

Pardaxin induces aggregation but not fusion of phosphatidylserine vesicles

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The effects on membranes of pardaxin, an amphipathic polypeptide, purified from the gland secretion of the Red Sea Moses sole flatfish *Pardachirus marmoratus* are dose-dependent and range from formation of voltage-gated, cation-selective pores to lysis. We have now investigated the interactions of pardaxin with small unilamellar liposomes. Light scattering showed that pardaxin (10^{-7} – 10^{-9} M) mediated the aggregation of liposomes composed of phosphatidylserine but not of phosphatidylcholine. Aggregation of phosphatidylserine vesicles was impaired by vesicle depolarization. Furthermore, pardaxin-mediated aggregation between fluorescent-labeled PS vesicles was accompanied by leakage of the vesicle contents, and not by fusogenic process within the aggregates. We suggest that pardaxin is a unique polypeptide, that induces vesicle aggregation and membrane destabilization, but not membrane fusion; the mechanism of the aggregation activity of pardaxin is related to its amphipathic properties.

Pardaxin; Phosphatidylserine vesicle; Phosphatidylserine aggregation

1. INTRODUCTION

Amphipathic polypeptides like gramicidin [1], alamethicin [2] or melittin [3] serve as general models for transmembrane channels or for pore forming membrane proteins. Recently, we have purified and characterized a novel pore forming, hydrophobic polypeptide from the secretion of the Red Sea Moses sole, named pardaxin [4]. Although its amino-terminal sequence differs from that of the pore forming polypeptides mentioned

above, the effects of pardaxin on membranes range similarly from an increase in the permeability to ions at low concentrations to lysis at high concentrations [4–7]. Furthermore, the increase in planar bilayer permeability by pardaxin was found to be voltage dependent [6] as is true for alamethicin [2] or melittin [3]. The detailed molecular structure of pardaxin pores and its interaction with artificial membranes are unknown. Thus, several questions about the interaction of pardaxin with artificial membranes remain: (i) Is its ability to produce aggregation related to its pore forming activity? (ii) Is the amino-terminal hydrophobic segment of pardaxin [4] sufficient to change membrane permeability and/or to induce phospholipid vesicle aggregation? (iii) Since vesicles pretreated with pardaxin form aggregates as qualitatively visualized under negative contrast electron microscopy [4], does this molecule qualify as a fusion protein? We have attempted to answer these questions by assaying for pardaxin induced aggregation and fusion of small unilamellar

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Abbreviations: N-NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine; N-Rho-PE, *N*-lissamine rhodamine-B-sulfonyl phosphatidylethanolamine; ANTS, 8-amino-naphthalene 1,3,6-trisulfonate; DPX, *p*-xylene-bis-pyridinium bromide; PC, phosphatidylcholine; PS, phosphatidylserine; PBS, phosphate buffered saline; PX, pardaxin; LUV, large unilamellar liposome; SUV, small unilamellar liposome

liposomes using light scattering, fluorescence energy transfer techniques, respectively. Our results indicate that pardaxin will aggregate liposomes made of PS but not PC. However, while it causes significant leakage of vesicle contents, pardaxin does not induce membrane fusion.

2. MATERIALS AND METHODS

2.1. Materials

Egg PC, PS from bovine brain, cholesterol and the fluorescent lipids N-NBD-PE and N-Rho-PE were purchased from Avanti Polar Lipids (Birmingham, AL). ANTS, DPX and dansyl-PS were purchased from Molecular Probes (Junction City, OR). Triton X-100 was purchased from Sigma (St. Louis, MO). The toxic secretion of the Red Sea Moses sole *Pardachirus marmoratus* (Pisces, Soleidae) was obtained according to the method of Clark and Chao [8] and pardaxin was isolated as recently described [4]. The NH₂-terminal segment of pardaxin: NH₂-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Gly-Ile-Glu-COOH was synthesized by the solid phase method [9].

All other chemicals were of the purest grade commercially available.

