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Penetration of Phospholipid Monolayers by Cardiotoxins†

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ABSTRACT: The monomolecular film technique was used to compare the specific interactions of four cardiotoxins from *Naja mossambica mossambica* with different phospholipids. We were able to demonstrate the interaction of cardiotoxins (10^{-7} M) with both neutral and negatively charged phospholipids up to very high surface pressures (45 dyn/cm). In the presence of a phospholipid monolayer, the surface activity of cardiotoxins became much greater than that observed at the air–water interface. Neurotoxins of the same venom do not penetrate a phospholipid film, even at low surface pressure (15 dyn/cm). The apparent molecular area of cardiotoxin III during its insertion into a negatively charged phospholipid film was quantitatively defined. As a function of surface pressure of the membrane around 25 dyn/cm, cardiotoxins may exist in two different configurations, "flat" (1400 Å^2) or "edgewise" (420 Å^2). This result could account for the lytic activity of this type of toxin.

Certain snake venoms, principally those of cobras (*Elapidae*), contain toxic proteins lacking enzymatic activity. Among these toxins, polycationic molecules called cardiotoxins (Sarkar, 1947), cytotoxins (Braganca et al., 1967; Patel et al., 1969), cobramines (Larsen & Wolff, 1968), or direct lytic factors (Condrea et al., 1964; Aloof-Hirsch et al., 1968) constitute a family of homologous proteins, generally composed of 60 amino acid residues (about 7000 daltons) and reticulated by four disulfide bridges.

Although interactions other than protein–lipid have been suggested (Vogt et al., 1970; Lin et al., 1975–1977), protein–lipid interactions are apparently involved in the fixation of cardiotoxins to the cell membrane since purified phospholipids inhibit their action with intact cells and reverse the inactivation of Na^+/K^+ dependent ATPase (Patel et al., 1969; Zaheer et al., 1975). Furthermore, studies on the fixation of tritiated cardiotoxin derivatives on axon membranes suggest a direct association of the toxin with lipid-type receptor structure since the number of fixation sites was found to be higher than the total number of membrane proteins (Vincent et al., 1976).

The direct study of cardiotoxin–lipid interactions thus seems to be a useful experimental approach toward the understanding of the biological effects of these molecules at the membrane level, especially concerning cellular lysis (Yang, 1974; Condrea, 1974). Studies of the variation of the intrinsic fluorescence of Trp_{11} of certain of these toxins led to the demonstration of their reversible fixation to bilamellar phospholipid vesicles (liposomes), which was inhibited by an ionic strength or pH effect (Dufourcq & Faucon, 1978; Vincent et al., 1978). The lipid–toxin complex with a stoichiometry of 7:1 lipid molecules/toxin molecule can apparently be formed only with phopholipids which are negatively charged at neutral pH ($K_d < 10^{-6}$ M).

The present report describes the use of the monomolecular film technique for the analysis of the specificity of interactions between the cardiotoxins of *Naja mossambica mossambica* and phospholipids. The apparent molecular area of these toxins during their insertion into a lipid film was quantitatively defined. A comparative study was performed simultaneously with neurotoxins from the same snake.

Materials and Methods

Toxins. The four cardiotoxins contained in the venom of *Naja mossambica mossambica* were purified in our laboratory. The fraction containing cardiotoxin activity obtained by filtration of the venom through Sephadex G-50 in 0.1 M ammonium acetate, pH 8.50, was further purified on Amberlite CG-50 in 0.45 M ammonium acetate, pH 7.30. The four cardiotoxins were progressively eluted with increasing concentrations of the salt, CTX I at 0.45 M, CTX II and CTX III at 0.6 M, and CTX IV at 0.8 M. On the basis of amino acid analyses, these proteins are identical with cardiotoxins V''_1, V''_2, V''_4, and V''_3 obtained from the same venom by Louw (1974a), but with a different method. The sequence of these cardiotoxins is known (Louw, 1974b,c).

The cardiotoxins thus purified were generally contaminated (0.05–0.45% w/w) by a phospholipase A_2 type activity, present in large quantities in the venom and acting synergistically with the cardiotoxins (Condrea, 1974; Louw & Visser, 1978). A contamination level lower than 0.0003% has been obtained by chromatography of cardiotoxin samples on antiphospholipase A_2 γ-globulins–Sepharose CL-4B (Delori & Tessier, 1980). Only this last cardiotoxin preparation was used all through this study, and we never observed phospholipase activity.

