Biochimica et Biophysica Acta, 945 (1988) 335–349
Elsevier

BBA 74188

Antibodies affect the ionic conductance of channels formed by amphotericin B in a lipid bilayer

Oleg V. Kolomytkin, Juri A. Mantzyghin, Natalya V. Swyatukhina

Institute of Biological Physics, Academy of Sciences of the USSR, Pushchino (U.S.S.R.)

(Received 22 December 1987)
(Revised manuscript received 28 July 1988)

Key words: Ion channel; Amphotericin B; Monoclonal antibody; Lipid membrane

For the first time poly- and monoclonal antibodies (class IgM) against the polyene antibiotic amphotericin B were obtained affecting the properties of a channel formed by the antibiotic and cholesterol in a lipid bilayer when amphotericin B was added to the solution at one (cis) side of the membrane. In the case of the symmetric distribution of cholesterol in the lipid bilayer, three molecules of monoclonal antibodies bind firmly to the channel at the trans-side of the membrane, thus strongly increasing the mean lifetime of the channel in the open state, and not changing practically the ion conductance of its open state. The antibodies did not alter the properties of these channels when added at the cis-side of the membrane as well as of the channels formed in the lipid bilayer when amphotericin B was added at both membrane sides. The antibodies obtained did not affect the conductance of channels in which amphotericin B and cholesterol were replaced with their analogs levorin and 5α-androstan-3β-one, which points to a high specificity of the immunoglobulins isolated. When cholesterol was present only in the cis-monolayer of the lipid bilayer and was absent in the trans-monolayer, the same monoclonal antibodies when added at the trans-side of the membrane blocked the conductance of the channel formed by adding the antibiotic to the solution at the cis-side of the bilayer. The obtained evidence is of interest in elucidating the general features of interaction of antibodies with the ionic channels of cellular and model membranes.

Introduction

Antibodies are a promising tool for studying and isolating ion channels of cellular membranes. However, such studies are difficult because the mechanism of antibody-channel interaction as well as the structure of cell membrane channels have not yet been understood enough. The investigation of this mechanism can be facilitated by the study of the action of antibodies on an ion channel with the well known structure. The ionic pore formed by the polyene antibiotic amphotericin B and cholesterol in a bimolecular lipid membrane was chosen to be such a channel.

The structure and properties of such a channel have been described in detail in Refs. 1–6. According to currently available conceptions, adding the antibiotic at one (cis) side of the lipid bilayer induces formation of 'halfpores' in the cis-monolayer of the membrane. The 'halfpore' can pierce the membrane for a short time to form an ion channel, its orientation with respect to membrane surface being not changed. It is believed that this channel is formed by eight molecules of amphotericin B and eight molecules of cholesterol

Correspondence: O.V. Kolomytkin, Institute of Biological Physics, Academy of Sciences of the U.S.S.R., Pushchino 142292, U.S.S.R.

both oriented normally to the membrane surface. Sterol molecules are situated between the antibiotic molecules. The cylindrical 'halfpore' has a diameter of about 0.8 nm lined with the hydrophilic groups of the amphotericin B lactone ring. The charged groups of the antibiotic (the carboxyl at C-16 and the amino sugar at C-19 as well as the 3-OH group of sterol) are in contact with the water from the cis-side of the membrane. At the same time, the OH group of the antibiotic at C-35 and the hydrocarbon chain of cholesterol at C-17 are situated at the trans-end of the 'halfpore'.

If amphotericin B is added at both sides of the lipid bilayer, the amphotericin B and cholesterol molecules form ionic channels which consist of two 'halfpores' occurring in the opposite monolayers of the bilayer and joined together by hydrogen bonds.

The knowledge of the structure of the amphotericin channel permits the mechanism of its interaction with antibodies to be studied in detail.

The tasks of the present study were: (1) to obtain antibodies against amphotericin B, (2) to define their class, (3) to estimate their effect on the amphotericin B channel in a lipid membrane, (4) to define localization of the antigen determinants on the channel, (5) to determine the specificity of the antibodies, (6) to estimate the stoichiometric channel-antibody ratio as well as to reveal: (7) whether one and the same antibody can increase or block the ion conductance of the channel depending on cholesterol distribution in the membrane.

Materials and Methods

Chemicals. Polyene antibiotics used are as follows: amphotericin B, nystatin and levorin from the Leningrad Institute of antibiotics, roflamycoin isolated by Dr. R. Schlegel et al. [7,8]; cholesterol from Serva, ergosterol and 5α-androstan-3β-one generously provided by Dr. De Hier; complete Freund's adjuvant from Sigma; class-specific antibodies from Sigma.

The fraction of bovine brain lipids containing 20% (w/w) cholesterol was obtained by the method described in Ref. 9, the fraction of bovine brain phospholipids by removing neutral lipids from the lipid fraction by means of resedimentation in acetone [10]. Asolectin was isolated by Dr. E.Ya. Kostetsky. The absence of cholesterol in the brain phospholipid fraction and in asolectin was testified biochemically by the method described in Ref. 11.

Preparation of polyclonal antibodies against amphotericin B. In order to reveal if it was possible to obtain antibodies changing the properties of the amphotericin B channels, rabbits (males of New Zealand strain, 2 kg weight) were immunized by a suspension of amphotericin B and lipids. The suspension was prepared as follows: amphotericin B up to a concentration of 2 mM and brain lipids up to a concentration of 40 g/l were added to 0.5 ml water solution of 0.15 M NaCl/5 mM Tris-HCl (pH 7.2). The obtained mixture was sonicated for 15 min by a UZDN-1 ultrasonic disperser (22 kHz, 50 W/cm^2). Then 0.5 ml of the complete Freund's adjuvant was added to the mixture under thorough stirring and the obtained suspension was injected to rabbits three times intraperitoneally and ones into lymphatic nodes. The interval between injections was two weeks. One week after the last injection 50 ml of blood was taken from each rabbit, water-soluble immunoglobulins were extracted by precipitation with ammonium sulphate [12].

Preparation of monoclonal antibodies. In order to define the class and specificity of immunoglobulins we prepared monoclonal antibodies against amphotericin B. Hybridomas were obtained according to the conventional technique [13]. Females of BALB/c mice were immunized with freshly-prepared liposomes with incorporated amphotericin B mixed with complete Freund's adjuvant (1 : 1, v/v) according to the above scheme. Each injection contained 20 μg of amphotericin B. Three days after the last injection the spleen cells were fused with myeloma Sp2 cells using polyethylene glycol (mol. wt. 1550 'Serva', the time of treatment with 50% (w/v) polyethylene glycol solution was 90 s). After fusion the cells were grown in a selective HAT medium. The hybridoma cells were cultured in Dulbecco's modified Eagle's medium (Serva) with addition of 10% fetal bovine serum (Serva, Gibco) and antibiotics.

The primary (mixed) clones were tested in 10 to 14 days with regard to the ability of antibodies

isolated from cellular supernatants to change membrane electroconductance in the presence of amphotericin B in the solution at one side of the lipid membrane. Among 50 primary clones, four were found to be positive. Subsequent recloning was carried out using mouse peritoneal macrophages by the limiting dilution method. In this way three monoclones were selected, whose antibodies increased the integral conductance of the membrane. However, the concentrations of these antibodies were taken different to produce an equal effect. In the present study one of these monoclones (HAB4) was used.

