Anomalous Proton Selectivity in a Large Channel: Colicin A[†]

Stephen L. Slatin,* Alan Finkelstein, and Paul K. Kienker

Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received September 17, 2007; Revised Manuscript Received November 14, 2007

ABSTRACT: Some of the bactericidal proteins known as colicins exert their toxic action by forming a large, nonselective channel in the inner membrane of target bacteria. The structure of this channel is unknown. It conducts large ions but has a much smaller conductance than would be expected for a channel of its deduced size. Here we report that the colicin channel, particularly the colicin A channel, is selective for protons over other cations (and anions) by many orders of magnitude. This was deduced from measurements of reversal potentials in pH gradients across planar lipid bilayers containing these channels. For example, in symmetric 0.1 M KCl with a pH 5/pH 8 gradient across the membrane, the reversal potential of colicin A is −21 mV, rather than 0. Such a result would be unremarkable for a narrow channel but is beyond explanation by current understanding of permeation for a channel of its diameter. For this reason, we re-examined the issue of the diameter of the channel lumen and confirmed that the lumen is indeed "too large" (∼10 Å) to select for protons by the amount that we measure. We are thus compelled to propose that an unorthodox mechanism is at work in this protein.

Proton-selective channels have been found in a variety of species and tissues and appear to play divergent roles. For some, such as the M2 protein of influenza virus and the voltage-dependent proton channels involved in the oxidative burst of phagocytic cells, H^+ is the relevant conducted ion; for others, such as the voltage-dependent Na^+ and K^+ channels, high proton selectivity is merely a byproduct of a narrow water-filled lumen, and other cations are more biologically relevant (reviewed in ref *1*). The one feature held in common by almost all of these channels is the narrowness of the conducting pathway, which contributes to their ability to select among ions. In fact, the highest proton selectivities are seen in proteins that appear to lack any open pore at all, such as the human Hv1 channel (*2*), which is homologous to the voltage-sensing domain of voltage-dependent cation channels but lacks any associated pore-forming domain. Here, we report on a rare example of a channel with paradoxically high proton selectivity that violates this generalization, the channel formed by colicin A.

Colicins are bacterial proteins that are toxic to sensitive strains of *Escherichia coli* (*3*, *4*). Some colicins kill their targets by forming ion-conducting channels, and these channels have proved to have highly unusual properties. They are monomeric and have a low conductance, yet they appear to have a large lumen. The details vary among the dozen or so known channel-forming colicins, but they appear to share fundamental properties (e.g., voltage-dependent gating, pH-dependent gating, and pH-dependent conductance), suggesting that they work by similar mechanisms (for reviews, see refs *5*−*7*). This inference is bolstered by the observation that the five solved structures of the water-soluble form of colicin channel-forming domains are qualitatively alike, consisting of a bundle of 10 α helices, two of which (H8 and H9) are highly hydrophobic and reside in the center of the bundle (*8*−*12*). Typical conductance values (in 1 M KCl at pH 7) range from 15 pS for colicin A to ∼50 pS for colicin Ia (*13*, *14*). In more physiological salts, the conductance is of course smaller, smaller than that of many highly selective eukaryotic channels, such as sodium and potassium channels (*15*), but colicin channels are not very selective (see below), conducting all monovalent cations and anions that have been tested and exhibiting little selectivity among them (*16*−*19*). They are also perplexingly large, on the order of 10 Å in diameter (*14*, *16*−*18*, *20*). Furthermore, these colicins can facilitate the transfer of much larger molecules, up to and including small proteins (*21*−*23*), presumably by a pathway different from that taken by the smaller ions. No structure has yet been determined, or even proposed, to account for this suite of properties. The observation that the channel appears to be formed from only a single protein molecule leads to the hypothesis that lipid molecules must also be involved (*24*, *25*).

To this list of collectively curious properties we now add another. In this paper, we show that colicin channels, and particularly colicin A channels, transport H^+ in preference to other monovalent ions, with a selectivity so high that it might be expected to be observed only in the very narrowest pores [a brief report of this has appeared (*26*)]. We arrived at this conclusion by examining the reversal potential of colicin channels in pH gradients. The large size and promiscuous ion selectivity of colicin channels are difficult, if not impossible, to reconcile with this high H^+ selectivity.

[†] This work was supported by NIH Grant GM 29210.

* To whom correspondence should be addressed. E-mail: slatin@aecom.yu.edu. Phone: (718) 430-3169. Fax: (718) 430-8819.

10.1021/bi701900x CCC: $40.75
Published on Web 01/19/2008

MATERIALS AND METHODS

Planar Bilayer Experiments. Folded planar bilayer membranes were formed at room temperature across an aperture (100−150 μm in diameter) in a Teflon partition as previously described (*27*). For most experiments, the hole was pretreated with 5 μL of 3% squalene in petroleum ether, and the lipid [asolectin (*27*), or other lipids as mentioned in the text] was dissolved in pentane at a concentration of 1% and layered (20 μL) on the surfaces of the chamber solutions. The membrane separated two 1 mL compartments, each containing a buffered (with one exception; see the text) salt solution. Electrical contact with the solutions was realized with agar bridges generally made up in the same solutions as the compartment solutions that they contacted; the other end of each bridge contacted a solution of 3 M KCl containing a Ag/AgCl electrode connected to the voltage clamp. This arrangement minimizes leakage of salt from the bridges into the chamber solutions. Voltages are given as the potential of the cis compartment, defined as the side to which colicin was added, with respect to that of the opposite trans compartment. When measuring reversal potentials in pH gradients, we subtracted from the raw measurement the voltage required to zero the current after the membrane was broken (usually <2 mV).

Purifying TEA-Cl, TEA_2-SO_4, and TEA-OH. For some preliminary experiments, tetraethylammonium chloride (TEA-Cl, Fluka) was recrystallized using standard methods (*16*). For most experiments, a 35% solution of TEA-OH (Aldrich) was stirred under vacuum for several hours to remove any volatile contaminants, such as NH_3, and the TEA concentration was determined by titration of the OH^- to be 2.72 M. This stock solution was then titrated with HCl or H_2SO_4, producing stocks of TEA-Cl and TEA_2-SO_4, respectively. Bath solutions for membrane experiments were made from these stocks, diluted to the desired concentration, and supplemented with HEPES buffer and EDTA stock solutions that had been titrated to an appropriate pH with vacuum-treated TEA-OH.

Miscellaneous. pD was measured with a standard Corning glass pH electrode. A correction of 0.4 pH unit was applied (*28*). Colicins A, Ia, and E1 were expressed in *E. coli* after induction with 0.5 μg/mL mitomycin C (Roche) and purified by ion exchange chromatography (*29*−*31*). Colicin B was supplied by V. Géli and colicin K by D. Baty, both at CNRS (Marseille, France). The T domain of diphtheria toxin and the PA_{63} fragment of anthrax toxin were obtained from R. J. Collier of Harvard Medical School (Boston, MA). Gramicidin and nystatin were purchased from ICN Biochemicals and Squibb (now Bristol Meyers Squibb), respectively. Tetrakis(2-hydroxyethyl)ammonium bromide was from Acros Organics.

RESULTS

Reversal Potentials of Colicin A Channels in a pH Gradient. The selectivity of colicin A for protons was examined by measuring the reversal potential (V_r)[1] in the presence of a pH gradient. Under some conditions, colicin A channels exhibit two different open states, which may have different reversal potentials: a "shallow" open state, from which the channel closes readily at moderate negative voltages (approximately −50 mV), and a "deep" open state, from which the channel closes only at much larger negative voltages (approximately −200 mV) (*29*, *32*). The reversal potential of the deep open state can be measured simply by allowing the shallow open-state channels to close (e.g., at −50 mV) and then finding the voltage that zeroes the current through the remaining, deep open-state channels. The reversal potential of the shallow open-state channels can be measured from the direction in which the tail currents decay as channels close: if $V > V_r$, the current becomes more negative as the channels close; if $V < V_r$, it becomes more positive; and if $V = V_r$, it is constant. Panels A and B of Figure 1 show experiments in symmetric 0.1 M KCl and a cis pH 5−trans pH 8 gradient across the membrane. The values for V_r measured by zeroing the current [−15 mV (Figure 1A)], or by tail currents [−23 mV (Figure 1B)], are different from each other, but the salient point is that both are very different from zero. Mean values from multiple experiments under these conditions were as follows: V_r(tail currents) = −21 ± 1 mV (n = 10) and V_r(zeroing) = −14 ± 1 mV (n = 6). Non-zero values were also measured when the direction of the pH gradient was reversed [e.g., 34 ± 2 mV (n = 4) in a pH 5/pH 3 gradient (Figure 1C)]. The large reversal potentials determined by both methods revealed that a significant fraction of the current through the channels is carried by ions that are asymmetrically disposed across the bilayer. Under these conditions, where the predominant current-carrying ions, K^+ and Cl^-, are at equal concentrations on both sides of the membrane, the only ions that could be responsible for the non-zero V_r are H^+, OH^-, charged species of the buffers used to control the pH, and, possibly, any contaminating ions that may be present in unequal concentrations across the bilayer.

