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Rapid Report

Phospholipase-like myotoxins induce rapid membrane leakage of non-hydrolyzable ether-lipid liposomes

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Abstract

Two phospholipase-like myotoxins – ammodytin L from *Vipera ammodytes* and myotoxin II from *Bothrops asper* – are shown to be able to induce leakage of liposomes made from non-hydrolyzable ether-linked phospholipids. This demonstrates that the cytolytic activity of these toxins is completely independent of any remaining enzyme activity or contamination with active phospholipases.

Key words: Phospholipase A_2; Myotoxin; Liposome; Membrane leakage; Snake venom

The importance of phospholipase activity for the cytotoxic reaction mechanism of toxic phospholipases in general is currently a much debated topic. There is now considerable evidence that phospholipid hydrolysis and cytotoxicity are at least partially independent effects, but this aspect of PLA_2 biochemistry is still highly controversial [1–11]. Phospholipase A_2 sequences from different sources show a highly conserved aspartate in position 49, which is known to be essential for Ca^{2+} binding and hydrolytic activity [1]. Exceptions to this rule has been found for a few snake venom toxins, that maintain up to 70% amino acid homology with other PLA_2s but have position 49 occupied by a different amino acid, such as lysine [12–14]. Although the Lys-49 protein was originally suggested to be a new type of active PLA_2 [12], subsequent work has questioned this assignment since only a very low enzyme activity was detected, and this residual activity has later been attributed to contamination with active PLA_2 [15]. If these toxins are indeed devoid of enzyme activity they obviously provide the proof that PLA_2 toxicity can be completely independent of catalytic activity; however, so far it has not been possible to exclude the presence of contaminating PLA_2 in the purified myotoxins.

We have recently studied the lipid-perturbing effect of the PLA_2-like proteins myotoxin II from *Bothrops asper*, in which position 49 is occupied by a lysine residue [14] and ammodytin L from *Vipera ammodytes*, where the aspartate 49 is replaced by serine [16]. Both myotoxin II and ammodytin L were found to provoke rapid and extensive release of the aqueous content of PC/PA liposomes [17]. The process was not dependent on Ca^{2+}, and took place without any detectable phospholipid hydrolysis, but the possibility of a catalytic or initiating role of PLA_2 activity was not excluded. To prove that cytolytic toxins with a PLA_2-like structure can operate by a mechanism completely independent of phospholipid hydrolysis, we have carried out leakage experiments using liposomes made of 1,2-*O*,*O*-dihexadecyl-*rac*-glycero-3-phosphocholine and 1,2-*O*,*O*-dihexadecyl-*rac*-glycero-3-phosphatidic acid, non-hydrolyzable analogues respectively of PC and PA. Finally, we have studied the lipid-perturbing properties of ammodytin L above and below the phase transition of ether-lipid liposomes.

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Abbreviations: CF, carboxyfluorescein; *O*-PC, 1,2-*O*,*O*-dihexadecyl-*rac*-glycero-3-phosphocholine; *O*-PA, 1,2-*O*,*O*-dihexadecyl-*rac*-glycero-3-phosphatidic acid; PC, phosphatidylcholine; PA, phosphatidic acid; PLA_2, phospholipase A_2; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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Clodomiro Picado, San José, Costa Rica, and ammodytin L from *Vipera ammodytes* by Prof. F. Gubensek, Jozef Stefan Institute, Ljubljana, Slovenia [18,19]. Carboxyfluorescein, tempocholine, and 1,6-diphenyl-1,3,5-hexatriene was from Molecular Probes (Eugene, OR). *Naja naja* phospholipase and different phospholipids were obtained from Sigma (St. Louis, MO), 1,2-*O*,*O*-dihexadecyl-*rac*-glycero-3-phosphocholine and 1,2-*O*,*O*-dihexadecyl-*rac*-glycero-3-phosphatidic acid were from Calbiochem (San Diego, CA) and used without further purification. Small unilamellar sonicated ether-lipid liposomes (*O*-PC/*O*-PA, 9:1, mol/mol) with an average diameter of 30–40 nm were made by the method of Huang [20], using a Branson bath sonicator. The phospholipid concentration was determined by the method of Ames [21]. 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+}.

Fluorescence experiments were made with an 4048 C SLM spectrofluorometer, as described previously [17]. Liposomes were made as described above, except that 50 mM of CF was included to monitor liposome leakage [22]. The liposomes were separated from non-encapsuled 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. Excitation was set at 480 nm and emission was detected at 540 nm. The results are expressed as:

$$\% \text{ CF leakage} = 100 \times [F - F_i]/[F_f - F_i]$$

were 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%.

