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Gating processes of channels induced by colicin A, its C-terminal fragment and colicin E_1 in planar lipid bilayers

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Abstract. The dependence on pH and membrane potential of the pore formed by colicin A and its C-terminal 20 kDa fragment has been measured using planar lipid bilayers. The single channel conductance of the pore formed by both colicin A and the fragment increases with pH with an apparent pK of 6.0. At pH 5.0 the gating by membrane potential of the channels formed by either colicin A or its fragment is identical. At the same pH, quite similar pore properties were found when using the related bacteriocin, colicin E_1. In agreement with previous studies, these data indicate that the protein structure containing the lumen of the pore resides in the 20 kDa C-terminal part of the colicin A and favours the recently proposed model, based on protein sequence analysis, which proposes that colicin A, E_1 and I_B C-terminal domains are folded in the same three-dimensional structure. However, it is also shown that colicin A and not its C-terminal fragment undergoes a pH dependent transition between an "acidic" and a "basic" form of the pore with an apparent pK of 5.3. The two forms of the pore differ by their gating charge but not by the channel size. These results suggest that there is a pH dependent association between the C-terminal domain carrying the lumen of the pore and another domain of the molecule which affect the pore sensitivity to membrane potential.

Key words: Colicins, channels, planar lipid bilayers

Introduction

Colicins are bacterial toxins which kill sensitive *E. coli* cells. Their mode of action comprises three steps: *1)* Binding to a specific receptor located in the outer membrane; *2)* Translocation across the membrane(s); *3)* Interaction with their target in the cell.

Most of them are large proteins of 60,000–70,000 molecular weight and the domains associated with the steps defined above are organized in three distinct regions of the polypeptide chain (for a review see Konisky 1982; Pugsley 1984a, b).

Colicin A, like colicins E_1, K and I_B, forms voltage-dependent pores thus leading to depolarization of the cytoplasmic membrane (Schein et al. 1978; Cleveland et al. 1983; Tokuda and Koninsky 1978; Pattus et al. 1983a; Seta et al. 1983). The ionophoric activity of these colicins has been localized in the C-terminal domain of the protein (Dankert et al. 1982; Ohno-Iwashita and Imahori 1982; Cleveland et al. 1983; Bullock et al. 1983; Martinez et al. 1983; Davidson et al. 1984a).

The affinity of colicin A and E_1 for lipid interfaces is drastically increased when the pH is lowered (Pattus et al. 1983b; Davidson et al. 1984b, 1985). Moreover colicin A, E_1 and also the non pore-forming colicins E_2 and E_3 are capable of promoting efficient fusion of phospholipid vesicles at acidic pH (Pattus et al. 1985a).

Circular dichroism studies on colicins A, E_1 and C-terminal fragments (Brunden et al. 1984; Pattus et al. 1985b) revealed that the protein domain responsible for pore formation is rich in α-helices. Different models of the structure of the pore, all of them built out of α-helical segments have been proposed (Guy 1983; Davidson et al. 1984a; Pattus et al. 1985b). Modified colicin A proteins, obtained by site-directed mutagenesis are now available (Cavard et al. 1986; Baty et al. personal communication). Comparison of the properties of these proteins will help to test the validity of the proposed models of the colicin pore and give new insights into the mechanism of voltage-gating of the pore. A better characterization of colicin A pore properties was necessary in order to obtain clear-cut conclusions on the effects of single point mutation or short deletions on pore activity.

In this study, it is shown that colicin A can adopt two different channel conformations. The equilibrium between the two conformations is pH dependent. At acidic pH, colicin E_1, as well as the C-terminal bromelain fragment, present quite similar pore properties to colicin A. A first analysis of the gating properties of the pores is proposed, based on a simplified open-closed model.

Material and methods

Bromelain was purchased from Boehringer, Mannheim. Asolectin (Sigma lecithin type II) was purified according to Kagawa and Racker (1971). Colicin A and its C-terminal bromelain fragment were prepared as described previously (Cavard and Lazdunski 1979; Martinez et al. 1983). Colicin E_1 was a generous gift from D. Cavard (Marseille).

Planar bilayers

Planar bilayers were formed from asolectin vesicles by monolayer apposition as described by Schindler (1980). Electrical measurements were according to Schindler and Feher (1976). Holding potential (V) was injected into the cis side and the trans side is maintained at virtual ground. In some experiments (fast current relaxation recordings) a Keithley 230 programmable voltage source was used to apply voltage steps (conversion time of the voltage source < 1 ms).

