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Biochimica et Biophysica Acta 1373 (1998) 163–169

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Membrane sterol composition modulates the pore forming activity of syringomycin E in human red blood cells

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Received 18 February 1998; revised 27 May 1998; accepted 5 June 1998

Abstract

The effect of lipopeptide antifungal agent, syringomycin E (SRE) on the membrane permeability of human red blood cells (RBCs) was studied. SRE added to RBCs above a concentration of 2×10^6 molecules/cell (50 μg/ml RBCs) caused a rapid and concentration dependent lysis of a small subpopulation of RBCs; the extent of this lysis remained unchanged as long as 100 min. During this time period the membranes of the unlysed cells had enhanced permeability for ions which was monitored by direct measurement of ^{86}Rb flux. Both the extent of cell lysis and ion transport rate showed linear relationships with SRE concentration demonstrating a random distribution of SRE molecules in red blood cells. The kinetics of the ^{86}Rb efflux suggested pore formation by syringomycin E. The pores had discrete life times and were eventually inactivated. The pores were also a pathway for efflux of monomeric haemoglobin. Alteration of the membrane sterol composition, i.e. depletion of cholesterol by 50% or partial ergosterol substitution of the cholesterol increased the SRE induced membrane permeability for ^{86}Rb by two orders compared to membranes with unaltered sterol composition. This modification of the sterol composition promotes the pore forming activity of this lipopeptide in the membrane.

Keywords: Syringomycin E; Pore formation; Red blood cell membrane; Sterol

1. Introduction

Syringomycin E (SRE) is a cyclic lipodepsipeptide produced by some strains of the phytopathogen, *Pseudomonas syringae* pv. *syringae* [1–4]. Besides contributing to plant diseases [5–9] SRE also exhibits antifungal activities [10,11].

SRE targets plant and fungal plasma membranes. It alters several membrane functions such as ion fluxes, membrane potential [12–14] and the activity of $(K^+ + H^+)$-ATPase [15–17]. SRE-induced lysis was observed with horse and sheep red blood cells [11,18] which was suggested to be related to SRE pore formation in the membrane. Hutchison et al. [18] suggested a colloid osmotic mechanism to explain the lytic effect of SRE in horse red blood cells. Recent molecular genetic studies with *Saccharomyces cerevisiae* indicated that lipids, particularly sterols and sphingolipids are involved in the action of SRE [19–22]. Feigin et al. [23] reported SRE-induced ion

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0005-2736/98/$19.00
PII: S0005-2736(98)00101-1

channels in planar lipid bilayers, and demonstrated that cholesterol affects the sensitivity to SRE by enlarging the energy barrier for channel formation rather than participating in channel formation itself. They found that cholesterol was more effective than ergosterol, the primary sterol of fungal cells. How sterols modulate the activity of SRE in natural cell membranes is not yet known.

In this work studies on the effects of SRE on ion transport kinetics were performed with human red blood cell (RBC) membranes. It is shown that SRE forms pores in the human RBC membrane and its pore forming activity is modulated by cholesterol.

2. Materials and methods

2.1. ^{86}Rb efflux

Human blood from healthy volunteers was stabilized with citrate buffer and stored at 4°C up to 3 days. SRE was purified to homogeneity as described previously [24]. Egg phosphatidylcholine (egg PC), cholesterol and ergosterol were from Sigma. All other chemicals used were of analytical grade.

Blood was loaded with ^{86}Rb for 1.5 h at 37°C. After centrifugation, the RBCs were washed 3 times with buffered salt (BS) solution (in mmol/l): 3.2 KCl, 138 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 27 sucrose, MOPS (pH 6.8) and then resuspended into the same solution. The RBC suspension was chilled to 20°C and maintained at this temperature.

