J. Membrane Biol. 84, 173–181 (1985)

The Journal of
Membrane Biology

Channels Formed by Colicin E1 in Planar Lipid Bilayers are Large and Exhibit pH-Dependent Ion Selectivity

Lynn Raymond, Stephen L. Slatin, and Alan Finkelstein
Departments of Physiology/Biophysics and Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

Summary. The E1 subgroup (E1, A, Ib, etc.) of antibacterial toxins called colicins are known to form voltage-dependent channels in planar lipid bilayers. The genes for colicins E1, A and Ib have been cloned and sequenced, making these channels interesting models for the widespread phenomenon of voltage dependence in cellular channels. In this paper we investigate ion selectivity and channel size—properties relevant to model building. Our major finding is that the colicin E1 channel is large, having a diameter of *at least* 8 Å at its narrowest point. We established this from measurements of reversal potentials for gradients formed by salts of large cations or large anions. In so doing, we exploited the fact that the colicin channel is permeable to both cations and anions, and its relative selectivity to them is a function of pH. The channel is anion selective (Cl^- over K^+) in neutral membranes, and the degree of selectivity is highly dependent on pH. In negatively charged membranes, it becomes cation selective at pH's higher than about 5. Experiments with pH gradients cross the membrane suggest that titratable groups both within the channel lumen and near the channel ends affect the selectivity. Individual E1 channels have more than one open conductance state, all displaying comparable ion selectivity. Colicins A and Ib also exhibit pH-dependent ion selectivity, and appear to have even larger lumens than E1.

Key Words colicins · channel size · ion selectivity · lipid bilayers

Introduction

Voltage-gated, ion-selective channels, familiar denizens of excitable cell membranes, are now known to inhabit diverse eucaryotic cells. Although the recently devised patch-clamp technique has permitted detailed kinetic studies at the single-channel level of several such channels (Sakmann & Neher, 1983), the molecular structures of the proteins which presumably form them are presently unknown. Several channel-forming antibiotics (such as monazomycin and alamethecin) have more or less well-understood structures, but it is not clear that these small molecules (with molecular weights of about 1000 daltons) are gated by the same mechanisms that pertain to the proteins of intrinsic membrane channels (Muller & Finkelstein, 1972; Heyer et al., 1976; Latorre & Alvarez, 1981).

The only voltage-dependent protein channels for which the primary structures are currently known are diphtheria toxin (Greenfield et al., 1983) and colicins E1, A and Ib—a related group of plasmid-encoded bacteriocins produced by *E. coli* (Yamada et al., 1982; Morlon et al., 1983; J. Konisky, *personal communication*). These latter water-soluble molecules bind to the outer membrane of target bacteria, which they then kill by forming leaky ion channels in the inner membrane (Schein et al., 1978). Colicin E1 can be produced in copious amounts, and its gene has been cloned and sequenced, thus making it amenable to genetic and chemical manipulations. Recently it has been shown (Cleveland et al., 1983) that the channel-forming capability of this 60,000 dalton protein resides in the 152 amino acid C-terminal portion produced by cyanogen bromide cleavage of the molecule. The remaining 43,000 dalton N-terminal piece is involved in binding to the outer membrane receptor and in subsequent translocation to the inner membrane (*see* Cramer et al., 1983, for review). This C-terminal fragment includes a 35 amino acid hydrophobic region near its C-terminal end which undoubtedly inserts in the membrane; a similar hydophobic region is found in colicins A and Ib. The rest of the sequence appears to be very hydrophilic, with many positive and negative charges.

This paper characterizes some of the properties of the channel formed by colicin E1 and its C-terminal fragments (produced by enzymatic or cyanogen bromide cleavage) in planar lipid bilayers, as part of a larger effort to implicate specific regions of the protein in particular actions of the channel. We show that the ion selectivity of the channel is controlled by titratable groups accessible from both sides of the membrane, and that the channel is large, with a lumen of at least 8 Å in diameter.

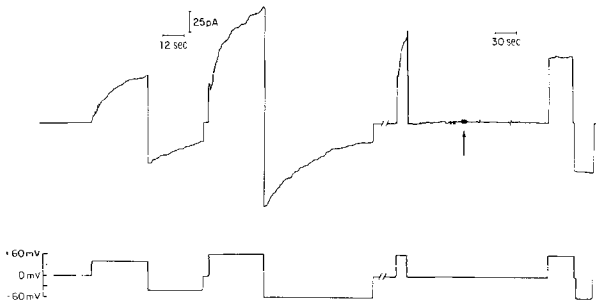

Fig. 1. Effect of membrane potential and pH on the turn-on and turn-off kinetics of colicin E1-induced conductance. An asolectin membrane separates symmetric solutions of 1 M KCl, 5 mM $CaCl_2$, 2 mM DMG, pH 4.3. About 1 min prior to the start of the record, colicin E1 was added to the *cis* compartment to a concentration of 150 ng/ml. Conductance is seen to turn on with *cis* positive voltage and to turn off with *cis* negative voltages. The kinetics are faster for +60 mV than for +40 mV. After the break in the record, the conductance is again turned on with +60 mV and the potential then returned to 0 mV. At the arrow, the pH of the *trans* and *cis* compartments are raised to pH 7.1. The conductance turned on by the previous +60 mV pulse is still present, but there is no further turn-on at +60 mV and no turn-off at −60 mV over the time intervals indicated. This illustrates that colicin E1 channel kinetics are slower at high pH (7.1) than at low pH (4.3)

These findings apply equally to channels formed both from the intact colicin E1 molecule and from its C-terminal fragments (produced by either trypsin, thermolysin or cyanogen bromide cleavage, as described by Cleveland et al., 1983). A preliminary report of some of these results appeared earlier (Slatin et al., 1983).