An antibody-linked immunosorbent assay for class-specific antibody activity showed that the class of the antibodies used is IgM [13]. The characterization of the antibodies will be given below.

Membrane formation. The Teflon cell had two compartments separated by a lavsan film with an aperture for the membrane. The diameter of the aperture was 100 μm. Membranes were assembled by Montal-Mueler's technique from two lipid monolayers [14].

Symmetric and asymmetric bilayers were used. The lipid composition of the membranes is given in figure legends. Asolectin and phospholipids from bovine brain were used for membrane formation. The results were analogous for both phospholipid types though they were essentially dependent on the distribution of cholesterol in the bilayer.

Asymmetric membranes were assembled from two different monolayers. The cis-monolayer was prepared from lipids containing 5% of cholesterol, the trans-monolayer from cholesterol-free phospholipids. A control experiment was performed to prove that the trans-monolayer of the bilayer thus prepared did not practically contain cholesterol either. The conductancies of two membranes were compared. The conductance of the asymmetric bilayer in the presence of 10 nM amphotericin B in the cis- and in the trans-compartments was low (approx. 1 pS (0.1 M KCl)) throughout the time of observation (30 min). The conductance of the symmetrical membrane assembled of two identical monolayers containing 2.5% cholesterol in the presence of 10 nM amphotericin B at the cis-compartment was low (approx. 1 pS) but increased up to approx. 1 nS (0.1 M KCl) after addition of 10 nM amphotericin B to the trans-compartment.

The high conductance of the latter membrane is explained by multiple channels consisting of two 'halfpores' situated in the opposite monolayers of the bilayer and joined with each other by hydrogen bonds. Each 'halfpore' is composed of eight amphotericin B and eight cholesterol molecules. Formation of the 'halfpores' was possible in both monolayers of the membrane because cholesterol was present in both monolayers as well. The low conductance of the first membrane denotes that there were practicaly no 'halfpores' in the trans-monolayer of the membrane. The fact that 'halfpores' were not formed indicates that cholesterol in the trans-monolayer of the membrane was practically absent. Therefore, under conditions of our experiment the cholesterol flip-flop in the membrane was not rapid and did not destroy the asymmetry of the bilayer for at least 30 min after membrane formation.

The transmembrane current was measured by a Keithley 301 electrometer amplifier. The membrane voltage was provided by an external source. The voltage sign in all figures is given for the cis-compartment in which amphotericin B was present. The trans-compartment contained no amphotericin B. The specific capacity of all bimolecular lipid membranes used was 9 ± 1 mF/m^2. The electroconductance of nonmodified lipid bilayers was below 1 pS.

All solutions in the measuring cell contained 0.1 M KCl/5 mM Tris-HCl (pH 7.4), 22°C. A peristaltic pump (LKB) was used to exchange the solution in the cell. This procedure did not destroy the membrane.

Effect of immunoglobulins on the lipid membrane. Control experiments showed that antibodies when added to one or both compartments up to a concentration of 1 g/l did not increase the conductance of nonmodified bilayers of the composition used.

Results

In order to find out how immunoglobulins influence the electroconductance of channels formed by one or two amphotericin B 'halfpores', the following experiments were performed.

Effect of specific antibodies on ionic channels formed by adding amphotericin B at two membrane sides

Bilayer membranes were formed from bovine brain lipids and 10 nM amphotericin B was added at both membrane sides. The membrane conductance increased by several orders of magnitude, which was due to formation of ion channels by two amphotericin B 'halfpores' in the lipid bilayer. Then the solutions in both compartments were replaced with amphotericin B-free ones. The membrane conductance remained practically the same. This indicates that the ion channels consisting of two 'halfpores' were firmly incorporated into the lipid bilayer. Subsequent addition of poly- and monoclonal antibodies (0.1 g/l) first at the one side, then at the other side of the membrane had no effect on the membrane conductance (measuring error ±1%) throughout the observation time (30 min).

Thus, the antibodies do not affect the electroconductance of ion channels formed by adding amphotericin B on two membrane sides.

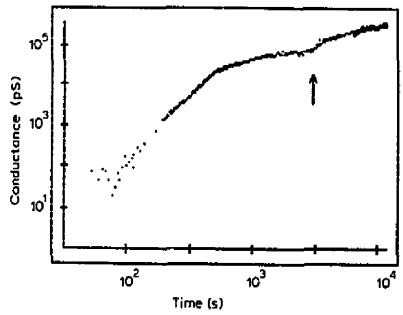

Fig. 1. Effect of monoclonal specific antibodies on the membrane conductance in the presence of 1 μM amphotericin B at the cis-side of a symmetric lipid bilayer. After adding amphotericin B to the cell, the membrane was allowed to stand about 20 min until the conductance reached a stationary level and then antibodies (0.7 mg/l) were added to the trans-compartment of the cell at a zero-time moment. The ordinate is the conductance increment after antibody addition. The arrow shows the time moment of the second addition of the same quantity of antibodies. The symmetric bilayer membrane was formed of bovine brain lipids containing 20% (w/w) cholesterol. The membrane voltage was 25 mV. Aqueous solution of 0.1 M KCl/5 mM Tris-HCl was in the cell.

Effect of specific antibodies on the integral conductance of a symmetric lipid bilayer with ion channels formed by adding amphotericin B at one membrane side

Ion channels were formed by adding 1 μM amphotericin B at the cis-side of the membrane only. In the cis-monolayer of the membrane, amphotericin B and cholesterol molecules formed a great number of 'halfpores' which were ion channels. It was naturally to expect that antibodies when added at the cis-side of the membrane would not affect the conductance of 'halfpores', since the orientation of 'halfpores' in the membrane did not practically change and antibodies had no effect on the conductance of ion channels formed by two joined 'halfpores'. This assumption was supported experimentally as well. Addition of poly- and monoclonal antibodies at concentrations of 0.125 and 0.02 g/l, respectively, to the cis-compartment of the cell had no effect on the current-voltage characteristics of the membrane throughout the observation (20 min).

Let us consider the changes in amphotericin B 'halfpore' conductance on addition of antibodies to the trans-compartment. Figs. 1–3 show the effect of antibodies added to that compartment on the integral current and current-voltage characteristics of the membrane in the presence of great amounts of amphotericin B in the cis-compartment. Addition of antibodies at the trans-side of the membrane caused an irreversible increase in membrane conductance by several orders of magnitude; after removal of antibodies and amphotericin B from the cell, the conductance remained practically unchanged throughout the observation time (10 min). These effects were qualitatively the same for both poly- and monoclonal antibodies.

