Membrane Permeabilization Induced by Cytolytic δ-Endotoxin CytA from *Bacillus thuringiensis* var. *israelensis*

Peter Butko,*,‡ Fang Huang, Marianne Pusztai-Carey,§ and Witold K. Surewicz||

Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

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ABSTRACT: CytA is a member of a functionally defined family of insecticidal δ-endotoxins occurring in parasporal crystals of *Bacillus thuringiensis* var. *israelensis.* We investigated the ability of CytA to permeabilize the membrane and release fluorescence marker molecules from unilamellar lipid vesicles. Both protoxin (27 kDa) and proteolytically activated toxin (24 kDa) were very effective in permeabilization of unilamellar lipid vesicles: concentrations as low as several nanomolar produced a significant effect. The toxin was about 2−3 times more effective than the protoxin. The concentration of CytA required for the same extent of calcein release in large unilamellar vesicles (LUV) was 5−10 times lower than that in small unilamellar vesicles (SUV). Both small (calcein) and large (fluorescein-dextrans, MW 3000 and 10 000) molecules were released from the vesicles by CytA with comparable single-exponential kinetics. The release was an all-or-none event, i.e., each vesicle either released all of its contents or remained completely intact. Binding of CytA to lipid membranes did not show appreciable cooperativity, the apparent binding constant (K_{app}) being on the order of 10^5 M^{-1}. The plots of kinetics of release vs bound protein/lipid ratio and the differential effects of CytA on LUV vs SUV indicate that at least 140 toxin molecules or 311 protoxin molecules must bind to an LUV before the latter starts losing its integrity. The necessity of adsorption of this relatively large number of toxin molecules to trigger permeabilization, together with the lack of discrimination in the size of the released marker molecules, suggests that the effect of CytA is a general, detergent-like, perturbation of the membrane rather than creation of small, well-defined, proteinaceous channels.

The δ-endotoxins are a large family of proteins that occur as crystalline inclusions in the spore-forming bacteria *Bacillus thuringiensis* (Bt)[1] (Hofte & Whiteley, 1989). These toxins are of a considerable interest as environmentally safe insecticides. A distinct subgroup of the Bt family of toxins are proteins produced by *Bacillus thuringiensis* var. *israelensis*. These proteins are highly toxic to the larvae of several dipteran species including mosquitos and black flies (Goldberg & Margalitt, 1977). The most active of several polypeptides found in parasporal crystals of *B. thuringiensis* var. *israelensis* (Chilcott & Ellar, 1988) is the 27 kDa protein known as CytA. This protein shows very little homology to the members of a larger Cry family of δ-endotoxins. In in vitro assays, CytA exhibits a strong cytolytic activity against a wide range of insect and mammalian cells, including mammalian erythrocytes, lymphocytes, and fibroblasts (Knowles & Ellar, 1987; (Thomas & Ellar, 1983a). This cytolytic activity is further enhanced upon proteolytic activation of the 27 kDa protoxin (CytA27) to the 24 kDa toxin (CytA24).

Studies with multilamellar liposomes (Drobniewski & Ellar, 1988; Haider & Ellar, 1989) and planar lipid bilayers (Knowles et al., 1989) have demonstrated that CytA interacts with phospholipids as previously suggested by Thomas and Ellar (1983b). The cytolytic action of the protein is thus largely caused by a nonspecific protein−lipid interaction, with a very limited role of specific surface receptors. This is in clear contrast to the postulated receptor-mediated insecticidal action of species-specific Cry δ-endotoxins (van Rie et al., 1989; English & Slatin, 1992). While it has been hypothesized that CytA acts by the formation of transmembrane ionic channels and/or pores (Knowles et al., 1989), molecular mechanisms of the toxin−membrane interaction remain largely unknown.

