propanidid, respectively. The influence of these two anaesthetics on membrane fluidity seemed to parallel their partition coefficients in decane and the erythrocyte membrane (Table 2). On the other hand, the local anaesthetic procaine demonstrated a relatively low partition coefficient in decane but an apparently high partition coefficient in the erythrocyte membrane, which might be due to binding of the positively charged anaesthetic to the negatively charged membrane. Procaine led to a reduction in the fluidity of the erythrocyte membrane. This result is in agreement with the ordering effect which procaine has at relatively low concentrations (10 mM) observed using ESR in lipid films containing a low percentage of cholesterol [19]. In addition, ouabain, a much more water-soluble cardiotonic steroid than althesin and other substances used, had the least influence on the lateral diffusion of DPP within the membrane. It decreased the fluidity of the erythrocyte membrane by 3.5%.

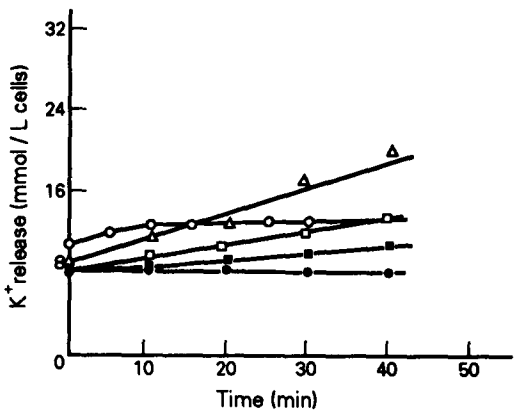

Fig. 1. The effect of anaesthetics and antibiotics on potassium transport. Washed erythrocytes were incubated at 37° with 0 μM (●), 20 mM procaine (■), 1 mM propanidid (□), 20 μM nystatin (○) or 1 mM althesin (△). The rate of K^+ effect was then monitored for 40 min.

The initial rate of potassium ion release from washed erythrocytes was stimulated in the presence of various drugs (Fig. 1). The general anaesthetics, althesin and propanidid, enhanced the initial K^+ efflux by 35 ± 3 and 21 ± 2%, respectively. The local anaesthetic, procaine, caused a very limited increase (9 ± 1%) in the release of K^+, even when used at a much higher concentration than the former two anaesthetics (Fig. 1). In addition, the polyene antibiotic, nystatin, showed a similar stimulatory effect on K^+ release to that demonstrated with amphotericin B [5]. At the concentrations studied,

no significant haemolysis started within 40 min of incubation. The solvents themselves, at the concentrations used to solubilize drugs, were found not to have any effect on potassium ion efflux.

DISCUSSION

The higher fluidizing effect of amphotericin B, compared with that of nystatin, on the non-polar phase of the erythrocyte membrane might be due to the combined effects of its higher partition coefficient and the higher affinity with which it binds [20, 21] membranous cholesterol. The fluidizing effect of both amphotericin B and nystatin may involve the removal of cholesterol, by complexing, from its interaction with neighbouring phospholipid molecules in the bulk lipid phase, which would lead to a reduction in the rigidifying effect [22–26] which cholesterol has on membranes. The disordering effects resulting from cholesterol removal, by amphotericin B and nystatin, might also cause a redistribution of the phospholipid molecules in the lipid annuli of membrane proteins, such as the anion transport protein and the (Na^+,K^+)-ATPase of the erythrocyte membrane, thus leading to an alteration in the fluidity characteristics of the microenvironment of the protein necessary for the conformation and activity it usually assumes. Moreover, there is no evidence for any direct interaction between these antibiotics and any membrane protein [27, 28]. It is very likely therefore that the stimulation of K^+ efflux by amphotericin B observed previously [5] and by nystatin, observed in this study, is due to the combined effects of formation transmembrane channel [2, 4] and perturbation of the fluidity of the hydrophobic milieu of the lipid bilayer, subsequent to channel formation by the antibiotics, which may affect the activity of the (Na^+,K^+)-ATPase. Perturbation of the fluidity characteristics of the erythrocyte membrane, observed in this study, might account at least partially for the inhibitory effect amphotericin B [5] and nystatin [6] have on the anion transport protein. In addition, the general anaesthetics, althesin and propanidid were found to be similar to amphotericin B and nystatin in their fluidizing effect on the erythrocyte membrane. The inhibitory effect on glucose and phosphate transport proteins [12] and the stimulatory effect on K^+ efflux, presumably due to (Na^+,K^+)-ATPase inhibition, of these two general anaesthetics might also be attributed to the observed perturbation in the normal fluidity of the membrane, which is necessary for these transport proteins to assume their usual conformation and activity. This does not exclude, however, the possibility of interaction of these anaesthetics with the less mobile lipid components more adjacent to anion and glucose transport proteins and to the (Na^+,K^+)-ATPase, or of direct interactions between the anaesthetic molecules and hydrophobic regions of the polypeptide chains. Moreover, the observed inhibition of (Ca^{2+},Mg^{2+})-ATPase activity of rat brain synaptosomes [29] and the stimulation of K^+ release across the rat erythrocyte membrane by procaine, presumably due to (Na^+,K^+)-ATPase inhibition, might be closely related to the alteration of membrane fluidity observed in this study. It is very likely, therefore, that the observed alterations in the fluidity of membranes, caused by different ionophoric and anaesthetic substances, contribute to the inhibitory activity on some transport systems in the membranes of red blood cells and possibly other cell types, including nerve tissue.