2.2. Preparation of liposomes

Small unilamellar liposomes composed of either pure PC or pure PS (5 mg/ml lipid concentration) were prepared according to established procedures [10] by sonication to clarity of the turbid suspensions (10–30 min) in a bath type sonicator (Laboratory Supplies, Inc., Hicksville, NJ). For most experiments the liposomes were prepared in PBS (Biofluids, Rockville, MD). Occasionally for studies involving membrane depolarization, the liposomes were made up in 150 mM KCl or 150 mM NaCl buffered with 10 mM Hepes and adjusted to pH 7.2 with KOH or NaOH, respectively. Several types of fluorescent liposomes were prepared, depending on the experimental requirements. For mixing of the lipid membranes and for aggregation studies, PC and PS (5 mg) in chloroform/methanol (1:1) were mixed with 50 µg each of N-NBD-PE or N-Rho-PE [11] or with 100 µg dansyl-PS, respectively. The mixtures were evaporated under N₂ to dryness and rehydrated in 1 ml of PBS. SUVs were then formed as described above. In order to measure mixing of the vesicle contents and leakage of the encapsulated dyes, liposomes were similarly prepared in 100 mM NaCl, 12 mM ANTS or 45 mM DPX and the preformed ANTS/DPX complex, respectively [12,13]. Following sonication of the mixtures the liposomes were purified by gel chromatography over PD10 (Pharmacia) columns equilibrated with Ca²⁺/Mg²⁺-free PBS.

2.3. Spectroscopic assay

All fluorescence measurements were performed at room temperature with a microprocessor controlled spectrofluorimeter (Fluorolog 2, Spex Industries, Metuchen, NJ). The sample chamber was equipped with a magnetic stirrer. Aggregation of the liposomes was measured using two independent assays. In the first one we measured changes in 90° light scattering at 340 nm using the spectrofluorimeter as a nephelometer. In the second assay the liposomes were labeled with 0.2 mol%

of dansyl-PS [14]. At this concentration the fluorescence of dansyl is essentially nonquenched. Since all the liposomes are labeled in their headgroup region, aggregation of the vesicles will result in a concentration dependent quenching of the fluorescence, as previously shown for other headgroup labeled lipids [15].

Vesicle fusion was measured using two independent fluorescent assays for mixing of the vesicle contents and merging of the lipid bilayer membranes, respectively. The mixing of the contents was monitored using the ANTS/DPX assay [12,13], incorporating ANTS and DPX into different vesicle populations. ANTS fluorescence was measured at $\lambda_{\text{ex}} = 384$; $\lambda_{\text{em}} > 530$.

Merging of the membranes was assayed according to Struck et al. [11]: 0.1 mol% each of NBD-PE and NBD-Rhodamine incorporated into the same vesicle membranes as fluorescence energy transfer donor and acceptor, respectively. NBD fluorescence was measured at 480 nm excitation and 530 nm emission with a 524 cuton filter in the emission light path. The excitation slits were kept narrow (1 mm) to reduce light scattering.

Vesicle leakage was assayed by monitoring the increase in the ANTS fluorescence due to the pardaxin induced, lipid membrane permeability changes and subsequent diffusion and dissociation of the ANTS/DPX, preincapsulated into the liposomes [12].

2.4. Other methods

Lipid concentrations were determined according to a modified Fiske-Subbarow method [16].

3. RESULTS

3.1. Pardaxin-induced aggregation of phosphatidylserine vesicles

The ability of pardaxin to cause aggregation of SUVs was tested by two different techniques. Addition of pardaxin to a suspension of dansyl-PS labeled PS vesicles produced a rapid decrease in the fluorescence intensity (fig.1). In contrast, when the same experiment was repeated using PC liposomes labeled with 0.2% mol dansyl-PS, no such quenching of fluorescence was observed (not shown). In a parallel set of experiments the effect of pardaxin on the 90° light scattering properties of SUVs were investigated (fig.2). Addition of 3×10^{-7} M PX to PC liposomes did not significantly change the light scattering signal (fig.2A). In contrast, adding of pardaxin to PS vesicles produces a rapid decrease in the light scattering signal which was followed by a slow, gradual increase (fig.2A). Qualitatively, similar, but far less pronounced effects were observed when using 100-fold higher concentration of the synthetic amino-terminal decapeptide (fig.2B). These results suggest that the intact pardaxin molecule, not only its synthetic

