THE SIZE OF MEMBRANE PORES: THE EFFECT OF NON-ELECTROLYTES ON THE CONDUCTANCE OF GRAMICIDIN*

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The effects of neutral polymers on ion channel conductance have been used in the past to estimate channel radius. We have measured the effect of Polyethylene-glycol and dextrans on gramicidin-D, a peptide ion channel. The availability of high resolution structures of gramicidin-A allows us to make a direct comparison between the characteristic radius obtained by these experiments and the radius of the channel obtained from the NMR structure. The effects of PEG on gramicidin are significantly different from those observed on other, wider channels, and the experiment suggests that the operational size of the gramicidin channel exceeds that seen in the NMR and crystal structures. Our data using non-dehydrating polymers such as dextrans, provide estimates of gramicidin channel size smaller than those obtained with PEGs and closer to those predicted by the NMR and crystal structures.

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1. Introduction

Many of the properties of biological membranes are conferred by proteins embedded in the lipid milieu. In particular, a number of membrane proteins form pores across membranes. Pores range in size from those which permit macromolecules to cross the membrane to those which permit the passage of just one class of ion. We use the word pore to indicate any structure that allows water-soluble molecules to cross the membrane; by convention pores

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that allow only ions to cross the membrane are called channels [1]. Such ion channels are an important class of biological molecules, involved in a wide range of cellular processes. Due to the technical difficulties in working with membrane associated proteins, the number of ion-channel structures that have been solved remain small compared to the wealth of structures available for soluble proteins. Whilst the structural information on ion channels may be comparatively small, the electrophysiological properties of single ion channels have been extensively investigated [2]. These studies show that most of the properties of ion channels can be explained in terms of the physical chemistry of ion flow in a narrow tube of water (the channel) that crosses the membrane.

A number of approaches have been used to study features of ion channel structure based on single channel conductance techniques. One approach is to probe the interior of a channel with neutral polymers of varying size, such as polyethylene glycol (PEG) or dextrans [3, 4].

In a typical PEG conductance experiment, channels are introduced into lipid bilayer membranes which are bathed in a solution containing a set concentration of non-electrolyte. Both PEG and dextrans lower the bulk conductivity of solutions in a manner that is independent of molecular weight but proportional to the mass of dissolved polymer [3]. The rationale behind the experiments is that when small polymers are added to the medium surrounding a channel, they are able to penetrate the channel lumen. The presence of polymer in the channel decreases the conductivity of the channel, just as it decreases the conductivity of the surrounding bulk solution. As the size of polymer is increased, it is increasingly excluded from the interior of the channel, resulting in a restoration of the channel's conductivity. By observing the effect of different sized polymers on the conductance of the channel, a characteristic cut-off radius can be obtained for the channel. Unfortunately, there is no agreed protocol for determining the cut-off radius and various experimental groups have used different methods making direct comparisons difficult [3, 4].

Large PEGs, which are excluded from the lumen of the channel, cause a slight increase in the observed channel conductance. PEGs increase the ion activity in the surrounding solvent, an effect that can be directly observed with a sodium electrode [5]. The increase in ion activity results in a similar increase in channel conductance. Dextrans do not increase ion activity and hence no increase in channel conductance is seen when large dextrans are added to ion channels. The increase in channel conductance seen in the presence of large PEGs can be used to calculate the access resistance of the channel and hence a value for the channel radius [3]. In the case of non-symmetrical channels, different sized PEGs can be added simultaneously to the *cis* and *trans* faces of the channel to obtain the radius of each side [6].

The HOLE [7] program can be used to predict the effects of non-electrolytes on the conductance of a channel, based solely on the atomic structure of the channel. The method allows a set of experimentally testable predictions to be produced for any channel structure. Such predictions can be used to check the validity of model structures, or to relate different channel structures to different conducting states of the channel [8]. So far, most polymer addition experiments have been carried out on channels for which we have limited structural information, such as alamethicin [3], or on channels where there is an ambiguity between the crystal structure and the structure of the channel in lipid bilayers [9], such as α-toxin [4, 10]. A comparison has been made between the experimental and HOLE predicted results for cholera-toxin β_5, with an encouraging degree of agreement [7].

