Calcium Ion Independent Membrane Leakage Induced by Phospholipase-like Myotoxins†

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ABSTRACT: The two snake venom myotoxins ammodytin L and myotoxin II, purified respectively from *Vipera ammodytes ammodytes* and *Bothrops asper*, have phospholipase-like structures but lack an Asp-49 in the active site and are without normal phospholipase activity. The interaction of these proteins with different types of liposomes indicated that the myotoxins were able to provoke rapid and extensive release of the aqueous content of liposomes. Leakage was measured by two different methods: fluorescence dequenching of liposome-entrapped carboxyfluorescein and ESR measurement of intravesicular TEMPOcholine reduction by external ascorbate. The process was independent of Ca^{2+} and took place without any detectable phospholipid hydrolysis. Nonmyotoxic phospholipases tested under the same conditions were unable to induce liposome leakage, which could be detected only when Ca^{2+} was added to the medium and with the concomitant hydrolysis of phospholipids. The kinetics of Ca^{2+}-dependent and Ca^{2+}-independent leakage were completely different, indicating two different mechanisms of interaction with the lipid bilayer. Studies using diphenylhexatriene as a probe of lipid membrane organization indicated that the myotoxins gave rise to a profound perturbation of the arrangement of the lipid chains in the membrane interior, whereas interaction of *Naja naja* phospholipase A_2 with the membrane surface did not affect lipid organization. On the basis of these results we suggest that a new type of cytolytic reaction mechanism is responsible for the effects of phospholipase-like myotoxins in vivo.

Snake venoms are known to contain several isoforms of PLA_2s[1] and phospholipase-like proteins which differ in terms of enzymatic activity, cellular target, and toxicity (Rosenberg, 1990). The snake venom PLA_2s from different species exhibit a high degree of sequence homology (Brunie et al., 1985; Waite, 1987) and show great structural similarity with mammalian pancreatic PLA_2s as far as the active-site region is concerned (Renetseder et al., 1985; Scott et al., 1990) but differ widely in their enzymatic and pharmacological properties (Rosenberg, 1990).

Recent analyses of snake venom toxins have revealed a class of these enzymes in which the aspartate residue in position 49, formerly thought to be invariant, is changed. This class of PLA_2-like protins includes at least four components (namely, the Lys-49-PLA_2s from *Agkistrodon piscivorus piscivorus*, *Bothrops atrox*, and *Trimeresurus flavoviridis* and the myotoxin II from *Bothrops asper*) in which position 49 is occupied by a lysine residue (Maraganore et al., 1984; Yoshizumi et al., 1990; Francis et al., 1991). This type of PLA_2 was originally reported to have a very low enzymatic activity and a reversed binding order of Ca^{2+} and phospholipid substrate compared to that of Asp-49-PLA_2 (Maraganore et al., 1984; Maraganore & Heinrikson, 1986). However, the group of de Haas has later disputed these data, affirming that all the residual enzymatic activity of Lys-49-PLA_2 must be due to contamination (van den Berg et al., 1989). The structure of the Lys-49-PLA_2 from *A. piscivorus*, obtained by crystallographic analysis, is very similar to that of the active PLA_2 from *Crotalus*, except for the calcium-binding loop, where the aspartate is known to be essential for cation binding (Holland et al., 1990). As far as their toxicological activities are concerned, neither Lys-49-PLA_2 from *A. piscivorus* nor myotoxin II shows any anticoagulant, hemolytic, or neurotoxic activity, but both have a cytotoxic effect when tested on isolated muscle preparations (Dhillon et al., 1987; Diaz et al., 1991) as well as in vivo (Lomonte & Gutiérrez, 1989).

Another PLA_2-like protein with myotoxic activity, ammodytin L, has been purified from *Vipera ammodytes* (Gubensek et al., 1980) and recently its cDNA and protein sequences were determined (Pungercar et al., 1990; Krizaj et al., 1991). Ammodytin L, which shows 90% nucleotide and 74% amino acid homology to neurotoxic PLA_2 from *V. ammodytes*, has the aspartate 49 replaced by serine as well as glycine 33 and tyrosine 28 replaced by asparagine and histidine, respectively, and lacks phospholipase activity. Thus it appears that there exists a group of cytolytic toxins with a highly conserved PLA_2-like structure but without the active-site Asp-49, which operate by a mechanism independent of phospholipid hydrolysis. We here show that two of these modified phospholipases, myotoxin II and ammodytin L, provoke instantaneous leakage of the aqueous content of liposomes. In order to elucidate the cytolytic mechanism, a

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[1] Abbreviations: PLA_2, phospholipase A_2; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; CF, carboxyfluorescein; DPH, 1,6-diphenyl-1,3,5-hexatriene; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane; PC, phosphatidylcholine; PA, phosphatidic acid; EDTA, ethylenediaminetetraacetic acid.

