

Probing Alamethicin Channels with Water-soluble Polymers. Size-modulated Osmotic Action

Igor Vodyanoy,*[‡] Sergey M. Bezrukov,*[§] and V. Adrian Parsegian*[¶]

*Division of Intramural Research, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; [‡]Office of Naval Research, Arlington, Virginia 22217-5000; [§]St. Petersburg Nuclear Physics Institute of the Russian Academy of Sciences, Russia 188350; [¶]Laboratory of Structural Biology, Division of Computer Research and Technology, National Institutes of Health, Bethesda, Maryland 20892 USA

ABSTRACT Contrary to expectations based on heightened solution viscosity, alamethicin channels appear to speed up in the presence of water soluble polyethylene glycols (PEGs) and dextrans. Specifically, added polymers reduce the probabilities of transition to higher-conductance states but do not change channel lifetimes. They thereby shorten the duration of current “bursts.” These modified probabilities and kinetics reveal the action of polymer osmotic stress to suppress channel formation. The osmotic action of large, fully excluded polymers shows that some 3,000 Å³ of water are taken up by the channel from the solution upon each transition to an adjacent higher-conductance state. The partial osmotic action of incompletely excluded polymers reveals the extent of exclusion for different-size polymers. The partial exclusion thus measured agrees remarkably well with estimates using data on reduction of single-channel conductance by current-impeding polymers. One can relate the degree of each polymer’s exclusion to its size and to the radius of the channel pore.

INTRODUCTION

Water-soluble polymers change many properties of ionic solutions, not only increasing solution viscosity but also affecting the activities of salts and water. Such polymers have been shown to alter characteristics of transmembrane ionic channels, in particular channel conductance and the probabilities of transition between different states:

1. From the osmotic action of very large polymers on the “open/closed” transitions in the “voltage-dependent anion channel” of mitochondria (VDAC or “mitochondrial porin”), it was possible to infer the change in polymer-inaccessible water volume when the channels switched from closed to open states (Zimmerberg and Parsegian, 1986). (The procedure also works to measure ion-specific channel open/close volume changes by using small, but excluded, solutes (Zimmerberg et al., 1990; Rayner et al., 1992)).

2. From polymer-induced changes in channel conductance, it was possible to gauge channel size in two ways: (i) through access resistance sensitive to polymers outside the channel (Bezrukov and Vodyanoy, 1993) and (ii) through the decrease in intrinsic single channel conductance caused by partly penetrating small polymers (Sabirov et al., 1991; Bezrukov and Vodyanoy, 1993).

In the case of alamethicin (Bezrukov and Vodyanoy, 1993), noise analysis of the open-channel current showed no indication of the specific binding between entrant polymers and the channel that one expects of channel “blockers.” It was concluded that the change in conductance could be best described as a change in internal conductivity whose depen-

dence on polymer concentration was like that of bulk solution conductivity as long as one corrects for the partial exclusion of polymers from the channel.

We now address the differences wrought by small versus large polyethyleneglycols (PEGs) on alamethicin channel dynamics. Again, nonpenetrating polymers exert an osmotic stress that allows one to measure changes in channel water volume upon transition. Here, with alamethicin, the effect of stress is to shorten the duration of current bursts. The successive stepping from “closed” to higher and higher conductance states is suppressed by osmotic stress. And the effect is incremental. It seems that a polymer-inaccessible 3,000-Å³ volume of water is created with each step to a higher conductance level.

Smaller molecular weight PEGs show fractional osmotic effects in proportion to their loss of exclusion from the channel. Partial penetration measured this way agrees remarkably well with the penetration inferred from changes in channel conductance, adding evidence for the idea that these successive states involve creation of new channels rather than enlargement of old ones.

Although PEG increases bulk solution viscosity, it has little effect on alamethicin kinetics beyond what is seen as osmotic action. Open-state dwell times are essentially unchanged despite 20-fold increases in bulk viscosity. Alamethicin dynamics are thus very different from those seen with water-soluble proteins, where solvent viscosity is an important determinant in dynamics and enzyme-substrate interaction (Gavish, 1986).

MATERIALS AND METHODS

“Solvent-free” membranes were prepared as described by Montal and Mueller (1972). The membrane forming solutions were 1- α -diphytanoyl lecithin (PC) and dioleoylphosphatidylethanolamine (PE) (Avanti Polar Lipids, Inc., Pelham, AL) used without further purification. Hexadecane in *n*-pentane (1:10) was used for aperture pretreatment. Natural alamethicin (The Upjohn

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Address reprint requests to Sergey M. Bezrukov at the National Institutes of Health, Bldg. 5, Room 405, Bethesda, MD 20892.

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Co., Kalamazoo, MI), purified as described by Balasubramanian et al. (1981), was added after membrane formation to one membrane bathing solution via ethanolic stock solutions. Analytical grade sodium chloride (Mallinckrodt, Inc., Paris, KY) was used to prepare aqueous solutions buffered at pH 6.2 by 2-(*N*-morpholino)-ethanesulfonic acid (molecular weight 213.2) (Calbiochem Corp., La Jolla, CA). PEGs of different molecular weights (Aldrich Chemical Co., Inc., Milwaukee, WI) and dextran 17,900 (Sigma Chemical Co., St. Louis, MO) were used to set specific weight/weight concentrations. Polymers were added to NaCl stock solutions in order to keep constant salt/water molar ratio.

In all experiments we used identical solutions on both sides of the bilayer. In this way a zero net transmembrane osmotic gradient was ensured that prevented the development of a hydrostatic pressure difference across the membrane.

Polymer-induced changes in solution osmotic pressures were measured with a vapor pressure osmometer (model 5100C; Wescor, Inc. Logan, UT). Cannon viscometers (Cannon Instrument Co., State College, PA) were used to determine solution viscosities. All measurements were made at room temperatures.