In all the experiments the colicins or the peptides were injected in the cis side under vigorous stirring at + 90 mV applied potential.

Results

Single channel conductances

Single channel conductances were deduced from the stepwise increase of current after colicin A injection in front of an asolectin phospholipid bilayer and from closing and opening kinetics after a potential step across a bilayer containing a few channel molecules (Fig. 1 A–C). Almost exclusively, one type of conducting step was found independent of the type of recording. In rare cases, channels, 6 or more times larger, opened transiently (Fig. 1 B). These types of channels are not stable. Their occurrence increases with the number of colicin A molecules inserted in the bilayer. The analysis presented below deals only with the first highly reproducible type of channel.

As shown in Fig. 2 A, single channel conductance is voltage and pH dependent. However, the in-

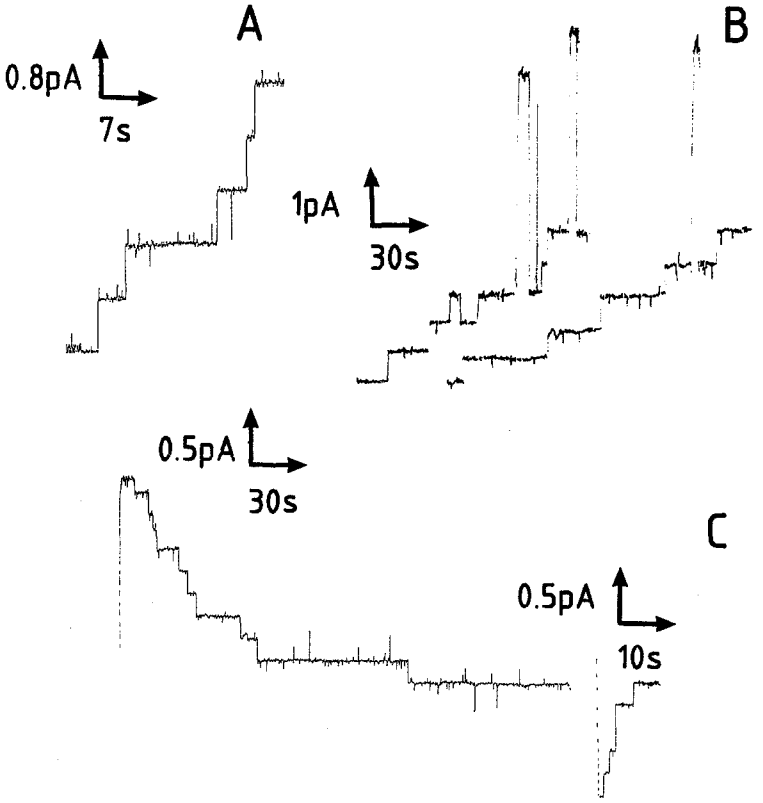

Fig. 1 A–C. Typical current fluctuations in planar bilayers containing colicin A channels. **A** Stepwise current increase after injection of colicin A (0.1 ng/ml) at 100 mV applied potential and pH 8.5. **B** Same conditions as **A** but pH 5.0. Note the occurrence of one or two rare large channel events. **C** Closing of colicin A channels after a +50 mV or −50 mV voltage step from 100 mV, pH 8.0. NaCl 1 *M*, $CaCl_2$ 5 m*M*, *tris*-acetate 10 m*M*

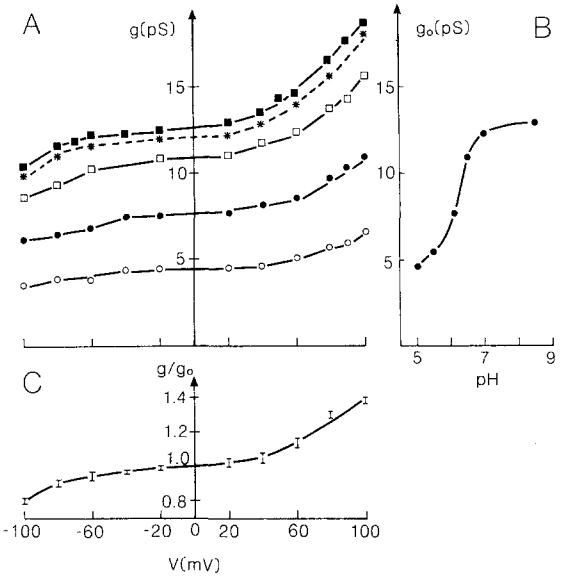

Fig. 2A–C. Influence of pH and voltage on single channel conductance. **A** Single channel conductance (g) as function of membrane potential. ○-○ pH 5.0; ●-● pH 6.1; □-□ pH 6.5; *-* pH 7.0; ■-■ pH 8.5. **B** Single channel conductance at 0 mV (g_0) as function of pH. **C** Normalized single channel conductance (g/g_0) as function of potential. Same conditions as in Fig. 1