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comparative study of myotoxin II and ammodytin L, the neurotoxic phospholipase β-bungarotoxin, and a hemolytic *Naja naja* phospholipase have been carried out. The results show different mechanisms of interaction with the liposome membranes for three types of toxins.

EXPERIMENTAL PROCEDURES

Materials

Crude, dried venom from Costa Rican *B. asper* was kindly provided by the Instituto Clodomiro Picado, Costa Rica, and the myotoxin II was purified as previously described by a two-step procedure (Gutiérrez et al., 1984; Lomonte & Gutiérrez, 1989). Ammodytin L from *V. a. ammodytes* was purified as described by Gubensek et al. (1980). β-Bungarotoxin made up by fraction β-3 of the preparation of Abe et al. (1977) was purified as described by the same authors. Carboxyfluorescein, TEMPOcholine, octadecylrhodamine B, and 1,6-diphenyl-1,3,5-hexatriene were from Molecular Probes Inc. (Eugene, OR). *N. naja* phospholipase and different phospholipids were obtained from Sigma (St. Louis, MO) and used without further purification. Some experiments were carried out using phosphatidylcholine purified from egg yolk according to Bloj and Zilversmit (1976). Some control measurements were made with liposomes composed of 1,2-di-*O*-hexadecyl-*rac*-glycero-3-phosphocholine/dicetyl phosphate (9:1). Purification and control of lipids by thin-layer chromatography were done according to Spinedi et al. (1987).

Methods

Preparation of Liposomes. Small unilamellar sonicated vesicles (SUVs) with an average diameter of 30–40 nm were made by the method of Huang (1969), using a Branson bath sonicator. Large unilamellar vesicles (LUVs) of an average diameter of 300–400 nm were made with a polycarbonate filter extrusion technique (Hope et al., 1986). Briefly, a few nanomoles of lipid dissolved in chloroform was dried under N_2 flow. Buffer was then added to the thin film of lipids, and after hydration the suspension was shaken vigorously. The multilamellar vesicles obtained in this way were extruded three times, above the transition temperature, through a 400-nm pore diameter filter (Nucleopore, Pleasanton, CA). The mean size of the liposome population was calculated using both exclusion chromatography and the fluorescence quenching method of Ralston et al. (1982). The phospholipid concentration was determined by the method of Ames (1966). Unless otherwise indicated, experiments were carried out with liposomes suspended in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA to avoid effects caused by spurious Ca^{2+}. PC/PA liposomes were prepared with a molar ratio of 9:1.

CF Leakage Experiments. Fluorescence experiments were made with an Aminco Bowman fluorescence spectrometer, equipped with a bath-operated circulation around the jacketed cuvette; the temperature of the mixture in the cuvette was routinely checked by a precision thermocouple thermometer. Liposomes, SUVs and LUVs, were made as described above, except that 50 mM CF was included to monitor liposome leakage (Wilschut et al., 1980). The liposomes were separated from nonencapsuled CF by gel filtration on a Sephadex G-75 column (20 × 1 cm). Leakage was induced by adding aliquots of toxins (5–10 μL) to a vesicle suspension (2 mL containing 60 nmol of total phosphorus) directly in the cuvette used for fluorescence determination, at 37 °C. Excitation was set at 480 nm and emission was detected at 540 nm, using a 3–73 cutoff filter (Corning) at the emission to reduce scatter contributions. The results are expressed as

$$\% \text{ CF leakage} = 100(F - F_i)/(F_f - F_i)$$

where F_i is the initial fluorescence before adding proteins, F is the fluorescence reading at different times, and F_f is the final fluorescence determined after addition of Triton X-100 (final concentration 0.1%).

Fluorescence Polarization Experiments. SUVs were made as described above, except that 0.01% (molar ratio) DPH in tetrahydrofuran was added to the lipids before drying. The final lipid concentration was 30 μM. Fluorescence polarization (P_{DPH}) measurements were carried out on a 4048 C SLM spectrofluorometer. Excitation was set at 360 nm and emission was detected at 450 nm. A 0–51 cutoff Corning filter was used at the emission. P_{DPH} was calculated by

$$P_{DPH} = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

$I_{\parallel}$ and $I_{\perp}$ being the fluorescence intensities recorded with the analyzing polarizer oriented parallel and perpendicular to the direction of the excitation beam, respectively (Shinitzky & Barenholz, 1978).

