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# A Novel Approach to Study the Geometry of the Water Lumen of Ion Channels: Colicin Ia Channels in Planar Lipid Bilayers

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**Abstract.** This paper describes a new approach to evaluate the inner structure (including a main constriction and its localization) of the water lumen of an ion channel. The method is based on the determination of channel filling by different nonelectrolyte molecules through each side of an ion channel. The method has two characteristic features that make its use attractive: (i) the possibility to ascertain the existence, localization and size of a narrow part inside an ion channel water lumen and (ii) the chances to determine the maximal size of both entrances of an ion channel and to obtain additional information about the geometry of its water lumen at the same time. Determinations were made on colicin Ia ion channels inserted into planar lipid bilayers. This channel was chosen because there is an apparent contradiction between its low single channel conductance and the large diameter of its water lumen. Our results show that the water lumen of the colicin Ia channel has a funnel-like structure with a small *trans*-entrance, with a diameter of about 1.0 nm, and a large *cis*-entrance, with a diameter of approximately 1.8 nm. A constriction with a diameter of approximately 0.7 nm is shown to be located close to the *trans*-entrance of the channel. The method can also be applied to patch clamp studies of single ion channels.

**Key words:** Methods — Nonelectrolytes — Ion channel — Colicin Ia — Planar lipid membranes

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## Introduction

The geometry of the water lumen of most ion channels is poorly understood and guessed mostly from experiments based on the determination of the voltage-dependence of blockade of single channel currents by large organic ions (Gadji-Zade & Silberstein, 1984; Borisova & Ermishkin, 1984; Hemsley & Busath, 1991; Villarroel, Burnashev & Sakmann, 1995; Bullock & Kolen, 1995). This methodology does not take into account electrostatic interactions between probing ions and ionogenic groups in the channel that can lead to uncertainties in the evaluation of the actual geometry of the ion channel.

Electron microscopy and X-ray diffraction studies (Olah et al., 1991; Guo & Mannella, 1993) rely on fixed ion channels, a procedure which could induce changes in the native structure of the channel-forming protein. A recently developed method, based on the scanning tunneling microscope, seems more appropriate (Kolomitkin et al., 1991) although the conditions required by the method are still far from ideal.

On the other hand, noncharged molecules have been used in experiments to determine the apparent size of polyene- and protein-induced single ion channels (Holz & Finkelstein, 1970; Silberstein, 1989; Krasilnikov, Sabirov & Ternovsky, 1991; Vodyanoy & Bezrukov, 1992; Krasilnikov et al., 1992; Sabirov et al., 1992, 1993; McKim & Hinton, 1994). These determinations are free from the limitations intrinsic to the methods mentioned above and the data correlate well with results from electron microscopy studies. However, they do not answer questions regarding the internal structure of an ion channel water lumen. We describe here a new method based

on the use of nonelectrolytes as molecular tools which can give results concerning the size of each entrance of an ion channel as well as the presence and apparent localization of structural constrictions inside an ion channel water lumen. The colicin Ia-induced ion channel in planar lipid bilayer membranes was chosen as a model because it has a water lumen of large apparent diameter associated with a low conductance of the open state (Nogueira & Varanda, 1988; Slatin et al., 1993; Krasilnikov et al., 1995). Considering these facts, the main purpose of the present investigation was to find and describe a method that could give information about the geometry of the water lumen of the colicin Ia-induced ion channel.

## Materials and Methods

Planar lipid bilayer membranes were formed at room temperature (25 ± 2°C) by the technique of Montal and Mueller (1972). Monolayers were spread from a 10 mg/ml solution of lipids (Asolectin-Type II, Sigma, purified by acetone extraction (Kagawa & Racker, 1971)) in n-hexane on the surface of two buffered salt solutions (4 ml) separated by a 25 μm thick Teflon partition in a Teflon chamber. After solvent evaporation the membrane was formed by raising the monolayers above the level of the hole (~0.1 mm in diameter) connecting the hemichambers through the partition. The hole was pretreated with a solution of 2% Vaseline in n-pentane. Lyophilized colicin Ia was a generous gift from Dr. S. Slatin (Albert Einstein College of Medicine, NY).

The composition of the standard solution bathing both sides of the membrane was: 1.77 M KCl, 2 mM EDTA, 10 mM $CaCl_2$, and 5 mM HEPES. pH was adjusted to 7.0 with 1.0 M KOH. In the experiments carried out to determine the channel geometry the above solution also contained 20% (w/v) of an appropriate nonelectrolyte. The following nonelectrolytes were used: ethylene glycol (Sigma), glycerol (Sigma), arabinose (Sigma), sorbitol (Sigma), maltose (Sigma), poly(ethylene glycol) (PEG) 300 (Sigma), PEG 400 (Sigma), PEG 600 (Riedel de Haen), PEG 1000 (Sigma), PEG 3000 (Loba Chema), and PEG 6000 (Reagen, Brazil). Polyethylene glycols were the molecules of choice in our experiments because in water solutions they have a spherical shape (Rempp, 1957; Mark & Flory, 1965). All nonelectrolyte polymers were additionally purified in the laboratory by anion-exchange chromatography using strong alkaline anion exchangers III or IV (Merck) to remove contaminating anion-groups. Other chemicals were analytical grade and were used without any additional purification. Twice-distilled water was used to prepare all solutions.

The current flowing through the bilayer was measured under voltage-clamp conditions with an operational amplifier (OPA 111) in the current to voltage configuration connected to the preparation via a pair of Ag/AgCl electrodes and salt bridges (3% agar with 3.0 M KCl). Bilayer formation was followed by continuously monitoring the capacitive current in response to square voltage pulses (±5–10 mV; ~500 Hz). Voltage pulses were applied to the *cis*-compartment of the chamber where colicin Ia was also added. The *trans*-compartment was connected to virtual ground. Current signals were monitored with a storage oscilloscope (Nicolet Instrument Corporation, Model 201) and recorded on a strip chart recorder (Hewlett Packard 70158 X-Y Recorder). Current traces were read by hand and single channel conductances were estimated by dividing the single channel current by the imposed voltage through the membrane. Recording of single channels was extended in time until a 25% increase in the amount of observed single channel events did not change the mean values of the channel conductances by more than 1% (0.6–0.8 pS). This criterion was used to decrease as much as possible the error in the channel size determination since we have experimentally found that a 1% alteration in the mean conductance value leads to a 3% error in the determination of the channel radius. This almost invariant mean value was used in the subsequent analysis. The relatively small amount of lower conductance steps (0–30 pS) was not considered in this mean value.

