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Gating Properties of Channels Formed by Colicin Ia in Planar Lipid Bilayer Membranes

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Summary. Colicin Ia forms voltage-dependent channels when incorporated into planar lipid bilayers. A membrane containing many Colicin Ia channels shows a conductance which is turned on when high positive voltages (> +10 mV) are applied to the *cis* side (side to which the protein is added). The ionic current flowing through the membrane in response to a voltage step shows at first an exponential and then a linear rise with time. The relationship between the steady-state conductance, achieved immediately after the exponential portion, and voltage is S-shaped and is adequately fit by a Boltzmann distribution. The time constant (τ) of the exponential is also dependent on voltage, and the relation between these two parameters is asymmetric around V_o (voltage at which half of the channels are open). In both cases the steepness of the voltage dependence, a consequence of the number of effective gating particles (n) present in the channel, is greatly influenced by the pH of the bathing solutions. Thus, increasing the pH leads to a reduction in n, while acidic pH's have the opposite effects. This result is obtained either by changing the pH on both sides of the membrane or on only one side, be it *cis* or *trans*. On the other hand, changing pH on only one side by addition of an impermeant buffer fails to induce any change in n. At the single-channel level, pH had an effect both on the unitary conductance, doubling it in going from pH 4.5 to 8.2, as well as on the fraction of time the channels stay open, $F_{(v)}$. For a given voltage, $F_{(v)}$ is clearly diminished by increasing the pH. This titration of the voltage senstivity leads to the conclusion that gating in the Colicin Ia molecule is accomplished by charged amino- acid residues present in the protein molecule. Our results also support the notion that these charged groups are inside the aqueous portion of the channel.

Key Words Colicin Ia · lipid bilayers · channels · gating charges · titration · pH

Introduction

Excitable tissues are typically characterized by the presence of ion channels in their membranes whose opening or closing can be controlled either chemically or by the electric field. An essential element in the functioning of a field-controlled channel is the rearrangement of charged gating structures in response to changes in the electric field. This process would be responsible, in the last instance, for a conformational change in the channel-forming protein leading to closing or opening of the channel. As expected, and predicted long ago by Hodgkin and Huxley (1952), the movement of these charged structures along the electric field generates a measurable "gating current" (Armstrong & Bezanilla, 1973; Keynes & Rojas, 1973).

Many attempts have been made to get a clear picture of these gated channels. From the literature it is evident that most of the information we have is concerned with the "selectivity filter" of various channels. With the patch-clamp technique many kinetic aspects of gated channels are now being resolved at the single-channel level (Sakmann & Neher, 1983). In recent years, cloning of the gene coding for the sodium channel-forming protein (Noda et al., 1984) and its sequencing has permitted rapid advances in the field. From a theoretical basis, the voltage dependence of a channel would require the presence of an "electric field sensor" in the protein moiety. Such an entity, commonly associated with the gating mechanism, could be represented either by dipoles or charged groups. The most straightforward way to distinguish between these two possibilities is to look at the degree of voltage dependence of the system as a function of pH. Variations induced by pH in the steepness of this voltage dependence would clearly indicate the presence of charged groups.

Colicins E1, A, Ia and Ib, on the other hand, are a group of bacteriocins, produced by *E. coli,* known to make aqueous channels in the inner bacterial membrane (Tokuda & Konisky, 1978). These proteins have molecular weights in the range 60,000 to 100,000 daltons and some of them have had their

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primary structure determined (Chan et al., 1985; Mankovitch, Hsu & Konisky, 1986). In fact it has been shown that Colicins E1, A and Ib have a similar C-terminal domain consisting of 35 to 48 uncharged amino acids, most probably involved in insertion into the lipid phase of the membrane (Slatin, Raymond & Finkelstein, 1986). With respect to Colicin Ia, we still lack information on the various domains of the protein involved in particular functions. As clearly shown for other Colicins (Schein, Kagan & Finkelstein, 1978; Weaver et al, 1981), Colicin Ia also forms voltage-gated ion channels in planar lipid bilayers.

The effects of pH have been tested previously on Colicin E1 and its C-terminal fragments. The most relevant results have shown that acidic pH (<5) increases activity in both planar bilayers and vesicles (Weaver et al., 1981; Davidson et al., 1984) and that pH can modulate selectivity (Raymond et al., 1985). Also dependent on pH is the conformation of the C-terminal tryptic peptide fragment (Brunden, Uratani & Cramer, 1984). Chemical modification of histidine residues has been reported to induce modifications in selectivity and single-channel conductance, depending on the pH of the solutions (Bishop et al., 1986).

In this paper we present results concerning the gating mechanism of the channel. By changing the pH of the solutions bathing the membrane, we could vary the degree of voltage dependence of the system and the single-channel conductance. This fact leads us to conclude that the voltage sensors in Colicin Ia are comprised of charged groups in the protein moiety and not dipoles. Using impermeant buffer, it was possible also to show that these sensors are localized inside the channel and not in the lipid phase.

Materials and Methods

Membranes were formed by the apposition of two monolayers across a hole in a Teflon® partition separating two aqueous solutions according to the method of Montal and Mueller (1972). Prior to membrane formation the partition was precoated with a 2% Vaseline® or a 2.5% squalene solution in pentane. Monolayers were spread from a solution of 1% asolectin, or 0.9% asolectin plus 0.1% DPPC in hexane. Asolectin is lecithin type II from Sigma and DPPC is diphytanoylphosphatidylcholine from Avanti Biochemicals Inc.

The aqueous solutions were (M): 0.1 KCl; 0.001 EDTA; 0.005 DMGA (dimethylglutaric acid) and 0.005 $CaCl_2$ or 0.5 KCl; 0.005 MES [2-(N-morpholino)ethanesulfonic acid]; 0.001 EDTA and 0.005 $CaCl_2$, final pH 6.2.

