Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1037–1041, February 1990
Biochemistry

In vivo properties of colicin A: Channel activity is voltage dependent but translocation may be voltage independent

(*Escherichia coli*/channel insertion/voltage-dependent gating)

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Communicated by Pierre Joliot, September 29, 1989 (received for review April 11, 1989)

ABSTRACT **The kinetics of K^+ efflux caused by colicin A in *Escherichia coli*-sensitive cells have been investigated by using a K^+-selective electrode. The order of magnitude of the rate of K^+ efflux per colicin molecule was comparable to that of ion channels. The dependence of K^+ efflux upon multiplicity, pH, temperature, and membrane potential ($\Delta\Psi$) was determined. The translocation of colicin A from the outer membrane receptor to the inner membrane and insertion into the inner membrane required a fluid membrane, but once inserted, the channel properties showed little dependence upon the state of the lipids. At a given multiplicity, the lag time before the onset of K^+ efflux was found to reflect the time required for translocation and/or insertion of colicin into the cytoplasmic membrane. Opening of the channel only occurred above a threshold value of $\Delta\Psi$ of 85 ± 10 and 110 ± 5 mV at pH 6.8 and 7.8, respectively. Conditions were designed for closing and reopening of the channel *in vivo*. These conditions allowed us to test separately the $\Delta\Psi$ requirements for translocation and channel opening: translocation and/or insertion did not appear to require $\Delta\Psi$. The channel formed *in vivo* featured properties similar to that of the channel in lipid planar bilayers.**

Colicins are toxins produced by and active against *Escherichia coli* and closely related bacteria. Their mode of action comprises three steps: (*i*) binding to a specific receptor located in the outer membrane, (*ii*) translocation across the membrane(s), and (*iii*) interaction of the colicin with its target in the cell. The largest group of colicins (A, B, E1, Ia, Ib, K, and N) comprises those that can form voltage-dependent channels in membrane model systems (1–3). The ionophoric activity of these colicins has been localized in the C-terminal domain of the protein (4). Recently, the three-dimensional structure of the soluble form of this domain has been determined at 2.5-Å resolution (5).

The properties of the channels formed in planar lipid bilayers by colicin A and by a 20-kDa fragment accounting for its C-terminal domain have been extensively studied (4, 6, 7). *In vivo*, the primary effects of these colicins are a collapse of the electrochemical gradient of protons and consequently an inhibition of active transport systems (for a review, see refs. 1 and 2). This led the authors to conclude that the killing activity of these colicins resulted from the formation of pores in the cytoplasmic membrane. Little is known about the molecular events between the initial adsorption to the outer membrane receptor and the final interaction with the cytoplasmic membrane: irreversible adsorption occurs even in deenergized cells, but an energized cytoplasmic membrane is required for the initiation of colicin activity (8, 9).

In this work we first demonstrated that colicin A can form voltage-dependent channels *in vivo* by analyzing the kinetics of K^+ efflux induced by the toxin (10, 11). We show that the colicin A channel required a threshold of membrane potential ($\Delta\Psi$) for opening. A coupled treatment of the cells with a protonophore to decrease $\Delta\Psi$ and then bovine serum albumin (BSA) to trap the protonophore and increase $\Delta\Psi$ again allowed the closing and reopening of the channel. Furthermore, translocation of colicin A from the outer membrane receptor to the inner membrane could be analyzed independently of the channel function and appears not to depend on $\Delta\Psi$.

MATERIALS AND METHODS

Growth of Bacteria and EDTA Treatment. *E. coli* GM1 cells were grown at 37°C in LB medium (12) to an OD_{650} of 0.5 (5×10^8 cells per ml). Cells were made permeable to the $\Delta\Psi$ probe by a mild Tris/EDTA treatment to prevent extensive loss of K^+ (13). Intact and EDTA-treated cells were washed and resuspended in 100 mM sodium phosphate buffer (pH 6.8) and kept on ice at a density of 5×10^{10} cells per ml (25 mg of cell dry weight per ml). They were used within the 2 hr following preparation.

Colicin A Preparation. Colicin A was prepared as previously described (14). Its concentration was calculated from the molecular extinction coefficient ($\varepsilon_{280} = 0.8\ cm^{-1} \cdot g^{-1} \cdot liter$; molecular mass = 63 kDa) and corrected, assessing its purity by gel scanning densitometry. The multiplicity is defined as the number of colicin molecules per cell.

