Articles

Pyrularia Thionin Binding to and the Role of Tryptophan-8 in the Enhancement of Phosphatidylserine Domains in Erythrocyte Membranes†

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ABSTRACT: Pyrularia thionin is a small, strongly basic peptide which interacts readily with cellular and synthetic membranes. With cells it induces hemolysis, depolarizes the cellular membrane with an accompanying influx of Ca^{2+}, and activates an endogenous phospholipase A_2. Evidence points toward a binding site involving phosphatidylserine (PS). This study shows that addition of the peptide to erythrocyte membranes as well as to vesicles formed from phospholipids isolated from erythrocyte membranes causes an enhancement of phospholipid domains which are made visible by the use of fluorescence digital imaging microscopy with fluorescent derivatives of PS (NBD-PS) and phosphatidylcholine (NBD-PC). Addition of thionin caused a large increase in NBD-PS domains, with an accompanying enrichment of NBD-PC in another separate domain. Double-labeling experiments performed with a Texas Red derivative of thionin show that the peptide binds to the domain enriched in NBD-PS. P thionin inactivated by modification of Trp-8 with *N*-bromosuccinimide lost the ability to enhance PS domains, although it bound to the membrane with the same affinity as native P thionin. This shows that binding to the membrane is not in itself sufficient to cause the NBD-PS and NBD-PC redistribution into domains.

Pyrularia thionin (P thionin)[1] is a strongly basic 47 amino acid peptide (mol wt 5280) isolated from the parasitic plant *Pyrularia pubera* (Vernon et al., 1985). It interacts readily with cellular membranes to cause hemolysis of erythrocytes (Osorio e Castro et al., 1989), inhibition of cell growth (Evett et al., 1986), depolarization of muscle and P388 cells with an accompanying influx of Ca^{2+} (Evans et al., 1989), and activation of an endogenous phospholipase A_2 in NIH 3T3 cells (Angerhofer et al., 1990). Hemolysis of erythrocytes follows Michaelis–Menten kinetics (Osorio e Castro & Vernon, 1989; Vernon & Rogers, 1992a), and binding studies indicate a specific binding site on erythrocytes, with each cell having approximately 1×10^5 sites (Vernon & Rogers, 1992b). Although the nature of the binding site is not definitely known, it is suspected to be a specific phospholipid domain containing phosphatidylserine. This is suggested since a protein receptor has been looked for but not detected (Evans, 1990), the thionin binds to DPPC liposomes containing 20 mol % PS more readily than liposomes containing the acidic phospholipids phosphatidylinositol or cardiolipin (Gasanov et al., 1993), and the number of binding sites on erythrocytes increases dramatically when erythrocytes are converted into ghosts in which the PS has redistributed to the outer leaflet (unpublished data).

The technique of fluorescence digital imaging microscopy has been used to determine the effect of added Ca^{2+} and cationic proteins upon the phospholipid organization of erythrocyte membranes (Haverstick & Glaser, 1987; Rodgers & Glaser, 1991, 1993). Using fluorescent-labeled phospholipids, the lateral distribution of specific phospholipids can be determined. Using this technique it was observed that erythrocyte membranes have an uneven distribution of phospholipids, demonstrating the presence of phospholipid domains in the native membrane. Such domains are not observed in vesicles prepared from extracted erythrocyte phospholipids, indicating that in the native membrane it is the proteins contained in the membrane which induce phospholipid domain formation. The purpose of this study is to examine the effect of the strongly cationic peptide P thionin on phospholipid distribution and domain formation in native erythrocyte and ghost membranes.

MATERIALS AND METHODS

Sample Preparation. Rabbit erythrocytes were isolated and ghosts were prepared as previously described (Haverstick & Glaser, 1987). The cells and ghosts were labeled for fluorescence digital imaging microscopy by monomer transfer of labeled phospholipids as previously reported (Rodgers & Glaser, 1991) using donor vesicles prepared by the ethanol injection method of Kremer et al. (1977). The amount of fluorescent phospholipid transferred to the erythrocyte membranes was less than 0.5% of the total phospholipid in the

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[1] Abbreviations: CTX, cardiotoxin; DMPA, dimyristoylphosphatidic acid; DMPC, dimyristoylphosphatidylcholine; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DOPS, dioleoylphosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; NBD-PA, 1-acyl-2-[[*N*-(4-nitrobenz-2-oxa-1,3-diazolyl)amino]caproyl]phosphatidic acid; NBD-PC, 1-acyl-2-[[*N*-(4-nitrobenz-2-oxa-1,3-diazolyl)amino]caproyl]phosphatidylcholine; NBD-PG, 1-acyl-2-[[*N*-(4-nitrobenz-2-oxa-1,3-diazolyl)amino]caproyl]-phosphatidylglycerol; NBD-PS, 1-acyl-2-[[*N*-(4-nitrobenz-2-oxa-1,3-diazolyl)amino]caproyl]phosphatidylserine; dansyl-PC, 1-acyl-2-[4-[*N*-[5-(dimethylamino)naphthalene-1-sulfonyl]amino]butanoyl]phosphatidylcholine; PBS, phosphate-buffered physiological saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; P thionin, Pyrularia thionin; SUV, small unilamellar vesicle.