The experiments described above used membranes containing large numbers of channels, leaving open the possibility that the non-zero V_r measured was due to an unknown cooperative interaction. To test this, we measured the reversal potential of single colicin A channels in pH 5/pH 8 and pH 5/pH 3 gradients (Figure 2). In a pH 5/pH 8 gradient (Figure 2A), we calculated a V_r of −18 mV by plotting, as a function of voltage, the current steps of several individual channels as they gated. This is in good agreement with the V_r of −15 to −21 mV determined from the corresponding macroscopic measurements (Table 1). For the pH 5/pH 3 condition, we were able to open two channels and measure the resulting currents at a series of voltages, above and below V_r, before the channels closed (Figure 2B) (this was possible because $V_r > 0$). A linear fit of the current−voltage curve (Figure 2B inset) gives a V_r of 39 mV, which is in accord with the macroscopic value of 34 ± 2 mV (n = 4). Thus, the properties of the single channel account for the macroscopic phenomenon.

H^+ versus OH^-. The sign of the reversal potential in a pH gradient provides no information to distinguish between H^+ and OH^- permeability, because H^+ and OH^- have opposite charges and opposite concentration gradients. However, we can evaluate the contributions of H^+ and OH^- by varying the absolute pH while keeping ΔpH fixed. As reported in Table 1, the magnitude of the reversal potential

[1] Abbreviations: TEA, tetraethylammonium; g_H, proton current; g_i, other ion current; V_r, reversal potential; P_n, permeability of ion n; GHK, Goldman−Hodgkin−Katz.

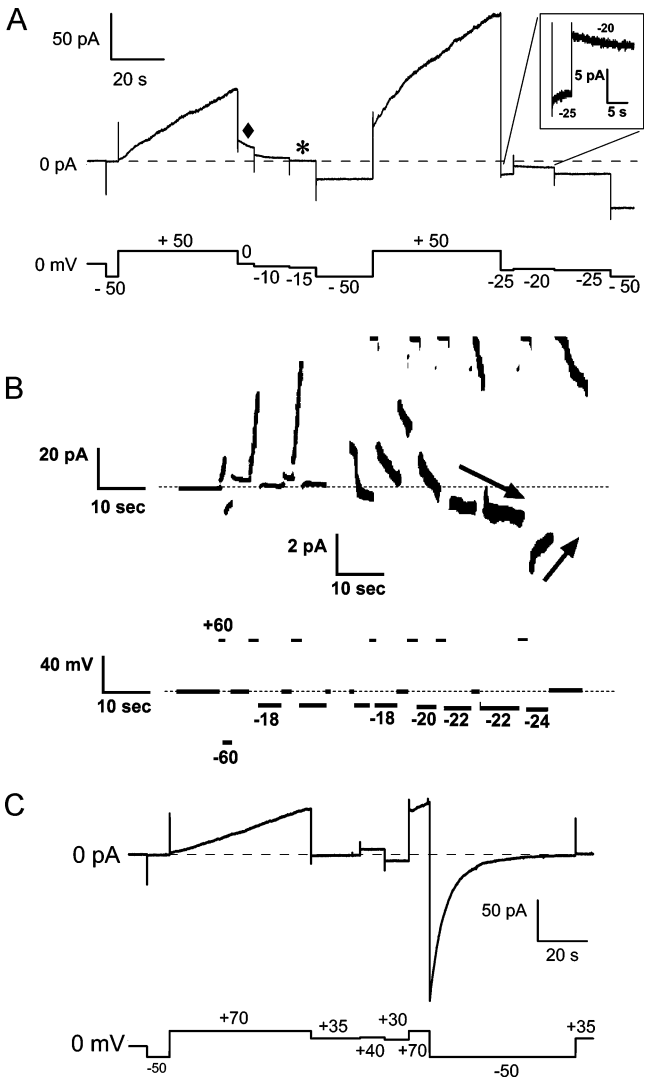

FIGURE 1: Determination of the reversal potential of colicin A in symmetric 0.1 M KCl with a pH gradient across the membrane. (A) Determination by zeroing the current in a pH 5/pH 8 gradient. $V_r = -15$ mV. The solutions are as follows: 0.1 M KCl, 5 mM $CaCl_2$, and 1 mM EDTA on both sides of the membrane and 20 mM malate (pH 5.0) (cis) or 20 mM HEPES (pH 8.0) (trans). Colicin A channels open at 50 mV. The channels carry a sizable positive current at 0 mV (♦); it is necessary to go to −15 mV to reach zero current (*). This is the reversal potential (V_r) for channels in the deep open state. Channels in the "shallow" open state slowly close at these test voltages ($V \sim -20$ mV). V_r values of these gatable channels can be estimated from the direction of the change in current as channels close, as indicated by the inset. (B) Determination by tail currents in a pH 5/pH 8 gradient. Conditions are as described in the legend of Figure 1A. Early in the record, a large number of colicin A channels were opened and closed by ±60 mV pulses and pulses to −18 mV, the approximate V_r. At the second current scale bar, the gain is increased by a factor of 10 to increase sensitivity near V_r. Now 60 mV pulses drive the current off scale, but the direction of the changing current near V_r is easily seen. The slanted arrows point out that the current changes in opposite directions at −22 and −24 mV, placing the V_r of the gating channels between these two values. Note that the absolute value of the current at −22 mV is <0 (dashed line), reflecting the presence of nongating conductance that reverses at a more positive voltage. (C) Determination by zeroing the current in a pH 5/pH 3 gradient (0.1 M KCl, 5 mM $CaCl_2$, 1 mM EDTA, and 20 mM malate). $V_r = 35$ mV. In this case, most of the conductance that reverses at 35 mV can be quickly turned off at −50 mV, so it represents channels in the shallow open state. Under these conditions, very few colicin A channels are in the deep, slow-gating, open state.

