The Adsorption of *Pseudomonas aeruginosa* Exotoxin A to Phospholipid Monolayers Is Controlled by pH and Surface Potential

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ABSTRACT The interaction of *Pseudomonas aeruginosa* exotoxin A (ETA) with lipid monolayers was studied by measuring the variation in surface pressure. ETA adsorbs to the monolayer, occupying an average area of ~4.6 nm^2 per molecule, up to a maximum density of one molecule per 28 nm^2 of lipid film, which corresponds roughly to the cross-sectional area of the toxin. This suggests that ETA molecules adsorb until they contact each other, but insert only a small portion into the lipid film. The kinetic process could be described by a Langmuir adsorption isotherm. The apparent association and dissociation rate constants were determined, as were their dependence upon toxin concentration, membrane composition, pH, and ionic strength. Two parameters were found to be paramount for this interaction: pH and surface potential of the lipid. It appears that ETA binding occurs only in a conformational state induced by low pH and is promoted by an electrostatic interaction between a positively charged region of the protein and the negative charge of acidic phospholipids. On the basis of a simple model, the salient features of ETA involved in its adsorption were derived: 1) the existence of a conformational state induced by the protonation of a group with pK 4.5 ± 0.2; 2) a positive charge of 1.9 ± 0.3 e.u. able to interact with the surface potential of the membrane; 3) the fraction of potential experienced by the protein in the activated state that precedes binding, ~80%; 4) the intrinsic adsorption and desorption rate constants, $k_a^0 = (4.8 \pm 0.3) \times 10^3\ M^{-1}\ s^{-1}$ and $k_d^0 = (4.4 \pm 0.4) \times 10^{-4}\ s^{-1}$. These rate constants are independent of pH and lipid and buffer composition, and provide a dissociation constant $K_d \sim 90$ nM.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic microorganism responsible for severe infections in immunocompromised human hosts, e.g., subjects exposed to severe burns, undergoing surgical operations, or affected by AIDS or cystic fibrosis (Pier et al., 1990). These bacteria release several virulence factors, including lipids, proteases, phospholipases, a cytotoxin (*Pseudomonas teruginosa* cytotoxin), two exo ADP-ribosyltransferases, and exotoxin A (ETA) (Liu, 1974; Woods and Iglewski, 1983). ETA appears to be the most potent component (Wick et al., 1990), and, interestingly, it was also shown to be crucial for plant infection by this pathogen (Rahme et al., 1995). Like diphteria toxin (DT), it blocks protein synthesis in eukaryotic cells by ADP-ribosylation of elongation factor 2 (Iglewski and Kabat, 1975; Iglewski et al., 1977). Research on ETA has also flourished because this toxin has been successfully used in recent years as a component of immunotoxins for cancer therapy and for the deletion of autoreactive or virus-infected lymphocytes (Pastan et al., 1992).

ETA is a single polypeptide chain of 613 amino acid residues, organized into three structurally and functionally distinct domains (Allured et al., 1986; Siegall et al., 1989): an amino-terminal domain that binds to a cell surface protein, the α_2-macroglobulin receptor (Kounnas et al., 1992); a carboxy-terminal enzymically active domain; and a central domain that assists membrane translocation. ETA enters via coated pits into the endosomal pathway, where it is cleaved by furin (Fryling et al., 1992), a membrane protease, to produce a 37-kDa fragment that includes the enzymic domain (Ogata et al., 1990, 1992). After reduction, the 37-kDa fragment is released into the cytoplasm (Ogata et al., 1990), where it begins its lethal activity.

Intracellular translocation of the 37-kDa ETA fragment is a crucial, yet poorly understood, step in cell intoxication. It requires proteolytic nicking at an exposed arginine-rich loop (between Arg^{274} and Arg^{279} (Ogata et al., 1992)) and reduction of the disulfide bond between Cys^{265} and Cys^{287} (Madshus and Collier, 1989). The arginine-rich loop and the disulfide bond link the first two α-helices of the translocation domain, which have hydrophobic and amphiphilic natures, respectively (Allured et al., 1986). It is possible that interaction of these two helices with the cell membrane might play a role both in the processing of the toxin and in its translocation.

Indeed, it has been shown that ETA interacts strongly with lipid vesicles, inducing permeabilization and aggregation in a lipid- and pH-dependent way (Farahbakhsh et al., 1986; Jiang and London, 1990; Menestrina et al., 1991; Rasper and Merrill, 1994; Zalman and Wisnieski, 1985), and is able to open pores in planar lipid membranes (Gambale et al., 1992). It shares these properties with other toxins of the A-B type, e.g., DT and tetanus neurotoxin (Montecucco and Schiavo, 1995; Montecucco et al., 1994; Olsnes et al., 1990). As for the other A-B toxins, permeabilization of lipid bilayers by ETA appears to be a complex event

Received for publication 23 December 1996 and in final form 21 May 1997.

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0006-3495/97/09/1468/11 $2.00

requiring several steps: 1) a pH-induced conformational transition exposing new hydrophobic regions; 2) a lipid-dependent binding to the membrane surface; 3) the insertion of one or more hydrophobic stretches of the polypeptide into the lipid phase; 4) the aggregation of several polypeptide entities in the lipid bilayer, with the formation of structures enabling the passage of otherwise impermeant molecules. Lipid monolayers are a very simple model system that can be conveniently used to study at least the first three steps in this chain, as was demonstrated for colicin A (Frenette et al., 1989; Pattus et al., 1983), DT (Demel et al., 1991), and tetanus neurotoxin (Schiavo et al., 1991). Using such films we have now obtained a quantitative analysis of the ETA-lipid binding process, determining the association and dissociation rate constants and their dependence upon pH, ionic strength, and membrane composition. Besides pH, the surface potential of the film is a major determinant of this interaction.

MATERIALS AND METHODS

Chemicals

Toxin

Pseudomonas aeruginosa exotoxin A (ETA) was purchased from the Swiss Vaccine Institute (Bern, Switzerland) as a lyophilyzed powder. A stock solution was prepared by dissolving it at 2 mg/ml in 10 mM Tris-HCl (pH 8.0). This solution was stored at 4°C without further purification and used within 1 week. According to the purchaser, this toxin is more than 97% pure by high-performance liquid chromatography, and after reconstitution in water has a mean lethal dose for mice of 15 ng/g of body weight. We determined that these samples had no hemolytic activity whatsoever on either human or bovine erythrocytes, confirming they were not contaminated by any other pseudomonal hemolytic toxin (Liu, 1974; Woods and Iglewski, 1983).

Solutions

The buffer solutions used were buffer A (for pH values between 6.0 and 7.0): NaCl 125 mM, HEPES 20 mM, EDTA 1 mM; buffer B (for pH values between 4.0 and 5.5): NaCl 125 mM, acetic acid 20 mM, EDTA 1 mM; and buffer C (for the experiments at variable ionic strength): NaCl 240, 125, 80, 40, 20, or 10 mM, acetic acid 20 mM, EDTA 1 mM, pH 5.0. The pH was always adjusted by adding NaOH; the amount of Na^+ added was taken into account in the calculations of the ionic strength.