Where indicated pH was made acidic by adding small volumes of 0.1 N HCl to he solutions, and basic by adding 10 mM Tris plus a small volume of 1 N KOH until the desired pH was achieved. When an impermeant buffer was necessary, we used the high molecular weight carrier ampholite Pharmalite (Pharmacia), buffering between pH 6.5 and 9.0. In some experiments pH was changed on only one side of the membrane and in others on both sides. This fact will be pointed out in conjunction with the specific experiment.

Electrical conductances of the membrane were measured under voltage-clamp conditions by using an operational amplifier (42J -Analog Devices) in the current-to-voltage converter configuration connected to the preparation via a pair of calomel electrodes.

Ionic currents flowing through the membrane, in response to voltage steps, were either directly recorded on paper or captured on a digital oscilloscope screen (Gould OS-4000) and then sent to a Physiograph (Narco MK IV) on a slower time scale. This last procedure was particularly used when measuring the time constants (τ) of the current response. Data were either analyzed by hand or digitized with the aid of a plotter (HP-digital plotter 7225) and fed into a computer (HP-1000) for later analysis. Voltage polarity is referred to the side of the membrane containing colicin (*cis* side). The colicin-free side is called the *trans* side. Colicin Ia was added directly to the aqueous solution to a final concentration of 1–5 μg/ml for the macroscopic experiments or 1–2 ng/ml for the single-channel experiments.

Colicin Ia was kindly supplied to us by Dr. Jordan Konisky (University of Illinois, Champagne-Urbana).

Results

Strategy

Similar to what has been described for Colicin A (Schein et al., 1978), the addition of a small amount of Colicin Ia to one of the solutions bathing a lipid bilayer membrane leads to a drastic increase in conductance.

Figure 1 shows the time course of the ionic current across the membrane in response to a +50 mV step of voltage. As can be seen, the current record shows two distinct components: 1) an exponential rise with time and 2) an approximately linear increase with time, starting late in the record. This linear increase was also described by Kagan (1981) for Colicin Ia-treated membranes, and most probably represents channel incorporation either directly from the aqueous solution or from the torus, as proposed by Kagan. Whatever the mechanism, it is very much evident that these two components can be clearly distinguished on the basis of their time course. In this paper we will be concerned with the first portion of the curve, since it alone represents the process of opening and/or closing of channels already in the membrane. Therefore, the steady-state current (I_∞) will be taken as the maximum level attained by the current immediately before the linear component becomes visible (arrow in Fig. 1). Reversing the voltage polarity to −50 mV reduces the ionic current to a value very close to zero. The residual voltage-independent conductance ob-

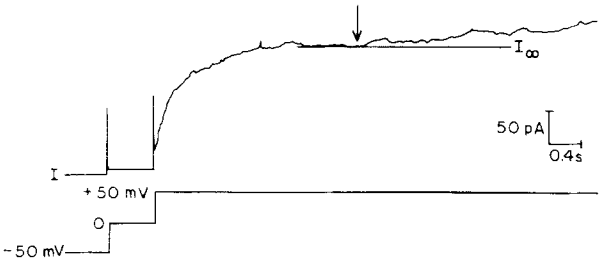

Fig. 1. Current response (I), as a function of time for a membrane treated with Colicin Ia (1 μg/ml) and pulsed to −50, 0 and +50 mV (lower trace). At −50 mV the current is practically zero. Arrow indicates where the linear increment starts, as well as the level of current taken as steady state (180 pA, marked I_∞). Solutions on both sides are at pH = 6.2, and the KCl concentration was 0.1 M. The time constant (τ) for the exponential portion is 0.3 sec

served at negative voltages was always subtracted from the total conductance observed at positive polarities. This voltage-dependent conductance is the result of aqueous channel formation in the membrane by the Colicin Ia molecule.

Assuming that these channels randomly open and close in response to the electric field imposed across the membrane, it can be shown that at equilibrium, i.e., after the current has reached a steady level, the relationship between conductance and applied voltage is described by an equation of the form (for a detailed derivation of this expression *see* Ehrenstein et al., 1974):

$$\frac{G_v}{(G_{\max} - G_v)} = \exp\left[\frac{-nq}{kT}(V - V_O)\right] \quad (1)$$

where: G_v is the steady-state conductance at a voltage V applied across the membrane, $G_{\max}$ is the maximum possible conductance, i.e., when all channels in the membrane are open; q is the electronic charge; k is Boltzmann's constant; T is the absolute temperature; V_O is the voltage where G_v = 1/2 $G_{\max}$ (*see* Ehrenstein et al., 1974, for a more complete definition of V_O) and n is the number of effective gating charges[1].

Therefore by plotting $\ln\left[\frac{G_v}{(G_{\max} - G_v)}\right]$ against voltage one can calculate both n, i.e., the degree of voltage dependence of the system, and V_O. Another way of measuring these parameters is to look at the time course of the current response as a function of the applied voltage. This analysis is restricted to the

[1] We use the term effective gating charges in order to emphasize the fact that what we measure is the result of charge movement either along the entire electric field or only a fraction of it.