With two exceptions, methods were as described by Bezrukov and Vodyanoy (1993). In the present study we also employ additional data-reduction procedures to measure relative state probabilities and mean dwell times in different states of the channel. These procedures were part of the general computer program for statistical analysis of single-channel recordings that was written in our laboratory. First, a 4,000-point sample was recorded into the computer memory to create a histogram of channel currents. Sampling frequency was chosen to be close to the 5-kHz corner frequency of a low-pass Bessel filter (Frequency Devices, 902; Frequency Devices, Haverhill, MA). Then, after positions of current histogram peaks were determined by eye using coordinates of the relative scale (Fig. 1), their values were put into the computer together with the values of "windows" in the current coordinate. The window was typically chosen to be three times broader than the corresponding peak width. For example, in the case of a particular histogram shown in the left side of Fig. 1, the positions of peaks were determined to be 2.2, 4.5, and 7.2 for levels 1, 2, and 3, respectively. The window widths were chosen equal to 1.0. Experimental points for the relative state probability were obtained as the ratios of the number of counts (points of the sample) in a window corresponding to this state to the number

of counts in the window corresponding to state 1. Mean dwell times were determined from the numbers of successive counts spent by channel current in a given window. The total number of counts in the window was then divided by a number of successive uninterrupted counts and by sampling frequency. The frequency of sampling/filtering was taken high enough to minimize errors in dwell time evaluation due to "cutting out" short times. Experiments performed with different sampling/filtering frequencies showed that for the channels studied the frequency of 5 kHz was not distorting the results of measurements by more than 5% of their value in comparison with 10-kHz sampling. Additional support for the validity of the sampling frequency choice was derived from noise measurements. In these measurements an eight-pole Butterworth filter (Frequency Devices 901) was employed, the corner frequency being chosen equal to 3/8 of the sampling frequency to escape signal aliasing. The spectra obtained from multi- and single channel recordings showed roll-off frequencies of 30–100 Hz giving no indication of faster processes. It should be mentioned here that the noise of the open channel has high-frequency contributions (Bezrukov and Vodyanoy, 1991 and 1993) but their intensity is many orders of magnitude less than the noise generated by switching of the alamethicin channel between different conductance states.

To characterize the polymers used in this study, we measured polymer-induced viscosity and osmotic pressure. Results plotted in Fig. 2 indicate that addition of 15 weight % of a polyethylene glycol increases initial viscosity of 1 M NaCl solution of 0.97 centistokes monotonically with increasing molecular weight. The relative increase in viscosity goes from 1.7 for PEG 200 to 25.4 for PEG 17,000. The polymer-induced osmotic tension, measured by vapor pressure, monotonically decreases with polymer molecular weight from PEG 200 to PEG 17,000. The results are in good agreement with the membrane osmometry data (Parsegian et al., 1986) that were used in the present study. The somewhat smaller values measured by the vapor pressure osmometer on large polymer solutions (the maximum deviation was about 20%) may be related to the high viscosity of these solutions that hinders vapor equilibration in the osmometer chamber.

To measure osmotic pressures plotted in Fig. 2 we dissolved polymers in salt-free water in order to eliminate the effects of changing salt activity on vapor pressure. As shown before (Bezrukov and Vodyanoy, 1993), polyethylene glycols increase dissolved salt activity due to water-binding properties of these polymers (Boni and Hui, 1987). Vapor pressure measure-

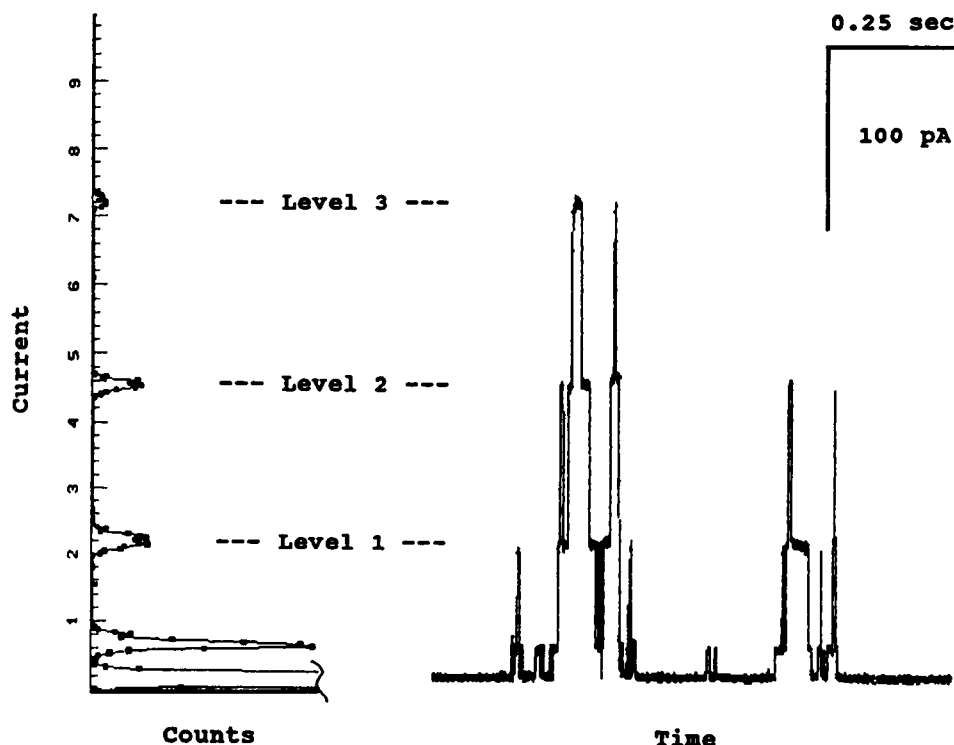


FIGURE 1 An example of a current histogram (relative units, left) and a single alamethicin channel current recording (right) at 100 mV of transmembrane voltage in 1 M NaCl solution. It is typical that channel activity occurs in "bursts." The lipid bilayer here was formed from 1- α -diphytanoylphosphatidylcholine (PC).