Fusion Assay. Intermixing of membrane components was estimated following the relief of self-quenching of octadecylrhodamine fluorescence (Hoekstra et al., 1984; Blumenthal et al., 1987). Two populations of SUVs were made as described above: one, containing 10 μM total lipid, was labeled with 6% (mol/mol) octadecylrhodamine, and the other, containing 50 μM total lipid, was unlabeled. Fusion was induced by adding aliquots of proteins (5–10 μL) to a vesicle suspension (1 mL of each population, 60 nM total phosphorus) directly in the cuvette used for fluorescence determination, at 37 °C. Excitation was set at 560 nm and emission was recorded between 570 and 600 nm. The fluorescence corresponding to the maximal fusion was determined as the fluorescence of a vesicle population (30 μM total phosphorus) with identical lipid composition but containing only 1.2% (mol/mol) octadecylrhodamine.

ESR Measurement of Leakage. Liposomes (SUVs, final lipid concentration 1 mM) were prepared as above with the Tris buffer containing 15 mM of the spin label TEMPOcholine, followed by dialysis against 1000 volumes of the same buffer without TEMPOcholine. The dialysis solution was changed twice, with a total dialysis time of 24 h. Samples were prepared by addition of toxins or other components to 50 μL of liposome suspension and subsequent mixing with 1 mL of ascorbate, final concentration 20 mM, at pH 7.4. The samples were measured in glass capillaries at room temperature, using a Bruker ESP300 instrument equipped with a high-sensitivity TM_{110} cavity and with the following instrument settings: 20-mW microwave power at 9.83 GHz, 1-G modulation, a scan time of 42 s, and a time constant of 20 ms. Normally four single 100-G scans were accumulated to obtain good signal-to-noise ratios. Leakage was determined by the loss of the TEMPOcholine signal due to reduction by ascorbate. Passive diffusion of TEMPOcholine and ascorbate across the liposome membranes was found to be negligible. To exclude possible effects of ascorbate on the various proteins, control samples were incubated for prolonged periods with the toxins before the addition of ascorbate.

Phospholipase Activity. Phospholipase activity was determined with an assay based on ^{14}C-labeled substrate as previously described (Rufini et al., 1990).

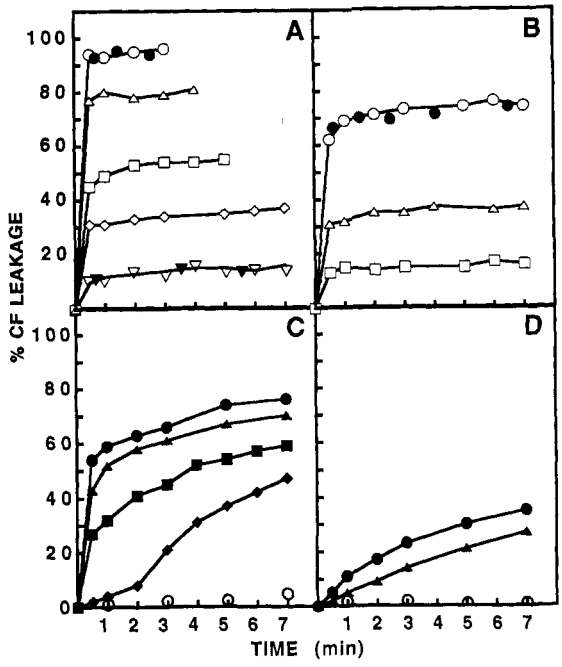

FIGURE 1: Time course of CF leakage induced by ammodytin L (A), myotoxin II (B), *Naja* PLA_2 (C), and β-bungarotoxin (D) from liposomes of an average diameter of 40 nm (final lipid concentration 60 nmol in 2 mL) of egg yolk PC/PA mixed in 20 mM Tris and 0.5 mM EDTA, pH 7.4. At time = 0, proteins were added to the SUV suspension, and the fluorescence of samples containing 1 μM (○), 500 nM (△), 250 nM (□), 125 nM (◇), or 62.5 nM (▽) of toxin was monitored as described under Experimental Procedures. The CF leakage in the absence of proteins was <1% after 10 min. Experiments were made in the absence (open symbols) or presence (closed symbols) of 1.5 mM Ca^{2+}.

