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# Voltage-Dependent, Monomeric Channel Activity of Colicin E1 in Artificial Membrane Vesicles

Arnold A. Peterson* and William A. Cramer
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

**Summary.** The dependence of colicin channel activity on membrane potential and peptide concentration was studied in large unilamellar vesicles using colicin E1, its COOH-terminal thermolytic peptide and other channel-forming colicins. Channel activity was assayed by release of vesicle-entrapped chloride, and could be detected at a peptide : lipid molar ratio as low as $10^{-7}$. The channel activity was dependent on the magnitude of a *trans*-negative potassium diffusion potential, with larger potentials yielding faster rates of solute efflux. For membrane potentials greater than −60 mV ($K^+_{in}/K^+_{out} \geq 10$), addition of valinomycin resulted in a 10-fold increase in the rate of $Cl^-$ efflux. A delay in $Cl^-$ efflux observed when the peptide was added to vesicles in the presence of a membrane potential implied a potential-independent binding-insertion mechanism. The initial rate of $Cl^-$ efflux was about 1% of the single-channel conductance, implying that only a small fraction of channels were initially open, due to the delay or latency of channel formation known to occur in planar bilayers.

The amount of $Cl^-$ released as a function of added peptide increased monotonically to a concentration of 0.7 ng peptide/ml, corresponding to release of 75% of the entrapped chloride. It was estimated from this high activity and consideration of vesicle number that 50–100% of the peptide molecules were active. The dependence of the initial rate of $Cl^-$ efflux on peptide concentration was linear to approximately the same concentration, implying that the active channel consists of a monomeric unit.

**Key Words** colicin E1 · ion channel · potential dependence · membrane vesicles

## Introduction

Colicin E1 belongs to a group of bactericidal proteins that act to depolarize cells by insertion into the cytoplasmic membrane and formation of a nonspecific monovalent ion channel. It has been inferred from the apparent first-order dependence of cell killing on colicin concentration that a single colicin molecule can kill the cell [14], although the fraction of cytotoxically active colicin molecules is ≤1–10% [12]. Colicin channel formation takes place in at least two steps: (i) binding and insertion of the water-soluble protein into the membrane, requiring an acidic pH in vitro [11, 23]; (ii) voltage-dependent activation of the channel involving gating [26] and perhaps further insertion of the protein into the membrane [7, 29]. While studies with both artificial planar membranes and vesicles have confirmed the acidic pH dependence of colicin activity, there is disagreement with respect to the magnitude of the voltage dependence in vesicles and planar membranes and between different groups using colicin E1 for vesicle studies. There is also an inconsistency in the potential dependence reported in vesicles for the different channel-forming colicins. The activity of the channel-forming colicins A, B, E1, and Ia is strongly voltage-dependent in planar membranes [5–7, 19, 25, 26]. This voltage dependence in the presence of potassium diffusion potentials has been found to be smaller in vesicles [8, 11, 16], with no dependence at all found in some studies on colicin A and Ia [17, 30].

The colicin E1 molecule, as well as colicin A [22], is divided into different domains [4, 7, 9], with the channel-forming domain of the molecule residing in the COOH-terminal region [7, 9]. Previous studies in this laboratory with colicin E1 and its COOH-terminal $M_r$ 20,000 tryptic fragment in vesicles demonstrated an enhancement of activity by a *trans*-negative membrane potential [8, 11, 16], although the magnitude of the dependence was small, a twofold increase in channel activity in the presence of a potassium diffusion potential of −60 mV relative to 0 mV, and a decrease in activity by a *trans*-positive potential.

In the present study, it is shown that the channel activity of colicin E1 and its COOH-terminal peptide, as well as other channel-forming colicins, can have a large (10- to 15-fold) dependence on

* *Current address:* Department of Bioengineering, University of Utah, Salt Lake City, UT 84112.