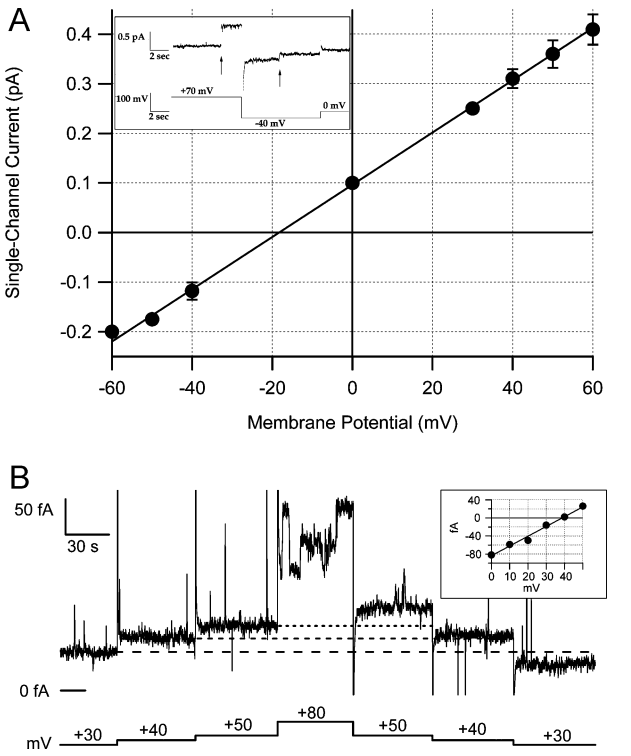

FIGURE 2: Colicin A single channels. (A) Single channels of colicin A were assessed under the same pH 5/pH 8 conditions as in Figure 1A, except that the asolectin membrane was made using the technique of Wonderlin et al. (*51*), with a 50 μm diameter aperture. The apparatus was shielded with sound shielding and a double Faraday cage and floated on an air table. Currents were recorded with a Medical Devices EPC-7 patch clamp amplifier and filtered at 10 Hz. The linear fit to the single-channel data reverses at −18 mV. The inset shows a channel opening at 70 mV (first arrow) and closing at −40 mV (second arrow). (B) Single channels of colicin A were assessed under the same pH 5/pH 3 conditions as in Figure 1C, except that the malate concentration was 5 mM. Due to the relatively low single-channel conductance under these conditions, it was critically important to account for the membrane's leak conductance. Colicin A (0.45 ng) was added to the cis solution ~40 min before the start of the record. In the first part of the record (steps to 30, 40, and 50 mV), there were no channels open, but there was a leak conductance of ~1.8 pS. The levels of the leak currents at 30, 40, and 50 mV are indicated by the long, medium, and short dashed lines, respectively. The step to 80 mV produced a somewhat complicated response that we shall describe in detail. After the initial capacitive transient decayed, there was only a leak current. Next, two channels opened at almost the same time, and they both closed 5 s later. (The 0.4 s delay between the openings is not visible in the figure.) Following this, a 41 fA channel opened, and 25 s later, a second, 48 fA, channel opened. The currents at 50, 40, and 30 mV were then recorded with these two channels remaining open. By comparing this total current at 50 mV with the corresponding leak current level (short dashed line), one can see that the (leak-subtracted) two-channel current is positive (26 fA). Similarly, the total current at 40 mV is only slightly positive (2 fA), relative to the leak current (medium dashed line), and the total current at 30 mV is negative (−16 fA), relative to the long dashed line. Thus, the reversal potential for the two-channel current is slightly less than 40 mV. In the inset, the leak-subtracted current is plotted against the membrane potential and fitted with a straight line. This gives a reversal potential of 39 mV, as well as a slope conductance of 2.2 pS for the two channels combined.

increases with a decrease in pH, suggesting that H^+ is the relevant ion. We should note that the KCl conductance of colicin A channels in symmetric pH solutions decreases by a factor of ~3 as the pH is lowered from 10 to 4 (*13*), so there is less KCl current (which, of course, reverses at 0

Table 1: Reversal Potentials of Colicin A Channels in Gradients of Three pH Units[a]

pH cis/trans	V_r (mV) by tail currents	V_r (mV) by zeroing
4/7	-40 ± 1 ($n = 3$)	-30 ± 1 ($n = 3$)
5/8	-21 ± 1 ($n = 10$)	-15 ± 2 ($n = 6$)
6/9	-10 ± 1 ($n = 3$)	-10 ($n = 1$)
7/10	-7 ± 0 ($n = 2$)	-2 ± 1 ($n = 2$)

[a] All measurements were taken in symmetric 0.1 M KCl, 5 mM $CaCl_2$, and 1 mM EDTA. Buffers were 20 mM malate (for pH 4 and 5), 20 mM HEPES (for pH 7 and 8), 8 mM MES (for pH 6), 20 mM Tris (for pH 9), and 20 mM CHES (for pH 10).

mV here) available to shunt the putative H^+ current as the pH is lowered. However, this effect is too small by itself to account for the pH dependence of V_r and therefore does not alter the inference that H^+, rather than OH^-, is the relevant ion.

Alternate Explanations for a Non-Zero V_r in a pH Gradient. Buffer molecules are generally present at much higher concentrations than H^+, and their conduction through the channel could, in principle, contribute to the measured reversal potential. At least one species of any buffer is itself an ion, and even with equal concentrations of buffer on both sides of the membrane, there will be a gradient of the buffer ions due to the pH gradient across the membrane. If different buffers are used, the gradients of each buffer ion will be practically infinite. If such ions carried a significant fraction of the current, they could give rise to large reversal potentials even in the absence of H^+ conductance. To examine the role of the buffer ions, we varied both the concentration and the identity of the buffers (Table 2). We also created a pH gradient without any added buffer, by adding small amounts of HCl to unbuffered solutions. Small effects of the buffers were observed, but in all cases, the proton anomaly was fully manifest. (Differences in the concentrations or species of buffers supporting a pH gradient are expected to alter somewhat the observed reversal potentials in a pH gradient due to their direct effect on the channel. The buffers are themselves permeant, and therefore, the pH profile in the lumen will depend on the buffering capacity on the two sides of the membrane and on the ability of the various buffering molecules to enter the lumen. Thus, any pH-sensitive chemical groups fully or even partially exposed to the lumen may see a slightly different pH under different buffering conditions, even in the same "bulk" pH gradient.)

Another possible concern is that all solutions exposed to air contain dissolved CO_2, which reacts with water to form $H_2CO_3 \leftrightarrow HCO_3^- + H^+$. In a buffered pH gradient, the bicarbonate ion will be asymmetric across the bilayer and could contribute to the reversal potential. Even though the concentration of HCO_3^- is only a few micromolar, the effect could be significant if bicarbonate were far more permeant than the other ions present. To examine this, we made fresh stock solutions of $KHCO_3$ and measured the reversal potential of a colicin A-treated membrane in a 0.1 M−1.0 M gradient at symmetric pH. We found a V_r of 34 mV, compared to a value of 42 mV when the same membrane was treated with the cation carrier nonactin. Thus, the channel is selective for K^+ over HCO_3^-, and bicarbonate from the air is unlikely to play an important role. We also looked at the effect of 5 mM $KHCO_3$ added to one side of colicin A-doped membranes in symmetric 0.1 M KCl at pH 5 and 8. As expected, the resulting changes in potential were small (≤ 2 mV).

Ammonium ion is a potential contaminant in our TEA solutions. To gauge whether it might account for the observed reversal potentials, we used nonactin, which can transport NH_4^+, but not TEA, across the membrane. In symmetric recrystallized 1 M TEA-Cl (pH 7.4), doping the membrane with nonactin increased the conductance by 30 pS. When symmetric 100 μM NH_4Cl was added, the conductance increased by 115 pS, putting an upper limit of less than 30 μM on any initial contamination with ammonium. Similar experiments with TEA_2-SO_4 prepared from vacuum-treated TEA-OH (see Materials and Methods) gave a limit of 10 μM ammonium. We then measured the reversal potential of a colicin A-containing membrane in a 0.1 M−1 M TEA-Cl gradient ($V_r = -5$ mV) and added NH_4Cl to both sides in an amount proportional to the salt concentration, so as to mimic any putative contamination. Adding 0.1/1 mM NH_4-Cl had essentially no effect [$\Delta V_r = 1 \pm 1$ mV ($n = 2$)]. Increasing the NH_4Cl concentration to 1 mM/10 mM shifted V_r by only 2.5 ± 1 mV ($n = 2$).

We also considered the possibility that the small volume ($\sim$1 μL) of the colicin A stock solution added to the cis side might create a gradient of some unknown, highly permeant ion. To test this, channels were incorporated into a membrane in 0.1 M KCl with a pH 5/pH 8 gradient; the cis compartment was then perfused with a fresh pH 5 bath solution to remove the hypothetical contaminant. The change in V_r was minor (<2 mV), thus allowing us to rule out this possibility.

Competing Ions. If H^+ were the only permeant ion, the reversal potential in a pH 5/pH 8 gradient would be -177 mV, the equilibrium potential for H^+. Other ions, at equal concentrations on both sides of the membrane, will tend to shunt V_r toward zero, to the extent that they contribute to the current. The simultaneous permeation by two or more ions can be described by two classes of models: those in which the ions can interact in the same channel and those in which there are at least two separate pathways for different ions. Due to the presumed large diameter of the colicin channel (see below), the former choice is unappealing, leading us to consider the latter. We suppose that the proton conductance, g_H, and the conductance of all the other ions collectively, g_i, are mediated by parallel, independent paths through the membrane. This simple model predicts a reversal potential

$$V_r = E_H g_H/(g_H + g_i)$$

when H^+ is the only asymmetric ion and E_H is its Nernst potential. We have two ways to control g_i: by varying the ion concentration (to a rough approximation, g_i is proportional to the concentration) or by the choice of cation species. [For instance, it is known that the single-channel conductance of colicin A in 1 M NH_4Cl is approximately twice that in KCl (*19*).] Increasing g_i, however it is done, is expected to decrease the magnitude of the reversal potential. Table 3 shows reversal potentials for colicin A channels measured in a pH 5/pH 8 gradient and with a variety of cation species and concentrations. The data qualitatively follow the expected trend. Experiments in NH_4Cl, however, form a notable