Measurements of the integral membrane conductance at amphotericin B concentrations below 2 μM and concentrations of monoclonal antibodies [mAb] higher than 0.7 mg/l are presented in Figs. 1 and 2. It follows from Figs. 1 and 2 that for the initial time period after addition of specific monoclonal antibodies the conductance increment is well approximated by a function:

$$G - g_0 \sim (g_0)^{\alpha} \cdot [\mathrm{mAb}]^{\beta} \cdot t^{\gamma}$$

where g_0 is the membrane conductance before antibody addition, t is the time after antibody addition, G is the membrane conductance at the

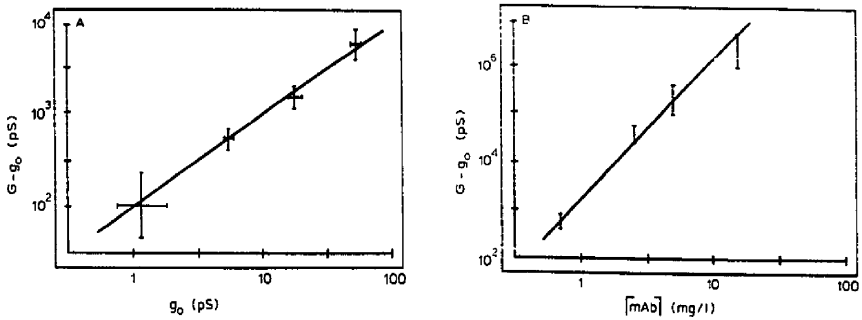

Fig. 2. Conductance of a lipid bilayer under various concentrations of amphotericin B and monoclonal antibodies in the cell. After adding amphotericin B to the cis-compartment, the membrane was allowed to stand about 20 min until the conductance reached a stationary level (g_0) and then antibodies were added to the trans-compartment. Conductance (G) was measured 2.5 min after addition of the antibodies. A symmetric bilayer was formed of bovine brain lipids containing 20% (w/w) cholesterol. The membrane voltage was 25 mV. Aqueous solution of 0.1 M KCl/5 mM Tris-HCl was in the cell. (A) Dependence of $(G - g_0)$ on (g_0). Monoclonal antibodies were added to the trans-compartment up to a concentration of 0.7 mg/l. (B) $(G - g_0)$ versus concentration of monoclonal antibodies in the trans-compartment [mAb]. $g_0 = 5.5 \pm 2$ pS.

time moments t. $\alpha = 1 \pm 0.2$, $\beta = 3 \pm 0.6$, $\gamma = 3 \pm 0.6$.

Characterization of antibodies

A question arises: do antibodies really induce an increase in membrane conductance or is the observed increase due to any minor substances present in the preparation used? To clarify the question, class-specific antibodies were used. The preparation of the monoclonal antibodies was incubated with class-specific antibodies for 2 h. Then the incubated mixture was added at the trans-side of the membrane in the presence of 1

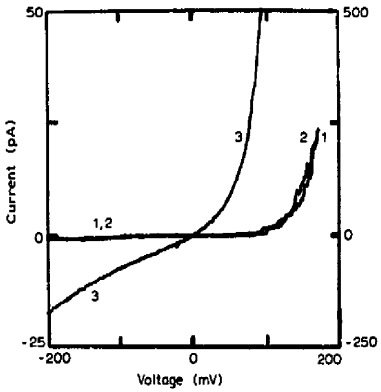

Fig. 3. Effect of antibodies on the current-voltage characteristics of a symmetric lipid bilayer formed of brain phospholipids (95% w/w) and cholesterol (5% w/w); (1) in the presence of 1 μM amphotericin B at the cis-side of the membrane; (2) 20 min after adding nonspecific immunoglobulins (100 mg/l) to the trans-compartment of the cell; (3) 20 min after adding specific polyclonal antibodies (25 mg/l) to the trans-compartment. Aqueous solution of 0.1 M KCl/5 mM Tris-HCl was in the cell. The membrane voltage increased at a rate of 5 mV/s. The current scale for curves 1, 2 is shown on the left, for curve 3 on the right.

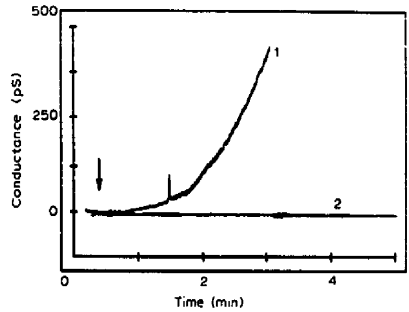

Fig. 4. Effect of monoclonal antibodies preincubated with class-specific antibodies on membrane conductance in the presence of 1 μM amphotericin B at the cis-side of the symmetric lipid bilayer. (1) 1.4 μg of monoclonal antibodies were preincubated for 2 h with 4 μg of anti-IgG in 100 μl of aqueous solution. (2) 1.4 μg of monoclonal antibodies were preincubated for 2 h with 4 μg of anti-IgM in 100 μl of aqueous solution. The arrow shows the time moment of addition of the preincubated mixture to the trans-compartment. The symmetric bilayer membrane was formed of bovine brain lipids containing 20% (w/w) cholesterol. The membrane voltage was 25 mV. Aqueous solution of 0.1 M KCl/5 mM Tris-HCl was in the cell.

μM amphotericin B in the cis-compartment. If the monoclonal antibodies were incubated with antibodies specific to the IgM class then the membrane conductance was low throughout the observation time (1 h). If the monoclonal antibodies were incubated with antibodies specific to other classes of immunoglobulins, the membrane conductance increased after addition of the incubated mixture to the membrane (Fig. 4). Therefore we can suppose that the increase in membrane conductance in Fig. 1 is due to monoclonal antibodies of the IgM class rather than due to other minor substances in the preparation.

In order to obtain another independent argument that amphotericin B and the prepared specific monoclonal antibodies tightly bind to each other on a lipid membrane the next experiment was performed. Lipids (18 g/l asolectin and 2 g/l cholesterol) were added to a solution containing 5 g/l monoclonal antibodies. The liposomes were prepared by means of sonication of the mixture using an ultrosonic disperser (22 kHz, 50 W/cm^2). Thus the antibodies were present inside and outside the liposomes. The antibody concentration outside liposomes was decreased 10^4-times by concentrating the liposome suspension on a filter with 0.1 μm pores (Millipore) and subsequent dilution of the suspension by a physiological solution.

The obtained liposomes containing antibodies inside vesicles were incubated with 10 nM amphotericin B for one hour.

The presence in the water solution of free amphothericin B molecules not bound with liposomes was testified by means of a lipid bilayer. First liposomes were removed from the solution. For that purpose the suspension was filtered through a XM50 Amicon filter. The filtrate was introduced into the cell where the lipid membrane was formed. The conductance of such membranes coincided with the conductance of a lipid bilayer without amphotericin B (approx. 1 pS). The low conductance of the membrane meant that the antibiotic concentration in the aqueous solution was essentially less than 10 nM [1,2].

In order to be certain that the low amphotericin B concentration was not due to binding of the antibiotic to the antibodies in the aqueous solution outside liposomes a control experiment was performed. A physiological solution containing 10 nM amphotericin B and 1 mg/l monoclonal antibodies was incubated without liposomes for 4 h. Then the procedure discribed above was performed. In this case the membrane conductance was 4 ± 3 nS. The high conductance meant that the ion channels in the bilayer were formed by free amphotericin B molecules presented in the aqueous solution. The presence of amphotericin B in the solution can be explained assuming that the binding of the antibiotic with the antibodies in the aqueous solution is absent. Therefore it can be supposed that amphotericin B is tightly bound with liposomes containing specific antibodies.