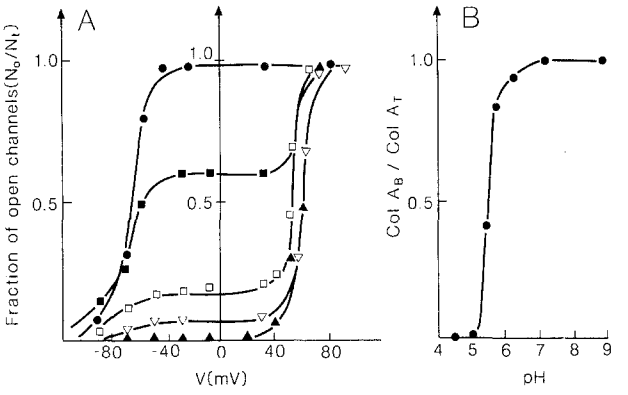

Fig. 3. **A** Plot of the fraction of open channels as function of voltage and pH. The number of channels in the open state $N_0 = G(V)/g(V)$, where $G(V)$ and $g(V)$ are the macroscopic and single channel conductances respectively. Above 80 mV applied potential $G(V)/g(V)$ is maximum, all the pores are in the open state ($N_T = G_{max}/g_{max}$). ●-● pH 5.0; ■-■ pH 5.45; □-□ pH 5.65; ▽-▽ pH 6.1; ▲-▲ pH 8.8. **B** Fraction of channels in the "basic form" as function of pH. This fraction (Col A_B/Col A_T) was calculated from Fig. 3A, assuming that, at 0 mV membrane potential, only the "acidic" form of colicin A channels contributes to the bilayer conductance. Col A_B/Col A_T = $1 - (N_0/N_T)$ $V = 0$

fluence of the transmembrane field on channel conductance $g(V)$ is the same independently of pH or the aqueous electrolyte (NaCl, KCl, NH_4Cl, RbCl; data not shown). As a consequence, normalized single channel conductances $g(V)/g(0)$ are only voltage dependent (Fig. 2, inset C). This reveals identical ion transfer kinetics within the channel whatever the composition of the aqueous phase. Ion transfer, quite ohmic between − 50 mV and + 40 mV, is markedly enhanced at potentials above 50 mV and decreased at negative potentials below − 70 mV. As found previously (Pattus et al., 1983a), single channel conductance is pH dependent. (An) amino-acid residue(s) with a pK of 6 influences single channel conductance (Fig. 2, inset C).

Macroscopic conductances

Steady state current voltage curve. The main experimental difficulty and source of errors with bilayer experiments and colicins is to reach a true steady-state level of conductance. As already discussed by Davidson et al. (1984a), the number of pores inserted in the bilayer increases almost indefinitely with time. However, after application of a potential higher than 70 mV for one hour, the rate of current increase due to the insertion of new colicin A molecules becomes negligible as compared to the kinetics of closing of the channels. In the following experiments quasi steady state curves were obtained, after reaching almost steady state current at 90 mV applied potentials, by decreasing the potential stepwise and recording the current until it reached a constant value. After the last recording at − 100 mV, a potential of 90 mV was applied again showed that the number of channels increased by no more than 5% during the recording of the current voltage curve. By assuming that macroscopic conductance arises only from the open state of the channels inserted in the bilayer, the number of conducting pores was calculated as the ratio of macroscopic conductance and single channel conductance at the same potential. Figure 3 shows the fraction of conducting pores (N_0/N_T) as a function of potential at different pH.

These curves strongly suggest that colicin A exhibits two forms of conducting pores whose relative amounts are pH dependent. At the extremities of the pH range, only one form is visible. The "basic" form (col A_B) is characterized by a switching voltage V_0^B of 50 ± 10 mV while the "acidic" form of the pore is characterized by a switching voltage V_0^A of -70 ± 10 mV. At intermediate pH ($5 < pH < 6$), both forms coexist. The relative proportions of the basic and the acidic form of the colicin A pore can be estimated from the fraction of open channels at 0 mV potential. As shown in Fig. 3B a group with a pK of 5.3 seems to affect the ratio of the two forms of colicin A pore.