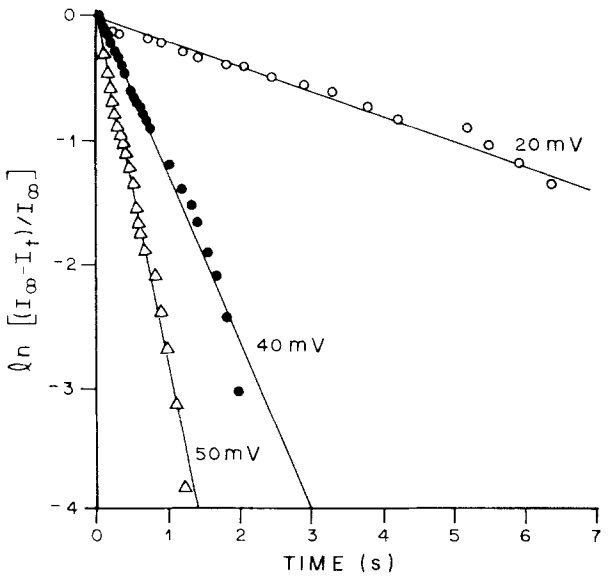

Fig. 2. Plot of $\ln[(I_\infty - I_t)/I_\infty]$ versus time for a membrane containing around 1000 channels and clamped at +20, +40 and +50 mV. Data refer only to the exponential portion of the current × time curve. I_∞ was taken as the value where the linear increment starts, indicated by the arrow in Fig. 1. Points were fitted by linear regression and the straight lines have correlation coefficients (r) equal to 0.994 at +50 mV, 0.991 at +40 mV and 0.995 at +20 mV, and time constants (τ) equal to 0.365, 0.775 and 4.995 sec, respectively. Bathing solutions had pH 6.2 and the KCl concentration was 0.1 M

first portion of the curve and its single exponential character can be seen by making a semilogarithmic plot of $[(I_\infty - I_t)/I_\infty]$ against time as shown in Fig. 2. The curves shown were obtained at three different voltages (+20, +40 and +50 mV) displaying the characteristic voltage dependence of their time constants (τ). Assuming that the channels already incorporated in the membrane obey a two-state model, the relationship between τ and voltage (V) will be given by (Erhenstein et al., 1974):

$$\tau = \frac{2\tau_{\max}}{\exp\left[\frac{-Q'(V - V_O)}{kT}\right] + \exp\left[\frac{Q''(V - V_O)}{kT}\right]} \quad (2)$$

where: $\tau_{\max}$ is the value of τ when $V = V_O$; V_O is the voltage at which half of the channels are open; Q' and Q'' are the total charges involved in the process of opening and closing the channel, respectively; k is Boltzmann's constant and T temperature (K).

Again, a semilogarithmic plot of τ against V allows us to calculate both V_O and the degree of voltage dependence of the system, i.e., Q' and Q''. On the other hand $Q' = n'q$ and $Q'' = n''q$ where n' and n'' are the effective number of charges and q is the electronic charge. Note also that the total number

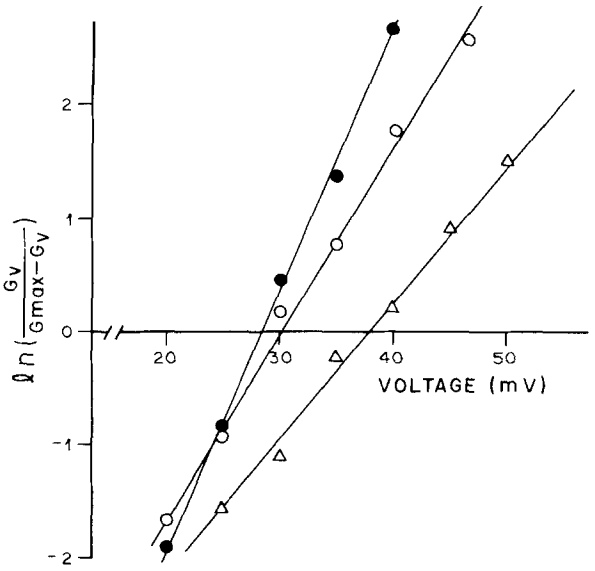

Fig. 3. Semilogarithmic plot of $\frac{G_v}{(G_{max} - G_v)}$ against voltage for a membrane treated with 0.8 μg/ml (final concentration) of Colicin Ia added to the *cis* side. The initial solutions had pH 6.2 (○) subsequently changed to 8.2 (△) or 4.5 (●) on both sides as described in Materials and Methods. KCl was at a concentration of 0.1 M. Points fitted by eye

of effective charges ($n = n' + n''$), calculated according to this method, should be equal to n obtained through steady-state measurements (*see* Harris, Spray & Bennett, 1981).

Assuming that n represents the number of effective gating charges moved during the opening/closing process, it is appealing to identify them either with dipoles or with charged groups of the amino acid residues of the protein. In order to distinguish between these two possibilities, we tested the effect of pH on n. Changes in n induced by variations in the pH of the bathing solutions would be an indication that the voltage sensors are charged groups.

Macroscopic Results

Figure 3 shows a semilogarithmic plot of $\frac{G_v}{(G_{max} - G_v)}$ versus voltage for a membrane containing many Colicin Ia channels. At first a G_v curve was obtained with the membrane separating solutions of pH 6.2 on both sides. The pH was then changed on both sides to 8.2 or 4.5 as described in Materials and Methods and conductances measured again for several applied voltages in the same membrane. Although highly desirable, it was not always possible to make all pH changes on the same membrane. The most common procedure was to start at pH 6.2 and then go to 8.2 or 4.5. Attempts to bring pH from 8.2 directly to 4.5 and vice-versa, resulted in breaking the membrane.

Table. Values of V_O, n and nFV_O as a function of pH[a]

pH	V_O (mV)	n	nFV_O (Kcal/mol)
4.5(9)	25.2 ± 3.6	6.2 ± 0.3	3.6 ± 0.6
5.1(12)	26.1 ± 3.9	5.9 ± 0.4	3.5 ± 0.6
5.8(10)	28.0 ± 2.6	5.6 ± 0.3	3.6 ± 0.5
6.2(10)	29.5 ± 2.8	5.3 ± 0.2	3.5 ± 0.3
6.8(11)	33.2 ± 3.1	4.2 ± 0.3	3.2 ± 0.3
7.4(8)	35.4 ± 3.6	3.6 ± 0.2	2.9 ± 0.3
8.2(12)	38.5 ± 4.1	3.1 ± 0.3	2.7 ± 0.2

[a] n was calculated as shown in Fig. 3. Numbers are mean ± SD; numbers in parentheses represent the number of experiments. F is the Faraday. Symmetrical change in pH as described in Materials and Methods.

