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# MECHANISM OF BLOCKAGE OF AMPHOTERICIN B CHANNELS IN A LIPID BILAYER

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## Summary

A number of organic compounds (non-electrolytes, tetraalkylammonia, etc.) with a molecular size of 6—8 Å decrease the conductance of ionic channels formed in the lipid bilayer by a polyene antibiotic amphotericin B. It is suggested that these compounds, upon entering the channel, block the passage of inorganic ions. The extent of conductance blockage by organic ions depends on the membrane potential and electrolyte concentration. In the presence of ionic blockers, for instance tetraethylammonium, amphotericin B-containing membranes assume some properties characteristic of excitable membranes, i.e. the current-voltage characteristic acquires the negative resistance region, and in response to a potential step activation followed by inactivation of conductance is observed. It is shown that the potential dependence of the blockage is due to interaction inside the channel of the blocker ion with penetrating ions, by a mechanism similar to that described by Armstrong ((1979) Q. Rev. Biophys. 7, 179—210) for blockage of squid axon potassium channels by ammonium derivatives.

## Introduction

In 1974 it was shown that permeability for non-electrolytes of cholesterol-containing membranes treated with amphotericin B decreases with the molecule size, and is already very low for glucose [1—3]. Permeability for non-electrolytes is proportional to induced ionic permeability. These facts suggested that amphotericin B produces pores in the membrane, about 8 Å in diameter, which are permeable for water, non-electrolytes, and ions. These channels have one conducting state (about 6 pS in 2 M KCl) permeable mostly for anions. It has been noted that if one of the ions is about 7 Å in size (e.g. acetylcholine chloride solution), conductance of the amphotericin B-containing

membrane is low compared to that in KCl [4]. To elucidate the mechanism of this phenomenon, we studied the effect of various salts added to 2 M KCl solutions. Preliminary experiments showed that even low concentration of organic compounds with the molecule or ion size about 7 Å suppressed the integral conductance of the membrane treated with amphotericin B. The drop in conductance might be caused by (1) disturbances in channel assembling, (2) shorter channel life, or (3) lower conductance of the open channel. The present work is concerned with effects of organic compounds on both the conductance of a many-channel membrane and the conductance of individual channels, in order to distinguish among the above-mentioned possibilities.

## Experimental

The membranes were formed on a 0.25 mm hole in a teflon cell from a solution of brain phospholipids (20 mg/ml) and cholesterol (1 mg/ml) in *n*-heptane. Routinely, the membrane separated two identical antibiotic solutions in 2 M KCl, pH 6.0, 21—23° C. Aqueous stock solutions of blockers supposed were used for addition to one of intensively stirred electrolyte solutions. The membrane current was measured with a Keithley 301 amplifier and recorded using an Endim XY-recorder when studying individual channels or storage oscilloscope in the case of integral conductance kinetics studies. Time resolution for individual channel studies was about 0.05 s; in the case of a large number of channels the membrane conductance was about $10^{-5}$ S and the resolution was better than 10 μs, which was achieved by compensating the capacitive current following the method of Sargent [5]. The potential of the outer relative to the inner solution was recorded.

## Results

*Blockage of the integral conductance*

After the establishment of a stationary conductance of a membrane the substance investigated was added to the outer solution, and the conductance was measured at a constant membrane potential. Fig. 1 shows the change of mem-

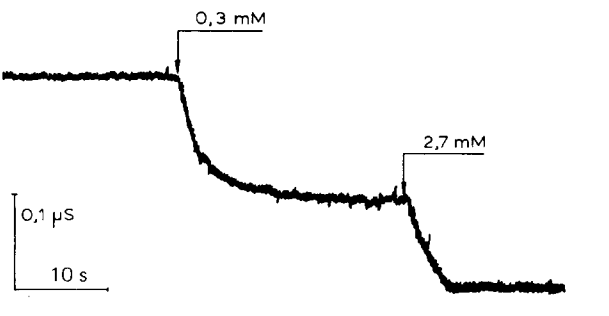


Fig. 1. Blockage of conductance of membrane with a large number of amphotericin B channels. At times indicated by arrows 0.3 or 2.7 mM tetraethylammonium (TEA) was added to the outer solution. Potential of the outer solution +150 mV, 2 M KCl, 0.07 μM amphotericin B, membrane area 0.05 $mm^2$. Current noise is due to intensive stirring of both solutions.

