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Sterol specific inactivation of gramicidin A induced membrane cation permeability [1]

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Channel inactivation, a time-dependent decrease of the high-cationic permeability induced by gramicidin A, has been found both in cholesterol containing red blood cell membranes and lipid bilayers (Schagina et al., (1989) Biochim. Biophys. Acta 978, 145–150). The rate of channel inactivation strongly depends on the phospholipid to cholesterol molar ratio of the membrane. The channel inactivation is suggested to be the result of an interaction between gramicidin and cholesterol in a stoichiometry of 1:5. Cholesterol dependent inactivation is shown also for gramicidin A analogs: tryptophan-*N*-formylated gramicidin A, *o*-pyromellitilgramicidin and malonylbisdesformylgramicidin. When cholesterol in the membrane is substituted by sitosterol, the inactivation of gramicidin-induced cation permeability is preserved, while in the presence of either ergosterol or 7-dehydrocholesterol no indication of the channel inactivation is observed. Thus, the structure of the 'B', ring, not the apolar tail of the sterol molecule, appears to be important in the inactivation process.

Introduction

It is well known that cholesterol has a significant effect on the cell membrane ionic permeability [1–3]. The decrease of cholesterol content in the red blood cell (RBC) membranes leads to enhanced passive permeability for K- and Na-ions [4]. Similar effect has been observed for K^+ and H^+ transport of mycoplasma cells [5]. On the other hand, the increase of the cholesterol level of the platelets membrane is followed by diminution of K^+-transport through Ca^{2+} activated K^+-channels [6]. In spite of the numerous experimental observations the mechanism of these cholesterol-dependent permeability changes of the plasma membranes is not clear yet.

The effect of cholesterol has been also shown on ion transport of bilayer lipid membranes (BLM) widely used as relatively simple model system to elucidate the mechanism of complex biological processes. Kasumov et al. [7] obtained a time-dependent ion permeability decrease of the cholesterol containing BLM modified by polyene antibiotics. Chanturia et al. [8] observed reduction of electric conductivity of model BLM modified by α-latrotoxin when the cholesterol content in the membranes was raised. The rate of the conductance decrease was found to be dependent on the cholesterol content.

In our previous paper [9], a new phenomenon has been described: a time-dependent decrease of the high cationic permeability induced by the well characterized channel former gramicidin A [10] both in RBC membranes and cholesterol containing BLMs.

In this paper, we are presenting data on the dependence of the inactivation process on the cholesterol content, both for gramicidin A-treated RBC membranes and artificial BLM, where it is possible to conduct much more detailed studies on the problem. In order to approach the understanding of the nature of the inactivation phenomenon, series of experiments with three gramicidin A analogs are performed and the effects of replacement of cholesterol with some other sterols are also studied.

Materials and Methods

Tracer exchange diffusion measurements on RBCs

Human blood from healthy volunteers stabilized by citrate buffer and stored at 4°C for no longer than 3

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[1] This paper is dedicated to Professor Imre Tarján on the occasion of his 80th birthday.

days was used. The blood was centrifuged and the red blood cells were washed twice. For washing and resuspending the cells, a solution of the following composition (in mmol/l) was used: 130 KCl, 20 NaCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 27.0 sucrose (pH 7). The suspension of washed red blood cells was mixed with liposomes of different lipid compositions in the same salt solution. 2.75 mg/ml albumin and 625 units/ml penicillin were added to the suspension and it was incubated at 30°C for 20 h.

Then, RBCs were separated from liposomes by centrifugation, washed three times and resuspended to obtain a haematocrit value (H) of 0.4. The suspension was put in a shaking bath and one of the antibiotics, gramicidin A or tryptophan-*N*-formylated gramicidin, was added to get 10^{-8} mol/l antibiotic concentration in the solvent of the suspension. This suspension was preincubated for 30 min at 35°C, then cooled down to 22°C and kept at this temperature. Radioactive tracer ^{86}Rb was added to the suspension at given time intervals after the preincubation. Samples of the suspension were taken, centrifuged and the radioactivity of aliquots of the supernatans was measured by the γ-scintillation counter (Gamma, Hungary).

The curves characterizing the cation influx are presented in semilogarithmic plots: $-\ln[(N_t/N_\infty) - 1]$ over time, where N_t is the amount of radioactive ions left in the external medium after a time period t and N_∞ is the amount left at the tracer equilibrium state. The slope of the curve is proportional to the rate coefficient of influx of the tracer cation.

