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Interaction of erythrocyte protein 4.1 with phospholipids. A monolayer and liposome study

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We have studied the interaction of purified human erythrocyte protein 4.1 with phospholipid membranes by monitoring both the increase in surface pressure of monolayers at the air/water interface and the change in permeability in liposomes to fluorescent molecules, in the presence of protein 4.1. Protein 4.1 penetrated into monolayers of brain phosphatidylserine (PS) and egg phosphatidylcholine (PC), even above surface pressures of 30 mN/m. Protein 4.1 increased the permeability of negatively charged PS, but not PC, liposomes, measured as the increase in fluorescence when encapsulated 1-aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *p*-xylenebispyridinium bromide (DPX) or carboxyfluorescein were released into the medium. The interaction of protein 4.1 with PS large unilamellar vesicles (LUV) was increased as the pH and the ionic strength were lowered, and decreased as the Ca^{2+} or Mg^{2+} concentrations and ionic strength were raised. In order to study the relevance of these measurements to the erythrocyte, we prepared LUV of synthetic lipid mixtures characteristic of both the inner and the outer membrane leaflets. Protein 4.1 increased the permeability of inner, but not outer, leaflet LUV at both pH 6.0 and 7.4. These observations suggest that negatively charged phospholipid domains around the protein 4.1 high-affinity protein-binding site(s) may contribute to the anchoring of protein 4.1 to the cytoplasmic surface of the red cell membrane.

Introduction

The human erythrocyte contains a fibrous meshwork of protein, known as the spectrin membrane skeleton, which lines the cytoplasmic surface of its membrane. This strong, yet flexible, structure is responsible for maintaining red cell shape, reversible deformability, and membrane structural integrity (for review see Ref. 1). It also restricts the lateral mobility of integral membrane proteins and may be involved in maintaining the transbilayer membrane asymmetry of the phospholipids [2–4]. Approximately equal molar amounts of phospholipids and unesterified cholesterol make up about 50% of the mass of the erythrocyte membrane. The transbilayer asymmetry of these phospholipids is manifested by the enrichment of

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Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; DPX, *p*-xylenebispyridinium bromide; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; LUV, large unilamellar vesicles; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino}-1-propanesulfonic acid; PI, phosphatidylinositol.

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the outer leaflet in choline-containing phosphatidylcholine (PC) and sphingomyelin, and the inner leaflet in the amino phospholipids phosphatidylethanolamine (PE) and PS [5,6]. The presence of membrane skeletal proteins in a large number of cell types (for review see Ref. 7), whose membranes are more complex than that of the erythrocyte, emphasizes the value of the erythrocyte membrane as a structural model for these cells.

Protein 4.1 is an essential component of the erythrocyte membrane skeleton. It appears as a single polypeptide of 78 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis utilizing a continuous buffer system [8], and can be resolved into a family of sequence-related peripheral membrane phosphoproteins by higher-resolution electrophoretic systems using a discontinuous buffer system [9]. The two major members of the protein 4.1 family, 4.1a and 4.1b (M_r = 80000 and 78000, respectively), are capable of binding to the ends of spectrin tetramers [10–12], and of stimulation a spectrin–actin interaction through the formation of a spectrin-protein 4.1-actin complex [13,14]. This ternary complex apparently links adjacent spectrin tetramers into a two-dimensional skeletal network.

When spectrin and actin are removed from the erythrocyte ghost by hypotonic extraction, 90% of the protein 4.1 remains attached to the membrane. The further addition of 1 M KCl is required to remove the protein from the membrane, suggesting that protein 4.1 has a high-affinity membrane attachment site which is separate from its interaction with spectrin and actin. Recent candidates for this site have been: (i) the sialoglycoprotein, glycophorin A [15]; (ii) the sialoglycoprotein, glycophorin C [16]; (iii) band 3 protein [17]; and (iv) phosphatidylserine domains [18–20]. Since there is evidence for specific, saturable, high-affinity protein-binding sites on the cytoplasmic surface of the erythrocyte membrane for proteins 4.1a and b [15,21], it is unlikely that protein 4.1 has high-affinity interactions with membrane phospholipids, although low-affinity interactions could occur, as in the case of spectrin [22].

Kimelberg and Papahadjopoulos [23] have shown that the ability of various soluble proteins to increase the permeability of PS membranes correlates directly with their ability to penetrate PS monolayers at an air/water interface. The authors proposed that the increase in permeability depended on hydrophobic associations of the protein with the PS film, and that these interactions were facilitated by initial electrostatic binding. They postulated an electrostatic attraction between the negatively charged PS monolayer and a globular protein possessing a hydrophobic interior and polar exterior with a net positive charge. Conformational changes in the protein would allow its hydrophobic portion to penetrate into the hydrocarbon region of the PS film.

We decided to investigate the possibility that protein 4.1 might behave in this fashion. In our initial experiments, the interactions of purified protein 4.1 with monolayers of the major erythrocyte membrane phospholipids were studied in a modified Wilhelmy surface balance. In this experiments, an increase in the surface pressure of a lipid monolayer composed of representative erythrocyte lipids was taken to indicate an interaction of these lipids with protein 4.1 from the subphase. Subsequently, we studied the ability of protein 4.1 to interact with liposomes and induce the leakage of entrapped materials. In these studies, an increase in fluorescence was taken to indicate some destabilizing interaction of protein 4.1 with the bilayer. We used two fluorescent probe systems: carboxyfluorescein and a combination of ANTS and DPX. We also examined the effect of lipid charge and composition, and the effect of calcium and magnesium.

Materials and Methods

Egg phosphatidylcholine (PC), 1,3-dioleoylglycerol, lysozyme (chicken egg white, grade I), α-casein, human serum albumin, diisopropyl fluorophosphate, L-histidine, Tes and dithiothreitol were purchased from Sigma (St. Louis, MO). Serum apolipoprotein A-I (a gift of Drs. Phoebe and Christropher Fielding) and α-casein were used as models to represent hydrophobic protein–lipid interactions [24,25], and lysozyme and human serum albumin were used to represent electrostatic interactions [23]. PS from bovine brain, sphingomyelin, dioleoyl-PE, and PI from bovine liver were from Avanti Polar (Birmingham, AL).