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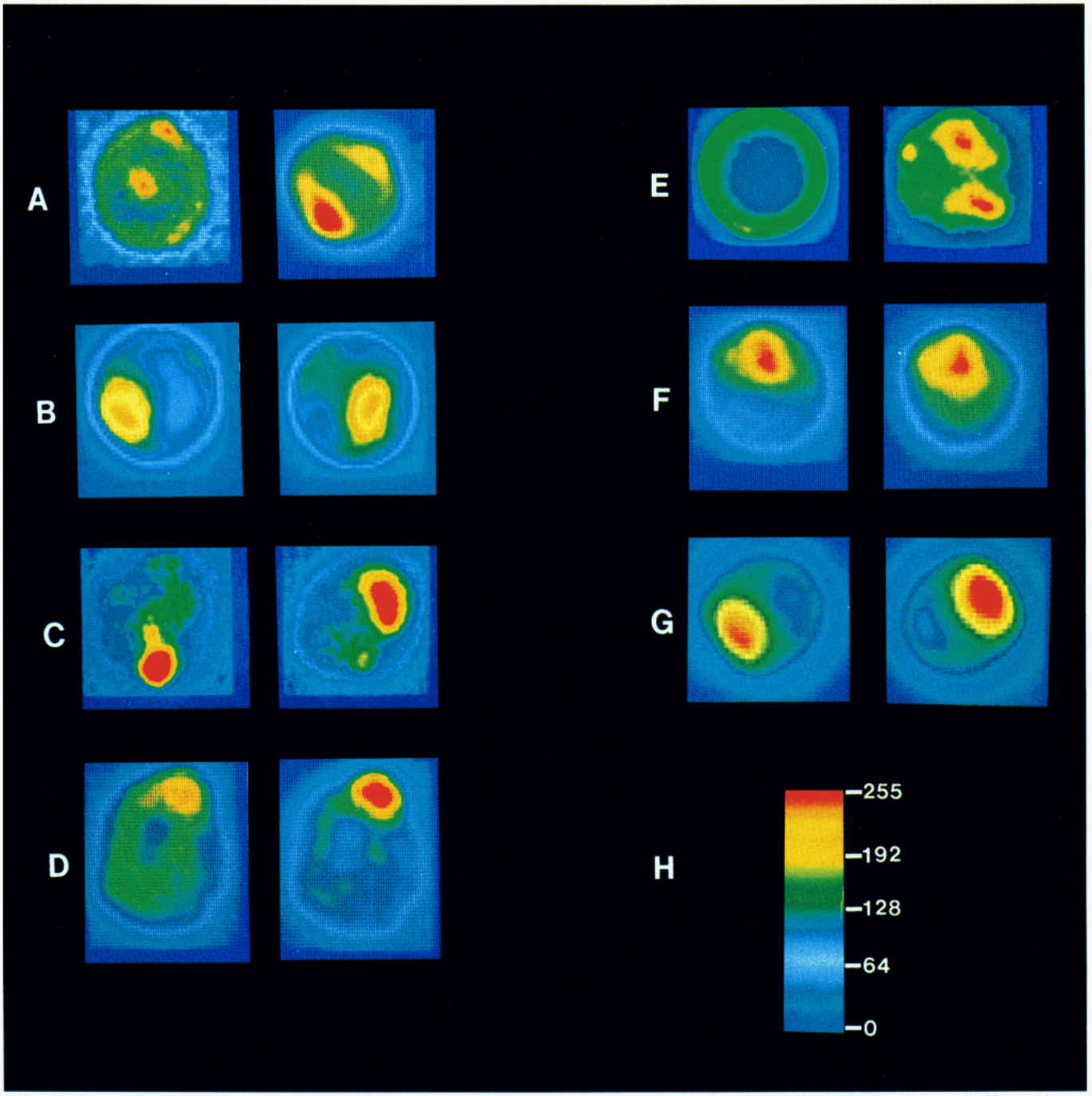

FIGURE 1: Fluorescence images showing enrichment of lipid domains induced by P thionin. (A, left) Fluorescence image of an NBD-PC labeled erythrocyte. (A, right) NBD-PC-labeled erythrocyte after the addition of P thionin. (B, left) NBD-PS-labeled erythrocyte ghost in PBS. (B, right) Dansyl-PC image of the same ghost. (C, left) NBD-PS-labeled ghost after P thionin (10 μM) was added. (C, right) Dansyl-PC image of the same ghost. (D) NBD-PS-labeled ghost at 2 min (left) and at 8 min (right) after thionin was added. (E, left) A vesicle made from extracted erythrocyte lipids labeled with NBD-PS. (E, right) NBD-PS image of a vesicle made from extracted erythrocyte lipids after P thionin was added. (F) A vesicle made from extracted erythrocyte lipids labeled with NBD-PS after an incubation with 5 μM Texas Red-labeled thionin. The NBD-PS image is on the left and the Texas Red-labeled thionin image of the same vesicle is on the right. (G, left) NBD-PC image of a vesicle formed with extracted erythrocyte lipids after incubation with 5 μM Texas Red-labeled P thionin. The Texas Red image of the same vesicle is on the right. (H) The pseudo-color scheme applied to the gray level values, which ranged from 0 (blue) to 255 (red). The sizes of erythrocytes, ghosts, and vesicles all were in the 6–8 μm range.

membranes. Since the ghosts were not sealed, both leaflets of the membrane were labeled. Samples for microscopy were suspended in PBS (137 mM NaCl, 3 mM KCl, 6 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The erythrocyte lipids used for forming large unilamellar vesicles were extracted by theprocedure of Rose and Oklander (1965), and the large unilamellar vesicles for viewing were formed as reported earlier (Haverstick & Glaser, 1987). All lipids used for microscopy were obtained and stored as previously described (Rodgers & Glaser, 1991), and the phospholipid concentrations were determined by colorimetric assay after perchloric acid digestion (Ames & Dubin, 1960).