Closer inspection of Fig. 3 reveals three main facts: 1) The experimental points can be well fitted by a single exponential type relation, as expected from Eq. (1), independent of the pH used; 2) as the pH is changed from 4.5 to 8.2, there is a shift in V_O to higher values; and 3) not only V_O varies but, more importantly in our case, n decreases with more alkaline pHs. For this particular membrane $n = 4.6$ and $V_O = 30$ mV at pH 6.2; $n = 3.2$ and $V_O = 36$ mV at pH 8.2; and $n = 5.8$ and $V_O = 27$ mV at pH 4.5.

The titration observed in Fig. 3 is reversible; i.e., if the experiment was started at pH 6.2 and then the pH changed to 8.2 or 4.5, both n and V_O return to the initial values upon returning the pH to 6.2. Another effect of pH was manifested on G_{max}; decreasing pH from 6.2 to 4.5 decreases G_{max} by a factor of two, and increasing it from 6.2 to 8.2 increases G_{max} 1.5 times. These variations are in part explained by alterations in the single-channel conductance (*see* Fig. 6).

Figure 4 (*a, c*) shows the relationship between (τ) and voltage, plotted according to Eq. (2). Two main facts arise from this Figure: 1st) the relationship is not symmetrical around V_O and 2nd) the degree of voltage dependence of the time constants is influenced by the pH of the bathing solutions. As before, acidic pH's lead to a stronger voltage dependence than more alkaline pH's.

The Table is a collection of values of n and V_O obtained on several different membranes, in experiments like those of Fig. 3. n clearly varies at least 2 units in going from pH 4.5 to 8.2. In other words, the system loses more than half of its voltage dependence at pH 8.2 in comparison with pH 4.5. Also shown in the Table is the product nFV_O which changes very little in the same pH range.

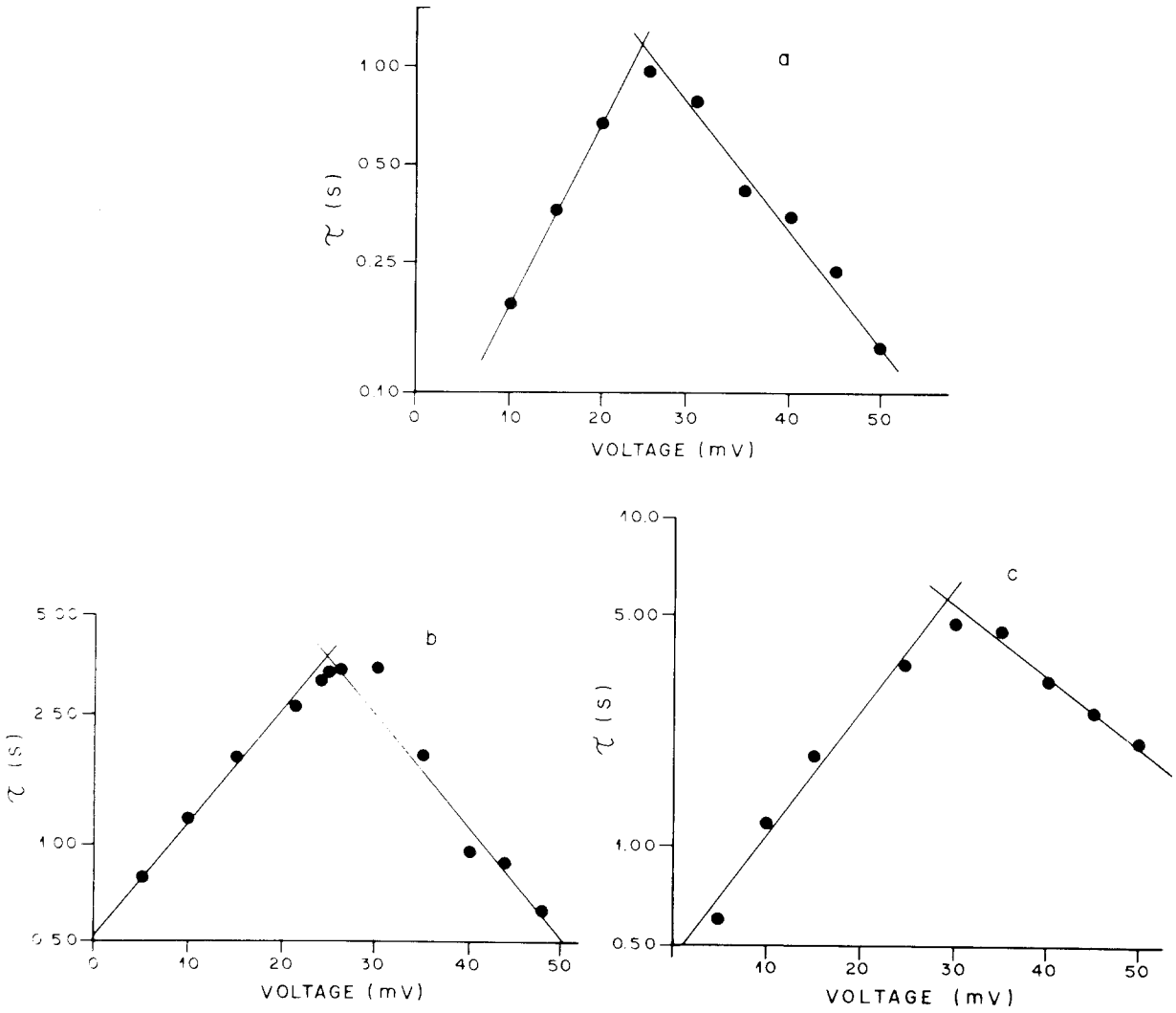

Fig. 4. Semilogarithmic plot of τ versus voltage across the membrane. τ was calculated from plots such as those shown in Fig. 2. In (*a*) the membrane had pH 5.2 on both sides ($n = 5.5$ and $V_O = 24$ mV): in (*b*) the pH was 6.2 ($n = 4.5$ and $V_O = 27$ mV) and in (*c*) the pH was 8.2 ($n = 3.8$ and $V_O = 33$ mV). The KCl concentration was 0.1 M in all cases. n and V_O were calculated according to the text. Curves shown were adjusted by eye on points far from V_O