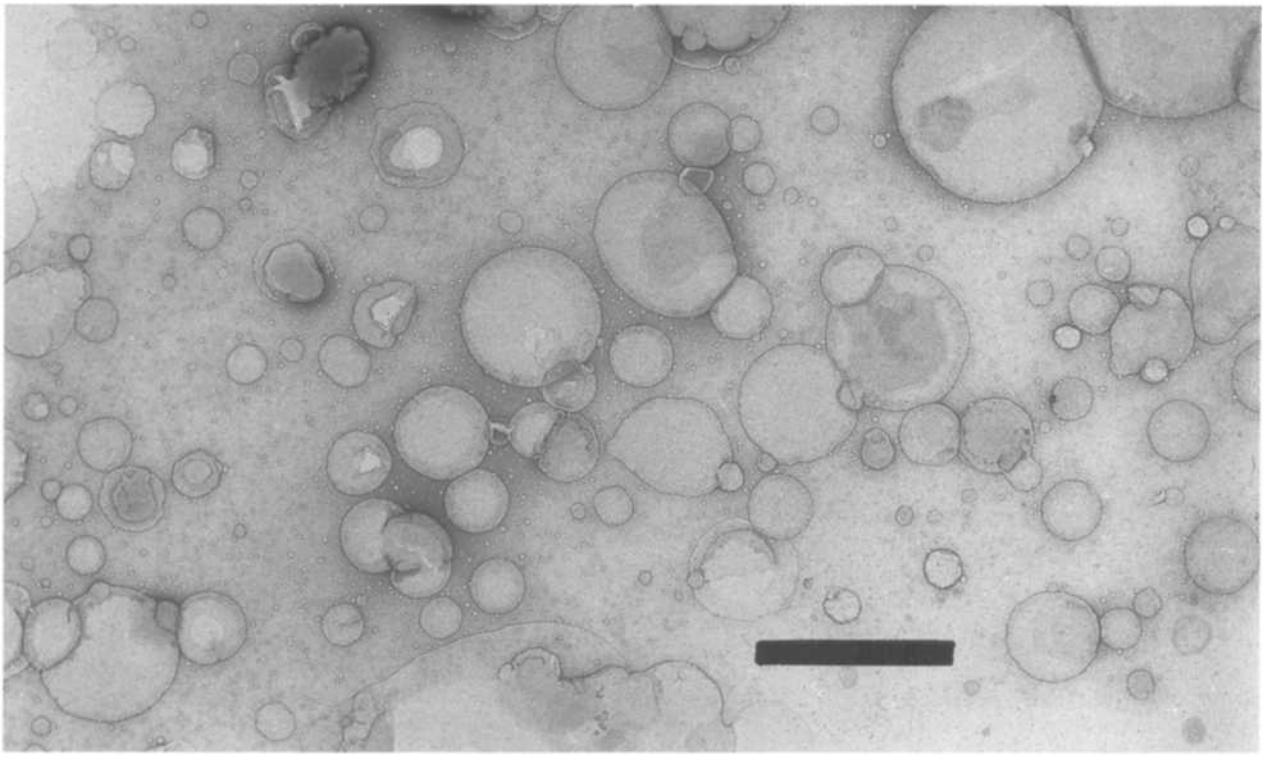

**Fig. 1.** Electron micrograph of large unilamellar vesicles. Bar represents 1 $\mu$m

membrane potential in artificial membrane vesicles. the amount of colicin E1 peptide required for channel formation is much lower than reported previously in the literature, and channel activity at a stoichiometry of less than one colicin peptide molecule per vesicle is first-order in the concentration of added peptide. A preliminary account of this work has been presented [24].

## Materials and Methods

### Preparation of Colicin E1 and Its Carboxyl-Terminal Peptide

Colicin E1 was prepared from *Escherichia coli* JC411 (ColE1) by the method of Cleveland et al. [7], except that 0.1 M $KHPO_4$, pH 7.0, was used for the final dialysis. The $M_r$ 18,000 COOH-terminal thermolytic peptide [20] was prepared by digestion of colicin E1 with a 1:125 (wt/wt) ratio of thermolysin to colicin in 1 M NaCl followed by size exclusion chromatography through a 1 × 25 cm Sephadex G-75 (Pharmacia, Uppsala, Sweden) column [4]. The channel-forming activity of the peptide decreased with storage at 4°C, and concentration-dependence curves were therefore measured with material no more than two days old. Samples of purified collicins B, A, and Ia were generously contributed by V. Braun and J. Konisky.

### Vesicle Preparation

Vesicles were formed from asolectin (Associated Concentrates, Woodside, NY) purified by the procedure of Kagawa and Racker [15]. Asolectin was dried from a chloroform solution, suspended at 20 mg/ml in 100 mM KCl, 1 mM $CaCl_2$, 10 mM dimethylglutaric acid, pH 5.0, sonicated to clarity in a bath sonicator, and twice frozen and thawed to room temperature, in contrast to previous studies [11] in which samples were prepared at pH 4.0 in the absence of $Ca^{2+}$ and sonicated following the freeze-thaw cycle. The phospholipid concentration in vesicles as determined by weight was verified by phosphate analysis [1].