In contrast, the ionophoric peptide antibiotics, gramicidin A and valinomycin, were found to reduce the fluidity of the non-polar phase of the erythrocyte membrane. This result is supported by results obtained using other physical techniques. Studies of gramicidin A–lipid model membranes, using deuterium NMR spectroscopy [26] and i.r. spectroscopy [25], indicated an ordering effect of low concentrations of gramicidin A at temperatures above the lipid transition temperature. Rice and Oldfield [26] explained their data on gramicidin A by suggesting that the lipid chains adjacent to the polypeptide are constrained in a twisted configuration within the crevices of the surface of the molecule. They proposed that this gramicidin A–lipid complex then presents a smooth cholesterol-like surface to the remainder of the lipids which it then orders.

It thus appears that the direction in which these ionophoric and anaesthetic compounds affect the fluidity of membranes seems to depend not only on the extent to which they partition in non-polar phases, their chemical nature and the configuration or the conformation which they assume in lipid bilayers but also on the type of neighbouring molecules with which they interact. Hence, when considering the use of anaesthetics or ionophores in clinical practice, such as in the treatment of sickle cell anaemia, one should take into consideration the changes they may induce in the physical properties of bio-membranes and the inhibitory effects they may have on the activities of different transport proteins.

Acknowledgements—The author would like to thank H. A. Gambo for his technical assistance. This work was supported by grant No. Bio/1409/26 from the Research Centre of the College of Science, King Saud University.

REFERENCES

1. Reynolds JEF (Ed.), *Martindale—The Extra Pharmacopoeia*, 29th Edn. pp. 417–420. The Pharmaceutical Press, London, 1989.
2. Teerlink T, De Kruijff B and Demel RA, The action of pimaricin, etruscomycin and amphotericin B on liposomes with varying sterol content. *Biochim Biophys Acta* **599**: 484–492, 1980.
3. De Kruijf B, Gerritsen WJ, Oerlemans A, Demel RA and Van Deeness LLM, Polyene antibiotic-sterol interactions in membranes of Acholeplasma Laidlawii cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. *Biochim Biophys Acta* **339**: 30–43, 1974.
4. Van Hoogevest P and De Kruijff B, Effect of amphotericin B on cholesterol-containing liposomes of egg phosphatidylcholine and didocosenoyl phosphatidylcholine: a refinement of the model for the formation of pores by amphotericin B in membranes. *Biochim Biophys Acta* **511**: 397–407, 1978.
5. Abu-Salah KM, Sedrani SH, Tobia AS and Gambo HA, Influence of amphotericin B on the transport of