The Student *t*-test was used to determine the significance of the difference between mean values of single channel conductances obtained under different experimental conditions.

The conductivity of each buffer solution was measured with a multi-range conductivity meter (HANNA Instruments-model HI 9033) at 25°C.

## Theoretical Considerations: The Concept of Channel Filling

### *Rationale*

The central idea on which our experiments were based is the determination of *channel filling* with different nonelectrolytes (NE) through each channel entrance. In this way different small NE are placed in contact with one of the entrances of the channel, while impermeant large NE are in contact with the other side of the channel. The method is based on the changes in ion channel conductance observed when distinct NE are added to the bathing solutions. This effect is due both to a decrease in the conductivity of the solutions and to the fact that the NE enter the channel lumen and impair the mobility of the ions inside the channel. As a corollary, molecules that do not enter the lumen should have no effect on single channel conductance. In this way, the dependence of single channel conductance on the hydrodynamic radii of different NE molecules can be used to determine the diameter of the entrances of an ion channel, the geometry of the channel lumen and a possible constriction inside it.

Nonelectrolytes have been used in the determination of the pore size of bacterial channels incorporated into planar lipid bilayers since 1988 (Krasilnikov et al., 1988) and an empirical permeability parameter was suggested as the main tool for obtaining the desired information (Krasilnikov et al., 1992; Sabirov et al., 1993). To expand the physical understanding of the use of NE in the estimation of a channel size, we will assume in this paper than an ion channel can be treated as an equivalent ohmic resistor with resistance ($R$). As a first approach, this assumption can be extended to all channels showing a liner current-voltage relationship. In our case $R$ can be seen as composed of two parts: one corresponding to the portion of the channel length filled with the NE ($F$) and the other corresponding to the portion without NE ($1 - F$). With these assumptions $R$ can be written as:

$$R = [F/(A\chi_i) + (1 - F)/(A\chi_0)] \quad (1)$$

where $A = \pi r^2/l$, $l$ is the length of the channel and $r$ its radius, and $\chi_0$ and $\chi_i$ are the conductivities of the solution without and with NE, respectively. Assuming that $A\chi_0$ is equal to the ion channel conductance in the presence of a solution without NE ($g_o$), it can be easily shown that the filling ($F$), will be given by:

$$F = [(g_o - g_i)/g_i]/[(\chi_0 - \chi_i)/\chi_i] \quad (2)$$

where $g_o$ is the single channel conductance in the presence of an impermeant NE or the single channel conductance in a solution without NE, $g_i$ is the single channel conductance in the presence of a solution containing 20% (w/v) of an NE with access to the channel interior from

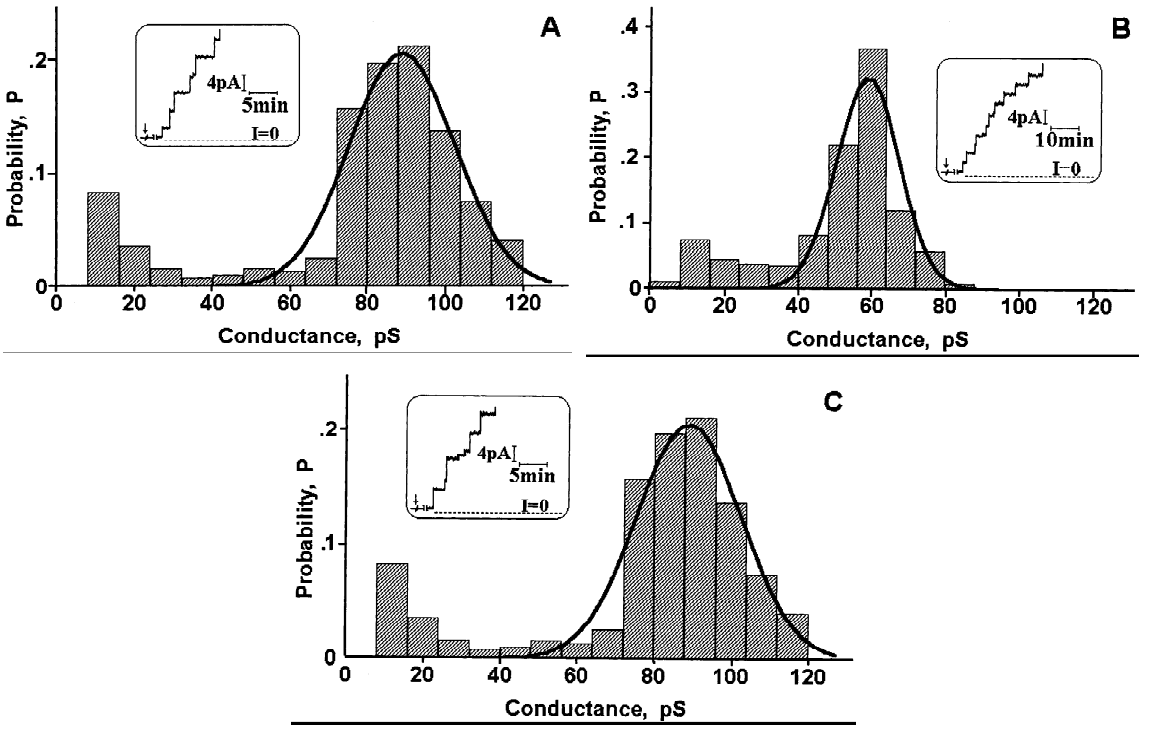


**Fig. 1.** Distribution of single channel conductances. Histograms were constructed from the observation of 409, 314 and 180 events for control solution (*A*), in the presence of 20% ethylene glycol (*B*) and in the presence of 20% PEG1000 (*C*), respectively. A total of 5–7 channels were present in each membrane and recording was stopped if any of the previously open channels temporarily closed; a new bilayer was formed and the experiment continued. *P* is the probability of finding a given conductance step. Bin size is 8 pS. The line indicates the fitting of a theoretical normal distribution to the main pool of events. The mean value of this distribution (peak) was taken as the single channel conductance for subsequent analysis. Colicin Ia was added (arrow in the inset) to the *cis*-compartment to a final concentration of 50–100 ng/ml and the bilayer clamped to +50 mV. The insets show original recordings of the single channel current vs. time and the broken line indicates the zero current level. In these particular experiments the bathing solutions contained: (*A*) 1.77 M KCl, 2 mM EDTA, 10 mM $CaCl_2$, 5 mM HEPES, pH = 7.0; (*B*) same as *A* plus 20% (w/v) ethylene glycol; (*C*) same as *A* plus 20% (w/v) PEG1000.