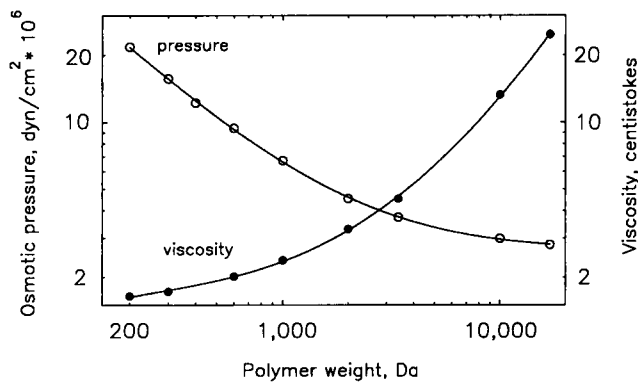


FIGURE 2 Measured osmotic pressure (left scale) and viscosity (right scale) of polyethyleneglycol/water solutions as functions of polymer molecular weight. Weight/weight concentration of PEGs was 15% for all solutions.

ments performed for PEGs dissolved in 0.1 and 1 M NaCl solutions demonstrated nonadditivity of polymer- and salt-induced effects. The increase in vapor pressure upon addition of PEG to salt solution was much greater (3.3 times for PEG 3,400 in 1 M NaCl) than upon addition to a distilled water. In the case of dextran, which was shown not to influence NaCl activity (Bezrukov and Vodyanoy, 1993), the readings of vapor pressure were additive within the accuracy of our measurements.

RESULTS

The action of 15 weight/weight % of PEG 20,000 on the conductance of a multichannel membrane is shown by Fig. 3. To obtain steady-state conditions, measurements were started an hour after membrane formation. The membrane was held at gradually reduced potentials (from +100 mV to +50 mV, alamethicin side positive) to maintain membrane current at the $\sim 10^{-9}$ A that corresponds to 10–20 simultaneously open channels. Then, after membrane conductance stabilized, membrane bathing solutions were switched (arrows) from polymer-free to polymer-containing several times without damaging the membrane (Bezrukov and Vodyanoy, 1993).

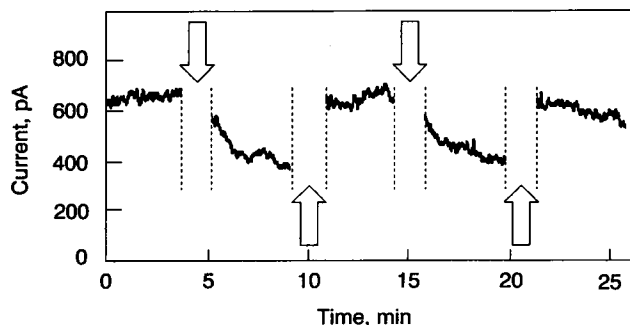


FIGURE 3 Effect of reversible perfusion of initial membrane-bathing solution (1 M NaCl) with polymer-containing solution (15 w/w % of PEG 17,000 in 1 M NaCl) on multichannel PC bilayer conductance induced by alamethicin at 50 mV of transmembrane voltage. Downward arrows correspond to polymer introduction; upward arrows signify perfusion with polymer-free solution.

Typically (downward arrows, Fig. 3), introduction of the polymer decreases membrane conductance. However, this change in membrane bathing solution also induces certain relaxation phenomena on a time-scale of several minutes. Return to a polymer-free solution (upward arrows) restores membrane conductance to its initial level.

Several effects contribute to the “integral” behavior of multichannel conductance in response to polymer addition. These can include: (a) Osmotic action itself, which is expected to shift the channel population to states of lower hydration (Zimmerberg and Parsegian, 1986). (b) The polymer-induced change in channel access resistance that modifies the integral conductance (Bezrukov and Vodyanoy, 1993). (c) The PEG binds water molecules and changes salt activity and channel conductance (Bezrukov and Vodyanoy, 1993). (d) Added polymer may significantly influence partitioning of alamethicin molecules between aqueous phase and membrane lipid thus changing the average number of active ionic channels.

In order to separate contributions from different mechanisms, most experiments were necessarily performed on single channels. Measurements were taken 10 min after solution change. The effect of PEG 3,400 on single alamethicin channels is shown in Fig. 4. As previously demonstrated (Bezrukov and Vodyanoy, 1993), PEG 3,400 is excluded from the alamethicin channel and thereby should impose an osmotic stress to collapse the channel to lower conductance states. The polymer-induced decrease in relative probability of higher states is clearly seen in Fig. 4.

The action of PEG 3,400 and PEG 2,000 on relative probabilities of levels 2–5 for three different solution conditions is shown in Fig. 5. The effect, measured as the logarithm of probability, is stronger for states of higher conductance and appears to be linear with the number of the level. This linearity suggests that polymer-inaccessible water volume of the channel increases by approximately equal amounts at each channel transition to a higher state (see Discussion).

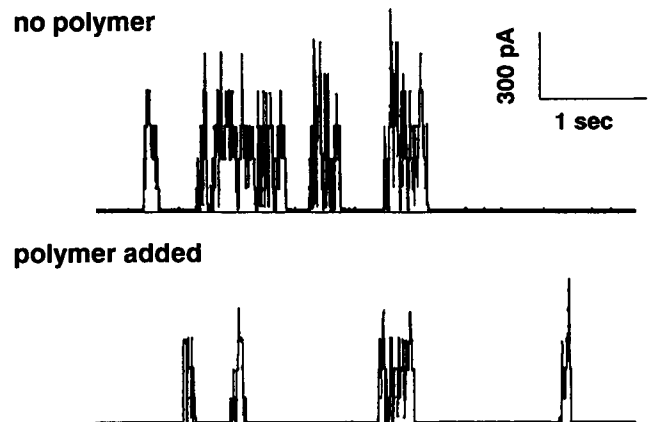


FIGURE 4 Examples of single channel recordings showing the “damping” effect of polymer addition on alamethicin channel behavior. Channels are reconstituted in PE bilayers in 1 M NaCl solution at 130 mV. Upper track, control recording. Bottom track, recording after addition of 15% PEG 3,400. Compare with multichannel recording in Fig. 3.

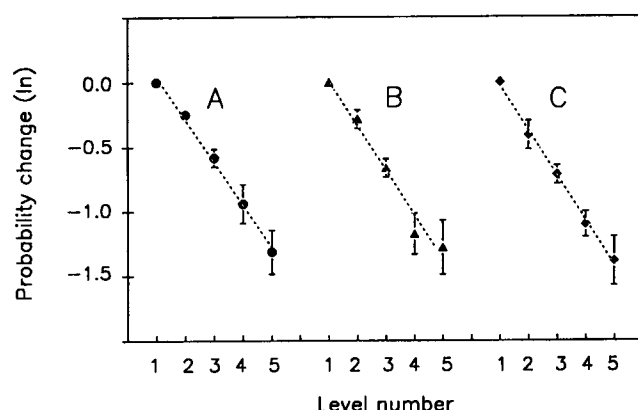


FIGURE 5 Changes in the relative level probability induced by 15% PEG concentration as functions of conductance level number. By definition (see Methods), the change in Level 1 relative probability is zero. Membranes were formed from PE in 1 M NaCl solution. (A) Effect of PEG 3,400 at 100 mV transmembrane voltage; (B) PEG 2,000 at 100 mV; (C) PEG 3,400 at 130 mV.