Determination of the K^+ Content of the Cells. The variations in the K^+ content of the cells (K^+_{in}) were determined by measuring the changes of the K^+ concentration in the external medium (K^+_{out}) with a K^+/valinomycin-selective electrode as previously described (10). To estimate the total K^+ content of the bacteria, the cation was released from the cells by a cold osmotic downshock. The cells (1×10^{10} per ml) were diluted 10-fold in ice-cold distilled water, maintained in water for 1 hr, and then centrifuged 2 min at $5000 \times g$. Under these conditions, the bacteria released 90–95% of their K^+ pool (15). The amount of released cation was then estimated with the K^+ electrode. K^+_{in} was calculated from the value of K^+_{out}.

Measurement of $\Delta\Psi$. $\Delta\Psi$ was determined from the accumulation of tritium-labeled tetraphenylphosphonium ion (TPP^+) (10 μM; 19.2 GBq/mmol) (16). The EDTA-treated cells were filtered on glass fiber filters. TPP^+ uptake was corrected for nonspecific binding by using the protonophore 3,3′,4′,5-tetrachlorosalicylanilide (TCS) (10 μM). $\Delta\Psi$ was calculated assuming a cytoplasmic volume of 1.1 μl/mg of cell dry weight (10).

The $\Delta\Psi$ and K^+ efflux measurements were performed in sodium phosphate buffer (100 mM) supplemented with 0.4% glycerol and 0.5 mM KCl.

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Abbreviations: TCS, 3,3′,4′,5-tetrachlorosalicylanilide; TPP^+, tetraphenylphosphonium ion; BSA, bovine serum albumin.

RESULTS

$\Delta\Psi$ Changes and Kinetics of K^+ Efflux Induced by Colicin A: Effect of the External pH. Energized EDTA-treated *E. coli* GM1 cells generate a $\Delta\Psi$ of 195 mV at pH 7.8 and 165 mV at pH 6.8 (Fig. 1*A*). Addition of colicin A induced a decrease of $\Delta\Psi$, the amplitude of which depended on the pH of the incubation medium but not on the amount of added colicin: the $\Delta\Psi$ values reached at steady state were 70 ± 10 mV (pH 6.8) and 120 ± 5 mV (pH 7.8) for colicin multiplicities of 3, 6, and 15. Replacing sodium by potassium in the buffer (K^+_{out} = 100 mM) did not significantly increase the amplitude of the depolarization (Fig. 1*A*), suggesting that colicin A was not behaving like a K^+ ionophore.

Freshly prepared *E. coli* GM1 cells retained 450–500 nmol of K^+ per mg of cell dry weight. When these cells were kept concentrated on ice, they lost K^+. However, in the presence of glycerol and 0.5 mM KCl, they reaccumulated K^+ by means of the TrkA transport system (17) (Fig. 1*B*). When the steady state of internal K^+ was attained, colicin A was added at a multiplicity of 10: the efflux of K^+ occurred after a lag time of about 30 sec at 37°C. Ten minutes later, the cells were irreversibly depleted of K^+. The initial rates of the delayed efflux were 180 ± 20 and 70 ± 20 nmol·mg^{-1}·min^{-1} at pH 6.8 and 7.8, respectively. EDTA-treated cells, which retained about 350 nmol of K^+ per mg, also induced the release of K^+ but at a smaller rate (85 ± 20 nmol·mg^{-1}·min^{-1} at pH 6.8) (Fig. 1*B*). The depolarization occurred on the same time scale as the K^+ efflux. The new steady state of $\Delta\Psi$ was attained at a time where the cells were depleted of K^+.