The cholesterol content of the RBC membrane was varied by treatment with liposomes prepared from egg-phosphatidylcholine (egg-PC) or mixture of egg-PC and cholesterol or ergosterol.

Liposomes preparation and sterol content determination in RBC membranes

Liposomes were prepared from a suspension of egg-PC (3 mg/ml) or a 1:1 mixture of egg-PC and cholesterol (1.5 or 3 mg/ml) or ergosterol (1.5 or 3 mg/ml) in the above described salt medium by sonication for 1 h at 12°C in a Ultrasonic disintegrator (MSE), then by centrifugation for 30 min at $21000 \times g$ at 4°C. The amount of lipid in the liposome suspensions was kept equal to that in RBC membranes.

Lipids were extracted from RBCs [11] and the sterol content of the extracts was determined by colour reaction [12]. For that, 1 ml of the extracted lipids was dried and 1 ml of chloroform, 0.2 ml of acetic anhydride and 0.02 ml of sulfuric acid were added to the lipids. Samples were incubated in complete darkness for 20 min, after which their cholesterol content was determined, measuring the absorbance at 660 nm in a Perkin-Elmer Lambda 15 UV/VIS Spectrophotometer. For determination of the ergosterol content in the preparations containing cholesterol as well, 0.02 ml of bidistilled water was added to the samples before the sulfuric acid addition. This simple modification led to a complete loss of the cholesterol-induced colour, while the efficiency of ergosterol-induced colour remained at 95%.

Electric conductance measurements on lipid bilayers

Planar BLM with an area of 10^{-5} cm^2 were formed by the method of Montal-Mueller [13] from ox brain lipids, dioleoylphosphatidylcholine (DOPC) or a mixture of these lipids and sterols. The electrical conductance of the lipid bilayers was determined by a standard reference technique [14] with the aid of the voltage clamp set-up constructed on the basis of a 41 K Analog Device (USA). The measurements were performed at 22°C. The bathing solutions contained 0.1 mol/l RbCl. Appropriate amounts of gramicidin A or one of its analogs (from an ethanolic stock solution) were added to the bathing solution of the BLM to obtain concentrations of the modifier as required and the solution was vigourosly mixed. The final ethanol concentration of the solution never exceeded 1% (v/v).

Ox brain lipids were obtained by the Folch method [11]. Gramicidin A, malonylbisdesformylgramicidin, DOPC, ergosterol, 7-dehydrocholesterol and sitosterol were from Sigma. *o*-pyromellitilgramicidin was a gift of Dr. P. Läuger (Konstanz, Germany), tryptophan-*N*-formylated gramicidin was a gift of Dr. H. Tournois (Utrecht, The Netherlands) and was also synthesized by Dr. A. Sokolova (St. Petersburg, Russia). Formylation of tryptophan residues reached 98% of the total content. All other chemicals used were of analytical grade.

Results and Discussion

The exchange diffusion of $^{86}Rb^+$ ions has been investigated on gramicidin A-treated RBC membranes of different lipid compositions. Fig. 1 shows the time courses of the exchange diffusion of ^{86}Rb. The gramicidin A was added to RBC suspensions to a final concentration of $1 \cdot 10^{-8}$ mol/l in the extra-cellular solution and then the dispersions of RBCs were divided into two portions. The ^{86}Rb tracer was added to the first one 40 min after the addition of gramicidin A, while to the second portion it was added after 120 min. The transport rate of tracer is characterized by the slopes of the kinetic curves. The ^{86}Rb flux registered 40 min after the gramicidin A addition to the RBC suspension with the inherent phospholipid·cholesterol molar ratio of 1:0.9 in the membranes (Fig. 1, curve B) indicated a single exponent diffusion process, but in the case of measurement of tracer exchange diffusion started 120 min after addition of gramicidin A to the