Cholesterol (recrystallized) was from Applied Sciences (State College, PA). Lipids were found to be chromatographically pure by thin-layer chromatography, except for PS, which was repurified by thin-layer chromatography [26]. Thin-layer chromatographic plates were from Whatman (type LK 5D, Clifton, NJ), DEAE-cellulose from Pierce (Rockford, IL), Sepharose 4B and Sephadex G-75 from Pharmacia (Piscataway, NJ), acrylamide, Tris, glycine, *N,N,N′,N′*-tetramethylethylenediamine, sodium dodecyl sulfate, ammonium persulfate and Coomassie blue R-250 from Bio-Rad (Richmond, CA), and carboxyfluorescein (purchased as chromatographically purified), ANTS, DPX from Molecular Probes (Eugene, OR). Water was double-distilled from glass.

Purification of proteins 4.1 and 2.1

Human erythrocyte proteins 4.1 and 2.1 were purified by the methods of Tyler and co-workers [11,12] with the modifications of Goodman et al. [10]. In brief, human erythrocyte ghosts were prepared essentially by the procedure of Dodge et al. [27], except that the lysing buffer consisted of 5 mM NaH_2PO_4-Na_2HPO_4/1 mM EDTA/0.4 mM diisopropyl fluorophosphate (pH 7.6). The ghosts were washed once with 0.1 mM EDTA/0.2 mM diisopropyl fluorophosphate (pH 8.0) and then incubated with this same buffer at 37 °C for 30 min to remove the skeletal proteins, spectrin and actin. In order to remove the majority of proteins 4.1 and 2.1 from the membrane, spectrin-actin-depleted inverted vesicles were incubated with 5 mM NaH_2PO_4-Na_2HPO_4/1 mM EDTA/1 M KCl/0.4 mM diisopropyl fluorophosphate (pH 7.6) at 37 °C for 60 min and then sedimented (300 000 × *g* for 30 min at 4 °C) (Beckman SW 41 rotor, Palo Alto, CA). The resultant supernatant, containing proteins 4.1 and 2.1, was dialyzed for 12–16 h at 4 °C against 5 mM NaH_2PO_4-Na_2HPO_4/1 mM EDTA/20 mM KCl (pH 7.6) (column buffer) and then loaded onto a DEAE-cellulose column (1.6 × 10 cm) previously equilibrated with the column buffer. The proteins were separated by elution with a stepped series of salt concentrations. Fractions containing the proteins were monitored by A_{280} readings and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis using the continuous system of Fairbanks et al. [8]. Protein 4.1 and 2.1 concentrations were determined by assuming $A^{1\%}_{280}$ values of 8.0 and 6.5, respectively [12]. Lipid extracts of protein 4.1 (0.6 mg) [28] were run on thin-layer chromatographs [26] which disclosed no phospholipid contaminants and less than 0.1% (by weight) free fatty acids. Protein 4.1 was denatured by heating to 50 °C for 20 min [21].

Purification of spectrin heterodimers

Human erythrocyte spectrin heterodimers were purified by the method of Goodman and Weidner [29] as follows. Spectrin heterodimers were extracted from human erythrocyte ghosts by a low-ionic strength extraction (0.1 mM EDTA/0.2 mM diisopropyl fluorophosphate (pH 8.0)) at 37 °C for 30 min. The crude spectrin extract was centrifuged (39 000 × *g* for 30 min at 4 °C) and the resultant supernatant loaded onto a Sepharose 4B column (2.5 × 90 cm), previously equilibrated with 20 mM Tris/1 mM dithiothreitol (pH 8.0). Spectrin-containing fractions were monitored by A_{280} readings, pooled, and then checked for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis [8]. Spectrin concentrations were determined by assuming an $A^{1\%}_{280}$ of 10.1 [12].

Lipids

Phospholipid concentrations were determined by phosphorus analysis [30]. Monolayers of each phospholipid were spread from a chloroform solution onto a 400 ml Teflon trough (surface area, 350 cm^2), and compression isotherms were performed as previously reported [31]. Areas ($Å^2$)/molecule at monolayer collapse were: (1) bovine brain PS, 60; (2) egg PC, 68; (3) bovine liver PI, 72; (4) dioleoyl PE, 55; and (5) 1,3-dioleoylglycerol, 67. In addition, the composition of the synthetic inner or outer leaflet mixtures was assessed by thin-layer chromatography [26] and phosphorus analysis [30].

Measurements of surface activity

Surface film pressure was measured with a platinum Wilhelmy dipping plate [31]. Each Teflon trough (surface area 350 cm^2, volume 400 ml; surface area 33 cm^2, volume 30 ml; or surface area 3.1 cm^2, volume 3 ml) was placed in a temperature-controlled chamber at 37 °C after being

scrubbed with phosphate-free detergent and rinsed exhaustively in tap water and distilled water. The appropriate volume of aqueous subphase (in excess of 400, 30 or 3 ml) was added to each trough and stirred, and its surface was cleaned by aspiration. Subphase buffer was 140 mM NaCl/20 mM Tris phosphate/0.5 mM EDTA/1 mM dithiothreitol (pH 6.0) (where maximal surface activity was found), except when the pH was varied. Monolayers of individual phospholipids or synthetic lipid mixtures were spread from chloroform solution onto the surface with a microsyringe. After a 10 min equilibration period, an appropriate amount of the protein to be analyzed was injected into the subphase and stirring was started. Surface pressure was continuously recorded for 1 h. Appropriate amounts of a synthetic lipid complex characteristic of either the inner or outer leaflet of the red cell membrane were also applied to form films with surface pressures equal to 0–35 mN/m at the air/water interface of a 30 ml Teflon trough. Based on the findings of Zwaal et al. [32], synthetic lipid combinations of PC/sphingomyelin/PE/PS (15 : 10 : 47 : 28, mol%) and PC/sphingomyelin/PE (44 : 44 : 12, mol%) were prepared to represent the inner and outer leaflet of the erythrocyte membrane, respectively. These are referred to hereafter as synthetic inner and outer leaflet mixtures. Cholesterol was also added to each mixture at a cholesterol/phospholipid molar ratio of 1 : 1. A set concentration of protein 4.1 (2.5 μg/ml) was introduced into the subphase and then stirred while the surface pressure (π) was recorded for 1 h. For the π-area isotherms, mixed monolayers of PS/protein 4.1 (1 : 1, w/w) and PS/α-casein (1 : 1, w/w) were dissolved in isopropanol/water (2 : 1, v/v) and spread onto a 400 ml Teflon trough, allowing a 10 min equilibration before monolayer compression. Control isotherms of pure PS, protein 4.1 and α-casein were also recorded. Isotherms were recorded with 20 mM Tris-acetate buffer at pH 6.0 with and without 10 mM Ca^{2+}.