Liposomes used in fluorescence polarization experiments were made as described above, except that 0.01% (molar ratio) of DPH in tetrahydrofuran was added to the lipids before drying. The final lipid concentration was 30 nmol/ml. Excitation was set at 360 nm and emission was detected at 450 nm. P_{DPH} was calculated by the equation:

$$P_{DPH} = [I_{\parallel} - I_{\perp}]/[I_{\parallel} + I_{\perp}]$$

$I_{\parallel}$ and $I_{\perp}$ being the fluorescence intensities recorded with the analyzing polarizer oriented parallel and perpendicular to the direction of the excitation beam, respectively [23].

For EPR measurement of leakage, liposomes (final lipid concentration 0.5 mM) were prepared as above with the Tris buffer containing 15 mM of the spin label tempocholine, followed by dialysis against the same buffer without tempocholine. Samples were prepared by addition of toxins or other components to 50 μl liposome suspension, and subsequent mixing with 1 μl 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. 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.

Table 1
Relative phospholipase activity of *Naja* PLA_2, ammodytin L and myotoxin II using liposomes made with dimyristoylphosphatidylcholine (DMPC) or with highly unsaturated phospholipids from rat brain

Phospholipase	Substrate	
	DMPC	rat brain phospholipids
Naja PLA_2	100	100
Ammodytin L	0.7	1.1
Myotoxin II	0.09	0.8

Phospholipase activity was determined as described under Materials and methods.

Phospholipase activity was determined with an assay based on ^{14}C-labelled substrate as previously described [24]. The purified toxins had very little phospholipase activity compared to an active PLA_2, but a residual activity of 0.5–1% was always found (Table 1). The toxins are known to copurify with active PLA_2s, and the complete absence of activity has apparently never been achieved [14–16].

Liposome leakage was measured by two different methods: fluorescence dequenching of liposome-entrapped carboxyfluorescein and EPR measurement of intravesicular tempocholine reduction by external ascorbate. Fig. 1 shows time-courses of the leakage of aqueous content from ether-liposomes, above and below T_m, induced by ammodytin L and monitored by the change in CF fluorescence. After the initial burst within the first 15 s the degree of leakage remained constant, even after prolonged incubation (not shown). Thus the lytic effect was practically instantaneous, and the toxins were not able to redistribute after the initial binding to one liposome, since this would have let to the gradual lysis of all liposomes. The extend of leakage and the kinetics were almost identical to the results obtained previously for normal PA/PC liposomes [17]. Under the same experimental conditions *Naja* phospholipase was not able to provoke any significant liposome lysis (Fig. 1). The temperature influenced the ability of ammodytin L to provoke CF leakage. In preliminary experiments we had determined the phase transition temperature, T_m, of the ether-lipid liposomes to be

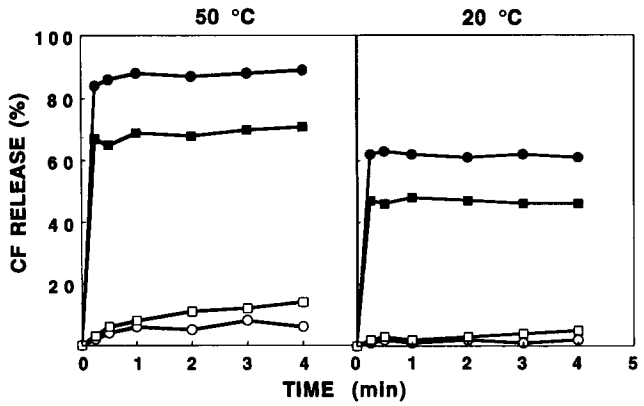

Fig. 1. Time-course of CF leakage induced by 1 μM (●) or 0.5 μM of ammodytin L (■), 1 μM *Naja* PLA_2 (□) or in the absence of proteins (○) from liposomes of *O*-PC/*O*-PA (9:1, mol/mol) in 20 mM Tris-HCl (pH 7.4). At time zero, proteins were added to the liposome suspension; the fluorescence of samples was monitored at two different temperatures: 50°C (left panel) and 20°C (right panel).

approximately 40°C, using P_{DPH} as previously described [17]. Leakage experiments were made at two different temperatures: 20°C (right panel) and 50°C (left panel), corresponding to below and above the gel to liquid-crystalline phase transition. The capacity of ammodytin L to induce liposome leakage appeared diminished by 30% at a temperature below the phase transition.

A parallel series of experiments were made using the spin label assay to determine leakage. Fig. 2 shows how the addition of ammodytin L to ether-liposomes caused leakage, observed as a decrease in the EPR signal of intravesicular tempocholine. Again the effect was too rapid to be followed in details and no subsequent changes were seen. Similar results were obtained for myotoxin II (not shown), whereas addition of active PLA_2 had no effect (Fig. 2). The absence or presence of 1 mM Ca^{2+} in the medium did not alter these results.