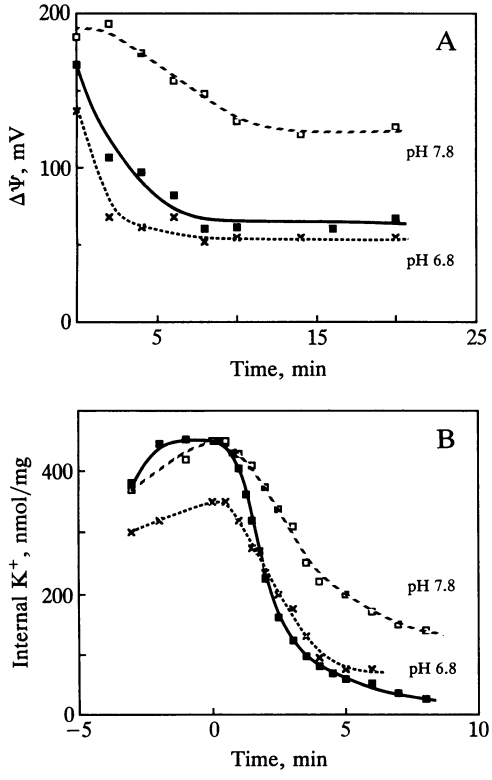

FIG. 1. $\Delta\Psi$ changes and K^+ efflux induced by colicin A. (*A*) EDTA-treated *E. coli* cells (2 × 10^9 per ml) were incubated at 37°C in sodium (■, □) or potassium (x) phosphate buffer (100 mM) at pH 6.8 (■, x) or pH 7.8 (□). [^{3}H]TPP$^+$ was added 10 min before the addition of colicin. Colicin (multiplicity = 15) was added at time 0. $\Delta\Psi$ values were the result of six independent experiments. (*B*) Colicin-induced K^+ efflux in intact (■, □) and EDTA-treated (x) cells. The cells (2 × 10^9 per ml) were incubated at 37°C in sodium phosphate buffer (100 mM). Colicin A was added at time 0, at a multiplicity of 10. The initial rate was measured in the linear part of the efflux curve.

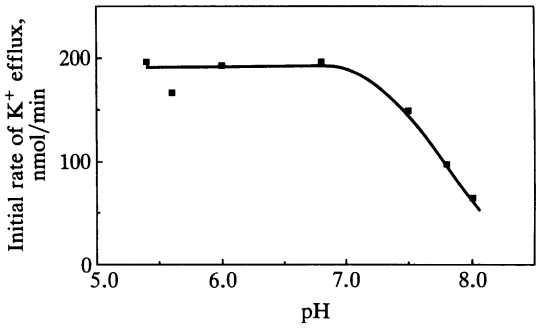

FIG. 2. Effect of external pH on colicin A-induced K^+ efflux. Intact cells retain 400 (pH 5.4) to 500 (pH 8) μmol of K^+ per mg. Experimental conditions were similar to those described in the legend to Fig. 1*B*. The multiplicity of colicin was 10. The initial rates refer to 1 mg of cell dry weight.

Fig. 2 shows that the initial rate of K^+ efflux first remained constant when the pH was varied between 5.4 and 6.8 and then decreased with increasing pH. The lag time between colicin addition and the efflux of K^+ was the same whatever the pH; under all conditions, the cells were depleted of K^+.

Effect of the Temperature on the Kinetics of K^+ Efflux. There was no efflux of K^+ between 4°C and 15°C, although colicin A, like other colicins, is able to adsorb to its receptor (18). When the temperature was increased from 15°C to 20–25°C (i.e., in the temperature range of the order/disorder transition of the lipid hydrocarbon chains) (19), the time required to initiate the efflux (lag time) decreased very rapidly from 450 sec to 150 sec (Fig. 3*A*). The lag time further decreased from 150 to 30 sec when the temperature was raised from 20–25°C to 42°C. Wendt (18) previously showed that colicin K, which shares a common mode of action with