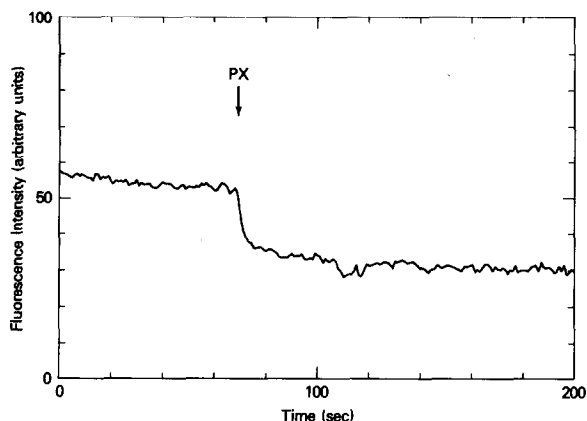


Fig. 1. Pardaxin-induced aggregation of PS vesicles labeled with dansyl-PS. Dansyl labeled ($20 \mu\text{M}$) liposomes were incubated in 150 mM KCl, 10 mM Hepes buffer, pH 7.2. The fluorescence of the lipid probe at this concentration is unquenched. Upon addition of pardaxin (PX, arrow) the fluorescence intensity is quenched by about 40% indicative of aggregation.

amino terminal decapeptide can aggregate phosphatidylserine vesicles, but not phosphatidylcholine vesicles.

The same techniques were used to explore the effects of the liposomal transmembrane potential on pardaxin induced liposome aggregation. Gramicidin (10^{-8} M) was added to liposomes loaded with 150 mM NaCl to generate a membrane potential (fig. 2C) which was positive on the external face of the membrane. Generation of a positive potential significantly reduced the rate of pardaxin-induced aggregation of PS vesicles compared to that of non-treated PS liposomes (fig. 2C). These results were independent of the species of monovalent cations and/or of the ionophore used since similar results were obtained when the same potential was generated using either monensin as a Na^+ ionophore, or KCl-loaded liposomes and valinomycin as a specific K^+ ionophore (not shown).

3.2. Evaluation of pardaxin-mediated leakage of vesicle content and possible vesicle-vesicle fusion

Previously we have shown that pardaxin is a pore forming polypeptide [4,5]. We therefore measured the effects of pardaxin on the leakage of vesicle contents from liposomes containing the