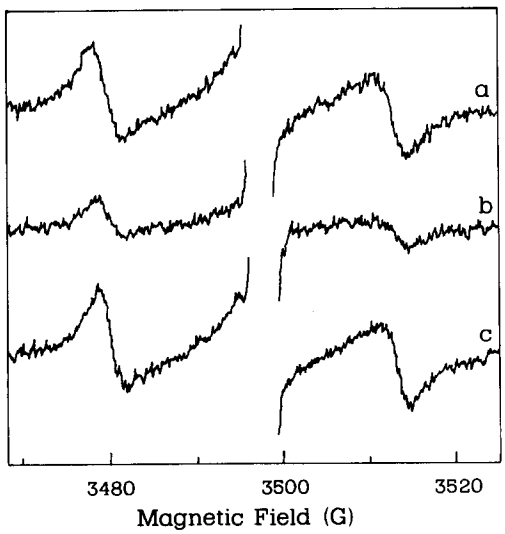

Fig. 2. EPR spectra of ether-linked PC liposomes loaded with tempocholine and suspended in ascorbate-containing buffer. The central parts of the spectra have been deleted to avoid overlap of the ascorbyl radical lines. (a) No additions; (b) after addition of 0.8 mg/ml ammodytin L; (c) after addition of 0.8 mg/ml *Naja* PLA_2 plus 1 mM Ca^{2+}.

Table 2
Effect of *Naja* PLA_2, ammodytin L and myotoxin II on the fluorescence polarization of DPH in *O*-PA/*O*-PC (9:1, mol/mol) liposomes at different temperatures

Temperature (°C)	Polarization parameter, P_{DPH}			
	control	*Naja* PLA_2	ammodytinn L	myotoxin II
31	0.400	0.392	0.380	0.377
37	0.383	0.381	0.364	0.369
44	0.307	0.305	0.313	0.311
51	0.165	0.169	0.197	0.201
58	0.100	0.102	0.129	0.123

To study the perturbative effects of the myotoxins on the liposome membrane, measurements were made at different temperatures using P_{DPH} to determine changes in membrane anisotropy (Table 2). Ammodytin L was able to affect the P_{DPH} compared to the control experiments both below and above the phase transition, but in different ways. Below T_m liposomes exposed to ammodytin L showed lower P_{DPH} values with respect to the control, indicating a more rigid lipid organization; whereas at a temperature above the phase transition ammodytin L increased the P_{DPH} values with respect to the control, corresponding to a lower degree of freedom for the motion of lipid molecules in the bilayer. No effects of *Naja* PLA_2 on P_{DPH} could be detected under the same experimental conditions, indicating that this enzyme did not perturb the organization of the ether phospholipids (Table 2).

The aim of this work was to demonstrate that the phospholipid membrane-damaging effect of PLA_2-like myotoxins does not involve any steps of phospholipase activity but proceeds trough a completely different mechanism. For this purpose we have chosen to test ammodytin L and myotoxin II using liposomes made of dialkyl-ether phospholipids, non-hydrolyzable substrate analogues for PLA_2. Substitution of ether linkages for ester linkages in these molecules does not affect lipid structure and bilayer conformation [25]. The results prove that the myotoxins have exactly the same effect in these liposomes as in vesicles made of acyl-ester phospholipids [17]. De Bose and Roberts have shown by NMR experiments that the binding of *Naja* PLA_2 to alkyl-ether PC vesicles accelerates phospholipid flip-flop and causes leakage of encapsulated Pr^{3+} [26]. They suggested that the change in flip-flop rate provokes a packing defect in the bilayer that allows increased permeability to small hydrophilic entities. However, this process is much too slow to account for the myotoxin-induced leakage seen here, and we do

not observe any change in the release of either tempocholine or CF when *Naja* PLA_2 is added to the vesicles. In the same way, ionomycin, an antibiotic able to translocate Ca^{2+} ions across cell and artificial membranes [27], failed to cause CF leakage in liposomes (data not shown). Clearly the damage caused by these myotoxins is more extensive than a simple change in membrane permeability or the formation of an ion channel.