brane conductance with time due to addition of tetraethylammonium. The beginning of the record corresponds to the initial stationary current. Following addition of tetraethylammonium, the membrane current rapidly drops down to a new stationary value, the duration of the drop corresponding to the time of tetraethylammonium diffusion through the unstirred layer. The current is decreased monotonically with the increase of tetraethylammonium concentration. In a similar manner, although with varied effectiveness, the membrane conductance is affected by a number of compounds close in size to tetraethylammonium, e.g. other ammonium derivatives, choline, acetylcholine, non-electrolytes such as glycerol, urea and sugars, as well as anions of propionate and α-naphthylsulfate. The effect of ionic blockers, when added on one side of the membrane, is a function of both magnitude and direction of the electric field. Fig. 2 shows steady-state current-voltage characteristics of the membrane in 2 M KCl without blocker and in the presence of 0.2 M ribose, 0.2 M acetylcholine chloride, and 0.4 M potassium propionate. Apparently, the addition of acetylcholine and propionate results in rectification. The cationic blocker is more effective when in the positive solution while anionic blocker shows greater effect in the opposite direction of the field.

*Effects on individual channels*

The action of acetylcholine chloride, potassium propionate, sodium naphthylsulfate, tetraethylammonium and phenyltrimethylammonium chlorides, ribose and urea on a single channel were studied. All these substances were found to decrease the conductance of individual channels. Fig. 3 shows

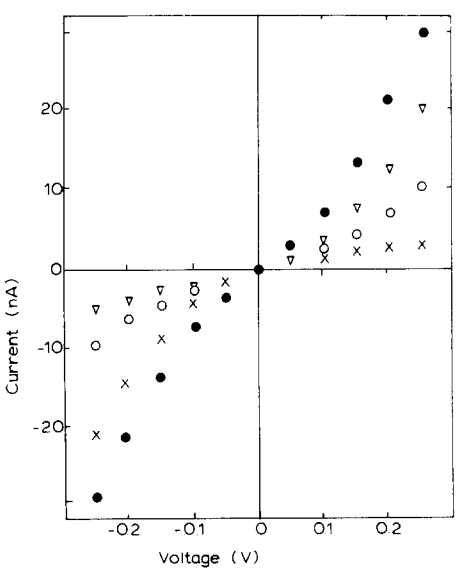


**Fig. 2. Current-voltage characteristics of membrane with amphotericin B without blocker (•), or with 0.2 M acetylcholine chloride (×), 0.4 M potassium propionate (▽) or 0.2 M ribose (○) in the outer solution. Each curve was taken on different membranes. Current scales of different membranes were adjusted so that current-voltage characteristics coincide before addition of a blocker. Membrane area 0.025 mm$^2$, 2 M KCl, 0.07 μM amphotericin B, 20° C, pH 6.4.**

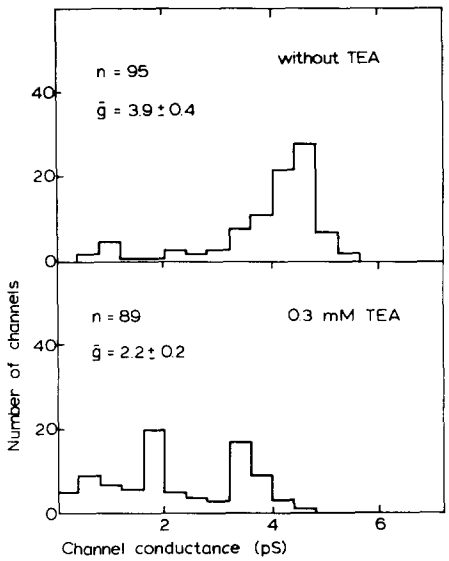


Fig. 3. Conductance density functions of individual channels without blocker (top) or with 0.3 mM tetraethylammonium in the outer solution (bottom), +150 mV, 2 M KCl.