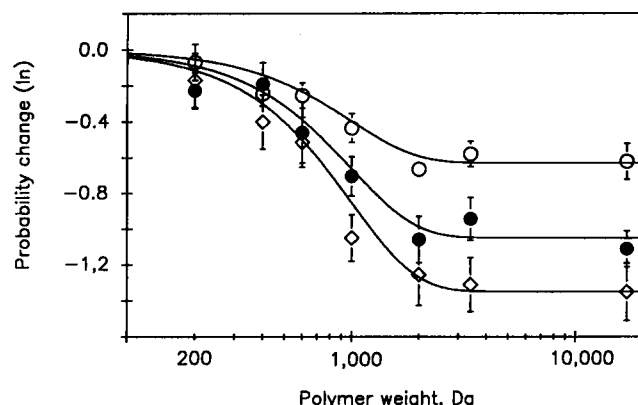


FIGURE 6 Dependence of the change in the relative level probability on polymer molecular weight. Changes were induced by polymer osmotic pressures of 4.5×10^6 dyn/cm². Level 3, open circles, level 4, filled circles, level 5, diamonds. Curves through the experimental points are drawn according to Eq. 8 with conductance-derived polymer partition coefficient. Bilayers were formed from PE in 1 M NaCl solutions. Transmembrane voltage was 100 or 110 mV.

The dependence of the osmotic action on polymer molecular weight is given in Fig. 6. Measurements were made at the same osmotic pressure from all polymers. That is, polymer concentrations were adjusted to give the same osmotic pressure of 4.5×10^6 dyn/cm². Corresponding weight/weight concentrations were: 2.9% of PEG 200; 5.3% of PEG 400; 6.8% of PEG 600; 9.0% of PEG 1,000; 12.7% of PEG 2,000; 15.0% of PEG 3,400; 19.1% of PEG 17,000. Fig. 6 shows that osmotic action begins to be lost for polymer molecular weights of 600–1,000 Da.

The effect of changing viscosity on the channel dynamics was studied by measuring state dwell times in the presence of different polymers. The results (Table 1) show no significant change of dwell time with added polymer. An increase of dwell times with applied voltage is clearly seen, but any polymer-induced effect is null within the error of measurement.

DISCUSSION

Addition of polymers changes several system parameters. In particular, it increases solution viscosity and induces osmotic stress on structures with polymer-inaccessible cavities. We argue that:

1. The dominant parameter governing the change in channel time behavior on polymer addition is polymer-induced osmotic pressure. It lowers higher conductance level probabilities to decrease the average duration of a channel "burst."
2. The extent of probability decrease induced by completely excluded polymers reveals some $3,000 \text{ \AA}^3$ of water taken up from bulk solution into the polymer inaccessible channel cavity upon each transition to a higher adjacent conductance level.
3. Data for partially excluded polymers show that PEG with molecular weights smaller than 1000 Da produce decreased osmotic responses. Partial osmotic action combined with thermodynamic analysis then provides an operational definition of polymer partition as it varies with polymer molecular weight.

TABLE 1 Channel dwell times at different levels in polymer-free and polymer-containing solutions

	Lipid	Voltage	Level 1	Level 2	Level 3	Level 4	Level 5
No polymer	PC	130 mV	3.3 ± 0.5	5.4 ± 0.5	5.5 ± 1.8		
PEG 200	PC	130 mV	3.7 ± 0.3	4.5 ± 1.1	4.6 ± 2.0		
No polymer	PC	100 mV	2.5 ± 0.3	3.4 ± 0.4	5.0 ± 1.4		
PEG 400	PC	100 mV	2.8 ± 0.5	3.8 ± 0.6	4.2 ± 1.7		
No polymer	PC	100 mV	2.7 ± 0.5	3.6 ± 0.5	5.5 ± 1.0		
PEG 2,000	PC	100 mV	2.7 ± 0.1	3.6 ± 0.2	4.4 ± 1.1		
No polymer	PE	100 mV	1.8 ± 0.3	3.0 ± 0.5	4.7 ± 0.9	5.1 ± 1.5	3.1 ± 1.5
PEG 2,000	PE	100 mV	2.0 ± 0.3	3.1 ± 0.4	4.5 ± 0.7	3.1 ± 1.0	2.3 ± 0.8
No polymer	PE	130 mV	2.1 ± 0.3	4.0 ± 0.4	6.3 ± 1.2	6.9 ± 1.6	4.3 ± 1.6
PEG 3,400	PE	130 mV	2.5 ± 0.3	4.2 ± 0.8	6.7 ± 1.2	5.1 ± 0.9	2.5 ± 0.5
No polymer	PC	150 mV	4.4 ± 0.7	7.6 ± 2.0	9.9 ± 2.6		
PEG 10,000	PC	150 mV	5.3 ± 0.6	7.3 ± 1.7	8.9 ± 3.1		
No polymer	PC	90 mV	2.2 ± 0.2	3.0 ± 0.4	4.4 ± 0.9		
PEG 17,000	PC	90 mV	2.3 ± 0.3	3.2 ± 0.4	4.0 ± 0.8		
No polymer	PC	90 mV	2.2 ± 0.2	3.2 ± 0.5	3.0 ± 1.1		
Dextran	PC	90 mV	2.3 ± 0.2	3.4 ± 0.8	2.3 ± 0.7		

Dwell times are given in milliseconds. The polymer concentration was 15 w/w % (added to 1 M NaCl) for all experiments.

4. Because the channel dwell time at a particular conductance level is insensitive to membrane-bathing solution viscosity, the kinetics of molecular rearrangements corresponding to channel transitions between different conductive states are likely dominated by the membrane matrix.

We will deal first with the osmotic stress of completely and partly excluded polymers; we will then examine nonosmotic phenomena, such as viscosity increase and the redistribution of voltage between channel and bathing solution, that go on with polymer addition.

