Membrane Insertion of *Escherichia coli* α-Hemolysin Is Independent from Membrane Lysis*

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5 The abbreviations used are: HlyA, α-hemolysin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid (ANTS) and p-xylene bis(pyridinium bromide) (DPX); WT, wild type; pro-HlyA, pro-α-hemolysin.

*Escherichia coli* α-hemolysin (HlyA) is a protein exotoxin that binds and lyases eukaryotic cell and model membranes in the presence of calcium. Previous studies have been able to distinguish between reversible toxin binding to the membrane and irreversible insertion into the lipid matrix. Membrane lysis occurs as the combined effect of protein insertion plus a transient perturbation of the membrane bilayer structure. In the past, insertion and bilayer perturbation have not been experimentally dissected. This has now been achieved by studying HlyA penetration into lipid monolayers at the air-water interface, in which three-dimensional effects (of the principal region inserting into the lipid bilayer) cannot occur. The study of native HlyA, together with the nonlytic precursor pro-HlyA, and of different mutants demonstrates that although some nonlytic variants (e.g., pro-HlyA) exhibit very low levels of insertion, others (e.g., the nonlytic mutant HlyA H859N) insert even more strongly than the lytic wild type. These results show that insertion does not necessarily lead to membrane lysis, i.e., that insertion and lysis are not “coupled” phenomena. Millimolar levels of Ca$^{2+}$, which are essential for the lytic activity, cause an extra degree of insertion but only in the case of the lytic forms of HlyA.

Among the many bacterial protein toxins known, α-hemolysin (HlyA) 6 from *Escherichia coli* has been studied in detail as a prominent member of the RTX protein family (for reviews see Refs. 1–4). It is a large (107 kDa) protein, containing 10 amphipathic α-helices that are located mostly near the N terminus (5, 6). In addition, a hydrophilic calcium-binding domain, consisting of 15 Gly- and Asp-rich nonapeptides repeated in tandem, characteristic of the RTX (repeats in toxin) family, is found close to the C-end of the protein (7–10). The presence of amphipathic and hydrophilic domains confers to the protein an overall amphiphilic character, which explains its tendency both to aggregate and to interact with membranes (6, 11, 12). Interaction with cell membranes occurs probably via specific receptors (13–15) and is responsible for the well known cytolytic effects of HlyA, actually the main pathogenic factor in extraintestinal infections by *E. coli* in humans. HlyA is secreted by the bacterium to the extracellular medium as a soluble protein, but upon interaction with a membrane, the toxin becomes inserted in the lipid bilayer, behaving thereafter as an integral membrane protein. This transition from soluble to membrane-embedded protein constitutes an interesting problem in the field of protein-lipid interactions that has been explored in this and other laboratories. The toxin has a lytic effect on pure lipid liposomes (16) and is able to form ion-permeable channels of large conductance on planar phospholipid bilayers (17, 18). Data obtained with photoactivable probes incorporated into the hydrophobic phase of the lipid bilayer indicate that the principal region inserting into the membrane lies within the N-terminal half of the toxin, between residues 177 and 411 (12). HlyA interaction with membranes can be reversible (adsorption) or irreversible (insertion). Until now insertion has been operationally identified with membrane lysis. Lysis requires the presence of Ca$^{2+}$ (8, 19) and a bilayer in the fluid state (20). Two fatty acyl chains, which make part of the mature form of HlyA, are also essential for the lytic effect (21).

Lysis, i.e. disruption of the membrane permeability barrier (e.g., hemolysis or efflux of aqueous liposome contents), has been used in the past as proof of toxin insertion into the membrane. However, a conceptual separation of (i) protein insertion and (ii) changes in lipid architecture, leading to the observed release of cell/vesicle contents, has been proposed (6, 16).

As a further step in the study of the HlyA membrane insertion process, we have examined the interaction of this toxin with lipid monolayers extended over an air-water interface. Lipid monolayers are a useful tool in the study of lipid-protein interactions (22–25). In particular, the monolayer technique allows the direct observation of the insertion phenomenon separated from further changes in lipid architecture. This occurs because, by design, the monolayer cannot undergo three-dimensional membrane restructuring that would be essential to alter the membrane permeability barrier. Thus the lipid monolayer studies allow a further dissection of the insertion process, as separated from the membrane lytic event(s). Our results with native and mutant toxins show that high levels of insertion can occur even in the case of mutants that are totally nonlytic, i.e. that insertion is independent from lysis. However, Ca$^{2+}$, which is essential for lysis to occur, does favor a further degree of insertion, only for the lytic variants of the toxin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Egg phosphatidyicholine (PC) and egg phosphatidylethanolamine (PE) were from Lipid Products (South Nutfield, UK). Cholesterol was supplied by Avanti Polar Lipids (Alabaster, AL). 8-Aminonaphthalene-1,2,3-trisulfonic acid (ANTS) and p-xylene bis(pyridinium bromide) (DPX) were from Molecular Probes (Eugene, OR).