conductance density functions for individual channels with 0.3 M tetraethylammonium and without. It can be seen that the substance not only decreases the channel conductance but also changes the shape of the density function. Apparently, the blocking efficiency varies with the initial conductance of the channel. The average channel conductance dropped in the presence of 0.3 mM tetraethylammonium 1.7 ± 0.4 times. The conductance of a membrane with about $10^4$ of such channels decreased under similar conditions 2-fold (Fig. 1), which is within the experimental error of the density function estimate. This indicates that tetraethylammonium affects only the conductance of the open channel rather than number of channels or their lifetime.

The above data may be explained by suggesting that blocker molecules or ions enter the pore and lock it up for a short period of time. If the time of the molecule's stay in the pore is short as compared to the time resolution for individual channels recording (approx. 100 ms), a decrease in the average channel conductance is observed. The channel conductance is proportional to the fraction of time for which the channel is free of the blocker. This time may be estimated from the rate of variation of the blockage extent in response to potential step on a membrane with a large number of channels. In this case time resolution is much better. Without a blocker, current response to voltage step is time independent. In the presence of an ionic blocker it is time dependent and the blockage occurs for 10 $\mu$s—10 ms depending on the membrane potential. At a high potential of the corresponding polarity the ion may stay in the channel for a rather long time (see below).

Fig. 4 presents examples of several ionic blocker effects on individual channels. Fig. 4A shows the effects of the most efficient cationic blocker, phenyltrimethylammonium. It can be seen that the open state conductance

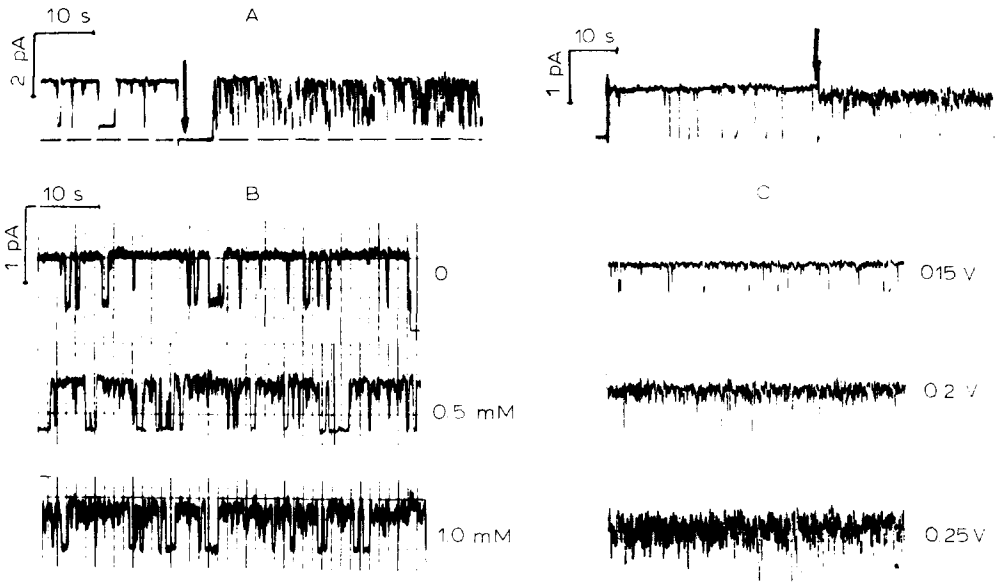


Fig. 4. Effect of same blockers on the single channel current. (A) Solutions contain 3.5 M KCl, 6 mM amphotericin B; pH 7.0, 23° C. At the time indicated by arrow 1 μM phenyltrimethylammonium was added to the solution at +200 mV, and the solution was stirred. During the blocker addition and stirring the current recorder was set to zero. (B) Top: channel in 2 M $KNO_3$ without blocker; middle: 0.5 mM naphthylsulfate; bottom: 1 mM naphthylsulfate in the solution at −200 mV. (C) Top: current through the channel in 2 M KCl without blocker, at the time indicated by arrow 10 μM tetraethylammonium was added to the solution at +200 mV. During the blocker addition and stirring the recorder was stopped and set to zero. Bottom: the same channel at different voltages.