Table 2: Reversal Potentials (in millivolts) of Colicin A Channels in Various Buffers As Determined from Tail Currents

malate/HEPES (20 mM)	pH 5−pH 8	symmetric 0.1 M TEA	-38 ± 1 ($n = 5$)
malate/HEPES (5 mM)	pH 5−pH 8	symmetric 0.1 M TEA	-38 ± 2 ($n = 2$)
malate/Tris (20 mM)	pH 5−pH 8	symmetric 0.1 M TEA	-50 ($n = 1$)
succinate/HEPES (20 mM)	pH 5−pH 8	symmetric 0.1 M TEA	-39 ($n = 1$)
malate/HEPES (20 mM)	pH 5−pH 8	symmetric 0.1 M KCl	-21 ± 1 ($n = 10$)
succinate/HEPES (20 mM)	pH 5−pH 8	symmetric 0.1 M KCl	-24 ± 1 ($n = 3$)
MES/HEPES (20 mM)	pH 5−pH 8	symmetric 0.1 M KCl	-23 ($n = 1$)
no buffer	pH 3.2−pH 4.6	symmetric 9 mM KCl	-65 ($n = 1$)

Table 3: Reversal Potentials (in millivolts) of Colicin A Channels in a pH 5/pH 8 Gradient with Symmetric Salts

	relative g[a]	measured V_r	expected V_r[b]
19 mM KCl	0.19	-35 ($n = 1$)	-74
0.1 M KCl	1	-21 ± 1 ($n = 10$)	−
1.0 M KCl	10	-4 ($n = 1$)	-2.4
0.1 M NaCl	1	-19 ($n = 1$)	-21
0.1 M TEA-Cl	0.23	-38 ± 1 ($n = 5$)	-66
0.1 M CsCl	0.5	-28 ± 2 ($n = 3$)	-38
0.1 M LiCl	0.7	-26 ($n = 1$)	-29
0.1 M NH_4Cl	2	0 ± 0 ($n = 2$)	-11
30 mM NH_4Cl	0.6	-3 ± 4 ($n = 11$)	-33
0.1 M KCl and 30 mM NH_4Cl	1.6	-7 ± 3 ($n = 9$)	-14

[a] These values are the relative single-channel conductances of colicin A in the various salts based on the single-channel conductance in 1 M salt (as measured by us) compared to the single-channel conductance in 1 M KCl. We assigned a value of 1 to the conductance in 0.1 M KCl and assigned a relative g to the other conditions by taking conductance to be proportional to salt concentration. [b] These values are the predicted reversal potentials, i.e., $V_r = -177$ mV $[g_H/(g_H + g_i)]$, where g_H is the proton conductance and g_i is the conductance of all the other ions collectively.

Table 4: Reversal Potentials (in millivolts) of Various Channel-Forming Colicins[a]

	pH 5−pH 3	pH 5−pH 8
A	34 ± 2 ($n = 4$)	-21 ± 1 ($n = 10$)
B	−	-15 ± 1 ($n = 3$)
K	−	-6 ($n = 1$)
E1	14 ± 0.5 ($n = 3$)	-3.5 ($n = 2$)
Ia	15 ± 0.3 ($n = 4$)	-3 ± 1 ($n = 10$)

[a] Values were determined by tail currents, except for that of colicin Ia under the pH 5/pH 3 condition and that for colicin E1, in which cases the slow gating of the conductance required that V_r be determined by zeroing the current. All solutions contained 0.1 M KCl, 5 mM $CaCl_2$, and 1 mM EDTA. The solutions were buffered with malate (pH 3 and 5) or HEPES (pH 8) at 20 mM, except for the colicin E1 and colicin Ia pH 5/pH 8 conditions, where the buffers were at 5 mM.

exception: in 0.1 M NH_4Cl, V_r was 0 mV, much smaller than expected, warranting further study.

Singular Effect of NH_4^+. Even at a lower concentration of NH_4Cl (30 mM), we found that V_r was still close to zero (Table 3). This corresponds to a channel conductance somewhat lower than that in 0.1 M KCl and thus might be expected to evoke a V_r more negative (−33 mV, by the model) than that in 0.1 M KCl (−21 mV, measured), rather than the far more positive one actually measured (−3 mV). When 0.1 M KCl and 30 mM NH_4Cl were present simultaneously, the absolute value of V_r (7 mV) was greater than that with 30 mM NH_4Cl alone (3 mV) (Table 3). Because of membrane-to-membrane variations, this effect is more clearly illustrated by starting the experiment in 30 mM NH_4Cl, measuring V_r, adding 0.1 M KCl, and repeating the measurement. Under these conditions, the absolute value of V_r increased by 10 mV ($n = 2$) when the KCl was added; i.e., the relative proton current increased with respect to the other ions, rather than decreased. This non-ohmic effect of NH_4Cl is an effect on the proton selectivity, rather than on the KCl current, since NH_4Cl does not have a similar non-ohmic effect on the reversal potential of colicin A channels in KCl gradients at symmetric pH (not shown).

We considered the possibility that NH_4Cl might be acting in this instance as a lipid-permeable buffer, rather than as an interacting ion in the channel. Under these conditions, the pH gradient across the membrane creates gradients of NH_3 and NH_4^+, since

$$NH_3 + H^+ \leftrightarrow NH_4^+$$

This reaction will deplete NH_3 on the low-pH side, creating a driving force for its flux across the bilayer. To the extent that it permeates, NH_3 from the high-pH side will cross the membrane and pick up a proton on the low-pH side, thus degrading the pH gradient at the surface, and consequently reducing the measured reversal potential. Of course, this effect should be negligible if the solutions are well-buffered and well-stirred, but we cannot rule out an effect in a thin, unstirred surface layer. In this picture, the ability of 0.1 M KCl to shift the measured V_r in 30 mM NH_4Cl to a more negative value would be a membrane surface effect and should not depend on the K^+ permeability of the channel. To test for such an artifact, we substituted TEA-Cl for KCl in the experiment described above. Colicin A is much less permeable to TEA^+ than to K^+, so TEA^+ should be much less effective than K^+ in shifting V_r if the effect is in the channel, but equally effective if it is at the membrane surface. We found that 100 mM TEA-Cl shifted V_r only −1.5 mV (± 1, $n = 2$), as compared to −10 mV for 100 mM KCl, thus showing that the anomalous NH_4^+ effect is not an artifact due to conditions in the unstirred layer.

Since NH_4^+ apparently acts in the channel, we investigated whether ions of the form R-NH_3^+ might have a similar effect. We looked at lysine (a hydrophilic molecule with two, widely separated, amino groups) and dodecylamine, a hydrophobic ion with one amino group. We found that symmetric 30 mM lysine had no effect on V_r in a pH 5/pH 8 gradient in 0.1 M KCl, nor did 100 μM dodecylamine. (We were constrained to use low concentrations of dodecylamine, since it is a detergent and thus detrimental to planar bilayers, but the local concentration may well have been higher than 100 μM.) Both of these compounds are larger than NH_4^+, which may be related to their ineffectiveness in this assay.

Other Colicins. We measured the reversal potential of four other channel-forming colicins in a pH gradient (Table 4). All four (colicins B, K, E1, and Ia) displayed high proton selectivity, but the reversal potential of only colicin B approached that of colicin A. The channel-forming domain

of colicin B is the most homologous to that of colicin A among this group (*8*, *33*).

Other Channels. We measured the reversal potential of diverse channels in symmetric 0.1 M KCl in the presence of a pH gradient. The gating properties of these channels required that the measurements be made by zeroing the current.

1. Gramicidin. Gramicidin is a small peptide channel that has a higher selectivity for protons than for other monovalent cations (*34*, *35*). We measured a reversal potential of −2.4 mV in a pH 5/pH 8 gradient, and +5 in a pH 5/pH 3 gradient, far less than for colicin A under these conditions.

2. Nystatin. The antifungal polyene nystatin forms cation-selective channels when added to only one side of a sterol-containing membrane (*36*), as we did in the presence of a pH 5/pH 8 gradient (in an 80% asolectin/20% ergosterol membrane). Under these conditions, the reversal potential was zero.

3. Channel-Forming Domain of Anthrax Toxin (PA_{63}). The structure of the prepore of this heptameric channel has been determined (*37*). The pore itself is a β barrel forming a lumen ~12 Å in diameter (*38*, *39*). We measured a reversal potential of −1.5 mV ($n = 2$) in a pH 5/pH 8 gradient and one of 1 mV in a pH 5/pH 3 gradient.

4. T-Domain of Diphtheria Toxin. The diphtheria toxin channel shares many peculiar properties with colicin channels (see the Discussion). In a pH 5/pH 8 gradient, the reversal potential was −2.4 mV, similar to that of colicin Ia, but far from that of colicin A. In a pH 5/pH 3 gradient, it was 5 ± 2 mV ($n = 3$).