Preparation of large unilamellar vesicles

Large unilamellar liposomes were prepared by reverse-phase evaporation according to Szoka and Papahadjopoulos [33], with some modifications [34], as follows: liposomes were each prepared with solutions containing (a) 50 mM carboxyfluorescein/2 mM L-histidine/2 mM Tes/10 mM NaCl/0.1 mM EDTA/(pH 7.4) (carboxyfluorescein-containing buffer), or (b) 45 mM DPX/12.5 mM ANTS/20 mM NaCl/2 mM Tes/2 mM L-histidine/1 mM EDTA (pH 7.4) (ANTS-DPX-containing buffer). The lipid (10 μmol) was dissolved in 1 ml of diethyl ether. 350 μl of carboxyfluorescein- or ANTS-DPX-containing buffer was added, and the mixture was sonicated for 5 min under argon. Diethyl ether was removed from the resulting emulsion by controlled flash evaporation, after which 650 μl of carboxyfluorescein- or ANTS-DPX-containing buffer was added and any residual diethyl ether was removed by further evaporation for 20 min. This mixture was extruded through polycarbonate membranes (Nucleopore, Pleasanton, CA) with 0.1 μm pores, under an argon pressure of approx. 60 lb/in^2, to form large unilamellar liposomes. Any multilamellar liposomes in the suspension were pelleted in an Eppendorf microcentrifuge (8000 $\times$ g for 20 min) (Brinkmann Model 3200, Toronto, Ont.). The liposomes were separated from nonencapsulated material on a Sephadex G-75 column (Pharmacia) equilibrated with either buffer A (100 mM NaCl/2 mM Tes/2 mM L-histidine/1 mM EDTA (pH 7.4)) for the carboxyfluorescein-containing PS or PC liposomes, or with buffer B (120 mM NaCl/2 mM Tes/2 mM L-histidine/1 mM EDTA (pH 7.4)) for the ANTS-DPX-containing liposomes. When the ionic strength of the buffer was varied, liposomes were each prepared with solutions containing 45 mM DPX, 12.5 mM ANTS, 2 mM Tes, 2 mM L-histidine, 1 mM EDTA and 0, 20, 135 or 575 mM NaCl. Buffer B contained 2 mM Tes, 2 mM L-histidine, 1 mM EDTA and 109, 130, 220 or 641 mM NaCl. Liposomal phospholipid concentrations were determined by phosphorus analysis [30]. Composition of mixed-lipid liposomes was assessed by thin-layer chromatography [26] and phosphorus analysis [30]. Vesicle size was determined by dynamic light scattering [35] using a Coulter Model N4 submicron particle size analyzer.

Measurements of liposomal leakage

The ability of protein 4.1 and other proteins to increase the permeability of LUV to carboxy-

fluorescein and ANTS/DPX was assayed in a spectrofluorometric system. Carboxyfluorescein self-quenches at high concentration within the liposomes, and DPX quenches the ANTS fluorescence when their intraliposomal concentrations are high. Release of fluorophores from the encapsulating liposomes produced an increase in fluorescence as the dyes became diluted. ANTS and DPX were used to study protein-induced membrane permeability changes because ANTS fluorescence is relatively independent of pH between pH 4.5 and 7.5 [36], and carboxyfluorescein was used as an independent validation of our ANTS-DPX results.

Fluorescence was measured in an SLM 4000 spectrofluorometer (SLM Instruments, Urbana, IL). Excitation was at 430 nm (carboxyfluorescein) or at 360 nm (ANTS). Emission was measured through a Corning 3-68 cutoff filter (carboxyfluorescein) (Corning Glass Works, Corning, NY) or through a Schott GG-435 cutoff filter (ANTS) (Melles Griot, Irvine, CA). The residual fluorescence of the liposomes containing either 50 mM carboxyfluorescein or 45 mM DPX and 12.5 mM ANTS was taken as 0% release. Maximal fluorescence, obtained after disruption of the liposomes with Triton X-100 (0.1%), was taken as 100% release. Leakage (percentage of maximal fluorescence) was measured in buffer A at pH 6.0 and 7.4 for the carboxyfluorescein/PS and carboxyfluorescein/PC liposomes, and in buffer B at pH 6.0 and 7.4 for the ANTS-DPX-containing liposomes. In these experiments the carboxyfluorescein or ANTS-DPX-containing liposomes were suspended in 1 ml of the appropriate buffer solution at a final concentration of 5 μM and the baseline fluorescence was recorded for 5 min. After 5 min, 0.010 ml of a predetermined amount of protein 4.1 was injected into the cuvette with constant stirring and the fluorescence was recorded for an additional 15 min. All experiments were conducted at 37 °C.

Results

Measurements of surface activity

In the surface activity experiments, an increase in surface pressure ($\Delta\pi$) of a lipid monolayer, particularly at high initial pressures ($\pi_i > 25$ mN/m), is taken to indicate an interaction of protein 4.1 from the subphase with the lipid film [23].