Lipids

The lipids used were egg phosphatidylcholine (PC), from Calbiochem (La Jolla, CA), and phosphatidic acid (PA) and phosphatidylglycerol (PG) from Avanti Polar Lipids (Pelham, AL). Purity was always more than 99%, according to the manufacturer. Before use they were dissolved in an ethanol:*n*-hexane solution (1:9 v:v) at a lipid concentration of 2 mg/ml. Different compositions were used as specified in the text; all binary mixtures were calculated on a molar basis.

Monolayer measurements

The monolayer experiments were performed with a commercial apparatus (Minitrough; KSV, Helsinki, Finland) enclosed in a plexiglass cabinet and connected to a computer. The surface pressure π of the lipid monolayer is defined as the decrease in surface tension γ, i.e., $\pi = (\gamma_o - \gamma_m)$, where γ_m and γ_o represent the surface tension of the water/air interface in the presence or absence of the lipid monolayer. It was measured by the Wilhelmy method, using a 2-cm roughened platinum plate, which ensures a zero contact angle. Before each experiment, the trough and the plate were thoroughly cleaned with hot water and organic solvents, followed by a final wash in bidistilled water. Solutions were stirred with a thin teflon-covered magnetic bar. All measurements were made at room temperature.

Determination of the molecular area occupied by the lipids

Compression isotherms were collected in a rectangular three-compartment teflon trough (zero-order type; Verger and de Haas, 1976; total volume 150 ml, surface of the central compartment 22.5 cm^2) that was equipped with two movable computer-controlled teflon barriers. The lipid solutions were added in small drops on top of the buffer surface (buffer C, pH 5.0) with a Hamilton microsyringe. Before the lipid was applied, any surface-active impurity was removed by repeated cycles of compression and suction of the top layer. Compression isotherms relating lipid molecular area with π were recorded by moving the barriers symmetrically at a speed of 20 mm/min. Collapse pressure was usually found to be above 40 mN/m.

Surface activity of the toxin

To minimize the amount of ETA required, these experiments were performed in a small, home-made, circular teflon trough (total surface 12.6 cm^2, total volume 20 ml). ETA was injected directly into the subphase with a Hamilton microsyringe through a hole in the trough wall. Before the toxin was applied, a lipid monolayer was prepared by adding lipid in small amounts until the desired initial surface pressure was reached. To attain a steady state, the monolayer was allowed to stand for at least half a hour before the ETA was injected. When the pH was step-changed during the experiment, its final value was measured with a microelectrode at the end of the observation. The time course of ETA-induced variations in surface pressure were least-square fitted to a single exponential. This procedure, applied to more than 80 kinetics obtained under all of the different experimental conditions we tried, provided an average correlation coefficient of 0.992 (SD = 0.011).

RESULTS AND DISCUSSION

The Langmuir monolayer technique was used to investigate the interaction of *P. aeruginosa* exotoxin A with lipid films. We observed that, under appropriate conditions, ETA can increase the surface pressure of lipid monolayers, suggesting that it can insert at least in part within the lipid molecules. Its effects on a lipid monolayer comprising PC and PG in a 1:1 molar ratio, for different values of pH, are shown in Fig. 1. The pressure increases at a rate that rises sharply when the pH becomes lower than 5.5 (Fig. 1 *A*). The time course of this interaction is well described by a mono-exponential relaxation (shown for the trace at pH 4.3 as a typical example), as one would expect from a simple adsorption isotherm. Under these conditions, the pH appears to be a major factor controlling partitioning, in fact, toxin insertion ensues only, and immediately, after the acidification of the reaction buffer (Fig. 1 *B*), whereas a reaction that had occurred in acidic conditions can be partly reversed by raising the pH (Fig. 1 *C*). The reversibility was tested with various values of final pH. We always observed a decrease that was proportional to the change in pH (ranging from 15% to 57% for final pH values between 5.9 and 10.5).

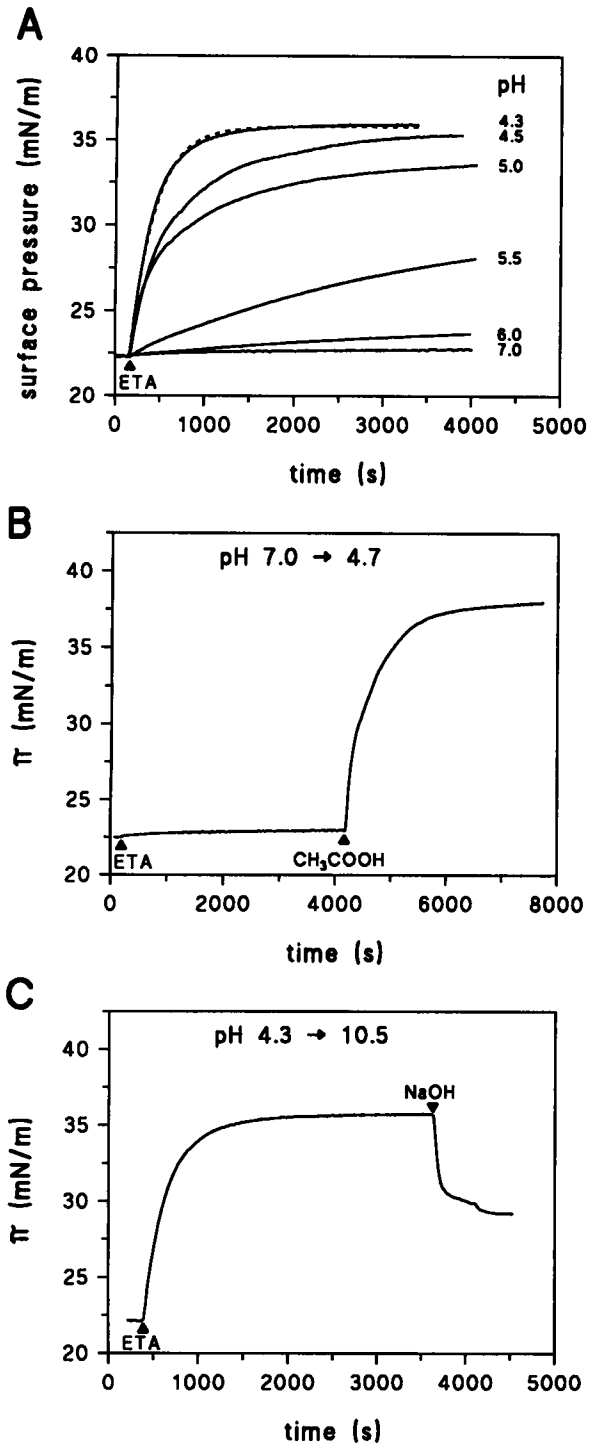

FIGURE 1 Adsorption of ETA to lipid monolayers monitored by measuring the surface pressure, π, of the film. (*A*) Adsorption isotherms were obtained with the following experimental conditions: lipid, PC/PG (1:1 molar ratio); toxin concentration, 40 nM; buffer A or B, depending on the indicated pH. A single exponential fit was superimposed on the upper trace (*dashed line*); best-fit parameters were $\Delta\pi_{ss}$ = 13.2 mN/m and τ = 280 s. All other traces could be fitted with a single exponential (not shown); the derived parameters are reported in Fig. 3. (*B*) Adsorption of ETA, which is negligible at pH 7.0, starts immediately after acidification to pH 4.7 by acetic acid. (*C*) ETA adsorbed to the film at pH 4.3 is substantially released upon addition of a base that raises the pH to 10.5. Other experimental conditions in *B* and *C* are the same as in *A*.