both sides or from only one of them, $\chi_o$ is the conductivity of the solution without NE or the conductivity of the virtual volume free of NE in a solution with NE, and $\chi_i$ is the conductivity of the solution containing 20% (w/v) of a given NE.

Equation 2 has a clear physical meaning, i.e., it describes the filling of a channel with NE. This equation is very similar to an empirical equation used for ion channel radius determination published elsewhere (Krasilnikov et al., 1991, 1992; Sabirov et al., 1993):

$$\nu = [(g_o - g_i)/g_o]/[\chi_0 - \chi_i)/\chi_0] \quad (3)$$

Where $\nu$ was defined as a permeability parameter, and the other symbols are the same as defined above.

## Results

### The Conductance of the Channel in the Presence of a Nonelectrolyte with Access to the Channel Interior

According to previously published data (Krasilnikov et al., 1995), and as shown in Fig. 1*A*, the conductance of the most frequently observed channel size formed by colicin Ia is in the range of 60 to 120 pS, in symmetrical 1.77 M KCl (pH = 7.0). In the presence of a small well permeant NE at both channel entrances the single channel conductance decreases, as evidenced in Fig. 1*B*. On the other hand, a large nonpermeant NE, PEG1000 for example, produces no effect on the single channel conductance (Fig. 1*C*). The mean value of the conductance, estimated from the main pool (Fig. 1), in the presence of NE on both sides of the channel will be referred to as $g^{both}$ in all experiments.

In the next set of experiments we measured the conductance of the channel with different NE in contact with each opening at the same time. Let us call the side of the channel that protrudes from the plane of the membrane into the *cis*-side (side of colicin addition) the *cis*-entrance and the other side the *trans*-entrance. To determine the filling from the *cis*-entrance a solution containing an impermeant NE (PEG 1000 in our experiments) is placed at the *trans*-entrance. The *cis*-side of the membrane will contain different NE molecules with hydrodynamic radii ranging from 0.26 nm (for the highly permeant ethylene

**Table 1.** Average single channel conductances in the presence of nonelectrolytes in the bathing solutions

| Nonelectrolyte | $g^{both}$ | $g^{cis}$ | $g^{trans}$ | $\chi$ |
|---|---|---|---|---|
| 1. None | 88.8 ± 13.6 (352) | | | 192.5 |
| 2. Ethylene glycol | 58.8 ± 8.9 (260) | 62.2 ± 11.9* (226) | 67.3 ± 7.6* (155) | 109.6 |
| 3. Glycerol | 56.0 ± 6.8 (264) | 59.4 ± 10.4* (109) | 65.6 ± 6.3* (108) | 105.8 |
| 4. Arabinose | 49.4 ± 10.1 (294) | 54.1 ± 8.7 (206) | 84.0 ± 13.0$^{\#}$ ( 80) | 105.8 |
| 5. Sorbitol | 56.6 ± 7.5 (221) | 60.9 ± 8.1 (180) | 84.6 ± 10.1$^{\#}$ (175) | 104.8 |
| 6. Maltose | 58.0 ± 10.7 (269) | 68.7 ± 10.6 (196) | 88.7 ± 11.7* (184) | 107.8 |
| 7. PEG300 | 65.0 ± 9.3$^{b}$ (188) | 65.5 ± 13.5$^{b}$ (284) | 87.8 ± 9.7* ( 88) | 95.7 |
| 8. PEG400 | 68.0 ± 8.6 (174) | 73.7 ± 10.1 (146) | 91.0 ± 9.75* ( 92) | 103.2 |
| 9. PEG600 | 83.4 ± 14.2$^{b}$ (274) | 82.8 ± 12.8$^{b}$ (193) | 89.0 ± 12.2* ( 85) | 100.6 |
| 10. PEG1000 | 88.5 ± 14.3*$^{b}$ (148) | 88.5 ± 14.3$^{b}$* (148) | 88.5 ± 14.3$^{b}$* (148) | 95.4 |
| 11. PEG3000 | 88.7 ± 12.0$^{b}$* (103) | 88.7 ± 12.3$^{b}$* ( 98) | | 102.0 |
| 12. PEG6000 | 91.6 ± 5.7$^{b}$* (112) | 91.6 ± 5.8$^{b}$* ( 88) | | 97.0 |

Single channel conductances (pS) are expressed as mean ± SD. The numbers in parentheses indicate the number of the single channel events counted in the main pool, which represented more than 80% of all the events observed in a given condition. $g^{both}$ was obtained in the presence of the same NE on both sides of the bilayer; $g^{cis}$ was measured in the presence of a given NE on the *cis*-side of the bilayer while PEG 1000 was present on the *trans*-side, and $g^{trans}$ refers to the conductance measured in the presence of a given NE on the *trans*-side of the bilayer while PEG 1000 was present at the *cis*-side. * and $^{\#}$ mark the neighboring values in columns with $P > 0.02$ (*t*-test). $^{b}$ marks neighboring values in rows with $P > 0.02$. In all other cases $P < 0.005$. $\chi$ (mS/cm) are the conductivities of the solutions. Other conditions are as described in the legend to Fig. 1 and in the text.

glycol) up to the radii of impermeant NE molecules. To determine filling from the *trans*-entrance the impermeant NE (PEG 1000) is now placed at the *cis*-side of the channel. These two maneuvers will give us the conductance of a single ion channel in the presence of the test NE at the *cis* ($g_i^{cis}$) or at the *trans* ($g_i^{trans}$) entrance of the channel. As a first approach, if the channel lumen behaves like a perfect cylinder we would expect $g_i^{cis}$ to have a value close to $g_i^{trans}$. On the contrary, if $g_i^{cis}$ and $g_i^{trans}$ are different from each other there is a high possibility that the channel entrances have different sizes. Moreover, these results also suggest the presence of a constriction inside the channel lumen. Its localization as well as the sizes of both channel entrances can also be established.