Influence of the composition of the monolayer. To determine whether this interaction was specific for PS, appropriate amounts of various lipids were applied to form 0–40 mN/m films at the air/water interface of a 30 ml circular Teflon chamber. Protein 4.1, at a final concentration of 2.5 μg/ml, was introduced into the subphase and then stirred while the surface pressure was recorded for 1 h. Fig. 1 shows data from such PS monolayers at 37 °C and pH 6.0, plotted as $\Delta\pi$ (at 1 h) versus π_i. The π_i intercept of a least-squares linear fit of these data with the π_i axis indicates the critical value above which no adsorption occurs. The $\Delta\pi$ intercept reflects the ability of the protein to absorb to an infinitely dilute lipid monolayer (practically speaking, a clean air/water interface). π_i intercepts for native and heat-denatured protein 4.1 were 36.0 ($r = 0.979$) and 28.1 mN/m ($r = 0.892$), respectively, reflecting the lower interaction of the heat-denatured preparation (Table II). Heat denaturation also caused a significant reduction in the ability of the protein itself to adsorb to the clean surface ($\Delta\pi$ intercept = 5.9 vs. 22.3 mN/m for the native protein). The values given in Table I demonstrate that PS, PC, dioleoyl PE, PI and 1,3-dioleoylglycerol appear to interact simi-

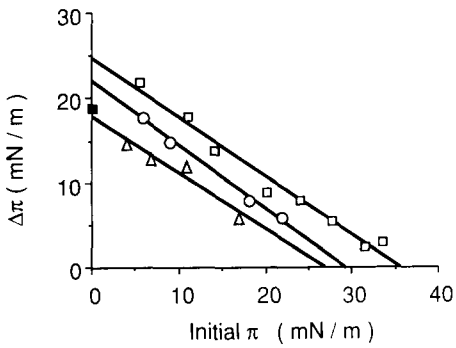

Fig. 1. Increase in surface pressure ($\Delta\pi$) of PS (□). PS/cholesterol (50:50, mol%) (○), and egg PC/cholesterol (50:50, mol%) (△) monolayers as a function of initial surface pressure (π_i). Protein adsorption to a surface without a lipid monolayer is also shown (■). Protein 4.1 (2.5 μg/ml) was introduced under each monolayer. Subphase buffer: 20 mM Tris-phosphate/140 mM NaCl/0.5 mM EDTA/1 mM dithiothreitol (pH 6.0). Temperature = 37 °C.

TABLE I

THE INTERACTION OF PROTEIN 4.1 WITH VARIOUS PHOSPHOLIPID MONOLAYERS

Data obtained from $\Delta\pi$ vs. π_i plots similar to those shown in Figs. 1 and 2. Conditions as in Fig. 1. Pairwise comparisons of slopes and intercepts (Newman-Keuls) disclosed no differences for which $P < 0.1$. DG, 1,3-dioleoylglycerol.

Phospho-lipid	π_i intercept (mN/m) [a]	$\Delta\pi$ intercept (mN/m) [a]	Slope [a]	r
PS	35.6 ± 2.4 -2.2	24.8 ± 1.0	-0.70 ± 0.05	0.987
PC	39.1 ± 2.9 -2.5	21.1 ± 0.8	-0.54 ± 0.05	0.987
PE	33.7 ± 1.8 -1.6	24.2 ± 0.8	-0.72 ± 0.05	0.993
PI	36.0 ± 3.6 -3.0	22.3 ± 1.1	-0.62 ± 0.07	0.979
DG	32.0 ± 2.2 -2.0	25.5 ± 1.0	-0.80 ± 0.06	0.982

[a] Mean ± S.E.

larly with protein 4.1. By Newman-Keuls analysis of variance, the slopes and $\Delta\pi$ intercepts did not differ among these lipids. However, PS and PC did not interact similarly with protein 4.1 when cholesterol (50 mol%) was present. The $\Delta\pi$ intercepts for PS/cholesterol (50 : 50, mol%) and egg PC/cholesterol (50 : 50, mol%) films were statistically lower than for pure PS and egg PC films, respectively, with the $\Delta\pi$ intercept for egg PC/cholesterol films being even lower than for PS/cholesterol films (Fig. 1). Protein 4.1 also adsorbed well to synthetic inner and outer leaflet mixtures at the air/water interface. Fig. 2 shows the data plotted as $\Delta\pi$ at 1 h versus π_i at pH 6.0. The $\Delta\pi$ and π_i intercepts were found to be 19.6 and 27.4 ($r = 0.989$) for the outer leaflet lipids and 23.8 and 32.9 ($r = 0.992$) for the inner leaflet lipids. This indicated a somewhat stronger interaction of the PS-containing inner leaflet with protein 4.1.

Effect of varying pH. Changes in surface pressure of PS monolayers spread to various π_i were measured at 1 h as a function of pH at different initial film pressures in the presence of a fixed concentration of protein 4.1 (2.5 μg/ml). When the lipid hydrocarbon chains were more widely spaced ($\pi_i = 5$ mN/m), $\Delta\pi$ was large (22 mN/m, pH 6.0), and when the monolayer was tightly packed ($\pi_i = 30$ mN/m), $\Delta\pi$ approached zero. Fig. 3 illustrates the data plotted as $\Delta\pi$ vs. pH, with separate curves for each π_i. The protein 4.1–PS interaction showed maximal surface activity near pH 6.0. Larger $\Delta\pi$ values were usually seen at each pH as π_i values were lowered.

Effect of varying the concentration of protein 4.1. Varying concentrations of protein 4.1 (2–28 μg/ml) were placed under PS films spread at a π_i of 5 or 15 mN/m. Fig. 4 shows $\Delta\pi$ at 1 h to be higher at higher protein 4.1 concentrations. This increase in $\Delta\pi$ was greater at lower pH (5.5 vs. 8) and at lower π_i (5 vs. 15 mN/m).

Pressure-area isotherms of mixed lipid/protein monolayers. From examining the π–area isotherms of spread PS and PS/protein 4.1 mixed films

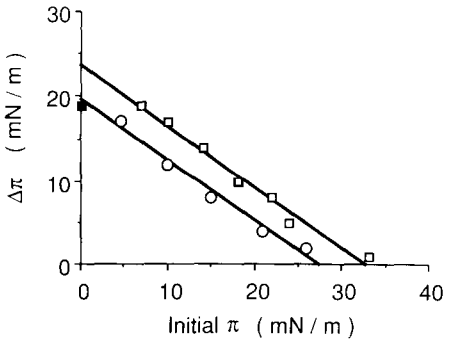

Fig. 2. Increase in surface pressure ($\Delta\pi$) of synthetic inner (□) and outer (○) red blood cell membrane lipid monolayers as a function of initial surface pressure (π_i). Protein adsorption to surface without a lipid monolayer is also shown (■). Experimental conditions as in Fig. 1.