With reference to traces like those in Fig. 1 *A*, we define π_i, $\pi(t)$, and π_{SS} as the surface pressure at the time of addition of ETA, at the time t after the addition and at steady state, respectively.

Dependence of adsorption on toxin concentration

The dose dependence of ETA adsorption at pH 5.0 is given in Fig. 2, where we have reported the rate of the transition, i.e., the reciprocal of the time constant, τ (Fig. 2 *B*), and the fractional increase of the surface pressure at steady state, θ_{ss}

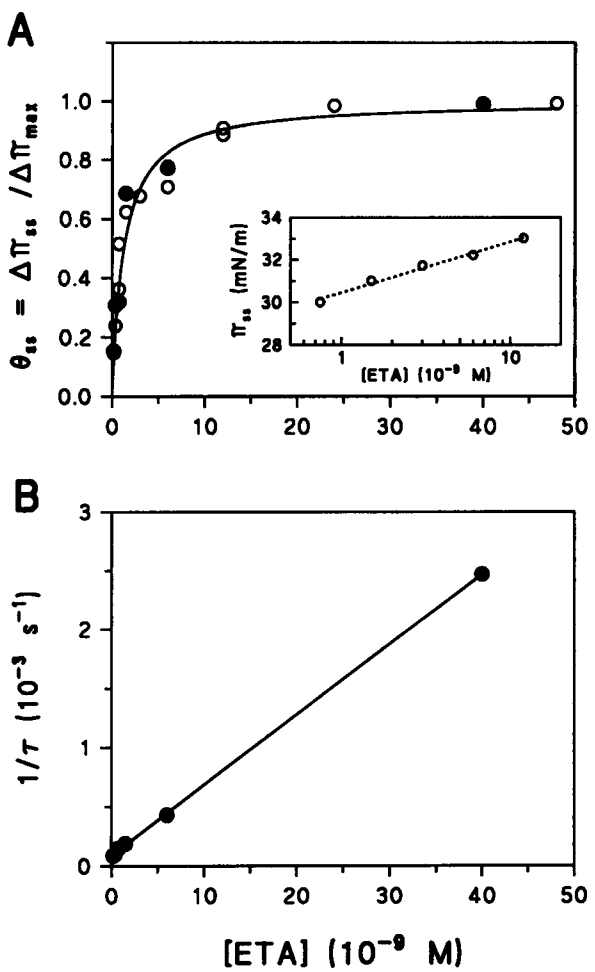

FIGURE 2 Dose dependence of the surface pressure increase induced by ETA. The ratio $\Delta\pi_{ss}/\Delta\pi_{max}$ and the reciprocal of the time constant of the transition are reported in *A* and *B*, respectively. Filled circles were obtained by a single-exponential fit of traces like those in Fig. 1, whereas open circles (only in *A*) were obtained by repeated additions of ETA to the same monolayer. Here $\Delta\pi_{max}$ was 13.8 mN/m. Other experimental conditions: lipid monolayer composed of pure PA at the initial surface pressure of 22 mN/m; buffer B, pH 5.0. Lines are best fit according to Eqs. 11 and 12. Values of pK_H, K, k_a, and k_d found are reported in Table 1. (*Inset*) Surface pressure as a function of the logarithm of ETA concentration. The straight line was used to calculate the portion of surface area occupied by each molecule according to the Gibbs equation (Eq. A3). The value obtained was 4.6 nm^2 (Appendix A).

(Fig. 2 *A*). θ_{ss} is defined as

$$\theta_{ss} = \Delta\pi_{ss}/\Delta\pi_{max} = (\pi_{SS} - \pi_i)/(\pi_{SS} - \pi_i)_{max} \quad (1)$$

where $\Delta\pi_{ss}$ is the surface pressure increase at steady state for the given conditions and $\Delta\pi_{max}$ its limiting value at increasing ETA concentration. For any point in Fig. 2, θ_{ss} and τ^{-1} were obtained by least-square fitting a single exponential to a trace like those in Fig. 1. In these experiments, to minimize the amount of ETA necessary, we used a pure PA monolayer (the most sensitive, in our hands). The observed dependence of θ_{ss} and τ^{-1} upon ETA concentration is consistent with a simple Langmuir adsorption isotherm. To show this, we first recall the general scheme for adsorption:

$$T + N_f \underset{k_d}{\overset{k_a}{\rightleftarrows}} N_o$$

Scheme I

were T represents free toxin, N_f and N_o are free and occupied toxin adsorption sites on the lipid film, and k_a and k_d are time-independent rate constants for adsorption and desorption. For the moment, these constants are called apparent because, as we will see later, they depend on several factors other than time.

Because we are far below the collapsing pressure, we can assume that the experimental observable $\Delta\pi(t) = \pi(t) - \pi_i$ is directly related to the degree of occupancy of the toxin-binding sites (which we call θ), through the following:

$$\theta(t) = \Delta\pi(t)/\Delta\pi_{max} = N_o(t)/N \quad (2)$$

where $\Delta\pi_{max}$ corresponds now to the situation in which all of the sites are occupied, and N is the total number of binding sites, i.e.,

$$N = N_f + N_o \quad (3)$$

Because the amount of toxin bound is relatively small (typically less than 10% as discussed in Appendix A), we can reasonably solve the system, assuming that T is actually all the toxin added. Therefore,

$$\theta(t) = \theta_{ss} \cdot (1 - \exp^{-t/\tau}) \quad (4)$$

$$\theta_{ss} = K \cdot T/(1 + K \cdot T) \quad (5)$$

$$\tau = 1/(k_a \cdot T + k_d) \quad (6)$$

$$K = k_a/k_d \quad (7)$$

were K is the apparent equilibrium constant and τ is the time constant of the transition. According to Eq. 4, the time course of ETA binding should follow a single exponential, as we have indeed observed (Fig. 1 *A*). Furthermore, Eq. 6 implies a linear dependence of τ^{-1} on ETA concentration, as was found (Fig. 2 *B*). From this plot one can derive k_a as the slope of the straight line, and k_d as the intercept. For a PA membrane, we found $k_a = (59 \pm 1) \times 10^3\ M^{-1}\ s^{-1}$, $k_d = (8.7 \pm 0.9) \times 10^{-5}\ s^{-1}$, and, through Eq. 7, $K = (6.8 \pm 0.6) \times 10^8\ M^{-1}$. The fact that k_d, albeit very small, is not zero confirms that the reaction is slowly reversible. Finally, the best fit of Eq. 5 to the points of Fig. 2 *A* provides a value of $K = (7.5 \pm 1.0) \times 10^8\ M^{-1}$, consistent with the value obtained from the rate constants.

A plot of the steady-state surface pressure π_{ss} versus the logarithm of ETA concentration, for experiments in which the toxin was repeatedly added to the same monolayer, is shown in the inset. This plot, which applies only to a restricted range of toxin concentration, was used to estimate the molecular area occupied by ETA according to the Gibbs equation (see Appendix A).