cell suspension, the flux of ^{86}Rb could be represented by a combination of two exponents (Fig. 1, curve B'). The rate coefficient obtained from the first exponent of this curve is about 4-times less than the rate coefficient of the ^{86}Rb exchange diffusion determined for gramicidin A modified RBC membranes after a 40-min preincubation with the antibiotic. When the phospholipid/cholesterol molar ratio of the cell membranes was lowered to 2:1, no change in the transport rates measured at the two subsequent time intervals could be found (Fig. 1, curves A and A'). The tracer achieved the equilibrium distribution between the intra- and extracellular media of the RBCs even when the flux measurement started 120 min after the gramicidin A addition. This observation indicates that no gramicidin channel inactivation appears in the cholesterol depleted RBC membranes. On the contrary, the enhancement of cholesterol content in the RBC membranes leads to more pronounced channel inactivation. As it can be seen from the curve C in Fig. 1 the second exponent indicating the channel inactivation process appeared already in the transport curve obtained after 40 min of the gramicidin addition to the RBC suspension with the phospholipid/cholesterol molar ratio in membranes equals to 1:1.3.

The effect of cholesterol on the gramicidin channel inactivation has been investigated further on cholesterol containing phospholipid bilayer membranes. The curves presented in Fig. 2 show the kinetics of the integral conductance of bilayers formed from DOPC or mixtures of DOPC and cholesterol with different molar

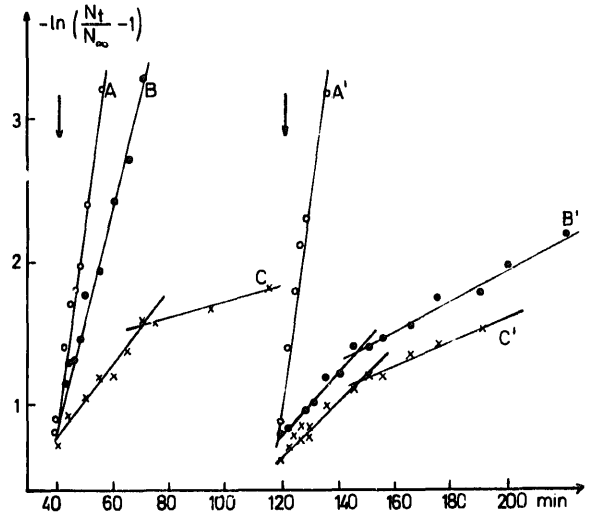

Fig. 1. Time courses of ^{86}Rb influx through gramicidin A-treated RBC membranes with different cholesterol content. The molar ratios of phospholipid/cholesterol in membranes to 2:1 (curves A, A'), 1:0.9 (curves B, B') and 1:1.3 (curves C, C'). Extracellular solution (in mmol/l): 130 KCl, 20 NaCl, 2.5 $CaCl_2$, 1 $MgCl_2$ and 27 sucrose (pH 7); H, 0.4; temperature 20°C; gramicidin A concentration: $1 \cdot 10^{-8}$ mol/l extracellular solution. Arrows show the tracer addition into the RBC suspension.

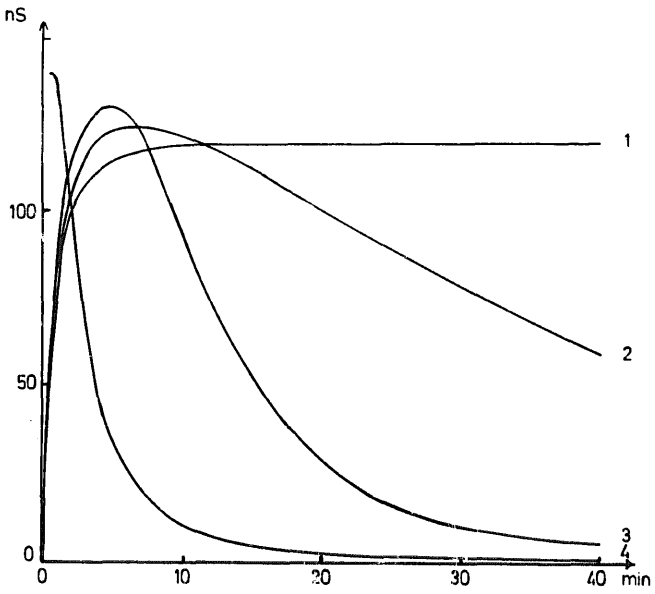

Fig. 2. Time courses of conductivity of gramicidin A-treated bilayers made from DOPC with cholesterol at different proportions. DOPC/cholesterol molar ratios were 1:0 (curve 1), 1:1 (curve 2), 1:2 (curve 3) and 1:3 (curve 4). Gramicidin A concentrations were $4 \cdot 10^{-10}$ mol/l (curves 1, 2, 3) and $2.5 \cdot 10^{-8}$ mol/l (curve 4). Area of BLM, 10^{-5} cm^2. Bathing solution was 0.1 mol/l RbCl. Gramicidin was added to the bathing solution at 0 min (curves 1, 2, 3) or just before BLM formation (curve 4).