Instrumentation and Image Processing. All experiments were done with the instrument described previously (Haverstick & Glaser, 1987; Rodgers & Glaser, 1991, 1993) except that the computer was replaced by a Macintosh IIci (Apple Computer Inc., Cupertine, CA) and a new charge-coupled-device video camera from Photometrics Ltd. (Tucson, AZ) was used. The image was visualized on an Apple color high-resolution RGB monitor. For Texas Red imaging, excitation

filters between 530 and 560 nm were used. Emission wavelengths >605 nm were used for image collection. The background field of illumination on all fluorescence images was subtracted, and the resulting images were normalized so that the final mean gray level values were 100 ± 4. A pseudocolor scheme was applied to the gray level values, which ranged from blue (0) to yellow to red (255 or above) (Figure 1H).

P thionin was prepared from nuts of *P. pubera* as described previously (Vernon et al., 1985). The dye Texas Red (Molecular Probes, Eugene, OR) was used to prepare a fluorescent derivative of P thionin. One milligram of Texas Red was dissolved in 2 drops of dimethylformamide, and a solution of 5 mg of P thionin in 1 mL of 0.025 M sodium borate, pH 8.8, was added with stirring. After 1 h at 25 °C the reaction mixture was added to a G10–120 Sephadex column (11-mL volume) and eluted with the borate buffer, protecting the solution from room light. A band of approximately 1.5 mL containing the dark purple conjugate separated from a slow-moving pink band (unconjugated Texas Red). The conjugate was collected from the column, and this material, having an absorbancy ratio A_{596nm}/A_{280nm} of 0.9 was used in the experiments. Using the molar extinction coefficient of 85 000 for Texas Red (Molecular Probes) and the absorption at 280 nm of Texas Red and the two Tyr and one Trp in P thionin (Fracki et al., 1992), it was calculated that about one in seven P thionin molecules was conjugated to Texas Red. As discussed below, the Texas Red derivative binds to the same site as native P thionin. Binding of the Texas Red-labeled thionin to erythrocyte ghosts was followed by measuring the fluorescence at 620 nm when excited by light at 596 nm.

Protein content was determined by the Lowry method. Hemolysis experiments involved a 1-h incubation at 37 °C of washed human erythrocytes in the presence of P thionin as previously described (Osorio e Castro & Vernon, 1989). For binding studies, native and Trp-8-modified P thionin were iodinated with ^{125}I, and binding was determined by the procedure reported earlier (Vernon & Rogers, 1992b). P thionin was modified at the Trp-8 position by oxidation of this residue with *N*-bromosuccinimide, as previously reported (Fracki et al., 1992). Briefly, 15 mg of P thionin was dissolved in 10 mL of 0.1 M acetate buffer, pH 4.0, and 250-μL increments of 5 mM *N*-bromosuccinimide were added until the absorbance at 280 nm no longer decreased, which occurred after four additions. This treatment inactivated the P thionin by 94% in terms of its ability to induce hemolysis of erythrocytes but did not significantly affect its binding to erythrocytes.

RESULTS

The effects of P thionin on erythrocyte membrane domains can be directly observed using fluorescence digital imaging microscopy. Shown in Figure 1A are NBD-PC-labeled erythrocytes before (left) and after (right) the addition of 10 μM P thionin. Without P thionin, there was a heterogeneous distribution of NBD-PC in the membrane with an enrichment of NBD-PC into large areas or domains. P thionin increased the radiance values of the PC-labeled domains. By using transmitted light, it was observed that the erythrocyte was lysed by P thionin in the time necessary to collect the fluorescence image. In further experiments to determine the effects of P thionin, ghosts rather than whole cells were used to avoid the quenching of hemoglobin and to avoid the problems posed by the biconcave shape of the intact cell.

The enrichment of lipid domains induced by P thionin was also seen in erythrocyte ghosts (Figure 1B,C). In ghosts