In the other control experiment liposomes containing immunoglobulins isolated from a nonimmunised rabbit were used instead of those containing specific antibodies. In this case the membrane conductance was 7 ± 4 nS. The high conductance can be explained if we suppose that the liposomes containing nonspecific immunoglobulins do not bind amphotericin B. Therefore amphotericin B and the obtained monoclonal antibodies specifically bind together on a lipid membrane.

The observed effects prove unambiguously that antibodies are firmly bound at the trans-side with the 'halfpores' formed by amphotericin B and cholesterol molecules in the lipid membrane.

Effect of nonspecific immunoglobins on the integral conductance of ion channels formed by amphotericin B in a symmetric lipid bilayer

Ionic channels were formed by adding 1 μM amphotericin B at the cis-side of the membrane only. In the cis-monolayer of the membrane, amphotericin B and cholesterol molecules formed a great number of 'halfpores' which were ion channels. The control experiment showed that addition of nonspecific immunoglobulins isolated from a nonimmunised rabbit to the trans-compartment did not change the current-voltage characteristics of the membrane throughout the observation time of 20 min (Fig. 3, curves 1 and 2). Another control experiment showed that addition of monoclonal antibodies of the IgM class against the histocompatibility antigen to the trans-compartment at a concentration of 10 mg/l did not increase the membrane conductance. The evidence

obtained points to the high specificity of antibodies isolated from animals immunised by amphotericin B.

The action of specific monoclonal antibodies on a single channel formed by adding amphotericin B on one membrane side

It is believed that an ion channel formed by a 'halfpore' is in the open state at moments when the 'halfpore' pierces the bilayer. If the 'halfpore' is situated entirely in the cis-monolayer at the membrane the channel is closed [3,5]. Proceeding from the abovesaid and considering that the interaction of antibodies with the 'halfpore' increases significantly the membrane conductance, it may be assumed that the interaction of antibodies with the 'halfpore' at the trans-side of the membrane increases the probability for the 'halfpore' to pierce the bilayer. This process must be responsible for the conductance increase.

In order to check this assumption, the effect of antibodies on a single channel was studied. Fig. 5a shows the record of jump-like changes in membrane conductance at a small amphotericin B concentration (10 nM) at the cis-side of the lipid bilayer. The kinetics of changeover and the amplitude of electroconductance of such a channel did not change throughout the observation period (15 min). The additional control experiment showed the following (unpublished data): (1) The mean current-voltage characteristics of the single amphotericin B channel at a low amphotericin B concentration (10 nM) can be made coincident with the integral current-voltage characteristics of the membrane at a high amphotericin B concentration (1 μM) by enlarging the current scale. (2) In the presence of the blocker tetraethylammonium (10 mM) in the solution the blocking coefficient for the open state of a single amphotericin B channel at low amphotericin B concentration (10 nM) was the same as for the integral transmembrane current at a high amphotericin B concentration (1 μM). Based on the above experimental evidence it can be assumed

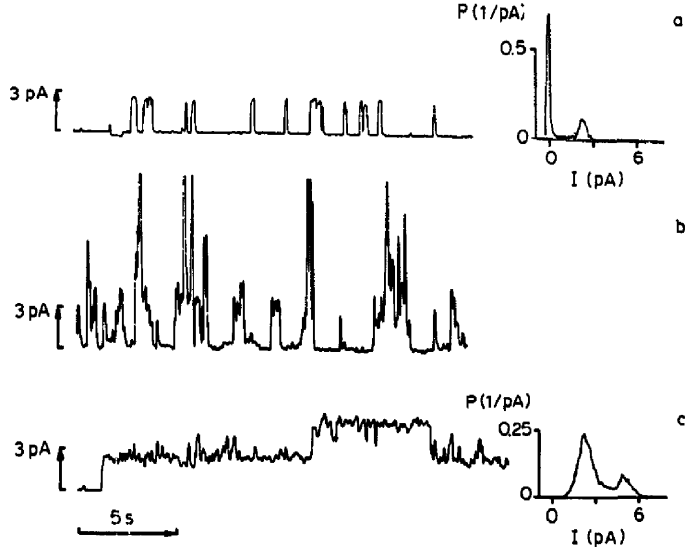

Fig. 5. Effect of specific monoclonal antibodies on a single amphotericin B channel in a symmetric membrane. (a) Records of trans-membrane current jumps in the presence of 10 nM amphotericin B in the cis-compartment and in the absence of antibodies in the cell; (b), (c) records of transmembrane current jumps 15 and 30 min, respectively, after adding monoclonal antibodies (1 mg/l) to the trans-compartment. The right parts show the probability density functions of current amplitudes during single channel events. The bilayer was formed of bovine brain lipids containing 20% (w/w) cholesterol. The membrane voltage was 0.325 V. The positive voltage sign was in the cis-compartment. Aqueous solution of 2 M KCl/5 mM Tris-HCl was in the cell.

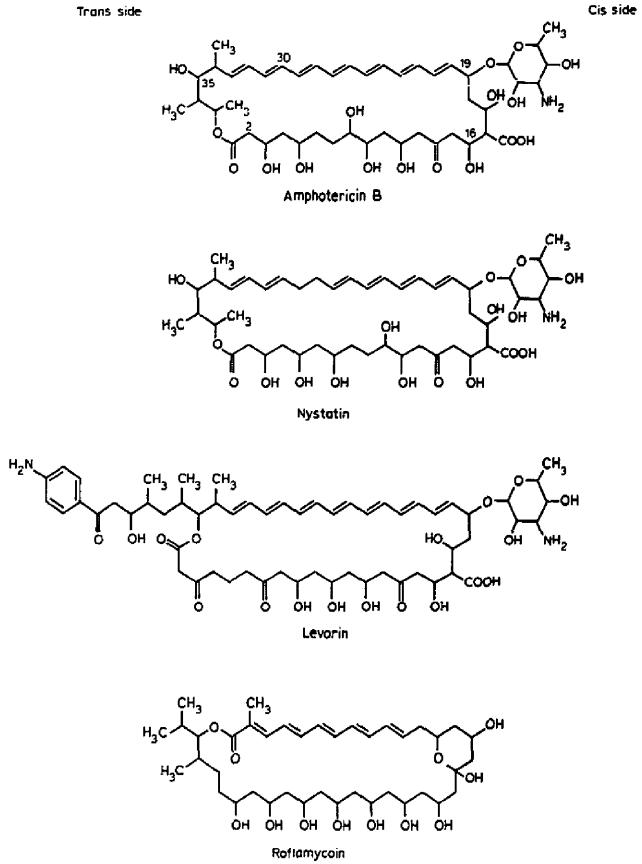

Fig. 6. Structural formula of the examined polyene antibiotics.

that the channels in the bilayer are identical at low (10 nM) and high (1 μM) amphotericin B concentrations in the solution.

After introduction of specific monoclonal antibodies at the trans-side of a membrane with a single channel, a change in the current switching kinetics was observed (Fig. 5, records (b) and (c)). About 0.5 h after addition of monoclonal antibodies to the trans-compartment, the transient ceased and current jumps as shown in Fig. 5c appeared. The kinetics and amplitude of these jumps did not change throughout the observation (15 min). It is seen that the amplitude of such jumps differed little from that of amphotericin channel without antibodies. At the same time, the probability for this channel to be open is much greater than that for the amphotericin channel. The increased noise in the record of current in Fig. 5c compared to the noise of an open channel without antibodies (Fig. 5a) may well be due to rapid switchings which are not registered because of the high time constant of the recording system (10 ms).