Materials and Methods

Planar phospholipid bilayer membranes were formed at room temperature by the union of two monolayers (Montal, 1974) of either asolectin [crude soybean phospholipid (lecithin type IIS from Sigma Chemical Co., St. Louis, Mo.)], from which neutral lipid was removed (Kagawa & Racker, 1971), or of purified phospholipids (Avanti Polar Lipids, Inc., Birmingham, Ala.). Monolayers were spread from 1% solutions of lipid in hexane, and after evaporation of the hexane, membranes were formed across a 50–150 micron hole in a Teflon® partition, previously painted with squalene (Sigma), separating buffered salt solutions. The membrane potential was clamped at known voltages and the current monitored by standard methods. For most experiments, protein from stock aqueous solutions was added, after the membrane was formed, to one compartment (the *cis*) to a final concentration of 0.05 to 5,000 ng/ml; the opposite (*trans*) compartment was held at virtual ground. For experiments with diphytanoylphosphatidylcholine (DPPC) membranes, protein was first dissolved in 0.5% octyl glucoside (Calbiochem-Behring, San Diego, Calif.) and then added either directly to the aqueous phase after the membrane was formed, or dissolved along with the lipid in hexane/ethanol (2 : 1) and spread in the *cis* monolayer. Colicin E1 and several of its C-terminal fragments were prepared in the laboratory of Dr. Cyrus Levinthal at Columbia University. Colicin A was a gift of Dr. S.E. Luria of M.I.T., and colicin Ib was a gift of Dr. J. Konisky of the University of Illinois.

Most ion selectivity experiments to determine the size of the channel lumen were performed on asolectin membranes at a pH close to 4.5, in order to insure reasonably fast turn-on and trn-off kinetics (illustrated in Fig. 1) while not compromising membrane stability. Membranes were formed in the presence of a 10 : 1 (1 M *vs.* 0.1 M—except where noted) concentration gradient of the appropriate salt solution, buffered with 2 to 5 mM dimethylglutaric acid (DMG) and also containing 5 mM $CaCl_2$ or $MgSO_4$ (for membrane stability) and 1 μM to 1 mM EDTA. The salts used were potassium chloride, tetramethylammonium (TMA) chloride (Eastman Organic Chemicals, Rochester, N.Y.), tetraethylammonium (TEA) chloride (Eastman), glucosamine chloride (Sigma), potassium glucuronate (Grade I, Sigma), potassium glutathione (reduced form, Sigma), and potassium NAD (Grade III-S, Sigma). The gradients used in experiments with potassium glucuronate and potassium NAD were 0.5 : 0.05 M and 0.7 : 0.07 molal, respectively, due to the limited solubility of these salts. Both TMA and TEA were purified once by dissolving in a minimum of boiling absolute EtOH with some activated charcoal, filtering the solutions while hot, and allowing them to cool slowly. Crystals were collected and washed with ice-cold dry acetone several times to remove any remaining yellow color and then dried under vacuum for two days. The TMA crystals were purified a second time by recrystallization from an EtOH/acetone solution.

Reversal (zero-current or cross-over) potentials for current through the channels formed by E1 or its fragments were determined from records of the current response during steps of voltage to different membrane potentials. The direction of the salt gradient (*cis* high, *trans* low; or vice-versa) was such that turn-on voltages (*cis* positive) would be of the same sign as that of the reversal potential. Thus, in experiments with large cations (in which the channel is anion-preferring), the salt gradient was *cis* to *trans*, whereas with large anions (in which the channel is cation-preferring), the salt gradient was *trans* to *cis*. The protocol in these experiments was to first apply a *cis* negative voltage to close any open channels, and then to step the voltage to a positive value to record the time-dependent change in current. The magnitude of this positive voltage relative to the reversal potential determines the direction of current flow (*cis* to *trans*—"positive," or *trans* to *cis*—"negative"); the voltage at which no time-dependent change in current is seen (and around which the current flow switches direction) is by definition the reversal potential. All measurements were corrected for electrode asymmetry.

Reversal potentials measured as described above were compared to the potential that would be measured if the channel were ideally selective for the preferred (smaller) ion, which was either K^+ or Cl^-. This "ideal" reversal potential is just the potential difference measured between the two salt solutions (1 and 0.1 M) separated by a cation- or anion-permselective membrane. For the potassium salts, the permselective membrane used was a nonactin-doped bilayer (nonactin added both *cis* and *trans* to a concentration of 1 to 5 μg/ml). For the chloride salts, however, no suitable anion-permselective membrane was available; therefore the potential difference between a Ag/AgCl electrode and a calomel reference electrode contacting the solution through an appropriate salt bridge (*see below*) was measured for each respective 1 and 0.1 M salt solution, and the difference between these two measurements was taken as the "ideal" reversal potential for chloride.