Table 1 shows the values of the above parameters for the colicin Ia channel. In this case $g_i^{trans} \geqslant g_i^{cis} \geqslant g_i^{both}$. Such asymmetry in distribution of conductances is monotonous and statistically significant. This together with the multiple channel incorporations as shown in Fig. 1 is only possible when all or the vast majority of the channels incorporate into the bilayer in a specific manner when they face *cis*-compartment with one side, and *trans*-compartment with another side. The asymmetric incorporation of colicin Ia channel in lipid bilayer is supported by our (*not shown*) and other published observations (Qiu et al., 1996) that all channels opened at +50 mV failed to turn off at −50 mV.

Analysis of the data also shows that even in the presence of small well permeant NE such as ethylene glycol and glycerol, the values of $g_i^{both}$ are almost always lower than the values of $g_i^{cis}$ and $g_i^{trans}$. This difference can result from a physical asymmetry in the channel and/or from an osmotic pressure imbalance between the *cis* and *trans* solutions. This latter effect may be present in our experiments because the osmotic pressure generated by a 20% solution of PEG1000 only partially compensates for the osmotic pressure generated by a 20% solution of NE with smaller molecular masses. The osmotic water flux established through the channel could, in principle, decrease the amount of NE inside the channel. However, the influence of this force should be small in our experiments since diffusional motion of particles should dominate over convective flow at all reasonable hydrostatis pressure differences across the membrane (Bezrukov, Vodyanoy & Parsegian, 1994). These investigators have shown that for a particle with a radius of 0.5 nm (close to size of the molecules used in the present experiments) the flow transient time and diffusion relaxation time become equal only at hydrostatic pressure differences of about $10^8$ Pa. This is much larger than observed in our experiments ($<10^7$ Pa).

Looking through the data one can see that $g_i^{trans}$ is often larger than $g_i^{cis}$. The differences observed between the values of $g_i^{cis}$ and $g_i^{trans}$ strongly suggest the existence of a physical asymmetry in the channel, since some factor has prevented its complete filling by the NE through one of its openings, but not through the other.

Table 1 also shows that $g_i^{cis}$ reaches a maximum for NE having the relatively large hydrodynamic radii ($R \geqslant$ 0.9 nm). The same trend can be observed for $g_i^{both}$. Hence the size of the ''*cis*''-entrance is close to the maximum value already established for the colicin Ia channel in contact with the same NE on both sides.

In contrast, $g_i^{trans}$ increased with hydrodynamic radii up to 0.5 nm (maltose) and then became practically constant at this maximum value. These facts are consistent with the hypothesis that the ''*trans*''-entrance of the co-

**Table 2.** Filling the colicin Ia channel with nonelectrolytes

| Nonelectrolyte | M.W.(Da) | $r$ (nm) | $F^{both}$ | $F^{cis}$ | $F^{trans}$ |
|---|---|---|---|---|---|
| 1. Ethylene glycol | 62 | 0.26 | 0.68 | 0.56 | 0.42 |
| 2. Glycerol | 92 | 0.31 | 0.72 | 0.60 | 0.43 |
| 3. Arabinose | 150 | 0.34 | 0.97 | 0.78 | 0.07 |
| 4. Sorbitol | 182 | 0.39 | 0.68 | 0.55 | 0.06 |
| 5. Maltose | 360 | 0.50 | 0.68 | 0.37 | 0.00 |
| 6. PEG300 | 300 | 0.60 | 0.36 | 0.35 | 0.01 |
| 7. PEG400 | 400 | 0.70 | 0.35 | 0.23 | 0.03 |
| 8. PEG600 | 600 | 0.80 | 0.07 | 0.08 | 0.00 |
| 9. PEG1000 | 1000 | 0.94 | 0.00 | 0.00 | 0.00 |
| 10. PEG3000 | 3000 | 1.44 | 0.00 | 0.00 | |
| 11. PEG6000 | 6000 | 2.50 | 0.03 | 0.03 | |

The NE were used at a concentration of 20% (w/v) in a solution containing: 1.77 M KCl, 0.002 M EDTA, 0.01 M $CaCl_2$, 0.005 M HEPES, pH 7.0. $F^{both}$, $F^{cis}$ and $F^{trans}$ are the ion channel filling in the presence of the same NE on both-, on the *cis* and on the *trans*-side of the bilayer, respectively. $F^i$ were calculated according to Eq. 2. $r$ are the hydrodynamic radii of NE taken from Krasilnikov et al. (1991, 1992) and Sabirov et al. (1993). The standard deviation of $r$ never exceeded 5% of its average value. Other conditions are as described in the legend to Fig. 1 and in Table 1.

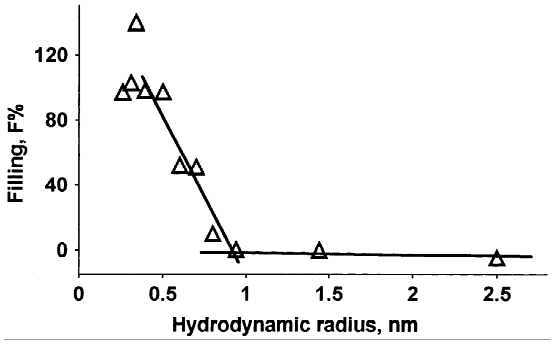


**Fig. 2.** The dependence of $F^{both}$% on the hydrodynamic radii of nonelectrolytes. $F^{both}$% (△) for each NE was calculated according to Eqs. 2 and 4. Lines are best fits to the experimental points. The horizontal lines connect the points measured in the presence of PEG1000, PEG3000 and PEG6000. Another linear regression was used to describe the points for radii ranging from 0.39 nm to 0.94 nm. The deviation bars were equal to or smaller than the symbols used. Hydrodynamic radii of NE were taken from Table 2. All other experimental conditions are as described in the legend to Fig. 1 and in Materials and Methods.

licin Ia channel has a much smaller radius than the ''*cis*''-one.