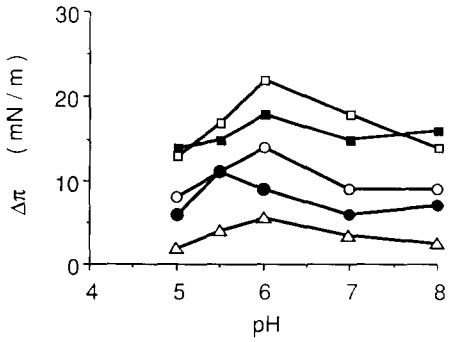

Fig. 3. Increase in surface pressure ($\Delta\pi$) of PS monolayers as a function of pH. Monolayers were spread at initial surface pressure (π_i) of 5 (□), 10 (■), 15 (○), 20 (●) and 30 (△) mN/m. Experimental conditions as in Fig. 1, except for pH.

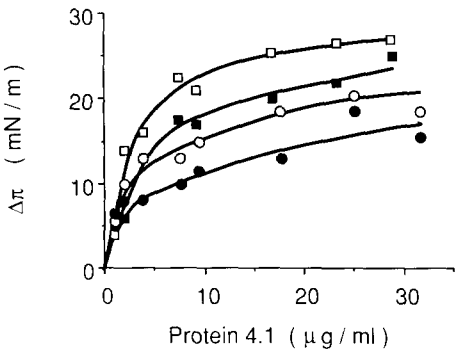

Fig. 4. Increase in surface pressure ($\Delta\pi$) of PS monolayers as a function of protein 4.1 concentration in the subphase. Monolayers were spread at initial surface pressures (π_i) of 5 mN/m over subphases of pH 5.5 (□), and 8.0 (■), and at π_i of 15 mN/m over pH 5.5 (○) and 8.0 (●). Experimental conditions as in Fig. 1, except for pH.

(1 : 1, w/w), we found that adding protein 4.1 expanded the PS isotherms (data not shown). Calcium (10 mM) produced small condensations of both the pure PS isotherms and the PS/protein 4.1 isotherm. Because the pure protein 4.1 monolayer collapsed at a π value lower than that of the pure PS film and the mixed PS/protein 4.1 film collapsed at an area/molecule ratio greater than the PS film, the protein was considered to be entrained in the surface by the lipid. From π–area isotherms of spread PS and PS/α-casein mixed films (1 : 1, w/w), wer found that adding α-casein produced a more expanded PS isotherm. This is consistent with the large π_i intercept for α-casein with PS films (40.7 mN/m) shown in Table II. In addition, pure α-casein alone had significant

TABLE II

THE INTERACTION OF VARIOUS PROTEINS WITH PHOSPHOLIPID MONOLAYERS

Data have been obtained from $\Delta\pi$ vs. π_i plots similar to those shown in Figs. 1 and 2. Conditions are as in Fig. 1. Pairwise comparisons of slopes and intercepts (Newman-Keuls) disclosed some differences, which are discussed in the text.

Protein	π_i intercept [a] (mN/m)	$\Delta\pi$ intercept [a] (mN/m)	Slope [a]	r
PS monolayer				
Protein 4.1	36.0 + 3.6 − 3.0	22.3 ± 1.1	−0.62 ± 0.07	0.979
Denatured protein 4.1	28.1 + 7.5 − 5.3	5.9 ± 0.7	−0.21 ± 0.05	0.892
Spectrin	35.3 + 3.5 − 3.2	21.5 ± 1.0	−0.61 ± 0.05	0.969
Protein 2.1	34.9 + 20.7 − 10.4	6.9 ± 1.7	−0.20 ± 0.09	0.778
Albumin	22.2 + 1.0 − 0.9	4.9 ± 0.1	−0.22 ± 0.01	0.998
Lysozyme	26.8 + 3.1 − 2.8	14.5 ± 0.5	−0.54 ± 0.04	0.975
α-Casein	40.7 + 2.6 − 2.4	20.6 ± 0.6	−0.51 ± 0.03	0.984
Apolipoprotein A-I [b]	27.7 + 2.3 − 2.1	23.9 ± 1.1	−0.86 ± 0.07	0.976
PC monolayer				
Protein 4.1	39.1 + 2.9 − 2.5	21.1 ± 0.8	−0.54 ± 0.05	0.987
Protein 2.1	36.1 + 5.1 − 3.7	7.3 ± 0.5	−0.20 ± 0.03	0.973
Apolipoprotein A-I [b]	23.2 + 0.9 − 0.9	25.6 ± 0.7	−1.10 ± 0.06	0.995
Spectrin [c]	22.0	20.3	−0.92	

[a] Mean ± S.E.

[b] Subphase buffer at pH 8.0.

[c] 2 points only.

inherent surface activity as shown by the $\Delta\pi$ intercept (20.6 mN/m) in Table II. These findings are typical of the interaction of a hydrophobic protein with a lipid monolayer [25].

Effects of various proteins. Table II shows the results of experiments performed to compare other proteins with protein 4.1. Protein concentrations were all 2.5 μg/ml, and conditions were otherwise the same as in Figs. 1 and 2. There were differences in the slopes and $\Delta\pi$ intercepts for some of the proteins by Newman-Keuls analysis of variance. The red cell skeletal protein, spectrin, under a PS monolayer at pH 6.0 had surface activity similar to protein 4.1; however, its interaction with a PC monolayer at pH 6.0 was less than that for protein 4.1. In comparison, protein 4.1, at lower π_i (less than 20 mN/m) interacted equally well with PS and PC (Tables I and II). Protein 2.1, another red cell skeletal protein, demonstrated little surface activity at pH 6.0 whether under PS or PC monolayers. Human serum albumin, as used previously to represent electrostatic interactions [23], showed little surface activity at pH 6.0 under a PS monolayer and behaved similarly to heat-treated protein 4.1 and protein 2.1. Lysozyme, used as an example of a basic protein at pH 6.0 and 7.4 (pI = 11.0) [37], demonstrated more activity than human serum albumin, heat-treated protein 4.1 and protein 2.1, but less than proteins 4.1, spectrin and α-casein. Serum apolipoprotein A-1 and α-casein were used to represent hydrophobic interactions [24,25]. Apolipoprotein A-1 interacted equally well with PS and PC at pH 8.0. It also behaved differently from protein 4.1 under a PS monolayer at pH 8.0. $\Delta\pi$ and π_i intercepts of 23.9 and 27.7 (r = 0.976) and 19.6 and 31.5 (r = 0.956) were obtained for apolipoprotein A-1 and protein 4.1, respectively. Lastly, α-casein and spectrin interacted similarly to protein 4.1 under a PS monolayer at pH 6.0.