**Purification of Wild Type and Mutant HlyA Proteins**—Wild type HlyA and mutant HlyA proteins were purified as described previously for the wild type (25). Exceptionally, HlyA H859N, which was expressed in smaller amounts, was purified by fast protein liquid chromatography using a Superdex HR-200 column instead of the Sephacryl S-500 gel.
filtration column (9). Pro-hemolysin was expressed using the plasmid pSU2726 (bla, hlyA+) (26, 27) in E. coli B121 (DE3). The bacterium was grown in Luria-Bertani liquid medium with 0.1 mg/ml ampicillin, at 37 °C, to 4°C. Isopropyl-1-thio-β-D-galactopyran (final concentration 0.5 mM) was then added, and expression was allowed to occur for 3 h. Cells were harvested by centrifugation (6000 × g, 10 min). The cell pellet was resuspended in “TES buffer” (10 mM EDTA, 0% (v/v) sucrose, 50 mM TRIS, pH 7.0) and stored at −20 °C overnight. The cells were thawed at 37 °C and treated with lysozyme (0.5 mg/ml), 25% benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride in “lysis buffer” (1 mM dithiothreitol, 0.1 mM EDTA, 0.5% (v/v) Triton X-100, 50 mM TRIS, pH 7.0). Cell lysis was allowed to occur for 45 min in an ice bath, followed by 25 sonication cycles (15 s of sonication at 12 μm amplitude + 25 s at rest) in a Soniprep 150 MSE probe sonicator. The suspension was centrifuged at 18,000 × g (20 min, 3°C), and pro-HlyA inclusion bodies, recovered in the pellet, were resuspended in “TU buffer” (6 M urea, 50 mM TRIS, pH 7.0). The sample in TU buffer was then applied to a DEAE-Sepharose ion exchange column (Amersham Biosciences) and eluted with a 0–150 mM NaCl gradient in the same buffer. Flow rate was 1 ml/min, and 5-ml fractions were collected. Pro-hemolysin eluted at 35 mM NaCl. The purified proteins were stored at −20 °C in 35 mM NaCl, 6 M urea, 50 mM TRIS-HCl, pH 7.0.

Large Unilamellar Vesicles—Large unilamellar vesicles (LUV) made of egg phosphatidylcholine:egg phosphatidylethanolamine:cholesterol (2:1:1) were prepared by extrusion through polycarbonate filters (Nuclepore, Pleasanton, CA), pore size 0.1 μm. Buffer was 150 mM NaCl, 20 mM TRIS-HCl, pH 7.0. The diameter of the resulting vesicles was 100 nm, according to quasi-elastic light scattering measurements. More details on the preparation of these vesicles can be found in Mayer et al. (28).

Release of Liposomal Contents—Leakage of vesicular aqueous contents was assayed with ANTS and DPX entrapped in the liposomes according to Ellens et al. (29). LUV were prepared in 70 mM NaCl, 12.5 mM ANTS, 45 mM DPX, 20 mM Tris-HCl, pH 7.0. Nonentrapped probes were removed passing the LUV through a Sephadex G-75 column, eluted with 150 mM NaCl, 20 mM Tris-HCl, pH 7.0. Assays were performed at 100 μM lipid in a total volume of 1 ml, with continuous stirring at 25 °C. Buffer was as above, with the addition of 10 mM CaCl₂. The assay was started by adding 10 μg/ml (96 nM) HlyA or mutant proteins. ANTS fluorescence was recorded continuously (λ ex = 355 nm; λ em = 520 nm). When leakage reached equilibrium, Triton X-100 was added (final concentration 0.1% w/v) to induce 100% release. Percent release was computed as shown in Equation 1.

\[
\text{% release} = \frac{(F_F - F_0)}{(F_{100} - F_0)} \times 100
\]

( Eq. 1)

where \( F_F \), \( F_{100} \) and \( F_0 \) were, respectively, the fluorescence intensity values observed after addition of HlyA, after addition of Triton X-100, and before any addition.