Microscopic Results

Decreasing the Colicin Ia concentration in the bathing solution to the ng/ml range makes it possible to observe discrete jumps in the current flowing through the bilayer. This observation lends support to the hypothesis that Colicin Ia-induced conductance is the result of aqueous channel incorporation into the membrane, as described by others (Schein et al., 1978; Kagan, 1981). Figure 5 shows the qualitative behavior of the channel as a function of the applied voltage. At +50 mV the channel stays open most of the time. Decreasing the voltage to +25 mV leads to a situation where the closed state predominates. In some traces at voltages smaller than +25 mV we could also see substates in the channel.

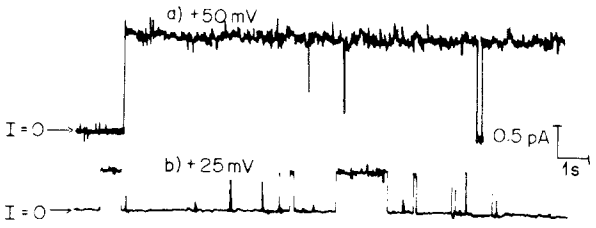

Fig. 5. Single-channel behavior of Colicin Ia at two different applied voltages: a) +50 mV and b) +25 mV. Upward deflections indicate channel opening. Bathing solutions had 0.5 M KCl and pH 6.2

Since this finding was not consistent in our experiments, we will not pursue it further here. The *I-V* relationship for such channels is shown in Fig. 6.

The ohmic behavior is clearly evidenced by the resulting linear relationship. The single-channel conductance is also pH dependent, being 44 pS at pH 8.2, 28 pS at 6.2 and 20 pS at pH 5.2 at a KCl concentration of 0.5 M.

Since, during the time course of most of our experiments more than one channel entered the membrane, especially at high positive voltages, we were unable to directly measure the rate constants for opening and closing the channels. For this reason we decided to express our results in terms of $F_{(v)}$, i.e., the fraction of time a given channel stays open. Clearly, in a membrane having more than one channel $F_{(v)}$ is given by:

$$F_{(v)} = \sum_{i=1}^{n} n_i^A \cdot t_i^A/(N \cdot T) \tag{3}$$

where: n_i^A is the number of open channels, t_i^A is the time during which these channels stayed open, N is the total number of channels and T is the total time of observation. N was checked before and after a given measurement by applying a large positive pulse known to open all channels in the membrane.

Assuming again a two-state model for the channel, it is easily shown that the fraction of time a given channel stays open $F_{(v)}$ should be related to the voltage across the membrane by the following equation (Ehrenstein et al., 1974):

$$F_{(v)} = \frac{1}{1 + \exp\left[\frac{-nq}{kT}(V - V_O)\right]} \tag{4}$$

where n, q, V, V_O, k and T have the same meaning as defined before.

As the above equation suggests, we have another way of obtaining V_O and n. Figure 7*A* shows a plot of $F_{(v)}$ against voltage for membranes at pH 5.2, 6.2 and 8.2. The experimental points are well fit by Eq. (4), and again the degree of voltage dependence varies with pH, n being 5.6, 3.16 and 1.5 at pH's 5.2, 6.2 and 8.2, respectively (Fig. 7*B*).

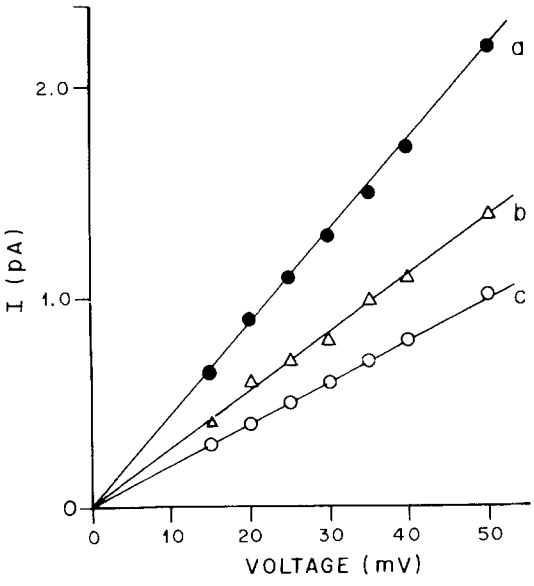

Fig. 6. Current-voltage relationships for a Colicin Ia channel obtained at pH 8.2 (*a*), 6.2 (*b*) and 5.2 (*c*). The single-channel conductances are 44, 28 and 20 pS for conditions *a*, *b* and *c*, respectively. Solutions had KCl at a concentration of 0.5 M

