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THE MOLECULAR BASIS OF THE SELF NONSELF SELECTIVITY OF A COELENTERATE TOXIN

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Coelenterates produce potent hemolysins inhibited by sphingomyelin (SM) Remarkably, instead of this lipid their membranes contain a phosphono analogue of it Using coelenterolysin (CL), a toxin produced by the sea anemone *Phymactis clematis*, we have examined a possible connection between these two peculiar traits Our experiments showed that, while SM binds this lysin and inhibits its hemolytic activity, the endogenous PnSL do neither In addition, liposomes made of bovine erythrocyte lipids are rapidly disrupted by CL, while those made of *P. clematis* lipids are completely resistant to it However, if small amounts of SM are added to the *P. clematis* lipids, the resulting liposomes become sensitive to CL Taken together, our results show for the first time that *substitution of SM by its phosphono analogue is the molecular basis for the selectivity of an anthozoan toxin* We therefore propose that exotoxin production and membrane composition are coadapted traits that confer on the coelenterates a significant evolutionary advantage

The search for new, pharmacologically active compounds in marine organisms and the understanding of the mechanisms underlying their action is today the subject of intensive research (1) An ancient and particularly succesful group of sea animals is that of the coelenterates, which include jelly fish, sea anemones and corals They produce venomous stinging organelles, the nematocysts, which play a role in capture of prey and defense (2) Hemolytic toxins associated with these structures have been described (3) We have recently found a hemolytic polypeptide in the coelenteron of the sea anemone *Phymactis clematis* Its activity, which we have designated *coelenterolysin* is unrelated to the nematocysts (4) A distinctive trait of a number of cnidarian hemolysins is that they are inhibited by sphingomyelin (SM) (5,6,7,8) and this holds true for coelenterolysin as well (4) It has been proposed that SM-inhibited coelenterate lysins bind to this lipid and that this binding is important to the hemolytic action, resulting in the formation of pores in the target membranes (6,9,10)

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The coelenterate cell membranes show further distinctive traits. On one hand, these organisms are one of the major phyla that expose unprotected membranes directly to the environment (11). Likewise, the epithelial cells lining the gastrovascular cavity are also exposed to the fluids contained therein. Some of these cells phagocytose the materials being digested to complete breakdown and absorption of nutrients (12). Ciliated cells create currents within the coelenteric fluids (13). Phagocytosis and ciliary activity are functions that require a direct contact with the sorrounding medium. In addition, in a number of coelenterates, the gonads also face the gastrovascular cavity and the gametes, as well as the larvae and juvenile forms in many cases, encounter a medium which can be prepared to rapidly lyse prey cells. These features indicate that mechanisms must exist to protect coelenterate cells and tissues from their own released lytic toxins.

Phosphonosphingolipids (PnSL), mainly ceramide aminoethyl phosphonate and N-methylated derivatives of it, are known to occur in large quantities in the coelenterates (14,15,16,17). The biological meaning of these rare compounds is as yet unknown. The phosphono bond makes them more resistant to both chemical and enzymic hydrolysis than the usual sphingomyelins (18). Strikingly, the latter are completely absent in coelenterates (17,19,20). The presence of PnSL and the absence of the usual SM appear unique to this phylum.

So far, no link has been proposed between the occurrence of SM-inhibited toxins and the peculiarities in the sphingolipid composition of the coelenterates. In this work, we have tested the hypothesis that these two features are closely connected. Our experiments strongly support the notion that this is indeed the case.

MATERIALS AND METHODS

Materials

Specimens of the sea anemone *Phymactis clematis* were collected from rocky areas of the South Atlantic coasts of Argentina. The sea anemones were kept in constantly aerated natural sea water (collected together with the organisms), under permanent illumination and at room temperature. They were bi-weekly fed small pieces of beef liver. Bovine erythrocytes were aseptically obtained and kept at 4°C in sterile Alsever solution. Chicken egg yolk SM was from Avanti Polar Lipids (Pelham, AL). Dioleoyl phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, cholesterol, cellulose phosphate and Silica Gel G were from SIGMA Chemical Co. (St. Louis, MO). 5(6)-carboxyfluorescein was from Eastman Kodak (Rochester, NY) or Molecular Probes (Eugene, OR). Sephadex G-75 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents and solvents used were of analytical grade.