### Chloride Efflux from Vesicles

70 $\mu$l of vesicles (1.4 mg lipid) were added to 14 ml of solution (0.1 mg lipid/ml final concentration) containing 100 mM $NaNO_3$, a mixture of choline nitrate and $KNO_3$ whose total concentration was 100 mM, with the $KNO_3$ at a concentration that defined the $K^+$-diffusion potential, 2 mM $CaNO_3$, 10 mM dimethylglutaric acid, pH 4.0–4.6 25°C, unless stated otherwise (*see* Fig. 5*C*). Choline nitrate was made from choline bicarbonate and $HNO_3$ and allowed to equilibrate overnight. Under these conditions, the pH profile of activity was alkaline-shifted compared to previous studies, so that the maximum channel activity occurred at approximately 4.6. Addition of valinomycin (15 nM final concentration) was used to generate a Nernst diffusion potential of 0 to 135 mV, inside negative. The magnitude of the potential-dependent

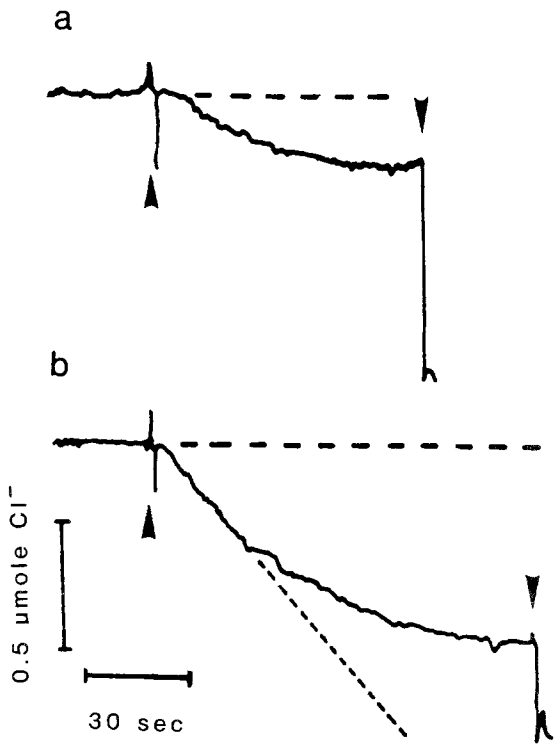


**Fig. 2.** Chloride efflux from vesicles caused by addition of different concentrations of the E1 COOH-terminal peptide. Addition (↑) of 9 ng (*a*), or 15 ng (*b*) of peptide to vesicles (14 ml; 0.1 mg lipid/ml; pH 4.05) with a valinomycin-induced potential of −135 mV. Addition of Triton X-100 (↓). The fine dashed line in *b* represents the initial rate of $Cl^-$ efflux

response (activity in the presence of valinomycin relative to that measured in its absence) was very sensitive to pH, and the highest ratios were often obtained near pH 4.4. Residual $Cl^-$ retained in vesicles after addition of colicin was released by addition of Triton X-100 (final conc., 0.1%). The colicin-induced $Cl^-$ efflux from vesicles was measured with a chloride-specific electrode (Radiometer, Copenhagen, Denmark).

### Electron Microscopy

Vesicles were negatively stained with 1% uranyl acetate on carbon-coated grids and observed with a Phillips 420 electron microscope. The measured size of the vesicles was multiplied by 0.75 to compensate for flattening of the vesicles on the grids [21].

## Results

### Vesicle Preparation

Electron microscopic analysis of vesicles formed by the freeze-thaw treatment revealed a range of vesicle diameters (20–1200 nm), with the majority of vesicles having diameters of approximately 150–400 nm (Fig. 1). Although there were a large number of small vesicles of approximately 20–25 nm diameter, the entrapped volume of these vesicles was negligible as verified by the lack of a measurable chloride release from the small unilamellar vesicles formed by sonication prior to the freeze-thaw treatment. For a population of 400 nm diameter vesicles, the measured entrapped chloride content of 0.7 μmol $Cl^-$/mg lipid, and the lipid concentration of 0.1 mg/ml used in the assays, correspond to a vesicle concentration of $2–4 \times 10^{10}$ vesicles/ml.