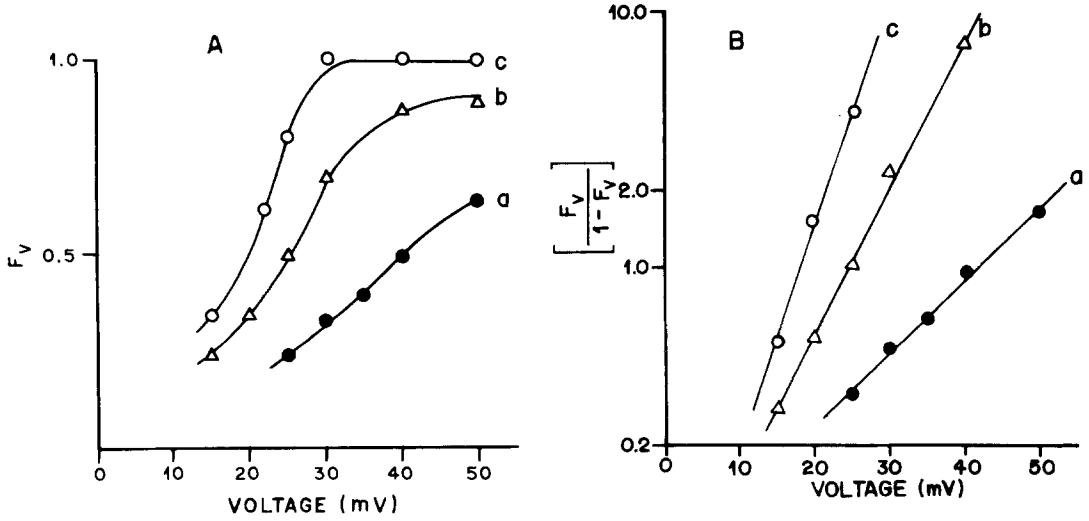

Fig. 7. (*A*) Fraction of time $F_{(v)}$ a channel stays open as a function of voltage. Curves *a*, *b* and *c* refer to solutions having pHs 8.2, 6.2 and 5.2, respectively, and were drawn by eye. (*B*) A plot of $\ln[F_{(v)}/(1 - F_{(v)})]$ linearizes the experimental points. At pH 5.2, $n = 5.6$ and $V_O = 20$ mV; at pH 6.2, $n = 3.16$ and $V_O = 30$ mV, and at pH 8.2, $n = 1.5$ and $V_O = 40$ mV. The KCl concentration was 0.5 M in all cases

Localization of the Electric Field Sensing Elements

The results shown in previous sections refer to measurements made on membranes having the same pH on both sides, i.e., under symmetric conditions. In an attempt to get some structural information on this channel, we also determined n and V_O under conditions of asymmetric pH. Experiments were performed in the following way. Initially the membrane separated solutions of pH 6.2 on both sides. A control G-V curve was obtained. Then, pH was changed to 4.5 or 8.2 either on the *cis* side only or on the *trans* side only. Figure 8 shows data for a membrane in which the pH was changed to 8.2 on the *cis* side, keeping the *trans* side at pH 6.2. Similar results were obtained by changing the pH on the *trans* side only.

Note that in changing the pH on only one side, we had to add 20 mM (final) of the indicated buffer to that particular side of the membrane[2]. Therefore the buffer capacity is higher on that side (20 mM) than on the one with the initial pH (5 mM). This fact leads us to the conclusion that the buffer must have access to the sensors in order to titrate them, and in this case it is reasonable to assume that they are in the aqueous phase of the channel. In order to test this hypothesis, we devised two kinds of experiments: a) Starting with symmetric pH (6.2 on both sides), we measured the conductance as a function of voltage. Then the pH was changed on only one side, as previously described. Another G-V curve was obtained. As expected, n and V_O changed in accordance with this new pH. Then the buffer capacity on the side with pH 6.2 was increased by adding MES or DMGA (20 mM final concentration), the pH was kept at 6.2, and n and V_O were again determined. b) In other experiments the pH was changed on only one side, but using an impermeant buffer which should not have access to the channel interior. Although the pH was effectively changed in the solution bathing the membrane, it is not significantly altered at the electric field sensors site, if that site lies within the channel, and therefore n and V_O should not change. Figure 9*A* and *B* show results of these kinds of experiments, respectively.

[2] In these experiments V_O could have changed also as a consequence of asymmetric surface potentials. This asymmetry would arise as a consequence of negatively charged lipids present in the membrane since Asolectin has around 8.8% cardiolipin (Kagawa & Racker, 1966). In this way different pH's on each side would produce asymmetrical screening of these charges and therefore asymmetrical charge densities, leading to a shift in the conductance-versus-voltage relationship.

Discussion

Colicins of the E1 class (A, K, Ia, E1) are well known for their conspicuous effect on the plasma membrane of bacterial cells. As a major result of their action, the cell loses potassium and depolarizes (Gould & Cramer, 1977; Davidson et al., 1984). Incorporation of these water-soluble proteins in lipid vesicles or planar lipid bilayer membranes led to the conclusion that they are able to form voltage-gated aqueous channels. The present results are concerned with the gating of the Colicin Ia molecule in planar bilayers. We chose to study this particular colicin because (1) its turn-on and turn-off kinetics are faster than those of other colicins; (2) because it is possible to clearly distinguish between channel incorporation and opening or closing of channels already in the membrane; and (3) insertion of this protein into the bilayer does not require an acidic pH. Furthermore, it can be obtained as a single purified protein in copious amounts. These facts make it a very profitable model for studying voltage-dependent conductance phenomena. Besides this the field has been enriched in the last years with the sequencing of several proteins, and in the case of

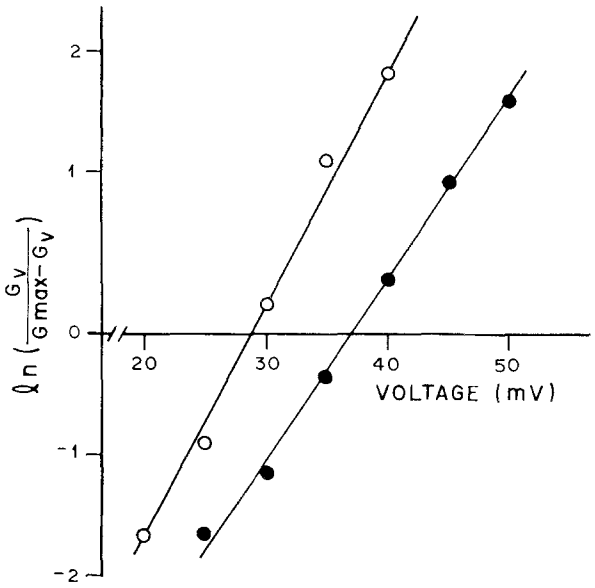

Fig. 8. Semilogarithmic plots of $\frac{G_v}{(G_{max} - G_v)}$ for a membrane initially with pH 6.2 on both sides (○). Subsequently the pH only on the *cis* side was changed to 8.2 (●). In the symmetrical condition, $n = 4.9$ and $V_O = 28$ mV; after changing the pH to 8.2 on the *cis* side, $n = 3.5$ and $V_O = 37$ mV. Final Colicin Ia concentration = 0.8 μg/ml. In this experiment the solution was initially buffered with 5 mM MES. pH was changed on the *cis* side by adding Tris to a final concentration of 20 mM. Therefore its buffer capacity is higher than in the *trans* compartment. The KCl concentration was 0.1 M