Similarly, the lack of both PEG conductance data and a relevant channel structure has made it difficult to validate the predictions HOLE provides of channel conductance. One way to overcome these problems is to apply the PEG addition methodology to channels of known structure. Here we report our initial results of both polymer addition experiments and HOLE conductance predictions on gramicidin, a peptide ion channel. The properties of gramicidin have been extensively studied, (for reviews see [11] and [12]). We also have a number of high resolution structures of gramicidin in its active, conducting form in lipid bilayers [13, 14].

2. Materials and method

2.1. Single channel recordings

A diphytanoylphosphatidylcholine (DPhPc, Avanti Polar Lipids) bilayer was formed across a 10–20 μm hole in a Teflon sheet, separating two Teflon chambers. 2 ml of buffer was added to each chamber, and a solution of DPhPc dissolved in pentane was added to each chamber. The lipid solution was prepared by evaporating a 25 mg ml^{-1} solution of DPhPc in chloroform under nitrogen, which was then re-dissolved in pentane. A bilayer formed across the hole by lowering and raising the level of the buffer solution on each side of the sheet past the hole. Gramicidin D (ICN) was incorporated into the bilayer. The buffer solution consisted of 1M KCl, 0.005 M Hepes (pH 7.4), to which was added 20% w/v of non-electrolyte. Channel current was measured using Ag/AgCl electrodes in voltage/clamp mode, at applied voltages of 50, 75, 100 and 125 mV. The current was recorded onto both a paper chart recorder and 4mm Digital Audio Tape at a 48.8 KHz sampling rate.

2.2. HOLE conductance prediction

The HOLE conductance prediction procedure was run on the solid state NMR gramicidin A structure [13] (PDB code 1MAG). The simplified AMBER [15] united-atom Van der Waals radii were used for the calculations, and predictions were made for 20% PEG and 20% dextran solutions. For 20% w/w PEG, $\Theta_{\text{peg}} = 1.18$ and $\Theta_{\text{non}} = 0.54$ [16] and for 20% w/w dextran $\Theta_{\text{peg}} = 1.00$ and $\Theta_{\text{non}} = 0.57$ [3].

2.3. Single channel data analysis

Single channel amplitudes were extracted from the current record by computer or by measuring transitions directly from the chart record. The single channel data was down sampled from 48.8 KHz to 200 Hz, and run through a software 5 Hz low pass digital filter. Single channel events were identified using a modified version of the Van Dongen [17] algorithm, described below.

The modified Van Dongen algorithm uses three detection criterion to distinguish between transitions, levels and background noise. Firstly, the whole record is differentiated using the central differences method (Eq.(1)).

$$\frac{\text{damp}_i}{dt} = \frac{\left(\text{amp}_{i+1} - \text{amp}_{i-1}\right)}{2\Delta t}. \tag{1}$$

Transition regions (*i.e.* channel opening or closure events) in the record were defined as regions where damp/dt exceeded $3\sigma_{(\text{damp}/dt)\,\text{noise}}$. Regions where damp/$dt$ was lower than this threshold value were defined as levels. Once this initial assignment was made, the trace was idealised by averaging the amplitude of each level along its length. The original Van Dongen algorithm used both forward and central differences to calculate damp/dt However, as the average lifetime of gramicidin channel events (typically 1–2 seconds) is much larger than Δt (0.005 seconds), the central difference method alone is sufficiently accurate to determine damp/dt.

At this stage the idealised trace contains a number of artifacts due to signal noise. Such artifacts can be distinguished from true channel events by examining both the size of the transition and the duration of the flanking levels. Transitions whose amplitude was smaller than $3\sigma_{\text{noise}}$were interpreted as being due to noise. The transition was removed from the record and the two flanking levels were concatenated into a new level. The amplitude of the new level was calculated by taking a duration-weighted average of the previous two levels. The transitions were removed in an iterative fashion, starting with the smallest transition.