CTX III (15 mg) was iodinated enzymatically with lactoperoxidase (Thorell & Johansson, 1971). A homogeneous fraction of diiodo-CTX III was obtained after chromatography on Amberlite CG-50 (specific radioactivity 14.8 mCi/mol).

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An Edman degradation of the first 12 amino acid residues unambiguously showed that only Tyr_{11} had incorporated the two atoms of iodine. The remaining two Tyr residues were essentially unmodified (unpublished experiments). Diiodo-CTX III is as toxic in mice as the native toxin (LD_{50} = 2.1 μg/g intravenous).

CTX III was reduced and methylated (CTX III RM) with the method described by Rochat et al. (1970). The neurotoxins contained in the venom (NTX I and NTX III) have already been purified in our laboratory (Rochat et al., 1974), and their sequences have been determined (Grêgoire & Rochat, 1977). Mellitin (3400 daltons) from bee venom (*Apis mellifera*) was a generous gift of Dr. Haberman.

Phospholipids. Short-chain phospholipids [dilaurylphosphatidylcholine (DLPC), dilaurylphosphatidylserine (DLPS), dilaurylphosphatidylethanolamine (DLPE), and dilaurylphosphatidylglycerol (DLPG)] were kindly furnished by Dr. Slotboom (Utrecht). Natural phospholipids were obtained from the Paul Pascal Research Center (Talence, France): chicken egg yolk phosphatidylcholine (PC) (Singleton et al., 1965) and phosphatidylserine (PS) from bovine brain (Rouser et al., 1963). Phospholipids labeled with ^{32}P were extracted from a culture of *Escherichia coli* grown in the presence of radiophosphate.

The phospholipid or lipid mixture in question was first dissolved in chloroform at a concentration of 2 mg/mL. It was then gently deposited at the air–water interface with a microsyringe in order to obtain a lipid monolayer.

The Aqueous Phase. Water which was double distilled over potassium permanganate was used to prepare all solutions of toxins (2 mg/mL) and buffers. The following buffer was most often utilized: 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), 140 mM NaCl, 0.5 mM KCl, 0.8 mM $MgCl_2$, and 2.5 mM $CaCl_2$; pH was adjusted to 7.5 with 5 M tris(hydroxymethyl)aminomethane (Tris). A low ionic strength buffer with 20 mM Tris–acetate, pH 7.5, was also utilized.

Before each experiment, contaminating surface active impurities were removed by simultaneous sweeping and suction of the interface. The system was designed so that the agitation of the aqueous phase with a magnetic stirring bar did not disturb the stability of the surface film.

Measurement of Surface Pressures and Surface Increases. Surface pressure (π) was measured with a platinum plate tensiometer according to the Wilhelmy method. Two types of Teflon troughs were used in a temperature-controlled (25 °C) chamber. One was cylindrical (6.3-cm diameter × 1.4 cm) for constant surface experiments and enabled surface pressure variations ($\Delta\pi$) to be determined. The second, three-compartment trough (Lairon et al., 1980) was used to measure surface changes (ΔS) with a surface barostat in constant pressure experiments (Verger & De Haas, 1973). The troughs were thoroughly washed with ethanol after each experiment since proteins were adsorbed on the Teflon walls. The platinum plate was washed with sulfochromic acid. All components were abundantly rinsed with double distilled water. When necessary, the film was directly recovered in scintillation vials by suction with a glass capillary (Rietsch et al., 1977), and radioactivity was determined (Packard Model 3002 Auto-γ). Surface radioactivity was quantitatively determined with an ionization chamber counter (Merlin Gêrin Ililog, Probe LMT 13 A P 7), with the probe place 4 mm above the air-water interface.

Results

Kinetics of Cardiotoxin Adsorption to the Air–Water Interface. Adsorption to the interface is reflected by the increase in surface pressure with time (Figure 1). The time required to obtain a maximum surface pressure value differed as a function of the toxin. CTX III, previously reduced and methylated, was adsorbed more rapidly and more strongly than the native toxins.