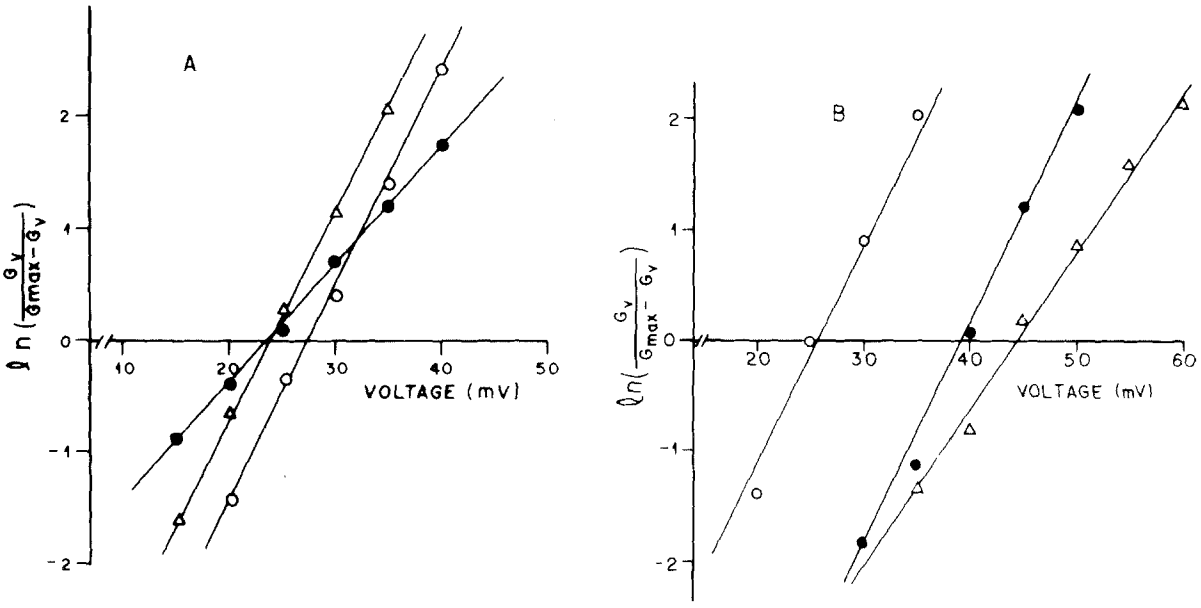

Fig. 9. Plots of $\ln\left[\frac{G_v}{(G_{max} - G_v)}\right]$ against voltage for membranes initially bathed by symmetrical solutions having pH 6.2 and a low buffer concentration (1 mM MES). Under these control conditions (○–○) both membranes (*A* and *B*) gave an n around 5.5 and V_O around 26 mV. In *A* the effect of increasing pH to 8.2 only on the *trans* side by adding 5 mM Tris plus KOH is shown (●–●); n decrease to 3.0 and V_O = 23 mV. Then the buffer capacity was increased on the *cis* side (keeping pH at 6.2) by adding MES to a final concentration of 20 mM; n increased to 5.2 and V_O is now 24 mV (△–△). (*B*) shows the same kind of experiment but using an impermeant buffer. Addition of 40 μl of Pharmalite plus KOH to the *cis* side increased its pH to 8.2, but had no significant effect on n (●–●). V_O, on the other hand, increased to 38 mV. Finally, Tris (10 mM final) was added also to the *cis* chamber, the pH remained at 8.2; n decreased to 3.6 and V_O increased to 44 mV (△–△). The buffer capacity of the solution containing Pharmalite was around 3.5 × 10^{-6} moles/pH_{unit} · ml. For comparison, the solution having 1 mM MES had a buffer capacity of 0.6 × 10^{-6} moles/pH_{unit} · ml. The KCl concentration was 0.1 M.

colicins particular attention has been given to Colicin E1 (Yamada et al., 1982; Chan et al., 1985; Raymond et al., 1985; Bishop et al., 1986; Slatin et al., 1986). In this case it is well established that the channel-forming domain is associated with the COOH-terminal end and that pH affects single-channel conductance, selectivity and insertion rate. As far as Colicin Ia is concerned, only recently its gene was cloned and the protein sequenced (Mankovich et al., 1986). In this paper we used an analysis based on an assumed equilibrium Boltzmann distribution for measuring n and V_O. As defined previously, n is a measure of the voltage sensitivity of the system.

This parameter should correspond, in a two-state model, to the number of effective gating charges moved during the process of opening or closing the channel. If we take a more general view independent of any kinetic model, it is also clear that this voltage sensitivity must be related to voltage sensors present in the channel-forming protein. Therefore increasing or decreasing the voltage dependence of the system means inducing variations on these sensors (gating charges).

At first sight a main criticism could be raised to this study: in considering the macroscopic results, it seems that a steady state is not reached in the current traces if the voltage is kept on for a long time and the paper run at low speed. Closer inspection of the records (Fig. 1) reveals, however, that the trace is composed of two distinct components. A fast one (around 1–2 sec), followed by a linear slow increment of the current with time. Our view is that the first portion of the curve represents the opening of channels already in the membrane and the second is due to channel incorporation into the bilayer. That this is so can be supported by the following argument: by taking the steady-state current of the first portion equal to the value at which the linear increment starts, not only the equilibrium data are well fit by a Boltzmann distribution, but the temporal evolution of the current is very well described by a single exponential (Fig. 2). This fact has two implications: 1st) it reassures us that the current is flowing through channels, already incorporated into the membrane, which are opening in response to the electric field, and 2nd) the system can be viewed, in principle, as a two-state channel.