SRE stock solutions (2 mg/ml in 10^{-3} mol/l HCl) were diluted with 6 ml of BS solution. An RBC suspension (14 ml) and the diluted SRE solution were mixed with rigorous stirring. The initial haematocrit values (H) of the RBC suspensions were between 0.3 and 0.4, the syringomycin E concentrations added to the suspensions were in the range of $2–15\times10^6$ molecules/cell (50–375 μg/ml RBCs). The suspension was incubated at 20°C in a shaking water bath. Samples of the suspension were taken at designated time intervals. The samples were centrifuged and the radioactivities of aliquots of the supernatant fluids were measured with a γ scintillation counter (Gamma, Hungary).

^{86}Rb transport was characterized by the amount of tracer found in the supernatant fluids after a time period t and expressed as the percentage of the total activity of the suspension (N_t). The tracer efflux was presented in a N_t versus t curve as well as in a semilogarithmic plot: $-\ln(1-N_t/N_\infty)$ over time, where N_∞ was the percentage of radioactivity in the external medium at the tracer equilibrium state. The slopes of the curves were proportional to the rate constant of efflux of tracer cation. From the rate constant (k), the permeability coefficient of the membrane (p) was calculated using the formula:

$$p = \frac{k\ V_i V_o}{S(V_i + V_o)} \quad (1)$$

where S is the total area of cell membranes, calculated as a product of the number of cells in 1 ml cell suspension (A) and the surface area of one cell (1.63×10^{-6} cm^2) [25], V_o is the extracellular volume of 1 ml cell suspension ($V_o = (1-H)$), and V_i is the intracellular volume of cells ($V_i = A\ 0.68\ V$, where V is the mean cell volume) [26].

RBC concentration, haemoglobin concentration, mean cell volume (V) and haematocrit value (H) were determined using an Automated Hematology Analyzer (Cobas Micros OT 18). The SRE induced haemolysis was calculated as the difference between the RBC concentrations of the untreated (A_0) and SRE-treated (A) RBC suspensions and expressed as a percentage of A_0.

2.2. Alteration of cholesterol content of RBC membrane

2.2.1. Liposome preparation

Liposomes were prepared from a suspension of egg PC (1.35 mg/ml) or a 1:1 (w/w) mixture of egg PC and ergosterol in BS solution by sonication for 1 h at 12°C in an ultrasonic disintegrator (MSE) followed by centrifugation for 30 min at $21\,000\times g$ at 4°C.

Human blood was centrifuged, and the RBCs were washed twice with BS solution and resuspended in the same solution. The suspension of washed RBCs was mixed with liposomes prepared from PC or PC/ergosterol (1:1). The amount of lipid in the liposome was equal to that in RBC membranes. Albumin, 2.75 mg/ml, 625 units/ml penicillin and ^{86}Rb tracer were

added to the suspension before incubation for 20 h at 35°C. The RBCs were separated from liposomes by centrifugation, washed 3 times with BS solution and resuspended to the same solution. The RBC suspension was chilled to 20°C and maintained at this temperature.

2.2.2. Sterol determination in RBC membranes

Lipids were extracted from RBCs [27] and the sterol content was determined by colour reaction as described previously [28]. Briefly, for cholesterol levels, lipid extracts (1 ml) were dried and 1 ml of chloroform, 0.2 ml of acetic anhydride and 0.02 ml of sulfuric acid were added. Samples were incubated in complete darkness for 20 min, and the cholesterol content determined, measuring absorbance at 656 nm in a Perkin-Elmer Lambda 15 UV/VIS spectrophotometer. For determination of ergosterol, the same procedure was applied except that 0.01 ml of bidistilled water was added to the samples before the sulfuric acid addition and the absorbance was measured at 680 nm. This simple modification led to the complete loss of cholesterol-induced colour, while the efficiency of ergosterol-induced colour remained at 95% level.

2.3. Calculation of the concentration of SRE modified cells

Under the effect of SRE there were three populations of cells in the RBC suspension: lysed cells, modified cells, the lysis survived, highly permeable cells having SRE pores in their membranes and unmodified cells, designated their concentrations l, m and u, respectively.