remained the same as without the blocker, frequent transitions to the closed state become apparent, however. As a result, the average channel conductance drops. Fig. 4B shows the effect of an anionic blocker, naphthylsulfate. The higher the blocker concentration the greater the current noise and the lower the average channel conductance. Fig. 4C indicates that at a constant cationic blocker concentration, the blocking extent grows with positive potential in the blocker solution.

Another confirmation to the dynamic plug hypothesis comes from comparing the blockage efficiency of molecules of different size. The hypothesis suggests that the blocking efficiency is a function of the molecule size, and that in a series of structurally related compounds there is an optimum size which is close to the channel diameter. Indeed, among non-electrolytes, most efficient blockers are five-carbon sugars (xylose, arabinose and ribose) with the molecular size of about 6 Å. Larger molecules of glucose and sucrose are less efficient. Urea and glycerol which are smaller can block the channel only at high concentration. As has been demonstrated earlier [1], permeability of the amphotericin B-containing membrane for non-electrolytes decreases with the molecule size, and is already very low for glucose. Thus, non-penetrating as well as easily penetrating substances are both poor blockers. Only those blockers are efficient whose molecules are close to the pore diameter in size and fit pore tightly. An optimum blocker size also exists in the tetraalkylammonium

series: tetraethylammonium is more efficient than tetramethyl- or tetrapropylammonium.

*The nature of the potential dependence of blockage*

Fig. 5A shows many-channel current-voltage characteristics of a membrane with amphotericin B in 0.2 M KCl without tetraethylammonium and at three different concentrations in one of the solutions. The higher the concentration the lower the membrane current. The curves are non-monotonical in the positive potential range (polarity in the blocker solution), and the maximum is shifted towards lower potential values as the tetraethylammonium concentration is increased. Fig. 5B shows logarithm of the blockage extent (ratio between the blocked and remaining currents) as a function of membrane potential, derived from the curves on Fig. 5A. All these functions are parallel straight lines. The degree of the potential dependence, which is determined by the slope of the lines, is thus irrespective of the blocker concentration. Relationship between the blockage extent ($B$) and the membrane potential ($V$) and blocker concentration in mM ($b$) may be described by the following empirical equation $B = 0.14 \cdot b^{0.8} \exp(+0.4\, VF/RT)$. The value of the coefficient in brackets is close to 0.4 for all the cationic blockers studied.

Let us assume that a blocker ion (b) enters the open channel (O) and shifts it to the closed state (C), i.e. $O + b \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} C$. The stationary blockage extent then is $B = \alpha b/\beta$. In response to step-wise change of the potential the fraction of blocked channels changes in accordance with:

$$dC_0/dt = -\alpha b C_0 + \beta C_c$$

where $C_0$ and $C_c$ are fractions of open and closed channels, respectively, and $\alpha$ and $\beta$ are blockage and releasing rate constants, which are functions of the new potential.

In accordance with the above equation, a step-wise change of the potential from $V_1$ to $V_2$ should result in exponential change of the blocked channel frac-