Lateral Pressure Measurements—Lateral pressure experiments were carried out with a MicroTrough S system from Kibron (Helsinki, Finland) at 25 °C and under constant stirring. The aqueous phase consisted of 1.0 ml of 20 mM Tris-HCl, 150 mM NaCl, pH 7.0. The lipid, dissolved in chloroform:methanol (2:1), was gently spread over the surface until the desired initial surface pressure was attained. The protein was injected with a micropipette through a hole connected to the subphase. The increment in surface pressure versus time was recorded until a stable signal was obtained. The figures show one of two closely similar independent measurements.

RESULTS

HlyA Adsorption at the Air-Water Interface—HlyA tendency to self-aggregate and its affinity for lipid bilayers strongly suggest that the toxin is a surface-active molecule (5, 16, 30). This is confirmed by its behavior in the Langmuir balance. HlyA injection in the aqueous phase leads to a rapid, dose-dependent increase in the lateral pressure at the air-water interface, which is interpreted as protein adsorption at the interface. Adsorption at an interface is the characteristic property of surface-active or interface-active substances (31). The time course of interface adsorption of HlyA can be seen in Fig. 1. The phenomenon is concentration-dependent, with both the rate of increase in surface pressure (dπ/dt) and the maximum increase of surface pressure (Δπ) increasing with HlyA concentration. Note that when referring to ”maximum increase of surface pressure” we only mean an operational value, i.e. the value at which the surface free energy does not further change by more than 1 mN/m in 5 min, after start the experiment. Fig. 2 summarizes the change in Δπ as a function of HlyA concentration. The increase in surface...
pressure appears to reach a plateau value of ~25 mN/m at ~50 nM HlyA (see summary of data in Table 1). Identical results are obtained in the presence and absence of Ca\(^{2+}\) showing that this cation, which is essential for the membrane lytic activity of HlyA (8), does not modify the surface-active properties of the protein at an air-water interface.

HlyA Insertion in a Lipid Monolayer at the Air-Water Interface—HlyA is known to insert irreversibly into fluid lipid bilayers in the presence of Ca\(^{2+}\) (20). HlyA is also able to insert into lipid monolayers extended at an air-water interface, at an initial surface pressure \(\pi_i \geq 25\) mN/m, as seen in Fig. 3. The experiment shown in Fig. 3 was performed with a monolayer containing egg phosphatidylcholine, egg phosphatidylethanolamine, and cholesterol at a 2:1:1 mol ratio, extended at the air-water interface of a Langmuir balance at an initial surface pressure \(\pi_i = 25\) mN/m. The lipid composition was chosen because it allows a high lytic activity when the toxin is applied to liposome preparations (16). The protein (96 nM) is injected into the subphase, in the presence or absence of Ca\(^{2+}\) at concentrations between 10 and 150 mM, or different initial surface pressures, between 25 and 35 mN/m (not shown).

The total change in lateral pressure \(\Delta \pi\) decreases linearly with \(\pi_i\), both in the presence and absence of Ca\(^{2+}\) (Fig. 4), but changes are consistently larger in the presence of Ca\(^{2+}\), with a difference in \(\Delta \pi\) of ~2 mN/m. Essentially similar results are obtained when HlyA is allowed to insert into pure egg PC, instead of the PC:PE:Ch monolayer (Fig. 4, inset). Above a certain \(\pi_i\) value insertion is no longer observed. Extrapolation of the \(\Delta \pi\) versus \(\pi_i\) lines to \(\Delta \pi = 0\) provides the so-called critical lateral pressure \(\pi_c\), or \(\pi_i\), above which no protein insertion occurs. \(\pi_c\) values for wild type HlyA are 34.6 and 37.3 mN/m, respectively, in the absence and presence of Ca\(^{2+}\) (see summary of data in Table 1). These values are above the usually accepted average value of ~30–35 mN/m for the lateral pressure of lipid monolayers in the cell membranes (22, 24), in agreement with the known ability of HlyA to insert into those membranes.