The effect of polyclonal antibodies isolated from amphotericin B immunized rabbits on a single amphotericin B channel was analogous to that of monoclonal antibodies. Thus, the evidence obtained supports our assumption that antibodies when interacting with the 'halfpore' at the trans-side of the membrane increase the probability for the 'halfpore' to extend through the mem-

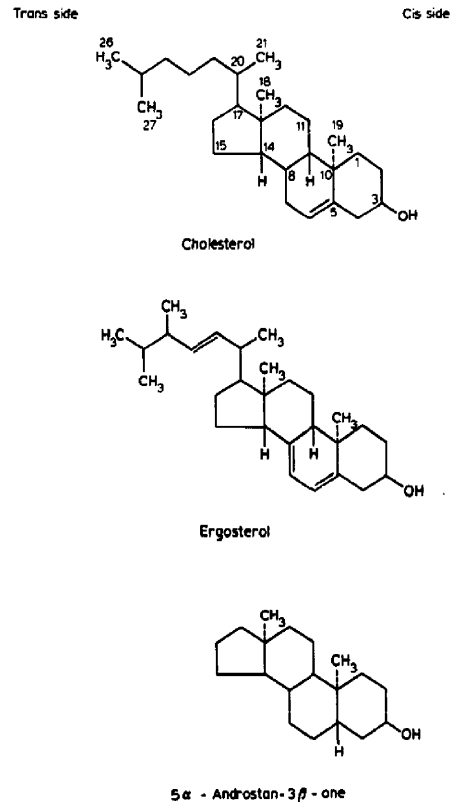

Fig. 7. Structural formula of the examined sterols.

brane, which leads to an increase in membrane conductance. Note that addition of poly- and monoclonal antibodies at concentrations of 0.1 and 0.02 g/l, respectively, to the cis-compartment of the cell had no effect on the channel properties.

To estimate the specificity of antibody action, let us consider the effect of antibodies on the channels formed by analogs of amphotericin B and cholesterol (Figs. 6 and 7) [7,8,15–18].

There experiments made it possible also to establish with more detail what fragments of the channel-forming molecules are involved in the antigenic determinant.

Effect of specific monoclonal antibodies on the electroconductance of symmetric lipid bilayer in the presence of amphotericin B and cholesterol analogs

The effect of the obtained monoclonal antibodies on ion channels formed by nystatin and cholesterol was analogous to the effect of antibodies of ion channels formed by amphotericin B and cholesterol. Thus, the fragment of the amphotericin B molecule different from the corresponding fragment of the nystatin molecule (in the middle of the molecule) seems not to be involved in the antigenic determinant. At the same time, the prepared antibodies did not change the conductance of channels formed by levorin and cholesterol. An amphotericin B molecule differs from a levorin one mainly by groups situated at the trans-side of the molecule. Hence, it may be assumed that the antigenic determinant involves groups from the trans-region of the molecule.

It should be noted that specific antibodies do not affect the conductance of channels formed by cholesterol and roflamycoin whose structure is substantially different from that of amphotericin B. This is also evidence that a fragment of the amphotericin B molecule is involved in the antigenic determinant.

Now we want to find out if cholesterol groups are involved in the antigenic determinant. For that purpose we studied the action of specific antibodies on the conductance of membranes containing ergosterol or 5α-androstan-3β-one instead of cholesterol in the presence of amphotericin B at the cis-side of the membrane. Note that ergosterol and 5α-androstan-3β-one are absent in the cellular membranes of rabbit and mouse [19].

The data presented in Fig. 8 suggest that the conductance of channels formed by amphotericin B and ergosterol is increased by the prepared monoclonal antibodies to a lesser degree than that of channels formed by amphotericin B and cholesterol (Fig. 3). Replacement of the solution in the cell by that without antibodies made the conductance of the membrane with ergosterol gradually decrease at a time constant of approx. 30 min. Therefore, unlike the channels formed by amphotericin B and cholesterol, the channels formed by amphotericin B and ergosterol bind antibodies reversibly. At the same time, specific antibodies had no effect on the conductance of channels formed by amphotericin B and 5α-androstan-3β-one.

The structure of ergosterol differs relatively little from that of cholesterol. Therefore, specific monoclonal antibodies affect (though weakly) the

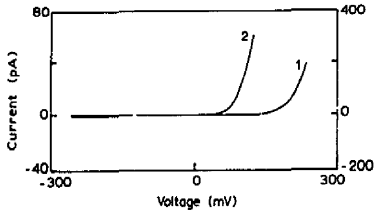

Fig. 8. Effect of specific antibodies on the current-voltage characteristics of a bilayer of brain phospholipids (85%) and ergosterol (15%) in the presence of 0.5 μM amphotericin B at the cis-side of the membrane. (1) Established current-voltage characteristics of the membrane in the presence of amphotericin B; (2) 30 min after adding antibodies (50 mg/l) to the trans-compartment of the cell. 0.1 M KCl/5 mM Tris-HCl was present in the cell. The membrane voltage increased at a rate of 5 mV/s. The current scale for curve 1 is shown on the left, for curve 2 on the right.

channels formed by amphotericin B and ergosterol. At the same time, the structures of 5α-androstan-3β-one and cholesterol strongly differ from each other (in particular, the hydrocarbon chain at C-17 in 5α-androstan-3β-one is absent). Therefore the antibodies do not influence the conductance of such channels. The results obtained indicate that the cholesterol groups situated in the trans-region of the molecule at C-17 play a key role in the channel-antibody interaction. Any change in these groups disturbs the channel structure so that the channel loses its ability to bind the antibodies obtained. This can occur without direct recognition between antibodies and sterol. But it can not be excluded also that these groups are involved in the antigenic determinant.

It follows from the aforesaid that both the groups belonging to the trans-end of the amphotericin B molecule and those situated at the trans-end of the cholesterol molecule are involved in the formation of the antigenic determinant accessible for the antibodies.

The experiments with channels formed of amphotericin B and cholesterol analogs are in support of the high specificity of antibody action.

Ion selectivity of channels formed by amphotericin B, cholesterol and specific antibodies

In order to estimate the selective properties of such a channel, the following experiments was performed. After formation of a symmetric bilayer, 1 μM amphotericin B was added to the cis-compartment, and specific antibodies to the trans-compartment. Then the zero-current potential was measured at different $[KCl]_1/[KCl]_2$, where $[KCl]_1$ is the salt concentration in the trans-compartment and $[KCl]_2$ is that in the cis-compartment (Fig. 9, curve 2). For comparison, an analogous relationship is presented for the case when at one membrane side 1 μM amphotericin B was present and at the other side antibodies and the antibiotic were absent (Fig. 9, curve 1). The both membranes were essentially potassium-selective. Dependence 2 (Fig. 9) measured for bilayers formed of brain lipids coincided with that for membranes composed of 95% asolectin and 5% cholesterol.