Osmotic stress

The concept of osmotic stress and associated water as a pair of conjugate variables was introduced into studies of ionic channels by Zimmerberg and Parsegian (1986). They showed that application of osmotic pressure shifts the equilibrium of a system toward the states corresponding to smaller volumes of polymer-inaccessible water. The quantitative measure of this shift provides information about the amount of associated water that changes upon transitions between channel states.

Our experiments with alamethicin channels show big changes in inaccessible water volumes upon transitions of the channel between states of differing conductance. Table 2 presents the volume changes, calculated for nonpenetrating polymers, that is polymers with molecular weights 2,000 Da or bigger, according to:

$$\Delta v_i = -kT\Delta \ln P_i / \Delta \pi_{\text{osm}}(W), \quad (1)$$

where k is Boltzmann's constant, T is absolute temperature, Δv_i the difference in volumes of polymer-inaccessible water between state i and reference state 1, P_i is the probability of state i relative to the probability of state 1, and

$\Delta \ln P_i$ the change in this relative probability due to added osmotic pressure $\Delta \pi_{\text{osm}}(W)$ induced by a polymer with molecular weight W . The mean volume of polymer-inaccessible water changing with each channel transition between adjacent states obtained from the data in Table 2 is $\langle \Delta v_{n \rightarrow n+1} \rangle = (3.3 \pm 1.0) 10^3 \text{ \AA}^3$.

Now we can estimate the total change in polymer-inaccessible water during a single-channel "burst" (see Figs. 1 and 4). It is clear that the average volume of channel-associated water is defined by the relative probabilities to find the channel at a particular conductance level during the burst. Recently it was shown that these probabilities depend strongly on membrane composition and correlate with lipid spontaneous curvature in the inverted hexagonal phase (Keller et al., 1993). In particular, states of higher conductance are more probable in PE, the lipid of higher curvature, than in PC, the lipid of lower curvature. Thus, the average amount of polymer-inaccessible water during the course of a burst is higher for alamethicin channels in PE than for those in PC lipids. It may be estimated as the probability weighted sum of water volumes at different conductance states. Assuming that state 1 requires the same amount of polymer-inaccessible water we can calculate the average volume v of inaccessible water intake during single-channel burst as

$$v = \langle \Delta v_{n \rightarrow n+1} \rangle \sum_i i P_i / \sum_i P_i, \quad (2)$$

where P_i is a relative probability of state i . Given the probability distribution of the different states in PC and PE bilayers, we obtain average change in water volumes of the alamethicin channel during a single "burst" of about 5,000 and 10,000 \AA^3 , respectively.

This volume is different from that of the amount of water taken up in the transition to a higher adjacent conductance

TABLE 2 Osmos-induced level probability change and corresponding polymer-inaccessible water volumes

Lipid	Polymer	P	Transition	$\Delta \ln P$	ΔU	Remarks
		10^6 dyn/cm^2			10^3 \AA^3	
PE	PEG 2,000	5.25	$1 \rightleftharpoons 2$	-0.285	2.2	15%
			$1 \rightleftharpoons 3$	-0.665	5.2	100 mV
			$1 \rightleftharpoons 4$	-1.175	9.3	1 M NaCl
			$1 \rightleftharpoons 5$	-1.280	10.1	
PE	PEG 3,400	4.5	$1 \rightleftharpoons 2$	-0.250	2.3	15%
			$1 \rightleftharpoons 3$	-0.585	5.4	100 mV
			$1 \rightleftharpoons 4$	-0.940	8.6	1 M NaCl
			$1 \rightleftharpoons 5$	-1.315	12.1	
PC	PEG 2,000	5.25	$1 \rightleftharpoons 2$	-0.448	3.5	15%
			$1 \rightleftharpoons 3$	-0.816	6.4	100 mV
						0.1 M NaCl
PC	PEG 3,400	4.5	$1 \rightleftharpoons 2$	-0.362	3.3	15%
			$1 \rightleftharpoons 3$	-0.725	6.7	100 mV
						0.1 M NaCl
PC	PEG 17,000	3.2	$1 \rightleftharpoons 2$	-0.287	3.7	15%
			$1 \rightleftharpoons 3$	-0.671	8.7	100 mV
						0.1 M NaCl
PC	Dextran	1.8	$1 \rightleftharpoons 2$	-0.180	4.1	20%
			$1 \rightleftharpoons 3$	-0.391	9.0	100 mV
						1 M NaCl

state, an incremental volume change that appears to be *independent* of the lipid type.

Hydrostatic pressure and osmotic stress

Hydrostatic pressure and total volume are another pair of conjugate variables describing the free energy of a system. Studies of hydrostatic pressure effects on reaction rates and equilibria yield information on system density changes accompanying the reaction (Asano and Le Noble, 1978).

The action of hydrostatic pressure on alamethicin channels in bilayer membranes was studied by Bruner and Hall (1983). Apparent activation volumes for both the formation and decay of conducting states were found to be positive and of comparable magnitude, about 100 Å³. The application of hydrostatic pressures of 100 MPa (1,000 atm) lengthened the life-times of the channel states by five to seven times and changed the kinetics of the onset of alamethicin conductance after a voltage pulse. The magnitudes of conductance levels were not altered by pressure. This observation demonstrates the rigidity of the alamethicin channel in a conductive state and is in accord with the rigidity observed under the osmotic stress of water-soluble nonpenetrating polymers (Bezrukov and Vodyanoy, 1993), where the change in conductance levels was well described by changes in channel access resistance and dissolved salt activity.

Osmotic stress and hydrostatic pressure are different system variables and probe different properties of the channel. Hydrostatic pressure experiments give some effective activation volume, the meaning of which can be very uncertain for complex systems (Asano and Le Noble, 1978). Interpretation of osmotic stress data is far more straightforward; it supplies information about the change in system hydration upon its transition to another state and, in the case of ionic channels can be particularly simple. It is the change in the water volume confined by a water-filled pore of a channel which large polymers can not enter.