The ^{86}Rb efflux through unmodified cells was negligible, only those tracer ions take part in the efflux which were initially in the modified and lysed cells: I $(m+l)$, where I is the amount of tracer ion in one cell after loading. ^{86}Rb distributed between the extracellular ($V_o = 1-H$) and the intracellular volume of modified cells ($V_i = m$ 0.68 V): $(m+1)$ $I = c_i V_i + c_o V_o$, where c_i and c_o are the intra- and extracellular tracer concentrations. At tracer equilibrium, $c_i = c_o$. The c_o value can be obtained from the activity (cpm) of the sample at the saturation level of the transport: $c_o = \text{cpm}/(1-H)$. Thus, the concentration of SRE modified cells was:

$$m = \frac{c_o(1-H)-lI}{I-0.68Vc_o} \quad (2)$$

3. Results and discussion

SRE addition to human RBCs above a concentration of 2×10^6 molecules/cell caused a lysis of a minor fraction of cells within 2 min. No increase in the lysed fraction size occurred at a particular SRE concentration for as long as 60 min (Fig. 1). The extent of lysis was a linear function of the toxin concentration ranging between 2×10^6 and 1.5×10^7 molecules/cell (Fig. 2).

SRE caused an increase in the ion permeability as monitored by direct measurement of ^{86}Rb (a tracer analog of K^+ ion) efflux on syringomycin E treated RBCs. Fig. 3A shows a typical time course of ^{86}Rb efflux measured for 100 min at a SRE concentration of 6×10^6 molecules/cell. ^{86}Rb efflux increased with time, however, since equilibrium tracer distribution between the intra- and extracellular spaces was not achieved during this time period. A semilogarithmic plot of the ^{86}Rb efflux showed that the results could not be fitted by one exponential curve but by a com-

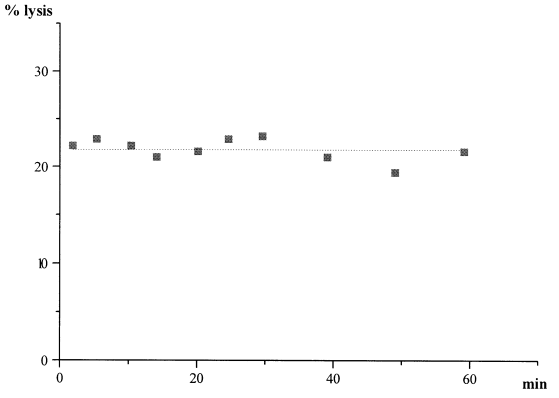

Fig. 1. Time dependence of SRE-induced haemolysis of RBCs suspended in BS solution (in mmol/l: 3.2 KCl, 138 NaCl, 1 CaCl, 1 MgCl, 27 sucrose, 5 MOPS (pH 6.8), temperature 20°C). Lysis was calculated as the difference between the RBC concentrations of SRE-untreated (A_0) and treated (A) RBC suspensions and expressed as the percentage of A_0. SRE concentration: 7×10^6 molecules/cell (175 μg/ml RBCs). SRE was added to RBC suspension at 0 min. Dashed line indicates the mean calculated lysis values.

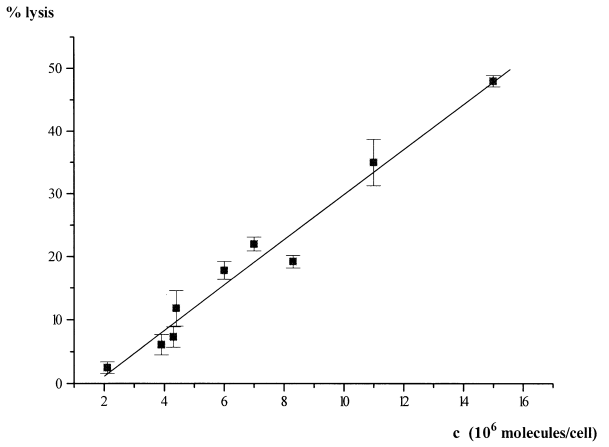

Fig. 2. Concentration dependence of SRE-induced haemolysis. Data points are the mean values of portion of haemolysed cells measured at different time periods within 2–100 min after addition of SRE to the RBC suspension. SRE concentration range: 2–15×10^6 molecules/cell (50–375 μg/ml RBCs). Error bars indicate standard deviations.

bination of at least two curves (Fig. 3B). The second exponent in the kinetic curve might be a result of channel inactivation [29,30]. This finding indicates that the SRE-induced enhanced ion permeability of human RBC membrane is not a result of general leakage produced by this toxic lipopeptide, but rather of the formation of pores, permeable for K and Rb ions. Such pores have discrete life times and are eventually inactivated.