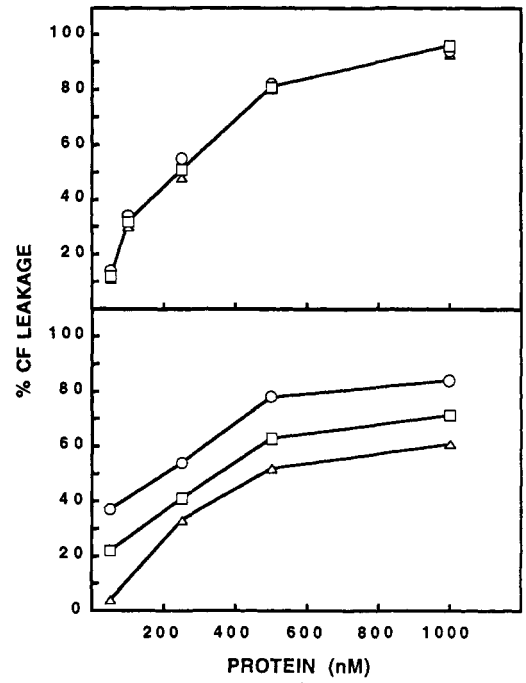

FIGURE 2: Rate of leakage of SUV aqueous content as a function of ammodytin L (top) and *Naja* PLA_2 (bottom) concentration. Experiments were done as described in Figure 1. The extent of leakage is shown after 1 min (△), 3 min (□), and 7 min (○).

RESULTS

Leakage Experiments. The cytolytic effect of myotoxins was demonstrated with an assay based on release of entrapped fluorescent probe from PC/PA liposomes. Figure 1 shows typical time courses of aqueous content leakage from SUVs induced by purified ammodytin L and myotoxin II, monitored by the relief in CF fluorescence self-quenching. In the concentration range of toxins tested, the CF release occurred within the first 15 s; with our experimental system it was impossible to follow faster events. After the initial burst the fluorescence level remained constant, indicating both that the effect was instantaneous and that the toxins were unable to redistribute after binding to the membrane since this would gradually cause lysis of the entire liposome population. The capacity of ammodytin L and myotoxin II to induce leakage was completely independent of Ca^{2+}: the presence of Ca^{2+} neither activated nor inhibited the leakage process. Under the same experimental conditions neither *Naja* phospholipase nor β-bungarotoxin was able to provoke lysis in the absence of Ca^{2+} (Figure 1). Only when 1.5 mM Ca^{2+} was added to the system could CF leakage be detected, but with very different kinetics compared to the myotoxin curves. For the *Naja* PLA_2 a rapid release of CF was seen at high protein concentrations, followed by a slow continuous increase of fluorescence; at low protein levels only the slow phase was observed. However, after prolonged incubation all concentrations of *Naja* PLA_2 lead to 90–100% release of CF (data not shown). With β-bungarotoxin the fast initial phase was never seen even at high protein concentrations, and on a molar basis this toxin was not very efficient in inducing leakage of liposomes (Figure 1).

Table I: Comparison of Different Myotoxic and Nonmyotoxic Snake Venom Activities[a]

	phospholipase activity		leakage induction		fusogenic activity	
	PC	PC/PA	Ca^{2+}	EDTA	Ca^{2+}	EDTA
β-bungarotoxin	–	+	+	–	+++	–
Naja PLA_2	++	+++	+++	–	++	–
ammodytin L	–	–	+++	+++	+++	++
myotoxin II	–	–	+++	+++	nd[b]	nd

[a] Experiments were carried out as described under Experimental Procedures. Phospholipase activity was assayed in the presence of 1.0 mM $CaCl_2$. Leakage and fusion induction was examined using PC/PA SUVs with or without 1.5 mM Ca^{2+} in a Tris buffer containing 0.5 mM EDTA. [b] nd, not determined.

When liposome leakage was expressed as a function of protein concentration, the activities of both ammodytin L and *Naja* phospholipase were seen to saturate as the concentration approached 1 μM (Figure 2). The percentage of CF leakage at different myotoxin doses did not change in time, while the *Naja* phospholipase activity was time-dependent at all concentrations tested, increasing with the incubation time. As expected, ammodytin L and myotoxin II did not show any measurable phospholipase activity, whereas the Ca^{2+}-dependent leakage induced by β-bungarotoxin and *Naja* PLA_2 was accompanied by extensive phospholipid hydrolysis (Table I). At high protein concentrations, the kinetics of free fatty acid production closely followed the curve seen in the liposome leakage experiments (Figure 3). The slow phase of the leakage process was not caused spontaneously by the accumulation of hydrolysis products but reflected the prolonged enzymatic activity, as seen by the inhibitory effect of EDTA, which blocked completely the slow phase of the leakage process when added 3 min after the active PLA_2s (results not shown). As an additional control, some experiments were carried out with liposomes composed entirely of ether-linked PC. These lipids are not substrates for PLA_2 and cannot be hydrolyzed, but the myotoxins could still induce almost complete leakage of the

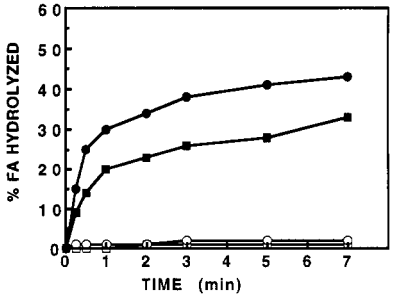

FIGURE 3: Time course of *Naja* PLA_2- and β-bungarotoxin-induced PC hydrolysis. SUVs containing egg yolk PC/PA (final lipid concentration 60 nmol in 2 mL) were incubated with 500 nM *Naja* PLA_2 (circles) or β-bungarotoxin (squares) in the same Tris buffer as in Figure 1, in the presence (closed symbols) or absence (open symbols) of 1.5 mM Ca^{2+}. Reaction was stopped by the addition of a mixture of chloroform and methanol (1:2) and products of hydrolysis were determined as described under Experimental Procedures.