It was important that the reversal potential measurements for the channel be accurately compared (to within one millivolt) to the "ideal" reversal potential, because our primary goal was to determine if an ion was totally excluded from the channel, not just to measure relative ion permeabilities. A number of factors may cause the magnitude of the reversal potential to be underestimated: 1) contamination by small, permeant ions. To eliminate such contamination, agar bridges (2%), each containing the appropriate test salt solution, were used to make electrical contact between the solutions bathing the membrane and the 2 M KCl baths into which the saturated KCl-filled calomel electrodes were dipped. This arrangement prevented the possibility of K^+ and Cl^- leakage into the test salt solutions. 2) Shunting from permeant divalent ions. A small amount (*ca.* 5 mM) of divalent cation (Mg^{++} or Ca^{++}) is needed to facilitate formation of bilayers and to maintain their stability. Doubling this concentration did not decrease the values of reversal potentials, thus indicating that Mg^{++} and Ca^{++} were not shunting the measured values. 3) An osmotic pressure difference across the bilayer, due to the significant difference in salt concentration on the two sides. This will cause water to flow across the bilayer, altering the salt concentrations at the membrane/solution interfaces, and thereby leading to an effective reduction in the gradient seen by the membrane. Osmotic water flow may also produce a streaming potential in the channel. To minimize this problem, glucose was added to the low (0.1 M) salt solution to raise its osmolality to that of the high (1 M) salt solution; in the absence of the glucose, reversal potentials were 2 to 3 millivolts smaller. To insure an accurate comparison of corresponding "channel" and "ideal" reversal potential measurements, both were made under conditions as identical as possible. In the case of the potassium salts, the conditions were essentially identical, since the only difference was whether the membrane was treated with protein or nonactin. For the chloride salts, although there was no membrane separating solutions in the "ideal" reversal potential measurements, all other conditions, particularly the composition of the agar salt bridges, were identical.

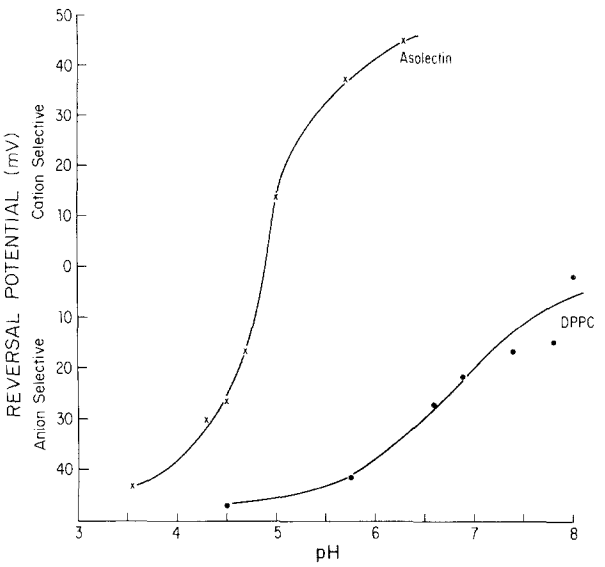

Fig. 2. The effect of pH and membrane composition on the ion selectivity of the colicin E1-induced conductance. The ordinate plots the reversal potential normalized to a 10 : 1 activity gradient of KCl (assuming that it is proportional to the logarithm of the KCl gradient). The curve marked asolectin was obtained on a single membrane. The pH initially was at the lowest value shown and then was raised symmetrically to the various indicated values. The asolectin membrane separated a 1 M KCl solution from a 0.1 M KCl solution that also contained 1.8 M glucose for osmotic balance. Both solutions also contained 5 mM $CaCl_2$, 2 mM DMG, and 1 μM EDTA. The DPPC membrane separated a 300 mM KCl solution from a 100 mM KCl solution. In addition, both solutions contained 5 mM $CaCl_2$, 5 mM DMG, and 1 mM EDTA. Note that the colicin E1-induced conductance switched from a chloride-preferring conductance at low pH to a potassium-preferring conductance at high pH in the asolectin membrane, but remained chloride-preferring in the DPPC membrane

Results

Effect of pH on Ion Selectivity

Membranes containing 10^3 to 10^5 channels of colicin E1 (or one of its C-terminal fragments) were exposed to gradients of KCl (1 M *vs.* 0.1 M) and the reversal potentials determined for a range of symmetric pH values. Figure 2 shows data obtained with membranes formed from asolectin, which contains about 20% negatively charged lipid, and from DPPC, a zwitterionic lipid with no net charge. The reversal potential in asolectin membranes (normalized to a 10-fold activity gradient of KCl) changes steeply from 30 mV anion selective to 41 mV cation selective, as pH is raised from 4.5 to 6.0, crossing zero selectivity at about pH 5. For DPPC membranes there is a similar trend in selectivity as a function of pH, but the channels remain anion selective throughout the pH range, even up to pH 7.0. In the presence of high levels of Ca^{++}, the asolectin curve is shifted entirely into the anion-selective region, as in the neutral membrane. These data indicate that the channel itself is a nonideal, anion-preferring channel, and that the degree of this preference is pH-dependent, ranging from about 16 : 1 (Cl^-/K^+) at pH 4.5 (as calculated by the Goldman-Hodgkin-Katz equation) to about 1 : 1 above pH 7.5. The cation selectivity observed at pH's above 5 in asolectin membranes is apparently a consequence of their negative surface potential, which concentrates cations and depletes anions in the double-layer region, and thus modifies the ion concentrations that the channels see from those in bulk solution. When the surface potential is screened by Ca^{++}, anion selectivity is restored.