Isotope Effect. We measured the V_r of colicin A when H_2O was replaced with D_2O. In symmetric 0.1 M TEA-Cl and a pD 5−pD 8 gradient, $V_r = -43 \pm 3$ mV ($n = 3$) by tail currents, essentially the same value obtained in H_2O under corresponding conditions [-38 ± 1 mV ($n = 5$)].

Role of Counterions. Since colicin channels are permeable to both cations and anions, the possibility exists that protons may interact with permeant anions in the channel. For example, the presence of an anion in the channel is crucial to the ability of the large channels formed by amphotericin B to conduct cations (*40*). To see if such a putative ion−ion interaction might be relevant to the high proton permeability of colicin A, we measured the V_r of colicin A channels in a pH 5/pH 8 gradient in the absence of permeant anions. We first verified that SO_4^{2-} is impermeant (not shown) and did the experiment in symmetric 50 mM K_2SO_4, which has the same potassium concentration as 0.1 M KCl. The result (−24 mV) was essentially the same as in the choride salt, ruling out the permeability of the anion being an important factor in the proton selectivity.

Size of the Lumen. Since the high value of P_H/P_K (see Discussion) is remarkable only because the lumen of colicin channels is thought to be large, we reinvestigated this parameter. Tetraethylammonium ion (diameter of ca. 8 Å) was shown to permeate colicin channels on the basis of experiments that measured nonideal reversal potentials in TEA-Cl concentration gradients (*16*). Fearing that possible contamination from NH_4^+ inadvertently introduced along with TEA may have corrupted such experiments, we extensively degassed TEA-OH stock solutions under vacuum. This should remove any volatile contaminants, including NH_3, and thus clear the solutions of any contaminating NH_4^+. We then verified that any remaining ammonium contamination was at most a few micromolar (see above). The degassed TEA-OH was then titrated with HCl to make a TEA-Cl stock solution, which was used in subsequent experiments. Notwithstanding these heroics, we measured a reversal potential of 18 mV for colicin A in a 1 M−0.1 M TEA-Cl gradient,[2] far from the ideal reversal potential for Cl^- that we would obtain if TEA^+ were impermeant [using the strict anion carrier di(pentafluorophenyl)mercury, we measured a reversal potential of 41 mV in the same membrane].

To continue beating this dead horse, we measured the reversal potential of colicin A in TEA_2-SO_4 gradients under various conditions. SO_4^{2-} is highly impermeant, so the channel should be TEA^+-selective over SO_4^{2-} if TEA^+ is permeant. In a 25 mM−125 mM TEA_2-SO_4 gradient, V_r was 18 mV (TEA^+ over SO_4^{2-}). We do not know what the reversal potential would be under the ideal conditions where TEA^+ is the only permeant species (in part because we do not know the activity coefficient of TEA^+ under these conditions), but the observation that $P_{TEA}/P_{SO_4} > 1$ confirms directly that TEA^+ is permeant.

Since TEA^+ is permeant, we examined the permeability of the larger cation tetrakis(2-hydroxyethyl)ammonium (aka tetrakis). Tetrakis is TEA hydroxylated at each of its four β carbons. Thus, it is larger than TEA^+, symmetric, and more hydrophilic than TEA^+. We used the (intermittently) commercially available bromide salt and measured the reversal potentials of colicins A and Ia in 0.1 M/0.5 M gradients of tetrakis. The V_r values for colicins A and Ia were -9 ± 3 mV ($n = 2$) and -17 ± 4 mV ($n = 2$), respectively, compared to -33 ± 3 mV ($n = 2$) for di(pentafluorophenyl)-mercury under the same conditions. Thus, tetrakis(2-hydroxyethyl)ammonium ion is not impermeant in either channel.

Finally, we considered that the proton permeability itself might shunt the reversal potential measured in a salt gradient at symmetric pH, shifting it away from ideality. Such an effect could lead to a false conclusion of permeability of an impermeant salt ion. However, above neutral pH (the condition used for most of the sizing experiments), the proton current in the parallel path model is far too small to alter the conclusions. In fact, at symmetric pH 9.5 and with a 0.1 M−1.0 M TEA-Cl gradient, where the fraction of charge carriers represented by protons is on the order of 10^{-9}, the reversal potential of colicin A is 40 mV short of ideal chloride selectivity [−10 mV vs −50 mV, as determined with di(pentafluorophenyl)mercury]. The comparatively low chloride selectivity reflects the pH dependence of ion charge selectivity (*16*), rather than shunting of the current by protons.

DISCUSSION

The Fundamental Observation. The fundamental experimental finding in this paper is that the reversal potential of colicin A channels in pH gradients is anomalously large. We believe that this is due to an unexpectedly high selectivity for protons compared to all other permeant ions, an interpretation that appears irreconcilable with current views of colicin channels and mechanisms of ion selectivity. Specif-

[2] At pH 7.9. The solutions also included 5 mM $CaCl_2$, 5 mM HEPES, and 0.5 mM EDTA. Ca^{2+}, $HEPES^-$, and EDTA have low permeability and do not influence V_r at these concentrations.

ically, colicin channels are large compared to protons, even hydrated protons, and they are sufficiently promiscuous to allow through both anions and cations. Colicin A allows TEA^+ (8 Å) to pass. Even larger ions, such as tetrakis(2-hydroxyethyl)ammonium ion (9 Å), still contribute to the current. It is difficult to envision any channel structure that could manifest the high H^+/K^+ selectivity ratio reported here that would not preclude the passage of such large ions.

In general, the observation of a non-zero reversal potential (the voltage at which the total current = 0) across a membrane separating asymmetric salt solutions implies that the membrane is not equally permeable to all of the permeant ions that are asymmetrically disposed across it. Thus, the fact that the reversal potential in colicin A channels is not zero under the conditions of Figure 1B ($\Delta pH = 3$, symmetric 0.1 M KCl) signifies that either H^+ or OH^- is selected by the channel over the other ions present; that the magnitude of the reversal potential is as large as it is [−23 mV, compared to a theoretical maximum of −177 mV for the case where H^+ (or OH^-) is the only permeant ion in the system] signifies that a substantial fraction of the current is carried by H^+ (or OH^-), since the measured reversal potential reflects a weighted average of all the ionic currents. Furthermore, since the concentrations of H^+ and OH^- are orders of magnitude lower than the concentration of the permeant ions K^+ and Cl^-, this means that the selectivity of the channel for H^+ (or OH^-) versus K^+ and Cl^- is inordinately large. We attribute the effect to H^+, rather than to OH^-, on the basis of the observation that the magnitude of the reversal potential increases with a decrease in pH for a given ΔpH. These qualitative statements can be recast as quantitative ones, but to do so, one must rely on a mathematical model of selectivity. For example, using the GHK equation (*15*), a reversal potential of −20 mV in a pH 5/pH 8 gradient and symmetric 0.1 M KCl implies that $P_H/P_K = 12{,}000$. However, the data are sufficiently robust that the inference of an anomalously high H^+ permeability is independent of the particular model chosen.

Robustness of the Fundamental Observation. Since the large reversal potentials we found lead naturally to a rather radical interpretation, we searched for alternative explanations that would be more palatable. In the following discussion, we will review possible alternative explanations of the data and explain our reasons for rejecting them, and then we will review a few experiments designed to characterize the reputed H^+ selectivity. There are, for example, several experimental artifacts that can give rise to non-zero voltages in the sorts of experiments described here, and these need to be taken into account before we give credence to our results. Standard methods easily account for artifacts generated by the measuring system (such as potentials arising at agar bridge junctions), and in any case, such artifacts can be ruled out by the observation that several channels tested, such as anthrax protective antigen fragment PA_{63}, have, essentially, zero reversal potential in a pH gradient. In addition, we note that high proton selectivity was also observed in single-channel experiments, so it cannot be attributed to any unspecified cooperative interactions among the channels.