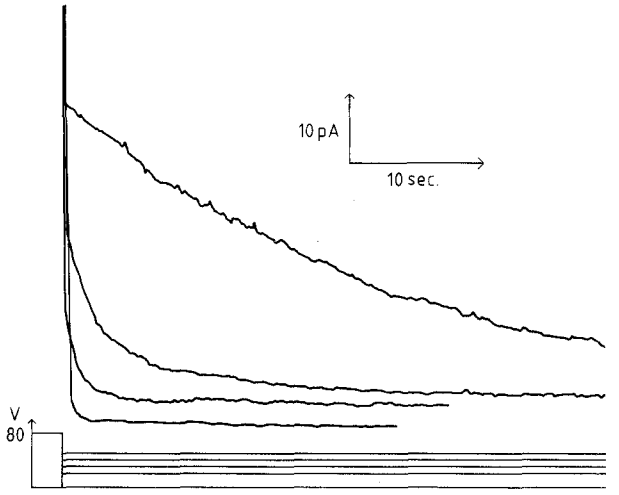

Fig. 4. Macroscopic current relaxation kinetics after voltage steps from 80 mV to 50 mV, 40 mV, 30 mV and 20 mV respectively. NaCl 1 *M*, $CaCl_2$ 5 m*M*, *tris*-acetate 10 m*M* pH 7.0

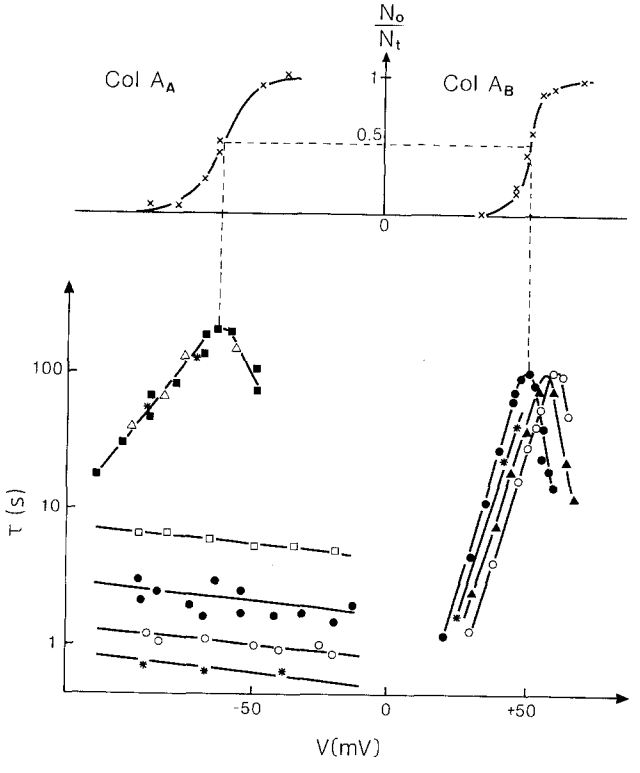

Fig. 5. pH and voltage dependence of the relaxation time constants. ▲-▲ pH 7.5; □-□ pH 9.0; ●-● pH 7.0; ○-○ pH 6.0; *-* pH 5.5; ■-■ pH 5.0; △-△ C-terminal bromelain fragment pH 8.5. Similar time constants were found with the C-terminal fragment at pH 5.5 and pH 5.0. The open state probability N_0/N_T of the "basic" and the "acidic" form of colicin A channels is shown on top. Note that the voltage where the relaxation time is maximum corresponds to the open state probability of 0.5

Kinetics of the closing process

As shown by Pattus et al. (1983a) the opening and closing kinetics after a voltage step from 100 mV is highly voltage dependent. The exponential decays of current shown in Fig. 4 are characterized by two parameters: the fraction of channels which remain in the open state at the end of the relaxation

$$N_0/N_T = I(t \to \infty)/I(t=0)$$

and the time constant of the exponential τ. The variation of N_0/N_T and τ with potential is shown in Fig. 5. The data presented in this figure again support the above assumption that the colicin A molecules can produce two different types of pores. The two forms of the channel differ not only in their switching voltage but also in their kinetic behaviour. The voltage dependence of the gating process appears more pronounced for Col A_B than for Col A_A. The time constant τ is maximal at the switching voltage. The value of this switching voltage is the same as the one determined from the pseudo steady state current voltage curve (Fig. 3). These two results indicate that the switching voltages are real values and not experimental artefacts due to the impossibility of reaching a real steady state conductance. Moreover, the different profiles obtained between pH 6 and pH 9 are superimposable differing only by the slight variations of the switching voltage from one membrane to another. The existence of an equilibrium between the two forms of the pore at intermediate pH is also indicated by the current relaxation curves. At intermediate pH ($5 < \text{pH} < 6$) the current relaxation curves can be decomposed in the sum of two exponentials according to the equation:

$$I(t) - I(t \to \infty) = I(t=0)\,[A\,e^{-t/\tau_A} + B\,e^{-t/\tau_B}], \qquad (1)$$

where A and B depend on the respective amounts of Col A_A and Col A_B channels in the bilayer.

Above pH 6, at negative potentials (well below the switching potential $V_0^B = +50$ mV) the time constants of the exponential decays vary only slightly with the applied potential (linear variation of the $\log\tau$, bottom left of Fig. 4). This lack of continuity of τ upon inversion of the transmembrane electrical field implies that closing of the channel is limited by a different closing step than in the positive voltage range. Here the movement of some "gating charge" is no longer the limiting step. Moreover, increasing the bulk pH decreases the closing rate.

Estimation of the gating charges of Col A_A and Col A_B pores

The voltage dependence of colicin A pores was analysed more quantitatively in terms of a two state

channel containing an "equivalent gating charge" as used to describe other channel forming protein pores (Hodgkin and Huxley 1952; Baumann and Easton 1980; Latorre et al. 1972; Ehrenstein et al. 1974).

Simplified model: For each type of colicin A channel ($Col A_A$ and $Col A_B$) it is assumed that the channel in the membrane is in a voltage dependent equilibrium between one conducting and one non-conducting state

$$(Col A)_0 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (Col A)_c .$$

The rate constants k_1 and k_{-1} vary with voltage according to Eyring's absolute rates theory.

$$k_1 = k_{01} \exp[Z \cdot \alpha \cdot f(V - V_0)], \quad (2a)$$

$$k_{-1} = k_{0-1} \exp[-Z \cdot (1-\alpha) \cdot f(V - V_0)], \quad (2b)$$

at $V = V_0$

$$k_{01} = k_{0-1} = k_0 . \quad (2c)$$

Z is the equivalent gating charge, which is considered here to be the same for both on and off processes.

α is the so called transfer coefficient and we assume here that $\alpha = 0.5$ as is usually done.

$f = F/RT$ with their usual meanings.

Note that the concept of "equivalent gating charges" might be replaced by that of variation of the dipole moment of the membrane-inserted protein with the electrical field (Schwarz 1978).

Under steady state conditions,

$$k_1/k_{-1} = N_0/N_c = \exp[f \cdot Z(V - V_0)] . \quad (3)$$

Applying Eq. (3) to the steady state conductance shown in Fig. 3 gives a value of 6 and 8 for the gating charge of $Col A_A$ and $Col A_B$ respectively (Z_A and Z_B). Due to the uncertainty of the steady state conductance measurements, these values should be considered as approximations. A value of 5 to 7 was obtained for Z_B by Schein et al. (1978) with colicin A. Analysis of the time constant data provides a second and more precise determination of the gating charge. The relaxation time constants are given by:

$$\tau = (k_1 + k_{-1})^{-1} \quad (4)$$

which leads to

$$\tau = [2k_0 \cdot \cosh(0.5 f \cdot Z(V - V_0))]^{-1} . \quad (5)$$

The best agreement with the experimental values is obtained with $Z_A = 4$ and $Z_B = 10$ which corresponds to rate constants at the switching voltage $k_A^0 = 2.5 \cdot 10^{-3}\ s^{-1}$ and $k_B^0 = 5 \cdot 10^{-3}\ s^{-1}$.

Pore properties of the C-terminal bromelain fragment of colicin A and of colicin E_1

As found previously (Martinez et al. 1983), the channel formed by the C-terminal bromelain peptide of colicin A (20,000 MW) is rigorously identical to the pore formed by colicin A at acidic pH (same gating charge, same single channel conductance). However, the voltage dependence of the pore remains unaffected upon raising the pH. No transition between an "acidic" and a "basic" form of the channel could be observed up to pH 9. In contrast single channel conductance is affected by an amino acid residue with a pK of 6 as found with the native colicin A pore.