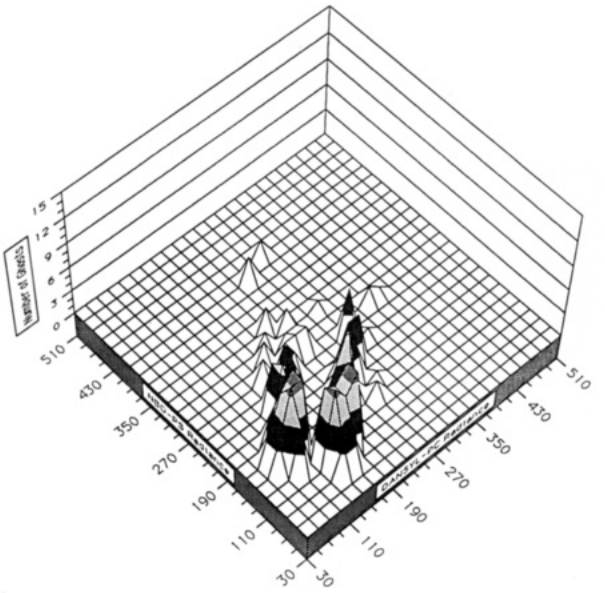

FIGURE 2: Distribution of domain radiance values for erythrocyte ghosts suspended in PBS buffer. In ghosts double-labeled with NBD-PS and dansyl-PC, the radiance value of the brightest pixel in the enriched domains (or the lowest pixel in the areas of depletion) of an image was measured, followed by measuring the corresponding pixel in the companion image. The domains in 70 ghosts were measured, and the distribution was normalized to 60 for comparison with Figures 3 and 4.

double-labeled with NBD-PS and dansyl-PC to which 10 μM P thionin was added, there was a large increase in NBD-PS (Figure 1C, left) and dansyl-PC (Figure 1C, right) domain radiance values.

The formation of PS-enriched domains was also followed as a function of time. Shown in Figure 1D is a single ghost labeled with NBD-PS at 2 min (left) and 8 min (right) after the addition of 10 μM P thionin. The effects of P thionin generally were apparent at the earliest time of observation. Approximately 2 min are needed to place the sample on a slide and to locate and capture an image. The enrichment of the domains continued for several minutes, with the domains stabilizing by 10 min. Each ghost had a distinctive pattern of domains with different sizes and degrees of enrichment. Some ghosts had a single large domain while others had multiple domains.

The changes in the radiance values of NBD-PS and dansyl-PC domains were quantitated by using a large population of double-labeled ghosts. In ghosts double-labeled with NBD-PS and dansyl-PC, the radiance value of the brightest pixel (or smallest pixel) in the area of enrichment (or depletion) of an image was measured, followed by measuring the corresponding pixel in the companion image. The graphs in Figures 2 and 3 show the distribution of radiance values for the double-labeled ghosts suspended in PBS (Figure 2) and PBS containing 10 μM P thionin (Figure 3).

The enrichment of lipid domains was measured by taking the ratio of the largest radiance value over the smallest radiance value within each image of the double-labeled ghosts (Rodgers & Glaser, 1993). In the absence of P thionin, the average value of the ratio was 2.7 for NBD-PS images and 2.6 for dansyl-PC images. For the ghosts with P thionin, however, the average value increased to 4.6 for the NBD-PS images and 3.3 for the dansyl-PC images. The higher degree of PS enrichment reflects the specific interaction between P thionin and the PS in the membranes.

The specific modification of Trp-8 of P thionin by *N*-bromosuccinimide resulted in a loss of hemolytic activity (Fracki

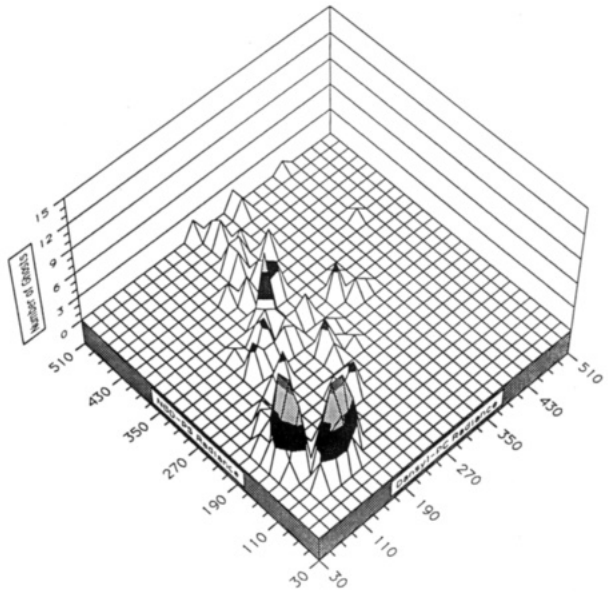

FIGURE 3: Distribution of domain radiance values for erythrocyte ghosts suspended in PBS buffer with 10 μM P thionin. The ghosts were double-labeled with NBD-PS and dansyl-PC as in Figure 2. The domains in 60 ghosts were measured.

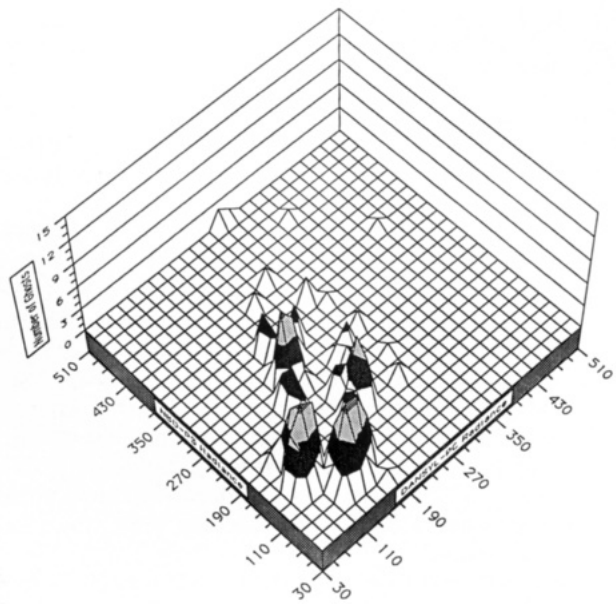

FIGURE 4: Distribution of domain radiance values in erythrocyte ghosts suspended in PBS buffer with 10 μM Trp-modified thionin. The ghosts were double-labeled with NBD-PS and dansyl-PC as in Figure 2. The domain in 66 ghosts were measured and normalized to 60.