Changes in either the *cis* or *trans* pH alone alter ion selectivity. This could arise from titratable groups well inside the lumen of the channel that see an average of the pH of the two bathing solutions. To test this hypothesis, we repeated the asymmetric pH experiments using buffers of various molecular

Table 1. Effect of *cis* and *trans* pH buffering on reversal potential (V_r)[a]

		V_r		V_r		V_r		V_r
A.								
cis	pH 3.5 LB[b]	−32	pH 8 LB	−19	pH 8 HB	0	pH 8 HB	+34
trans	pH 3.5 LB		pH 3.5 LB		pH 3.5 LB		pH 8 LB	
B.								
cis	pH 4 LB	−25	pH 3.5 LB	−2	pH 3.5 LB	+8	pH 8 HB	+32
trans	pH 4 LB		pH 8 LB		pH 8 HB		pH 8 HB	
C.								
cis	pH 3.5 HB	−34	pH 3.5 HB	−8	pH 3.5 HB	+2		
trans	pH 3.5 LB		pH 7.5 LB		pH 8.8 HB			
D.								
cis	pH 3.5 LB	−31	pH 8.5 LB	+3				
trans	pH 3.5 HB		pH 3.5 HB					

[a] The four panels show four experiments in which the pH of the bathing solutions was changed asymmetrically. All experiments were done in the presence of a 1 : 0.1 M KCl gradient and symmetric 5 mM $CaCl_2$. Experiments in panels A and D used the thermolysin C-terminal fragment of colicin E1; experiments in panels B and C used the trypsin C-terminal fragment of colicin E1. Specific additional conditions were: A. Initially: *cis* and *trans* 5 mM calcium glycerate, pH 3.5; then add to *cis* 3 mM Bis Tris Propane (BTP), final pH 8; then add to *cis* 100 mM Tris, pH 8. The last condition (V_r at symmetric pH 8) represents the average of several experiments. B. Initially: *cis* 5 mM DMG, pH 4, *trans* 2 mM DMG, pH 4; then add to *trans* 3 mM BTP, final pH 8; then add to *trans* 20 mM Tris, pH 8; then add to *cis* 20 mM Tris, final pH 8. C. Initially: *cis* 200 mM calcium glycerate, pH 3.5, *trans* 5 mM calcium glycerate, pH 3.5; then add to *trans* 4 mM BTP, final pH 7.5; then add to *trans* 6 mM BTP, final pH 8.8. D. Initially: *cis* 5 mM potassium glucuronate, pH 3.5, *trans* 200 mM calcium glycerate, pH 3.5; then add to *cis* 1 mM BTP, final pH 8.5.

[b] Definitions: LB = low concentration of relatively impermeant buffer; HB = high concentration of relatively permeant buffer; negative reversal potentials indicate anion selectivity; positive reversal potentials indicate cation selectivity.

sizes which either could (e.g., glycerate and Tris) or could not (e.g., Bis Tris Propane and glucuronate) easily penetrate the channel. We concluded from such experiments that the selectivity is controlled by separate titratable groups (probably carboxyl groups, as judged from titration curves in DPPC membranes) near both ends of the channel and within the lumen. Our basis for reaching this conclusion can be seen in Table 1, which shows the results of a few such experiments. Consider, for example, panel B. The channels start off anion selective at symmetric low pH, but become less so when the *trans* pH is raised to 8 with a small amount of Bis Tris Propane buffer. The pH profile in the lumen is unknown in this configuration. Large amounts of Tris buffer are then added to the *trans* compartment (third condition) without changing the pH from 8, and this makes the channel cation selective, presumably because the high concentration of Tris (which is small enough to easily pass through the channel but hydrophilic enough, even in the neutral form, to be excluded from the lipid phase) is now dominating the lumenal pH and holding it near 8. However, maximal cation selectivity cannot be achieved by adding even larger amounts of Tris pH 8 to the *trans* side. This occurs only when the *cis* pH is also raised to 8, as shown. The remaining panels show similar experiments in which the lumenal pH is clamped with penetrating buffers from the *cis* and *trans* sides at different pH values. In no case is it possible to eliminate the contribution made to the selectivity by the pH of any one of the three regions (i.e., *cis* end, *trans* end and lumen) by controlling the pH of the other two. This strongly suggests that there are different groups being titrated in each of the accessible regions.

Colicins A and Ib, which have primary sequences grossly similar to that of colicin E1 but with very little homology (Morlon et al., 1983; J. Konisky, *personal communication*) show a pH-dependence of K^+ *vs.* Cl^- selectivity similar to that seen with E1. For colicin A, however, the selectivity is unaffected by *trans* pH (*data not shown*).