This type of data can also be used to determine the apparent sizes of the channel entrances by analyzing the relationship between $g^i$ and the NEs hydrodynamic radii. However, to obtain a more precise measurement of the channel radius one can use either the empirical permeability parameter ($v$) or the filling ($F$) as suggested in the ''Theoretical considerations'' section. Table 2 shows the calculated values of $F$. One can see that in spite of equilibrium between the bathing solution and the solution inside the ion channel lumen the concentration of NE inside the channel is lower than that in the bulk solution. Although this can be caused by several effects, these effects may be overlooked by simply assuming that the filling of the channel by two of the smallest NE, namely ethylene glycol and glycerol, is close to the maximum possible level. This assumption allows us to calculate the filling in terms of percentage ($F$%) as follows:

$$F\% = 2F_i/(F_1 + F_2)*100\% \quad (4)$$

where $F_i$ is the filling in the presence of a given NE and $F_1$ and $F_2$ represent filling in the presence of ethylene glycol and glycerol in the bathing solutions, respectively. This procedure minimizes the error in the determination of filling. The results of the dependence of $F^{both}$% on the hydrodynamic radii of the NE are shown in Fig. 2. One can see that if the size of the NE did not exceed 0.5 nm, $F$% was always close to 100%, as is the case for ethylene glycol ($r$ = 0.26 nm), glycerol ($r$ = 0.31 nm), sorbitol ($r$ = 0.39 nm) and maltose ($r$ = 0.5 nm). $F$% for arabinose exceeded 100% probably because this NE is producing particular effects on the conductance of the channel different from those of the other small NE mentioned above. The reason of such an influence of arabinose is not clear to us. One may speculate that it is reflecting a specific interaction between arabinose and the channel, occurring most likely in a constriction inside the channel lumen whose size is close to the size of the arabinose molecule. Aside from this deviation, increasing the hydrodynamic radius causes a decrease in the filling parameter. In this way PEG 300 and PEG 400 are able to fill about half of the channel and a much smaller volume is occupied by PEG600. PEG with larger molecular mass (and larger hydrodynamic size) do not enter the channel at all.

## Filling the Ion Channel through One of its Entrances: Establishing the Size of the Channel Entrances and Localizing a Constriction

Let us now analyze how the percentage value of the channel filling through its *cis* ($F^{cis}$%) and *trans*-entrances ($F^{trans}$%) depends on the hydrodynamic radii of the NE. Calculations of the parameters were done using Eqs. (2) and (4). Briefly, the use of $F$% ($F^{cis}$% and $F^{trans}$%) should allow us to find the maximum size of both entrances of the channel as well as the size and (unlike $F$) the localization of the constriction on the channel length coordinate. The results are presented in Fig. 3.

One can see in Fig. 3 that if the channel is filled from its *trans*-entrance ($F^{trans}$%) the maximum filling is

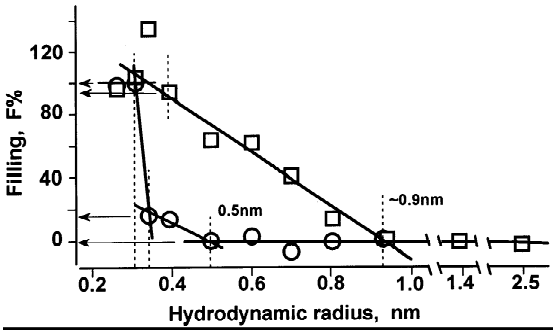


**Fig. 3** The dependence of $F^{cis}$% (□) and $F^{trans}$% (○) on the hydrodynamic radii of nonelectrolytes. The values of $F^{cis}$% and $F^{trans}$% of each NE were calculated as described in the text. The lines are least-square fits to the experimental points. For $F^{cis}$% one was fitted to the points for NE with radii ranging from 0.5 nm to 0.94 nm and another to the points obtained with PEG1000, PEG3000 and PEG6000. For $F^{trans}$% one line fitted the points from 0.34 nm to 0.5 nm and another horizontal line fitted all points from 0.5 to the largest radius used. The deviation bars are equal to or smaller than the symbols used. Broken lines indicate the values of the radius of colicin Ia-induced water pores. Arrows indicate the localization of the transition points on the channel length coordinate (*F*%). Hydrodynamic radii of NE were taken from Table 2. All other experimental conditions are as described in the legend to Fig. 1 and in the text and Materials and Methods.

achieved only with ethylene glycol ($r = 0.26$ nm) and glycerol ($r = 0.31$ nm). Increasing the radius to 0.34 nm (arabinose), caused a sharp decrease in *F*% to a value of about 15%. A less pronounced slope is seen for molecules with sizes between those of arabinose and maltose ($r = 0.34$–$0.5$ nm). Molecules with radii larger than 0.5 nm did not enter the channel from the *trans*-entrance at all ($F^{trans}\% \sim 0$). Therefore, we may assume that the limiting size for the radius of the *trans*-entrance of the channel should not exceed 0.5 nm.

When the channel was filled from its *cis*-entrance ($F^{cis}$%) maximal filling was observed in the presence of ethylene glycol, glycerol and sorbitol. Filling by arabinose was anomalous, being more than 100%. This anomaly was not observed when the channel was filled with arabinose coming from the *trans*-side, suggesting the existence of a specific site for interaction of arabinose with colicin Ia present on the *cis*-side of the channel. From the data, we may conclude that the maximum radius of the *cis*-entrance should be equal to 0.9 nm (Fig. 3), a value about twice that of the radius of the *trans*-entrance.