Influence of calcium, magnesium and ionic strength. PS or PC monolayers were spread at π_i = 10 mN/m and a fixed concentration of protein 4.1 (2.5 μg/ml) was added to the subphase. The interaction of protein 4.1 with PS was unaffected by Ca^{2+} at either pH 6.0 or 8.0 (13, 14, 13 mN/m at pH 6.0 and 9, 13, 9 mN/m at pH 8.0, in the presence of 0, 1 and 10 mM Ca^{2+}, respectively). The interaction of protein 4.1 with PC was also unaffected at these pH values (11, 11, 11 mN/m at pH 6.0 and 10, 12, 8 mN/m at pH 8.0, in the presence of 0, 1 and 10 mM Ca^{2+}, respectively). In addition, the interaction of protein 4.1 with PS monolayers was unaffected by Mg^{2+} at either pH 6.0 or pH 8.0 (18, 19, 19 mN/m at pH 6.0 and 16, 14, 14 mN/m at pH 8.0, in the presence of 0, 1 and 10 mM Mg^{2+}, respectively). This interaction was not affected by varying the ionic strength of the subphase buffer at pH 6.0 or at pH 8.0 (I = 1.5, 21.5, 71.5, 161.5, and 321.5 mM, yielding 17, 17, 17 18, and 19 mN/m at pH 6.0 and 15, 18, 16, 18, and 16 mN/m at pH 8.0, respectively).

Measurements of liposomal leakage

Protein 4.1 induced leakage of coencapsulated ANTS and DPX or carboxyfluorescein from large unilamellar vesicles. The release of these fluorophores into the medium resulted in an increase in fluorescence.

Permeability of liposomes to ANTS-DPX. ANTS-DPX fluorescence measurements in Fig. 5 show that protein 4.1 (4 μg/ml) induced more rapid leakage from PS than from PC liposomes. This pattern was seen both at pH 6.0 and pH 7.4. Fig. 6 shows the rates of ANTS-DPX release (percentage maximal fluorescence/min; % F_{max}/min) after the addition of various concentrations

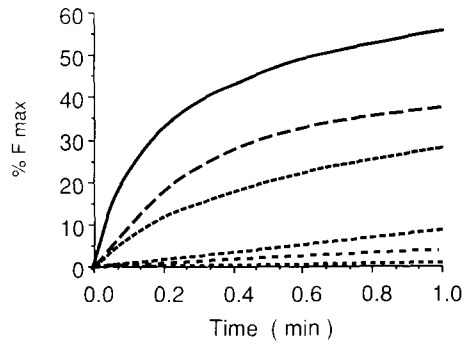

Fig. 5. Time-course of ANTS/DPX release by protein 4.1 from PS, PS/cholesterol, and PC liposomes. Protein 4.1 (4.0 μg/ml) was injected into buffer containing liposomes (5 μM). Curves, starting from top (solid line): PS liposomes at pH 6; PS, pH 7.4; PS/cholesterol (77:23, mol%), pH 6; PS/cholesterol, pH 7.4; PC, pH 6; and PC, pH 7.4. Buffer: 2 mM Tes/2 mM L-histidine/1 mM EDTA/120 mM NaCl. Temperature = 37°C.

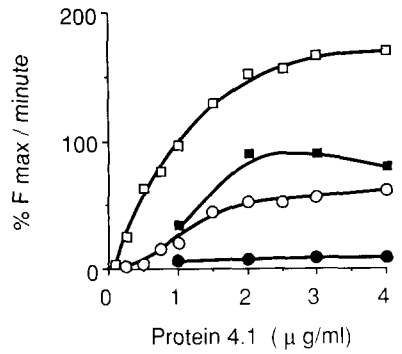

Fig. 6. Rate of ANTS-DPX release (% maximal fluorescence/min) by protein 4.1 from PS liposomes at pH 6.0 (□) and 7.4 (■), and from PS/cholesterol (77:23, mol%) liposomes at pH 6.0 (○) and 7.4 (●) versus protein 4.1 concentration. Experimental conditions as in Fig. 5.

of protein 4.1 (0–4 μg/ml) to pure PS or PS/cholesterol vesicles. The initial rates of release were calculated from the maximal slopes of the fluorescence vs. time curves after the addition of protein. Higher initial rates of release were seen at pH 6.0. The rates of ANTS-DPX release from pure PC vesicles were substantially lower. Specifically, the rates produced by 4, 10 or 24 μg protein 4.1/ml were 2, 2 and 2 at pH 7.4, and 4, 4 and 6 at pH 6.0. When pure PS vesicles were preincubated with Ca^{2+} or Mg^{2+}, protein 4.1 (4 μg/ml) was less able to increase membrane permeability at pH 6.0 and at pH 7.4. However, Ca^{2+} caused a greater decrease in the % F_{max}/min at each pH. The rates produced by 0, 0.5, 1 and 2 mM Ca^{2+} at pH 6.0 were 260, 120, 90 and 50% F_{max}/min and by 0, 0.5, 1 and 2 mM Mg^{2+} at pH 6.0 were 260, 210, 200 and 120, respectively. At pH 7.4, the rates produced by the above Ca^{2+} and Mg^{2+} concentrations were 70, 38, 30 and 15, vs. 76, 48, 36 and 31, respectively. An upper limit of 2 mM Ca^{2+} was used because higher concentrations produce vesicle fusion which would have caused the release of contents [38]. In addition, the interaction of protein 4.1 with PS decreased as the ionic strength was raised at both pH 6.0 and 7.4 (data not shown). When PS vesicles containing cholesterol (23 mol%) were used, protein 4.1 produced fluorescent patterns at pH 6.0 and 7.4 which were intermediate between the results obtained with pure PS vesicles and pure PC vesicles (Fig. 5). In addition, the amounts of probe released at the end of 1 min were greater at pH 6.0 than at pH 7.4 (Fig. 5). Fig. 6 shows the rates of ANTS-DPX release after the addition of various concentrations of protein 4.1 to PS vesicles containing cholesterol (23 mol%). Higher initial rates were observed at pH 6.0. Preincubation of these vesicles (37°C, 3 min) with increasing concentrations of Ca^{2+} or Mg^{2+} at pH 6.0 or 7.4 resulted in a corresponding decrease in membrane permeability upon the addition of protein 4.1 (4 μg/ml). The rates produced by 0, 0.5, 1 and 2 mM Ca^{2+} at pH 6.0 were 120, 50, 40 and 30% F_{max}/min, and by 0, 0.5, 1 and 2 mM Mg^{2+} at pH 6.0 were 130, 86, 60 and 40. At pH 7.4, the rates produced by these Ca^{2+} and Mg^{2+} concentrations were 17, 10, 8 and 5, vs. 20, 14, 10 and 8, respectively. In this case, both divalent cations decreased equally the ability of protein 4.1 (4 μg/ml) to increase permeability at pH 6.0 and 7.4. When cholesterol was higher than 23 mol%, leakage was negligible at pH 6.0 and 7.4.