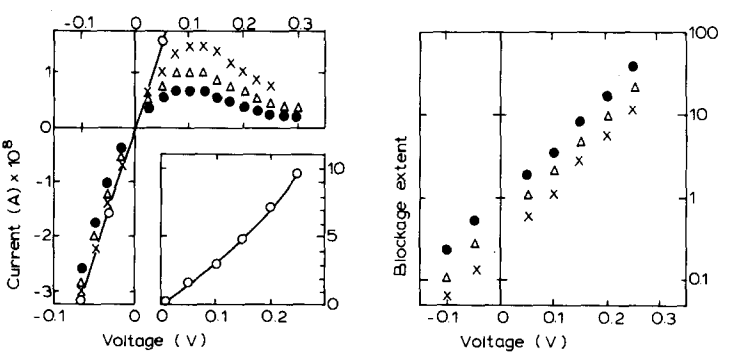


Fig. 5. Blockage of membrane current in 0.2 M KCl as a function of membrane potential. Left. Current-voltage characteristics of membranes with 0.1 $\mu$M amphotericin, pH 7.0, 20°C: without tetraethylammonium (○), or with 3 mM (X), 6 mM (△) and 12 mM tetraethylammonium (●) in one of the solutions. Potential of the blocker-free solution is taken for zero. Right. Logarithm of the blockage extent (ratio between the blocked and remaining currents) as a function of membrane potential.

tion with time, the time constant being

$$\tau^{-1} = (\alpha b + \beta)$$

where $\alpha$ and $\beta$ are functions of only $V_2$. The model satisfactorily describes $B(V)$ curves (Fig. 5) when $\alpha/\beta \approx \exp(0.4\ VF/RT)$, suggesting that the rate constants (at least one of them) are potential dependent. The $\alpha(V)$ and $\beta(V)$ functions can be found using the time constant and the stationary blockage extent.

Fig. 6 shows the response of the membrane to a potential step. The response is single exponential, and $\tau$ is a function of $V_2$ and blocker concentration, irrespective of the initial potential. Fig. 7 shows $\alpha b$ and $\beta$ values for two tetraethylammonium concentrations calculated for various $V_2$ values. The entrance rate constant, $\alpha b$, is independent of the membrane potential and is indeed proportional to $b$. The release constant $\beta$ is dependent exponentially on $V_2$ and slightly dependent on $b$, decreasing about 1.6 fold following a 10 fold decrease in $b$. As a result, the dependence $B(b) = \alpha b/\beta$ is sublinear. The dependence of $\beta$ on the blocker concentration may be accounted for by different affinity of channels to blocker ions. In the case of low $b$, channels with high affinity are blocked mainly, while channels with poor affinity remain open. Accordingly, when $b$ is further increased, the $\beta$ values observed will be lower. This hypothesis is also confirmed by the deformation of the histogram (Fig. 3) upon blockage.

We have supposed that the blockage extent of a given channel is determined by its selectivity: the higher the anion-cation selectivity the better blockage. In this case the selectivity of a membrane with a large number of channels will be somewhat lower after introduction of a cationic blocker, as a result of greater blockage of more selective channels. This was indeed observed. Zero-current potential on an amphotericin B-containing membrane separating 0.5 M and

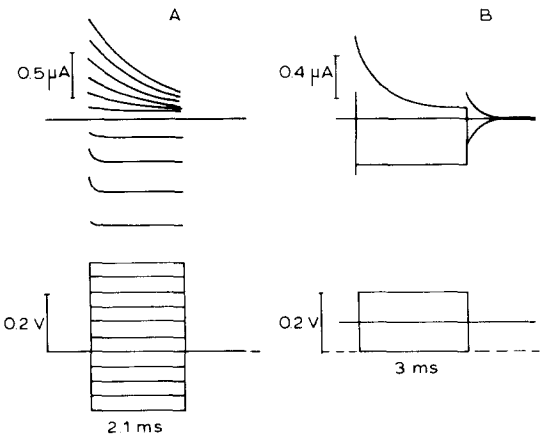


Fig. 6. Response of amphotericin B-containing membrane with cationic blockers in outer solution to rectangular pulses of potential. (A) 2 M KF, 0.1 μM amphotericin B in both solutions; 0.1 mM phenyltrimethylammonium. (B) 2 M KCl; 0.1 μM amphotericin B in both solutions; 0.6 mM tetraethylammonium. Voltage programs are shown in bottom.