et al., 1992). Earlier experiments on extensively iodinated P thionin showed uniform loss of all cellular responses (Evans et al., 1989), and the later data show that it is modification of Trp-8 under these conditions of extensive iodination which leads to inactivation of P thionin. The ability of the Trp-8-modified thionin to induce domains was investigated by using ghosts double-labeled with NBD-PS and dansyl-PC. After 10 μM modified thionin was added, there was only a slight increase in the PS and PC domain radiance values (Figure 4) in comparison to the large changes in domain enrichment caused by native P thionin. The average values for the enrichment of domains were 2.9 for NBD-PS and 2.7 for dansyl-PC. Importantly, these results indicate that P thionin had lost almost all of its activity to enhance lipid domains after the modification. The small shift in domain radiance values after the addition of the modified thionin is likely due to slight residual cellular activity remaining after the modification (Fracki et al., 1992).

The effects of P thionin on vesicles made from extracted erythrocyte lipids were also investigated. Figure 1E shows vesicles labeled with 0.5% NBD-PS with (right) and without (left) P thionin. In the presence of 5 μM P thionin, large PS domains were formed in the vesicle. No lipid domains were formed after the addition of the modified thionin.

The data presented above indicate that the strongly basic P thionin interacts with negatively charged lipids in the membranes to form domains similar to those that happen with calcium or cytochrome *c* (Haverstick & Glaser, 1989). When P thionin was labeled with Texas Red and when vesicles made from extracted lipids with NBD-PS were used, the induced PS domains were found to be colocalized with P thionin domains (Figure 1F). PC was excluded from the Texas Red-labeled P thionin domains (Figure 1G). In vesicles formed with 10% DOPS, 89.5% DOPC, and 0.5% NBD-PC, PC was excluded from P thionin domains as well (Figure 5A). The same result was observed with erythrocyte ghosts; PS colocalized with Texas Red-labeled thionin while PC was enriched predominantly in another domain (data not shown). Importantly, no domains were found in vesicles formed entirely with egg PC and labeled with NBD-PC, indicating again that the primary reaction of P thionin is with NBD-PS in the membrane. At the concentration of Texas Red-labeled thionin used for these experiments (5 μM), there was some fluorescence energy transfer between NBD and Texas Red, which caused some quenching of the NBD fluorescence (approximately 30%). This prevented the quantitation of the effects of the labeled thionin on PS domains. When Texas Red-labeled thionin was added to whole erythrocytes or ghosts without the addition of a fluorescent-labeled phospholipid, similar Texas Red-labeled thionin domains were observed as in Figure 1F or 1G (data not shown). Thus, the thionin-induced domains were independent of the presence of labeled phospholipids. Finally, in the presence of P thionin, PS domains were visualized when the PS concentration was reduced to 0.3% in DOPC vesicles. This was the lowest concentration of PS that was tried.

The ability of P thionin to form domains with different negatively charged phospholipids was also investigated. P thionin readily binds to large unilamellar vesicles containing acidic phospholipids, showing a preference for vesicles composed of PS. Suspensions of 5 mg/mL vesicles containing only PC bound less than 1% of added radiolabeled P thionin (1 μg/mL). Vesicles prepared separately from PI, PG, PA, and PS bound 17%, 44%, 79%, and 96%, respectively, of the labeled P thionin.

When vesicles containing either NBD-PS or NBD-PA were viewed with the fluorescence microscope, intense domains were formed in the presence of Texas Red-labeled thionin (Figure 5B,C). The domains of NBD-PS or NBD-PA colocalized with the Texas Red-labeled thionin domains. For the PS and PA vesicles with 2 μM P thionin, the average value of the ratio of the largest radiance value over the smallest value within a vesicle was 4.1 for both the NBD-PS and NBD-PA images. The colocalization of NBD-PG domains with the Texas Red-labeled thionin domains was also observed (Figure 2D). The average value of the ratio of the largest to smallest radiance value was 2.6 for NBD-PG after the addition of 2 μM P thionin. When Texas Red-labeled thionin was added to vesicles containing PI, the fluorescence of the Texas Red-labeled thionin was hardly visible on the vesicles, apparently due to the weak binding of P thionin to the vesicles. NBD-PI was

