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Membrane action of colicin E1: detection by the release of carboxyfluorescein and calcein from liposomes

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Colicin E1 induces the efflux of carboxyfluorescein and calcein from liposomes whose phospholipid composition is similar to that of *Escherichia coli*. This colicin action takes place at protein-to-liposome ratios and within pH ranges that are physiologically meaningful. Colicin-induced permeability of carboxyfluorescein is not limited to the initial phase of colicin membrane interaction but is sustained thereafter. Colicin E1 requires negatively charged phosholipids in the liposomal membrane in order to bind and induce efflux.

Introduction

Colicins are plasmid-coded proteins that kill sensitive strains of *Escherichia coli* [1]. The cellular target of the E1 class of colicins (E1, K, A, Ia and Ib) is the cytoplasmic membrane [2,3]. A valuable insight into the mechanism of action of these colicins came from the studies of Schein et al. [4] who reported that colicin A formed voltage-dependent, ion-permeable channels in planar membranes. The voltage dependence of colicin channels was later confirmed in liposomes [3,8,9]. Certain experiments with bacterial cells suggest that this may also be the case in vivo [3]. Although a channel-like action of these colicins in membranes is now firmly established, the maximum size of molecules that can permeate through (i.e., the channel size cut-off) has so far been a controversial issue. Original studies with liposomes [5,6] and bacterial cells [5] have demonstrated that, in addition to small ions, molecules as large as sucrose and glucose 6-phosphate readily permeated membranes in the presence of colicins. In contrast, Uratani and Cramer [7] subsequently reported a significantly smaller channel size cut-off for colicin E1 reconstituted into liposomes composed of a single synthetic phospholipid.

In this study we show that colicin E1, under physiologically meaningful conditions, induces the efflux of carboxyfluorescein (M_r 378) and calcein (M_r 622) from large unilamellar phospholipid vesicles. Moreover, this channel effect is not limited to the initial phase of colicin-membrane interacation but is sustained. The efflux of these fluorophores provides a simple and highly sensitive assay for the membrane action of colicins.

Materials and Methods

Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). Large unilamellar vesicles (10 μmol phospholipid/ml) were prepared by reverse-phase evaporation [10] with an aqueous solution (2 mM 2{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid (Tes), 2 mM L-histidine (pH 7.4), 0.1 mM EDTA), containing either 50 mM carboxyfluorescein (East-

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man, Rochester, NY; purified on Sephadex LH-20; Pharmacia, Uppsala, Sweden), or 35 mM calcein. The vesicles were extruded through polycarbonate membranes of 0.1 μm pore diameter (Uni-Pore, Bio-Rad, Richmond, CA) to achieve a homogeneous size distribution. The vesicles were separated from the non-encapsulated fluorophores by gel filtration on Sephadex G-75 (elution buffer: 100 mM NaCl/2 mM L-histidine/2 mM Tes (pH 7.4)/0.1 mM EDTA).

Carboxyfluorescein and calcein are self-quenched at the concentrations encapsulated in the liposomes. Their efflux from the liposomes and dilution into the medium result in a large increase in their fluorescence [11,17].

Multilamellar vesicles (MLV) were prepared by drying the lipids under vacuum, hydrating the thin film with elution buffer and vortexing under argon for 10 min.

Fluorescence measurements for carboxyfluorescein and calcein were made in an SLM-4000 spectrofluorometer (SLM Instruments, Urbana, IL). The excitation wavelength was 493 nm (1 nm slit-width) and the emission above 530 nm was detected by the use of a Corning (Corning, NY) 3-68 cut-off filter. The residual fluorescence of the fluorophore-containing vesicles after gel filtration was set to 0%. 100% fluorescence was obtained by lysing the vesicles with 0.1% Triton X-100 (Sigma).