It follows from curves 1 and 2 (Fig. 9) and the Goldman equation [20] that the membranes in both cases were permeable for K^+ as well as for Cl^- ions, though the permeability coefficient for Cl^- was lower than that for K^+. The selective properties of the channel are determined by the small number of charged groups which are situated at the channel entrance at the cis-side of the membrane [2–4]. At the same time, antibodies

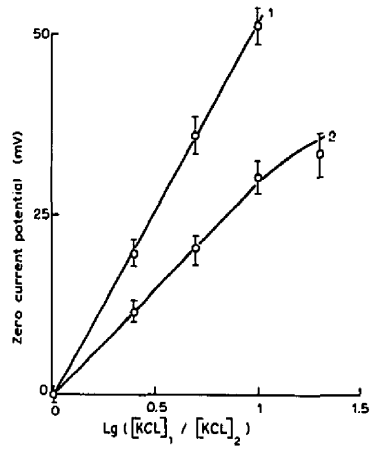

Fig. 9. Dependence of the zero current potential on the logarithm of the ratio of KCl concentrations in the opposite cell compartments. The cis-compartment contained 1 μM amphotericin B/0.1 M KCl, the trans-compartment specific antibodies at a concentration of zero (curve 1) and 25 mg/l (curve 2)/various [KCl]. A symmetric bilayer membrane was formed of bovine brain lipids containing 20% (w/w) cholesterol.

interact with groups located at the trans-end of the channel. Comparison of curves 1 and 2 (Fig. 9) shows that such attachment of antibodies causes the ratio of permeability coefficients of K^+ and Cl^- (P_{K^+}/P_{Cl^-}) decrease. Nevertheless, this ratio for a channel formed by a 'halfpore' and antibodies is more than unity, as distinct from the symmetric channel assembled from two amphotericin 'halfpores'.

It should be noted that antibodies added to the cis-compartment did not change the value of P_{K^+}/P_{Cl^-}.

Blocking of ion channels formed by amphotericin B, cholesterol and antibodies with tetraethylammonium

Tetraethylammonium efficiently blocks the current through an amphotericin 'halfpore' while entering the channel from the cis-side. The channel block is inefficient when tetraethylammonium is added at the trans-side of the membrane only, therefore it may be assumed that the entry of the blocker to the channel from the trans-side is difficult [5].

Let us follow how the blocking action of tetraethylammonium changes after antibodies have bound to the trans-end of the amphotericin channel. Fig. 10 shows current-voltage characteristics for the membrane in the presence of amphotericin B in the cis-compartment and of antibodies in the trans-compartment of the cell (curve 1) and after addition of 10 mM tetraethylammonium to the cis-compartment (curve 2). It is seen that tetraethylammonium blocked the current mainly at positive voltages in the cis-compartment. Analogous results were observed for asymmetric amphotericin channels without antibodies [5].

Thus, the attachment of antibodies to the trans-end of the 'halfpore' did not essentially change the blocker entry to the channel from the cis-side and the accompanying block of the ion current.

Now consider the block of current on addition of tetraethylammonium to the trans-compartment. The block of current through a 'halfpore' becomes efficient only at high tetraethylammonium concentrations (0.1 M) in the trans-compartment. It should be emphasized that at the same tetraethylammonium concentration in the trans-compartment there was no block of the current through

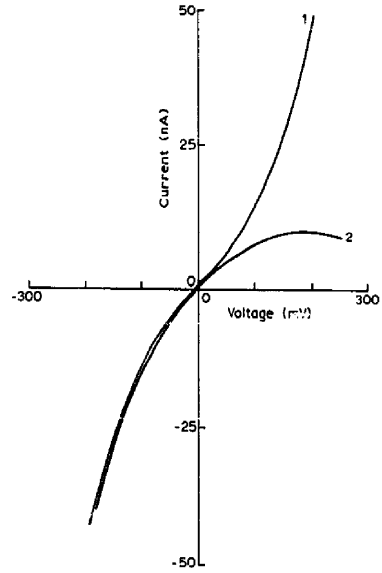

Fig. 10. Block of the transmembrane current by tetraethylammonium in the presence of amphotericin B (2 μM) at the cis-side and of antibodies (25 mg/l) at the trans-side of the bilayer formed of bovine brain lipids: (1) before addition, (2) after addition of 10 mM tetraethylammonium to the cis-compartment. The symmetric bilayer membrane was formed of bovine brain lipids containing 20% (w/w) cholesterol.

the channel formed by the antibiotic and antibodies (with an accuracy of ±5%).

Hence, the interaction of antibodies with the trans-end of an asymmetric channel leads to inability for the blocker molecule to enter the channel from the trans-side and to block the ion current.

Effect of monoclonal antibodies on a channel in an asymmetric bilayer

When cholesterol was present only in the cis-monolayer of the membrane the action of antibodies on the ion permeability of amphotericin channels was opposite to that in the case of the symmetric bilayer. Specific monoclonal antibodies added to the trans-compartment reduced the integral ion conductance of the membrane in the presence of a high amphotericin B concentration (1 μM) in the cis-compartment (Fig. 11).

At the low amphotericin B concentration (1 nM) in the cis-compartment a characteristic noise

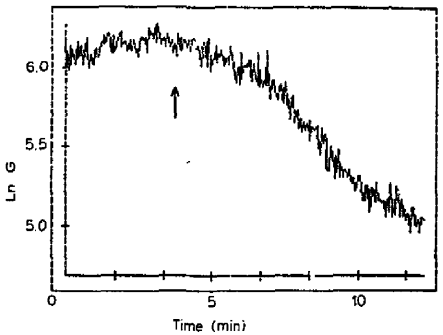

Fig. 11. Decrease in the conductance of an asymmetric lipid membrane with time in the presence of 1 μM amphotericin B at the cis-compartment after adding 1 mg/l of specific monoclonal antibodies to the trans-compartment of the cell. The arrow shows the time moment of adding antibodies. The bilayer was assembled of two different monolayers. The cis-monolayer was formed of 95% (w/w) brain phospholipids and 5% (w/w) cholesterol, the trans-monolayer of asolectin without cholesterol. The membrane voltage was 20 mV, the positive sign was in the cis-compartment. The ordinate is the logarithm of the conductance measured in pS. 0.1 M KCl/5 mM Tris-HCl was in the cell.

in the membrane conductance was observed. Distinct conductance jumps were not registered, probably, due to the short life time of the channel in the open state and the high time constant of the recording system. After addition of antibodies to the trans-compartment, the conductance decreased to that of an unmodified lipid bilayer (approx. 1 pS).

At the same time, antibodies added to the cis-compartment at a concentration of 30 mg/l did not affect the membrane conductance.

Thus, the antigenic determinant which makes antibodies block the conductance when bound to them is present in the channel only at the trans-side.

Discussion

The polyclonal antibodies specific for amphotericin B were obtained earlier by immunization with a protein conjugate of the D-lysyl amphotericin B methyl ester [21]. The results presented in our work show that amphotericin B can be an immunogen themself. It is known that substances possesing immunogenic properties have a molecular mass of more than approx. 3000 Da [23]. Amphotericin B and cholesterol have molecular masses of approx. 1000 and 386 Da, respectively. Hence, these substances by themselves are not immunogens. We believe that the immunogen is an asymmetric channel formed by eight amphotericin b and eight cholesterol molecules or some other complex of amphotericin B with lipids or proteolipids. The molecular mass of such a channel (or other complexes) is sufficiently great to provide its immunogenicity.