Sampling of coelenteric fluid: The liquid contained in the coelenteric cavity of specimens of *Phymactis clematis* was gently withdrawn through the oral opening as described before (4). The fluid was filtered through Whatman N° 1 filter paper and used fresh or aliquoted and kept at -20°C.

Purification of coelenterolysin: Purified preparations of coelenterolysin were obtained following a slight modification of the procedure described before (4). The steps involved precipitation with 80 % saturated ammonium sulfate, ion exchange chromatography on cellulose phosphate and gel filtration on Sephadex G-50.

Assay of hemolytic activity: Hemolytic activity was assayed by two different methods: i) determination of the amount of toxin needed to cause 100% hemolysis of a 0.3% v/v suspension of bovine erythrocytes after 1h incubation at 37°C in serial dilutions of the samples, carried out in 96-well microtiter plates, or ii) spectrophotometric continuous recordings of the changes in apparent absorbancy at 500 nm when erythrocyte suspensions were exposed to the toxin, as described before (4).

Preparation of lipid extracts: *Erythrocyte lipids:* An aliquot of 5 ml of bovine blood was washed thrice in 0.9% NaCl. The erythrocytes were finally resuspended in 2 ml of the same solution and extracted according to the procedure of Bligh and Dyer (21). The lower phases containing the lipids were filtered, taken to dryness under nitrogen and weighed repeatedly until constant weight. The lipids were finally dissolved in chloroform at a concentration of 10 mg/ml and stored at -20°C under nitrogen. *Lipids from sea anemones:* *P.clematis* specimens frozen at -20°C were cut into small pieces and homogeneized in an Ultraturrax tissue homogeneizer for 1 min. Lipids were then rapidly extracted, weighed and stored as above.

Isolation of PnSL: Sea anemone lipids extracted as above were separated by thin layer chromatography on Silica Gel G plates, using chloroform/acetic acid/methanol/water (75:25:5:1.5, v/v) as solvent mixture (22). The lipid spots were visualized by brief exposure to iodine vapors. The spot corresponding to PnSL was identified in parallel chromatograms by a selective staining method for phosphonolipids (23). This lipid was scraped off the plates and eluted from the Silica Gel with methanol/chloroform (2:1).

Preparation of liposomes: Liposomes containing 100 mM 5(6)-carboxyfluorescein (CF), a concentration at which fluorescence (λ_{exc490}-λ_{emm520}) is strongly quenched, were prepared as follows. The lipids were placed on round bottom tubes with rotation under a stream of nitrogen to create an even film. Then, 100 mM CF in 10 mM Tris-HCl, pH 7.4, was added so that the lipid concentration was, in all cases, 10 mg/ml. After addition of washed sea sand, the samples were vortexed vigorously and, then, sonicated in a Branson 100 bath sonifier for 5 min. An aliquot of 100 ul of this suspension was then applied onto a 1x 20 cm Sephadex G-75 column, which was eluted with 10 mM Tris-HCl / 100 mM NaCl, pH 7.4, to separate free from liposome-entrapped CF. The latter elutes as a distinct brown peak in the void volume, which is neatly separated from the bright green band corresponding to free CF.

In the cases where the effect of supplementation with SM or PnSL was studied, these lipids were added in chloroform solutions in the indicated quantities before the films were formed.

Kinetics of liposome disruption: This was followed spectrofluorometrically on a Turner 430 instrument, equipped with a thermostatized cuvette holder. Liposomes were diluted with 10 mM Tris-HCl/ 100 mM NaCl (1:20) and after a base-line recording of the fluorescence (λ_{exc490}-λ_{emm520}), coelenterolysin was added and the changes in absorbancy were recorded.

RESULTS AND DISCUSSION

Hemolytic assays are widely used tests for cytolytic activity (24,25). As a first approach, we have employed this methodology to investigate the behavior of *Phymactis clematis* coelenterolysin (CL). Like other coelenterate hemolysins, CL is inhibited by SM (4). No inhibition is observed with other lipids like dioleoyl phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or cholesterol (data not shown).