The plus/minus Ca\(^{2+}\) difference in \(\Delta \pi\) can be measured at varying Ca\(^{2+}\) concentrations. A Ca\(^{2+}\) titration curve is shown in Fig. 5, together with the Ca\(^{2+}\) dependence of HlyA-induced vesicle leakage (leakage data taken from Ref. 9). It is interesting that a plateau for \(\Delta \pi\) is reached at ~100 \(\mu\)M Ca\(^{2+}\), because this is precisely the Ca\(^{2+}\) concentration required for optimum lytic activity of HlyA (8).

Monolayer Insertion of HlyA Variants—A number of HlyA mutants and variants, whose activity on red blood cells and liposomes has been tested in our laboratory, were used in monolayer insertion experiments. The HlyA precursor pro-\(\alpha\)-hemolysin (pro-HlyA) differs from the mature protein only in that it lacks the two fatty acyl residues in Lys-564 and -690. It does not cause hemolysis or lipidosome efflux of aqueous contents (27). Its behavior in the Langmuir balance is very different from that of the mature protein (Fig. 6); maximum \(\Delta \pi\) at the air-water interface is lower, by about 5 mN/m (Fig. 6A), and in the case of pro-HlyA, Ca\(^{2+}\) has the effect of decreasing \(\Delta \pi\) by ~2 mN/m. However, the

![FIGURE 3. Time course of insertion of HlyA into a lipid monolayer at an initial lateral pressure \(\pi_i = 25\) mN/m. The experiment was performed in the presence (continuous line) or in the absence (dotted line) of calcium (10 mM Ca\(^{2+}\)). Lipid composition was egg phosphatidylcholine:egg phosphatidylethanolamine:cholesterol (2:1:1 mol ratio). Initial protein concentration in the subphase was 96 nM.](image-url)

![FIGURE 4. Maximum increase in lateral pressure after HlyA insertion in a lipid monolayer as a function of initial lateral pressure. Measurements were performed in the presence (closed circle) or absence (open circle) of calcium, as shown in Fig. 3. Experimental details are also as in Fig. 3. Inset, a comparison of the results obtained with PC:PE:Ch (closed circle) and with pure PC monolayers (open circle), in the presence of Ca\(^{2+}\).](image-url)

![TABLE 1. A comparison of the adsorption and insertion steady state data for native and mutant or modified HlyA. \(\Delta \pi\) and \(\pi_c\) are given in mN/m.](image-url)

| Protein                  | \(\Delta \pi^{a}\) | Slope\(^{b}\) | \(\pi_c^{b}\) | \(\Delta \pi^{c}\) | Slope\(^{c}\) | \(\pi_c^{c}\) |
|--------------------------|---------------------|--------------|--------------|---------------------|--------------|--------------|
| HlyA (wild type)         | 25.6                | -0.664       | 34.6         | 24.0                | -0.680       | 37.3         |
| Pro-HlyA                 | 20.9                | -0.511       | 44.9         | 29.0                | -0.517       | 45.6         |
| HlyA H859N               | 29.3                | -0.511       | 44.9         | 29.0                | -0.517       | 45.6         |
| HlyA H859D/D863G         | 26.2                | -0.79        | 30.8         | 25.3                | -0.893\(^{d}\) | 35.8\(^{d}\) |

\(^{a}\) Protein adsorption to the air-water interface. Data are from experiments similar to those shown in Fig. 1.

\(^{b}\) Insertion into a PC:PE:Ch monolayer. Data are from the experiments in Figs. 4 and 6 – 8. Insertion data for pro-HlyA are not included because insertion was very poor, and the data could not be properly fitted to a straight line (see Fig. 6).

\(^{c}\) 50 mM Ca\(^{2+}\). See text for details.
main difference between the precursor and the mature protein is that
the former is virtually unable to insert into the lipid monolayer, either
with or without Ca$^{2+}$ (Fig. 6B).

An HlyA mutant has been constructed (9) in which His-859 has been
replaced by Asn, using site-directed mutagenesis. The resulting mutant
HlyAH859N is totally inactive, both on red blood cells and on lipop-
osomes, presumably because of its high tendency to aggregate. A $\Delta \pi$
versus $\pi$, plot (Fig. 7) shows again important differences with the wild
type (Fig. 4). $\pi$, is higher, as is $\Delta \pi$ at comparable surface pressures,
suggesting an increased capacity of the mutant over the wild type to
insert into membranes, and Ca$^{2+}$ has no effect (Table 1). It is interesting
that this mutant, with a very high capacity to become inserted into cell
membranes, judging from its high $\pi$, is totally inactive because of its
high self-aggregation properties (i.e. low free-monomer concentration).