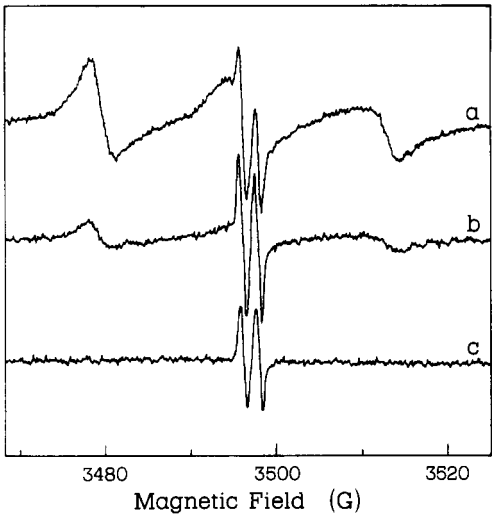

FIGURE 4: ESR spectra of TEMPOcholine-loaded egg yolk PC/PA liposomes (1 mM) in the presence of extracellular ascorbate (a). Upon addition of 400 μg/mL ammodytin L, the three-line TEMPOcholine signal partially disappeared (b). Total reduction of the spin label was seen after addition of 0.1% Triton X-100 (c). Further details are as described in Experimental Procedures.

aqueous contents from such liposomes (data not shown). To exclude the possibility of specific interaction between the toxins and the fluorescent probes, a series of analogous experiments were made using electron spin resonance to measure the leakage process. In this experimental system, membrane leakage was measured through the reduction of an intravesicular spin label by externally added ascorbate. In agreement with the fluorescence experiments, both myotoxin II and ammodytin L caused an instantaneous decrease of the spin label ESR signal in Ca^{2+}-free samples (Figure 4). The degree of reduction was proportional to the toxin concentration, and no further decrease was seen after the first minute. In contrast, *Naja* PLA_2 did not have any effect on the entrapped spin label in the absence of Ca^{2+}; reduction occurred only after addition of Ca^{2+}, and in this case the remaining spin label signal disappeared gradually during a 30-min period (results not shown).

Further studies were made to examine the influence of the structure and composition of the liposomes on the leakage process. In order to determine the effect of vesicle bilayer curvature, experiments were carried out using LUVs of an average diameter of 300–400 nm. Measurements with these liposomes gave CF leakage kinetics identical to those observed with SUVs (Figure 5). The main difference appeared to be

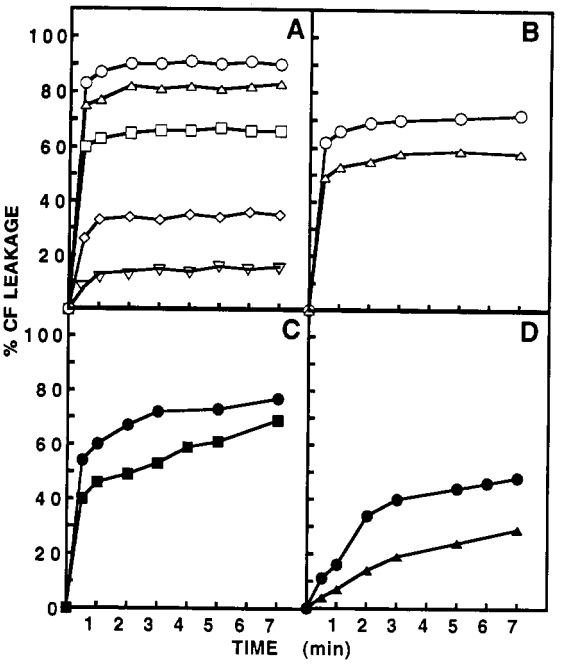

FIGURE 5: Time course of egg yolk PC/PA LUV leakage induced by ammodytin L (A), myotoxin II (B), *Naja* PLA_2 (C), and β-bungarotoxin (D) monitored by the CF-quenching method. Experiments were carried out as described in Figure 1, except that liposomes were of an average diameter of 400 nm. The symbols represent the same protein concentrations as in Figure 1.