Once the small transition had been removed, short duration levels were then examined. Levels with a duration shorter than 0.1 seconds were re-

moved from the record, as they were likely to be noise rather than the longer lived gramicidin channels. The artifact removal process was repeated until no further artifacts were detected. The magnitude of each channel transition was then calculated by measuring the difference between adjacent levels. Only channel opening events were measured; the closures were discarded to prevent the same event being measured twice. Conductance histograms were calculated for gramicidin in the presence of each non-electrolyte. Mean single channel conductances were calculated by fitting a gaussian curve to the main peak in each histogram.

3. Results

A typical section of single channel recording is shown in figure 1. A conductance histogram for gramicidin in 20% PEG 300 is shown in figure 2. Although the main population of channels have conductances of around 17 pS, there is a broad band of channels that have conductances which fall below the main distribution which have conductances in the 4–12 pS range. The low conductance band is likely to have two causes.

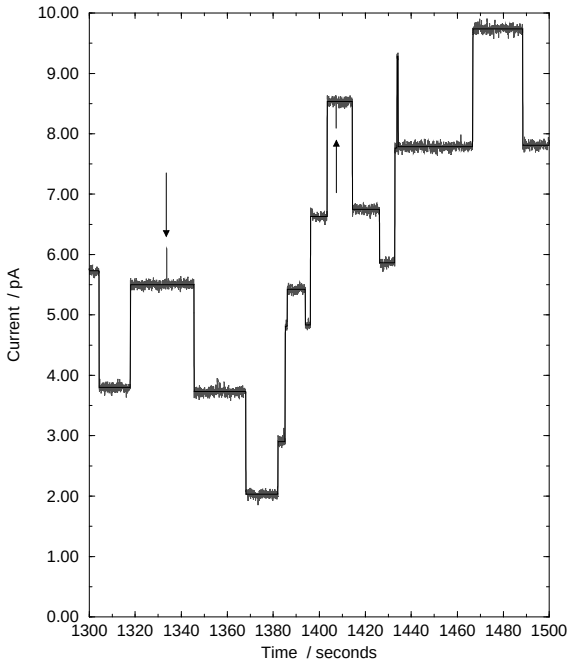

Fig. 1. Current record record for gramicidin in 1M KCl. The applied voltage was 100mV. The idealised trace is superposed in black. The two events highlighted by the arrows are ignored in the idealised trace and have been attributed to noise.

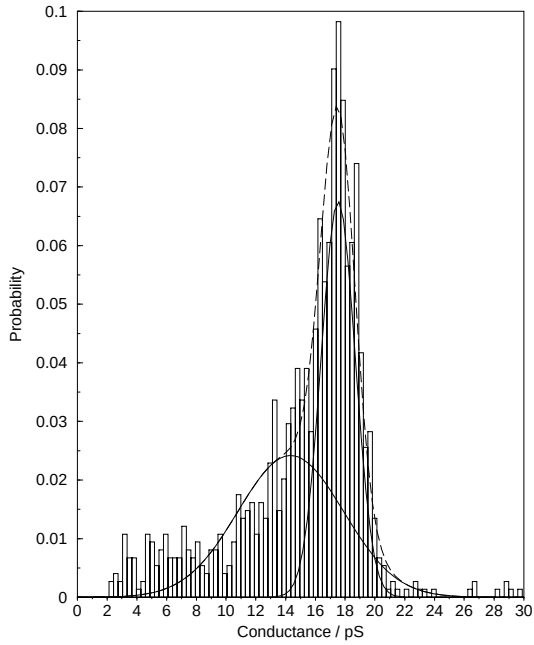

Fig. 2. Conductance histogram for gramicidin D in 20% PEG 300. Two gaussian curves were fitted to the data and the single channel conductance calculated from the main peak. The black dashed line is the sum of the curves.