Dependence of adsorption on pH and membrane composition

Toxin insertion depends on various factors, as, for instance, pH and membrane composition. The pH dependence of ETA binding to membranes composed only of neutral lipids or also containing negatively charged lipids (PG and PA) is compared in Fig. 3. In any case, the surface pressure varies sigmoidally with the pH, but the inflection point (i.e., the apparent pK of the reaction) depends on the lipid composition and becomes higher when negatively charged lipids are introduced. This implies that, at any given pH, monolayers with acidic lipids are more sensitive to the toxin than neutral ones.

It is known that around pH 4.5 ETA undergoes a conformational change that increases its hydrophobicity and its tendency to partition into lipid membranes (Farahbakhsh and Wisnieski, 1989; Farahbakhsh et al., 1986; Jiang and London, 1990; Menestrina et al., 1991; Rasper and Merrill, 1994; Sandvig and Moskaug, 1987). Remaining within the framework of the Langmuir theory, we could use this notion to describe the pH dependency of ETA action simply by assuming that the toxin in solution is in equilibrium between two forms, one protonated (T^+) and the other unprotonated (T^o), and that only the protonated form can interact with lipids. Accordingly, reaction scheme I must be replaced by the following two schemes:

$$T^o + H^+ \underset{k_-}{\overset{k_+}{\rightleftarrows}} T^+$$

Scheme II

$$T^+ + N_f \underset{k_d}{\overset{k_a^*}{\rightleftarrows}} N_o$$

Scheme III

with

$$T^o + T^+ = T \quad (8)$$

Scheme II describes toxin protonation, k_+ and k_- being, respectively, the protonation and deprotonation rate constants. We define $K_H = k_+/k_-$ as the equilibrium constant

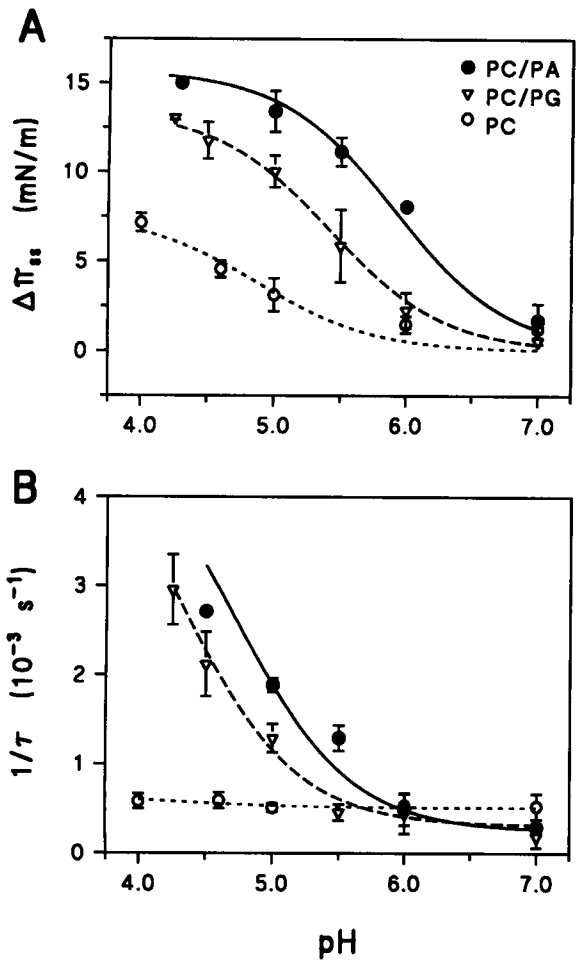

FIGURE 3 pH dependence of the surface pressure increase induced by ETA with various lipid compositions. The surface pressure increase at steady state ($\Delta\pi_{ss}$) and the reciprocal of the time constant of the transition are reported in *A* and *B*, respectively. Both parameters were obtained by a single-exponential fit of traces like those in Fig. 1. Other experimental conditions: lipid monolayer of the indicated composition (all binary mixtures were 1:1 on a molar basis); initial surface pressure 22 mN/m; toxin concentration, 40 nM; buffer A or B, depending on the indicated pH. Points are the mean ± SD of two or three experiments. Lines are best fit according to Eqs. 11 and 12. Values of pK_H, K^*, k_a^*, k_d, and $\Delta\pi_{max}$ found are reported in Table 1.

for the protonation reaction. In Scheme III, the new version of Scheme I, k_a^* is the adsorption rate constant of protonated ETA, which is now independent of the pH, but still dependent on the lipid composition.

If we further assume that the protonation reaction is fast compared to the interaction with lipids (see Appendix B), we can account for the pH effect simply by substituting into Scheme III the equilibrium value of T^+:

$$T^+ = T \cdot J \tag{9}$$

with

$$J = K_H \cdot [H^+]/(1 + K_H \cdot [H^+]) \tag{10}$$

From Scheme III, using Eqs. 8–10 we now obtain:

$$\theta_{ss} = K^* \cdot T^+/(1 + K^* \cdot T^+) = K^* \cdot J \cdot T/(1 + K^* \cdot J \cdot T) \tag{11}$$

and

$$\tau = 1/(k_a^* \cdot T^+ + k_d) = 1/(k_a^* \cdot J \cdot T + k_d) \tag{12}$$

where

$$K^* = k_a^*/k_d \tag{13}$$

Values of K_H, K^*, k_a^*, and k_d obtained from the least-squares fit of Eqs. 11–13 to the experimental points of Figs. 2 and 3 are given in Table 1. It appears that K_H is indeed relatively constant, as expected for an intrinsic property of the protein, whereas all of the other parameters depend strongly on the lipid composition of the membrane and, in particular, on the content of negative lipids. From K_H we can derive the pK of the protonable group of the toxin as

$$pK_H = \log_{10} K_H \tag{14}$$

We obtain an average value of $pK_H = 4.5 \pm 0.2$, which is consistent with previous values reported by us (Menestrina et al., 1991) and by others (Farahbakhsh and Wisnieski, 1989; Farahbakhsh et al., 1986; Jiang and London, 1990; Rasper and Merrill, 1994; Sandvig and Moskaug, 1987), and suggests that the protonable group could be a carboxyl group. Inspection of Fig. 3 indicates that the intrinsic pK coincides with the apparent pK in the case of neutral lipids (PC), but is smaller than the apparent pK for negatively charged lipids. This effect is entirely due to the lipid dependence of the binding constant K^*.

Besides being dependent on the lipid composition, the maximum pressure increase induced by ETA depends on the initial surface pressure of the monolayer (π_i), as detailed in Table 2. Clearly, at lower values of π_i, there is a higher effect; however, even monolayers with a π_i of ~36 mN/m are penetrated by ETA, albeit to a much reduced extent.