Effect of Ion Size on Selectivity

It is possible to exploit the channel's permeability to both anions and cations to determine the pore size at its narrowest point. Channel selectivity was measured in a series of three different chloride and three different potassium salt gradients, in which the

counterion was a larger cation or anion, respectively. If the larger ion cannot pass through the lumen, then the channel should exhibit ideal selectivity for the smaller ion, regardless of the sign of its ionic charge or the pH of the solution.

Selectivity measurements were generally made on three separate membranes for each of the salt solutions tested, and results agreed to within ± 1 mV in most cases. Figure 3 illustrates a determination of the permeability of glucosamine relative to chloride (the *cis* compartment contained the higher salt concentration). For positive voltages less than 45 mV, current increased in the negative direction, whereas for voltages greater than 45 mV, the current increase was positive. At 45 mV, the reversal potential, no current increase was seen (since the driving force was zero). (The nonzero current at the reversal potential is due to a nonspecific leak.) The "ideal" chloride reversal potential for this salt gradient was 47 mV.

In some experiments, the direction of current flow was biphasic for pulses near the reversal potential. For example, in a KCl gradient, upon switching from negative to positive voltage, the current initially flowed in a negative direction (*trans* to *cis*), as though the pulse were still smaller than the reversal potential, but then current flow reversed after 20 to 30 sec. In these cases the early phase was ignored in determining the reversal potential. This biphasic current response occurred for pulses in a range of approximately ± 3 mV around the reversal potential.

Surprisingly, of all the large ions used to determine pore size, only glutathione had a significant pharmacological effect on the channel formed by E1. In the presence of this ion, turn-on of channels was irreversible; that is, channels did not turn off at negative voltages. The method described above for determining reversal potentials could therefore not be used in this instance, since increases in current around the reversal potential are too small to detect in the presence of a large background conductance. Instead, the reversal potential was determined from the voltage at which total current was zero.

Results of selectivity experiments for each salt are shown in Table 2. For large cations, the reversal potential for the E1 channel approaches the "ideal" reversal potential for chloride, as the size of the cation increases. One must be careful, however, in interpreting a reversal potential that is close to ideal as indicating that a large cation (or anion) is impermeant. For example, notice that at pH 4.5, where the channel is intrinsically anion-preferring, the reversal potential measured for glucosamine chloride (−44.5 mV) differs from the ideal Nernst potential for chloride (−47 mV) by two to three millivolts, but

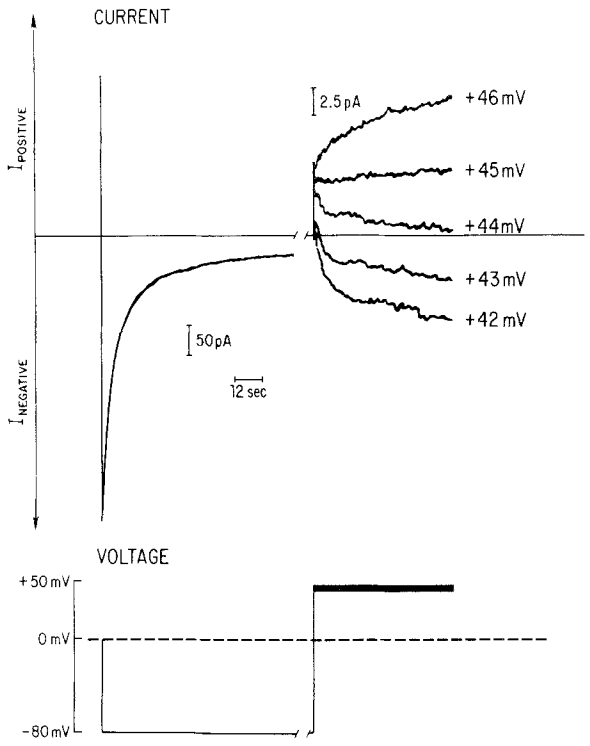

Fig. 3. Illustration of the method used to determine reversal potentials. An asolectin membrane separated a *cis* solution of 1 M glucosamine chloride from a *trans* solution of 0.1 M glucosamine chloride that also contained 1.8 M glucose for osmotic balance. Both solutions also contained 5 mM $CaCl_2$, 5 mM DMG, 1 μM EDTA, pH 4.5. Colicin E1 was added to the *cis* compartment to a concentration of 4.5 μg/ml. The right half of the figure shows a superposition of current records corresponding to the various *cis* positive voltages indicated. Before each record was taken, the colicin E1-induced conductance was turned off by a *cis* negative voltage of 80 mV, as illustrated in the left half of the figure. The reversal potential was clearly around 45 mV. (The small current at this voltage is a consequence of a nonspecific leakage conductance that sometimes develops in membranes exposed to large concentrations of colicin E1.)

at pH 6, where the channel is intrinsically cation-preferring, the reversal potential is only −37.5 mV, indicating significant glucosamine permeability. Similarly, the anion glutathione appears impermeant at pH's slightly above 5, but shows significant permeability at pH 4.3. Both size and charge determine ion permeability, and this must be carefully considered in using ions to size a channel (*see* Discussion).