### Concentration-Dependence and Activity

The initial rate of efflux and amount of chloride released from vesicles was measured as a function of the concentration of the colicin E1 COOH-terminal peptide added to the vesicles (Fig. 2). The rate of chloride efflux from vesicles is proportional to the number of open colicin channels, whereas the amount of $Cl^-$ released is proportional to the number of vesicles containing channels. Chloride efflux could be detected at a peptide : lipid ratio as low as $10^{-7}$. The amount of $Cl^-$ released from vesicles increased as a function of the concentration of added peptide, with approximately 75% of the $Cl^-$ released by 0.7 ng peptide/ml (Fig. 3*A*). Higher concentrations of peptide released less $Cl^-$ per peptide, indicating a saturation of channels/vesicle. If the adsorption of the peptide to the vesicle population is assumed to obey Poisson statistics, then an average of one active channel/vesicle would exist when 63% of the $Cl^-$ was released. This occurs at a peptide concentration of $2 \times 10^{10}$ peptide molecules/ml, which agrees closely with the number of vesicles calculated for a diameter of 400 nm, indicating that 50–100% of the peptide molecules were active. Calculation of the vesicle number using a smaller average vesicle diameter results in a larger number of vesicles and hence on the average, less than one peptide molecule per vesicle at 63% $Cl^-$ release. Regardless of the membrane potential or initial rate, the total amount of $Cl^-$ released was dependent only on the peptide concentration. In the absence of E1 peptide there was little or no $Cl^-$ release from the vesicles. Approximately 5% of the entrapped $Cl^-$ was not released by a 1000-fold higher peptide concentration, possibly representing chloride that was entrapped within inner compartments of multilamellar vesicles.

The initial rate of chloride efflux from vesicles, measured over the first 10 sec, was linearly dependent on the amount of added peptide for concentrations ≤ 0.7 ng/ml (Fig. 3*B*). The time-averaged specific rate of chloride efflux was calculated to be $3 \times$

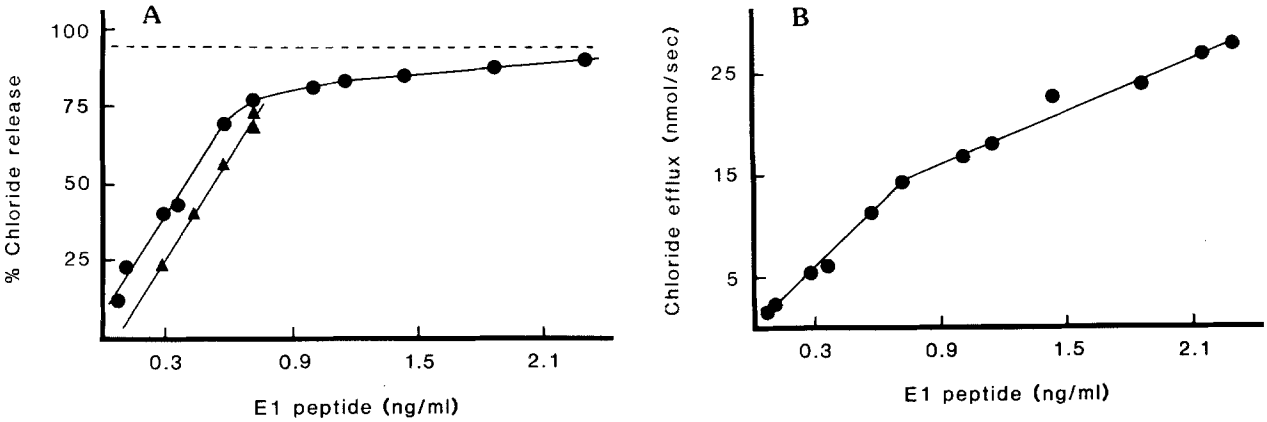


**Fig. 3.** Concentration dependence of the extent (*A*) and initial rate (*B*) of chloride efflux from vesicles. (*A*) Chloride release was calculated from the amount released by E1 peptide and by subsequent addition of Triton X-100. 100% $Cl^-$ release equals approximately 1 $\mu$mol $Cl^-$. The dashed line represents the maximum $Cl^-$ released by a 1000-fold excess of peptide. (*A*) and (*B*): pH, 4.0; $K^+$-diffusion potential, −135 mV. Data represent two different experiments and are representative of many trials