Since PnSL closely resemble SM (Fig.1), it was of particular interest to determine if, like SM, PnSL could also block the hemolytic activity of CL. Fig. 2 shows that no inhibition of hemolysis takes place when the phosphono analogue is added to the incubation mixture. Thus, CL reacts very differently towards these two very similar lipids. Further, we found that the mechanism underlying this difference is the inability of CL to bind PnSL. If sonicated SM (0.1 mg/ml) is added to a CL solution containing 100 HU/ml and after 1 min, the resulting suspension is centrifuged to remove the SM liposomes, less than 1% of the initial hemolytic activity remains in the supernatant, suggesting that the CL bound to the SM and was precipitated together with it. If an identical experiment is carried out using 0.1 mg/ml of bovine erythrocyte lipids, instead of SM, no hemolytic activity is recovered in the supernatant. In

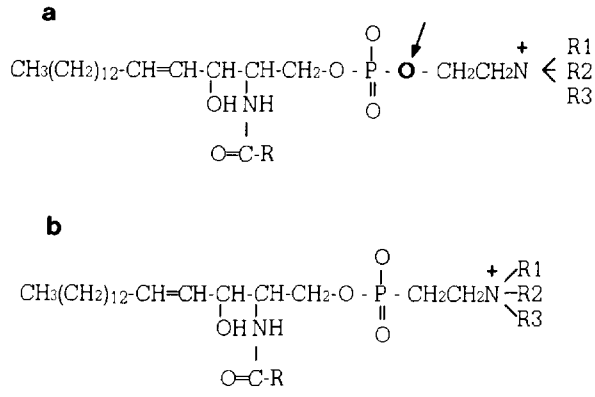

Fig. 1. Structure of **a)** *sphingomyelins (SM) and* **b)** *phosphonosphingolipids (PnSL).* R1, R2 and R3 designate -H or $-CH_3$ groups. In SM, the latter predominate, while in PnSL, the amino group is mainly unsubstituted or monomethylated. R is a long chain aliphatic group. The arrow points the oxygen atom that distinguishes phosphoryl SM from their phosphono analogues.

sharp contrast, addition of 1 mg/ml of purified PnSL or total sea anemone lipids, cause no decrease in hemolytic activity of the supernatants of the CL solution. We conclude that CL binds to SM and erythrocyte lipids but not to PnSL or sea anemone total lipids.

Our observations suggest that the polar head region of the sphingolipids, particularly the oxygen bridge that is present in SM but absent in PnSL, plays a critical role in the interaction with the toxin. It is also possible that differences in the extent of N-methylation at the polar head group may also be important, since the normal SM of mammalian origin is commonly trimethylated (26), whereas in PnSL the amino group is, predominantly, unsubstituted or monomethylated (18). However, the likelihood of this possibility is weakened by the fact that phosphatidylethanolamine, as mentioned, is not an inhibitor of CL.

Membrane disruption mechanisms can be conveniently studied using liposomes containing concentration-quenched fluorescent dyes. This technique is based on the increase in fluorescence by dilution, following leakage of the dye from the liposome into the sorrounding medium (27). A similar approach has already been used to study the lipid requirements of another anthozoan cytolysin (28). Using 5(6)-carboxyfluorescein as entrapped fluorescent dye (29), we established that CL can rapidly disrupt liposomes made of bovine erythrocyte lipid extracts (Fig. 3a). By contrast, no carboxyfluorescein leakage is induced when *P. clematis* lipids are used for the preparation of liposomes (Fig. 3b). Therefore, the lipid composition by itself can fully account to explain the selective lytic action on red cells and not on sea anemone tissues.