Firstly, the conductance experiments were carried out with gramicidin D, the naturally occurring gramicidin mixture produced by *Bacillus brevis*. The mixture contains approximately 80% gramicidin A and minor amounts of gramicidins B and C [11]. Gramicidins B and C, although similar in structure to gramicidin A, have conductances that are respectively 38% and 10% lower than that of gramicidin A [18]. Secondly, even highly purified gramicidin can form channels which possess a wide range of lower than normal conductances [19]. These "mini channels" are thought to be caused by gramicidin adopting either an alternative side-chain conformation [20], or a pore structure [12]. It is likely that both of the presence of gramicidins B and C and the formation of "mini channels" account for the presence of the second peak in the conductance histograms.

The low conductance band in the experimental data was ignored in our data analysis. Only the mean conductance of the main peak in the channel population was used to calculate the effect of polymer addition. The change in channel conductance in response to the addition of non-electrolytes is summarised in Table I. The experimental values and the HOLE prediction are shown in figure 3.

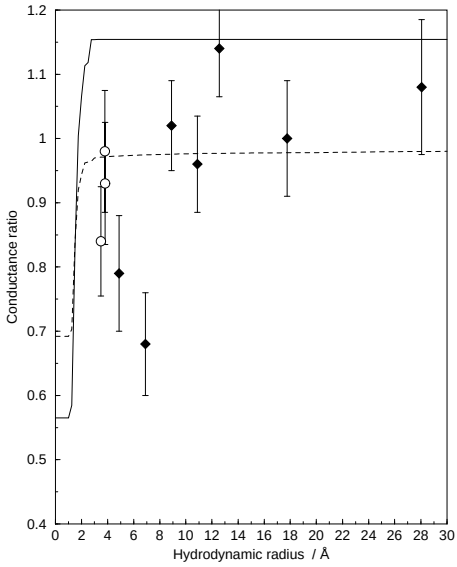

Fig. 3. Comparison of the experimental conductance data with the HOLE predictions. The dextran prediction is shown as a dashed line and the PEG prediction as a black line. The experimental PEG points are shown as black diamonds and the dextran results as black circles. Error bars are 1 standard deviation.

TABLE I

The effect of non-electrolytes on PEG conductance

Substance	Hydrodynamic radius	Conductance ratio	Error
KCl	–	1.0	–
Peg 300	4.9	0.85	0.09
Peg 600	6.9	0.76	0.08
Peg 1000	8.9	0.95	0.07
Peg 1500	1.09	0.96	0.075
Peg 2000	1.26	1.12	0.075
Peg 4000	1.78	1.04	0.9
Peg 10000	2.81	1.14	0.105
Xylitol	3.5	0.84	0.085
Sorbitol	3.8	0.67	0.095
Glucose	4.0	0.87	0.095

Our present data is rather noisy and further experiments are required to fully characterise the channel. However our data does contain some surprising features. Both PEG 300 and PEG 600 decrease the conductance of gramicidin, even though their hydrodynamic radius is too large to allow them to fit inside the channel. The anomalous results for the smaller PEGs results in a large over-estimation for the radius of the channel. The cut-off radius calculated using the Krasilnikov method [10] yields a value of 10.3Å, whilst the Bezrukov [3] method gives a radius of 8Å. However, the pore radius of the NMR gramicidin structure is only 1.15–1.5Å [21].

The effects of dextran addition on gramicidin conductance resemble those of PEG addition. The non-penetrating dextrans, such as glucose, have little effect on conductance and the smaller penetrating sugars lower channel conductance. Although the dextran data is incomplete, the present values suggest a cut-off radius of around 4Å(both Krasilnikov and Bezrukov methods), a value which is much greater than the radius of the channel shown in the NMR structure, but which is closer than the value predicted by the PEG addition experiments.

4. Discussion

The effects non-electrolytes on gramicidin conductance are very different to those which we would expect. Both PEGs and dextrans which are apparently too large to penetrate the channel were still able to lower the conductance of gramicidin, resulting in an overestimation of channel size. It is not possible to say whether the sizes of other channels measured using PEG have been overestimated, as in most cases we do not have experimental structures with which to compare the results. However, in the case of alamethicin [3], both PEG and dextrans were used to probe the channel, and both sets of experiments yielded the same value for the channel radius. In this light it may be that the disparity between the PEG and dextran results is peculiar to very narrow channels such as gramicidin.