Fig. 4 shows the first exponents of ^{86}Rb efflux kinetic curves obtained at different extracellular syringomycin E concentrations (2×10^6–6×10^6 molecules/cell). The rate constant for ^{86}Rb efflux from untreated RBCs was found to be 1×10^{-4} min^{-1}. With 2×10^6 molecules/cell the rate constant (2.5×10^{-3} min^{-1}) was about 4 times lower than at 6×10^6 molecules/cell (rate constant is 1×10^{-2} min^{-1}). The linear relationships of both the extent of cell lysis and ion transport rate with SRE concentration (Figs. 2 and 4) suggest a random distribution of SRE molecules in red blood cells. Cells with SRE above a critical threshold concentration lysed, while the structural integrity of the remainder of cells was maintained and the syringomycin E formed pores in these latter cells had an increased permeability for ions.

It is significant that the change in the permeability for ^{86}Rb with syringomycin E treatment was relatively small even at a concentration of 6×10^6 molecules/cell, which suggests that relatively few pores are formed by this lipopeptide in the membrane. The tracer transport, that is the free diffusion of ^{86}Rb ions through these few pores is inhibited before getting to the equilibrium tracer distribution by channel inactivation.

A minor portion of RBCs exposed to syringomycin E showed a rapid and concentration dependent lysis; the extent of this lysis remained unchanged as long as 100 min. However, during this time period,

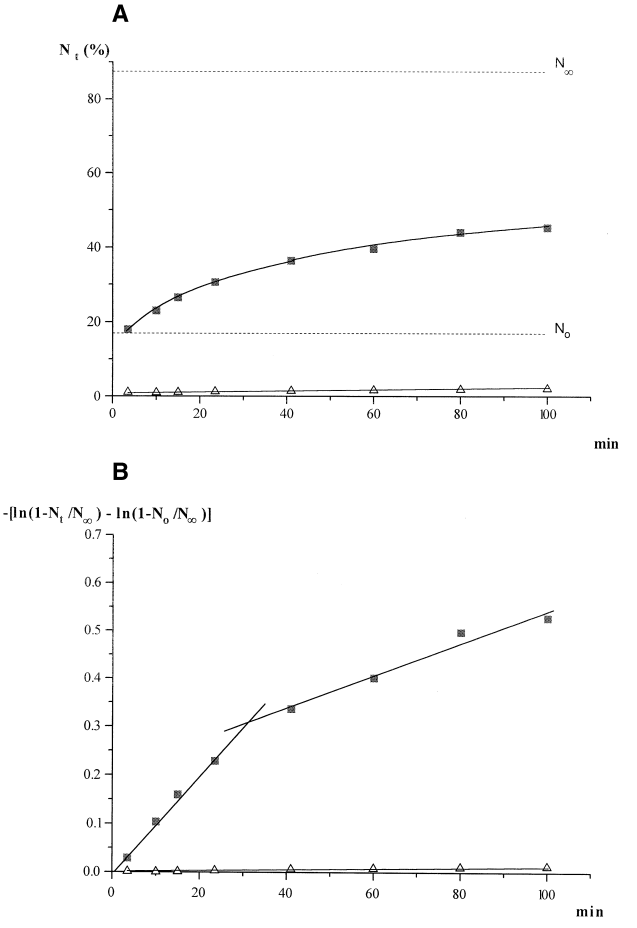

Fig. 3. (A) Effect of SRE on the ^{86}Rb efflux through RBC membranes. RBCs were suspended in BS solution (for composition see Fig. 1). The ordinate gives the amount of radioactive ions effluxed expressed as the percentage of the total activity of the RBC suspension (N_t). N_∞ is the percentage amount of ^{86}Rb in the extracellular solution at tracer equilibrium. N_o is the percentage value of the ^{86}Rb due to lysis and contamination. SRE concentration: △, 0; ■, 6×10^6 molecules/cell (150 μg/ml RBCs). SRE was added at 0 min. (B) Semilogarithmic representation of the kinetic data given in A. N_o is the percentage value of ^{86}Rb due to contamination and lysis.