Fusion of liposomes was followed by an assay for the dilution of fluorescent lipid probes from 'labelled' vesicles [12,13]. Labeled vesicles contained 0.6 mol% each of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine, and were mixed with unlabelled vesicles at a 1 : 1 ratio (2.5 nmol lipid/ml each). In this assay, resonance energy transfer from NBD to Rhodamine is decreased when the probes are diluted in the unlabelled membrane as a result of fusion [12,13]. Fluorescence was detected at 530 nm with an excitation wavelength of 450 nm. Aggregation of the vesicles was monitored simultaneously by 90° light scattering in a second emission channel, using a Melles-Griot 450 nm band-pass filter. The fluorescence of 5 nmol/ml vesicles containing 0.3 mol% of each probe was set as the maximum fluorescence.

Colicin E1 was prepared as described by Schwartz and Helinski [14] except that the last CM-Sephadex step was omitted.

Results

Addition of colicin E1 to large unilamellar liposomes resulted in a rapid efflux of carboxyfluorescein. Liposomes were composed of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin (PE/PG/cardiolipin), with a molar ratio of 70 : 25 : 5 in order to mimic the phospholipid composition of *Escherichia coli* membranes [15]. Addition of heat-denatured colicin E1, colicins E2 and E3, or bovine serum albumin at comparable concentrations did not cause any carboxyfluorescein efflux.

Nature of the colicin-induced carboxyfluorescein efflux

Fig. 1 depicts the time course of the carboxyfluorescein efflux induced by the addition of five different concentrations of colicin E1 to lipo-

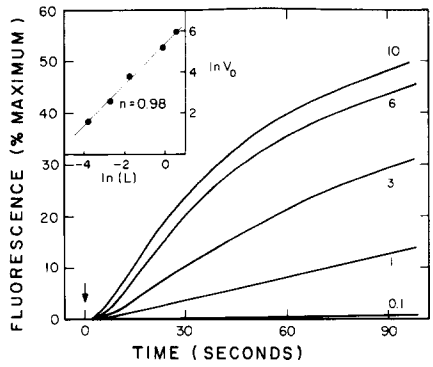

Fig. 1. The time course of carboxyfluorescein efflux from large unilamellar PE/PG/cardiolipin (70 : 25 : 5) liposomes upon addition of colicin E1 ($t = 0$) into the medium. Liposome concentration: 1 nmol phospholipid/ml. Colicin concentrations: 0.1, 1, 3, 6, 10 μg/ml. Temperature is 25°C and the pH is 7.4. The efflux of carboxyfluorescein is given as the % of maximum fluorescence obtained after detergent lysis of the liposomes. Inset: logarithm of the initial velocity of carboxyfluorescein efflux vs. the logarithm of the liposome concentration (L). Colicin E1 concentration: 3 μg/ml. Liposome concentrations: 0.018, 0.06, 0.18, 0.9, 1.8 nmol phospholipid/ml. The initial velocities are calculated by multiplying the liposome concentration (normalized for the lowest liposome concentration) with the fraction of the maximum fluorescence per min obtained at each liposome concentration.

somes. The most likely explanation for the efflux of carboxyfluorescein is the formation of channels by colicin E1 in the liposome membrane. However, the possibility that the release of contents may be the result of colicin-induced aggregation and fusion of liposomes was not ruled out in previous studies [5–7]. We therefore addressed this question by examining the liposome concentration dependence of the initial velocity (V_0) of carboxyfluorescein efflux. V_0 was determined as the steepest slope of each efflux curve such as those shown in Fig. 1. The logarithm of V_0 versus the logarithm of liposome concentration (ln L) gave a straight line with a slope of 0.98, indicating that the rate of colicin-induced carboxyfluorescein efflux has a first-order dependence on liposome concentration. A second-order dependence would have indicated a liposome-liposome contact-dependent release of contents [13]. We also tested the possibility of colicin-induced aggregation and fusion by 90°-light scattering and the resonance energy transfer assay, respectively. Liposomes neither aggregated nor fused in the presence of 5–50 μg/ml colicin E1 in the pH range 5–7.4 (data not shown). The highest colicin-to-liposome ratio used in these experiments was 10-times higher than that employed in Fig. 2. These experiments rule out liposome aggregation and fusion as the cause of colicin E1-induced carboxyfluorescein efflux.