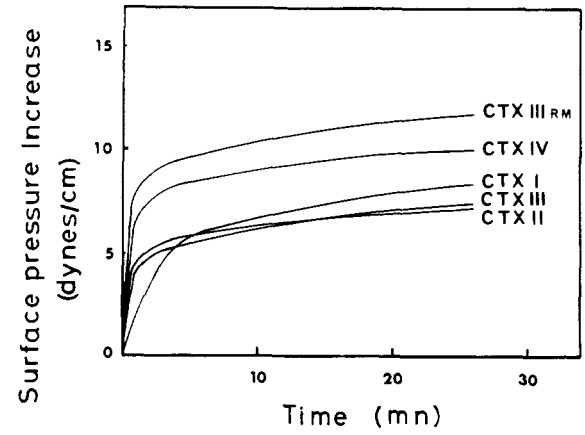

FIGURE 1: Adsorption kinetics of different cardiotoxins to an air-water interface. [CTX] = 10^{-6} M.

Cardiotoxin Penetration of a Lipid Monolayer. We first verified that, unlike detergents, the cardiotoxins did not solubilize the lipid films. CTX III at a concentration of 10^{-5} M was injected under a film of a total ^{32}P-labeled lipid extract of *E. coli* (82% PE, 15% PG) and spread at an initial pressure (π_i) of 30 dyn/cm. After 25 min, surface radioactivity was found to be unchanged whereas mellitin (10^{-5} M) led to an 8% decrease within 5 min.

The penetration of cardiotoxin molecules into a lipid film of DLPS was accompanied by a notable increase of surface pressure (Figure 2). This increase was greater with lower initial pressure of the film. The observed kinetics were nonlinear, reaching a plateau within several minutes (Figure 2a). This is in agreement with existing data on the penetration of proteins in the monolayer films (Macritichie, 1978). At low ionic strength and in the absence of calcium, however, penetration kinetics present a transitory phase of overpressure whose amplitude decreases and whose duration increases when the toxin concentration in the aqueous phase decreases. Final equilibrium pressure was nonetheless observed to be identical in all cases (Figure 2b). This phenomenon was observed with all the cardiotoxins employed, except in the presence of neutral phospholipids.

Critical Pressures for Cardiotoxin Penetration. The results in Figure 2a may be otherwise presented: the maximum value of surface pressure increase can be shown as a function of the initial film pressure at which the toxin was injected into the aqueous phase. A critical pressure for penetration may thus be defined; it corresponds to the extrapolated value of initial pressure beyond which there is no increase in surface pressure. This was performed for the different cardiotoxins as well as for mellitin (Figure 3a), enabling the penetration powers of these toxins in DLPS to be determined: CTX III RM $\ll$ CTX I = CTX II = CTX III $\ll$ mellitin < CTX IV.

The behavior of cardiotoxins was different as a function of the phospholipid forming the film. In the case of CTX III, it was found to interact with all the phospholipids tested, but the strongest interactions were observed with negatively charged molecules (Figure 3b). The very high critical pressure of 45 dyn/cm was obtained for DLPG, which is nevertheless lower than the collapse pressure of the film. In the case of natural PS with long and unsaturated fatty acyl chains, critical