In the present work, we describe detailed studies on the mechanism of CytA-induced release of self-quenching fluorescent markers from large (LUV) and small (SUV) unilamellar vesicles. Our data indicate that binding of a relatively large number of protein molecules is required to trigger the breakdown of the permeability barrier of LUV. Once initiated, the release of entrapped dyes proceeds via the all-or-none mechanism, with the kinetics showing little dependence on the size of the marker molecule. These results suggest that vesicle permeabilization may occur largely as a result of a nonspecific membrane perturbation or a detergent-like action of CytA and is not necessarily due to formation of ion-specific proteinaceous channels.

MATERIALS AND METHODS

Materials. Egg yolk L-α-phosphatidylcholine type V-E, NaCl, EDTA, and buffers were from Sigma Chemical Co. (St. Louis, MO). Calcein and FITC-dextrans (molecular

* To whom correspondence should be addressed.

‡ Present address: Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, 180 Longwood Ave., Boston, MA 02115. Telephone: 1-617-432-0578. Fax: 1-617-731-1541. E-mail: butko@warren.med.harvard.edu.

§ Present address: Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44106.

|| Present address: Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106.

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[1] Abbreviations: Bt, *Bacillus thuringiensis*; FITC, fluorescein isothiocyanate; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles.

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weights of 3000 and 10 000) were from Molecular Probes, Inc. (Eugene, OR). CytA was isolated from cultures of *Bacillus thuringiensis* var. *israelensis* obtained from Institut Pasteur (Paris, France). Briefly, the bacteria were lysed and parasporal crystals separated and solubilized in 10 mM dithiothreitol, 50 mM Na_2CO_3/HCl, pH 9.5. The CytA27 protoxin was purified by HPLC on a Mono-Q ion-exchange column (Pharmacia LKB, Uppsala, Sweden) using a linear gradient from 10 to 800 mM of the NH_4HCO_3 buffer, pH 8.5 (Chilcott & Ellar, 1988). The CytA24 toxin was obtained by tryptic digestion of CytA27 and purified by HPLC on a Protein Pak SP 5PW column (Millipore, Milford, MA) using the same elution conditions as above. The purity of the preparations was routinely checked by SDS−PAGE and occasionally by mass spectroscopy and N-terminal sequencing. The proteins were lyophilized and stored at −20 °C.

Vesicle Preparation. Calcein or FITC-dextrans were dissolved in distilled water, and the pH was adjusted to 7.4 with 1 M NaOH. The final concentrations of calcein, FITC-dextran 3000, and FITC-dextran 10000 were 80 mM, 10 mM, and 5 mM, respectively. LUV were prepared by extrusion (MacDonald et al., 1991). Lipid (5 mg) in chloroform was dried under a stream of nitrogen. The resulting thin film of dried lipid was hydrated in 0.5 mL of the fluorescence dye solution. The lipid suspension was vortexed, twice frozen and thawed, and extruded 21 times through a stack of two polycarbonate filters (pore diameter 100 nm) in a LiposoFast extruder (Avestin, Inc., Ottawa, Ontario). To remove the unentrapped dye, 0.2 mL of the LUV suspension was passed through a Sephadex G-50 column (1 cm × 30 cm) with the elution buffer containing 50 mM HEPES, 100 mM NaCl, and 0.3 mM EDTA, pH 7.4. The final lipid concentration was determined by an inorganic phosphate assay (Ames, 1966). SUV were prepared by sonication of the initial lipid suspension with a Sonifier 350 tip sonicator (Branson Sonic Power Co., Danbury, CT) at 4 °C for 20 min. The extravesicular dye was removed by gel filtration as described above.

Release of the Dye. CytA-induced leakage from vesicles was examined using self-quenching fluorescent dyes (Allen, 1984; Weinstein et al., 1984). With this technique, vesicles are prepared with a highly concentrated (therefore self-quenched) fluorescent compound (calcein or FITC-dextran) entrapped. As the dye leaks from the vesicles, it becomes diluted (dequenched), and as a result, an increase in fluorescence is observed. The extent of vesicle leakage as a function of protein concentration was measured as follows. Aliquots additions of CytA were sequentially added to a stirred suspension of dye-loaded vesicles at 20 °C, and the changes in fluorescence were monitored using an SLM 8000C spectrofluorometer equipped with double monochromators (SLM Instruments, Urbana, IL). The excitation and emission wavelengths were 494 and 520 nm, respectively, slit widths 4 nm. At the end of the run, Triton X-100 was added to a final concentration of 0.03% to determine the level of fluorescence when all the calcein is released from the vesicles. The extent of calcein release was calculated as the fluorescence at a given protein concentration divided by the fluorescence in the presence of Triton X-100.