$10^4$ $Cl^-$/peptide-sec, assuming that all peptide molecules are active and contribute equally to the efflux. However, this would be the actual single channel conductance only if all channels were simultaneously in the open state throughout the initial 10 sec of efflux. The latter assumption is known to be incorrect, as discussed below, since individual colicin single channels are expected to show a long delay or latency of channel opening after peptide addition. A similar value for the specific rate of efflux was calculated when the initial part of the efflux curve shown in Fig. 2*b* was fit to an exponential function with a decay time constant of 40 sec (*not shown*). The specific rate of efflux decreased above a peptide concentration of 0.7 ng/ml, the change in activity occurring at about the same concentration as the saturation of total chloride release in Fig. 3*A*. This suggests that the increase in rate below 0.7 ng peptide/ml (Fig. 3*B*) results mainly from an increase in the number of vesicles containing channels, while the rate increment above this level, occurring with a smaller slope as a function of concentration, is caused by an increase in the number of vesicles containing more than one peptide.

The linear dependence of the rate of $Cl^-$ efflux on the concentration of peptide implies that the number of peptide molecules required to form an active channel, the molecularity [3, 31], is one. The molecularity can also be calculated from the slope of a line drawn through the same data presented on a log-log plot (as in ref. 31). At peptide concentrations less than 0.7 ng/ml ($4 \times 10^{-11}$ M) the molecularity was 1 (slope, 0.99; correlation coefficient, 0.997).

## Dependence on Membrane Potential

The change in rate of chloride efflux in the presence of a constant amount of peptide is proportional to the rate of formation of open channels. The rate of formation or gating of colicin channels is markedly dependent on the presence of a negative *trans*-membrane diffusion potential (Figs. 4 and 5). Efflux of chloride from vesicles by E1 peptide was very small until a potential was imposed by addition of valinomycin to vesicles in which an inside-out $K^+$ gradient was present (Fig. 4*a* and *b*). The measured rates of chloride efflux involved no correction, since the instrument response and mixing times were small (~1 sec) compared to the rate of $Cl^-$ efflux, as shown by the response to added detergent (Fig. 2) or to calibration pulses of added chloride (*not shown*). Chloride efflux in the presence of both E1 peptide and valinomycin starts without delay following imposition of a membrane potential if the peptide was added prior to the addition of valinomycin (Fig. 4*b*). However, a measurable delay in onset of the maximum rate of chloride efflux was observed when the peptide was added after valinomycin (Fig. 4*c*), suggesting the presence of a slow, potential-independent step in the binding/insertion of the colicin channel prior to gating of the channel by a membrane potential.

The rate of chloride efflux caused by colicin E1, the COOH-terminal peptide, or colicin B increased by a factor of 10–15 in the presence of a potential of −135 mV (Fig. 5). A similar dependence on membrane potential was also observed with colicins A and Ia (*data not shown*). The factor

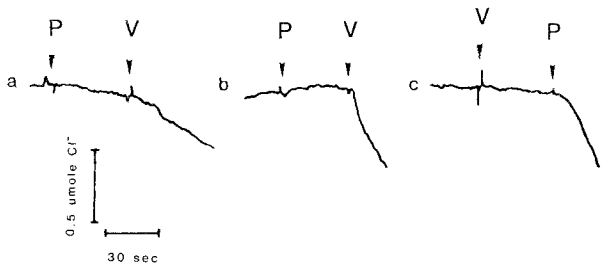


**Fig. 4.** The rate of chloride efflux induced by E1 peptide from vesicles having different membrane potentials: (*a*) 14 mV (100 mM $K^+$ inside, 58 mM $K^+$ outside); (*b,c*) 68 mV (100 mM $K^+$ inside, 7 mM $K^+$ outside). 5 ng/ml of peptide (*P*) were added to vesicles at pH 4.4, either prior to (*a,b*) or after (*c*) the addition of valinomycin (*V*)

of 10–15 for stimulation of the chloride efflux rate can be calculated (i) from the ratio of the rates in the presence of valinomycin at −135 mV relative to 0 mV (filled circles), or (ii) from the value of the $K^+$ gradient corresponding to the potential of −135 mV in the presence (filled circles) relative to the absence (open circles) of valinomycin. The data of Fig. 5*B* was obtained with a peptide concentration (5 ng/ml) higher than that (0.7 ng/ml) corresponding to the change in specific rate of chloride efflux, but the dependence of efflux rate on membrane potential was similar at the low peptide concentrations. In the absence of valinomycin, there was a slight increase in the background rate of colicin-induced chloride release in the presence of a large potassium gradient, suggesting that the salt asymmetry of the internal and external solutions may develop a small potential across the vesicle membrane in the absence of valinomycin.