Dependence of adsorption on the surface potential of the monolayer

From the dependence of the equilibrium and rate constants on the lipid composition (Table 1), it appears that the surface potential of the lipid monolayer is involved in increasing the affinity of ETA for the membrane. The facilitating role of the negative surface potential was confirmed by two independent experiments: the dependence of the adsorption on the amount of negatively charged lipid present (Fig. 4), and the dependence on the ionic strength of the solution (Fig. 5). Clearly, increasing the percentage of negative charges in the lipid layer, which increases the surface potential, promotes both the extent and the rate of insertion of ETA, although, even with pure PA, no insertion was ever observed at pH 7.0 (Fig. 4). Conversely, both the extent and the rate of increase of ETA-induced surface pressure are diminished at higher ionic strength, which

TABLE 1 Kinetic and equilibrium parameters regulating the adsorption of ETA to lipid monolayers, derived from the best fit of the model to the experimental results*

Figure (variable)	Fitted curve	$\Delta\pi_{max}$ (mN/m)	Q (e.u.)	δ	K (10^8 M^{-1})	K^* (10^8 M^{-1})	K^0 (10^8 M^{-1})	k_a (10^3 M^{-1} s^{-1})	k_a^* (10^3 M^{-1} s^{-1})	k_a^0 (10^3 M^{-1} s^{-1})	k_d (10^{-3} s^{-1})	k_d^0 (10^{-3} s^{-1})	pK_H
Fig. 3	PC ($\Delta\pi_{ss}$)	17.0 ± 1.3	—	—	—	0.21 ± .02	0.21#	—	—	—	—	—	4.6 ± .2
(pH)	PC (τ)	—	—	—	—	0.07 ± .03	0.07#	—	3 ± 1	3#	0.51 ± .02	0.51#	4.5 ± .6
	PC/PG ($\Delta\pi_{ss}$)	15.0 ± 0.6	—	—	—	2.8 ± 0.4	—	—	—	—	—	—	4.3 ± .2
	PC/PG (τ)	—	—	—	—	4.0 ± 1.2	—	—	98 ± 6	—	0.25 ± .07	—	4.5 ± .2
	PC/PA ($\Delta\pi_{ss}$)	16.5 ± 0.6	—	—	—	5.2 ± 1.0	—	—	—	—	—	—	4.6 ± .2
	PC/PA (τ)	—	—	—	—	5.0 ± 1.0	—	—	120 ± 7	—	0.24 ± .07	—	4.7 ± .2
	PA§ ($\Delta\pi_{ss}$)	16.5 ± 1.0	—	—	—	15 ± 7	—	—	—	—	—	—	4.2 ± .5
	PA§ (τ)	—	—	—	—	23 ± 6	—	—	320 ± 10	—	0.14 ± .04	—	4.3 ± .2
Fig. 2	PA ($\Delta\pi_{ss}$)	—	—	—	7.5 ± 1.0	34¶	—	—	—	—	—	—	—
([ETA])	PA (τ)	—	—	—	6.8 ± 0.6	31¶	—	59 ± 1	258 ± 5¶	—	0.09 ± .01	—	—
Fig. 6	k_a^* (τ)	—	1.8 ± .2	0.8 ± .1	—	—	0.11‖	—	—	4.8 ± .3	—	—	—
(ψ)	k_d (τ)	—	1.9 ± .6	0.8 ± .1	—	—	0.11‖	—	—	—	—	0.44 ± .04	—
	K^* (τ)	—	2.0 ± .2	—	—	—	0.13 ± .01	—	—	—	—	—	—
	K^* ($\Delta\pi_{ss}$)	—	1.9 ± .2	—	—	—	0.11 ± .01	—	—	—	—	—	—
Intrinsic values**		16.3 ± .8	1.9 ± .3	0.8 ± .1			0.12 ± .01			4.8 ± .3		0.44 ± .04	4.5 ± .2

*The experimental conditions are described in the respective figures. The parameters $\Delta\pi_{max}$, K^*, k_a^*, k_d, and pK_H were derived by a best fit of Eqs. 11 and 12 to the pH dependence of ETA adsorption; K, k_a and k_d by a best fit of Eqs. 5 and 6 to the dose dependence of ETA adsorption; Q, δ, K^0, k_a^0 and k_d^0 by a best fit of Eqs. 15–17 to the surface potential dependence of the parameters K^*, k_a^* and k_d.

#Because with PC the surface potential is zero, in this case $K^0 = K^*$, $k_a^0 = k_a^*$, and $k_d^0 = k_d$ as reported; however, these values were not used to calculate the average intrinsic values given in the last row.

§This fit was not shown in Fig. 3.

¶K^* and k_a^* were derived from K and k_a, using the average value of $pK_H = 4.5$, as obtained above.

‖This value of K^0 was obtained as k_a^0/k_d^0.

**These average intrinsic values were used to generate the theoretical curves in Figs. 4, 5, and 7.

TABLE 2 Binding of ETA to lipid monolayers of variable composition as a function of the initial surface pressure π_i^*

[PA]# (%)	π_i§ (mN/m)	π_f§ (mN/m)	$\Delta\pi_{ss}$§ (mN/m)	τ^{-1}¶ (10^{-1} s^{-1})	A_L^i‖ (nm^{-2})	A_L^f‖ (nm^{-2})	n_L/n_T**
50	12.1	28.1	16.0	1.48	0.98	0.66	—
	22.2	34.6	12.4	1.50	0.73	0.60	35.4
	35.8	40.7	4.9	0.59	0.58	0.53	—
75	12.0	26.3	14.3	1.58	0.87	0.62	—
	22.2	36.8	14.6	1.70	0.66	0.52	32.9
	36.0	40.9	4.9	1.21	0.51	0.47	—
100##	12.1	33.5	21.4	1.29	0.75	0.47	—
	22.3	35.8	13.5	2.12	0.60	0.46	34.1

*ETA, 40 nM, was added in the subphase (containing buffer B at pH 5.0) of lipid monolayers composed of different binary mixtures of PC and PA.
#Percentage of PA present.
§π_i and π_f are the initial and the final surface pressure before and after ETA addition, and $\Delta\pi_{ss}$ is the steady-state increment ($\pi_f - \pi_i$).
¶τ is the time constant of the transition derived from a single-exponential fit as in Fig. 1.
‖A_L^i and A_L^f are the average areas occupied by the lipid molecules before and after toxin adsorption, directly determined from the lipid compression isotherms.
**n_L/n_T is the number of lipid molecules per toxin molecule bound, given by $A_m/(A_L^i - A_L^f)$, where A_m is the interface area occupied by the toxin molecule. A_m was obtained applying the Gibbs equation only for the case of π_i = 22 mN/m (Fig. 2 *A*, *inset*); therefore values of n_L/n_T are reported only for that case.
##With monolayers composed of pure PA, it was impossible to reach π_i = 36 mN/m without collapsing the film.

actually lowers the surface potential by accumulation of counterions at the interface (Fig. 5).

An effect mediated by the lowering of the local pH at the interface due to electrostatic attraction of protons seems to be unlikely in this case for several reasons: 1) the desorption rate constant, k_d, also depends on the lipid, which should not be the case if the effect is only to increase T^+ locally; 2) the local pH extends over a region whose thickness is estimated by the Debye-Hückel distance (~0.5 nm under our typical experimental conditions; Eisenberg et al., 1979), which is much smaller than the diameter of the ETA molecule (~6 nm; Allured et al., 1986); 3) no interaction is observed at pH 7.0, even with PA membranes that have a surface pH of ~5.2 and should thus be sensitive.