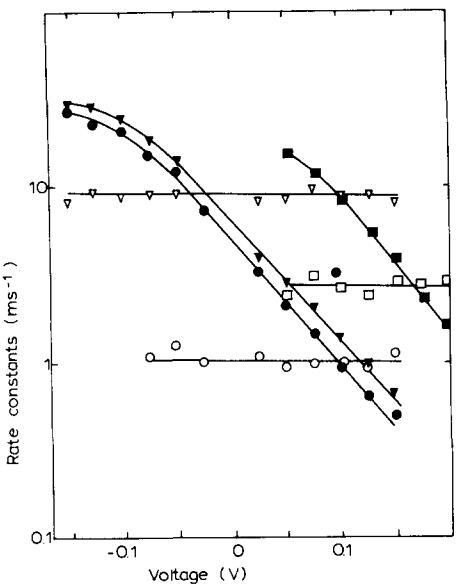


Fig. 7. Dependences of $\alpha b$ and $\beta$ on the membrane potential. With 2 M KCl: ▽, $\alpha b$ 6 mM tetraethylammonium; ▼, $\beta$ 6 mM tetraethylammonium; ○, $\alpha b$ 0.6 mM tetraethylammonium; ●, $\beta$ 0.6 mM tetraethylammonium. With 0.2 M KCl: □, $\alpha b$ 0.6 mM tetraethylammonium; ■, $\beta$ 0.6 mM tetraethylammonium.

2.0 M KCl solutions dropped from 25 mV to 21 mV after addition of 6 mM tetraethylammonium to one solution.

The blockage extent is noted to be about 2.5 fold higher in 2 M KCl than that in 0.2 M KCl solutions. When salt concentration was varied only in one solution it became apparent that the blocking efficiency depends mostly on KCl concentration in the solution without the blocker. Indeed, a change in the KCl concentration in the blocker solution from 0.2 to 1.0 M has almost no effect on the blockage ($B = 4.2$ and $4.0 \pm 0.2$, respectively) while the same change in the other solution leads to a rise in the blocking efficiency about 1.7 fold. This effect may be easily explained by supposing that the blocker ion is electrostatically retained in the pore by a permeating anion, which enters the channel from the other solution. This suggestion is supported by the fact that in 2 M $KNO_3$ solution the blockage by the same concentration of tetraethylammonium is three times worse, and in 2 M KF it is 1.7 times better, than in 2 M KCl. It has been reported that the anion-cation selectivity of amphotericin channels grows in the order $KNO_3 < KCl < KF$ [4]. The same hypothesis explains greater blocking ability of organic cations as compared to non-electrolytes of the same size.

It is suggested, therefore, that the blocker cation, after entering the channel, interacts with an anion that enters the channel from the opposite solution. The anions interact with the pore walls on one hand, and with the blocker cation on the other, thus decreasing the probability of blocker release by the pore. In this case the probability of release should be inversely proportional to the probability that the $Cl^-$ is in the channel. The latter probability, in turn, is determined by the membrane potential and the chloride concentration, and for not very

high KCl concentration is proportional to [Cl] $\exp(\gamma FV/RT)$. When $\gamma = 0.4$ this model fits well with the relationship between the stationary blockage extent and the membrane potential (Fig. 5), and with the release rate constant as a function of membrane potential and electrolyte concentration (Fig. 7). Experimentally obtained relationship between $B$ and KCl is, however, sublinear. The reason for that becomes clear when considering the dependency of $\alpha$ and $\beta$ on KCl concentration. Fig. 7 shows that $\beta$ varies almost proportionally to $[KCl]^{-1}$. However, the entrance rate constant $\alpha$ also depends on KCl concentration, it grows three fold upon decreasing KCl concentration from 2 M to 0.2 M.

## Discussion

Our data show that the blockage of lipid bilayer conductance in the presence of amphotericin B by organic molecules and ions 6—8 Å in size is due to the entering of these molecules or ions into the channel, which prevents the passage of inorganic ions. The duration of blocker stay in the channel is a function of the energy of its interaction with the channel walls, and is therefore molecular size dependent. For ionic blockers, the stay duration is also a function of the energy of their interaction with inorganic ions inside the channel.