Permeability of PS and PC liposomes to carboxyfluorescein. In these experiments, a different fluorescent probe, carboxyfluorescein, was encapsulated inside LUV and used to study the ability of protein 4.1 to induce leakage. Carboxyfluorescein was used as an independent validation of our ANTS-DPX results. Fig. 7 shows that protein 4.1 (10 μg/ml) induced leakage, with higher fluorescence recorded at each time point at the lower pH of 6. Due to the protonation of carboxyfluorescein at lower pH values, a basal leakage

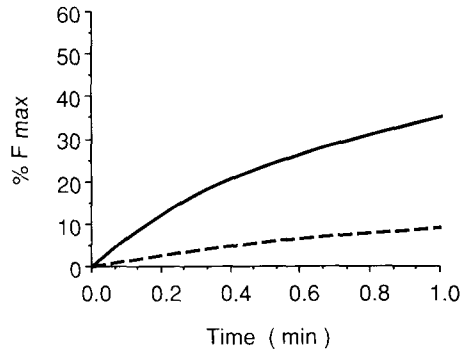

Fig. 7. Time-course of carboxyfluorescein release by protein 4.1 from PS liposomes at pH 6.0 (solid line) and pH 7.4 (dashed line). Experimental conditions as in Fig. 5, except that the NaCl concentration was 100 mM.

rate of 4%/min occurred at pH 6 (but not at pH 7.4). The initial rates of carboxyfluorescein release (%F_{max}/min) from pure PS vesicles after the addition of various concentrations of protein 4.1 (0–10 μg/ml) were 1, 14, 28, 40 and 60 at pH 6.0 and 0, 2, 5, 9 and 14 at pH 7.4; that is, they were higher at pH 6.0. In contrast, the rates of carboxyfluorescein release from pure PC vesicles were much lower. Specifically, the rates produces by 5, 10 or 24 μg protein 4.1/ml were 2, 1 and 0 at pH 7.4 and 4, 3, and 4 at pH 6.0. In order to determine the effect of divalent cations on the protein 4.1–PS interaction, pure PS vesicles were preincubated (37 °C, 3 min) with increasing concentrations of Ca^{2+} or Mg^{2+} (0–2 mM). After the addition of protein 4.1 (3 μg/ml), a decrease in vesicle permeability at pH 6.0 was seen when either divalent cation concentration approached 2 mM. A 50% decrease in %F_{max}/min was obtained with either 2 mM Ca^{2+} or 2 mM Mg^{2+}. At pH 7.4, neither Ca^{2+} nor Mg^{2+} had any effect on the protein 4.1–PS interaction.

Permeability of synthetic inner or outer leaflet lipid mixtures. To study the relevance of these measurements to the erythrocyte, synthetic lipid mixtures characteristic of either the inner (PS/sphingomyelin/PC/PE/cholesterol, 14 : 5 : 7.5 : 23.5 : 50, mol%) or the outer (sphingomyelin/PC/PE/cholesterol, 22 : 22 : 6 : 50, mol%) leaflet of the red cell membrane were used to prepare LUV. In addition, liposomes similar to these mixtures (PS/sphingomyelin/PC/PE, 28 : 10 : 15 : 47, mol% and sphingomyelin/PC/PE, 44 : 44 : 12, mol%) but containing no cholesterol were also prepared. Experiments were carried out at pH 6.0 and at pH 7.4. The addition of protein 4.1 (20 μg/ml) did not cause any permeability changes in the synthetic outer leaflet mixtures at either pH, whether cholesterol was present or not. Protein 4.1 also did not induce leakage of the inner leaflet mixture without cholesterol at pH 7.4. Protein 4.1 did induce permeability changes in the inner leaflet mixture at both pH 6.0 and at pH 7.4 as well as in the inner leaflet mixture without cholesterol at pH 6.0 (Fig. 8). Inner leaflet vesicles with (23 mol%) and without cholesterol released the same amount of fluorescent probe. The presence of Ca^{2+} or Mg^{2+} (1 mM) decreased the amount of probe released from the inner leaflet vesicles (50 mol% cholesterol) at pH 6.0 and 7.4.

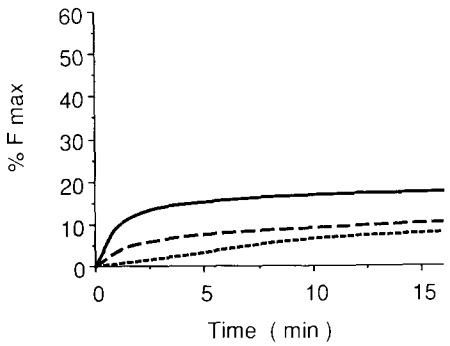

Fig. 8. Time-course of ANTS-DPX release by protein 4.1 from liposomes characteristic of the inner leaflet of the red cell membrane. Curves, starting from the top (solid) line: liposomes of PS/sphingomyelin/PC/PE/cholesterol (14 : 5 : 7.5 : 23.5 : 50, mol%), pH 6.0; same liposomes except at pH 7.4; and liposomes of PS/sphingomyelin/PC/PE (28 : 10 : 15 : 47, mol%), pH 6.0. Experimental conditions as in Fig. 5, except for 20 μg/ml protein 4.1.