P_{DPH} in *O*-PC/*O*-PA liposomes is not affected by *Naja* PLA_2, while ammodytin L induces intermolecular disordering of the bilayer in the liquid-crystalline phase, and increases the order above the lipid phase transition. This behaviour is the same as seen for acyl-ester phospholipid liposomes [17], indicating that the myotoxin interact in a similar way with both ether-linked and ester-linked phospholipids. In this respect the effect of the toxin on the membrane is similar to that of an intrinsic membrane protein [28]. On the basis of these data we suggest that ammodytin L and myotoxin II bind to and penetrate the membrane, leading to destabilization of the bilayer structure. The results obtained using fluid and solid liposomes (i.e. above and below the phase transition) suggest that an increase in membrane fluidity promotes the ammodytin L damaging activity. Yasuda's group report similar results using the pore-forming a-toxin from *Staphylococcus aureus*, and ascribes this phenomenon to the different topology of the toxin in the membrane depending on the lipid fluidity [29]. Irrespective of the reaction mechanism, it can be concluded that the phospholipase-like myotoxins exert their toxic effects completely independent of an eventual residual phospholipase activity.

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1. References

[1] Waite, M. (1987) in The Phospholipases: Handbook of Lipid Research (Waite, M., ed.), pp. 155–241, Plenum Press, New York.
[2] Gutiérrez, J.M., Chaves, F., Gené, J.A., Lomonte, B., Camacho, Z. and Schosinsky, K. (1989) Toxicon 27, 735–745.
[3] Chwetzoff, S., Tsunasawa, S., Sakiyama, F. and Ménez, A. (1989) J. Biol. Chem. 264, 13289–13297.
[4] Rosenberg, P. (1990) in Handbook of Toxinology (Shier, W. and Mebs, D., eds.), pp. 67–277, Marcel Dekker, New York.
[5] Kasturi, S., Rudrammajl, L.M.S. and Gowda, T.V. (1990) Immunology 70, 175–180.
[6] Yang, C.-C. and Chang, L.-S. (1991) Biochem. J. 280, 739–744.
[7] Diaz, C., Gutiérrez, J.M., Lomonte, B. and Gené, J.A. (1991) Biochim. Biophys. Acta 1070, 455–460.
[8] Bouchier, C., Boyot, P., Tesson, F., Trémeau, O., Bouet, F., Hodgson, D., Boulain, J.-C. and Ménez, A. (1991) Eur. J. Biochem. 202, 493–500.
[9] Babu, A.S. and Gowda, T.V. (1991) Toxicon 29, 1251–126.
[10] Lomonte, B., Gutiérrez, J.M., Ramirez, M. and Diaz, C. (1992) Toxicon 30, 239–245.
[11] Fletcher, J.E. and Jiang, M.-S. (1993) Toxicon 31, 669–695.
[12] Maraganore, J.M., Merutka, G., Cho, W., Welches, W., Kezdy, F.J. and Heinrikson, R.L. (1984) J. Biol. Chem. 259, 13839–13843.
[13] Yoshizumi, K., Liu, S.-Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S. and Khiara, H. (1990) Toxicon 28, 43–54.
[14] Francis, B., Gutiérrez, J.M., Lomonte, B. and Kaiser, I.I. (1991) Arch. Biochem. Biophys. 284, 352–359.
[15] Van den Bergh, C.J., Slotboom, A.J., Verheij, H.M. and De Haas, G.H. (1989) J. Cell. Biochem. 39, 379–390.
[16] Krizaj, I., Bieber, A.L., Ritonja, A. and Gubensek, F. (1991) Eur. J. Biochem. 202, 1165–1168.
[17] Rufini, S., Cesaroni, P., Desideri, A., Farias, R., Gubensek, F., Gutiérrez, J.M., Luly, P., Massoud, R., Morero, R. and Pedersen, J.Z. (1992) Biochemistry 31, 12424–12430.
[18] Lomonte, B. and Gutiérrez, J.M. (1989) Toxicon 27, 725–733.
[19] Gubensek, F., Ritonja, A., Zupan, J. and Turk, V. (1980) Period. Biol. 82, 443–447.
[20] Huang, C.H. (1969) Biochemistry 8, 344–355.
[21] Ames, G. (1966) Methods Enzymol. 8, 115–116.
[22] Wilschut, J., Düzgüneş, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021.
[23] Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 525, 367–394.
[24] Rufini, S., Pedersen, J.Z., Desideri, A. and Luly, P. (1990) Biochemistry 29, 9644–9651.
[25] Burns, R.A., Friedman, J. and Roberts, M.F. (1981) Biochemistry 20, 5945–5950.
[26] DeBose, C.D. and Roberts, M.F. (1983) J. Biol. Chem. 258, 6327–6334.
[27] Liu, C.M. and Herman, T.E. (1978) J. Biol. Chem. 253, 5892–5901.
[28] Owicki, J.C. and McConnell, H.M. (1979) Proc. Natl. Acad. Sci. USA 76, 4750–4754.
[29] Tomita, T., Watanabe, M. and Yasuda, T. (1992) J. Biol. Chem. 267, 13391–13397.