Table II: Effect of Negatively Charged Phospholipids on Toxin-Induced Lysis[a]

	SUVs		LUVs	
	PC	PC/PA	PC	PC/PA
myotoxin II	40	96	82	91
ammodytin L	24	74	60	70
β-bungarotoxin[b]	4	34	7	42
Naja PLA_2[b]	68	60	82	73

[a] Percentage of leakage from CF-loaded liposomes after 5-min reaction time. Protein concentration 1 μM. [b] With 1.5 mM Ca^{2+} added.

in the extent of leakage provoked by a certain dose of toxins: the LUVs were much more susceptible than SUVs.

In Table II is shown the effect of the presence of charged phospholipids on the leakage activity of the toxins. In the case of *Naja* phospholipase the leakage was not affected by the presence of 10% (mol/mol) phosphatidic acid, whereas the neurotoxic PLA_2 β-bungarotoxin was strongly dependent on the presence of negative charges on the liposome surface. In the same way, when enzymatic activity was concerned, the *Naja* PLA_2 could hydrolyze both zwitterionic and anionic substrates with the same efficiency, whereas β-bungarotoxin required the presence of net negative charges in the micellar substrate (Table I). In Ca^{2+}-free medium, both myotoxins were affected by the presence of phosphatidic acid that strongly enhanced the CF release from SUVs; the same result was seen with LUVs, although the effect was less marked (Table II). For both myotoxins the leakage kinetics in pure PC vesicles were the same as in mixed PC/PA liposomes (Figure 6); the kinetics in SUVs and LUVs were identical (data not shown).

Fluorescence Polarization Experiments. In order to study the perturbative effects of PLA_2 and myotoxic proteins on the bilayer membrane, temperature variation experiments were made using P_{DPH} as a parameter to monitor overall changes in membrane anisotropy. Using SUVs made of dimyristoyl-PC containing 10% (mol/mol) dimyristoyl-PA in a Ca^{2+}-free

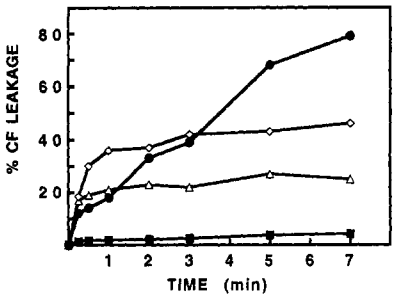

FIGURE 6: Time course of egg yolk PC SUV leakage induced by ammodytin L (△), myotoxin II (◇), *Naja* PLA_2 (●), and β-bungarotoxin (■) monitored by the CF-quenching method in absence (open symbols) or presence (closed symbols) of 1.5 Ca^{2+}. Protein concentration was 1 μM. Experiments were carried out as described in Figure 1.

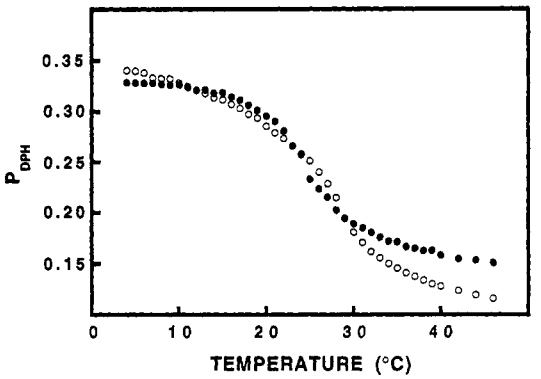

FIGURE 7: Effect of ammodytin L on the temperature variation of the fluorescence polarization parameter P_{DPH} in PC/PA SUVs containing 0.1% DPH. (○) Control liposomes; (●) plus 500 nM ammodytin L.

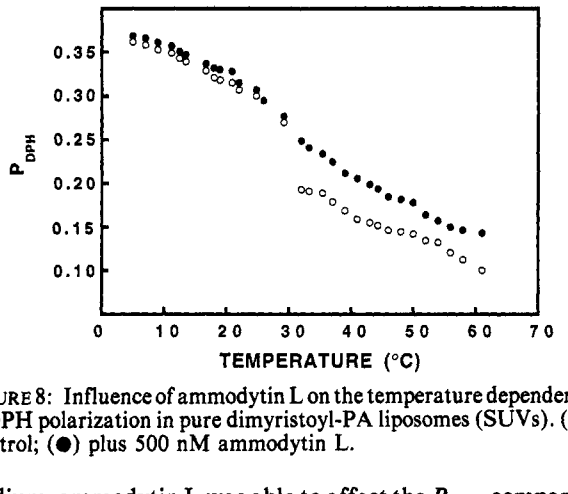

FIGURE 8: Influence of ammodytin L on the temperature dependence of DPH polarization in pure dimyristoyl-PA liposomes (SUVs). (○) Control; (●) plus 500 nM ammodytin L.