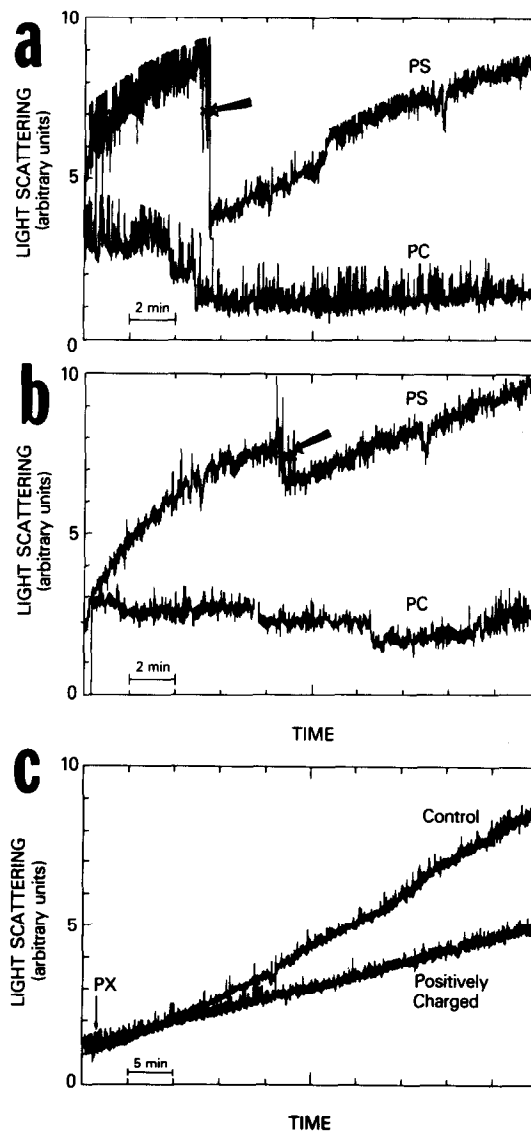


Fig. 2. Light scattering measurements of liposomal aggregation, induced by pardaxin (A) and the synthetic decapeptide (B) and the effect of gramicidin-induced liposomal diffusion potential on pardaxin-mediated aggregation of phosphatidylserine liposomes (C). (A,B) $100 \mu\text{M}$ phosphatidylserine (PS) or phosphatidylcholine (PC) SUV were incubated in PBS, pH 7.3, 3×10^{-7} pardaxin (A) or 4×10^{-5} M, NH_2 -terminal synthetic decapeptide (B) were added. 90° light scattering of the suspension was measured as described in section 2. (C) 10^{-8} M gramicidin was added to a sample of PS, Na^+ loaded liposomal suspension ($100 \mu\text{M}$) in 0.3 M sucrose, 3 mM Tris, pH 7.3, therefore inducing an inside/outside Na^+ concentration gradient [35]. This gradient produces a positive charge on external liposomal surface. 2 min after gramicidin addition, 3×10^{-7} M pardaxin (PX, arrow) was added and the aggregation of vesicles was monitored by light scattering at 340 nm.

ANTS/DPX complex. Pardaxin below 10^{-10} M did not induce vesicle leakage. At pardaxin concentrations between 10^{-9} and 10^{-8} M there was a small, slow leakage of ANTS/DPX; at 10^{-7} M pardaxin and above the rate and extent of leakage increased dramatically (fig.3). However, pardaxin-induced leakage was not complete even at 10^{-6} M since disruption of the vesicles after addition of 0.1% (v/v) Triton X-100 increased further the fluorescence signal (fig.3).

Polypeptides that aggregate PS liposomes and concomitantly induce leakage are likely to be fusogenic [17,18]. To test this hypothesis we assayed for liposome fusion using two independent techniques which measured the mixing of the contents and merging of the membranes, respectively. Pardaxin at concentrations exceeding 10^{-8} M, induced a small, but measurable leakage of the vesicle contents, as shown above. However, there was no measurable mixing of contents using the ANTS/DPX fusion assay (fig.4A). The presence of 10^{-8} M pardaxin did not impair the fusion competence of these liposomes since subsequent addition of 2 mM calcium induced membrane fusion manifested by the decrease in ANTS fluorescence upon mixing of the vesicle contents (fig.4A).

In some systems, hemifusion of liposomes has been demonstrated in which the outer monolayer

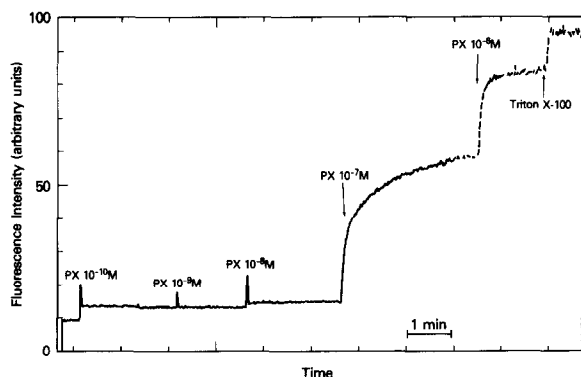


Fig.3. Pardaxin-induced leakage of PS vesicles. PS liposomes were loaded with ANTS/DPX during sonication and purified by gel filtration as described in section 2. Liposomes, 20 μ M, were incubated in 150 mM KCl, 10 mM Hepes buffer, pH 7.2, and pardaxin was added at the times and concentrations indicated in the figure. The first evidence for leakage was noticed at 10^{-8} M. Vesicle lysis was not complete even at 10^{-6} M pardaxin since addition of 0.1% (v/v) Triton X-100 induced additional release of the ANTS/DPX complex.