ratios. Gramicidin A added to the bathing solution of the bilayer produced a sharp increase of the membrane conductance. In the case of the DOPC bilayer (Fig. 2, curve 1) a stable high-conductance value was registered for at least 40 min after the gramicidin A addition with no sign of the conductance decrease. But the presence of cholesterol in the bilayer caused an exponential conductance decay following the gramicidin induced increase of the membrane conductance (Fig. 2, curves 2–4). The rate of the conductance decrease of gramicidin A modified BLM appeared to be strongly dependent on the phospholipid:cholesterol molar ratio in the bilayer. The smaller the phospholipid:cholesterol molar ratio, the faster the conductance decrease was. In a double-logarithmic representation the relation between the half-time ($t_{1/2}$) of the conductance decay and the molar fraction of cholesterol in the membrane $[C_{cholesterol}/(C_{cholesterol} + C_{DOPC})]$ is obtained as a linear function with a slope of 5.3 (Fig. 3). These kinetic data seem to be formally in agreement with a process of a fifth-order reaction with respect to cholesterol.

In Fig. 4, the $t_{1/2}$ values of the integral conductance decay of the gramicidin A treated bilayers formed from the mixture of ox brain lipids or DOPC and cholesterol at 1:2 molar ratio are shown as a function of gramicidin A concentration. According to the data presented, the value of $t_{1/2}$ does not depend on the species of phospholipid and is constant in a gramicidin A concentration range as wide as 10^{-11}–10^{-7} mol/l.

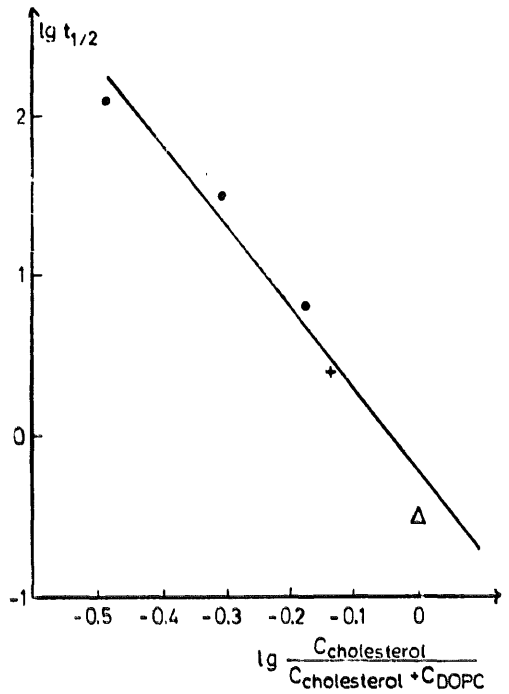

Fig. 3. The dependence of half-time of gramicidin A induced conductance decrease on the molar fraction of cholesterol in bilayer-forming lipid solutions. Gramicidin A concentrations were: 10^{-7} mol/l (△); $2.5 \cdot 10^{-8}$ mol/l (+) and $4 \cdot 10^{-10}$ mol/l (●), respectively.

This finding evidences that the inactivation process with respect to gramicidin A may be considered as a first-order reaction.

Our data on the dependence of inactivation rate constants on the molar fraction of cholesterol in the membrane (see Fig. 3), as well as on the independence of $t_{1/2}$ on the gramicidin A concentration (see Fig. 4), enable us to suggest that, in order to get channel inactivation, 1 gramicidin A molecule should interact with five cholesterol molecules.

Gershfeld and co-workers [15,16] have shown that the structure of the bilayer membranes formed from a mixture of phospholipids and cholesterol is not homogeneous and it strongly depends on its cholesterol content. In the membrane containing more than 33 mol% of cholesterol, there are cholesterol domains and domains containing phospholipids and cholesterol in a molar ratio of 2:1.