The precise significance of the SM absence among the lipids of this coelenterate was determined by supplementing *P. clematis* lipids with pure exogenous SM and, then, studying the sensitivity to CL of the resulting liposomes. Fig. 4a shows that as little as 1% w/w SM is enough to confer on the liposomes significant sensitivity to the lysin. This effect is intensified by increasing the SM concentration further (Fig. 4b and c). If, instead of SM, other lipids, including cholesterol, dioleoyl phosphatidylcholine, egg phosphatidylethanolamine, bovine phosphatidylserine, are added, no

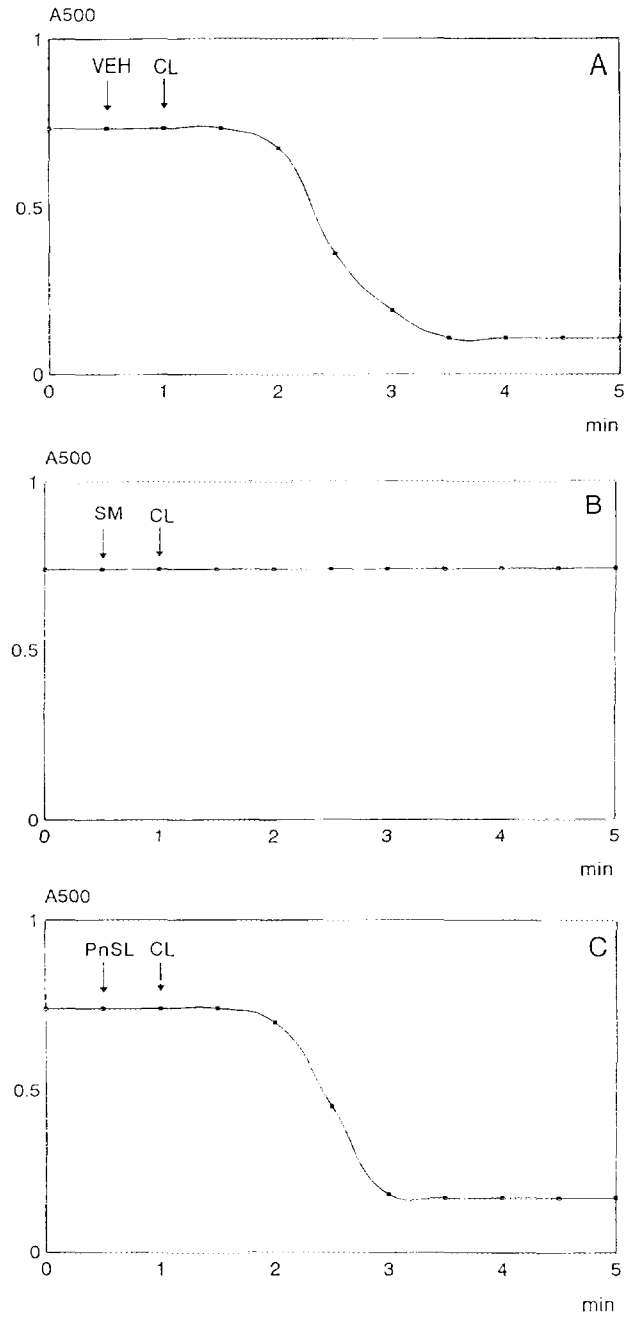

Fig. 2. Effect of SM and PnSL on the hemolytic activity of coelenterolysin. The course of hemolysis of bovine red cell suspensions induced by coelenterolysin (CL) was followed spectrophotometrically at 500 nm. Plot **A** shows a control experiment in which only 50 µl of vehicle (VEH), 20 mM Tris-HCl/ 150 mM NaCl, pH 7.4 (TBS), were added before CL (10 HU_{100}). In plots **B** and **C**, 50 µl of 2 mg/ml sphingomyelin in TBS (SM) or 50 µl of 2 mg/ml of purified phosphonosphingolipid from *P. clematis* (PnSL), respectively, were added before CL. The final volume in all cases was 1 ml. Note that while SM causes inhibition of hemolysis, PnSL is without effect.