The magnitude of the dependence of the chloride efflux rate on membrane potential varied with the external salt concentration. Increasing the $Ca^{2+}$ concentration in the external buffer from 0 to 4 mM or the monovalent ion concentration from 100 to 250 mM decreased the rate of chloride efflux both in the presence and absence of valinomycin. However, as the rate in the absence of valinomycin approached zero (at the higher salt concentrations) the ratio of the two rates and the resulting potential-dependence became large. Thus, the presence of $Ca^{2+}$ and high ionic strength appear to inhibit channel formation or leaks in the absence of a membrane potential.

The channel-forming activity of colicin B [25] showed a strong dependence on the membrane potential even at low (100 mM) external salt concentrations (Fig. 5*C*). Thus, at low ionic strength where colicin E1 caused chloride efflux both in the absence and presence of a membrane potential, colicin B (and colicin A, *data not shown*) formed active channels only in the presence of a potential. It should be noted that even with a large excess of colicin B (400 ng/ml) channel formation was limited at small membrane potentials.

## Discussion

Experimental conditions have been established for which a relatively large and reproducible potential-dependence of colicin channel-forming activity in vesicles can be obtained. It is shown in the present work that the ionic strength and presence of calcium are critical. In solutions of 0.1 M monovalent salt the channel-forming ability of colicin B is markedly potential-dependent, although under these conditions colicin E1 spontaneously forms channels or leaks in the absence of a membrane potential. The higher channel-forming activity of colicin E1 in the absence of a membrane potential observed in a previous study [11] may indicate that at low salt concentrations colicin E1 is able to insert into the membrane in an open channel conformation, or once inserted, to readily switch between open and closed states. Thus, a *trans*-positive membrane potential may have been needed to close the channel [11]. By increasing the monovalent salt concentration to 0.2 M and adding 2 mM calcium ions, ionic conditions closer to those of the periplasmic space, the rate of channel formation by colicin E1 in the absence of a potential was greatly reduced and the potential-dependent channels were the predominant form. Such a system is essential for the study of structure and topography of proteins whose insertion or structure is thought to be dependent on membrane potential.

Colicin channel activity in vitro is dependent on pH, the colicin changing from a water-soluble protein at neutral pH to that of a membrane protein at acidic pH values [10]. This suggests the possibility of ionizable groups affecting the partitioning of the channel domain between the lipid membrane and water [10, 11], as indicated by the change in accessibility of colicin to protease by potentials of different polarity imposed across planar membranes [7]. A model may then be envisioned in which some of the acidic amino acids are protonated at low pH values, allowing a more energetically favorable insertion of the protein into a lipid environment. A *trans*-negative potential may assist in translocating positively charged protein segments across or into the membrane [7]. The differential placement and arrangement of charges in the amino acid sequence of the various colicins would then be a key factor affecting