The data in Figs. 3, 4 and 7 also show that n can be varied by changing the bathing solution pH. Going to more alkaline pH leads to a reduction in the voltage sensitivity of the system. Not only n varies, but also V_O increases with alkalinization. In this case the shift in V_O with pH cannot be attributed simply to a surface potential difference, since pH was equally changed on both sides of the membrane. The Table lists n for several pH's and various membranes. It is clearly seen that in going from pH 4.5 to 8.2 n changes by approximately 3 units. These data also suggest an apparent pK around 6 for the charged group. Combining this with the fact that alkalinization leads to a decrease in n, we can argue that the groups involved should be positive. We have no way of deciding how much of the electric field is sensed by the gates. The crucial point that arises from these data is that the voltage dependence of the system can be titrated. This is possible only if we assume that the voltage sensors are represented by charged groups in the protein molecule.

Using a completely different approach, namely selective enzymatic digestion of the Colicin E1 molecule, Slatin et al. (1986) arrived at a similar conclusion. The energetic problems involved in translocation of bulky charged groups across the membrane can be overcome if we assume that they are located inside the aqueous environment of the channel, as our result suggests, and if the movement is made through a small fraction of the electrical field. This is in contrast to the conclusion of Raymond et al. (1986) that regions outside the channel-forming domain in Colicin E1 modulate gating and selectivity. Another difference between Colicin E1 and Ia is in the pH dependence of the single-channel conductance. Whereas Colicin E1 changes its conductance very little in the pH range 4–6 (Raymond et al., 1986), Colicin Ia more than doubles its conductance in going from pH 5 to 8 (Fig. 6). Although we have not enough data on selectivity, this result suggests that it is changing *pari passu* with the conductance. Colicin Ia single-channel conductance is in general higher than the reported values for either Colicin E1 or its tryptic peptides. At pH 6.2 and 0.5 M KCl the single-channel conductance of Colicin Ia is around 28 pS, while it is 20 pS for Colicin E1 in 1 M KCl and pH 6.0 (Bishop et al., 1986; Raymond et al., 1985, 1986). These results suggest that although being structurally similar, the functioning of these colicins have individual peculiarities.

By ethoxiformilation of the histidine residues, Bishop et al. (1986) were able to change the selectivity and single-channel conductance of the COOH-terminal tryptic peptide of Colicin E1. This effect may indicate the participation of this amino acid in controlling the permeability properties of the channel. Although we have no data concerning the specific channel-forming domain in the Colicin Ia molecule, it is worth noting the existence of histidine residues in position 338 and 410, which could also be involved in determining selectivity and channel conductance. Another point of interest is the presence of hydrophilic residues near the COOH-termini (Mankovich et al., 1986) which could be involved in lining the channel interior.

Our results point in the same direction as those of Bowen, Tam and Colombini (1985) where they report a loss of voltage dependence in the channel-forming protein extracted from outer mitochondrial membrane (VDAC) at very high alkaline pH (>10). According to their results the apparent pK of the group involved in gating would be 10.6. In the case of Colicin Ia, the apparent pK is around 6, suggesting again the involvement of histidine residues. It is our view that these numbers merely suggest a particular group involved. In fact the high pH needed in order to cancel the voltage dependence observed in Bowen's experiments put some constraints to the interpretation of their data.

Similar experiments with sodium and potassium channels of excitable tissues have led to no firm conclusions about the chemical nature of the gating particles. Results concerning variations in intra- or extracellular pH's have been generally explained by assuming an effect on membrane surface charges (Hille, 1968; Schauf & Davis, 1976; Carbone et al., 1978; Iigima, Ciani & Hagiwara, 1986). On the other hand, Schauf (1983) showed that reduction of external pH from 7.3 to 5.5 in *Myxicola* axons lowered the gating current associated with the sodium channels. Neumcke, Schwarz and Stampfli (1980) show the opposite effect of pH on the channels of myelinated fibers, i.e., decreasing external pH increases the voltage dependence of the system. Using a different approach, namely treatment of the *Myxicola* axon with carbodiimide which reacts with carboxyl groups, Mozayeva, Naumov and Nosyreva (1984) report a 40% reduction in the voltage sensitivity of the sodium channel. The lack of effect on n observed in most studies could be explained either by assuming that a low permeant buffer was used in changing pH or that its concentration was too low to appreciably change the pH at the site of the charges. The latter is particularly true in experiments where the effects of variations in the intracellular pH were analyzed (Schauf & Davies, 1976; Byerly & Moody, 1986).

Our experiments also permit conclusions on the possible location of the gating charges. It is quite reasonable to assume that these charged groups should be preferentially in a polar environment. The location of the charges inside the aqueous channel

is energetically favored by the high dielectric constant of this region in relation to the lipid bilayer. To prove this point we performed the experiments shown in Figs. 8 and 9. First, it is clear that the effect of pH on n is seen even if it is changed on only one side of the membrane. What counts here is the buffer capacity. Second, the use of an impermeant buffer on only one side for modifying the pH does not change n. By using a completely different approach, Fernandez, Bezanilla and Taylor (1982) have shown that the gating particles of the sodium channels move in a medium not affected by maneuvers known to alter the lipid matrix of the membrane. This suggests that in this system, too, the gate should be inside the channel, or at least protected from the lipid environment.

Another point of interest bears on the fact that whenever n changes, V_O also changes. In other words, if n increased, V_O decreased and vice-versa, leaving the product nFV_O practically constant in the pH range 4.5 to 8.2 (Table). This means that the energetic barrier between the open and closed state in the absence of a field is very little affected by pH.

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