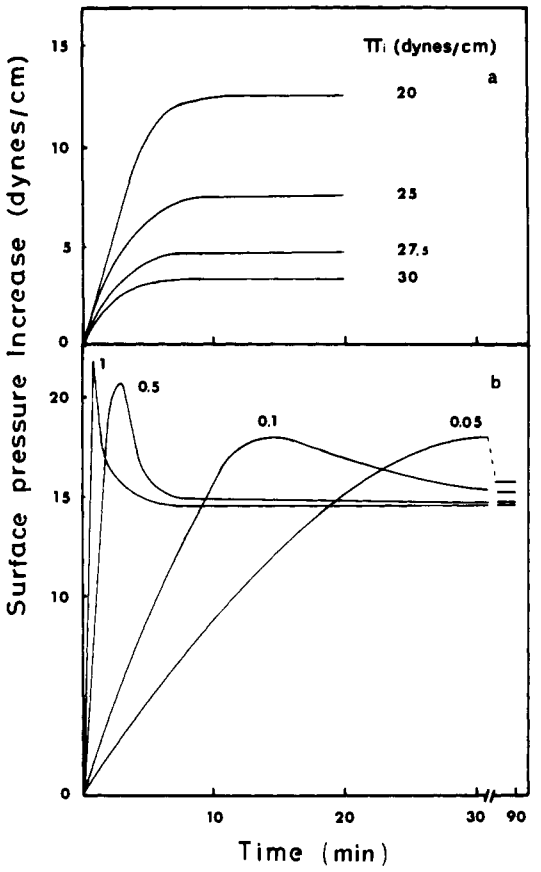

FIGURE 2: Kinetics of surface pressure increase, related to cardiotoxin penetration of a phospholipid film. (a) Hepes buffer containing 2.5 mM Ca^{2+}; [CTX III] = 10^{-7} M; DLPS film at variable initial surface pressure (π_i). (b) Tris buffer without Ca^{2+}; [CTX III] = 1, 0.5, 0.1, and 0.05 × 10^{-6} M; DLPS film at an initial pressure of 25 dyn/cm.

pressure was higher than that obtained for DLPS. Finally, if the negative charge density of a PS film was decreased by dilution with a neutral phospholipid, such as PC, the critical pressure also decreased.

The penetration powers of CTX III and mellitin in variously charged films were compared (Figure 3c). At the same concentration, the penetration power of mellitin was observed to be greater than that of CTX III, regardless of the nature of the lipid film. The penetration of CTX III, however, was more dependent on the charge of the film than was that of mellitin.

When neurotoxins I and III were utilized at identical concentrations (10^{-7} M), no increase of surface pressure could be observed with films of PC or PS, even at an initial surface pressure of 15 dyn/cm. Under conditions which were particularly favorable for an interaction (calcium-free aqueous phase and low ionic strength, 20 mM Tris–acetate, pH 7.5), only NTX III weakly penetrated ($\Delta\pi$ = 5 dyn/cm) a PS film spread at an initial pressure of 15 dyn/cm.

Adsorption Isotherm of CTX III. The Gibbs equation $a = -(1/RT)/(d\gamma/d \ln c)$ was applied to the penetration of CTX III into a DLPS film. The apparent molecular area can be directly determined from the value of a, representing the interfacial excess of CTX III. Thus, the apparent molecular area of one molecule of CTX III in a film of DLPS was found to be 320 Å^2.

Direct Determination of the Apparent Molecular Area of Diiodo-CTX III in a DLPG Film. The number of toxin molecules associated with the film was determined by the use of uniformly ^{125}I-labeled diiodo-CTX III. Combined with the surface increase at constant pressure, we were thus able to