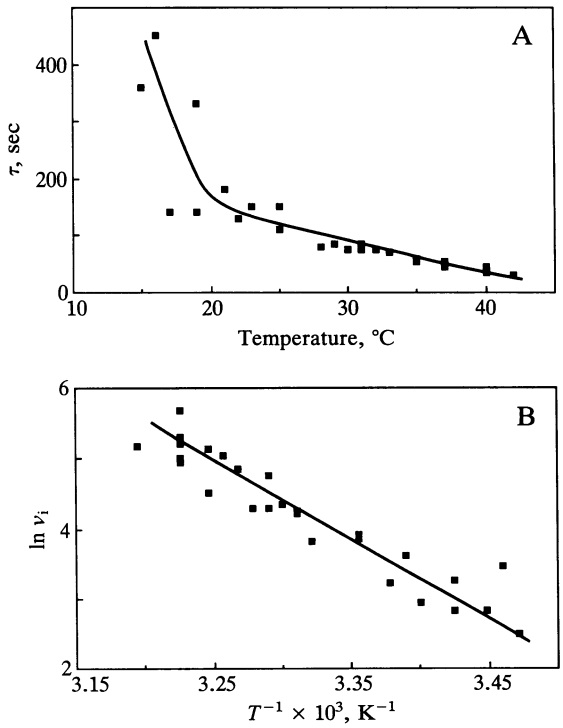

FIG. 3. Effect of the temperature on colicin A-induced K^+ efflux. The cells (not treated with EDTA) were incubated in sodium buffer at pH 6.8. Other experimental conditions were identical to those described in the legend to Fig. 1*B*. The colicin multiplicity was 10. (*A*) Effect of the temperature on the lag time before K^+ efflux. The lag time (τ) is defined as the time that separates colicin addition from the onset of K^+ efflux. (*B*) Arrhenius representation of the initial rate of K^+ efflux. The rate of efflux (v_i) is expressed in nmol·mg^{-1}·min^{-1}.

colicin A, adsorbs to 99.5% of the cells within 1 min at a multiplicity of 10 and at 10°C. Thus, at least in the temperature range of the order/disorder transition, the adsorption was complete several minutes before K^+ efflux started. It is likely that the time that separates adsorption from efflux corresponds to the time required for the translocation of colicin between the two membranes and to its insertion in the cytoplasmic membrane. Fig. 3*B* represents the Arrhenius plot of the initial rate of K^+ efflux for temperatures between 15°C and 42°C. The plot is well fitted by a straight line with a Q_{10} of 2.9 (activation energy = 73 kJ/mol).

The Initial Rate of K^+ Efflux Is Dependent on Colicin Multiplicity. Fig. 4*A* shows that the initial rate of K^+ efflux first increased with the number of colicin added and then saturated at a value of 320 ± 40 nmol·min^{-1}·mg^{-1} when the multiplicity was >20–30. Fig. 4*B* shows that the lag time before K^+ efflux decreased with increasing multiplicity and remained constant when the multiplicity was >20. It is likely that the decreasing lag time reflects the increasing probability of interaction between colicin A and its receptor/translocation site. The constant lag time observed at multiplicities >20 may again correspond to a saturation of the number of colicin molecules able to reach the cytoplasm (see *Discussion*).

Taken together these experiments suggest that colicin A is also able to form channels in the bacterial cytoplasmic membrane. The addition of one colicin molecule to one bacteria induced an efflux of 3×10^5 K^+ ions per sec (Fig. 4*A*). This is larger than the K^+ flux through the K^+ transport system TrkA (17) and is of the order of magnitude of the fluxes of ions through channels. Furthermore, the value of the Q_{10} (2.9) is representative of the temperature dependence of the ion fluxes through several channels, including those involved in the uptake of phage DNA (10, 11, 20).

Voltage Dependence of the Colicin A Channel Function. *(i) Voltage dependence of the K^+ efflux induced by colicin A.* The kinetics of K^+ efflux and $\Delta\Psi$ were measured at 37°C in EDTA-treated cells, incubated at pH 6.8, that were depolarized before the addition of colicin by variable concentrations of the protonophore TCS. $\Delta\Psi$ was determined before the addition of colicin A by using [^{3}H]TPP$^+$. Great care was taken in the binding corrections, which were estimated by using a TCS concentration of 10 μM, a concentration known to completely uncouple the cells (16, 21). Fig. 5*A* shows that the initial rate of K^+ efflux remained relatively constant (80 ± 10 nmol·mg^{-1}·min^{-1}) for values of $\Delta\Psi$ ranging from 160 mV (TCS = 0 μM) to 140 mV (TCS = 0.2 μM) and then decreased for $\Delta\Psi$ values smaller than 130 mV (TCS = 0.5 μM). Furthermore, when $\Delta\Psi$ was decreased below 130 mV, the efflux was not complete and the bacteria retained about 250 nmol of K^+ per mg of dry weight (Fig. 5*B*). The $\Delta\Psi$ value reached at this steady state was 80 ± 10 mV (Fig. 5*B*). When $\Delta\Psi$ was decreased below 85 ± 10 mV, there was no efflux (Fig. 5*A*). The decrease of the initial rate of K^+ efflux with $\Delta\Psi$ was also observed when the EDTA-treated cells were incubated at pH 7.8, but the efflux was completely blocked below 110 ± 5 mV (Fig. 5*A*). It should be pointed out that these threshold values of 85 and 110 mV correspond to the steady state of $\Delta\Psi$ measured after colicin addition (see Fig. 1*A*).