The kinetics of calcein release were measured as follows. To a suspension of LUV (1.2 μM lipid) with entrapped calcein were added various concentrations of CytA, and the resulting increase in fluorescence (emission at 520 nm, excitation at 495 nm) due to calcein release was recorded until saturation was reached. In the pH dependency experiments, the assays were performed in 100 mM NaCl buffered with 10 mM HEPES (pH 7.4), 10 mM CAPS (pH 10), or 10 mM citrate (pH 4). The exponential fitting was carried out with the Origin software (MicroCal, Inc., Northampton, MA).

Mechanism of Release. To distinguish between the two possible mechanisms of release (either partial release, when each vesicle in the suspension releases a fraction of its calcein, or all-or-none release, when a fraction of vesicles releases all of their calcein while the rest of the vesicles retain all of their calcein at the initial concentration), we used the method devised by Weinstein et al. (1981, 1984). The principle of the method is to let the vesicles release various fractions of the dye (depending on protein/lipid ratio), and then separate them from the released dye and measure how much calcein (i.e., fluorescence) they retained. Experimentally, vesicles with entrapped 80 mM calcein were incubated at the indicated protein/lipid ratio with CytA24 or CytA27. After 15 min, the suspensions were gel-filtered on PD-10 Sephadex G-25 columns (Pharmacia LKB, Uppsala, Sweden) in order to separate the released dye from the vesicles. The fluorescence of the vesicles was measured before and after addition of Triton X-100. We found that in our experiments, the PD-10 column did not separate the free dye perfectly. A small amount of external, dequenched, calcein would contribute a large fraction to the total fluorescence, in addition to the low fluorescence of the entrapped quenched dye. Therefore, the signal from the free calcein was eliminated by addition of 0.5 mM Co^{2+}, a quencher of calcein fluorescence (Oku et al., 1982). With this correction for the residual external calcein, the ratio R of fluorescence before and after addition of Triton was calculated as

$$R = F_C/(F_T - F_0 + F_C) \quad (1)$$

where F_0 and F_C is the fluorescence in the absence and presence of Co^{2+}, respectively, and F_T is fluorescence in the presence of 0.15% Triton X-100.

The experimental data were compared to the simulations for partial and all-or-none release. The simulated curves were constructed from the known fraction of released calcein at each protein to lipid ratio and a calibration curve of fluorescence as a function of the entrapped calcein concentration.

Binding of CytA to the LUV. The dye release experiments can provide information from which estimates of binding parameters can be obtained. We followed the method and data treatment described by Matsuzaki et al. (1989a,b, 1991). The maximum slope, k', of calcein release (expressed as the percent of total calcein per minute) was determined at various protein and lipid concentrations. A horizontal line drawn at a certain value of k' crosses the experimental curves at points whose coordinates provide the protein and lipid concentrations, $[P]_0$ and [L], respectively, that yield the given leakage rate k'. It is assumed that, at various concentrations, the same leakage rate is brought about by the same ratio r of the membrane-bound peptide to total lipid. The conservation equation relates $[P]_0$, [L], and r as

$$[P]_0 = [P]_f + r[L] \quad (2)$$

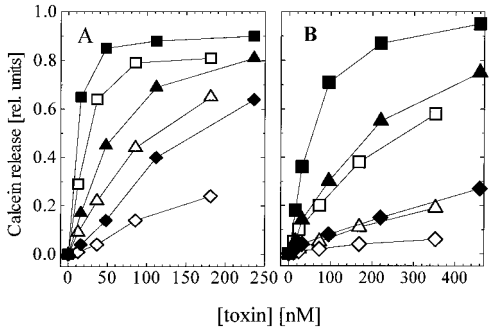

FIGURE 1: Extent of CytA-induced calcein release from LUV (A) and SUV (B) as a function of CytA concentration. The extent was normalized to 1 for total dye release so that the ordinate represents the fraction of total dye released. Open and filled symbols denote CytA27 and CytA24 protein, respectively. Lipid concentrations were 0.6, 3, and 12 μM (squares, triangles, and diamonds, respectively).