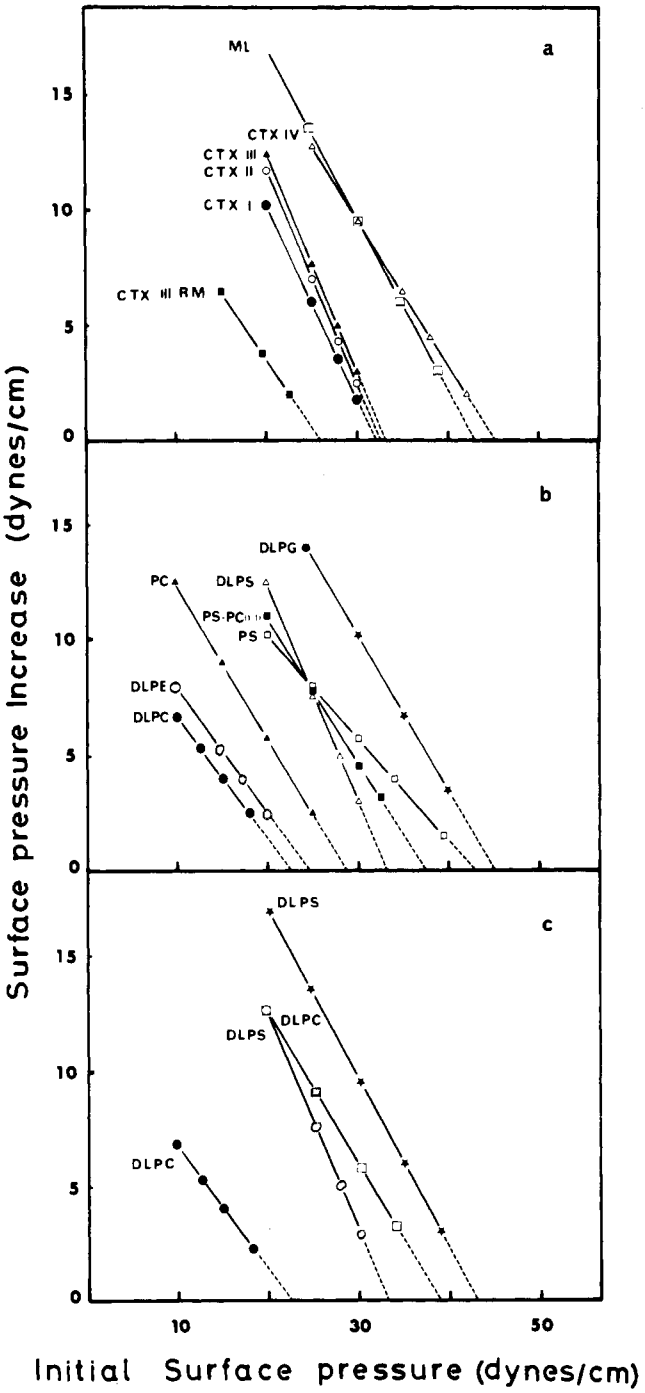

FIGURE 3: Increase in maximum value of surface pressure as a function of initial film pressure. (a) Different cardiotoxins and mellitin in DLPS film: CTX I (●), CTX II (○), CTX III (▲), CTX IV (△), CTX III RM (■), and mellitin (□). [Toxins] = 10^{-7} M. (b) CTX III in various phospholipid films: DLPC (●), DLPE (○), PC (▲), DLPS (△), PS–PC (1:1) (■), PS (□), and DLPG (☆). [CTX III] = 10^{-7} M. (c) CTX III and mellitin in neutral and negatively charged phospholipid films: CTX III/DLPC (●), CTX III/DLPS (○), mellitin/DLPC (□), and mellitin/DLPS (☆). [Toxins] = 10^{-7} M.

determine the apparent molecular area of diiodo-CTX III during its interaction with a film of DLPG. This value is apparent for two possible reasons: first, because of an influence of the phospholipid molecules, in direct interaction with cardiotoxin, to the surface increase (ΔS); second, because the surface radioactivity measured represents not only those cardiotoxin molecules directly responsible for the surface increase but also an unknown amount of protein present close to the monolayer. At the end of each experiment, more than 95% of the initial quantity of toxin was found in the aqueous