The results of PEG addition experiments on a larger channel, cholera toxin β_5, are shown in figure 4. For comparison the experimental data for gramicidin is overlaid on the cholera toxin data, which it closely resembles. This comparison could lead to the conclusion that the dimensions of both channel are identical. However, as is clear from figure 5, the radius of cholera toxin is much greater than that of gramicidin.

How do small PEGs and dextrans alter the ability of ions to permeate through gramicidin if they are unable to fit inside the channel? The obvious explanation is that other effects, in addition to steric ones, affect gramicidin conductance.

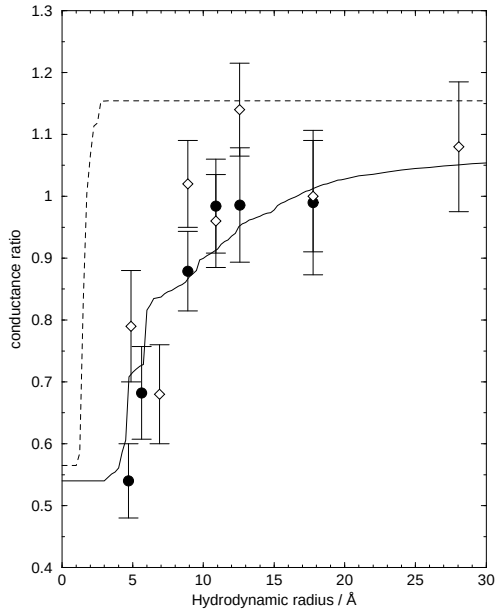

Fig. 4. A comparison of the cholera toxin β_5 experimental data (circles) and HOLE prediction (Black line) with the gramicidin data (diamonds) and prediction (dashed line).

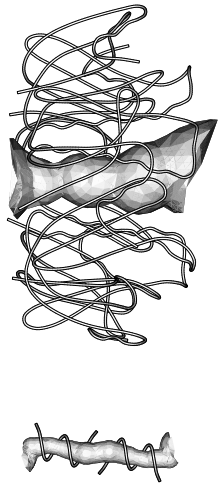

Fig. 5. A HOLE diagram of the pores in cholera toxin (top) and gramicidin (bottom). Both molecules are drawn to the same scale. The protein backbone is shown as a ribbon and the channel is shown as a solid surface. The channel radius of cholera toxin is clearly greater than that of gramicidin. This figure was generated using MOLSCRIPT [25].

One possibility is that PEG interferes with the ion binding site of gramicidin [22]. Prior to transport down the lumen of gramicidin, permeant ions are de-solvated. This process occurs at an ion binding site at the mouth of the channel, where water ligands are exchanged for carbonyl groups from the gramicidin backbone. Such a mechanism could explain how non-penetrating PEGs could lower channel conductance. In order to test this hypothesis, we are endeavouring to repeat PEG addition experiments in the presence of ions with a larger de-solvation energy than K^+, such as Li^+or Na^+.

Our experiments also bring into question the suitability of gramicidin as a model ion channel. Ideally, we expect small channels to behave in a similar fashion to larger channels. It is possible that PEG lowers the conductance of all ion channels by its non-steric mechanism, but that the effect is small compared to the change in conductance caused by PEGs steric interaction with the channel. Thus the non-steric PEG interaction may only become significant when experiments are performed on channels which have very low conductances, such as gramicidin. The use of non-permeant molecules to facilitate closure of gated ion channel by an osmotic mechanism [23] also seem more clear cut with larger channels than smaller ones [24].

In summary our experiments suggest that determining size of channels with cut off radii > 10Å using non electrolytes is reliable and predictable from the published structure using the computational approach of the HOLE program. In the limit of very narrow channels, such as gramicidin, we cannot be confident that this approach remains applicable. It may be more appropriate to use large channels of known structure, such as the porins, as test systems for our methodology.

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