As previously reported [5,6], colicin E1 did not induce a detectable efflux of [^{14}C]inulin (M_r 5000), indicating that vesicle lysis did not occur within the range of the colicin concentration used (data not shown).

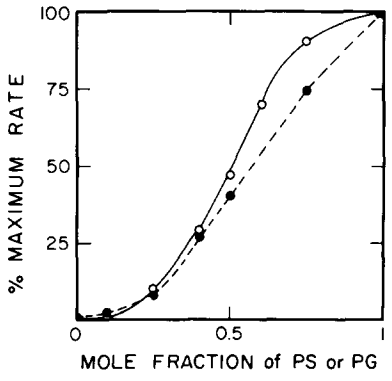

Fig. 2. Dependence of colicin-induced carboxyfluorescein efflux rates on the mole fraction of PS (○) or PG (●) in PC liposomes. Liposome concentration: 2 nmol phospholipid/ml. Colicin concentration: 2 μg/ml. Initial rates of efflux with pure PS and PG liposomes were 175 and 120 (% max. fluorescence/min), respectively. Other conditions were as in Fig. 1.

TABLE I

PHOSPHOLIPID DEPENDENCE OF COLICIN E1 BINDING TO MLV

Colicin E1 (0.1 mg/ml) was preincubated with MLV (2 μmol phospholipid/ml) of PE/PG/cardiolipin or PC composition for 2 min at pH 7.4 and centrifuged. Control preincubation was with elution buffer instead of MLV. 30 μl of the supernatant were added to 1 ml of carboxyfluorescein-containing, large unilamellar liposomes (PE/PG/cardiolipin, 1 nmol phospholipid/ml) to assay for colicin activity, as in Fig. 1.

Phospholipid composition of MLV	Activity in supernatant V_0 (% max. fluorescence/min)	% Activity removed by MLV
Control	17 (100%)	–
PE/PG/cardiolipin	0 (0%)	100
PC	16 (94%)	6

Lipid specificity

We observed that colicin E1 required the presence of negatively charged phospholipids in the liposomal membrane in order to induce carboxyfluorescein efflux [16]. Addition of colicin to liposomes made of only phosphatidylcholine (PC) did not lead to any carboxyfluorescein efflux, even at high concentrations where saturating initial velocities were observed for PE/PG/cardiolipin liposomes. Fig. 2 shows that carboxyfluorescein efflux rates induced by colicin E1 increased with the mole fraction of phosphatidylserine (PS) or phosphatidylglycerol (PG) in PC liposomes at pH 7.4

Next we wanted to establish whether the lack of colicin effect on PC liposomes could be due to the inability of colicin E1 to bind to this type of membrane. Two batches of multilamellar vesicles (MLV) were prepared without any carboxyfluorescein encapsulated in them. One batch was composed of PE/PG/cardiolipin and the other was made of only PC. Both types of MLV were preincubated with colicin E1 at pH 7.4 for 2 min and then centrifuged in an Eppendorf centrifuge for 10 min at 4°C. In this experiment MLV were chosen because they are easily separated from the

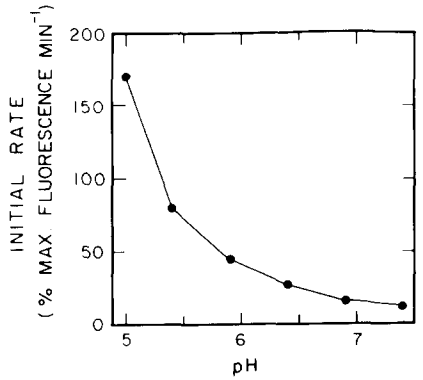

Fig. 3. pH dependence of colicin E1 activity. Calcein-containing liposomes were prepared as described. Initial rates of calcein effluxes were measured as in Fig. 1. Data shown are with 1 nmol phospholipid/ml and 3 μg colicin/ml.

supernatant by a relatively short centrifugation. The supernatants were tested for colicin activity by adding a portion of them to carboxyfluorescein-containing large unilamellar liposomes composed of PE/PG/cardiolipin. Results in Table I show that MLV composed of PE/PG/cardiolipin removed 100% of colicin activity from the supernatant while MLV made of PC removed only 6%. Together, these results indicate that colicin E1 neither induces carboxyfluorescein efflux in, nor binds to a significant degree to vesicles composed of only PC at pH 7.4.