Discussion

Although our studies at the air/water interface indicated that there was an interaction between phospholipid monolayers and protein 4.1, this interaction did not seem to be of an electrostatic nature. Table I shows that protein 4.1 interacted similarly with both charged PS and neutral phospholipid monolayers, but not with PS or egg PC monolayers which contained cholesterol (50 mol%, Fig. 1). When the pH of the subphase buffer under a PS monolayer was varied at constant π_i, the variation in $\Delta\pi$ with pH was small (Figs. 3 and 4). In addition, the interaction was not affected by varying the ionic strength of the subphase buffer at pH 6.0 or 8.0, or by varying the Ca^{2+} and Mg^{2+} concentrations at either pH 6.0 or 8.0.

Both spectrin and protein 4.1 interacted with PS monolayers, whereas protein 2.1 did not (Table II). These results are consistent with other studies of lipid–protein binding [20] and phospholipid–spectrin interactions in the surface balance [22]. Heat denaturation, under conditions reported previously to reduce substantially the interaction of protein 4.1 with membranes [21], caused a significant reduction in the surface activity of protein

4.1 ($\Delta\pi = 5.9$ for heat-treated vs. 22.3 for native at $\pi_i = 0$) (Table II). This may be due to a structural change in the protein (e.g., the formation of higher order oligomers) impeding adsorption to the surface. The relative surface activities of the various proteins were consistent with published results [23–25].

In contrast to the monolayer studies, the membrane permeability studies using liposomes containing fluorescent probes showed that the interaction of protein 4.1 with negatively charged PS was pH-dependent, and that there was essentially no interaction with zwitterionic PC, regardless of the pH. Similar results were obtained with carboxyfluorescein and ANTS-DPX, and are consistent with the results of binding assays published by Sato and Ohnishi [18]. Both Ca^{2+} and Mg^{2+} caused a decrease in the interaction of protein 4.1 with PS or PS/cholesterol (77 : 23, mol%) vesicles. With pure PS vesicles, Ca^{2+} caused a greater decrease in the rate of release of contents than did Mg^{2+}, providing additional evidence for an electrostatic interaction in this liposome system. This finding agrees with the previously reported association constants of Ca^{2+} and Mg^{2+} for PS [39,40], although the results do not indicate whether this effect is due to the interaction of the divalent cation with PS or with the protein itself.

The rates and extent of leakage from the PS/cholesterol liposomes (77 : 23, mol%) were much lower than those values obtained from pure PS liposomes. This finding parallels the reduced surface activity of protein 4.1 under a PS/cholesterol (50 : 50, mol%) monolayer. One possible explanation for this is that cholesterol condensed the lipid bilayer by filling the potential space provided by double bonds at the second position fatty acids, and thereby prevented protein 4.1 insertion. The ability of cholesterol to impair protein-induced leakage from unilamellar liposomes containing unsaturated phospholipids has been previously reported [41].

On the other hand, the amount of fluorescent probe released from synthetic inner leaflet vesicles containing cholesterol (50 mol%) at pH 6.0 was more than that released when either cholesterol was absent (Fig. 8) or when 23 mol% was present (data not shown). At pH 7.4, protein 4.1 caused leakage only from inner leaflet liposomes containing 50 mol% cholesterol, and not from those containing 23 mol% or no cholesterol. However, these inner leaflet vesicles had a very different lipid composition (PC/ sphingomyelin/ PE/ PS/ cholesterol, 7.5 : 5 : 23.5 : 14 : 50, mol%) than the PS/cholesterol vesicles (77 : 23, mol%) described above. It is possible that this high proportion of cholesterol (50 mol%) induced a lateral phase separation of the lipids in the inner leaflet mixture, thereby creating PS-rich and cholesterol-depleted domains capable of interacting with protein 4.1. It is also interesting that protein 4.1 did not induce leakage from the PS-free synthetic outer leaflet vesicles at pH 6.0 or at pH 7.4, in the presence or absence of cholesterol. One can conclude that both the amount of cholesterol and the phospholipid and fatty acid compositions of the lipid mixtures are important in determining the interaction of protein 4.1 with synthetic lipid mixtures.

In summary, protein 4.1 penetrated into PS monolayers and increased the permeability of PS liposomes, whereas it did not change the permeability of PC liposomes, even though it penetrated into PC monolayers. The differences between our surface activity and liposomal permeability measurements of PC may be explained by the fact that protein–lipid interactions could occur in a liposomal system without producing leakage. The reason for our detecting an interaction between protein 4.1 and PC in the surface balance while others [18] have not reported appreciable binding of ^{125}I-protein 4.1 to PC is not clear; however, those authors did find some binding of ^{125}I-protein 4.1 to PC-containing liposomes which lack PS. In the process of adsorbing to, and interacting with, a lipid film at an air-water interface, protein 4.1 probably undergoes a conformational change allowing its hydrophobic portion to penetrate the hydrocarbon region of a lipid film. The conformation of this protein under these experimental conditions may be quite different from the form which associates with lipid vesicles in an aqueous solution. Therefore, the interaction of protein 4.1 with a lipid monolayer at an air/water interface may well be qualitatively different from the association of labeled protein 4.1 with phospholipid bilayer vesicles in aqueous suspension. In contrast to the surface film measurements, the fluorescence studies do provide evidence for an electrostatic

interaction between PS and protein 4.1.

Since protein 4.1 has both acidic and basic segments [42], it is likely that the latter interact with regions of the membrane rich in PS. This process may be followed by insertion of part of the molecule into the hydrophobic core of the bilayer, resulting in partial breakdown of the permeability barrier of the membrane. This could represent a transient destabilization of the membrane, or a sustained leakage [43]. The interaction of protein 4.1 with PS vesicles suggests that negatively charged phospholipid domains around the high-affinity protein-binding site(s) of protein 4.1 may contribute to the anchoring of the protein to the erythrocyte membrane.

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