For these reasons, we would rather envisage a direct electrostatic interaction between ETA and the membrane. Thus we assume that the energetics of interaction also has a coulombic component due to the attraction between a positive charge on ETA (which we call Q and express in electronic units) and the negative surface potential (ψ) on the lipids (Heymann et al., 1996). According to usual reaction rate theory, we can thus write

$$k_a^* = k_a^0 \cdot \exp(-\delta \cdot Q \cdot e\psi/kT) \quad (15)$$

$$k_d = k_d^0 \cdot \exp[-(\delta - 1) \cdot Q \cdot e\psi/kT] \quad (16)$$

and, recalling Eq. 13:

$$K^* = K^0 \cdot \exp(-Q \cdot e\psi/kT] \quad (17)$$

where δ is the fraction of potential sensed by the protein in the activated state which precedes binding; k_a^0 and k_d^0 are the intrinsic adsorption and desorption rate constants (independent of lipid and pH); and $K^0 = k_a^0/k_d^0$ is the intrinsic equilibrium constant. Introducing the surface potential, evaluated according to the Guy-Chapman-Stern theory (see Appendix C), we can see that the constants have the required exponential dependence on ψ (Fig. 6). The best fit of Eqs. 15–17 provided the intrinsic constants listed in Table 1, a protein charge $Q = +1.9 \pm 0.3$, and a fraction $\delta = 0.8 \pm 0.1$.

Interestingly, these same parameters can be used to predict rather satisfactorily the effects observed by changing the percentage of PA in the membrane (Fig. 4), or the concentration of Na^+ in solution (Fig. 5). It appears even clearer when the data are replotted as a function of the surface potential (Fig. 7). Thus a rather simple model appears to adequately account for all of the observed pH effects (Figs. 1–3) and to correctly predict, without introducing any new adjustable fit parameter, the results of new experiments (Figs. 4 and 5). It is also consistent with previous reports on the lipid dependence of pore formation in lipid vesicles that is stimulated by negatively charged lipids (Menestrina et al., 1991; Rasper and Merrill, 1994) and inhibited by even small amounts of positively charged lipids (Farahbakhsh and Wisnieski, 1989).

Implications for the mechanism of ETA intoxication

To evaluate the physiological relevance of the finding that the surface potential can increase ETA binding by up to two orders of magnitude, one should note that our results imply that such an effect is due only to the surface charge of the lipid film and not to the particular lipid composition. In fact, cells normally have only a small amount of acidic phospholipids on their outer leaflet (Connor et al., 1989; Op den Kamp, 1979) (mainly PA and phosphatidylinositol (Gascard et al., 1991)), but have nonetheless a substantial negative surface potential contributed by other components, such as glycoproteins and glycolipids. Thus it appears likely that

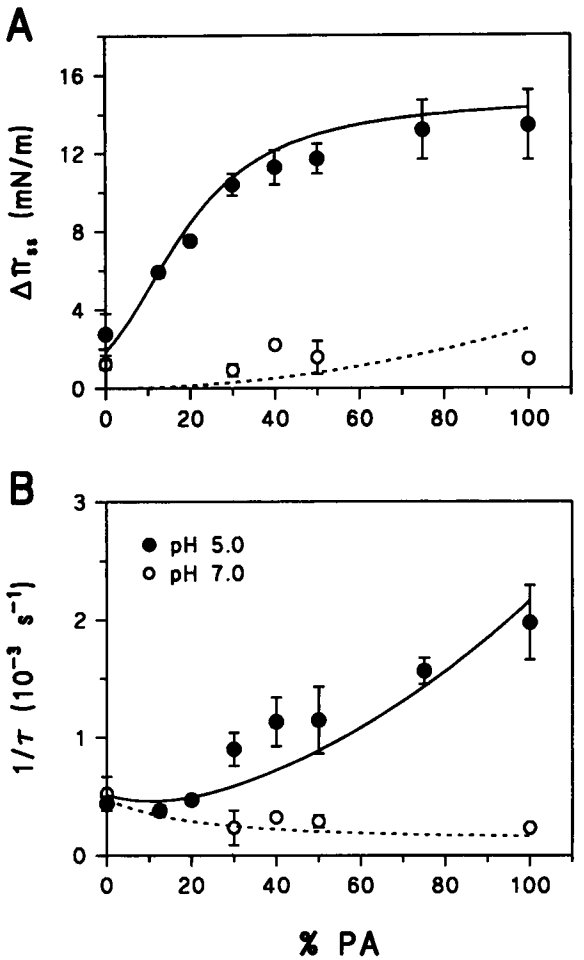

FIGURE 4 Dependence of the lipid adsorption of ETA on the percentage of acidic phospholipid present in the monolayer. $\Delta\pi_{ss}$ and τ^{-1}, obtained by a single-exponential fit of traces like those in Fig. 1, are reported in *A* and *B*, respectively. Other experimental conditions: lipid monolayer formed by a binary mixture of PC and PA (the amount of PA is indicated), at an initial surface pressure of 22 mN/m; toxin concentration, 40 nM; buffer A, at pH 7.0, or buffer B, at pH 5.0. Points are the mean ± SD of two or three experiments. Lines are the predictions of our model (Eqs. 11–13 and 15–17), with the average values of pK_H, K^0, k_a^0, k_d^0, Q, δ, and $\Delta\pi_{max}$ taken from the last line of Table 1. ——, pH 5.0; – – –, pH 7.0. We wish to emphasize that no adjustable parameter was used here.

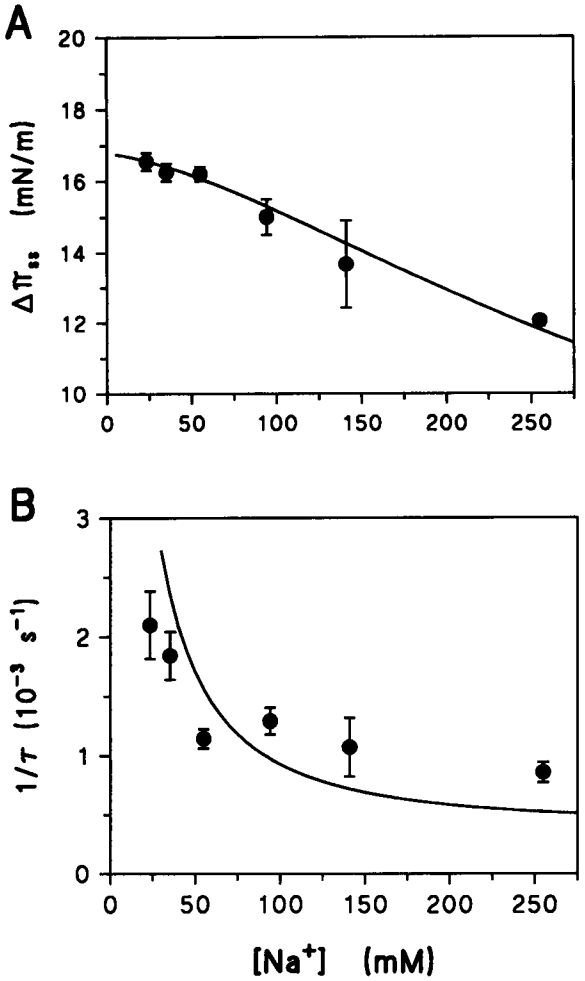

FIGURE 5 Dependence of the lipid adsorption of ETA on the ionic strength of the medium. $\Delta\pi_{ss}$ and τ^{-1}, obtained by a single-exponential fit of traces like those in Fig. 1, are reported in *A* and *B*, respectively. Other experimental conditions: lipid monolayer composed of a binary mixture of PC and PA (1:1 molar ratio) at the initial surface pressure of 22 mN/m; toxin concentration, 40 nM; buffer C, pH 5.0, with variable NaCl concentration. Points are the mean ± SD of two or three experiments. Lines are the predictions of our model, with the average values of pK_H, K^0, k_a^0, k_d^0, Q, δ, and $\Delta\pi_{max}$ taken from the last line of Table 1. Note that here, as in Fig. 4, no adjustable parameter was used.