(ii) The colicin A channel can be closed and reopened in the bacterial cell. The experiments described in Fig. 5 suggest that the colicin channel may exist in a closed configuration when $\Delta\Psi$ is decreased below a threshold of 85 ± 10 mV. Is it then possible to open it again by restoring $\Delta\Psi$? $\Delta\Psi$ was increased by the addition of BSA that has been previously

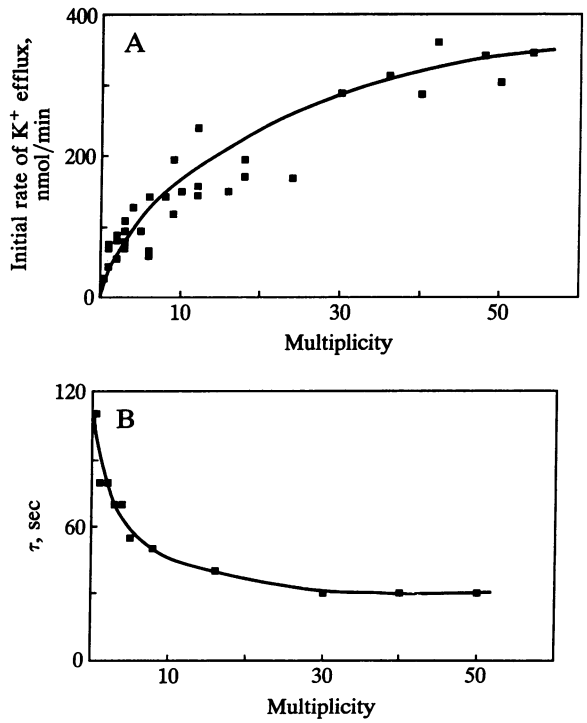

FIG. 4. Effect of the multiplicity of colicin A on the initial rate of K^+ efflux and on the lag time. Experimental conditions were identical to those described in the legend to Fig. 3 except for the temperature, which was set at 37°C.