Figure 3 also shows that the dependence of $F^{trans}$% on the hydrodynamic radii of the NE is characterized by three distinct slope regions, contrary to what was seen both for $F^{cis}$% and $F^{both}$% (Figs. 2 and 3) where a single line was enough to connect all points from 0.31 nm until 0.94 nm. For $F^{trans}$% a first sharp decline in F% is seen for molecular sizes between 0.31 nm and 0.34 nm, when it reaches a low value of about 15%. A second region of interest is located between arabinose (0.34 nm) and maltose (0.5 nm), where $F^{trans}$% decreases less abruptly and reaches a constant value close to zero. For NE with radii larger than 0.5 nm $F^{trans}$% stays constant. Therefore, we are forced to conclude that a molecule with a radius of 0.31 nm or less should pass freely through the channel and that the narrowest portion of the ion channel lumen, a constriction, should have a radius measuring 0.31 nm to 0.34 nm.

## Discussion

### The Geometry of the Colicin Ia Channel

The geometrical parameters of the channel can be derived from out data by the following line of reasoning. Let us compare two possible structures for an ion channel pore, i.e., cylindrical and funnel-shaped. Let us assume that the solution inside the channel is in thermodynamic equilibrium with the bathing solutions. Then for an ideal cylindrical structure all permeant NE should fill the channel to the same maximum extent. This fact will hold up to the point where the radius of a particular NE becomes equal to the radius of the channel. After this point, we expect a sharp decrease in the extent of filling. Hence for an ideal cylindrical structure the NE used in this study should behave as two distinct groups; one which will fill the channel completely and another that will not fill the channel at all. Only a few NE, or even none of them, should fill the channel partially. An apparent incomplete filling ability is possible only for polymers having a wide dispersion in molecular mass. By the same reasoning, for a funnel-like structure we expect to find several NE able to completely fill the channel, given that the hydrodynamic sizes of the molecules are smaller than the size of the narrowest part of the channel. On the other hand, when the hydrodynamic size of the NE molecules becomes larger than the size of the largest entrance of the channel, the NE will not fill the channel at all and intermediate-sized NE will fill the channel to an extent inversely related to their sizes.

The depth of the channel reached by those intermediate-sized NE will depend on the angle between the funnel walls and on the hydrodynamic size of the NE molecules. As a first approximation, the depth can be considered to be equal to the value of the filling (*F*%). The dependence of *F*% on the hydrodynamic size of the molecules with incomplete filling ability should reflect the geometry of the channel lumen. This dependence has to be linear for an ideal conical channel. This seems to be the case for $F^{cis}$% as shown in Fig. 3, indicating that the colicin Ia channel should have a funnel-like structure. If a constriction of a smaller size than any of the entrances is present inside the channel lumen then the maximal depth of the channel reached by and filled with NE with partial filling ability must reflect the localization of that constriction inside the channel lumen. NE

with molecules smaller than the size of the constriction should lead to a stepwise increase in *F*% until a maximum value is reached. Hence, the dependence of *F* on the hydrodynamic radii of the NE should be discontinuous, as is the case for $F^{trans}$% shown in Fig. 3. This fact is a clear indication that the colicin Ia channel has a constriction.

## Localizing the Constriction—Cylinder Model Approach

With the above reasoning in mind, we can now correlate the slope of the lines seen in Fig. 3 with a varying radius of the channel along its length. In other words, *F*% can be taken to represent a channel length coordinate (in percentage) where a value of 0% for $F^{cis}$% represents the ''*cis*-entrance'' and a value of 0% for $F^{trans}$% represents the ''*trans-entrance.*'' This distance can be taken from the *F*%-axis. In this way, the *cis*-filling experiment demonstrated that the radius of the channel lumen is wide at this entrance ($r \sim 0.9$ nm) and gradually decreases until the narrowest part of the channel is reached. The actual size of that zone and its localization can be established in the following way. As seen before, a molecule with a hydrodynamic radius equal to 0.39 nm is able to fill ~90% of the channel, while all NE with $r \leqslant 0.31$ nm are able to completely fill the channel. The maximal depth of the channel reached by the NE with incomplete filling capacity can be considered to be equal to the distance from the entrance to the constriction zone in the channel lumen. The radius of this zone should now be equal to the radius of the smallest NE that do not pass freely through the channel and do not fill it completely; $r = 0.39$ nm in our case.

The *trans*-filling experiment, in turn, showed that the radius of the *trans*-entrance of the channel started at ~0.5 nm and then decreased to 0.34 nm. Since the maximal depth of the channel reached by the NE with incomplete filling capacity ($r = 0.34$–$0.39$ nm) did not exceed 15%, the predicted constriction must be located at a distance ~15% from the *trans*-entrance. The size of the constriction can be derived from the following line of reasoning. As one can see in Fig. 3 ($F^{trans}$%), the stepwise increase in channel filling from 15% to 100% was observed when the hydrodynamic radii of the NE decreased from 0.34 nm to 0.31 nm only. Hence, from this trans-filling experiment the minimum value of the ion channel radius at the constriction zone can be considered to be equal to 0.34 nm.

## Localizing the Constriction—Cone Model Approach

As a first approximation, the above assumption of a cylindrical pore led us to picture the colicin Ia channel as having two different entrances, resembling a conical

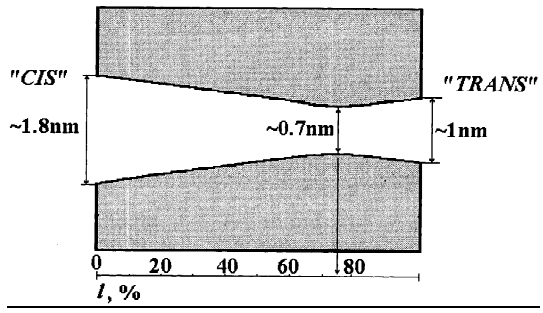


**Fig. 4.** An inside view of the colicin Ia channel lumen. *l* is the channel length in % starting from the *cis* entrance (0%) and ending at the *trans*-entrance (100%). The constriction (diameter ~0.7 nm) is situated at ~75% of the channel length.

structure. Hence, in this section we will use a cone as a model of the channel and will provide conclusions regarding the localization of the barrier. In the presence of an NE with incomplete filling ability at the *cis*-side the filling of a conical structure will be determined by:

$$F = A/(B + C) \tag{5}$$

where $A = (r_i^3 - r_c^3)/tg\alpha$ is the channel volume filled with NE; $B = (r_{cis}^3 - r_c^3)/tg\alpha$ is the channel volume taken from the *cis*-entrance to the constriction; $C = (r_{trans}^3 - r_c^3)/tg\beta$ is the channel volume taken from the *trans*-entrance to the constriction, $r_i$ is the hydrodynamic radius of the NE, $r_c$ is the radius of the channel lumen at the constriction point, $r_{cis}$ and $r_{trans}$ are the radii of the *cis*- and *trans*-entrances of the channel, and $tg\alpha = (r_{cis} - r_c)/l_{cis}$; $tg\beta = (r_{trans} - r_c)/l_{trans}$; $l_{cis}$ and $l_{trans}$ are the distances from the *cis*- and from the *trans*-entrance to the constriction, respectively.