once inside the endocytic vesicle, and after acidification of this compartment to pH 5.5 (Gruenberg and Howell, 1989), ETA can bind to the lipid surface, as a preliminary step necessary for translocation. As we have previously proposed (Menestrina et al., 1991), the positively charged region of the protein responsible for the electrostatic binding to the lipid membrane is most probably the loop encompassing residues 274 to 279, which includes three arginines (at positions 274, 276, 279), His^{275}, and a proline. In fact, two steps are required to produce the enzymically active fragment of ETA that is translocated to the cytoplasm: 1) this loop must be proteolytically nicked after Arg^{274} by the membrane protease furin (Fryling et al., 1992; Ogata et al., 1990); 2) the disulfide bond between Cys^{265} and Cys^{287} that links the two α-helices connected by this loop must be reduced (Ogata et al., 1990, 1992; Wick et al., 1990). Both events are more likely if the loop enters into close contact with the membrane. The charge value that we have observed, which is around 2, is actually consistent with the involvement of the stretch 274–276, because the histidine in position 275 might be deprotonated, even at low pH, because of the close proximity to the two positively charged arginine residues. According to the G-C-S theory, the fraction of potential experienced by the protein charge in the active state that precedes binding (~80%) is compatible with a location of this group ~0.1 nm away from the lipid surface, implying a rather small conformational change between the active and bound states.

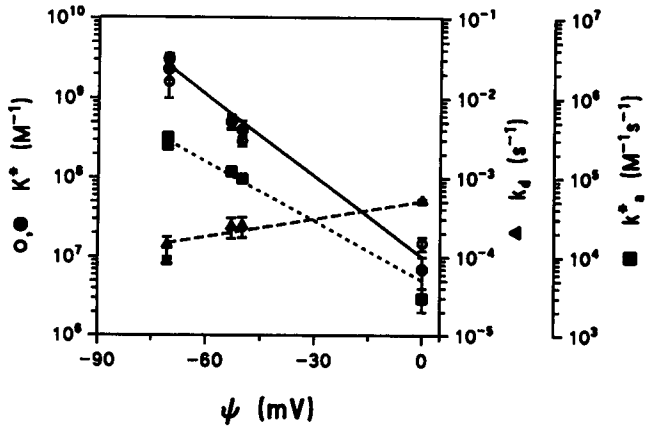

FIGURE 6 Dependence of the equilibrium and rate constants (K^*, k_a^*, k_d) on the surface potential of membranes with different lipid composition. Values of the constants were obtained as best fit of the data in Figs. 2 and 3 (as reported in Table 1). Open symbols came from the analysis of $\Delta\pi_{ss}$ (*A* in those figures), whereas closed symbols came from the analysis of τ (*B*). The lipid surface potential ψ was calculated according to the Guy-Chapman-Stern theory (Appendix C). Lines are best fit according to Eqs. 15–17, providing the values of K^0, k_a^0, k_d^0, Q, and δ reported in Table 1. Because of the lack of points in the interval between 0 mV and −45 mV, the slope of the lines has some uncertainity; accordingly in Table 1, we reported twice the SE of the fit as the error on parameter Q.

APPENDIX A: ESTIMATION OF THE MOLECULAR AREA OCCUPIED BY THE TOXIN

It is possible to calculate the portion of area occupied by a surfactant molecule at the air/water interface via the Gibbs equation (Seelig, 1990):

$$\Gamma = -c \cdot (\delta\gamma/\delta c)/RT = -(\delta\gamma/\delta \ln c)/RT \qquad \text{(A1)}$$

where Γ is the excess amount of amphiphilic molecules accumulated per unit area at the interface, c is the concentration of the amphiphile in the bulk solution, and R and T have their usual meaning. Although originally derived for a ternary system comprising air, water, and the amphiphile, this equation was also used to determine the molecular area occupied by a macromolecule adsorbing to a preformed lipid monolayer and was validated by more direct methods (Bougis et al., 1981). Accordingly, we will use it in this way for ETA. Because the toxin concentration is very low, we can neglect its activity coefficient (Seelig, 1990). Therefore, the surface area occupied by one inserted toxin molecule, A_m, is given by

$$A_m = 1/\Gamma N_A \qquad \text{(A2)}$$

where N_A is Avogadro's number. Because, according to Eq. 1, $\delta\gamma = -\delta\pi$, it follows that

$$\delta\pi = RT/A_m N_A \cdot \delta \ln c \qquad \text{(A3)}$$

i.e., that A_m is inversely related to the slope of a graph of π versus ln c. Such logarithmic dependence appears to hold in our case, at least in a certain range of ETA concentration (Fig. 2 *A*, *inset*). From the slope of this graph we obtained A_m = 4.6 nm^2. This value is ~50% larger than that found for cardiotoxin, a molecule of m.w. 7000 (Bougis et al., 1981), but only one-fourth that of colicin A (Pattus et al., 1983), and much smaller than that of DT (~30 nm^2; Demel et al., 1991). This suggests that only a small portion of the ETA molecule is actually inserted into the lipid monolayer. From A_m we can estimate n_T, the number of ETA molecules bound to the monolayer under experimental conditions that ensure complete binding (e.g., a monolayer comprising an equimolar mixture of PC and PA, pH 5.0 and 40 nM ETA). Calling A_t the total area of the monolayer, ΔA the part of this area occupied by the toxin, A_L^i and A_L^f the