medium, ammodytin L was able to affect the P_{DPH} compared to the control experiments. The abrupt decrease of P_{DPH} between 20 and 25 °C, due to the gel to liquid-crystalline phase transition, was shifted to lower values, and all values of P_{DPH} above the phase transition were increased with respect to the control, indicating a lower degree of freedom for the motion of lipid molecules in the bilayer (Figure 7). In pure dimyristoyl-PC liposomes, no significant effect of ammodytin L was detected (not shown). When SUVs made of pure dimyristoyl-PA were used, the phase transition was practically abolished by ammodytin L, and above this temperature all P_{DPH} values were higher than found for the control (Figure 8). In the same experimental conditions, no effects of *Naja* PLA_2 on P_{DPH} could be detected, indicating that this enzyme, at least in the absence of Ca^{2+} ions, did not perturb the organization of membrane phospholipids (results not shown).

DISCUSSION

The results presented in this work demonstrate that two myotoxins, ammodytin L and myotoxin II, interact with phospholipid membranes in a way to induce leakage of liposome by a Ca^{2+}-independent mechanism. The pathogenesis of the toxins has not been described in details, but myotoxin II was reported to cause release of creatine and creatine kinase from muscle tissue, both in vivo and in vitro (Lomonte & Gutiérrez, 1989; Gené, personal comunication). Ammodytin L was recently shown to prevent fusion of myoblasts to myotubules and even to destroy the established myotubules (Krizaj et al., 1991). We therefore suggest that the lytic effect characterized here is responsible for the myotoxicity of this class of toxins.

The leakage process was studied by two independent assays using two different spectroscopic techniques: in this way it could be excluded that the activity of the toxins was conditioned by the assay system. A singular feature of the leakage induced by the phospholipase-like myotoxins is the absolute independence of Ca^{2+}. In contrast, nonmyotoxic, catalytically active phospholipases do not provoke any leakage at all in the absence of calcium, and their strict requirement for Ca^{2+} is strongly correlated with their enzymatic activity, wheras both myotoxins are enzymatically inactive. Furthermore, the kinetics of myotoxin-induced leakage are very rapid, monophasic (or possibly hyperbolic), and concentration independent. In spite of the high homology with the primary structure of PLA_2, the myotoxins operate by a completely different molecular mechanism. Myotoxin II has previously been shown to associate with cell membranes in a charge-dependent manner and to require negative surface charges for interaction with liposomes (Diaz et al., 1991). This is confirmed, in our experimental procedure, by the smaller release of CF observed for pure PC liposomes compared to mixed PC/PA vesicles; however, leakage still occurs, so the requirement for negative charges is not absolute but may have a facilitating role in binding of the toxins to the membrane. Consistently, the kinetics of the leakage process do not change in the absence of PA. A necessity for the presence of negative charges is seen for both enzymatic and fusogenic activities of the neurotoxic phospholipase β-bungarotoxin (Rufini et al., 1990), while the hemolytic *Naja* PLA_2 does not have any special charge requirements (Pluckthun et al., 1985).

The ability of PLA_2 hydrolysis products—free fatty acids and lysophospholipids—to enhance membrane permeability has been shown in both model systems and intact cells (Lovstad, 1986; Osorio e Castro et al., 1990), and we ascribe the CF release induced by *Naja* PLA_2 and β-bungarotoxin in our experimental system to this well-known phenomenon. But as far as the myotoxins are concerned, where no or very little enzymatic activity is detected, the cytotoxic effect cannot be explained by hydrolysis products. Likewise, it is obvious that the leakage effect described in this work does not involve interaction with membrane proteins, but it is possible that a protein target exists for the myotoxins in vivo. This might be the reason for the specificity of these toxins; negatively charged phospholipids are not common in the outer layer of plasma membranes (Devaux, 1991). It should be emphasized that all four venom proteins studied here are cytotoxic since they disrupt the cell membrane integrity, but only the myotoxins appear to interact specifically with muscle tissue membranes. *Naja* PLA_2 is a hemolytic toxin, whereas β-bungarotoxin is a highly specific presynaptic neurotoxin. It is likely that specific receptors exist for the myotoxins as well, but at present there is nothing known about the mechanism which enables a myotoxin to recognize its target membrane.