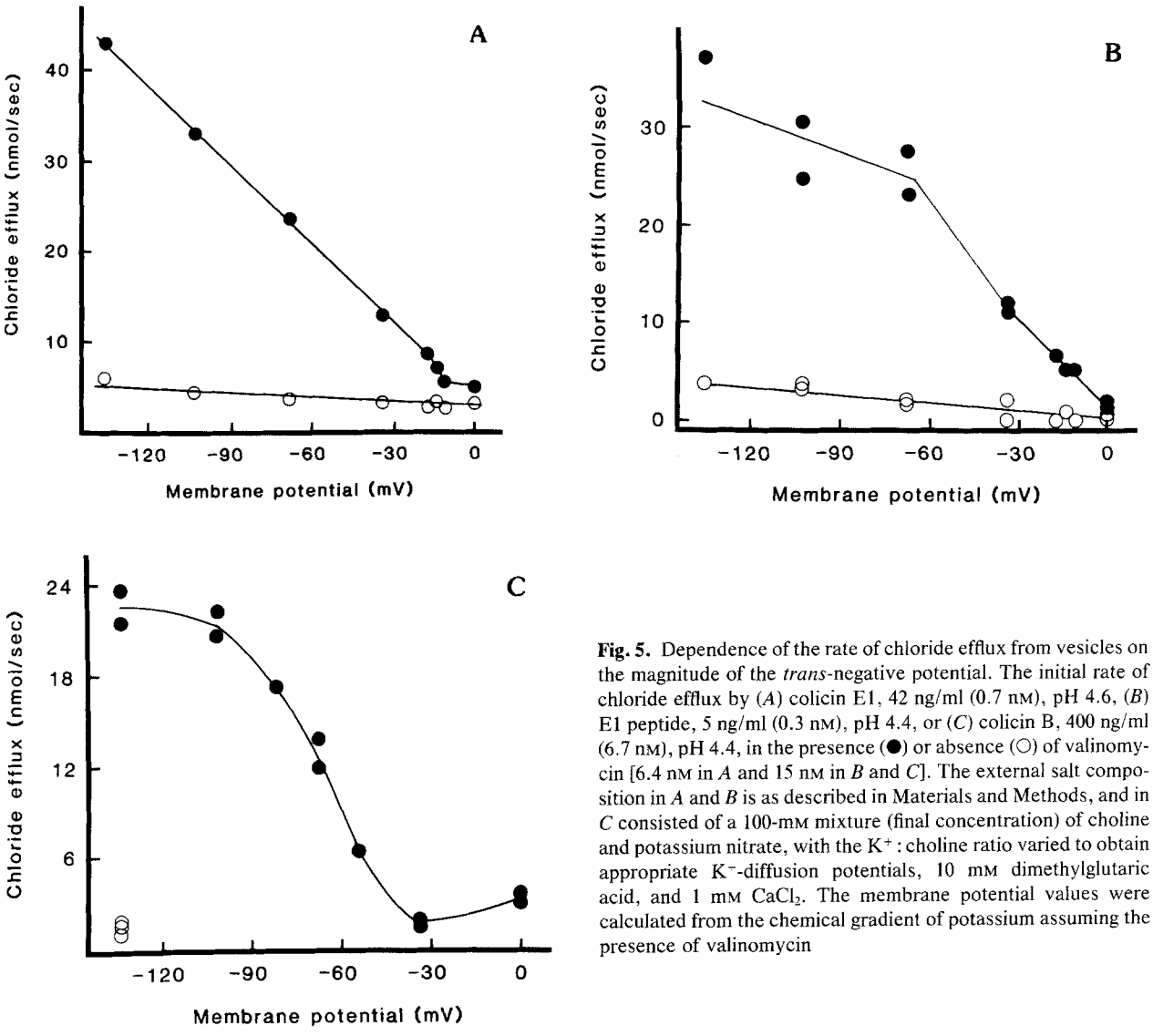


**Fig. 5.** Dependence of the rate of chloride efflux from vesicles on the magnitude of the *trans*-negative potential. The initial rate of chloride efflux by (*A*) colicin E1, 42 ng/ml (0.7 nM), pH 4.6, (*B*) E1 peptide, 5 ng/ml (0.3 nM), pH 4.4, or (*C*) colicin B, 400 ng/ml (6.7 nM), pH 4.4, in the presence (●) or absence (○) of valinomycin [6.4 nM in *A* and 15 nM in *B* and *C*]. The external salt composition in *A* and *B* is as described in Materials and Methods, and in *C* consisted of a 100-mM mixture (final concentration) of choline and potassium nitrate, with the $K^+$ : choline ratio varied to obtain appropriate $K^+$-diffusion potentials, 10 mM dimethylglutaric acid, and 1 mM $CaCl_2$. The membrane potential values were calculated from the chemical gradient of potassium assuming the presence of valinomycin

differences in pH and membrane potential dependence. Thus, considering the differences in voltage dependence of colicins E1 and B in 0.1 M salt, it is thought that the arrangement of charged amino acids involved in the gating response varies among the different colicins.

The colicin E1 peptide concentrations used here are lower by at least a factor of 30 compared to those of peptide or colicin used previously by ourselves and others [11, 16], and by a factor of $10^3$ compared to those used in another study with artificial membrane vesicles in which a molecularity of one was inferred [3].[1] In the present work, nearly all of the entrapped chloride was released from the vesicles by the addition of a 1 : 140,000 (wt/wt) ratio of peptide/lipid. This results in an approximately 1 : 1 ratio of colicin/vesicle for a calculated average vesicle diameter of 400 nm. Thus, most of the molecules are active, ensuring that the procedures for preparing the peptide, as well as those for assaying its activity, do not degrade or produce an inactive molecule. Using substoichiometric amounts of colicin per vesicle also shows that the total amount of chloride released is directly dependent on the amount of colicin added. Thus, once colicin binds to a vesicle, it does not come off and then bind to another.