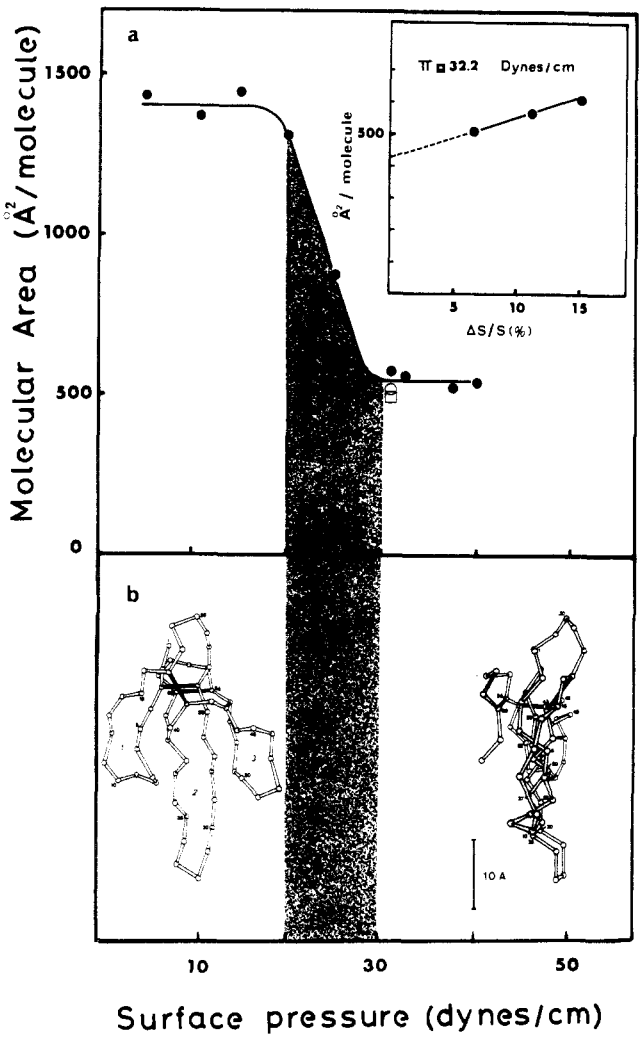

FIGURE 4: (a) Variation with surface pressure of the apparent molecular area of diiodo-CTX III (0.9×10^{-7} M) in DLPG (●), DLPS (○), and PS (□) films. (Insert) Variation of the apparent molecular area for different values of the relative surface increase ($\Delta S/S$) obtained at 0.7, 0.9, and 1.8×10^{-7} M of diiodo-CTX III. DPLG film at 32.2 dyn/cm. (b) Drawing of three-dimensional structure of snake short neurotoxin, "flat" (left) and "edgewise" (right), according to Feldmann (1976). The hatched area represents the transition region (20–30 dyn/cm) between the "flat" and "edgewise" orientations.

phase. This confirms that, as in the case of lipolytic enzymes (Verger, 1980), only a small proportion of the total quantity of protein $\simeq$3%) is associated with the lipid film. In order to reduce experimental errors of measurement of this molecular area generated by the progressive formation of a mixed lipid–cardiotoxin film, we always measured $\Delta S/S$ values lower than 15%. Under these conditions, the apparent molecular area presents two characteristic values of 1400 and 550 Å^2 for respective pressures lower than 20 dyn/cm and higher than 30 dyn/cm, with an abrupt change within this interval of surface pressures (Figure 4a). The apparent molecular area of diiodo-CTX III in DLPS and PS films at a pressure of 32.2 dyn/cm is of the same order of magnitude as that observed for a DLPG film (Figure 4a). Extrapolation to zero surface increase ($\Delta S/S = 0$) gives an apparent molecular area of 420 Å^2 for a DLPG film at a surface pressure of 32.2 dyn/cm (insert, Figure 4a).

Discussion

Surface Activity of Cardiotoxins. This activity reflects the power of cardiotoxins to adsorb and/or to penetrate an interface. It was determined by the variations of surface pressure. Surface activity at the air–water interface is low (10–12 dyn/cm) compared to the lytic peptide of bee venom, mellitin (Habermann, 1972), which has been described as a "protein detergent" whose film could attain an equilibrium pressure of 24.5 dyn/cm (Sessa et al., 1969). Mellitin is thus considered one of the strongest natural surface active agents at an air–water interface. The fact that denatured, i.e., reduced and methylated, CTX III had greater surface activity than native toxins may be explained by an increased flexibility of polypeptide chains resulting from the loss of three-dimensional structure. Its hemolytic capacity is nil (M. Tessier et al., unpublished results), which shows that surface activity at the *air–water* interface is not directly related to hemolytic power.

In the presence of a phospholipid monolayer, the surface activity of cardiotoxins becomes much greater than that observed at the air–water interface (Figure 2). Mellitin, on the other hand, exhibits comparable surface activities in the presence or absence of phospholipids at the air–water interface. These observations, demonstrated in the cases of *Naja naja oxiana* cardiotoxin (Ksenzhek et al., 1979) and mellitin (Sessa et al., 1969), suggest that, unlike mellitin, the interactions between cardiotoxins and phospholipids are more specific.