Table 1: Concentrations of CytA Required for 30% Release of Calcein from LUV and SUV[a]

[lipid] (μM)	24 kDa LUV	24 kDa SUV	28 kDa LUV	27 kDa SUV
0.6	7	26	12	125
3	30	96	53	600
12	87	490	280	2400[b]

[a] The units are nanomolar. [b] Extrapolated value, since it fell outside the experimental range of concentrations.

where $[P]_f$ is the concentration of free protein. The plot of $[P]_0$ vs [L] is a straight line with the slope r and intercept $[P]_f$. If one plots r vs $[P]_f$, the binding isotherm is obtained, whose slope is the apparent binding constant K'_{app}.

RESULTS

Extent of Calcein Release from LUV and SUV. Figure 1A demonstrates several features of the CytA-induced release of calcein from LUV. First, the extent of calcein release is not always 100%; the saturation level appears to be proportional to the protein concentration and inversely proportional to the lipid concentration. Second, the CytA24 toxin is about twice as effective as the CytA27 protoxin in eliciting the same magnitude of response.

From Figure 1B, it is apparent that when calcein is entrapped in SUV instead of LUV, both the toxin and protoxin are significantly less efficient in releasing the dye, and the difference in the efficiency between CytA24 and CytA27 is even more pronounced (5-fold). Table 1 summarizes some quantitative data, namely, the concentrations of CytA required for 30% release of calcein.

When the data of Figure 1 are replotted as calcein release vs toxin/lipid ratio in a double-logarithmic scale, one obtains plots formally identical to Hill plots (Figure 2). The Hill coefficients (the slopes of the linear part of the curves at low toxin/lipid ratios) were 1.02 ± 0.05 and 0.89 ± 0.05 in the case of LUV and SUV, respectively. The values are reasonably close to 1, which suggests that there is no cooperativity between CytA molecules in calcein release.

Kinetics of Calcein Release. A one-exponential rising curve, $F(t) = (F_{max} - F_0)[1 - \exp(-kt)]$, was fitted to the data. The fitting parameters were the rate constant k and the maximum fluorescence F_{max}. Some preparations of vesicles were less stable than others and exhibited a nonzero slope in the time course of fluorescence due to spontaneous

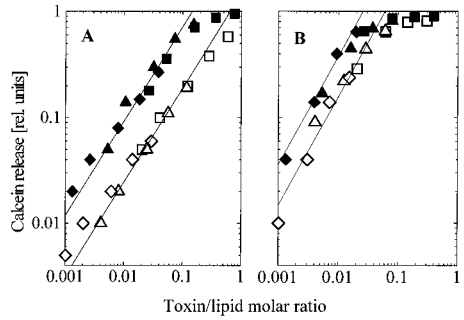

FIGURE 2: Double-logarithmic plots of the extent of CytA-induced calcein release from LUV (A) and SUV (B) as functions of toxin/lipid ratio (Hill plots). The data of Figure 1 were replotted, using the same symbols as in Figure 1.

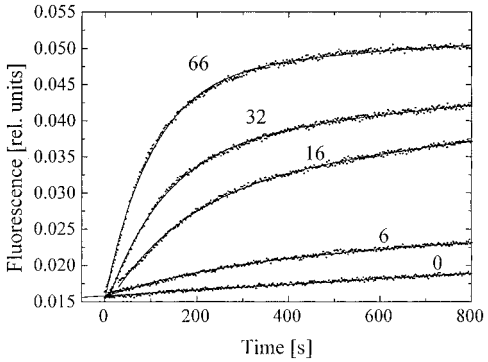

FIGURE 3: Kinetics of fluorescence increase due to the CytA24-induced calcein release from LUV. The cuvette contained 1.2 μM lipid and the indicated concentrations of CytA24 (in nM) in 50 mM HEPES, 100 mM NaCl, pH 7.4. Points are experimental data, and solid lines are one-exponential fits (see text).