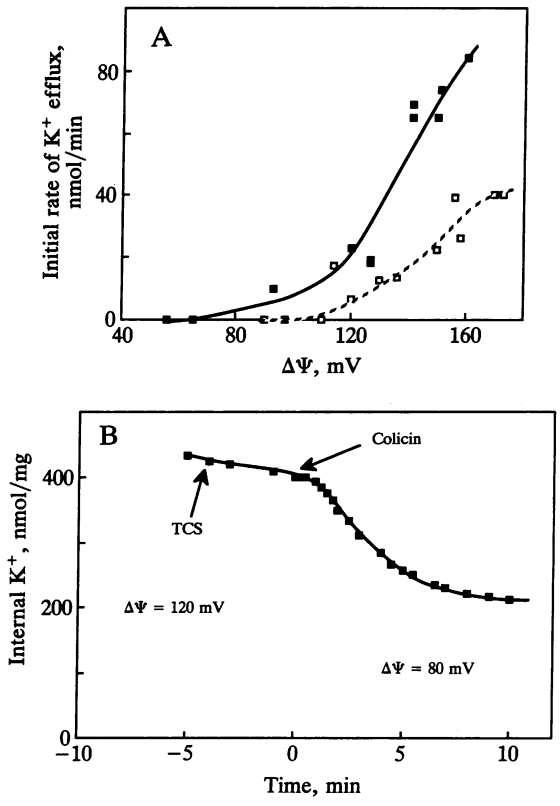

FIG. 5. Dependence of the rate of colicin A-induced K^+ efflux on the value of $\Delta\Psi$. (*A*) EDTA-treated cells were incubated at 37°C in sodium phosphate buffer at pH 6.8 (■) or pH 7.8 (□) in the presence of various concentrations of TCS. $\Delta\Psi$ was measured on an aliquot of this bacterial suspension before the addition of colicin A (multiplicity = 10). $\Delta\Psi$ values were the result of six independent experiments. The initial rate of K^+ efflux was measured as described in the legend to Fig. 1*B* except for the calibration of the electrode, which was performed in the presence of the different concentrations of TCS. Control experiments showed that TCS did not affect significantly the K^+ content of the cells. (*B*) Kinetics of K^+ efflux induced by colicin A (multiplicity = 10) after addition of TCS (0.5 μM). $\Delta\Psi$ was measured on an aliquot of the cell suspension before the addition of colicin A and at the end of colicin-induced K^+ efflux.

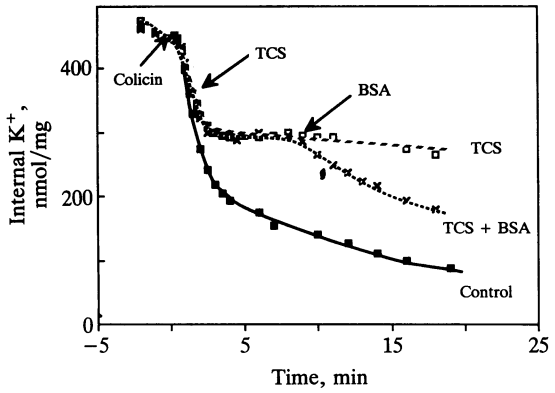

FIG. 6. Effect of successive additions of the protonophore TCS and BSA on colicin A-induced K^+ efflux. Intact *E. coli* cells were incubated at 37°C in sodium phosphate buffer (100 mM) at pH 6.8. Colicin A was added at time 0 at a multiplicity of 6. ■, Control; □, TCS (4 μM) was added 75 sec after the onset of K^+ efflux; ×, TCS (4 μM) and BSA (6 μM) were added 75 sec and 10 min after colicin A, respectively.

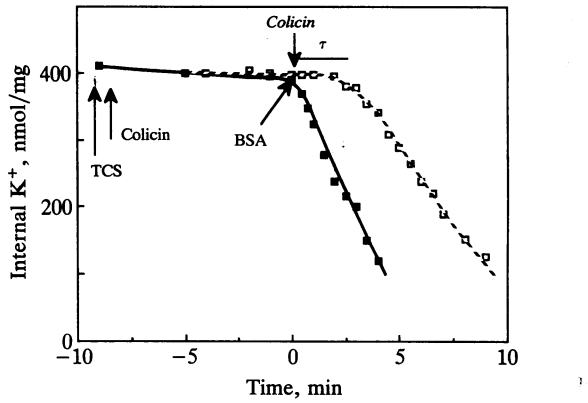

FIG. 7. Effect of successive addition of TCS, colicin A, and BSA on K^+ efflux. Intact *E. coli* cells were incubated at 25°C in sodium phosphate buffer (100 mM). □, Colicin was added at a multiplicity of 12 at time 0; K^+ efflux started after a lag time of 3 min. ■, Cells were first incubated with TCS (4 μM). Colicin was added 5 min later. The cells were then incubated for an additional 10 min and BSA (6 μM) was added to trap TCS at time 0. K^+ efflux started 5 sec after BSA addition.

used to desorb amphiphilic molecules from membranes (22) and to trap fatty acids freed from triglycerides by the action of lipase in lipid monolayer studies (23). Fig. 6 shows the effect of successive additions of TCS and BSA on the colicin-induced K^+ efflux. Colicin A was added to the cells at a multiplicity of 6; 75 sec after the onset of K^+ efflux, TCS was added so that $\Delta\Psi$ was decreased below 80 mV: the efflux of K^+ stopped a few seconds later. This state could be maintained for at least 20 min. BSA (6 μM) was then added. Under these conditions, $\Delta\Psi$ was reincreased to 122 mV (Table 1) and the efflux of K^+ started again (Fig. 6). Since this efflux occurred immediately after BSA addition, this suggests that the channel-forming domain of colicin A had not been pulled out from the cytoplasmic membrane after the first depolarization. Control experiments show that in the absence of colicin, BSA affected neither $\Delta\Psi$ (Table 1) nor the K^+ content of the cells (data not shown).