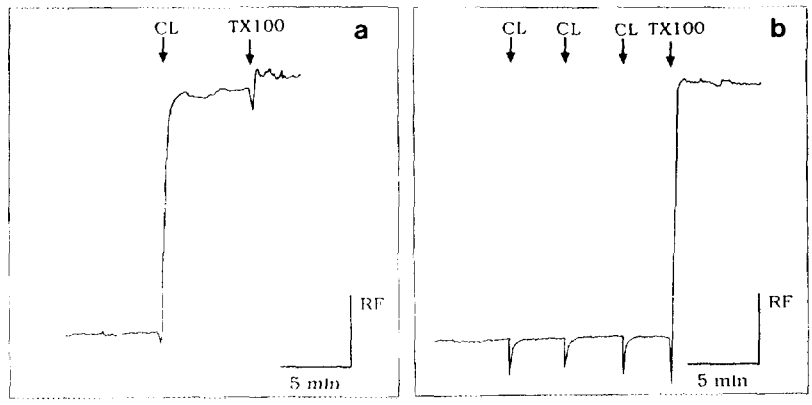

Fig. 3. Differential response of liposomes made of **a)** *bovine erythrocyte lipids and* **b)** *P.clematis lipids, upon exposure to coelenterolysin (CL).* These liposomes, containing concentration-quenched 5(6) carboxyfluorescein, were exposed to 10 HU_{100} of CL, in a final volume of 2 ml, at the indicated time points. Fluorescence (λ_{exc490}-λ_{emm520}) was continuously recorded. Increases indicate release of the dye. At the end of each experiment, 50 µl of 1% Triton X-100 (TX100) were added to release to induce complete release of entrapped carboxyfluorescein. While erythrocyte lipid liposomes rapidly released the dye immediately after the first CL addition, those made of sea anemone lipids did not respond despite several consecutive additons of the toxin. Both kinds of liposomes were disrupted upon TX100 addition.

sensitizing effects are observed (data not shown). This indicates that the effect is highly specific with regard to SM.

Based on the present work with *P. clematis*, we propose that the lack of SM, the presence of PnSI and the production of an exotoxin like CL have evolved as closely related traits enabling these organisms to attack membranes of other members of the animal kingdom while their cells remain tolerant to their own harmful secretions. In previous studies, we found a cytolytic phospholipase C

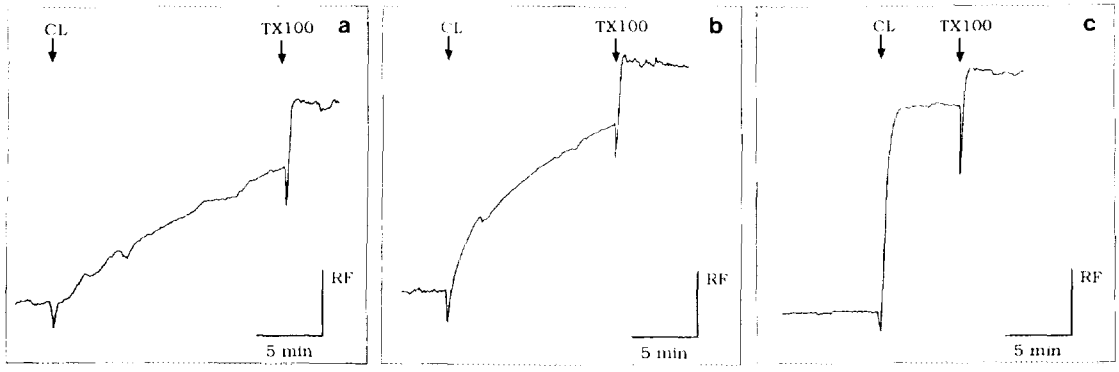

Fig. 4. Effect of supplementation of P. clematis lipids with SM on the sensitivity of liposomes to coelenterolysin (CL). The conditions of these experiments are similar to those in Fig. 3, except that 1% w/w (**a**), 10% w/w (**b**) or 30% w/w (**c**) of SM was added to sea anemone lipids before the liposome formation. Comparison with Fig.3b shows sharp differences, demonstrating that addition of SM makes the sea anemone lipid liposomes sensitive to the toxin.

secreted to the medium by the ciliated protozoon *Tetrahymena thermophila* (30). We proposed (31) that the tolerance of this naked cell to this enzyme was due to the extraordinary abundance of glycerophosphonolipids in its plasma membrane (32). Thus, the occurrence of peculiar lipid compositions can be related, now in two very distant phyla, to possible evolutionary advantages resulting from the ability to tolerate otherwise noxious exotoxins.

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