The transitory overpressure effect observed in the absence of calcium (figure 2b) may be explained by the superposition of two antagonizing actions: on the one hand, the penetration of the toxin into a film of negatively charged phospholipids leads to an increase of surface pressure; on the other hand, the supply in situ of positive charges from the cardiotoxin itself (isoelectric point greater than 11) leads to condensation of the lipid film. The latter well-known phenomenon has already been described in the case of numerous cationic substances (ions, proteins, antibiotics, polylysine, etc.) which are responsible for a decrease in the electrostatic repulsion between the anionic groups of phospholipids. Thus, in the presence of calcium, the lipid film is already in a precondensed state, and only the penetration phase is manifested by simpler kinetics (Figure 2a). Furthermore, the lower amplitude of surface pressure variation observed with cardiotoxins in the presence of calcium may be related to the capacity of these ions to dissociate the cardiotoxin–phospholipid complex (Dufourcq & Faucon, 1978; Vincent et al., 1978). These dissociating effects of calcium ions may be explained by the condensation of the phospholipid film as well as by a competition for negative charges at the phospholipid interface.

The classification of cardiotoxins with respect to their critical pressure for penetration into a DLPS film agrees with that established by Tessier et al. (M. Tessier et al., unpublished experiments), using either spectrofluorometry in a liposome system or hemolysis. CTX IV distinguishes itself from the three other cardiotoxins in that it is systematically more active.

Prior studies on cardiotoxin fixation to liposomes, determined by intrinsic tryptophan fluorescence (Dufourcq & Faucon, 1978; Vincent et al., 1978), were unable to detect the formation of complexes with neutral phospholipids. In the present case of monomolecular films, however, we were able to demonstrate the interaction of cardiotoxins with both neutral and negatively charged phospholipids. The lack of interaction with PC liposomes may be due to the existence of a surface pressure at the lipid–water interface of these structures (Fulford & Peel, 1980) which is greater than the critical pressure for cardiotoxin penetration.

The critical pressures for penetration into different types of phospholipid films were observed to be much higher in the cases of negatively charged phospholipids. This indicates the predominance of electrostatic-type interactions (Dufourcq &

Faucon, 1978). Hydrophobic-type interactions were also manifested since the utilization of natural long-chain unsaturated phospholipids reinforced affinity (Figure 3b). Furthermore, the passage of Trp_{11} of CTX II from a hydrophilic to a more hydrophobic medium is characterized by variations in quantum yield and the maximum wavelength of fluorescence emission (Dufourcq & Faucon, 1978). These experimental results agree with the idea of a true penetration, causing an increase in the surface pressure of a monomolecular film.

Comparison between Cardiotoxins and Neurotoxins. Sequence homologies exist between cardiotoxins and neurotoxins I and III from the same venom. It is thus surprising to observe that the latter do not penetrate a lipid film, even at low surface pressure (15 dyn/cm). The explanation of this basic difference may be found in the analysis of sequences and of three-dimensional structure. We may adopt the working hypothesis that the three-dimensional structure of cardiotoxins (Visser & Louw, 1978; Drake et al., 1980; Lauterwein et al., 1977, 1978) is similar to those established for short-chain neurotoxins (Low et al., 1976; Tsernoglou & Petsko, 1976). As indicated by Dufton & Hider (1977), a clear hydrophobicity difference appears between the first loop of the cardiotoxins and that of the neurotoxins. The hydrophobicity index (HI), calculated according to Segrest & Feldmann (1974), for the sequence 6–11 of CTX II and NTX III is 3.8 and 0.4, respectively. Thus, according to the interaction model proposed by Faucon et al. (1979), there is an initial step of electrostatic interaction between the basic residues located near the hydrophobic region of the first loop and the phosphate or carboxylic groups of the phospholipids. There would then be penetration of this hydrophobic loop into the bilayer. In the case of neurotoxins, however, only the first step would occur, thus explaining the absence of surface pressure changes.

Apparent Molecular Area of Cardiotoxins. We utilized two independent approaches, Gibbs equation and direct measurement (Figure 4a), to determine the experimental value. The order of magnitude of apparent molecular area of CTX III and diiodo-CTX III at high pressures (greater than 30 dyn/cm) is comparable (320 and 420 Å^2). The observed difference in molecular area found with the two methods does not seem to be significant. The chemical nature of the phospholipid does not appear to influence the apparent molecular area determined at pressures greater than 30 dyn/cm (Figure 4a).