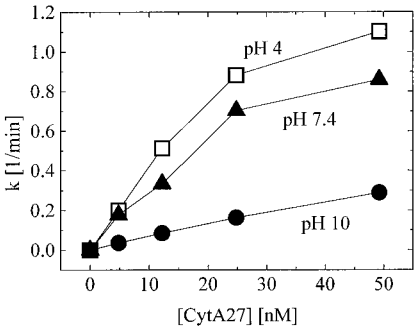

FIGURE 4: Rate constants k of the calcein fluorescence increase as a function of the CytA27 concentration at three different pHs. The rate constants were determined by curve-fitting to the measured kinetic data as in Figure 3. Total lipid concentration was 1.2 μM.

leakage of calcein. This was accounted for by adding a linear term to the exponential. Excellent fits were obtained for time intervals of up to 1000 s if an initial lag period of 20−50 s was excluded from the fittings. The results for CytA24 are shown in Figure 3. Similar experiments were carried out with CytA27 at pH 7.4, 4, and 10. A single rate constant [reciprocal of the time in which fluorescence reaches 63%, i.e., the fraction $(1 - 1/e)$, of its saturation value] was sufficient to describe the kinetics of the fluorescence increase in each case. The rate constants for release by CytA24 and CytA27 did not differ substantially. Figure 4 shows the concentration dependency of the rate constant. There is no indication of sigmoidicity at low toxin concentrations, which confirms that there is no cooperativity between the toxin molecules in calcein release. In experiments with CytA27,

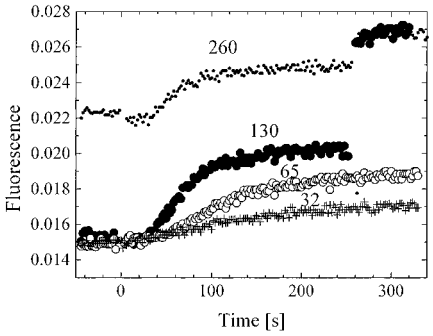

FIGURE 5: Kinetics of fluorescence increase due to CytA24-induced release of fluorescein-dextrans with molecular weights 3000 and 10 000 from LUV. The cuvette contained about 5 or 10 μM lipid (3000 or 10 000 dextran, respectively) and the indicated concentrations of CytA24, added at time zero, in 50 mM HEPES, 100 mM NaCl, and 0.3 mM EDTA, pH 7.4. For the two uppermost curves at the time 250 s, Triton X-100 was added to the final concentration 0.05%. Dots denote the data with dextran 10 000 and 260 nM toxin. Other symbols denote the data with dextran 3000 and the toxin concentrations of 32 nM (crosses), 65 nM (open circles), and 130 nM (filled circles).

low pH slightly increased the rate constant, while high pH decreased it significantly (Figure 4).

Release of FITC-Dextrans. In these experiments, concentrations of fluorophore entrapped in the vesicles were significantly lower than in the calcein release experiments. Consequently, the fluorescence signal was lower, and, more importantly, the difference between the fluorescence levels of the probe before and after release was smaller due to the decreased self-quenching inside the vesicles. Therefore the fluorescein dextran data were not subjected to the kinetic analysis as the calcein data. Nevertheless, Figure 5 demonstrates that the high molecular weight polymers also are released from the vesicles by the action of CytA.