More sinister artifacts may be caused by other ions in the system, whether knowingly or unknowingly added. If colicin A has a high permeability to such an ion, asymmetries in its concentration could cause a large reversal potential. Any nontitratable contaminants in the salts used or the (double-distilled) water are not suspect since their concentrations were always equal across the bilayer. We ruled out the buffer ions themselves by varying the buffer species and buffer concentrations. If they were the source of the large potentials, then varying them in this way would greatly change or eliminate the reversal potential measured in a given pH gradient. It did not. In fact, the malate/Tris buffer arrangement in Table 2 would give rise to a potential opposite in sign to the one we observe, if the malate anion and/or the Tris cation were highly permeant. We also measured a large reversal potential (−65 mV) in a pH gradient (pH 3.2−pH 4.6) established in the absence of buffers. We considered that bicarbonate ion, naturally present in all solutions exposed to air, might be responsible, since its concentration would be a function of pH. To test this, we added $KHCO_3$ in the presence of KCl and found only small effects. We furthermore determined that colicin A is selective for K^+ over HCO_3^-, indicating that bicarbonate lacks the high permeability that would be necessary to skew the potential under our conditions. Finally, we considered that a titratable ion contaminant could form an ion gradient even if it was introduced into the chamber symmetrically (e.g., as a contaminant of the symmetric salt). The most likely ion in this category is NH_4^+, often present as a minor contaminant in salts. The colicin A channel has an only 2-fold higher conductance in NH_4Cl than in KCl (*19*), suggesting (albeit not proving) that NH_4^+ is not capable of producing the observed V_r values. We verified that a 0.1 mM−1 mM NH_4Cl gradient added to a 0.1 M−1 M TEA-Cl gradient barely shifted the reversal potential of colicin A channels by a millivolt, showing that NH_4^+ is not anomalously permeant (but see below). These experiments offer no alternative interpretation that can explain away the observed reversal potentials indicating proton selectivity.

Two Open States of the Colicin A Channel. Colicin A channels have two open states that are easily distinguished by their gating characteristics, even though they have the same, or nearly the same, conductance (*29*, *32*). A fraction (whose value depends on the particular conditions of the experiment) of a population of open channels is in a deep open state that is slow to close at moderate negative voltages, while the remainder of the channels close with a time constant of a few seconds. This circumstance means that we could use the method of tail current reversal to determine V_r values for only channels in the shallow open state. For channels in the deep open state, it was more practical to measure V_r by determining the voltage at which the DC current was zero. We found that the fast-gating channels were more proton-selective than the slow-gating channels (−21 mV vs −15 mV at the pH 5/pH 8 gradient in symmetric 0.1 M KCl). The null current method measures the V_r of the total membrane conductance and thus might include any nonspecific leaks along with the slow-gating colicin conductance, which would lead to a lower measured value of V_r. However, the slow-gating channels can usually be closed with a large negative voltage, allowing us to ascertain whether the conductance in question is indeed solely due to open colicin channels. Thus, we believe that the difference between the V_r values of the two states is real, i.e., not an artifact of the measuring technique; however, we are much

more impressed by the difference between either V_r and zero than that between the two V_r values. The essential finding, then, is that both of these states possess the anomalous proton conductance. Moreover, since we do not have even a hypothetical structure for either state, we can draw no conclusions about the difference in their V_r values and will not consider the subject further.

Colicin A versus Other Tested Channels. The high proton selectivity of colicin A channels is unequalled in the other colicins we examined, as well as in the other channels we used as controls. All evidence suggests that the channel-forming colicins share essentially the same structure in their soluble form, and the same gating mechanism, a mechanism that involves the translocation of a large fraction of the channel-forming domain to the far side of the membrane (*7*). Little is known about the structure of the open channel, but it does not seem rash to suppose that the structures of the various colicin channels will likewise turn out to be similar. In those colicins which have been examined, conductance, lumen size, and salt selectivity are qualitatively similar. Nevertheless, the proton selectivity of colicin A is much higher than that of any of the other colicins we tested (except for that of colicin B, the most homologous to A), which somewhat decouples this phenomenon from the basic structure of the channel. To verify the exceptional nature of the colicin A proton selectivity, we tested three channels with wide lumens and distinct structures, a polyene (nystatin), a β barrel (the PA_{63} fragment of anthrax toxin), and another protein with an α-helical structure in solution (the T-domain of diphtheria toxin). As expected, none exhibited the colicin A phenomenon. T-Domain, interestingly, had a non-zero (−3 mV) V_r in a pH 5/pH 8 gradient, comparable to that of colicin E1 or Ia under similar conditions. T-Domain bears many similarities to colicin channels, including the apparent ability of a single molecule to form a channel with a wide lumen and to translocate substantial segments of attached protein (*41*−*43*). To the extent that proton selectivity reports on the structure of the pore, the data imply that the T-domain channel is more like the colicin E1 or Ia channel than the colicin A channel.

Finally, we looked at gramicidin, a well-studied peptide channel that has a high selectivity for protons versus other monovalent cations (reviewed in ref *1*). Its lumen is only 4 Å in diameter, and it excludes all anions and all but the smallest cations. Protons are believed to permeate via a Grotthuss mechanism, hopping from water molecule to water molecule along the confined, single-file water wire that fills the lumen. Despite these constraints, gramicidin is only 10−100-fold selective for protons versus other monovalent cations, as determined under conditions different from those of our experiments here (*34*). We measured −2.4 mV in a pH 5/pH 8 gradient and 100 mM KCl, compared to −21 mV for the colicin A channel under the same conditions. Given the different ionic conditions and the inherent difficulties in measuring reversal potentials near 0 mV, our gramicidin results are consistent with those in the literature. It is striking to note that colicin A exhibits a far higher P_H/P_K than this single-file cation channel.

Colicin A versus Other Channels with High Proton Conductance. High proton selectivity in a wide channel, while first observed in colicins (*26*), is not unique to them. It has also been reported in the TRPV1 channel (*44*, *45*), a member of the Trp family of receptors, which play a variety of important roles in signal transduction. The biological significance of the high proton selectivity of TRPV1 is not yet clear, nor is it known whether the phenomenon is manifested in any other Trp channels. The reported P_H/P_K of TRPV1 (>1000), and its concurrent permeability to TEA^+ (*44*), are reminiscent of colicin A, although TRPV1 is a cation-selective channel, whereas colicin A is permeable to both cations and anions. The structures of the two channels are presumably quite different (e.g., Trp channels are thought to be tetramers, whereas colicin A is a monomer), and it of course remains an open question whether similar mechanisms are responsible for the high proton selectivity.

Colicin A and TRPV1 are, at least for the moment, rare examples of high proton selectivity in a wide channel. Many proton-selective channels have now been described (for a review, see ref *1*), and several mechanisms have been proposed to operate in them; however, a highly restricted conductance pathway is a persistent theme. Some (of which gramicidin is the paradigm) support a water wire, which may allow the passage of small cations, but (unlike colicin A) not anions or large cations. The single-file string of water molecules in the lumen is inherently selective for protons, which can diffuse through faster than can other ions, by exploiting the fast rotational diffusion of the water, as do protons in bulk water (where protons likewise have a comparatively high mobility). However, this mechanism confers a less than 10-fold mobility boost (*46*, *47*) and cannot account for our results in the absence of other effects. Members of another class of channels, including F_o, a component of membrane ATPases, and bacteriorhodopsin, the light receptor of bacterial purple membrane, conduct protons by mechanisms that involve the proton binding and unbinding to specific chemical groups (such as $R\text{-}COO^-$) along the way (*1*). The particular pathway may involve other steps, such as passage through a water wire, but selectivity is dominated by the near absolute requirement for a proton as ligand; thus, these channels are highly selective for protons. These channels are several orders of magnitude more proton-selective than are water wire channels (*1*). It is not clear how mechanisms such as these can account for the properties of colicin A, which is permeable to anions and large cations.

A telling feature of many proton channels is the fact that their conductance is hypersensitive to a shift from H_2O to D_2O; i.e., it is larger than would be expected on the basis of the properties of bulk water. Proton mobility in water is ∼1.4 times D^+ mobility in D_2O, but the ratio of currents in H_2O to D_2O is ∼2-fold in, for example, the M2 channel (*48*) and the voltage-gated proton channel of alveolar cells (*49*). This is generally interpreted to mean that the rate-limiting step for conductance in these channels is due to a mechanism involving protonation and deprotonation of particular chemical groups, rather than diffusion in water. In contrast, the P_H/P_D ratio in gramicidin, where conductance is via a water wire, is only 1.37 (*50*), similar to what would be expected for bulk water. We see no decrease in the proton selectivity of colicin A in D_2O compared to H_2O, suggesting that the D^+ current is close in magnitude to the H^+ current, and that protons are not primarily conducted via a protonation−deprotonation mechanism.