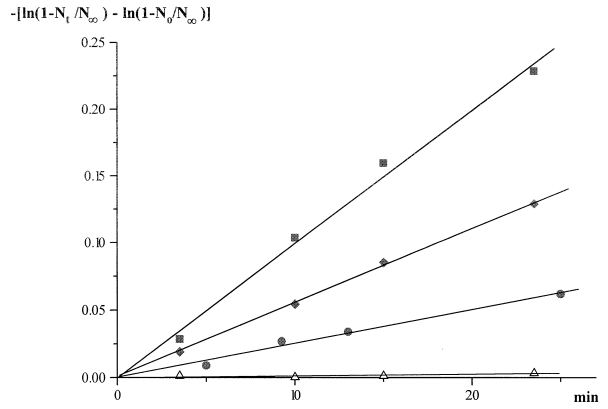

Fig. 4. Concentration dependence of SRE-induced ^{86}Rb efflux through RBC membranes. SRE concentrations in molecules/cell (μg/ml RBCs): △, 0; ●, 2×10^6 (50); ◆, 4×10^6 (100); ■, 6×10^6 (150). SRE was added at 0 min. N_o is the percentage value of ^{86}Rb due to contamination and lysis. For experimental conditions see Fig. 3.

haemoglobin efflux was also observed with the SRE treated RBC membranes (Fig. 5). The time course and kinetics of haemoglobin efflux were similar to that of ^{86}Rb efflux. Hutchison et al. [18] estimated the radius of SRE channels in horse erythrocytes between 0.6 and 1 nm. Similar size 1 nm SRE channel radius was determined in model lipid bilayer membranes [31]. Assuming SRE pores to have a pore radius of about 1 nm, it is suggested that haemoglobin can pass through the SRE induced pores in a monomeric form since the estimated radius of the haemoglobin monomer (assuming a spherical form) is about 1.5 nm [32]. Furthermore, the intracellular monomeric haemoglobin concentration is about 10^{-6} mol/l (for this estimation a tetramer/dimer $K_d = 4\times10^{-6}$ mol/l and a dimer/monomer $K_d = 3\times10^{-9}$ mol/l were taken [33]).

The similar time courses of both ^{86}Rb and haemoglobin effluxes and the long lasting increased permeability of the SRE treated membrane without further cell lysis might indicate that the same pathway was used for both ion and haemoglobin effluxes. We suggest that both pass through the SRE pores by diffusion that does not lead to lysis.

The role of sterols in SRE-induced ion permeability changes was studied. To facilitate these studies, the sterol composition of the RBC membrane was altered. With liposome treatment the RBC membrane sterol composition was either depleted of cholesterol by 50% or 30% replaced by ergosterol. Fig. 6 (curves a, b) show the time courses of ^{86}Rb efflux from these RBCs exposed to SRE at a concentration of 6×10^6 molecules/cell. For comparison the kinetic curve of ^{86}Rb efflux of RBCs with unaltered sterol composition (phospholipid:cholesterol molar ratio 1:0.9) and treated with the same concentration of SRE is shown (curve c in Fig. 6). Sterol composition alteration did not significantly effect the SRE induced RBC lysis, i.e. 23% of the liposome treated RBCs lysed within 2 min after SRE exposure com-