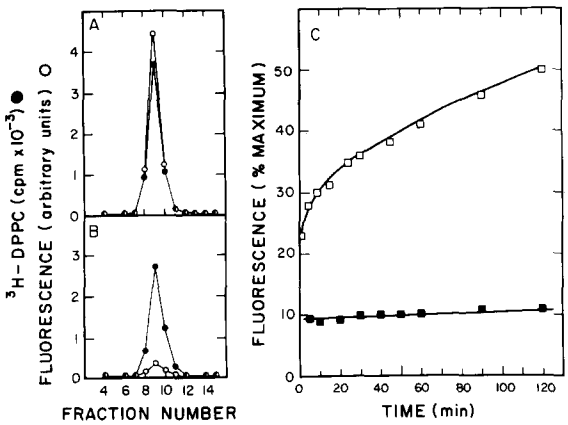

Fig. 4. Elution profile of liposomes after gel filtration. A: control liposomes. B: liposomes preincubated with colicin E1. Determination of phospholipid by [^{3}H]DPPC counts (●) and liposome-encapsulated carboxyfluorescein by Δ(fluorescence) after detergent lysis (○). C: time course of carboxyfluorescein release after gel filtration. Control liposomes (■); liposomes preincubated with colicin E1 (□). Liposome concentration: 100 nmol phospholipid/ml. Colicin concentration; 10 μg/ml. Other conditions were as described in the text.

pH Dependence of calcein efflux

The pH dependence of colicin-induced fluorophore efflux was determined by using vesicles containing calcein, since the efflux of carboxyfluorescein from liposomes and its fluorescence are highly pH dependent [17,18]. At pH 5, the rate of colicin-induced calcein efflux was over an order of magnitude higher than at pH 7.4 (Fig. 3). Increase in the channel activity of colicin E1 in asolectin vesicles at acidic pH conditions has been reported previously [9]. This increase has been attributed to an enhanced binding of this colicin to liposomes at low pH [19]. Increased binding of colicin A to asolectin vesicles at low pH has also been demonstrated [20].

Temporary versus sustained action

It is conceivable that the carboxyfluorescein efflux is induced only during the initial interaction (binding and/or insertion) of colicin E1 with liposomes. Once colicin molecules insert into the membrane, there may not be a sustained permeability to carboxyfluorescein. In the present assay, such a temporary colicin action could lead to a complete carboxyfluorescein efflux due to the limited internal volume of each vesicle. To differentiate between temporary and sustained colicin action, the following series of experiments were performed.

Large unilamellar liposomes containing 50 mM carboxyfluorescein inside as well as in the medium were preincubated with colicin E1 for 15 min at 25°C. Control liposomes were preincubated without colicin E1. Liposomes were composed of PE/PG/cardiolipin with a trace amount of tritium-labelled dipalmitoylphosphatidylcholine ([^{3}H]DPPC). After preincubation, these liposomes were passed through a gel filtration column (Sephadex G-75) to remove the external carboxyfluorescein. Fractions were collected (0.6 ml), and the carboxyfluorescein fluorescence and radioactivity in each fraction were determined. The fluorescence of carboxyfluorescein that can be re-

leased by Triton was also measured in order to determine the amount of liposome-entrapped fluorophore. Radioactivity and fluorescence of liposome-entrapped carboxyfluorescein of each fraction are shown in Fig. 4 (A and B). In liposomes preincubated with colicin E1, the entrapped carboxyfluorescein was reduced drastically. A temporary permeability during the initial colicin-membrane interaction would not have resulted in carboxyfluorescein loss from these liposomes because during the preincubation period carboxyfluorescein was present outside as well as inside.