On the other hand, in bilayer membranes having small amounts of gramicidin (as it is in our experiments), one gramicidin molecule is surrounded by up to 12 phospholipid molecules [17]. The interaction of one gramicidin molecule with 5 cholesterol molecules, the channel inactivation, is more probable in the cholesterol domains than in the phospholipid-cholesterol domains, since the gramicidin molecules in the latter domains may be protected by the surrounding phospholipid molecules from the interaction with cholesterol and remain active. Gramicidin channels may be formed in both domains. At higher cholesterol/phospolipid ratios, more cholesterol domains are in the bilayer, more channels are in these domains and the inactivation process is more pronounced.

Our findings are in accordance with the results obtained by Gasset et al. [18]. On the base of NMR studies of lipid dispersions containing cholesterol, DOPC and gramicidin A, the authors suggested a preferential gramicidin A–cholesterol interaction at and above 25°C.

Thus, assuming that a gramicidin A-cholesterol interaction is responsible for the channel inactivation, the question arises as to what the nature of this interaction is. In order to answer it, studies were carried out with some analogs of gramicidin and different sterols.

One may suppose that the result of gramicidin A–cholesterol interaction is a shift in dimer-monomer equilibrium of the gramicidin A molecules. In accordance with the widely accepted Urry model of conducting $\beta^{6,3}$ helical gramicidin dimer [19], such a shift may result in a decreasing number of acting gramicidin channels. But in conductance measurements performed on bilayer membranes formed from DOPC/cholesterol at a molar ratio of 1:2 and modified by malonylbisdesformylgramicidin, a covalently stabilized gramicidin A dimer which could not dissociate to monomers showed that in this case the inactivation process still existed with a rate which was only 2-times smaller than for gramicidin A (Table I). This finding excludes a casual role of dissociation in channel inactivation in the membrane.

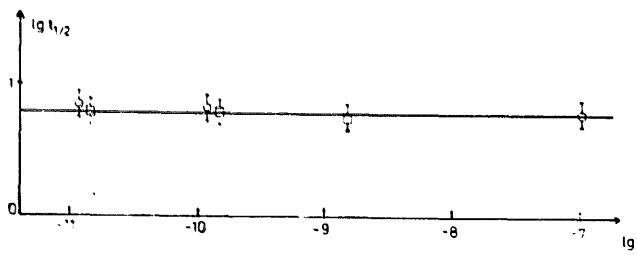

Fig. 4. The dependence of half-time of bilayer conductance decay on gramicidin A concentration. Bilayers were made from mixtures of either DOPC (□) or ox brain lipids (○) with cholesterol. The phospholipid/cholesterol molar ratio equal to 1:2.

TABLE I

Half-time of channel conductance decay in BLM prepared from DOPC and cholesterol in a molar ratio of 1:2 and modified by different gramicidin A analogs

Modifier	$t_{1/2}$ (min)
Gramicidin A	6.4 ± 0.4
Malonylbisdesformylgramicidin	14.3 ± 1.1
Tryptophan-*N*-formylated gramicidin	1.8 ± 0.2
o-Pyromellitilgramicidin	5.3 ± 0.4

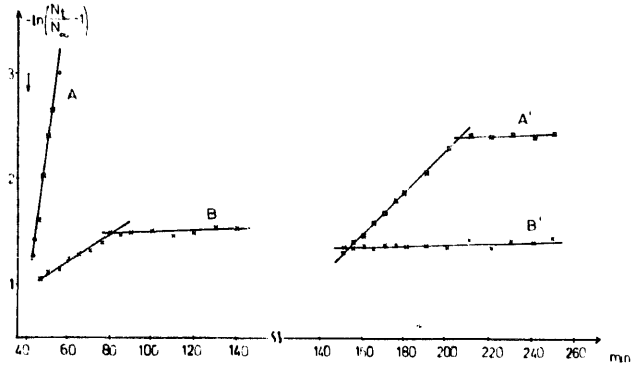

Fig. 5. Time-courses of ^{86}Rb influx through RBC membranes with inherent cholesterol content treated by gramicidin A (curves A, A') and tryptophan-*N*-formylated gramicidin (curves B, B'). Arrows show the tracer addition into the RBC suspension.