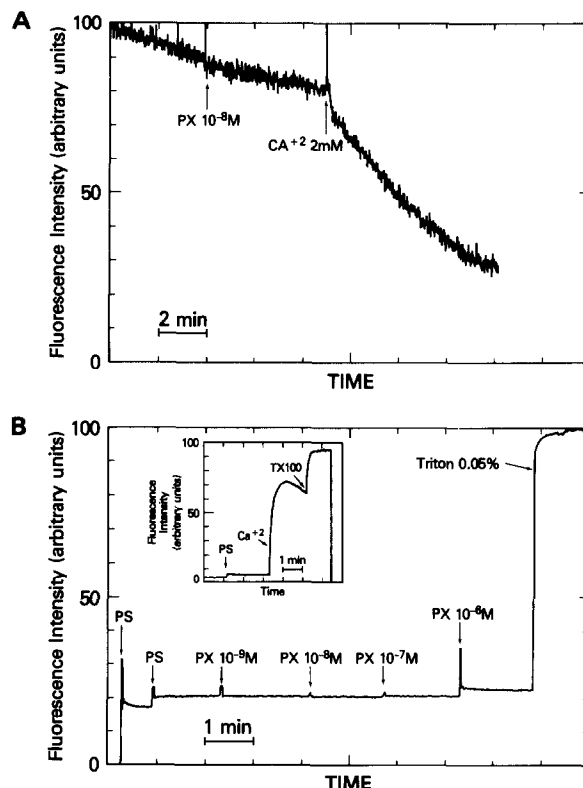


Fig.4. Pardaxin does not induce PS vesicle fusion. Fusion of PS vesicles was independently assayed using two complementary techniques: (A) mixing of vesicle content; (B) mixing of membrane lipids. (A) SUV, PS vesicles containing 12 mM/ANTS and 45 mM/DPX, respectively, were prepared by sonication and purified by gel chromatography. The fusion assays were performed as described [12,13]. 20 μ M each of ANTS and DPX containing SUVs were incubated in 150 mM KCl, 10 mM Hepes buffer, pH 7.2. Addition of 10^{-8} M pardaxin (PX, arrow) caused a minor decrease in the fluorescence, probably due to leakage (cf. fig.3). However, addition of 2 mM Ca^{2+} resulted in a time-dependent reduction in the fusion signal, typical for the occurrence of membrane fusion. (B) SUV, PS vesicles containing 0.1 mol% each, NBD-Rhodamine PS in the membranes were prepared by sonication. Coincubation of 10 μ M of the labeled vesicles with a 5-fold excess of unlabeled PS-SUV (arrow, PS) did not change the fluorescence of the donor. Similarly, addition of up to 10^{-7} M pardaxin (PX, arrows) did not alter NBD fluorescence. The slight increase in NBD fluorescence at 10^{-6} M pardaxin is probably related to its surfactant properties [20]. However, this surfactant property did not cause sizable disruption of the membrane as seen from the steep increase in NBD fluorescence upon addition of 0.1% Triton X-100. The fusogenic properties of these vesicle preparations is shown in the inset. Addition of 5 mM Ca^{2+} induced a rapid mixing of the membranes resulting in the increase in NBD fluorescence. Addition of 0.1% (v/v) Triton X-100 yielded maximal NBD dilution.