In a similar experiment, liposomes containing carboxyfluorescein both inside and outside were preincubated with colicin E1 and subsequently passed through a column as described. All liposomes were collected in one fraction (4 ml) and kept at 25°C thereafter. At various times small aliquots were taken to determine their carboxyfluorescein fluorescence with and without Triton addition. Fluorescence levels without Triton were expressed as the percent of maximum fluorescence obtained by Triton addition. This fluorescence level did not change with time significantly in control liposomes. In contrast, there was a rapid increase of carboxyfluorescein fluorescence in liposomes preincubated with colicin E1 (Fig. 4C). The fluorescence reached approximately 25% of the maximum level in 3 min after the removal of external carboxyfluorescein by gel filtration. Afterwards, it continued to increase at a slower rate for the next 2 h, indicating that the colicin-induced carboxyfluorescein permeability was sustained.

Discussion

In this paper we present a simple and highly sensitive fluorescence assay based on carboxyfluorescein and calcein efflux to study the membrane action of E1 class of colicins. Our experiments show that colicin E1 induces the efflux of carboxyfluorescein (M_r 378) and calcein (M_r 622) from large unilamellar liposomes whose phospholipid composition closely resembles that of bacterial membranes. Colicin effects are readily observed within a physiological pH range. The ratio of colicin molecules to liposomes sufficient to induce a minimum efflux is similar to that used in other liposome assays [3,5–9]. For example, this ratio is approximately 10 for calcein efflux at pH 5 where colicin E1 had the highest activity in the pH range tested. (Protein-to-phospholipid ratio is approx. $1:10^4$.) Against bacteria, one killing-unit of colicin typically consists of ten to a few hundred molecules, depending on the particular preparation. Therefore, the amount of colicin per liposome used in the present study was quite reasonable.

Previously it was demonstrated that the E1 class of colicins increased the permeability of liposomal membranes to a variety of ions and small molecules (Rb, SO_4, PO_4, choline, leucine, sucrose and glucose 6-phosphate), but not to inulin or dextran [5,6]. In addition, we had also documented colicin-mediated entry of sucrose and exit of α-methylglucosephosphate in sensitive bacteria [5]. We had interpreted these results as the best evidence to indicate that colicins directly mediated a high and sustained permeability in the cytoplasmic membrane for molecules of the size of sucrose and glucose 6-phosphate, as well as for small ions.

Subsequently, Uratani and Cramer [7] have reported that colicin E1 channels reconstituted into dimyristoylphosphatidylcholine (DMPC) vesicles had a size discrimination against non-electrolytes of the size of glycerol or larger. This observation is in apparent agreement with some of the planar membrane results which had been interpreted to mean that colicin channels would permeate only small ions [21,22]. However, the present demonstration that colicin E1 induces a sustained carboxyfluorescein permeability in liposomes at reasonable protein-to-vesicle ratios and at physiological conditions therefore argues against the conclusions in Ref. 7 and supports the original results on colicin channel size cut-off in liposomes [5,6]. In addition, recent experiments with planar membranes [23] now show that molecules as large as NAD (M_r 663) do permeate through colicins E1, A and I_b, which is also in agreement with the original liposome studies [5,6]. We believe that the small size cut-off reported for colicin E1 in Ref. 7 might have been estimated as a result of rather non-physiological experimental conditions.

Although the term 'channel' is generally used for the membrane action of the E1 class of col-

icins, it is not clear whether observed ion and solute fluxes take place through an aqueous pore within the protein. As pointed out earlier [5] it is conceivable that fluxes actually take place through packing defects at the protein-lipid interface of the membrane-inserted colicin molecules, thus manifesting a channel-like effect. Regardless of the exact nature of colicin channels, recent results with colicin E1 are consistent with a single molecule forming a functional channel in liposomal membranes [24,25].

Pattus et al. [20] have reported that colicin A induces aggregation and partial fusion of small unilamellar vesicles (500 Å diameter) composed of asolectin at pH below 5. Our experiments demonstrate that colicin E1 does not cause any aggregation or fusion of liposomes under the conditions used in our efflux measurements.

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