$\Delta\Psi$ Is Not Required for the Translocation and/or Insertion of Colicin A in the Cytoplasmic Membrane. The cells were incubated at 25°C in the presence of colicin at a multiplicity of 12. Under these conditions, the lag time before K^+ efflux, which is about 3 min (Fig. 7), mainly corresponds to the time required for translocation and insertion of colicin in the cytoplasmic membrane (see above). In a parallel experiment, the cells were first depolarized with TCS (4 μM, $\Delta\Psi < 80$ mV). Colicin A was added 5 min later, and the cells were incubated for an additional 10 min to allow the adsorption (which occurs normally in deenergized cells) (8) and eventually the translocation and insertion steps. No K^+ efflux occurred under these conditions. Then, BSA was added to trap TCS; 5 sec later a K^+ efflux of the same amplitude as that of the control was observed. The absence of a lag time before K^+ efflux in the TCS/BSA-treated cells as compared to the delayed efflux in the control experiment suggests that the adsorption and translocation steps had occurred even in the depolarized cells.

Table 1. Effect of TCS, BSA, and colicin on $\Delta\Psi$

Exp.	Conditions	$\Delta\Psi$, mV
1	Control	130
2	6 μM BSA	129
3	4 μM TCS + 6 μM BSA	122
4	Colicin A	68
5	Colicin A + 6 μM BSA	74
6	4 μM TCS + colicin A + 6 μM BSA	60

EDTA-treated *E. coli* cells (2×10^9 per ml) were incubated at 37°C in sodium phosphate buffer at pH 6.8 in the presence of 4 μM TCS (experiments 3 and 6) or in the absence of TCS (experiments 1, 2, 4, and 5). Colicin A was added 10 min later at a multiplicity of 15 (experiments 4–6), and the cells were incubated again for 10 min. They were then washed twice in the buffer in the absence (experiments 1 and 4) or presence of 6 μM BSA (experiments 2, 3, 5, and 6) and resuspended in the buffer containing 0.5 mM KCl and 0.4% glycerol for an additional 10 min. $\Delta\Psi$ was significantly lower in the control (experiment 1) as compared to the value given in Fig. 1*A* because of the successive washing steps.

In an additional experiment, *E. coli* cells were incubated at 37°C in the presence of 4 μM TCS; colicin A was added 10 min later; and the cells were again incubated for 10 min. They were centrifuged and washed twice in the presence of 6 μM BSA to remove TCS (see above) and nonadsorbed colicin molecules. The cells were then resuspended in sodium phosphate buffer at pH 6.8 containing 0.5 mM KCl and 0.4% glycerol and incubated at 37°C for 10 min in the presence of the $\Delta\Psi$ probe. Table 1 shows that after a treatment with TCS and BSA *E. coli* cells retain a $\Delta\Psi$ of 122 mV. On the other hand, $\Delta\Psi$ was decreased to 60 mV when the cells were successively treated with TCS, colicin, and BSA.

CONCLUSIONS AND DISCUSSION

So far, quantitative and kinetic analyses on the mode of action of pore-forming colicins have mainly been restricted to model membrane systems (1, 2, 24). *In vitro* studies with cytoplasmic membrane vesicles have been described, but they could not account for the early step in the colicin uptake pathway (25).

The main result of this study is the direct demonstration that colicin A can form voltage-dependent channels *in vivo* that could be closed and reopened at will. These conclusions are based on the analysis of the kinetics of colicin A-induced K^+ efflux, which allowed us to have access to the properties of the channel once colicin A was inserted in the cytoplasmic membrane. The existence of a switching voltage for channel opening and closing *in vivo* was inferred from the following observations: (*i*) colicin A could depolarize the sensitive cells to a plateau value of 85 ± 10 mV at pH 6.8, which is also the value required to observe the onset of K^+ efflux; (*ii*) this threshold was the same whatever the colicin multiplicity between 3 and 15; and (*iii*) decreasing $\Delta\Psi$ below 85 mV after colicin addition by addition of TCS resulted in an immediate arrest of the efflux; reincreasing $\Delta\Psi$ above 85 mV by the

addition of BSA reinitiated the efflux. It should be stressed that the value of $\Delta\Psi$ required for channel opening *in vivo* is compatible with that determined for the "basic" form of colicin A channel in planar lipid bilayers (7).