The extrapolated value of 420 Å^2 is consistent with structural data established for short-chain neurotoxins by radiodiffractometry (Kimball et al., 1979). In this case, it may be imagined that it is primarily the first hydrophobic loop of the cardiotoxin that penetrates edgewise the phospholipid–water interface. It is difficult to see how any other mode of penetration could occur without seriously perturbing the molecular stereochemistry. At low pressures (less than 20 dyn/cm), however, the value of 1400 Å^2, slightly overestimated since it was not determined by extrapolation to $\Delta S/S = 0$, is consistent with the maximum area of the projection of the cardiotoxin molecule (Figure 4b). It is interesting to note that the value of 1400 Å^2/molecule, or 1.1 m^2/mg of protein, corresponds to the surface density of a β-leaflet polypeptide chain spread on an air–water interface (Macritchie, 1978). It should also be borne in mind that this β-leaflet structure is predominant in the secondary structure of cardiotoxin (Visser & Louw, 1978; Drake et al., 1980). Thus, as a function of surface pressure of the membrane, cardiotoxins may exist in two different configurations, "flat" or "edgewise" (Figure 4b).

Finally, one of the most unexpected results of this work was the finding that the apparent molecular area of the cardiotoxin molecule presented only two characteristic values. The transition between these two values occurred in a very narrow range of surface pressures (25 ± 5 dyn/cm). At the surface pressures existing at the level of an erythrocyte membrane, about 30 dyn/cm (Demel et al., 1975), it may be imagined that the first hydrophobic loop penetrates the membrane and the molecule is in an "edgewise" configuration. It is not impossible that the lytic activity of this type of toxin would depend on its capacity to reorient itself "flat" in relation to the plane of the membrane.

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Formation of Asymmetric Phospholipid Membranes via Spontaneous Transfer of Fluorescent Lipid Analogues between Vesicle Populations†

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ABSTRACT: A method is presented for generating artificial lipid vesicles bearing an asymmetric distribution of either of the fluorescent lipid analogues 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine or 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-phosphatidylcholine, in which the fluorescent lipid is located predominantly in either the outer or inner leaflet of the vesicle bilayer. The procedure is based on the observation that these lipid analogues undergo rapid spontaneous transfer (exchange) between vesicle populations [Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783–2789]. When an excess of nonfluorescent acceptor vesicles is mixed with small unilamellar vesicles containing 5 mol % fluorescent lipid, approximately 50% of the fluorescent lipid is transferred to the acceptor vesicles, whereas if fluorescent multilamellar vesicles are used, only approximately 10% of the analogues is available for transfer. These fractions of fluorescent lipid available for intervesicular transfer correspond closely to the amount of phospholipid residing in the outermost leaflet of the donor vesicles, suggesting that only fluorescent lipids present in the outer surface of the vesicles can spontaneously transfer between vesicle populations. Evidence demonstrating that the movement of the fluorescent lipid between vesicle populations is the result of a net transfer process rather than lipid exchange is also presented. A novel assay based on resonance energy transfer is described for determining the size of the exchangeable fluorescent lipid pool, a measure of the degree of asymmetry of these preparations. Finally, for demonstration of the usefulness of asymmetric vesicles in distinguishing various pathways of vesicle–cell association, preliminary results are presented on their interactions with Chinese hamster fibroblasts in vitro.

The asymmetric distribution of the various lipid classes in the plasma membranes of cells is emerging as a general feature of membrane structure [reviewed in Bergelson & Barsukov (1977), Rothman & Lenard (1977), Op den Kamp (1979), and Van Deenen (1981)]. In a number of studies, the choline-containing lipids have been found to be enriched on the external leaflet of the membrane lipid bilayer, while the acidic and amino-containing lipids are enriched on the inner half of the membrane. Several questions of general interest relating to the maintenance of this asymmetry remain to be explored. It is of interest, for example, to determine whether or not lipid asymmetry is conserved during fusion of two asymmetric membranes with one another. In addition, there exists the possibility that exchange of lipids into the plasma membrane of a cell from an exogenous source such as lipoproteins is an asymmetric process, resulting in the modification and/or maintenance of cell surface lipid asymmetry.

In principle, it should be possible to begin to address such questions using "asymmetric" lipid vesicles in which a particular lipid is restricted to the inner or outer leaflet of the vesicle bilayer. The distribution of that lipid following vesicle–vesicle fusion (Papahadjopoulos et al., 1979), vesicle–cell fusion (Pagano & Weinstein, 1978), or vesicle–cell exchange

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