lipids merge effectively without concomitant mixing of vesicle contents [19]. To test this possibility, PS liposomes doped with NBD-PE and Rhodamine-PE were used in a lipid mixing assay [21]. Addition of pardaxin, up to 10^{-6} M, did not induce mixing of the membrane lipids of PS liposomes, as ascertained by the NBD/Rhodamine assay (fig.4B). Control liposomes were shown to undergo fusion upon addition of 5 mM calcium (fig.4B, inset). Previous studies have indicated that at these high concentrations pardaxin might act as a surfactant [20]; however, its mechanism of action seems to differ from that of other detergents, such as Triton, which completely disrupt the vesicles, resulting in an abrupt increase in the NBD fluorescence [21].

4. DISCUSSION

Pardaxin is a well characterized single chain, amphipathic polypeptide [4-7,9] which readily interacts with biological and artificial lipid membranes. Pardaxin binds to and spontaneously inserts into the membranes to form voltage-gated pores [4-7]. In this study we present evidence that pardaxin rapidly aggregates phosphatidylserine (but not phosphatidylcholine liposomes) in a voltage-dependent fashion, and induces membrane destabilization, as manifested by the leakage of their contents, and yet our results clearly demonstrate that pardaxin does not fuse PS liposomes. In this respect, pardaxin differs from another pore forming oligopeptide, alamethicin, which was shown to fuse phosphatidylcholine liposomes [22]. Our results seem even more unexpected, since membrane aggregation and destabilization have hitherto been interpreted as being characteristic for fusogenic molecules such as Ca^{2+} [23], polylysine [24] or for oligopeptides, such as mellitin [17] or a synthetic copolymer of glutamic acid-alanine-leucine-alanine [25].

True membrane fusion, as opposed to hemifusion [26] requires besides membrane destabilization mixing of both the membrane lipids and the vesicle contents [27]. Using a non-lytic concentration of 10^{-8} M pardaxin, we clearly demonstrate that aggregation of the liposomes is accompanied by a slight leakage of the vesicle contents (fig.3) without concomitant merging of the bilayer membranes and mixing of the liposomal contents

(fig.4). However, pardaxin-aggregated liposomes remain sensitive to calcium triggered fusion (fig.4). Therefore, under our experimental conditions, pardaxin neither resembles calcium/membrane binding proteins which inhibit liposome fusion, such as prothrombin, parvalbumin or calmodulin [28] nor does it belong to the class of fusion proteins, like clathrin [29] or synexin [30,31] which were shown to induce and/or enhance fusion of artificial and biological membranes.

Close inspection of the molecular structure of pardaxin reveals that this oligopeptide contains a single hydrophobic, helical segment at the amino terminal; by contrast, the carboxy-terminal is composed mainly of charged amino acids (serine, glycine, glutamic acid), rendering this portion of the molecule highly hydrophilic [6]. In line with previous findings and computer modeling [9], we propose, that, while the hydrophobic segment will be easily inserted into lipid membranes (pore formation), the aggregation properties of pardaxin are due to electrostatic interactions of the carboxy-terminus with neighboring acidic liposomes. Hence the preference of PS over PC and the modulating role of the transmembrane electrical potential (fig.2).

However, to induce membrane destabilization and ultimately fusion, it does not seem to suffice to aggregate neighboring membrane surfaces via hydrostatic interactions. Rather, fusion requires both dehydration and membrane destabilization as suggested for the fusogenic action of calcium [23,26] and also that of viral glycoproteins [32,33].

Pardaxin, however, seems to lack a second hydrophobic domain which could insert into opposing bilayers to further destabilize and fuse the aggregated membrane complexes. Such multiple hydrophobic domains have been found in, e.g., synexin [34], which indeed aggregates and fuses chromaffin granules [31]. We believe that continued studies of amphipathic, lipid binding proteins, such as pardaxin, which do aggregate and destabilize, but do not fuse lipid membranes, will eventually contribute to our understanding of the molecular basis required for protein-mediated fusion in biological systems.

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