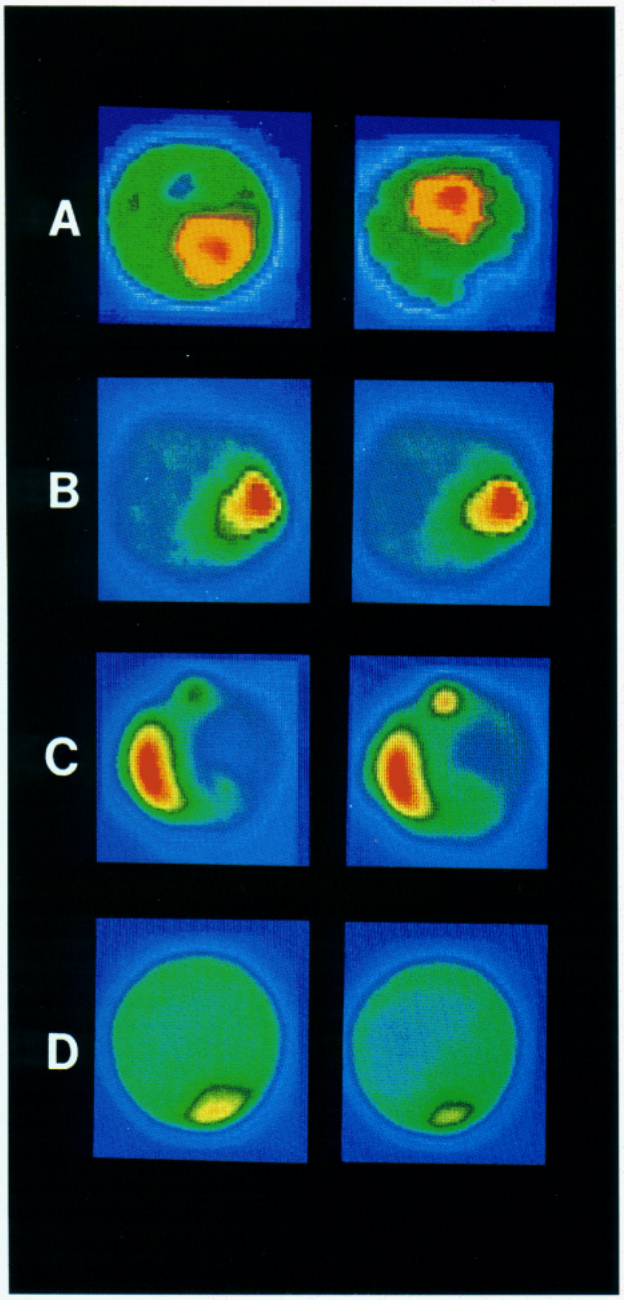

FIGURE 5: Fluorescence images showing enrichment of lipid domains in lipid vesicles induced by P thionin. (A, left) NBD-PC image of a vesicle formed with 89.5% DOPC, 10% DOPS, and 0.5% NBD-PC after the addition of 5 mM Texas Red-labeled P thionin. (A, right) Texas Red-labeled thionin image of the same vesicle. (B, left) NBD-PS image of a vesicle formed with 89.5% DOPC, 10% DOPS, and 0.5% NBD-PS after 5 mM Texas Red-labeled thionin was added. (B, right) Texas Red-labeled thionin image of the same vesicle. (C, left) NBD-PA image of a vesicle made from 89.5% DOPC, 10% DOPA, and 0.5% NBD-PA after the addition of Texas Red-labeled thionin. (C, right) Texas Red-labeled thionin image of the same vesicle. (D, left) NBD-PG image of a vesicle formed with 89.5% DOPC, 10% DOPG, and 0.5% NBD-PG after the addition of 5 mM Texas Red-labeled thionin. (D, right) Texas Red-labeled thionin image of the same vesicle.

not available, so its distribution could not be observed. These results indicate that P thionin has the strongest effects on the distribution of PS and PA. Since PS is the only negatively charged phospholipid present in substantial amounts in the erythrocyte membrane, it appears that P thionin binds predominantly to PS in this membrane.

Our proposal that P thionin binds to the NBD-PS-enriched domain and that Trp-8 on the peptide is required for domain