Using the experimental values of *F* one can calculate the ratio $tg\beta/tg\alpha$, which is equal to:

$$tg\beta/tg\alpha = K = F_i(r_{trans}^3 - r_c^3)/[(r_{cis}^3 - r_c^3) - F_i(r_{cis}^3 - r_c^3)] \tag{6}$$

The mean value of *K* was found to be $0.89 \pm 0.52$ ($n = 7$). Then the ratio $l_{cis}/l_{trans} = K(r_{cis} - r_c)/(r_{trans} - r_c)$ should be 3.25 or close to a 75:25 ratio in terms of percentage. Therefore, by assuming a conical model the predicted constriction would have to be placed at a distance ~25% from the *trans*-entrance. From these findings the following picture emerges for the colicin Ia channel (Fig. 4).

## Nonelectrolytes in Contact with Both Sides of the Channel: Comparing Methodologies

In this section we will compare results of ion channel radius determination obtained from the relationship between conductance and hydrodynamic radii ($g - r$), from the empirical permeability parameter (*v*, defined in Eq.

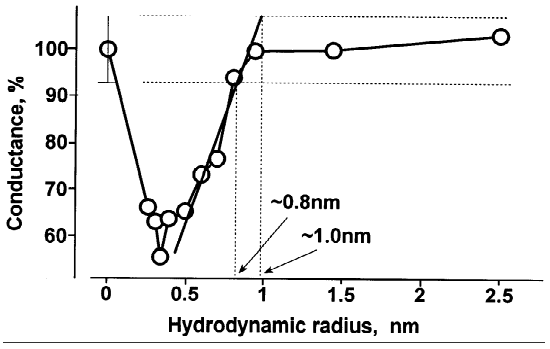


**Fig. 5.** Single channel conductance ($g^{both}$%) depends on the hydrodynamic radii of the nonelectrolytes added to both sides of the bilayer. Single channel current events were recorded in symmetrical solutions containing 20% (w/v) of a test NE and the conductance was measured as indicated in the legend to Fig. 1. The solid line is a best fit to the experimental points in the radius range of 0.5 and 0.94 nm. The horizontal dashed lines indicate the boundaries of the half deviation of the highest observed conductance. Arrows indicate minimal and maximal values of the apparent radius of the colicin Ia-induced water pore. All other conditions are as described in Materials and Methods. Hydrodynamic radii of NE were taken from Table 2.

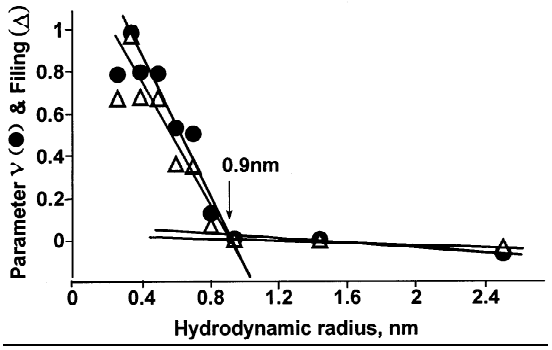


**Fig. 6.** The dependence of $F^{both}$ and of parameter $\nu$ on the hydrodynamic radii of nonelectrolytes. $F^{both}$ (△) and the permeability parameter $\nu$ (●) for each NE were calculated according to Eqs. 2 and 3. The quasi-horizontal lines connect the points measured in the presence of PEG1000, PEG3000 and PEG6000. Another linear regression was used to describe the points for radii ranging from 0.39 to 0.94 nm. The deviation bars are equal to or smaller than the symbols used. The arrow indicates the maximal value of the radius of colicin Ia-induced water pores. Hydrodynamic radii of NE were taken from Table 2. All other experimental conditions are as described in the legend to Fig. 1 and in Materials and Methods.

3) and from filling ($F$). It is implicit in all three methods that the channel radius will be considered equal to the minimum hydrodynamic radius of a given NE, whose presence in the bathing solutions does not decrease the ion channel conductance and which, therefore, does not enter the channel mouth at all. Figure 5 shows the relationship between $g$ and $r$ for a colicin Ia channel. As can be seen, NE with hydrodynamic radii not exceeding 0.5 nm induced a significant reduction in the ion channel conductance (*see also* Fig. 1), which follows very closely the decrease in the conductivities of the bathing solutions. Increasing the radii of the nonelectrolytes from 0.5 to 0.8 nm decreases their influence on the conductance because their ability to enter the ion channel is progressively impaired. NE molecules with radii equal to or larger than 0.9 nm did not decrease the channel conductance at all. Hence from this result one can draw the conclusion that the colicin Ia channel should have a radius with an upper limit in the 0.8 to 1.0 nm range.

Figure 6 shows results for the colicin Ia channel obtained in the presence of the same NE on both sides of the bilayer and analyzed according to Eqs. 2 and 3. As expected, both $F$ and $v$ are in the range 0.3–1.0 for small NE such as ethylene glycol, glycerol, arabinose, sorbitol and maltose. Further increases in the size of the NE decreases $F$ and $v$, until a practically constant low limit close to zero is attained. From this plot the radius of the larger entrance of the channel should correspond to the hydrodynamic radius observed at the transition point between the falling line and the quasi-horizontal portion of the plot. As can be seen, both parameters give practically the same result (~0.9 nm) for the radius of the larger entrance of the channel, which is close to the size of PEG 1000 (radius = 0.94 nm) and are more accurate than those obtained from the $g - r$ relationship. In addition, there is a striking similarity in the maximal channel size (~0.9 nm) calculated from the analysis of $F^{both}$, $v$ (Fig. 6) and $F^{cis}$% (Fig. 3). This fact allows us to conclude that measurements of single channel conductance with different NE present on both sides of the bilayer is a reliable method for the determination of the size of the larger entrance of an ion channel.