An additional mutant contains two substitutions, namely Asp instead
of His-859 and Gly instead of Asp-863 (HlyA H859D/D863G). This
mutant differs from the wild type in that it requires Ca$^{2+}$ concentrations
2 orders of magnitude higher (≈20 mM) to achieve a similar degree of
lytic activity (10). The $\Delta \pi$ versus $\pi$, plot of this mutant is very similar
to that of the wild type (Fig. 8 and Table 1), except that a higher Ca$^{2+}$
concentration is required. Fig. 8A shows that, at the air-water interface,
the behavior of the protein is similar in the presence of either 10 or 50
mM Ca$^{2+}$. Larger $\Delta \pi$ values are observed in the absence of Ca$^{2+}$, par-
ticularly at low protein concentrations. The inset in Fig. 8B dem-
strates that the insertion of H859D/D863G into a lipid monolayer in the
presence of 50 mM Ca$^{2+}$ is similar to that of the wild type in the presence
of 10 mM Ca$^{2+}$. Furthermore, Fig. 8B shows that monolayer insertion
of the double mutant in the absence of Ca$^{2+}$ is clearly lower than that of
the WT under the same conditions (Figs. 8B and 4, compare open sym-
bols). A Ca$^{2+}$ titration experiment is depicted in Fig. 9, in which the
plus/minus Ca$^{2+}$ difference in $\Delta \pi$ is plotted as a function of Ca$^{2+}$ con-
centration in the subphase. A plateau is observed above 20 mM Ca$^{2+}$,
and again the Ca$^{2+}$ dependence of $\Delta \pi$ happens to parallel precisely the
Ca$^{2+}$ dependence of the lytic activity of this mutant (10).

Insertion Kinetics—The time courses ( $\pi$ versus time plots) of HlyA
adsorption onto the air-water interface and of HlyA insertion into lipid
monolayers can be fitted in most cases to an exponential equation of the
form $\pi_t = \pi_i + (\pi_\infty - \pi_i) (1 - e^{-kt})$, where $\pi_i$, $\pi_\infty$, and $\pi_t$ correspond,
respectively, to the maximum surface pressures in the steady state after
protein addition, at time $t$ after protein addition, and before protein
addition. After an apparent lag time ("induction time" (32, 33)), a steep
increase is observed, whose slope is interpreted as the rate constant $k$
of protein adsorption or insertion (33, 34). A number of such rate con-
stants are collected in Table 2. In turn, the lag time indicates a coopera-
tive facilitation of the toxin penetration process as the initial insertion
proceeds, which is shortened with the increase of toxin concentration in
the subphase.

Adsorption data are collected at $\pi_i = 0$ mN/m, i.e. in the absence of
lipids. The rates are similar for WT HlyA and pro-HlyA, suggesting that
the fatty acids present in the WT, but not in its precursor, do not modify
the surface-active properties of the protein. Adsorption of H859N is
slower, by about one-half, and this would be expected from a protein
that has a higher tendency to aggregate than the WT (9). The double
mutant H859D/D863G also adsorbs slowly, but in this case no data are
available on its aggregation properties. Adsorption rates are hardly, if at
all, modified in the presence of Ca$^{2+}$. Bakás et al. (20) has shown that the
reversible adsorption step of toxin insertion into membranes was
Ca$^{2+}$-insensitive.
The rates of insertion into PC:PE:Ch monolayers at $\pi$, of either 25 or 30 mN/m have also been computed (Table 2). For WT HlyA the insertion is slower than adsorption, as expected from the different complexity of the phenomena, and the rate decreases with increasing initial pressure, also as expected. Insertion is slower in the presence of Ca$^{2+}$, which can be due to the “compacting” effect of this cation on the conformation of HlyA.

The $\pi$ versus time curves of pro-HlyA insertion could not be fitted to the exponential equation; $r^2$ correlation coefficients <0.9 were observed; thus $k$ values are not shown in Table 2. This is probably related to the very low ability of pro-HlyA to insert into lipid monolayers (Fig. 6). Mutant H859N inserts into lipids both in the absence and in the presence of Ca$^{2+}$. In the former case insertion is slower than absorption, as observed for the WT. However, and opposite to what was found with the WT, H859N insertion is faster, by 4-fold, in the presence of Ca$^{2+}$ than in its absence. This is remarkable in view of the complete absence of lytic activity of this mutant, both in the absence and in the presence of Ca$^{2+}$ (9). Finally, for the double mutant H859D/D863G, the $\pi$ versus time plots could not be properly fitted to the equation for experiments in the absence of Ca$^{2+}$, but as expected from its lytic behavior (10), the rate of insertion in the presence of 50 mM Ca$^{2+}$ was very similar to that of the WT in the presence of 10 mM Ca$^{2+}$.