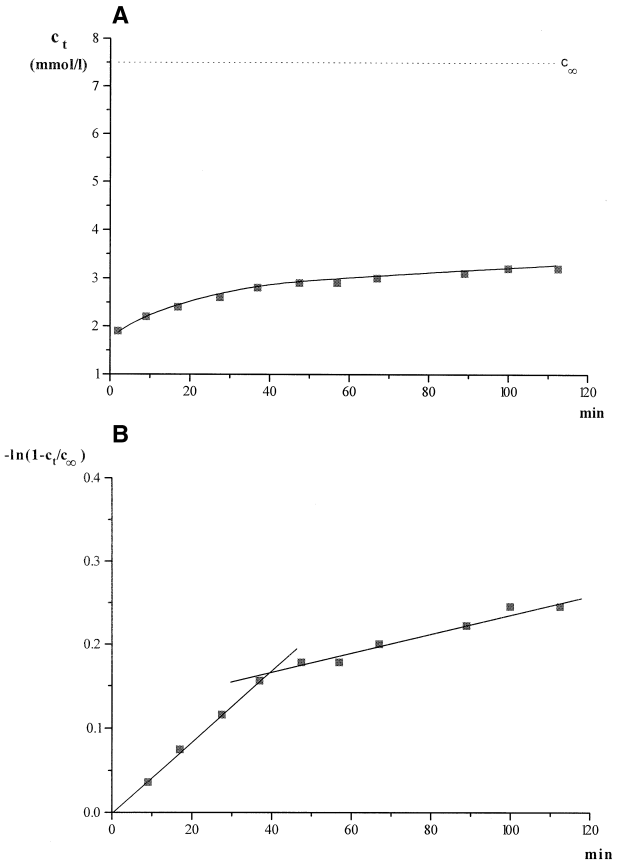

Fig. 5. (A) Time course of SRE-induced haemoglobin efflux through RBC membrane. RBCs were suspended in BS solution (for composition see Fig. 1). The ordinate gives the extracellular haemoglobin concentration. c_∞ is the haemoglobin concentration at equilibrium when haemoglobin is distributed between the lysis survived cells and the extracellular solution of the RBC suspension. The first data point measured at 2 min after SRE addition is the extracellular haemoglobin concentration due to lysis. SRE concentration: 6×10^6 molecules/cell (150 μg/ml RBCs). SRE was added at 0 min. (B) Semilogarithmic representation of the kinetic data given in A. The haemoglobin concentration due to lysis was considered as background and was subtracted.

pared to 18% lysis for RBCs with unaltered sterol composition. However, the ion transport kinetic curves were markedly different. SRE treatment of both cholesterol depleted and the partial ergosterol substituted membranes resulted in a rapid increase in the membrane permeability followed by a cessation of ^{86}Rb efflux that did not achieve an equilibrium tracer distribution. The permeability coefficient for ^{86}Rb was higher by approximately two orders ($p = 5 \times 10^{-7}$ cm/s) with sterol modification compared to unaltered sterol composition ($p = 6 \times 10^{-9}$ cm/s). This observation suggests that the modification of the sterol composition causes structural alterations in the membrane which promote the SRE pore formation in the membrane.

^{86}Rb efflux in the sterol modulated cells ceased within 10–15 min after SRE exposure at a level far from the tracer equilibrium distribution (Fig. 6, curves a, b). Furthermore, when the ^{86}Rb efflux ceased haemoglobin transport was still evident (Fig. 7) with kinetics resembling that found on membranes with unaltered sterol composition (see Fig. 5B). An explanation for these results is that there are three populations of cells: lysed, unmodified and SRE/sterol modified cells with the last having pores in their membranes that allowed ^{86}Rb transport to reach an equilibrium. The proportion of modified cells was