A myotoxic effect could be obtained simply through a Ca^{2+} channel or ionophore action. However, the penetration of CF or TEMPOcholine/ascorbate requires a substantial opening of the membrane, and moreover liposomes were found to release encapsulated peroxidase after incubation with myotoxin II (Diaz et al., 1991). The kinetics of membrane leakage provide some information on the mechanism at the molecular level. The kinetics are too fast to be explained by an unspecific increase in bilayer permeability, as seen with membrane fluidizers, or by a mechanism similar to that of thiol-activated bacterial toxins, which would involve diffusion and association of toxins within a membrane bilayer (Bhakdi et al., 1985). Rapid kinetics have been described for several cytolytic agents such as melittin, gramicidin S, and alamethicin (Portlock et al., 1990); but the effects of this class of "pore-formers" are generally concentration dependent, giving sigmoidal dose–response curves, and leakage is inhibited by high levels of Ca^{2+} (Bashford et al., 1986). In the case of the myotoxins the dose-dependence curves do not indicate any form of cooperativity; the kinetics are identical at low and high protein concentrations. Moreover, divalent ions do not inhibit the leakage process, in contrast to their effects on many other membrane-damaging agents such as hemolytic viruses, complement, and Triton X-100 (Bashford et al., 1986; Alder et al., 1991). It is therefore necessary to search for a different type of explanation of the leakage phenomenon.

Using DPH as lipid membrane probe, we find that ammodytin L induces intermolecular disordering of PA bilayers in the liquid-crystalline phase; the perturbed lipids can no longer undergo a cooperative transition. In this respect the effect on the phase transition of the toxin is similar to that of an intrinsic membrane protein: above a critical protein concentration the sharp lipid phase transition is abolished (Owicki & McConnell, 1979). In contrast, the binding of *Naja* PLA_2 to liposome does not have any prominent effect on the phase transition (data not shown). This indicates that some feature of the membrane structure itself could be involved in myotoxic action. Abolition of the thermotropic transition has previously been reported, for different types of negatively charged liposomes, as an effect induced by *Naja mossambica* cardiotoxin (Faucon et al., 1983). A model that explains the molecular mechanism of the low molecular weight cardiotoxins from *Naja* and *Laticauda* snakes has been proposed by the group of de Kruijff (Batenburg et al., 1985). It was suggested that highly positively charged domains of the polypeptides reside in the interior of an inverted micelle structure, which thereby is stabilized in the membrane (Batenburg et al., 1985). The consequent formation of a nonbilayer structure could explain the CF leakage from liposomes. In connection with this model, it should be kept in mind that the two basic myotoxins studied here contain highly positively charged domains (Pungercar et al., 1990; Francis et al., 1991), which could stabilize nonbilayer structures in the membrane.

The ability of toxins with a high molecular weight, as well as botulinum and diphteria toxins, to penetrate into and perturb lipid bilayers has been explained by a mechanism based on an unfolding/refolding cycle in which a pivotal role is played by pH changes (Zhao & London, 1988; Jiang et al., 1991). This model involves binding of the predominantly hydrophilic surface region of these proteins on the membrane bilayer, followed by a conformation change which exposes hydrophobic sites. In the case of PLA_2-like myotoxins, the high level of homology with the *A. piscivorus* Lys-49 toxin suggests a structure very similar to that of the group II PLA_2s (Holland et al., 1990), where the binding of protein to the interface is most probably not accompanied by a conformation change (Jain et al., 1991). Moreover, it is difficult to assume nonreductive conformation changes in proteins so rich in disulfide bridges as the PLA_2-type toxins.

Since the myotoxins used in this work are highly basic, the numerous positively charged lysyl groups may interact with the polar phosphate headgroups of the phospholipids; the charge neutralization may favor hydrophobic interactions and the penetration of the protein into the membrane with a consequent perturbation of the lipid chains. The amphiphilic transition could be induced by the binding in the modified active site of a phospholipid molecule which cannot be hydrolyzed. This hypothesis assumes that the role of Ca^{2+} in the substrate binding in active phospholipase is only connected with neutralization of the negative charges of Asp-49. This assumption is supported by elegant work by the group of de Haas showing that pancreatic PLA_2 mutants, with Asp-49 substituted by Lys or Gly, bind to micelles with the same affinity as the wild-type PLA_2 (van den Bergh et al., 1989). Only the Asp-49 protein becomes an active enzyme in the presence of Ca^{2+}, but it is not necessarily given that phospholipid hydrolysis is the primary event in PLA_2 toxicity. In fact, an important question that arises from this work is the relationship between hydrolytic and cytolytic activities of the PLA_2 toxins. In the cases of nigexine (Chwetzoff et al., 1989) and myotoxin I (Diaz et al., 1991), convincing evidence has shown that their cytotoxic activity is at least in part independent of the phospholipase reaction, and several authors dissociate the pharmacological effect of venom PLA_2 from the enzyme activity (Kasturi et al., 1990; Babu & Gowda, 1991). The definitive answer to this question awaits the isolation of further phospholipase-like toxins lacking Asp-49 and the examination of PLA_2 mutants where Asp-49 has been substituted through DNA recombination techniques; such studies are currently being pursued in our laboratories.

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