Mechanism of the Release. The fluorescence ratio *R*, defined in eq 1, was measured at three CytA concentration, as described under Materials and Methods. The experimental data were compared to the simulations for partial and all-or-none release. The simulated curves were constructed from the known fraction of released calcein at each protein to lipid ratio and a calibration curve of fluorescence as a function of the entrapped calcein concentration. Qualitatively, with the all-or-none mechanism, one does not expect a change in the fluorescence ratio *R*, since the releasing vesicles release all calcein (and thus become invisible for the assay) while the remaining vesicles retain calcein at the same concentration as in the control vesicles in the absence of the toxin. Consequently, *R* as a function of toxin concentration would be a straight horizontal line. In the case of partial release, all vesicles would release a fraction of their calcein, and the lower dye concentration inside the vesicles would result in dequenching. The fluorescence ratio *R* would increase with increasing percentage of release, i.e., with increasing toxin concentration. The results shown in Figure 6 unequivocally prove that CytA releases calcein from vesicles by the all-or-none mechanism.

Binding of CytA to the LUV. The maximum slope k' of calcein release (expressed as percent of total calcein per minute) was determined at various protein and lipid concentrations. A series of straight lines (eq 2 under Materials and Methods) for six values of k' was constructed; the correlation coefficients were always greater than 0.97 (not

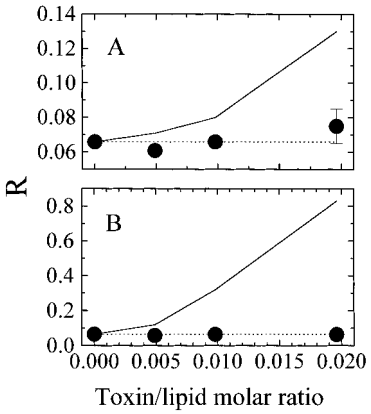

FIGURE 6: Fraction *R* of fluorescence retained in the vesicles as a function of CytA concentration. (A) CytA27 protoxin; (B) 24 kDa toxin. *R* is the ratio of fluorescence before and after addition of Triton X-100, calculated according to eq 1. Solid and dotted lines are the predictions of the partial and all-or-none mechanism of release, respectively. Circles are the experimental data, with the standard deviation shown at the last point of panel A. In panel B, the standard deviation is less than the size of the symbols. Experiments were performed in 50 mM HEPES, 150 mM NaCl, and 0.3 mM EDTA, pH 7.4.

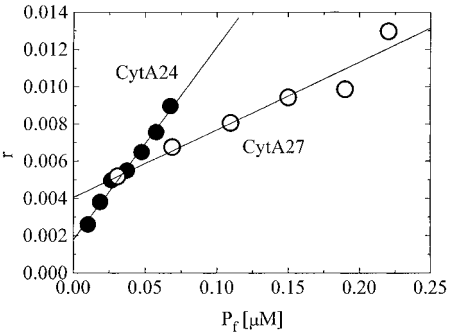

FIGURE 7: Binding isotherm for the interaction of CytA with PC LUV. Ratios *r* of the membrane-bound protein to total lipid and the concentrations [P_f] of free protein were determined at various protein and lipid concentrations as described under Materials and Methods. The data were fitted with eq 2.

shown). The resulting binding isotherms for both CytA24 and CytA27 are in Figure 7. The slope of the isotherm is the apparent binding constant K'_{app}. In an LUV suspension, only the outer monolayer, which represents the fraction 0.54 of total lipid, is available for binding. This is taken into account by introducing a corrected $r_{corr} = r/0.54$. The corrected values K_{app} ($=K'_{app}/0.54$) for binding of CytA27 and CytA24 to the surface of an LUV were $(0.67 \pm 0.07) \times 10^5$ M^{-1} and $(1.93 \pm 0.11) \times 10^5$ M^{-1}, respectively. Thus, the CytA24 toxin binds to LUV approximately 3 times tighter than the CytA27 protoxin.