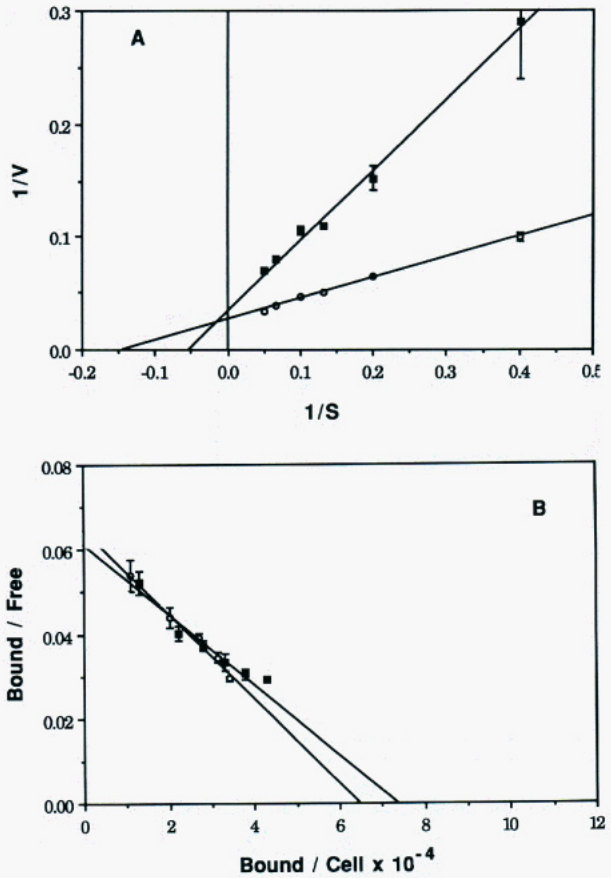

FIGURE 6: Similar binding properties of P thionin and Trp-8-modified P thionin to human erythrocytes. (A) Double-reciprocal plots of the velocity (% hemolysis in 1 h) induced by varying concentrations of P thionin (μg/mL) added to human erythrocytes in the presence of 60 μg/mL Trp-8-modified P thionin: (○) P thionin and (■) P thionin plus 60 μg/mL Trp-8-modified P thionin. (B) Scatchard plot for binding of ^{125}I-labeled P thionin (○) and similarly labeled Trp-8-modified P thionin (■) to human erythrocytes. All experiments were run in triplicate, and the error bars represent SEM. When the error bar falls within the symbol, it is not shown.

formation is valid if both the Trp-8-modified P thionin and the Texas Red derivative bind to the same site as native P thionin. That this is the case for Trp-8-modified P thionin is shown by the data in Figure 6. The Trp-8-modified P thionin lost 94% of its hemolytic activity. The relationship between Trp modification and hemolysis activity is shown in the reference of Fracki et al. (1992). More extensive reaction with *N*-bromosuccinimide was avoided since it caused bromination of the two Tyr residues in the molecule. When the inactivated P thionin was added to a hemolysis assay along with native P thionin, it inhibited P thionin hemolytic activity. Plotting the data so obtained as a double-reciprocal plot (Figure 6A) shows that the inhibition caused by the modified P thionin was competitive to native P thionin. For this experiment the calculated K_m is 1.28 μM, and the K_i value is 4.6 μM. These values are in agreement with those from previous experiments (Osorio e Castro et al., 1989; Vernon & Rogers, 1992a). Iodination of the Trp-8-modified P thionin with ^{125}I allowed a comparison of the binding properties of the native and modified P thionin to erythrocytes, using the procedure described previously (Vernon & Rogers, 1992b). Figure 6B contains the data obtained, showing very little difference in the binding properties of the two peptides. Carrying out a binding experiment with native ^{125}I-labeled P thionin in the presence of nonlabeled modified P thionin showed a competition between the two for the same binding site (data not shown) similar to the situation for P thionin and CTX (Vernon

& Rogers, 1992b). Earlier hemolysis experiments showed that extensively iodinated P thionin, in which the Trp-8 is modified, and native P thionin compete for the same site on the erythrocyte membrane (Osorio e Castro et al., 1989).

Similar experiments cannot be done with erythrocytes using the Texas Red derivative of P thionin since the preparation used in these studies contained unmodified P thionin, which itself would cause hemolysis. Also, the hemoglobin in erythrocytes interferes with the fluorescence binding assay. Experiments with ghosts are complicated by the fact that the unmodified P thionin gives a nonlinear binding response at low peptide concentrations. Addition of 60 μg/mL inactive Trp-8-modified P thionin to 10 μg/mL Texas Red-labeled thionin caused a 24% inhibition of Texas Red-labeled thionin binding to ghosts, as determined from fluorescence measurements. The percent inhibition decreased at higher concentrations of Texas Red-labeled thionin, going to 16% at 30 μg/mL, indicative of competitive binding. Thus, both the Trp-8-and the Texas Red-modified thionins bind to the same site as the native P thionin.

DISCUSSION

Using the technique described here, it was shown earlier that erythrocyte membranes as well as erythrocyte ghost membranes contain areas enriched in PC and in PS (Rodgers & Glaser, 1991, 1993). Since domains were not observed in vesicles formed from extracted erythrocyte phospholipids, the presence of proteins in the erythrocyte membranes is the directing force for the enrichment of phospholipids into domains. Extensive documentation exists for phase separations induced by adding Ca^{2+} (Hartmann et al., 1977; Hui et al., 1983; Haverstick & Glaser, 1987; Kouaouci et al., 1985) or basic proteins such as myelin basic protein (Boggs et al., 1977) and cytochrome *c* (Birrell & Griffith, 1976) as well as polylysine (Hartmann et al., 1977; Laroche et al., 1988) to bilayers containing acidic phospholipids. There is evidence that prothrombin induces the formation of domains rich in negatively charged phospholipids (Mayer & Nelsestuen, 1981), but this is disputed by Tendian and Lentz (1990). Also, annexin VI induces extensive clustering of acidic phospholipids in a Ca^{2+}-dependent manner, which involves the lateral movement of phospholipids in the membrane (Bazzi & Nelsestuen, 1992). Protein kinase C is another protein which binds to bilayers in a Ca^{2+}-dependent manner (Bazzi & Nelsestuen, 1991), and this protein has a specific requirement for PS for its activity. The best comparisons for our observed effects of P thionin on membranes are those reported for the strongly basic cardiotoxins (CTX) of approximately 60 amino acids isolated from cobra venoms. In all cellular responses studied to date, CTX from *Naja naja kaouthia* mimics P thionin and binds competitively to the same membrane site (Vernon & Rogers, 1992a,b). The data of Dufourcq and Faucon (1978) show that CTX binds to negatively charged phospholipids, but not to PC, and that binding to mixtures of phospholipids results in a lateral phase separation between negative and neutral phospholipids. A more recent study by Desormeaux et al. (1992) involving infrared spectroscopy shows that CTX from *Naja mossambica mossambica* interacts with an equimolar mixture of DMPC and the negatively charged DMPA to induce lateral phase separation, with one phase containing DMPA-rich domains perturbed by CTX while the second phase is composed of regions enriched in DMPC. Our experiments show that P thionin behaves in a similar manner, binding to NBD-PS-enriched domains to induce movement of other phospholipids within the bilayer to enhance the enrichment of both NBD-PS and NBD-PC in distinct domains. The continued enhancement of domain formation over a 10-min period shows a slow movement of phospholipids within the bilayer as a consequence of P thionin binding.