Ion−Ion Interactions Involving NH_4^+. The observation that V_r in a pH 5/pH 8 gradient with 30 mM NH_4Cl (as the only salt) is 0 mV rather than the expected −32 mV (Table 3) suggests that NH_4^+ interferes with the proton selectivity, as if it were a low-affinity blocker of a separate proton pathway. However, since the reversal potential is not 0 mV when 0.1 M KCl is present along with 30 mM NH_4Cl, NH_4^+ is not acting by blocking a proton pathway separate from the K^+Cl^- pathway. The fact that the selectivity of the channel to protons increases, rather than decreases, when the competing K^+ and Cl^- ions are added back into the chamber is a form of anomalous mole fraction effect and is diagnostic of ion−ion interaction in the pore (see, for example, ref *15*). NH_4^+ may bind to a site in the channel at or near a crucial proton-binding site, interfering with access to it by protons. K^+ ions in the channel would then increase the off-rate of NH_4^+ from the site, allowing better access by protons. Other schemes might be imagined, but all such interactions must occur in a tightly restricted volume so that the free energy of one ion in the pore is strongly affected by another one. One can imagine that these interactions are occurring in a separate $H^+/K^+/NH_4^+$ pathway. That is, the very observation of high proton selectivity in the colicin A channel suggests that the conduction pathway for ions is highly restricted, and the further observation that permeant ions H^+, K^+, and NH_4^+ interact in the pore is perfectly consistent with a restricted conduction pathway. The difficulty is reconciling such a restricted pathway with the permeability to large molecules. In fact, high concentrations of nonelectrolytes, such as ethylene glycol, do not increase the reversal potential in pH gradients (not shown). These compounds, which replace water in the regions of the pore that they can access and thus reduce conductance, would be expected to do just that if the proton pathway were separate, small, and inaccessible to the nonelectrolyte. This observation means that the proton pathway is just as susceptible to the nonelectrolyte as the ion pathway and thus is probably not fully separate from it.

Is the Proton Selectivity Anomaly a Problem? The data show that the colicin A channel selects for protons over other ions with an efficiency almost unknown outside of dedicated proton channels, but that in itself would not be a "problem" were it not for the fact that colicin channels are known to have large lumens. Since this point is so crucial to defining the situation, it behooved us to verify that the lumen is indeed large. We did this by repeating one old experiment and by conducting one new one. Both used quaternary ammonium ions as size probes. These ions are monovalent cations whose tetrahedral symmetry minimizes ambiguities in matching the probe diameter to the channel diameter; i.e., they cannot assume extended, if rare, structures that could account for a disproportionate fraction of any current they carry through the channel. TEA^+ (8 Å) has been shown to permeate colicin channels on the basis of the nonideality of the chloride selectivity in TEA-Cl concentration gradients (*16*), but such experiments could be confounded by permeant contaminants, the most likely of which is ammonium ion, which well might contaminate ammonia derivatives such as TEA^+. To eliminate ammonium, and any other volatile contaminants, we pumped on the TEA-OH stock solution at room temperature under vacuum before using it to prepare the TEA-Cl salt solutions. Nevertheless, reversal potentials measured using these solutions were more than 20 mV short of the ideal chloride potential. Finally, we measured a significant cation-selective reversal potential in a TEA-SO_4^- gradient. Taken together, these data make it difficult to avoid the conclusion that TEA^+ is permeant.

We also looked at reversal potentials in gradients of the larger ion tetrakis, which is essentially TEA hydroxylated at each of its four β carbons. Tetrakis is larger than TEA by ~1 Å, and its hydroxyls ensure that it is highly hydrophilic and is not able to penetrate the lipid bilayer. We find that it too passes through the colicin A channel. Thus, we cannot avoid the conclusion that the lumen is indeed at least as large as 9 Å in diameter, which is unmistakably larger in diameter than a single file of water molecules. Of course, this would not be a problem concerning the proton selectivity if protons had another pathway (it is still a problem for modeling the channel as a monomer), but we could find no evidence to support the existence of another pathway. Accordingly, we must answer the question posed above in the affirmative: the proton selectivity anomaly is indeed a problem.

CONCLUSIONS

Our main finding is that colicin channels, particularly colicin A channels, can exhibit an enormous selectivity for protons over other cations, on the order of 10^4 under certain conditions. Nevertheless, there is good evidence that the pore formed by colicin A is at least 10 Å in diameter, far wider than that of any other channel with a proton selectivity that is so high. In fact, under some pH conditions, P_H/P_K for colicin A is orders of magnitude higher than that found in "water-wire" proton-selective channels, such as gramicidin. Rather, the P_{H^+}/P_{K^+} of colicin A approaches that of the "hydrogen-bonded chain" (see ref *1*) proton channels, "channels" whose high selectivity for protons is due to a bottleneck in the ion pathway, where protons are required to bind and unbind to specific side groups on the protein to travel through the channel. The protons advance through the bottleneck by temporarily forming hydrogen bonds with a series of appropriately oriented H^+ acceptor and donors. Since other monovalent cations cannot form such bonds, they cannot cross. However, in contrast to the results presented here, hydrogen-bonded chain proton channels exhibit a large isotope effect, since the protons are unrestrained by water in the rate-limiting process. Of course, such a bottleneck, or even a water wire, would not be expected to admit molecules the size of, say, TEA, which readily pass through colicin A channels. Nevertheless, the high selectivity that we measure strongly suggests that H^+ is "seeing" a very narrow channel, even though colicin A also allows larger ions to pass. This view is reinforced by the "anomalous mole fraction" effect seen with K^+ and NH_4^+. Such an effect implies a strong interaction among the transported ions in the channel and is thought to be due to competition for a specific binding site, or at least for a very small volume of space, within the channel (*15*). These observations force us to take seriously the idea that the channel seen by small cations is narrow and to infer that the larger ions are moving through a different pathway. It follows judiciously that such pathways should be separated in either space or time from the H^+/small cation pathway.

ACKNOWLEDGMENT

We thank Dr. Zhengyan Wu for assistance with the experiments depicted in Figure 2A.