Another useful piece of information can be obtained from the plot of leakage rate k' vs ratio *r* (Figure 8). Plots for both CytA27 and CytA24 yield straight lines which extrapolate to nonzero intercepts on the r axis. The intercepts are interpreted as threshold values of *r*, below which there is no appreciable leakage. In other words, the bound protein to lipid ratio *r* must reach a threshold value for LUV to start releasing calcein. The threshold values were determined as 0.0038 ± 0.0006 protein/lipid and 0.0017 ± 0.0002 protein/lipid for CytA27 and CytA24, respectively. A vesicle with an average outer diameter of 100 nm consists of 83 000 lipid molecules (assuming the area per molecule 0.7 nm^2 and membrane thickness 4 nm). With this information, one can

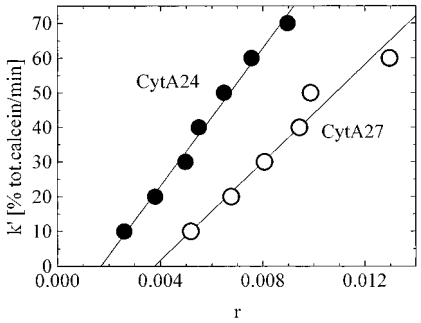

FIGURE 8: Dose−response curve relating leakage rate k' to the molar ratio r of bound protein to lipid. The parameters k' and r were determined (see Materials and Methods) from the data set used in Figure 7.

calculate from the threshold values of r that in order for the vesicle membrane to start losing its integrity (i.e., releasing calcein), on the average 311 ± 47 protoxin (27 kDa) or 140 ± 16 toxin (24 kDa) molecules must bind to a vesicle. The outer monolayer of an LUV consists of approximately 44 800 lipid molecules. Thus, at the threshold value of r, there is approximately 1 protoxin per 140 lipids and 1 toxin per 320 lipids in the outer monolayer.

DISCUSSION

At a low toxin/lipid ratio, the release of calcein from LUV is slow (Figure 3) and, at saturation, does not reach the 100% value (Figures 1 and 3). The latter fact can be accounted for by two mechanisms: either all the vesicles release part of their contents (partial release) or a fraction of vesicles releases all their contents (all-or-none release). The first mechanism implies a temporary permeabilization of the membrane, which, although difficult to envisage, is in principle possible. It was suggested, for instance, by Blumenthal et al. (1986) that this mechanism is operating in permeabilization of liposomes by adenovirus. Partial release can also be induced by raising the temperature to and above the phase-transition temperature of the lipid (Weinstein et al., 1981; Ueno et al., 1991). In that case, however, disordering of lipid packing plays a role, in addition to the presence of the release-inducing molecule. Our samples were always thermostated at 20 °C. Recently, a glycoalkaloid-induced transient disruption of the lipid bilayer was reported by Keukens et al. (1992). The all-or-none mechanism has been encountered much more often, with a variety of peptides and proteins, such as the GALA peptide of Parente et al. (1990), magainin 2a (Grant et al., 1992), and α-hemolysin (Ostolaza et al., 1993). We felt it was important to provide a clear proof for either mode of action of CytA. To that aim, we employed the method of Weinstein et al. (1981, 1984) with a small modification (see Materials and Methods). Figure 6 compares the experimental results with the prediction of both mechanisms. The data unequivocally show that CytA releases calcein via the all-or-none mechanism.

Then the question arises why CytA cannot release calcein from all the vesicles. We suggest here two possible reasons: first, adsorption of the toxin molecule to the membrane may include an irreversible step, so that once tightly bound, the toxin cannot redistribute among the vesicles; and second, a certain number of toxin molecules must accumulate in the membrane before the vesicle starts to leak its contents. These two characteristics of binding to lipid membranes are not unprecedented. For example, irreversible binding of HDL (Weinstein et al., 1981), glycoalkaloid α-chaconine (Keukens et al., 1992), and exotoxin A from *Pseudomonas aeruginosa* (Menestrina et al., 1991) to liposomes has been reported, and Matsuzaki et al. (1989) and Grant et al. (1992) observed that a minimum number of bound magainin molecules is required for vesicle leakage. The minimum number of CytA molecules per lipid required for induction of leakage was determined from the data in Figure 8 as 0.0038 ± 0.0006 CytA27/lipid and 0.0017 ± 0.0002 CytA24/lipid. The values are of the same order of magnitude as those for the antimicrobial peptides hypelcin A (Matsuzaki et al., 1989) and tachyplesin I (Matsuzaki et al., 1991), but an order of magnitude lower than those for magainins (Matsuzaki et al., 1989; Grant et al., 1992). Incidentally, from their experiments on the effect of CytA27 on insect Malpighian tubules, Maddrell et al. (1988) concluded that the toxic effect develops only when a sufficiently large number of CytA molecules attach to the membrane.