We can add also that in some sense the alamethicin channel is more sensitive to osmotic pressure induced by excluded polymers than to hydrostatic pressure because osmotic stress acts on at least order-of-magnitude bigger characteristic volumes. Indeed, the volume of water that enters an opening channel is an altogether different quantity from the small volume change that goes with the change in system density upon channel transition between conductance states.¹

¹ The distinction between applied hydrostatic pressure P and controlled water activity μ_w as independent system variables has been discussed in detail by Parsegian and Zimmerberg (1993). In particular, Eq. 4 of that reference gives a Gibbs-Duhem relation showing clearly that sensitivity to hydrostatic pressure depends on system volume (or weight density), while sensitivity to water activity depends on the number of solute-excluding water molecules associated with the channel (as in Eq. 6 of the present text). There is no reason why changes in density should be identical to the change in number of associated water molecules. Hydrostatic (Conti et al., 1982;

Osmotic effects induced by partly excluded polymers

To discuss osmotic stress effects for small polymers, we will first extend the theoretical treatment of Zimmerberg and Parsegian (1986) and Zimmerberg et al. (1990) to the case of partially excluded polymers.

We will consider the osmotic stress effects for an ionic channel with partial entry of polymers starting with a general statement that the ratio of probabilities to find the channel in states ι and m may be written as

$$P_{\iota}/P_m = \exp[-(\mu^{\iota} - \mu^m)/kT], \quad (3)$$

where μ^{ι} and μ^m are free energies of states ι and m . The osmos-induced change in this ratio is found through change in free energies of the states. Treating these states separately with N_w and N_p being the numbers of water molecules or polymer monomer units going into either state we have

$$\begin{aligned} d\mu^{\iota} &= -N_w^{\iota}d\mu_w - N_p^{\iota}d\mu_p, \\ d\mu^m &= -N_w^m d\mu_w - N_p^m d\mu_p, \end{aligned} \quad (4)$$

where μ_w and μ_p are chemical potentials of water and polymer (normalized per monomer). Using the Gibbs-Duhem equation for the coupling between water and polymer activity

$$n_w d\mu_w + n_p d\mu_p = 0, \quad (5)$$

we have

$$d(\mu^{\iota} - \mu^m) = -(N_w^{\iota} - N_w^m) \left[1 - \frac{(N_p^{\iota} - N_p^m) n_w}{(N_w^{\iota} - N_w^m) n_p} \right] d\mu_w, \quad (6)$$

where n_w/n_p is the water/polymer mole ratio in the bathing solution. The second term in square brackets of Eq. 6 is a partition coefficient $p(c, W)$, describing the partitioning between channel interior and membrane-bathing solution of polymers of molecular weight W and solution concentration c . First, considering the limit when $p(c, W)$ is independent of polymer concentration, $p(c, W) = p(W)$, and neglecting small changes induced by polymer partitioning to the term $(N_w^{\iota} - N_w^m)$, we can calculate the total change in free energy balance as

$$\begin{aligned} \int_0^{n_p, \Delta\pi_{\text{osm}}(W)} d(\mu^{\iota} - \mu^m) \\ = (v_{\iota} - v_m)[1 - p(W)]\Delta\pi_{\text{osm}}(W), \end{aligned} \quad (7)$$

where $v_{\iota} - v_m$ is the water volume change associated with the channel transition from state m to state ι in polymer-free solution. Here we used equations $d\mu_w = -v_w d\pi_{\text{osm}}$ between μ_w and π_{osm} , where v_w is water partial molar volume, and $v_{\iota} - v_m = (N_w^{\iota} - N_w^m) \cdot v_w$.

Bruner and Hall, 1983) and osmotic (Zimmerberg and Parsegian, 1986; Zimmerberg et al., 1990; Rayner et al., 1992) stresses probe different properties of the system.

More careful integration of Eq. 6 should take into account a nonlinearity of polymer partitioning into the channel versus polymer concentration and consequent reduction in the number of water molecules, $N_w^t - N_w^m$, due to substitution of water by polymer.²

Eqs. 3 and 7 yield a formula

$$\Delta \ln P_i = -(v_i - v_1) \Delta \pi_{\text{osm}}(W)[1 - p(W)]/kT, \quad (8)$$

which describes the change in relative probability of level i induced by osmotic stress from polymers of different molecular size.

Taking the reduction in channel conductance as a measure of polymer penetration,³ we obtain the polymer partition coefficient, $p(W)$, independently from the osmotic data. Then using this $p(W)$, we predict what should be the osmotic response due to partial exclusion. These predictions (Fig. 6, *lines*) are in remarkable agreement with experimental data (Fig. 6, *points*) on partial osmotic action. From these lines we calculate volume changes Δv_3 , Δv_4 , and Δv_5 equal to $5.8 \cdot 10^3$, $9.6 \cdot 10^3$, and $12.3 \cdot 10^3 \text{ \AA}^3$, respectively.

In this way the polymer-size dependence of osmotic effects allows one to evaluate the degree of polymer exclusion from the alamethicin channel. At the same time the size contour of exclusion lets one gauge the size of the pore independently of estimations using the osmotic stress of fully excluded large polymers.

Non-osmotic effects

Polymer addition itself, in a way that is not directly related to osmotic stress, may affect channel probability distribution. The most important such effect of polymer addition is, in our opinion, the redistribution of applied electric potential divided between the ion channel proper and the region of access resistance. As was found previously (see Table 2 of Bezrukov and Vodyanoy, 1993), the relative contribution of the alamethicin channel access resistance grows with the conductance level. Nonpenetrating polymers that reduce the conductivity of bulk solution outside the channel decrease the electric potential drop across the channel itself more ex-

tensively for higher conducting states (Fig. 7 schematically illustrates the potential distribution). Therefore, one should take into account the voltage dependence of the channel dwell time at each conductance level. In voltage-jump current-relaxation measurements of alamethicin-induced conductance, this dependence is seen as an increase in the fast relaxation time with voltage. According to data reported by Boheim and Kolb (1978), the fast-relaxation time is approximately doubled when transmembrane voltage grows by 60 mV (alamethicin side positive). A similar conclusion about dwell time voltage dependence can be made from Table 1 of the present paper. A somewhat higher voltage sensitivity is reported by Latorre and Alvarez (1981) for alamethicin in glycerolmonooleate/cholesterol bilayers, namely a 5-fold increase over a 60-mV range.