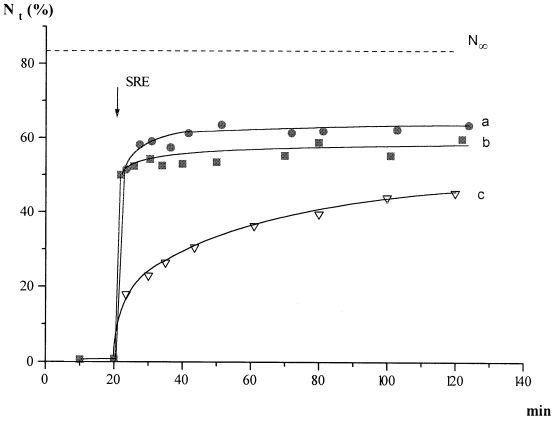

Fig. 6. Effect of sterol composition of RBC membrane on SRE-induced ^{86}Rb efflux. Cholesterol content of the membrane: ●, substituted with ergosterol by 30%; ■, depleted by 50%; ▽, unaltered. The ordinate gives the amount of radioactive ions effluxed expressed as the percentage of the total activity of RBC suspension (N_t). N_∞ is the percentage amount of ^{86}Rb in the extracellular solution at tracer equilibrium. SRE concentration: 6×10^6 molecules/cell (150 μg/ml RBCs). Addition of SRE is indicated by the arrow.

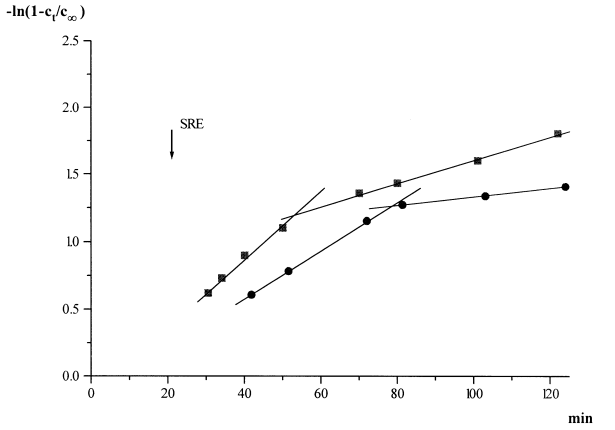

Fig. 7. Effect of sterol composition of RBC membrane on SRE-induced haemoglobin efflux (semilogarithmic representation). Cholesterol content of the membrane: ■, depleted by 50%; ●, substituted with ergosterol by 30%. SRE concentration: 6×10^6 molecules/cell (150 μg/ml RBCs). Addition of SRE is indicated by the arrow.

calculated and found to be dependent on the toxin concentration (at a SRE concentration of 3×10^6 molecules/cell 24% of the cells were modified, at 6x10^6 molecules/cell 37% was modified).

The different transport kinetics obtained for ^{86}Rb ions and for haemoglobin through SRE modified RBC membranes give additional indication of the SRE pore inactivation. The small ^{86}Rb ions diffusing through the pores approach soon the equilibrium tracer distribution between the intracellular space of the modified cells and the extracellular fluid of the RBC suspension. The larger size haemoglobin molecules have lower efflux rate, thus it takes longer time to get to that equilibrium distribution; in the meantime their efflux rate is decreased further by the decreasing number of pores due to pore inactivation. On the other hand the rate of free diffusion is inversely proportional to the size of the solute, thus the ratio of permeability coefficients, p_{Rb}/p_{Hb} should be equal to the ratio of radii of haemoglobin and Rb ion. Taking 1.5 nm as the radius of the haemoglobin monomer and approx. 0.4 nm as that of Rb ion (with one water shell near the ion), the Hb/Rb size ratio is about 4. However, p_{Hb} determined from the first exponent of the kinetic curves for haemoglobin efflux is about 25 times less than that for Rb ion. This discrepancy may be explained by friction of haemoglobin on pore walls, as the pore size is about the size of haemoglobin molecule.

The different ion permeability increasing effects of SRE on the sterol altered and unaltered RBCs shows that reducing the cholesterol content or substituting by ergosterol promotes the pore forming activity of this lipopeptide in the membrane. The permeability of the membrane for ^{86}Rb was two orders higher than that of the membranes with unaltered sterol composition. These results agree with previous observations [23] that the sensitivity of lipid bilayers to SRE decreased in the presence of cholesterol.

Acknowledgements

This research was supported by grants from the Hungarian Ministry of Welfare (ETT, No. 740/93; ETT, No.486/96), the Russian Ministry of Sciences (No. 8f), the Russian Fund for Basic Research (No. 97-04-49623), the Utah Agricultural Experiment Station (UTA 601), and the Willard L. Eccles Foundation.

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