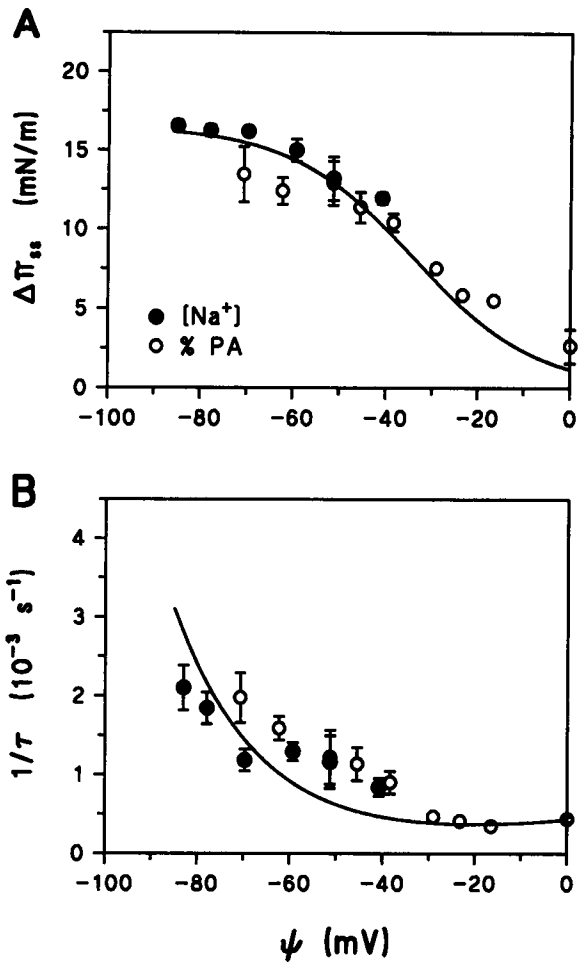

FIGURE 7 Direct dependence of the surface pressure increase induced by ETA on the surface potential ψ of the monolayer. $\Delta\pi_{ss}$ and τ^{-1} are reported in *A* and *B*, respectively, as a function of the surface potential ψ. ψ was varied by changing either the PA content of the monolayer (○, replotted from Fig. 4) or the ionic strength of the medium (●, replotted from Fig. 5) and calculated according to the G-C-S theory (Appendix C). Points are the mean ± SD of two or three experiments. The straight lines are the predictions of our model, with the average values of pK_H, K^0, k_a^0, k_d^0, Q, δ, and $\Delta\pi_{max}$ taken from the last line of Table 1, and no other adjustable parameter.

average molecular areas occupied by the lipid before and after toxin adsorption, and n_L the total number of lipid molecules, we have

$$n_L = A_t/A_L^i \qquad \text{(A4)}$$

$$\Delta A = n_L \cdot (A_L^i - A_L^f) \qquad \text{(A5)}$$

and finally,

$$n_T = \Delta A/A_m = A_t \cdot (A_L^i - A_L^f)/(A_L^i \cdot A_m) \qquad \text{(A6)}$$

Using A_L^i and A_L^f as directly obtained from the respective lipid compression isotherms (Table 2), we calculated $n_T = 45 \times 10^{12}$. From this number we could derive three important notions: 1) The percentage of ETA bound with respect to that added was 9.2%, which is similar to values found for cardiotoxin (Bougis et al., 1981) and DT (Demel et al., 1991), and indicates that most ETA remains in solution. 2) The average number of lipid molecules per bound toxin molecule was 35.4, very similar to the limiting value found by studying the adsorption of anti-hapten IgG to hapten-lipid

containing monolayers (Tamm and Bartoldus, 1988). This number did not depend significantly on the percentage of PA present, at least in the range of 50–100% (Table 2). 3) The total area occupied by one toxin molecule, i.e., A_m plus the surface of the coordinated lipid annulus, was 28 nm^2, which is very similar to the area occupied by DT in compressed protein films (Demel et al., 1991), and corresponds to the cross section of ETA as calculated from a radius of 3.0 nm derived from its crystal structure (Allured et al., 1986). This suggests that saturation of ETA adsorption actually occurs when the toxin molecules approach physical contact in the subphase.

APPENDIX B: ESTIMATION OF THE PROTONATION TIME OF ETA

The assumption that the protonation reaction is always at equilibrium with respect to the lipid adsorption is warranted by the exceedingly high rate of diffusion of protons in water. In fact, the literature reports that the rate of protein protonation is in the range of 10^{10} to 10^{11} M^{-1} s^{-1} (Gutman and Nachliel, 1985; Gutman et al., 1983). From this and the observed K_H, a lower limit for the rate of deprotonation, k_-, can be calculated to be 3×10^5 s^{-1} in our case. Because the protonation time constant is given by

$$\tau_H = 1/(k_+ \cdot [H^+] + k_-) \qquad \text{(B1)}$$

an upper limit of π_H is therefore attained, using $k_+ = 10^{10}$ M^{-1} s^{-1}. Under our experimental conditions, π_H is always less than 10 μs, i.e., ~7 orders of magnitude smaller than the observed rate constants for toxin-lipid interaction (see Figs. 1–5).

APPENDIX C: ESTIMATION OF THE SURFACE POTENTIAL OF THE LIPID MONOLAYER

The local electrical potential at the surface of a phospholipid monolayer containing acidic lipids was calculated according to the Guy-Chapman-Stern (G-C-S) theory (Aveyard and Haydon, 1973), as discussed, for example in Eisenberg et al. (1979) and McDaniel et al. (1984). Both screening and specific binding of counterions (protons and Na^+ ions in our case) were taken into account.

If we call ψ the surface potential, H and H_o the concentrations of protons at the surface of the lipid film and in the bulk solution, C and C_o the surface and bulk concentrations of Na^+ ions, σ and σ_{max} the actual and the maximum charge densities of the monolayer, and K_H and K_C the dissociation constants for the binding to the lipid of H^+ and Na^+ ions, respectively, we get the following set of expressions:

$$\sinh(e\psi/2kT) = B\sigma/C_o^{1/2} \qquad \text{(C1)}$$

$$\sigma = \sigma_{max}/(1 + H/K_H + C/K_C) \qquad \text{(C2)}$$

$$H = H_o \exp - (e\psi/kT) \qquad \text{(C3)}$$

$$C = C_o \exp - (e\psi/kT) \qquad \text{(C4)}$$

where e is the charge of the electron, k and T have their usual meaning, and B is a constant given by

$$B = 1/(8N_A\epsilon\epsilon_o kT)^{1/2} \qquad \text{(C5)}$$

with N_A again representing Avogadro's number and $\epsilon \cdot \epsilon_o$ the dielectric constant of water. Values of K_H and K_C can be found in the literature. From a recent review (Cevc, 1990), we took $pK_H = 3.5$, 2.9, and ≤ 1 for the cases of PA, PG, and PC, respectively, and $K_C = 1.25$ M and 1.54 M for the binding of Na^+ to PA and PG (PC does not appreciably bind monovalent cations at these concentrations). σ_{max} was calculated by using one electron charge per acidic lipid in the composition and using the following molecular areas for the phospholipids: PC = 0.89 nm^2, PG = 0.87 nm^2, and PA = 0.60 nm^2. These values were all directly evaluated from the respective compression isotherms for the initial pressure of 22 mN/m (and are consistent with published data). In this first approach we did not take into consideration the small decrease in σ_{max} resulting from the adsorption of the positively charged protein molecules.

Equations C1–C5 were solved numerically (the problem is equivalent to finding the roots of a fourth-order equation), with the appropriate values for the parameters. In this way we determined ψ as a function of H_o, C_o, and σ_{max} for all of the different lipid and buffer compositions, which we then introduced into Eqs. 15–17 to produce the theoretical curves of Figs. 4–7.

We thank Cesare Montecucco for useful discussions in the course of these experiments and for a critical reading of the manuscript, and Susan Struthers and Stephen Trueman for checking the English.

This work was financially supported by the Consiglio Nazionale delle Ricerche, the Istituto Trentino di Cultura, and the Human Capital and Mobility Programme of the European Community (contract CHR X-CT 93-0255).

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