**DISCUSSION**

**Amphipathic Proteins at the Air-Water Interface**—Figs. 1 and 2 show the adsorption of HlyA at an air-water interface, thus demonstrating the surface-active nature of this protein. To interpret these results adequately, they were compared with the corresponding data obtained with other amphipathic peptides or proteins under similar conditions (Table 3). The maximum change in surface pressure at saturating protein/peptide concentrations varies from 6 to 26 mN/m for the 8 cases under consideration, without any discernible pattern. The molecular masses of the peptides/proteins in Table 3 vary also widely, between 3 and 177 kDa. However, when the “corrected $\Delta \pi$” or $\Delta \pi$ per residue is computed (Table 3), its values are seen to decrease more or less uniformly with increasing molecular weights. This is probably an indication that not all the amino acid residues are actually adsorbing at the interface, except for the smaller peptides. In the larger proteins, such as HlyA or adenylate cyclase toxin, it is known that purely hydrophilic domains exist

**FIGURE 7.** Maximum increase in lateral pressure after insertion of the inactive mutant HlyA H859N in a lipid monolayer, as a function of initial lateral pressure. Measurements were performed in the presence ($\bullet$) or absence (○) of calcium, and experimental conditions are as shown in Fig. 3.

**FIGURE 8.** A, maximum increase in lateral pressure caused by the H859D/D863G HlyA double mutant adsorbed at an air-water interface. The increase in lateral pressure ($\Delta \pi$) is plotted as a function of initial protein concentration in the subphase. $\square$, no added Ca$^{2+}$; $\blacklozenge$, 10 mM Ca$^{2+}$; $\blacksquare$, 50 mM Ca$^{2+}$. Other conditions are as in Fig. 1. B, maximum increase in lateral pressure after insertion of H859D/D863G in a lipid monolayer and as a function of initial lateral pressure. Measurements were performed in the presence ($\bullet$) or absence (○) of 50 mM calcium, and other conditions are as shown in Fig. 3. Inset, a comparison of the results of insertion of WT HlyA in the presence of 10 mM Ca$^{2+}$ ($\blacklozenge$) and the insertion of H859D/D863G in the presence of 50 mM Ca$^{2+}$ ($\bullet$).

**FIGURE 9.** Calcium dependence of HlyA H859D/D863G insertion into lipid monolayers (○) and of HlyA H859D/D863G-induced leakage of lipid vesicles ($\bullet$). Insertion data are derived from experiments as shown in Fig. 3, with an initial lateral pressure of 25.5 mN/m, and varying CaCl$_2$ concentrations in the subphase. Leakage data are redrawn from Fig. 18 in Cortajarena et al. (10).
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| Table 2 | Rate constants (k) of HlyA (wild type and variants) insertion into lipid monolayers at the air-water interface |
|---------|-----------------------------------------------------------------------------------------------------------|
| Protein | Initial pressure πᵢ | Rate constants k × 10⁸ |
|         | mN/m | 0 mm Ca²⁺ | 10 mm Ca²⁺ |
| HlyA     | 0⁺  | 104 (0.99) | 108 (0.99) |
| HlyA     | 25⁺ | 58.7 (0.98) | 43.1 (0.99) |
| HlyA     | 30⁺ | 42.9 (0.90) | 32.9 (0.98) |
| Pro-HlyA | 0⁺  | 117 (0.99)  | 94.9 (0.99) |
| H859N    | 0⁺  | 48.0 (0.99) | 53.1 (0.99) |
| H859N    | 30⁺ | 19.9 (0.93) | 88.6 (0.94) |
| H859D/D863G | 0⁺  | 60.1 (0.99) | 46.5 (0.99) |
| H859D/D863G | 28⁺ | ND<sup>a</sup> | 55.1 (0.95)<sup>a</sup> |

<sup>a</sup> Adsorption was to the air-water interface.

<sup>b</sup> Insertion into a PC-PE-Ch (2:1:1 molar ratio) monolayer extended at the indicated πᵢ.