The potential-dependent blockage of potassium channels by quarternary ammonia, which leads to anomalous rectification and inactivation of conductance, has been described by Armstrong [6,7]. In his experiments the blockage extent was also dependent on the concentration of the penetrating ion in the blocker-free solution: an increase in $K^+$ concentration from 10 to 440 mM decreased the time of recovery from inactivation from 36 ms to 6 ms, the membrane potential in both the cases being equal to —60 mV. The author explains the observed dependence by electrostatic pushing of the blocker cation out of the channel by $K^+$ that enter the channel from other side of axon. In our case, the tetraethylammonium ion, on the contrary, is retained in the channel by the attraction to the penetrating anion, which results in the increase of blockage about 5 fold when KCl concentration in the blocker-free solution is raised from 20 mM to 1 M. It appears that there is not only a qualitative but also a quantitative similarity between the blockage mechanisms in the two permeability systems.

The fact that ammonium derivatives in a similar manner block both the potassium channels of the axon and the anion-selective channels in the lipid bilayer in the presence of amphotericin B indicates that the blocking ability is determined by the correspondence between the sizes of the blocker and the mouth of the channel. Indeed, the tetraethylammonium ion is close in size to both hydrated $K^+$ and $Cl^-$ and appears to fit well to the mouth of potassium channels, as well as to the amphotericin channel, which is estimated to be 8 Å in diameter [1,2]. On the other hand it would seem that cationic blockers should not be able to enter an amphotericin B channel whose anionic selectivity is apparently determined by positive potential induced by OH-dipoles in the pore. It is possible that this potential is appreciable only in the middle part of the pore thus preventing cations from passing through and is negligible at the mouth. The slight dependence of the entrance rate constant of blocker ions

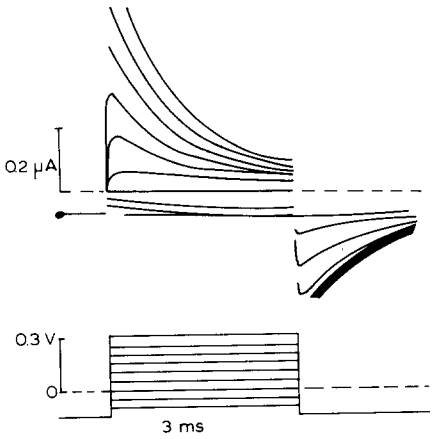


Fig. 8. Membrane current change in response to step-wise change of the potential. All solutions contain 2 M KCl, 0.1 μM amphotericin, 0.1 mM phenyltrimethylammonium, 22°C. Pulse duration 3 ms, the potential changes from −150 mV to +300 mV.

upon the membrane potential may be regarded as an evidence for the location of the blocker ion at the pore mouth.

Addition of a cationic blocker to both solutions might block both channel mouths. Since the release rate constant depends on the polarity of membrane potential, however, only the entrance at the positive solution is blocked. Following a step-wise polarity change, the probability of the $Cl^-$ being in the channel drops abruptly, the blocker detaches and the conductance increases. Simultaneously the blockage of the opposite mouth begins. Superposition of the two exponential processes results in the activation-inactivation conductance kinetics shown in Fig. 8. The activation rate grows with the potential, which is understandable considering that chloride concentration in the channel is a function of the potential. The inactivation rate, however, is determined by a potential-independent constant and is the same for all the potential values.

## References

1 Holz, R. and Finkelstein, A. (1970) J. Gen. Physiol. 56, 125—145
2 Andreoli, T.E., Dennis, V.W. and Weighl, A.M. (1969) J. Gen. Physiol. 53, 133—156
3 De Kruijff, B., Gerritsen, W.J., Oerlemens, A., Demel, R.A. and van Deenen, L.L. (1974) Biochim. Biophys. Acta 339, 30—43
4 Ermishkin, L.N., Kasumov, Kh.M. and Potseluyev, V.M. (1976) Nature 262, 698—699
5 Sargent, D.E. (1975) J. Membrane Biol. 23, 227—247
6 Armstrong, C.M. (1974) Q. Rev. Biophys. 7, 179—210
7 Armstrong, C.M. (1969) J. Gen. Physiol. 54, 553—575