It is known that the tryptophan residues of gramicidin molecules appear to play an important role in the cation-conducting properties of the gramicidin channels [10]. When the DOPC bilayer is modified by a tryptophan-*N*-formylated gramicidin a 10-times less single-channel conductance is recorded than on a gramicidin A modified bilayer [20]. Similarly, the ultraviolet photolysis of tryptophan residues in the gramicidin A molecule [21,22] leads to a diminished channel conductivity. Special experiments have been made with tryptophan-*N*-formylated gramicidin on RBC membranes with a inherent phospholipid/cholesterol molar ratio and on cholesterol containing phospholipid bilayers. The kinetic curves of ^{86}Rb exchange diffusion after a 40-min and a 140-min preincubation of the RBC suspension with tryptophan-*N*-formylated gramicidin and with gramicidin A are presented in Fig. 5. The low transport rate of the exchange diffusion of ^{86}Rb ions, as well as the presence of the second exponent in the kinetic curve in about 2 h after addition of the gramicidin analog to the cell suspension, may be considered as an indication for the induction of the less permeable ionic channels possessing a faster inactivation process in comparison to the case of gramicidin A modified RBC membranes.

Inactivation of tryptophan-*N*-formylated gramicidin induced cation permeability was also observed on BLM formed from a mixture of DOPC and cholesterol in a molar ratio of 1:2. The half-time of the conductance decay was found to be 1.8 min. This value is about 3-times less than that found for gramicidin A modified bilayer membranes prepared from the same lipid mixture ($t_{1/2} = 6.4$ min, Table I).

The third gramicidin analog studied was *o*-pyromellitilgramicidin, containing three negative charges on the C-end of the molecule. It was shown that this analog also possesses cholesterol-dependent inactivation. For the membranes made of DOPC with cholesterol in molar ratio 1:2, the inactivation observed with the $t_{1/2}$ was 5.3 min (Table I).

TABLE II

Half-time of channel conductance decay in gramicidin A modified BLM prepared from DOPC and sterol in a molar ratio of 1:3

Sterol	$t_{1/2}$ (min)
Cholesterol	2.4 ± 0.2
Sitosterol	2.6 ± 0.3
Ergosterol	∞ [a]
7-Dehydrocholesterol	∞ [a]

[a] No inactivation.

Since the three gramicidin analogs modified in different parts of the molecule showed cholesterol-dependent inactivation process only with some variations in the $t_{1/2}$ values, in the next experiments the action of some analogs of the other interacting molecule, cholesterol, was studied. In particular 7-dehydrocholesterol, which is quite similar to cholesterol except for the presence of a second double bound in the 'B' ring, sitosterol which differs from cholesterol in the branching of its hydrophobic tail and ergosterol, which has additional double bounds, both in the 'B' ring and in the branched tail.

As it can be seen from the data of Table II, the substitution of cholesterol by sitosterol does not influence the channel inactivation. Very similar electric conductance decays have been observed both on the DOPC + cholesterol and on the DOPC + sitosterol BLMs, whereas the channel inactivation is abolished completely by substituting cholesterol with either 7-dehydrocholesterol or ergosterol. Similarly, no channel inactivation has been found on RBC membranes when the cholesterol in the membrane is partially substituted by ergosterol (Fig. 6). These results evidence that pres-

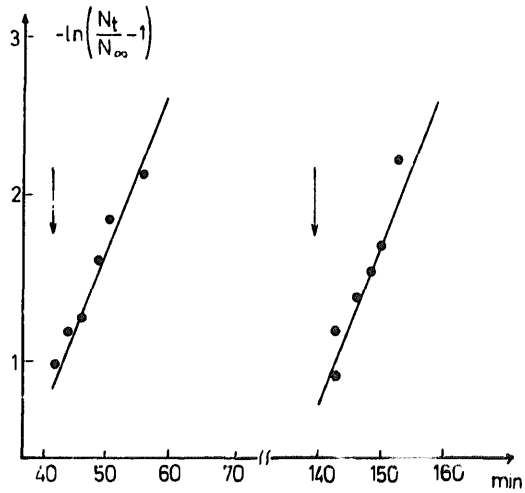

Fig. 6. Time-courses of ^{86}Rb influx through RBC membranes when 36% of RBC cholesterol was substituted by ergosterol. Arrows show the tracer addition into the suspension.

ence of the second double bound in the 'B' ring of the sterol, but not the tail structure, preserves the development of the inactivation process.

From the results obtained it is possible to suggest that the inactivation of gramicidin-induced ion permeability is the result of the interaction between cholesterol and the gramicidin channel and that the 'B' ring structure of the sterol molecule has an important role in this process.

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