Using the results of access resistance measurements (Bezrukov and Vodyanoy, 1993), we can estimate the PEG-induced difference in voltages acting across the channel itself. In 1 M NaCl solutions at 100 mV of applied voltage, the potential drop on the channel itself is 93 mV at Level 1 and 86 mV at Level 3. Addition of 15% PEG will increase access resistance and decrease resistance of the channel itself (due to the PEG-induced change in salt activity) reducing the voltages to 88 and 77 mV, correspondingly. Thus, in experiments with nonpenetrating PEGs the difference in voltages on the channel itself between Level 1 and Level 3 is 11 mV, whereas in the control experiment it is about 7 mV. The deviation in voltage conditions between experiment and control is then 4 mV. This difference will produce only a small effect. Specifically, if a 60-mV voltage shift gives a twofold change in fast-relaxation time, then 4 mV should lead to a change of $2^{4/60}$ that equals to $\exp(0.046)$ or $\sim 5\%$. Of course, this secondary voltage effect could be important, even dominate for ionic channels that are highly voltage-sensitive.

We also considered several other possibilities of nonosmotic polymer action on channel state probabilities. First, polymer addition may influence the solution-membrane partition coefficient for alamethicin, changing the number of

² To evaluate a correction to our simplifications, we integrated Eq. 6 using the empirical dependence of partition coefficient on polymer concentration for PEG 400. First, the data on channel conductance versus polymer concentration (Fig. 3 of Bezrukov and Vodyanoy (1993)) were used to calculate the polymer effect on the conductance of the channel proper. Then, the polymer-induced conductance reduction was approximated by a second-order polynomial, and an empirical relation for the partition coefficient as a function of polymer concentration, $p(c, W)$, was obtained. This relation was used in Eq. 6 for the second term in square brackets. A volume correction term describing the reduction in the number of water molecules in the channel due to polymer partitioning was also introduced. The result of integration showed that the simplified Eq. 7 gives only 13% smaller values of the integral. For clarity here, we defer consideration of this correction to later studies.

³ To do this we assume that open-channel conductance (corrected for access resistance and heightened salt activity) decreases linearly with internal polymer concentration as does the conductivity of polymer solutions in the concentration range of 0–15% (Bezrukov and Vodyanoy, 1993).

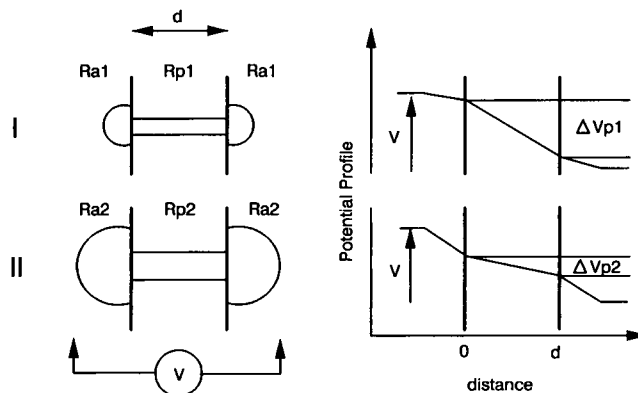


FIGURE 7 Redistribution of electric potential across a membrane containing a single channel switching between low (*top*, *I*) and high (*bottom*, *II*) conductance states. R_a , access resistance, R_p , channel proper resistance. Higher channel conductance incurs greater relative access resistance R_a/R_p and consequent decrease of the voltage drop ΔV_p across the channel itself.

active ionic channels in the bilayer. To minimize the error from a possible change in the number of the channels we have used only the change in a *relative* level probability, that is in the probability to find a channel at a certain level divided by probability to find it at the Level 1.

Second, addition of polymers influences the solution molar ionic concentration and activity as well as its dielectric properties. Changing salt concentration produces a measurable change in the alamethicin channel probability histogram (Hall, 1975). As shown recently (Bezrukov and Vodyanoy, 1993), nonpenetrating PEGs slightly increase salt concentration inside the channel. PEG added at 15% weight/weight concentration to 1 M NaCl solution elevates the ionic concentration inside the channel by 17%. The change in the relative probability of higher conductance states may be estimated from sensitivity of these probabilities to the concentration of salt in the bathing solution. Our experiments showed that a twofold increase in NaCl concentration does not change relative probabilities of levels 2 and 3 by a factor exceeding $\exp(0.3)$. This fact renders negligible any correction due to a 17% salt concentration increase.

Third, the presence of PEGs may, in principle, affect the bilayer itself and thereby change the channel probability histogram. That possibility also does not seem to be the case. Multiple experiments supply evidence against any direct interaction of PEG molecules with bilayer phospholipids (Gawrisch et al., 1988).

Viscosity effects

Typical recordings of current through the channel in polymer-free and polymer-loaded solutions are shown in Fig. 4. The decreased probabilities of higher states driven by osmotic stress "speed up" the channel to produce an effect opposite to that expected from polymer-induced solution viscosity increase.

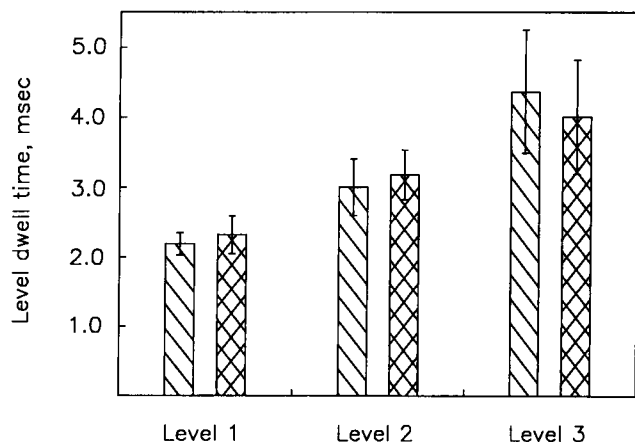


FIGURE 8 Comparison of channel dwell times at different levels in polymer-free solution (left bar in a pair) and solution containing 15% of PEG 17,000 (right bar). A 25-fold increase in viscosity does not induce any change of the channel dwell time. Results are shown for PC bilayers in 1 M NaCl.

Table 1 and Fig. 8 present a comparison of channel dwell times. Even a 25-fold increase in membrane bathing solution viscosity with the biggest PEG used has no measurable effect on dwell times in any state. The same statement applies to the case of small penetrating polymer PEG 200 which increases solution viscosity by a factor of 1.7.