The high conductance of a symmetric lipid membrane (with amphotericin B in the cis-compartment) after adding antibodies to the trans-compartment is explained by the increased probability for the channel to be in the open state. Such an increase in the probability is due to the binding of an amphotericin B 'halfpore' by antibodies at the trans-side of the membrane rather than due to formation of channels assembled of two amphotericin 'halfpores'. The equal amplitudes of channel conductance before and after antibody addition suggest a fine mechanism of interaction between immunoglobulins and the 'halfpore'. These conclusions were made based on the evidence obtained in studies of a single channel, of selective properties of the amphotericin B - antibody channel as well as of the asymmetric block of such channels by tetraethylammonium.

Let us consider the mean channel-antibody stoichiometric ratio in the complex responsible for membrane conductance. An amphotericin channel has a rotational 8-fold symmetry [3]. Hence it may be assumed that its valency in the reaction with the antibodies is eight. Fig. 12 shows schematically on the same scale a channel with three antibody molecules of IgM class bound to it at the transside of the membrane. The channel - antibody size ratio was taken from Refs. 3 and 22. The antibodies in the figure are presented so that they do not hide the channel entrance, since the amplitude of channel conductance and its selectivity for K^+ and Cl^- change only slightly on binding of antibodies to the channel. It follows from Fig. 12 that for the steric reasons no more than three antibody molecules can bind to one channel.

Antibodies of IgM class when bound to an antigen have a valency from 1 to 10 [22]. Due to the polyvalency of antigens and immunoglobulins,

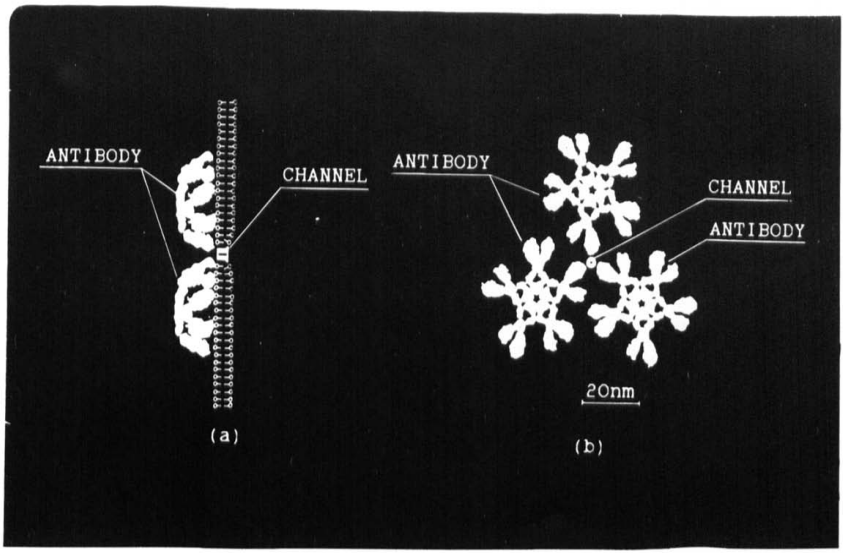

Fig. 12. Schematic representation of an amphotericin B channel and three antibody molecules bound. On the left, only two antibody molecules bound to the channel are shown for clarity.

a channel-antibody complex can vary its stoichiometry. Here two extremes should be discriminated: (1) the antibody concentration exceeds much the antigen concentration, (2) the antibody concentration is much lower than the antigen concentration. In the first case complexes must be formed consisting of one channel with the maximum number of antibodies bound. In the second case the complexes formed consist of one antibody molecule associated with the maximum number of channels [23]. Suppose that (1) membrane conductance is determined by a complex consisting of m channels and n antibody molecules, (2) the channels, the antibodies and the intermedeate products of the channel-antibody reaction bind with each other only by a specific interaction of the antigenic determinants with the variable regions of antibodies, (3) the nonspecific aggregation is absent. Theoretical analysis showed that under the assumptions made the integral conductance increment of the membrane with incorporated channels in the initial time period after antibody addition increases as follows:

$$G - g_0 \sim [\mathrm{Ch}]^m \cdot [\mathrm{mAb}]^n \cdot t^{m+n-1}$$

where [Ch] is amphotericin B channel concentration in the bilayer. According to the experimental evidence, the conductance rises linearly with increasing g_0 and as a function of the third degree of [mAb] and t with increasing antibody concentration and time. Membrane conductance before antibody addition g is proportional to [Ch]. Hence, it may be assumed that for the [Ch] and [mAb] used the complex determining membrane conductance consists of one channel with three antibody molecules bound. The reaction resulting in the formation of this complex in the initial period after adding antibodies are as follows:

$$\mathrm{Ch} + \mathrm{mAb} \rightarrow (\mathrm{Ch\text{-}mAb})$$

$$(\mathrm{Ch\text{-}mAb}) + \mathrm{mAb} \rightarrow (\mathrm{Ch\text{-}mAb_2})$$

$$(\mathrm{Ch\text{-}mAb_2}) + \mathrm{mAb} \rightarrow (\mathrm{Ch\text{-}mAb_3})$$

where Ch is the ion channel; mAb is the monoclonal antibody; (Ch-mAb), ($\mathrm{Ch\text{-}mAb_2}$), ($\mathrm{Ch\text{-}mAb_3}$) are the complexes of channel with one, two or three antibody molecules, respectively. The formation of the structure ($\mathrm{Ch\text{-}mAb_3}$) (Fig. 12) seems to increase very much the probability for the channel to be in the open state (Fig. 5).

Thus, the antibody concentration [mAb] used exceeds much the channel concentration [Ch] so

that finally complexes are formed consisting of one channel with the maximum number of antibodies bound to its antigenic determinants. It may be assumed that the three antibody molecules shown in Fig. 12 can form, due to mutual attraction, a common structure constituting an ion 'halt ore' tightly bound with the 'halfpore' formed on amphotericin B and cholesterol in the membrane.

Let us consider the possible mechanism for the block of channel conductance by antibodies in an asymmetric lipid membrane. A characteristic noise in membrane conductance registered at low amphotericin B concentrations at the cis-side of such membranes in the absence of antibodies may be due to the short lifetime of the channel in the open state in comparison with the time constant of the recording system (10 ms). The short lifetime of the channel in the open state may be explained as follows: In the closed state, a 'halfpore' of antibiotic and cholesterol is situated in the cis-monolayer enriched by cholesterol. When the channel opens the 'halfpore' pierces the lipid bilayer and hence contacts with the trans-monolayer where cholesterol is absent. Then cholesterol from the channel migrates to the trans-monolayer, which leads to destruction of the channel thus shortening its lifetime in the open state. The possibility of such a mechanism follows from Ref. 24 where it was demonstrated that cholesterol can detach from the symmetric amphotericin channel thus making it close.