<sup>c</sup> Experiments in the presence of 50 mM Ca²⁺.

<sup>d</sup> The experimental data could not be properly fit to the equation.

**Table 3**

A comparative study of the adsorption of different peptides and proteins at the air-water interface

Δπ figures below correspond to the maximum change in surface pressure at saturating peptide/protein concentrations.

| Peptide/protein | Molecular mass | No. residues | Δπ<sub>t</sub> | Corrected Δπ | Ref. |
|-----------------|---------------|-------------|-------------|--------------|-----|
| δ-Lysine        | 3             | 26          | 26          | 1.000        | 36  |
| Melittin        | 3             | 26          | 18          | 0.692        | 37  |
| Defensin A      | 4             | 40          | 18          | 0.450        | 38  |
| Cardiotatin     | 6.8           | 68          | 6           | 0.100        | 39  |
| Equitoxin II    | 19.8          | 179         | 7           | 0.039        | 40  |
| Trichosanthin   | 27            | 247         | 10          | 0.041        | 33  |
| Apolipoprotein  | 54            | 345         | 14.7        | 0.042        | 41  |
| HlyA            | 107           | 1024        | 24          | 0.023        | Figs. 1 and 2<sup>a</sup> |
| Adenylate cyclase | 177          | 1706        | 19          | 0.011        |     |

<sup>a</sup> A. L. Cortajarena, unpublished data.

monolayer technique has been extensively used in the study of membrane protein insertion (for reviews see Refs. 22–24). In the case of HlyA, this method may be particularly adequate to cause membrane lysis, because we have proposed that HlyA becomes inserted only in the outer monolayer (at least in phospholipid vesicles).

The above results, see summary in Tables 1 (steady state) and 2 (kinetic data), indicate that WT HlyA becomes inserted with very similar parameters both in the presence and absence of Ca²⁺, although it is known that only in the former case does lysis occur. Quantitatively similar is the situation with the mutant H859D/D863G, although in this case insertion in the absence of Ca²⁺ cannot be fitted to a monoexponential curve. The totally nonlytic mutant H859N is nevertheless easily inserted in the presence and in the absence of Ca²⁺. At the other end of the insertion spectrum, the also nonlytic pro-HlyA hardly achieves monolayer insertion (Fig. 6). The ensemble of the data is compatible with the hypothesis that insertion is an essential step for lysis, but the data also demonstrate unequivocally that insertion does not necessarily correlate with lytic capacity. The monolayer studies clearly reveal that insertion and lysis are two conceptually and experimentally different concepts.

**Calcium Dependence of Protein Effects**—The data in Figs. 5 and 9 underline the parallelism of the Ca²⁺ dependence of HlyA insertion into lipid monolayers and induction of lytic effects in membranes. Even when the Ca²⁺ requirements for lysis in a mutant (HlyA H859D/D863G) exceed by 2 orders of magnitude those of the wild type, exactly the same increased requirements are observed for monolayer insertion. Moreover, the plots in Figs. 6 and 7 and the data in Table 2 clearly indicate that inactive forms of the protein, which are known not to bind Ca²⁺ (5, 9), also do not show a Ca²⁺-dependent interaction with the monolayer. Thus, monolayer measurements reveal a particular mode of Ca²⁺-dependent protein insertion, presumably as a consequence of a Ca²⁺-dependent conformational change (35), that when occurring in a lipid bilayer will lead to vesicle leakage or cell lysis. This is coherent with the following observations: (a) vesicle leakage requires Ca²⁺ binding to HlyA prior to interaction with membranes (19), and (b) Ca²⁺ binding is accompanied by characteristic changes in intrinsic fluorescence (i.e. characteristic conformational changes) of wild type or mutant HlyA when the protein is able to induce membrane lysis (8, 10). Fluorescence-detected conformational changes, insertion into lipid monolayers, and vesicle leakage vary in parallel with Ca²⁺ concentration. These data are in agreement with the observations of Schindel et al. (11) and Hyland et al. (12) on membrane interaction of HlyA. It should be added here that Ca²⁺ effects on insertion, although being clear and significant, are only quantitative in nature, i.e. Ca²⁺ does not allow the insertion of a protein that would otherwise not penetrate the monolayer. HlyA does penetrate the lipid monolayer in the absence of Ca²⁺ (Table 2); however, only in the presence of the cation does the extra increase in Δπ occur that leads to an irreversible insertion in bilayers (20).

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