The time-average initial rate of chloride efflux was $3 \times 10^4$ $Cl^-$/peptide-sec for addition of 0.07–0.7 ng peptide/ml (Figs. 2*b* and 3*b*), in the presence of a membrane potential of −135 mV. Since channel for-

[1] A molecularity of one for colicin E1 has also been demonstrated in small unilamellar vesicles (C. Levinthal, *personal communication*).

mation has a delay or latency of the order of tens of seconds or minutes [29], the above value for the initial rate greatly underestimates the single-channel conductance in vesicles. The relation of this specific efflux rate to the true channel conductance in vesicles depends on (i) the delay or latency for channel opening, (ii) the time in which an individual open channel depletes the chloride content of a single vesicle, and (iii) the characteristic closing time of the channel after the vesicle is depolarized.

Regarding points ii and iii, collapse of the potential would occur on a msec-time scale after a channel is inserted and opened if the single-channel conductance in vesicles is the same as that measured in planar bilayers, i.e., $2–3 \times 10^6$ ions/sec [2, 6, 7]. The channel closing time at 0V is on the order of a minute, so that after opening, a channel would remain open during the times (1 msec and 1 sec, respectively) required for depolarization and depletion of the entrapped $Cl^-$ from a single vesicle ($\sim 2 \times 10^6$ ions in a 400-nm vesicle), again assuming a single-channel conductance of $2–3 \times 10^6$ ions/sec. After depolarization, the efflux is driven by the chloride concentration gradient, initially 200-fold.

The meaning of the latency in channel formation is not clear. Whether it represents a slow and statistically broad range of times for gating of inserted channels, a slow process of insertion prior to gating, or protein translocation and insertion that is an intrinsic part of the gating process [29], is not known. The delay or latency in channel formation can be roughly estimated from the time it takes to reach steady-state conductance in planar membrane experiments upon imposition of a *trans*-negative potential. The problems with such an estimate are (i) that it is very dependent on pH and potential, and (ii) that a steady-state conductance can only be obtained at pH 3.5 [29]. At the pH values used in the present experiments, 4.0–4.6, it is not possible in planar membranes to measure a characteristic half-time for channel formation because, for reasons not understood, a steady state is never attained. The half-time required for the colicin thermolytic peptide prepared in our laboratory to attain its steady-state conductance at pH 3.5 and an imposed *trans*-negative potential of −40 mV in planar membranes was between 1 and 3.5 min (3 trials).[2] Comparing these times to the somewhat smaller half-time of 0.5–0.7 min observed for $Cl^-$ efflux from vesicles (Fig. 2*B*) at a higher pH and a larger membrane potential indicates that the measurement of conductance in vesicles over the initial 10 sec of efflux will yield a value substantially less than the true single-channel conductance.

The linearity of the initial rate of chloride release at peptide concentrations less than 0.7 ng/ml implies that the peptide acts as a monomer, since the concerted action of monomeric units to form an active oligomer would generate a sigmoidal function. It has been suggested that the portion of colicin active in channel formation may be too short to form channels as a monomer [13, 22], and recent data indicate that an active unit may be as short as 88 amino acids [18]. Although models for a monomeric channel employing *trans*-membrane $\beta$-sheet may be structurally sufficient using such a short peptide [28, 32], the possibility that the killing unit of both colicin and the COOH-terminal peptide might be a preformed dimer or oligomer should be considered. Sedimentation studies showed colicin E1 to be predominantly monomeric at pH 7 [27] with the *caveat* that a few percent dimer or oligomer would probably not have been detected. One might propose that the >10-fold increase of the in vitro activity obtained at acidic pH [10] could arise from increased formation of an active dimer. This hypothesis was tested by measurement at pH 4 and 7 of the molecular size of colicin E1 through low angle *X*-ray scattering. Using concentrated (5–10 mg/ml) solutions, the colicin was found to consist of 60–70% monomer with the rest higher order aggregates. A difference in the monomer content of the pH 4 and pH 7 samples could not be distinguished, providing no evidence at present for a pH-dependent monomer-dimer equilibrium.[3]

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