Little is known about the mechanism of colicin A translocation and insertion. Localization of labeled colicin A in different membrane fractions has been unsuccessful since colicin A binds spontaneously to membranes (26). This is why we chose a more indirect approach. A coupled treatment with TCS and BSA to depolarize and repolarize the cells in a few seconds allowed us to discriminate between the energy requirement for translocation and channel opening. From the comparison of the lag time required to initiate K^+ efflux under energized and deenergized conditions, we concluded that one of the steps following adsorption (i.e., translocation or insertion in the cytoplasmic membrane) occurs in deenergized cells. At present time, we do not know if colicin A is translocated and inserted into the deenergized membrane as previously demonstrated in model membrane studies at acidic pH (26–28). Should this be the case, then the threshold of $\Delta\Psi$ would only be necessary for channel opening. Alternatively, after translocation, colicin A may remain at the surface of the cytoplasmic membrane in electrostatic interaction with negatively charged phospholipid head groups, as recently proposed (5, 26). Then $\Delta\Psi$ would serve both for insertion into the inner membrane and channel opening.

A decrease in temperature delayed the K^+ efflux and even prevented it below 15°C (the temperature of the order/disorder transition of the lipid hydrocarbon chains) (19). On the other hand, the temperature had no effect on the Q_{10} of K^+ efflux between 15°C and 42°C. This suggests that the conformation of the lipid hydrocarbon chains affects the rate of translocation/insertion of the channel and may even prevent the translocation but that, once inserted in the cytoplasmic membrane, the properties of the channel are not very dependent on the state of lipids.

The activity of the colicin channel displayed a similar dependence with regard to channels formed in planar lipid bilayers: the rate of K^+ efflux decreased when the pH was increased above 7.0. The hypothesis of an increased protease susceptibility of colicin A at alkaline pH was ruled out. Indeed, the characteristic cleavage of colicin A by the outer membrane protease Cpr (29) was not increased at alkaline pH (data not shown). Furthermore, the lag time before K^+ efflux was not modified by pH; since this lag time is strongly dependent on multiplicity (Fig. 4*B*), this suggests that the number of colicin molecules forming the channel was the same whatever the pH. We are thus facing different nonexclusive hypotheses: (*i*) if the channel is permeant to protons, which is likely (30), the rate of K^+ efflux should depend on the rate of H^+ influx, which could be itself pH dependent; (*ii*) the insertion of the pore-forming domain into the cytoplasmic membrane may be pH dependent; and (*iii*) the conformation of colicin A channel may be pH dependent. This hypothesis is supported by the results obtained on isolated colicin (7).

It has been repeatedly observed that the killing efficiency of various colicins, including those forming pores, follows single-hit kinetics at low multiplicity and deviates from linearity when the multiplicity is increased above 20–30 (2, 18, 31–34). The fact that the initial rate of K^+ efflux first increased linearly with multiplicity and leveled off at multiplicities higher than 15–20 suggests that colicin A behaves like the other pore-forming colicins. There are three possible and nonexclusive reasons why such a saturation is observed: (*i*) the number of functional receptor sites should be rather limited because both BtuB and OmpF are required for adsorption (35, 36); (*ii*) the number of colicin molecules able to reach the cytoplasmic membrane could be limited due to the restricted number of efficient receptor/translocation sites; and (*iii*) since the opening of the colicin channels is voltage dependent, if all adsorbed colicin molecules do not reach the cytoplasmic membrane simultaneously, the depolarization induced by the first inserted colicin molecules may prevent the insertion or opening of the channels of late arrivers.

We thank Drs. A. Ghazi, C. Houssin, G. Leblanc, J. P. Mazat, F. Pattus, and E. Shechter for helpful suggestions and stimulating discussions. We thank M. Leroux and M. Kniebiehler for excellent technical assistance and M. Payan for preparation of the manuscript. This work was supported by the Centre National de la Recherche Scientifique and by the Fondation pour la Recherche Medicale.

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