The conductance block by antibodies seems to occur as follows. An antibody binds to the open channel at the trans-side of the membrane. Cholesterol migrates from the channel to the trans-monolayer of the membrane, because of which the channel is destroyed. A channel fragment containing the antibiotic and possessing no transmembrane ion conductance is left bound to the antibody. If we take the binding of antibodies with the channel to be the limiting stage and the quantity of amphotericin B in the membrane to be constant, the membrane conductance after antibody addition must decrease as follows: $G \approx \exp(-K \cdot [\text{mAb}] \cdot t)$ where K is a constant. The exponential decrease in conductance observed in the experiment (Fig. 11) is evidence that the above blocking mechanism is true.

An alternative mechanism for blocking the conductance by antibodies may work on the principle of a plug closing the entry to the channel. However, such a mechanism is unlikely, since in this case the activation of conductance by antibodies in case of a symmetric lipid bilayer would hardly to be explained.

We found out that antibodies affect the conductance of an amphotericin B 'halfpore' only when added to the trans-side of the membrane and do not change the conductance when added to the cis-side. Now consider if this phenomenon is common for membrane-active substances.

It should be emphasized that in the present work not only monoclonal but also polyclonal antibodies isolated from animal blood were used. The experiments with total immunoglobulins showed that antibodies contained in the blood of immunized animal possess none of the following properties: (a) affect the ion conductance of an amphotericin 'halfpore' when added to the cis-side of the membrane; (b) affect the electroconductance of a channel assembled of two 'halfpores'. This result indicates most probably that antibodies against the cis-end of the 'halfpore' and, hence, against the symmetrical channel at the ends of which charged amino- and carboxyl groups of amphotericin B molecules occur are not produced at all. Indeed, proceeding from the structure of the channel, it is difficult to believe that antibodies would interact with the groups at the cis-end of the channel without changing its mean ionic conductance. Based on the aforesaid, the channel-antibody complex may be schematically represented as shown in Fig. 12.

Earlier we have studied the effect of antibodies on an ion channel formed in a lipid bilayer by β-latrotoxin [25]. The same asymmetry of their action was found as in the case of the 'halfpore' of polyene antibiotic amphotericin B. The antibodies against β-latrotoxin decreased the conductance of the channel formed by β-latrotoxin while binding to the channel at the trans-side of the membrane. At the same time, these antibodies, when added at the cis-side of the membrane, did not influence channel conductance.

It can be supposed that the found asymmetry of the action of immunoglobulins is inherent in many of membrane-active substances.

Based on the evidence obtained it may be hypothesized that in B-lymphocytes producing antibodies against such membrane-active substances the following process takes place: The membrane-active substance incorporates into the outer membrane of B-lymphocytes. The antibodies are present in the cytoplasm and bind irreversibly to this substance on the inner surface of the outer membrane of the cell. The subsequent increase in ion conductance of the B-lymphocyte membrane results in producing antibodies, other necessary conditions being provided [26]. Thus, water-soluble antibodies play the role of antigenic receptors of B-lymphocytes. Those antibodies released by the B-lymphocyte to the external solution can bind antigens (for example, amphotericin B 'halfpores' present in lipid membranes).

The scheme suggested is substantially different from the conventional viewpoint that the binding of antigens to a B-lymphocyte occurs on the receptors located on the external side of the outer membrane of the cell so that antibodies do not contact with the inner surface of the outer membrane of B-lymphocytes [23]. According to our hypothesis, such a binding takes place on the inner surface of the outer membrane of B-lymphocytes and the receptors of the antigen are the antibodies themselves. It is possible that our hypothesis is correct only for the antigens soluble in lipid membranes, whereas water-soluble and cell-surface antigens bind to B-lymphocytes as it has been described earlier [23]. The elucidation of the real mechanism for antibody production requires further study.

Acknowledgement

The authors wish to thank Professor L.N. Ermishkin for his helpful discussions in the course of this investigation.

References

1 Andreoti, T.E. (1973) Kidney Int. 4, 337–345.
2 Finkelstein, A. and Holz, R. (1973) in Membranes (Eisenman, C., ed.), Vol. 2, pp. 377–407, Marcel Dekker Inc. New York.
3 De Kruÿff, B. and Demel, R.A. (1974) Biochim. Biophys. Acta 339, 57–70.
4 Ermishkin, L.N., Kasumov, Kh.M. and Potzeluev, V.M. (1976) Nature 262, 698–699.
5 Brutian, R.A. (1982) Biophyzika 27, 646–649 (in Russian).
6 Bolard, J. (1986) Biochim. Biophys. Acta 864, 257–304.
7 Schlegel, R., Thrum, H., Zielinski, J. and Borovski, E. (1981) J. Antibiotics 34, 133–130.
8 Schlegel, R., Grigorjev, P. and Thrum, H. (1982) Stud. Biophys. 92, 135–140.
9 Hara, A. and Radin, N.S. (1978) Anal. Biochem. 90, 420–426.
10 Labarca, P., Coronada, R. and Miller, C. (1980) J. Gen. Physiol. 76, 397–424.
11 Kates, M. (1972) Techniques of Lipidology, pp. 78–117, North-Holland Amsterdam/American Elsevier, New York.
12 Kabat, E.A. and Mayer, H.M. (1964) Experimental Immunochemistry, pp. 497–518, Charles C. Thomas Publisher, Springfield, IL.
13 Zelig Eshhar (1985) in Hybridoma Technology in the Biosciences and Medicine (Springer, T.A., ed.), pp. 1–40, Plenum Press, New York and London.
14 Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3561–3566.
15 Borovski, E., Zielinski, J., Ziminski, T., Falkowski, L., Kolodziejczyk, P., Golik, J., Jereczek, E. and Aldercreutz, H. (1977) Tetrahedron Lett. 45, 3909–3914.
16 Lancini, G. and Parenti, F. (1982) Antibiotics, pp. 142–143, Springer-Verlag, Berlin.
17 Zielinski, J., Borowy-Borowski, H., Golik, J., Gumieniak, J. Ziminski, T. and Kolodziejczyk, P. (1979) Tetrahedron Lett. 20, 1791–1794.
18 Borisova, M. and Kasumov, Kh. (1978) Stud. Biophys. 71, 197–202.
19 Krebs, E.M. (1981) Lipids of Cellular Membranes, pp. 18–20, 58–61, Science, Leningrad (in Russian).
20 Goldman, D.E. (1943) J. Gen. Physiol. 27, 37–60.
21 Little, J.R., Little, K.D., Plut, E., Koldin, M. and Kobayashi, G.S. (1984) Antimicrobal Agents and Chemotherapy 26, 824–828.
22 Feinstein, A. and Beale, D. (1981) in Structure and Function of Antibodies (Glynn, L.E. and Steward, M.W., eds.), pp. 148–196, John Wiley and Sons, Chichester.
23 Friemel, H. and Brock, J. (1983) Grundlagen der Immunologie, pp. 14–32, 95–127, Akademic-Verlag, Berlin.
24 Kasumov, Kh.M., Borisova, M.P., Ermishkin, L.N., Potseluev, V.M., Silberstein, A.Ya. and Vainshtein, V.A. (1979) Biochim. Biophys. Acta 551, 229–237.
25 Kolomytkin, O., Kasymov, Sh. and Salichov, Sh. (1988) Gen. Physiol. Biophys. 7, 157–163.
26 Cambier, J.G., Monroe, J.G., Coggeshall, K.M. and Ransom, T. (1985) Immunol. Today 6, 218–222.