The results of experiments with large anions (*see* Table 2) are less straightforward than those with large cations; reversal potentials do not increase monotonically with ion size. Thus, the reversal potentials are further from ideal for glutathione and NAD than for glucuronate, a smaller anion. This is probably a consequence of the former two

Table 2. Effect of ion size on anion and cation selectivity as measured by reversal potentials for 10 : 1 concentration gradients[a]

Salts	pH	Reversal potential (mV)[b]	Ideal Reversal Potentials		Molecular weight of organic ion (daltons)
			Cl^- (Ag/AgCl electrode)	K^+ (Nonactin)	
K^+ Cl^-	4.3	−27	−50.5		
	6.0	+37		+54	
TMA^+ Cl^-	4.5	−39.5	−47		79
TEA^+ Cl^-	4.5	−42	−48.5		130
Glucosamine$^+$ Cl^-	4.5	−44.5	−47		179
	5.8	−37.5[c]			
K^+ Glucuronate$^-$	4.4	+38		+52.5	194
	6	+50			
K^+ Glutathione$^-$	4.3	+31.5		+53	307
	5.1	+51			
K^+ NAD^-	4.2	+27[d]		+49	663
	5	+46			

[a] The gradient used was 1 M *vs.* 0.1 M except for experiments with glucuronate and NAD, in which the gradients were 0.5 : 0.05 M and 0.7 : 0.07 molal, respectively.
[b] In this table, a minus sign is used to designate anion selectivity and a plus sign to designate cation selectivity.
[c] For colicins A and Ib, this was −15.5 and −29.5 mV, respectively, at pH 6.0.
[d] For colicins A and Ib, this was +27.5 and +33 mV, respectively.

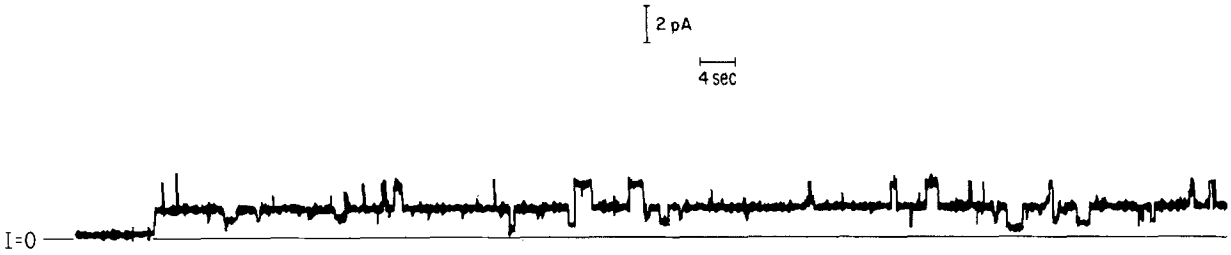

Fig. 4. Example of single-channel activity induced by colicin E1. The membrane was formed from a 10 : 1 weight ratio of asolectin/cholesterol that contained in the monolayer 0.3 μg of the cyanogen bromide C-terminal fragment of colicin E1. It separated symmetric solutions of 1 M KCl, 3 mM DMG, 5 mM $CaCl_2$, 1 mM EDTA, pH 4.0. A *cis* positive voltage of + 50 mV was present throughout the record

ions having two negative charges (and one positive charge), whereas the latter ion has a single negative charge (*see* Discussion).

Channel sizing experiments were also carried out with colicins A and Ib. These two colicins exhibit a trend in ion selectivity as a function of pH that is similar to that of colicin E1. Reversal potentials were measured at pH 6 (a cation-selective pH) for glucosamine chloride and at pH 4.2 (an anion-selective pH) for potassium NAD (*see* Table 2). The results demonstrate that like colicin E1 channels, these channels are also permeable to glucosamine and NAD.

Single-Channel Measurements

Figure 4 is a record of a membrane containing only two active colicin E1 channels. It can be seen here that a channel has several conductance levels. The "main" level is about 24 pS (in 1 M KCl, pH 4.0), and there are several smaller substates. The reversal potentials of these various channel states in a KCl gradient are roughly the same, and agree with the value in Table 2, obtained on membranes containing thousands of channels. The conductance in 1 M KCl of the main single-channel state does not change significantly between pH 4.0 and 6.0, a

range where the ion selectivity is changing steeply. This finding suggests that channel conductance and selectivity are controlled by different regions of the channel, since they are fairly independent of each other. Apparently, the titratable groups affecting selectivity do not determine the conductance state—they are not the rate-limiting steps for an ion traversing the channel.

Discussion

The El family of colicins are a group of proteins especially well suited for study as model membrane channels. They mimic many of the interesting properties of intrinsic membrane channels, and are available in comparatively large quantities. They are water soluble and yet insert spontaneously and irreversibly into lipid bilayers, thus also providing a model system for studying the mechanism of protein insertion in, and transport through, cell membranes. But most importantly, several of these colicins have been cloned and sequenced, and are susceptible to genetic and chemical manipulations. In this paper we characterize two properties—lumen size and pH modulation of selectivity—which will have to be incorporated into any complete model of the colicin channel.