## The Contradiction Between Single Channel Conductance and the Size of the Colicin Ia Channel

Measurements reported by several authors (Raymond, Slatin & Finkelstein, 1985; Kayalar & Duzgunes, 1986; Slatin, 1988; Bullock, Kolen & Shear, 1992) have established that the colicin E1 channel has a diameter larger than 0.8 nm. However, the exact size and details of the structure of the water lumen of the colicin-induced water pores have not been determined. More recently Krasilnikov et al. (1995), analyzing the $v - r$ dependence, found that PEG with a molecular weight $\geqslant$1000 Da did not enter the channel formed by colicin Ia. This finding led to the suggestion that the diameter of this channel should have an upper limit close to 1.85 ± 0.1 nm. Taking this value into consideration and assuming that the colicin Ia channel can be viewed as a cylinder with a length close to the width of a lipid bilayer (~5 nm), under our experimental conditions (1.77 M KCl in the bathing

solutions) we would expect a single channel conductance of about 9000 pS. However, the actual mean conductance of the colicin Ia channel is almost one hundred times lower (~90 pS; Table 1). To explain such a difference we must assume that the colicin Ia channel has some kind of a barrier against ion movement located inside the lumen of the channel. It seems unlikely that the barrier could be based on electrostatic forces because in the presence of 1.77 M KCl the distance of charge influence should be very small (Debye length ~0.23 nm) and also because the current-voltage relationship for the colicin Ia channel is linear (Nogueira & Varanda, 1988). Based on these considerations, a physical constriction (or barrier) was assumed to exist inside the ion channel lumen (Krasilnikov et al., 1995).

However, the geometrical parameters of the channel derived from our results (*see* Fig. 4) are still considerably larger than expected from the value of the actual single channel conductance (d ~ 0.2–0.3 nm; for a channel length ~5 nm). In our opinion, this discrepancy may result from an oversimplification of the theoretical approach used to calculate channel radius from its conductance, which does not take into account the fact that ions in the channel may be subject to the action of image forces (Markin & Chizmadzhev, 1974; Sizonenko, 1995) or that friction forces hinder ion movement (Antonov, 1982).

## Problems with the Method and Possible Applications

The method described in this paper has some inherent difficulties that can interfere with the final results, namely:

(1) The first of them is the assumption that the hydrophilic NE are inert and do not interfere with the functioning of the ion channel-membrane system. This kind of influence should not be widespread among NE since we used several NE and only arabinose showed an anomalous behavior.

(2) Another difficulty is the requirement for high accuracy in the determination of the mean single channel conductance because we have experimentally found that a change of 1% in the mean value of the single channel conductance leads to a 3% change in the value of $F$.

(3) For channels having much larger conductances and radii than colicin Ia channel the access resistance may become a problem. In our case the contribution of the access resistance, calculated as $R_{acc} = 1/(4r\chi)$ (where $r$ is the radius of the channel entrance and $\chi$ is the solution conductivity (Hall, 1975)) is minimal and should contribute no more than 0.3 pS to the measured conductance. Furthermore, introducing $R_{acc}$ into the filling equation does not change it meaning significantly, as shown below. The actual conductance of the colicin Ia channel ($g^c$) can be written as:

$$g^c = g[g^{acc}/(g^{acc} - g)] \quad (7)$$

where $g$ is the experimental value of the single channel conductance and $g^{acc}$ is the sum of the solution access conductances at the *cis*- and at the *trans*-entrances of the channel. Taking this into consideration, Eq. [2] describing the filling can now be written as:

$$F = [(Cg_o - g_i)/g_i]/[(\chi_0 - \chi_i)/\chi_i] \quad (8)$$

where $C = [g_0^{acc}/(g_0^{acc} - g_0)] \times (1 - g_i/g_i^{acc})$ and the indexes ''$o$'' and ''$i$'' indicate values obtained in the absence and in the presence of NE in the solutions.

From the above equation one can see that the access resistances can have a significant effect on the experimentally determined channel conductance only if $g^{acc}$ is of the same order of magnitude as $g$. This is clearly not the case for the colicin Ia channel. In fact, it can be seen that the access resistances can displace the relationship between $F$ and hydrodynamic radii on the $Y$-axis but should not alter the main feature of this relationship, i.e., the minimal size of NE, which is not able to decrease the channel conductance and therefore does not enter the channel lumen. Hence, the sizes of channel openings derived from $F$ against $r$ dependence could not be affected by the access resistances at all.

(4) A last concern is related to the commercial preparations of polymers which can have a wide dispersion in molecular mass (Scherrer & Gerhardt, 1971). However, the standard deviation in molecular mass and in hydrodynamic radii of NE, obtained from viscosimetric studies (Krasilnikov et al., 1991, 1992; Sabirov et al., 1993), is within the same small range (3–5%). This suggests that the accuracy of the determination of the geometrical parameters of the ion channel should fall within the same range. The accuracy with which we can localize the constriction within the pore also depends on the difference in geometry between an actual channel water lumen and an assumed ideal structure used in the calculation of $F$.

It is worth noting that this method can be used in patch-clamp studies of ion channels. Hence, by using the ''inside-out'' or the ''outside-out'' configuration one can simply add a given NE to the solution external to the pipette and measure single channel conductances. The channel will be filled from the ''intracellular'' or from the ''extracellular'' side depending on which configuration is being used, i.e., ''inside-out'' or ''outside-out,'' respectively. It is possible to obtain results analogous to $g_i^{trans}$ and $g_i^{cis}$ as described in our experiments. Of course, the method may be directly applied only to channels having large water pores that are large enough to accommodate the nonelectrolytes. The chloride channel complex, supposedly related with the mitochondrial porin channel observed in different cells (Thinnes et al., 1990), and chemo-activated channels (acetylcholine re-

ceptor channel in particular) seem to be good candidates because of the large apparent diameter of their pores [2–4 nm for porins and 1–2 nm for acetylcholine receptor channel; Krasilnikov et al. (1996) and Unwin (1995), respectively].

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