phosphate, sulphate and potassium ions across the human erythrocyte membrane. *Acta Haem* **79**: 77–80, 1988.
6. Abu-Salah KM, Some effects of nystatin on phosphate and sulphate transport across the membrane of the human erythrocyte. *Med Sci Res* (UK) **12**: 526–527, 1984.
7. Goodall MC, Thickness dependence in the action of gramicidin A on lipid bilayers. *Arch Biochem Biophys* **147**: 120–135, 1971.
8. Pressman BC, Biological applications of ionophores. *Annu Rev Biochem* **45**: 501–530, 1976.
9. Ovchinnikov Yu A, Membrane active complexsones. *FEBS Lett* **44**: 1–21, 1974.
10. Bielawski J and Kwinto B, The influence of gramicidin A and valinomycin on the permeability of mammalian erythrocytes. *Acta Biochem Pol* **22**: 269–277, 1975.
11. Schagina LV, Blasko K, Grinfeldt AE, Korcher YE and Lev AA, Cholesterol-dependent gramicidin A channel inactivation in red blood cell membranes and lipid bilayer membranes. *Biochim Biophys Acta* **978**: 145–150, 1989.
12. Heinemann SH and Sigworth FJ, Estimation of Na^+ dwell time in gramicidin A channel. Na^+ ions as blockers of H^+ currents. *Biochim Biophys Acta* **987**: 8–14, 1989.
13. Abu-Salah KM, Hampton KK and Findlay JBC, The effects of general anaesthetics on glucose and phosphate transport across the membrane of the human erythrocyte. *Biochim Biophys Acta* **688**: 163–168, 1982.
14. Blaustein MP and Goldman DE, Competitive action of calcium and procaine on lobster axon. *J Gen Physiol* **49**: 1043–1063, 1966.
15. Hanahan DJ and Ekholm JE, The preparation of red cell ghosts. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 31, pp. 168–170. Academic Press, New York, 1974.
16. Hirs CHW, Detection of peptides by chemical methods. In: *Methods in Enzymology* (Ed. Hirs CHW), Vol. 11 pp. 325–329. Academic Press, New York, 1967.
17. Storch J and Schachter D, Dietary induction of acyl chain desaturases alters the lipid composition and fluidity of rat hepatocyte plasma membranes. *Biochemistry* **23**: 1165–1170, 1984.
18. Zachariasse KA, Vaz WLC, Sotomayor C and Kuhnle W, Investigation of human erythrocyte ghost membranes with intramolecular excimer probes. *Biochim Biophys Acta* **688**: 323–332, 1982.
19. Buder KW, Schneider H and Smith ICP, The effects of local anaesthetics on lipid multilayers. *Arch Biochem Biophys* **154**: 548–554, 1973.
20. Demel RA, Grombag FJL, Van Deenen LLM and Kinsky SC, Interaction of polyene antibiotics with single and mixed lipid monomolecular layers. *Biochim Biophys Acta* **150**: 1–14, 1968.
21. Norman AW, Demel RA, DeKruyff B, Geurts Van Kessel WSM and Van Deenen LLM, Studies on the biological properties of polyene antibiotics: comparison of other polyenes with filpin in their ability to interact specifically with sterol. *Biochim Biophys Acta* **290**: 1–14, 1972.
22. Cooper RA, Leslie MH, FischKoff S, Shinitzky M and Shattil SJ, Factors influencing the lipid composition and fluidity of red cell membranes *in vitro*: production of red cells possessing more than two cholesterols per phospholipid. *Biochemistry* **17**: 327–333, 1978.
23. Giraud F, Claret M, Bruckdorfer R and Chailley B, The effects of membrane lipid order and cholesterol on the internal and external cationic sites of the Na^+–K^+ pump in erythrocytes. *Biochim Biophys Acta* **647**: 249–258, 1981.
24. Ladbrooke BD and Chapman D, Thermal analysis of lipids, proteins and biological membranes. *Chem Phys Lipids* **3**: 304–356, 1969.
25. Lee DC, Durrani AA and Chapman D, A difference infrared spectroscopic study of gramicidin A, almethecin and bacteriorohodopsin in perdeuterated dimyristoylphosphatidylcholine. *Biochim Biophys Acta* **769**: 49–56, 1984.
26. Rice D and Oldfield E, Deuterium nuclear magnetic resonance studies of the interaction between dimyristoylphosphatidylcholine and gramicidin A. *Biochemistry* **18**: 3272–3279, 1979.
27. Dekruijff B and Demel RA, Polyene antibiotic–sterol interactions in membranes of Acholeplasma laidlawii cells and lecithin liposomes III. Molecular structure of the polyene antibiotic–cholesterol complex. *Biochim Biophys Acta* **339**: 57–70, 1974.
28. Kitajima Y, Sekiya T and Nozowa Y, Freeze-fracture ultrastructural alterations induced by filipin, pimaricin, nystatin and amphotericin B in the plasma membranes of Epidermophyton, Sacharomyces and red blood cells. A proposal of model for polyene-ergosterol complex-induced membrane lesions. *Biochim Biophys Acta* **445**: 452–465, 1976.
29. Garcia-Martin E and Gatierrez-Merino G, Local anaesthetics inhibit (Ca^{2+} + Mg^{2+})-ATPase activity of rat brain synaptosomes. *J Neurochem* **47**: 668–671, 1986.