Lumen Size

Our main finding is that the colicin E1 channel has a large lumen of *at least* 8 Å diameter. We established this from measurements of reversal potentials for gradients formed by salts of large cations or large anions. Such large ions as glucosamine$^+$ and NAD$^-$ are not excluded from the channel. The estimate of 8 Å is a conservative one, moreover, as NAD can adopt many different conformations in solution, and it is only the conformation having the smallest possible diameter, as determined from the CPK model of the molecule, which measures 8 Å (in this conformation the length is *ca.* 27 Å). In fact, the average diameter seen by the channel is probably much larger, *ca.* 12 to 16 Å. Whatever the ambiguities inherent in assigning "minimum" or "average" diameters to molecules which can adopt a myriad of conformations in solution, the fact that the reversal potential for each of the molecules tested is significantly different from ideal puts definite constraints on model-building. Any model of the colicin E1 channel must possess a lumen through which glucosamine, glucuronate, glutathione and NAD can readily pass. As an aside, these molecules are as large or larger than those which have measurable permeability through the acetylcholine channel (Huang et al., 1978; Dwyer et al., 1980), a channel which is considered large by physiological standards.

Sizing of a channel with electrolytes is complicated by the effects of electrostatic forces on permeability.[1] A more direct method of estimating pore size is to measure nonelectrolyte permeability, which is dominated primarily by steric factors (although short-range forces cannot be totally neglected). Unfortunately, we could not incorporate sufficient numbers of E1 channels in planar bilayers to make nonelectrolyte flux measurements feasible. There is evidence, however, from studies on the acetylcholine channel (Huang et al., 1978; Dwyer et al., 1980), to suggest that the use of electrolytes to size a channel has its advantages. In particular, Huang et al. (1978), using isotope fluxes to examine selectivity of the acetylcholine channel for both ions and nonelectrolytes, found that small charged amines (e.g. methylamine) showed permeabilities comparable to that of sodium, whereas the uncharged forms of the same molecules were essentially impermeant. For channels which discriminate among ions on the basis of charge, therefore, measuring selectivity for electrolytes can be a more sensitive method of determining pore size than measuring nonelectrolyte fluxes. In fact, for a channel like colicin E1 which allows both cations and anions to pass and whose relative selectivity to them is a function of pH, it is possible to increase the sensitivity of the sizing experiments even further: the channel can be biased in favor of the larger ion on the basis of charge, simply by measuring selectivity at the appropriate pH. The potassium glucuronate results shown in Table 2 illustrate this point. At pH 6, the channel appears nearly ideally selective for potassium and therefore impermeant to glucuronate, but when the pH is lowered to 4.4, it shows significant permeability to glucuronate.

The largest ion we used to size the channel, NAD, gives a reversal potential still far from ideal, indicating that the E1 channel lumen is larger than this molecule (i.e. greater than 8 Å in diameter). It is difficult to determine how much larger, however, since the reversal potential measured for any given ion is a function of ion-protein charge interactions as well as ion size. For instance, the reversal poten-

[1] A particularly striking illustration of the truth of this statement is our finding that despite the large size of the colicin E1 channel lumen, the single-channel conductance in 1 M KCl is only 24 pS, a value less than that for the 4 Å diameter gramicidin A channel.

tial obtained for glucuronate, with a molecular weight of 194 daltons, is closer to ideal than is that obtained for glutathione (mol wt 307 daltons), or for NAD (mol wt 663 daltons). However, when reversal potentials were measured for these same salts on the channels formed by the CNBr fragment, the measurements were correlated with ion size (*data not shown*). Since both glutathione and NAD have two negative and one positive charge (whereas glucuronate has just one negative charge), the reversal potentials measured for glutathione and for NAD on channels formed by the whole colicin may be anomalous. That is, these anions may be concentrated at the channel mouth due to charge interactions with the N-terminus (which is not directly involved in channel formation), resulting in unusually high anion permeability. We would like to have extended these permeability measurements to include larger ions, in order to find at least one which is impermeant. However, we were unable to come up with additional candidates which fit the criteria of bearing a net charge of ± 1, being water soluble, and being available in gram quantities at a reasonable cost.

Our finding of a very large channel lumen contrasts with that of Uratani and Cramer (1981), who studied the retention of nonelectrolytes in dimyristoylphosphatidylcholine vesicles into which colicin E1 had been incorporated below their phase transition. They found a permeability cut-off at glycerol, whose molecular weight is only 92 daltons. It is not clear from the unusual conditions they employed in incorporating E1 into these vesicles that functional channels were actually present in the vesicle membrane.

The biphasic current response seen at voltages close to the reversal potential suggest that there may be different "populations" of channels dominating the conductance, as a function of time, after pulse initiation. These populations may represent entirely different channels (e.g. dimers versus monomers) or just different conformational states of the same channel reflected in fluctuations in lumen size. Either explanation fits with data from single-channel records which show a heterogeneous distribution of single-channel conductances that have the same voltage dependence. In any case, the variation in macroscopic reversal potential is only $\pm$ 3 mV, too small to detect on the single-channel level.

Reversal potentials measured for colicins A and Ib indicate that these channels may have even larger lumens than colicin E1. The results can be compared in a qualitative way since all three channels favor the larger ion on the basis of charge at the pH of the selectivity experiments (although colicins A and Ib are overall more cation-preferring than E1 in the pH range of 4 to 5.5). However, lumen size is not proportional to reversal potential in any straightforward manner, and therefore we cannot say how much bigger the lumens of colicins A and Ib are.