In contrast to further claims (Davidson et al. 1984b, 1985) colicin E_1 was found to form pores up to pH 9.0 provided that a sufficient amount of protein was added to the cis compartment (> 10 ng/ml^{-1}). At pH 7 and above, the switching voltage seems to be less than −160 mV. No real quantifications of the current voltage curve could be obtained at these pH's due to the magnitude of the time constant of the process. At pH 5.0 the voltage dependence of colicin E_1 pores is remarkably similar to that of colicin A pores. The same switching voltage was found (−70 mV ± 10 mV), but somewhat higher time constants (200 s and 500 s for colicin A and colicin E_1 respectively).

Discussion

The results presented here demonstrate that colicin A and its 20 kDa C-terminal fragment create, in phospholipid planar bilayers, channels which are as strongly voltage dependent as channels from excitable membranes. In agreement with our previous study (Martinez et al. 1983), the identity of the single channel conductance at any pH and of the channel gating charge at acidic pH between colicin A and its C-terminal fragment indicates that the channel lumen is built by the 20 kDa C-terminal end of the polypeptide chain. Moreover, the very similar pore properties displayed by colicin E_1 at pH 5.0 favour the assumption based on amino acid sequence comparison that colicin E_1, A and I_B C-terminal domains are folded in the same tertiary structure and as a consequence have the same pore structure (Pattus et al. 1985b).

The pH dependence of the single channel conductance of colicin A or its C-terminal fragment may be a consequence of a pH dependence of the pore selectivity as demonstrated with colicin E_1 (Raymond et al. 1985). Preliminary experiments with

Na^+ and Cl^- ions have demonstrated that colicin A shows some cation selectivity at neutral pH and anion selectivity at acidic pH in asolectin membranes (data not shown). Our data suggest that acidic residues (asp or glu) modify selectivity and single channel conductance.

Conformational investigations, recently carried out, have demonstrated that the C-terminal domain of colicin A is rich in α-helices, especially in solvent of low polarity (Pattus et al. 1985 b). Structure predictions and calculations of hydrophobic moment and hydrophobicity profiles on colicin A, I_B and E_1 sequences have allowed selection of two or three helical segments of this domain which might be involved in pore formation (Pattus et al. 1985 b). These helices correspond to residues 481–501, 520–540, 548–572 in the colicin A sequence. According to these predictions, the most probable acidic residues which could alter single pore conductance upon protonation are glu 524 and 526 and asp 576 and 575, glu 524 and asp 575 being conserved in the three colicin sequences (A, E_1 and I_B).

More surprising is the difference observed in the voltage dependence of the pores produced by colicin A and its C-terminal fragment. Our results show that colicin A can adopt two different conformations depending on the pH of the medium. One conformation, the so-called "acidic form" has the same and close similarity to the colicin E_1 pore. The second conformation, the "basic" form displays a completely different voltage dependence although its single channel conductance is not distinguishable from the C-terminal fragment single channel conductance.

A similar shift of the switching voltage upon lowering the pH from 8.5 to 5.0 has been observed recently with channels induced by the mushroom toxin phallolysin (Wilmsen et al. 1985). In this case, however, the switching voltage continuously increases with pH. A linear relationship between pH and voltage indicates a direct influence of the ionisation of amino acid residues on the channel gating by membrane potential.

With colicin A, the lack of continuity of the switching voltage shift, the coexistence of two switching voltages at intermediate pH and the different behaviour of the colicin A and its fragment indicates an indirect effect of the pH and ionisation state of the amino acid residues with a pK of 5.3. Such results could be explained by the interaction of a second domain on the colicin A sequence with the channel structure at neutral and basic pH. Titration with an apparent pK of 5.3 would correspond to the dissociation of the two domains leading to a pore with identical properties to the pore formed by the C-terminal fragment alone.

In vivo studies on the interaction of monoclonal antibodies on native and modified colicin A by genetic engineering suggest that such interaction may occur (Cavard et al. 1985). The mapping of the antigenic sites indicates that the C- and N-terminal regions constitute surface domains protruding from the general surface structure. Moreover, the monoclonal antibodies directed against the COOH terminal region activate the binding of colicin A to its receptor and the in vivo killing activity. Although the model used to quantitate the gating properties of the pores over-simplifies the actual voltage dependence of the colicin A channel, it shows unambiguously that colicin A displays two forms of channels differing by their dependence on voltage. Comparison of the "equivalent gating charge" calculated according to this model, together with comparison of single channel conductance in different salt solutions and ionic selectivity will provide an easy and general description of the effects of point mutations or deletions on the channel function leading to a better understanding of the structural aspects of ionic channel functions.

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