As shown in Figure 4, the ability of P thionin to induce domain enhancement is lost when the Trp-8 residue on the peptide is modified by oxidation with *N*-bromosuccinimide. This treatment inactivates the peptide in terms of its ability to cause hemolysis of erythrocytes (Fracki et al., 1992). A similar modification of Trp-8 is caused by extensive iodination which also modifies Trp-8 (Fracki et al., 1992). P thionin treated in this way is inactive for all known cellular responses (Evans et al., 1989). By reference to the known structure of the closely related α_1-purothionin, Trp-8 is contained in the hydrophobic helical region of the amphipathic molecule which interacts with the hydrophobic domain of phospholipid membranes (Teeter et al., 1990). Our data indicate that it is the insertion of this portion of the P thionin molecule into the phospholipid bilayer which perturbs the bilayer and induces the observed movement of phospholipids which results in the enhancement of phospholipid domains.

P thionin and cardiotoxin from snake venoms induce very similar cellular responses (Vernon, 1992; Harvey, 1991). Both peptides are strongly basic and are held in a very compact and stable structure by four disulfide bonds. Both bind to the same site on erythrocyte membranes to cause hemolysis (Osorio e Castro & Vernon, 1989). They also cause membrane depolarization, Ca^{2+} influx into cells, and activation of phospholipase A_2 and are cytotoxic to cells. Cardiotoxins have been extensively studied in terms of their interaction with cell membranes and synthetic phospholipid bilayers (Harvey, 1991). Four cardiotoxins have been isolated from *N. mossambica mossambica*, and three of these have Trp at position 11 in the first loop of the peptide (Bougis et al., 1983). This Trp residue is at the active site as shown by the fact that it is protected from trypsin action when cardiotoxin is bound to membranes, and fluorescence changes show that the Trp is inserted into the hydrophobic region of the membrane. Our data are in agreement, showing that Trp-8 of P thionin is necessary to induce phospholipid movement in the membrane to form specific domains, leading to the observed cellular responses.

There is now ample evidence for the binding of P thionin to PS in natural and synthetic membranes, but the question remains open concerning the mode of binding of P thionin to native erythrocytes. A conclusion that P thionin binds to PS in the erythrocyte membrane requires that a small portion of the PS in the membrane be exposed on the outer leaflet. It is well-known that PS in erythrocyte membranes is concentrated on the inner leaflet, but the extent of this concentration is uncertain. Some reports say categorically that all PS is on the inner leaflet, and others report some PS on the outer leaflet. A recent review by Devaux (1992) states "...however, after 20 years of investigation we still do not know if 0 or 10% of PS is in the outer monolayer...". Each method for measuring external PS has its limitations in terms of specificity, access to phospholipids, extent of reaction, etc. PS on the inner leaflet of erythrocytes redistributes readily to the outer leaflet when cytoplasmic Ca^{2+} is elevated, and the continued segregation of PS to the inner leaflet requires an active ATP-driven translocase (Williamson et al., 1992). It was shown by Connor et al. (1990) that the distribution of fluorescent NBD derivatives of phospholipids accurately reflects their asymmetric distribution in erythrocyte membranes, and such

membranes show slight prothrombinase activity. This indicates some exposure of PS in the outer leaflet. Other experiments (Connor et al., 1992) show there is a separate slow translocase which moves PS from the inner to the outer leaflet in an ATP-dependent process. When P thionin was added to whole cells labeled with NBD-PC, there was a change in the NBD-PC domains (Figure 1A), which supports the idea that there is endogenous PS in the outer leaflet and the movement of NBD-PC reflects the formation of the PS/P thionin domains. Therefore, it is reasonable to assume that there is some PS in the outer leaflet, and it is a question of how much is there. If only 1% of the 4×10^7 PS molecules in an erythrocyte membrane were located on the outer leaflet, that would be sufficient to accommodate the binding sites for P thionin, which is in the range of 10^5 per cell (Vernon & Rogers, 1992b), even if there is more than one PS molecule bound per added P thionin. A question remains concerning the size and distribution of the PS domains which bind P thionin to native erythrocytes. The number of NBD-PS domains seen in our study varies from one to several per cell, and these could be formed by subsequent movement of PS within the membrane after the initial binding process. Further experiments are needed in this important area.

In our case it is reasonable to assume there are sufficient PS molecules on the outer leaflet to initially bind P thionin, resulting in a subsequent movement of PS (and also PC) within the membrane to form the observed domains of varying size, which increase in intensity for 8–10 min. P thionin can react with small concentrations of PS in bilayers as shown for vesicles containing DOPC and only 0.3% PS.

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