REFERENCES

1. Decoursey, T. E. (2003) Voltage-gated proton channels and other proton transfer pathways, *Physiol. Rev. 83*, 475−579.
2. Ramsey, I. S., Moran, M. M., Chong, J. A., and Clapham, D. E. (2006) A voltage-gated proton-selective channel lacking the pore domain, *Nature 440*, 1213−1216.
3. Konisky, J. (1982) Colicins and other bacteriocins with established modes of action, *Annu. Rev. Microbiol. 36*, 125−144.
4. Pugsley, A. P. (1984) The ins and outs of colicins. Part II. Lethal action, immunity and ecological implications, *Microbiol. Sci. 1*, 203−205.
5. Cramer, W. A., Heymann, J. B., Schendel, S. L., Deriy, B. N., Cohen, F. S., Elkins, P. A., and Stauffacher, C. V. (1995) Structure-function of the channel-forming colicins, *Annu. Rev. Biophys. Biomol. Struct. 24*, 611−641.
6. Lakey, J. H., and Slatin, S. L. (2001) Pore-forming colicins and their relatives, *Curr. Top. Microbiol. Immunol. 257*, 131−161.
7. Cascales, E., Buchanan, S. K., Duché, D., Kleanthous, C., Lloubès, R., Postle, K., Riley, M., Slatin, S., and Cavard, D. (2007) Colicin biology, *Microbiol. Mol. Biol. Rev. 71*, 158−229.
8. Parker, M. W., Pattus, F., Tucker, A. D., and Tsernoglou, D. (1989) Structure of the membrane-pore-forming fragment of colicin A, *Nature 337*, 93−96.
9. Wiener, M., Freymann, D., Ghosh, P., and Stroud, R. M. (1997) Crystal structure of colicin Ia, *Nature 385*, 461−464.
10. Elkins, P., Bunker, A., Cramer, W. A., and Stauffacher, C. V. (1997) A mechanism for toxin insertion into membranes is suggested by the crystal structure of the channel-forming domain of colicin E1, *Structure 5*, 443−458.
11. Vetter, I. R., Parker, M. W., Tucker, A., Lakey, J., Pattus, F., and Tsernoglou, D. (1998) Crystal structure of a colicin N fragment suggests a model for toxicity, *Structure 6*, 863−874.
12. Hilsenbeck, J. L., Park, H., Chen, G., Youn, B., Postle, K., and Kang, C. (2004) Crystal structure of the cytotoxic bacterial protein colicin B at 2.5 Å resolution, *Mol. Microbiol. 51*, 711−720.
13. Pattus, F., Cavard, D., Verger, R., Lazdunski, C., Rosenbusch, J., and Schindler, H. (1983). Formation of voltage dependent pores in planar bilayers by colicin A, in *Physical Chemistry of Transmembrane Ion Motions* (Spach, G., Ed.) pp 407−413, Elsevier, Amsterdam.
14. Krasilnikov, O. V., Yuldasheva, L. N., Nogueira, R. A., and Rodrigues, C. G. (1995) The diameter of water pores formed by colicin Ia in planar lipid bilayers, *Braz. J. Med. Biol. Res. 28*, 693−698.
15. Hille, B. (2001) *Ionic Channels of Excitable Membranes*, 3rd ed., Sinauer Associates, Inc., Sunderland, MA.
16. Raymond, L., Slatin, S. L., and Finkelstein, A. (1985) Channels formed by colicin E1 in planar lipid bilayers are large and exhibit pH-dependent ion selectivity, *J. Membr. Biol. 84*, 173−181.
17. Bullock, J. O., Kolen, E. R., and Shear, J. L. (1992) Ion selectivity of colicin E1: II. Permeability to organic cations, *J. Membr. Biol. 128*, 1−16.
18. Bullock, J. O., and Kolen, E. R. (1995) Ion selectivity of colicin E1: III. Anion permeability, *J. Membr. Biol. 144*, 131−145.
19. Pressler, U., Braun, V., Wittmann-Liebold, B., and Benz, R. (1986) Structural and functional properties of colicin B, *J. Biol. Chem. 261*, 2654−2659.
20. Krasilnikov, O. V., Da Cruz, J. B., Yuldasheva, L. N., Varanda, W. A., and Nogueira, R. A. (1998) A novel approach to study the geometry of the water lumen of ion channels: Colicin Ia channels in planar lipid bilayers, *J. Membr. Biol. 161*, 83−92.
21. Slatin, S. L., Nardi, A., Jakes, K. S., Baty, D., and Duché, D. (2002) Translocation of a functional protein by a voltage-dependent ion channel, *Proc. Natl. Acad. Sci. U.S.A. 99*, 1286−1291.
22. Jakes, K. S., Kienker, P. K., Slatin, S. L., and Finkelstein, A. (1998) Translocation of inserted foreign epitopes by a channel-forming protein, *Proc. Natl. Acad. Sci. U.S.A. 95*, 4321−4326.
23. Kienker, P. K., Jakes, K. S., Blaustein, R. O., Miller, C., and Finkelstein, A. (2003) Sizing the protein translocation pathway of colicin Ia channels, *J. Gen. Physiol. 122*, 161−176.
24. Slatin, S. L. (1988) Colicin E1 in planar lipid bilayers, *Int. J. Biochem. 20*, 737−744.
25. Zakharov, S. D., Kotova, E. A., Antonenko, Y. N., and Cramer, W. A. (2004) On the role of lipid in colicin pore formation, *Biochim. Biophys. Acta 1666*, 239−249.
26. Kienker, P., Slatin, S. L., and Finkelstein, A. (2002) Colicin channels have a shockingly high proton permeability, *Biophys. J. 82*, 555a.
27. Qiu, X.-Q., Jakes, K. S., Kienker, P. K., Finkelstein, A., and Slatin, S. L. (1996) Major transmembrane movement associated with colicin Ia channel gating, *J. Gen. Physiol. 107*, 313−328.
28. Glasoe, P. K., and Long, F. A. (1960) Use of glass electrodes to measure acidities in deuterium oxide, *J. Phys. Chem. 64*, 188−193.
29. Slatin, S. L., Duché, D., Kienker, P. K., and Baty, D. (2004) Gating movements of colicin A and colicin Ia are different, *J. Membr. Biol. 202*, 73−83.
30. Qiu, X.-Q., Jakes, K. S., Finkelstein, A., and Slatin, S. L. (1994) Site-specific biotinylation of colicin Ia: A probe for protein conformation in the membrane, *J. Biol. Chem. 269*, 7483−7488.
31. Jakes, K. S., Abrams, C. K., Finkelstein, A., and Slatin, S. L. (1990) Alteration of the pH-dependent ion selectivity of the colicin E1 channel by site-directed mutagenesis, *J. Biol. Chem. 265*, 6984−6991.
32. Collarini, M., Amblard, G., Lazdunski, C., and Pattus, F. (1987) Gating processes of channels induced by colicin A, its C-terminal fragment and colicin E1, in planar lipid bilayers, *Eur. Biophys. J. 14*, 147−153.
33. Pilsl, H., and Braun, V. (1995) Strong function-related homology between the pore-forming colicins K and 5, *J. Bacteriol. 177*, 6973−6977.
34. Myers, V. B., and Haydon, D. A. (1972) Ion transfer across lipid membranes in the presence of gramicidin A. II. The ion selectivity, *Biochim. Biophys. Acta 274*, 313−322.
35. Hladky, S. B., and Haydon, D. A. (1972) Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel, *Biochim. Biophys. Acta 274*, 294−312.
36. Kleinberg, M. E., and Finkelstein, A. (1984) Single-length and double-length channels formed by nystatin in lipid bilayer membranes, *J. Membr. Biol. 80*, 257−269.
37. Petosa, C., Collier, R. J., Klimpel, K. R., Leppla, S. H., and Liddington, R. C. (1997) Crystal structure of the anthrax toxin protective antigen, *Nature 385*, 833−838.
38. Nassi, S., Collier, R. J., and Finkelstein, A. (2002) PA63 channel of anthrax toxin: An extended β-barrel, *Biochemistry 41*, 1445−1450.
39. Blaustein, R. O., Lea, E. J., and Finkelstein, A. (1990) Voltage-dependent block of anthrax toxin channels in planar phospholipid bilayer membranes by symmetric tetraalkylammonium ions. Single-channel analysis, *J. Gen. Physiol. 96*, 921−942.
40. Borisova, M. P., Brutyan, R. A., and Ermishkin, L. N. (1986) Mechanism of anion-cation selectivity of amphotericin B channels, *J. Membr. Biol. 90*, 13−20.
41. Senzel, L., Huynh, P. D., Jakes, K. S., Collier, R. J., and Finkelstein, A. (1998) The diphtheria toxin channel-forming T-domain translocates its own N-terminal region across planar bilayers, *J. Gen. Physiol. 112*, 317−324.
42. Slatin, S. L., and Kienker, P. (2003). Colicin channels and protein translocation: Parallels with diphtheria toxin, in *Pore Forming Peptides and Protein Toxins* (Menestrina, G., Ed.) Harwood Academic Publishers, Basingstoke, U.K.
43. Gordon, M., and Finkelstein, A. (2001) The number of subunits comprising the channel formed by the T domain of diphtheria toxin, *J. Gen. Physiol. 118*, 471−480.
44. Hellwig, N., Plant, T. D., Janson, W., Schäfer, M., Schultz, G., and Schaefer, M. (2004) TRPV1 acts as proton channel to induce acidification in nociceptive neurons, *J. Biol. Chem. 279*, 34553−34561.
45. Vulcu, S. D., Liewald, J. F., Gillen, C., Rupp, J., and Nawrath, H. (2004) Proton conductance of human transient receptor potential-vanilloid type-1 expressed in oocytes of *Xenopus laevis* and in Chinese hamster ovary cells, *Neuroscience 125*, 861−866.
46. Bernal, J. D., and Fowler, R. H. (1933) A theory of water and ionic solution, with particular reference to hyrodgen and hydroxyl ions, *J. Chem. Phys. 1*, 515−548.

47. Robinson, R. A., and Stokes, R. H. (1959) *Electrolyte Solutions*, Butterworths, London.
48. Mould, J. A., Li, H. C., Dudlak, C. S., Lear, J. D., Pekosz, A., Lamb, R. A., and Pinto, L. H. (2000) Mechanism for proton conduction of the M(2) ion channel of influenza A virus, *J. Biol. Chem. 275*, 8592−8599.
49. Decoursey, T. E., and Cherny, V. V. (1997) Deuterium isotope effects on permeation and gating of proton channels in rat alveolar epithelium, *J. Gen. Physiol. 109*, 415−434.
50. Chernyshev, A., Pomès, R., and Cukierman, S. (2003) Kinetic isotope effects of proton transfer in aqueous and methanol containing solutions, and in gramicidin A channels, *Biophys. Chem. 103*, 179−190.
51. Wonderlin, W. F., Finkel, A., and French, R. J. (1990) Optimizing planar lipid bilayer single-channel recordings for high time resolution, *Biophys. J. 58*, 289−297.

BI701900X