It is interesting to note the significantly decreased efficiency of CytA in SUV as compared to LUV (Figure 1). Comparing the surface areas of LUV with the outer diameter 100 nm and SUV with the outer diameter 15 nm reveals that, at the same total lipid concentration, there is approximately 14 times more SUV than LUV. If the dye release is governed by the number of CytA molecules accumulated per vesicle, one would expect that to release the same fraction of the dye from SUV would require 14 times more CytA than from LUV. On the other hand, if it is simply the surface density of the adsorbed CytA that matters, one would expect that the requirement for CytA in SUV would be indistinguishable from that in LUV. (Although the surface area of an individual LUV is about 11 times larger than that of SUV, the total surface of all the SUV in the suspension is 1.2 times larger than that of LUV at the same lipid concentration.) Table 1 shows that the ratio of the CytA concentration causing 30% release in SUV to that in LUV is between 8.5 and 10 for CytA27 protoxin and between 3.5 and 5.5 for CytA24 toxin. Since the numbers are significantly different from 1, these considerations suggest that for the same extent of calcein release from SUV and LUV, it is not sufficient to keep the same protein/lipid ratio (or protein/vesicle surface area) in the two systems. An important factor seems to be the protein/vesicle ratio, and it is conceivable that, at a given surface density of adsorbed protein, an SUV does not have sufficiently large surface area to harbor enough proteins to induce leakage. Indeed, the outer monolayer of an SUV with diameter 30 nm contains only about 4000 lipid molecules (considering the molecular area 0.7 nm^2). At the threshold density of CytA27 and CytA24 in LUV, there is 1 protoxin per 140 outer lipid molecules and 1 toxin per 320 outer lipid molecules (see Results). If we apply these numbers to SUV, it turns out that the whole SUV surface would contain only 29 protoxin and 13 toxin molecules, which may not be sufficient for formation of the membrane-damaging aggregates. Alternatively, differences between LUV and SUV in lipid packing and surface tension may play a role: these parameters may influence the conformation and diffusibility of CytA on the membrane surface and, consequently, its ability to aggregate.

Our data suggest that it is the aggregates, not individual molecules, of CytA that induce breakdown of the membrane

permeability barrier. In that respect, it is interesting to note that we did not detect any cooperativity in binding of CytA to the membrane: the plot of rate constants as a function of CytA concentration (Figure 4) does not exhibit sigmoidicity at low toxin concentrations, and the Hill coefficients from Figure 2 are close to 1. Also, the single-exponential kinetics of calcein release (Figure 3) are consistent with the membrane association of CytA being a simple one-step process.

The CytA-induced release of such large molecules as 10 000 kDa dextran (Figure 5) proceeds with kinetics comparable to those for calcein (Figure 3) or even $^{86}Rb^{+}$ (Knowles & Ellar, 1987). This, together with the requirement for adsorption of a large number of toxin molecules before onset of leakage, argues against the toxin-induced specific ion channels which were observed by Knowles et al. (1989) on bilayer lipid membranes. It is possible that apparent cation-selective channels are only the first step in permeabilization of the membrane. Alternatively, it is not always straightforward to decide whether discreet conductance steps are due to formation of channels or due to much less specific perturbation of bilayer structure mediated by proteins (Blumenthal & Klausner, 1982). More solid evidence for or against specific channels would come from a distribution histogram of the conductance steps, which has not been published, or even better, from a physical proof for the protein spanning the bilayer, which will be the subject of our subsequent paper.

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