This observation is in good agreement with results reported by Ollmann et al. (1988) on lipid translational diffusion. Only minor effects, less than a factor of two on lipid diffusion, were found in phospholipid bilayer membranes of vesicle suspensions subject to more than 100-fold increase in solution viscosity. The conclusion was drawn that the viscosity of the fluid bounding the lipid bilayers is of minor importance for the diffusion of membrane constituents.

Still, a strong dependence of water-soluble protein structural fluctuations on solution viscosity has been reported for several systems (Gavish, 1986) where the rate of protein conformational change was found to be slowed significantly by increased solvent viscosity. Taken together these observations permit us to discriminate between different models suggested for alamethicin channel formation (Latorre and Alvarez, 1981). We conclude that channel transitions between different conducting states are restricted to events in the membrane matrix whose "viscosity" does not change appreciably with polymer addition.

To sum up, osmotic pressure seems to be the dominant parameter governing the changes in alamethicin channel probability histogram and time behavior. Application of osmotic stress changes the channel pattern, damps higher conductance states and effectively reduces duration of the "bursts." Though osmotic stress does not produce any measurable distortion of the channel structure or dwell time in any particular conducting state, the decrease in probability of higher states reveals some $3,000 \text{ \AA}^3$ of water intake/release upon each transition to an adjacent state. The incomplete osmotic action of partly penetrant, smaller polymers is quantitatively consistent with polymer penetration measured from diminished channel conductance.

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REFERENCES

- Asano, T., and W. J. Le Noble. 1978. Activation and reaction volumes in solution. *Chem. Rev.* 78:407-489.
- Balasubramanian, T. M., N. C. E. Kendrick, M. Taylor, G. R. Marshall, J. E. Hall, I. Vodyanoy, and F. Reusser. 1981. Synthesis and characterization of the major component of alamethicin. *J. Am. Chem. Soc.* 103: 6127-6132.
- Bezrukov, S. M., and I. Vodyanoy. 1991. Electrical noise of the open alamethicin channel. In *Noise in Physical Systems and 1/f Fluctuations*. T. Musha, S. Sato, and M. Yamamoto, editors. Ohmsha, Ltd., Tokyo. 641-644.
- Bezrukov, S. M., and I. Vodyanoy. 1993. Probing alamethicin channels with water-soluble polymers. Effect on conductance of channel states. *Biophys. J.* 64:16-25.
- Boheim, G., and H.-A. Kolb. 1978. Analysis of the multipore system of alamethicin in a lipid membrane. I. Voltage-jump current-relaxation measurements. *J. Membr. Biol.* 38:99-150.

- Boni, L. T., and S. W. Hui. 1987. The mechanism of polyethylene glycol-induced fusion in model membranes. *In* Cell Fusion. A. E. Sowers, editor. Plenum Press, New York. 301–330.
- Bruner, L. J., and J. E. Hall. 1983. Pressure effects on alamethicin conductance in bilayer membranes. *Biophys. J.* 44:39–47.
- Conti, F., R. Fioravanti, J. R. Segal, and W. Stuhmer. 1982. Pressure dependence of the potassium currents of the squid giant axon. *J. Membr. Biol.* 69:35–40.
- Gavish, B. 1986. Molecular dynamics and the transient strain model of enzyme catalysis. *In* The Fluctuating Enzyme. G. P. Welck, editor. John Wiley, New York. 263–339.
- Gawrisch, K., K. Arnold, K. Dietze, and U. Schulze. 1988. Hydration forces between phospholipid membranes and the polyethylene glycol induced membrane approach. *In* Electromagnetic Fields and Biomembranes. M. Markov and M. Blank, editors. Plenum Press, New York. 9–18.
- Hall, J. E. 1975. Toward a molecular understanding of excitability. Alamethicin in black lipid films. *Biophys. J.* 15:934–939.
- Keller, S. L., S. M. Bezrukov, S. M. Gruner, M. W. Tate, I. Vodyanoy, and V. A. Parsegian. 1993. Probability of alamethicin conductance states varies with nonlamellar tendency of bilayer phospholipids. *Biophys. J.* 65: 23–27.
- Latorre, R., and O. Alvarez. 1981. Voltage-dependent channels in planar lipid membranes. *Physiol. Rev.* 61:77–150.
- Montal, M., and P. Mueller. 1972. Formation of bimolecular membranes from lipid monolayers and study of their properties. *Proc. Natl. Acad. Sci. USA.* 65:3561–3566.
- Ollmann, M., A. Robitzki, G. Schwarzmann, and H.-J. Galla. 1988. Minor effects of bulk viscosity on lipid translational diffusion measured by the excimer formation technique. *Eur. Biophys. J.* 16:109–112.
- Parsegian, V. A., R. P. Rand, N. L. Fuller, and D. C. Rau. 1986. Osmotic stress for the direct measurement of intermolecular forces. *Methods Enzymol.* 127:400–416.
- Parsegian, V. A., and J. Zimmerberg. 1993. Channels under osmotic pressure. *In* Thermodynamics of Membrane Receptors and Channels. M. B. Jackson, editor. CRC Press, London. 390–405.
- Rayner, M. D., J. G. Starkus, P. C. Ruben, and D. A. Alicata. 1992. Voltage-sensitive and solvent-sensitive processes in ion channel gating. Kinetic effects of hyperosmolar media on activation and deactivation of sodium channels. *Biophys. J.* 61:96–108.
- Sabirov, R. Z., O. V. Krasilnikov, V. I. Ternovsky, and P. G. Merzliak. 1991. Influence of non-electrolytes on ion mobility in an aqueous solution volume and in the channel cavity. Determination of pore radius from electrical measurements. *Biologicheskie Membrany.* 8:280–291 (in Russian).
- Zimmerberg, J., F. Bezanilla, and V. A. Parsegian. 1990. Solute inaccessible aqueous volume changes during opening of the potassium channel of the squid giant axon. *Biophys. J.* 57:1049–1064.
- Zimmerberg, J., and V. A. Parsegian. 1986. Polymer inaccessible volume changes during opening and closing of a voltage-dependent ion channel. *Nature (Lond.).* 323:36–39.