Control of Selectivity

When the current is carried in the channel by small ions of both signs, the selectivity between these ions is very sensitive to pH and surface charge. A charged membrane is expected to alter the ratio of cations to anions near (within a Debye length) its surface from that in the bulk solutions. An indifferent (i.e. inherently nonselective) channel in such a membrane might "see" more cations than anions, and, in effect, show cation selectivity. If the channel were, however, highly selective for cations over anions (due to internal structural features of the channel), the measured selectivity would be quite insensitive to changes in the cation/anion ratio at the mouth. Colicin E1 lies between these two extremes. It has a preference (though not absolute) for anions over cations, but since it passes both, the measured selectivity is sensitive to conditions at the surface. This implies that the mouth of the channel "sees" at least part of the double layer, suggesting it is not more than a few angstroms from the membrane surface.

The situation in a neutral membrane is not complicated by surface potential effects on ions (including H^+). Our data indicate that titratable groups on the colicin E1 protein modify the selectivity of the channel in the neutral DPPC membrane from about 16 : 1 (Cl^-/K^+) to about 1 : 1 (over a pH range where DPPC has no net charge). The pH on both sides of the membrane is important. We reasoned that if titratable groups within the lumen of the channel were responsible for the selectivity, then the observed selectivity would be dominated by the pH of whichever side had a permeant buffer, if the other side had a relatively impermeant one (such as glycerate *vs.* Bis Tris Propane). The results show that the amount of buffering on either side *does* affect the selectivity, implying that there are indeed titratable groups in the channel lumen which modulate selectivity. However, no amount of buffering from either side could entirely eliminate the effect of the pH on the opposite side. This means that there are also titratable groups near the channel ends that contribute to the selectivity. The pH range over which this titration occurs suggests (but does not prove) that these are acidic residues (Asp or Glu).

This work was supported by NIH grant GM29210-07 and by NIH Training grant T32GM7288 from NIGMS.

References

Cleveland, M., Slatin, S., Finkelstein, A., Levinthal, C. 1983. Structure-function relationships for a voltage-dependent ion channel: Properties of COOH-terminal fragments of colicin E1. *Proc. Natl. Acad. Sci. USA* **80:**3706–3710

Cramer, W.A., Dankert, J.R., Uratani, Y. 1983. The membrane channel-forming bacteriocidal protein, colicin E1. *Biochim. Biophys. Acta* **737:**173–193

Dwyer, T.M., Adams, D.J., Hille, B. 1980. The permeability of the endplate channel to organic cations in frog muscle. *J. Gen. Physiol.* **75:**469–492

Greenfield, L., Bjorn, M., Horn, G., Fong, D., Buck, G., Collier, J., Kaplan, D. 1983. Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage. *Proc. Natl. Acad. Sci. USA* **80:**6853–6857

Heyer, E., Muller, R., Finkelstein, A. 1976. Inactivation of monazomycin-induced voltage-dependent conductance in thin lipid membranes. II. Inactivation produced by monazomycin transport through the membrane. *J. Gen. Physiol.* **67:**731–748

Huang, L.M., Catterall, W.A., Ehrenstein, G. 1978. Selectivity of cations and nonelectrolytes for acetylcholine-activated channels in cultured muscle cells. *J. Gen. Physiol.* **71:**397–410

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation: XXV. Reconstitution of particles catalyzing $^{32}P_i$-adenosine triphosphate exchange. *J. Biol. Chem.* **246:**5477–5487

Latorre, R., Alvarez, O. 1981. Voltage-dependent channels in planar lipid bilayers. *Physiol. Rev.* **61:**77–150

Montal, M. 1974. Formation of bimolecular membranes from lipid monolayers. *Methods Enzymol.* **32:**545–554

Morlon, J., Lloubes, R., Varenne, S., Chartier, M., Lazdunski, C. 1983. Complete nucleotide sequence of the structural gene for colicin A, a gene translated at a non-uniform rate. *J. Mol. Biol.* **170:**271–285

Muller, R., Finkelstein, A. 1972. Voltage-dependent conductance induced in thin lipid membranes by monazomycin. *J. Gen. Physiol.* **60:**263–284

Sakmann, B., Neher, E. 1983. Single Channel Recording. Plenum, New York

Schein, S., Kagan, B., Finkelstein, A. 1978. Colicin K acts by forming voltage-dependent pores in phospholipid bilayer membranes. *Nature (London)* **276:**159–163

Slatin, S., Raymond, L., Finkelstein, A. 1983. Colicin E1 makes large, anion selective channels in planar lipid bilayers. *Biophys. J.* **41:**363a

Uratani, Y., Cramer, W. 1981. Reconstitution of colicin E1 into dimyristoylphosphatidylcholine membrane vesicles. *J. Biol. Chem.* **256:**4017–4023

Yamada, M., Ebina, Y., Miyata, T., Nakazawa, T., Nakazawa, A. 1982. Nucleotide sequence of the structural gene for colicin E1 and predicted structure of the protein. *Proc. Natl. Acad. Sci. USA* **79:**2827–2831

Received 27 July 1984; revised 25 October 1984