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The interaction of lung annexin I with phospholipid monolayers at the air/water interface

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Abstract

Lung annexin I (LAI), a calcium-ion-dependent phospholipid-binding protein, has been shown earlier to cause aggregation and fusion of bilayered vesicles containing phospholipids found in lung surfactant, and to be a very likely factor in the assembly of lung surfactant into the lamellar bodies stored in the Type II cell. In this study, we have measured the accumulation of LAI into spread monolayers of some major lipid components of lung surfactant, dipalmitoyl-phosphatidylcholine (DPPC), dipalmitoyl-phosphatidylglycerol (DPPG), palmitoyl-oleyoyl-phosphatidylglycerol (POPG), and selected mixtures, as a function of calcium-ion concentration and surface concentration (degree of packing) of the phospholipid monolayer. The ability of LAI to significantly penetrate such monolayers was calcium-ion-dependent and only occurred in the presence of DPPG or POPG. The relative extent of penetration into DPPG and POPG was directly related to the available free area in the monolayer, penetration being greater with POPG. Fluorescence microscopy measurements revealed that DPPC mixed with either DPPG or POPG caused a change in surface phase behavior in a manner believed to be related to certain types of bilayer fusion. A chemical breakdown product of LAI, LAI-bp, previously found not to cause aggregation and fusion of bilayers, did not exhibit comparable monolayer penetration or surface phase separation to LAI.

Keywords: Lung surfactant; Phospholipid; Annexin; Monolayer; Protein-lipid interaction

1. Introduction

The annexins are a group of structurally related, calcium-dependent, phospholipid-binding proteins that are widely present in plant and animal tissues [1–5]. To-date, ten homologous proteins found in human tissues have been identified suggesting that they function in a broad range of biological processes. Many common in-vitro activities have been found for these proteins; however, their exact biological functions are not yet known [6]. Annexin I, of interest in the present study, has been hypothesized to be involved in mediating membrane fusion [7–12]. It has been suggested that through Ca^{2+}-dependent binding to anionic phospholipids, oligomerization or

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conformational changes in the protein at a phospholipid–water interface trigger aggregation of vesicles and bilayer fusion [12]. Aggregation and fusion experiments, in vitro, using chimeric annexins and mutant annexin I, have demonstrated the ability of annexin I to promote membrane aggregation and fusion. These processes are sensitive to changes in the structure of the N-terminal domain, suggesting that the N-terminus plays an important role in its ability to promote fusion [13]. A number of studies have examined molecular events taking place during the interaction of several annexins with model phospholipid bilayer and monolayer systems. It has been shown, for example, that Annexins V and VI primarily bind to anionic phospholipid headgroups through an interaction with calcium ions that are bound to polypeptide loops at specific sites in the annexin [14–19]. Such an interaction was shown to give rise to a two-dimensional organization of the annexin at the phospholipid–water interface, closely related to the corresponding three-dimensional crystal structure, suggesting minimal penetration of the protein into the phospholipid monolayer or bilayer [14]. Other studies indicate the strong possibility of an additional direct interaction of the protein with the phospholipid, either via a tryptophan residue exposed upon calcium-binding [20–22] or a conserved basic site (most likely a conserved lysine residue) on the annexin and the acidic phospholipids [23]. Recent studies on annexin IV with pyrene-PG/PC vesicles, using a fluorescence energy-transfer assay [24] or with supported bilayers of POPC and POPG using fluorescence recovery after photobleaching (FRAP) [25], measured lateral lipid diffusion and changes in the lipid bilayer structure brought about by the presence of the annexin. It was concluded that annexin IV induced a surface phase separation, dependent on the presence of POPG and that such structural changes in the bilayer might be related to the effects of annexins on bilayer fusion.

Recently, Tsao has purified and characterized a 36 kDa calcium-dependent phospholipid-binding protein with a pI of 6.0 from a rabbit lung cytosolic fraction and has shown it to have homology with human annexin I [26]. In vitro, this lung annexin I, LAI, significantly inhibits phospholipase A_2 activity (normally associated with inflammation) at phospholipid interfaces containing anionic phospholipids [27,28] and causes aggregation and fusion of a variety of negatively charged unilamellar vesicles, but has no effect on neutral phosphatidylcholine (PC) vesicles [29].

In vivo, LAI is richly present in alveolar epithelial type II cells which synthesize, store, and secrete pulmonary surfactant complex [26]. The annexin I levels in fetal rabbit lungs also markedly increased at late gestational ages, suggesting a role in lung development [26]. In vitro, LAI has been demonstrated to have a high affinity binding to PG, the most abundant anionic phospholipid in lung surfactant [29]. In view of these properties, it was concluded that LAI in type II cells might have a role in the assembly of lamellar bodies, the multilayer membranous surfactant storage organelles. LAI also has been found in bronchoalveolar lavage (BAL) fluids from humans [30,31] and animals [26,32]. In BAL fluid from humans, the annexin I level increased in response to corticosteroids [30]. This property, in combination with LAI inhibition of phospholipase A_2, suggests a role for this protein in inflammation suppression [30]. It also has been shown that LAI in BAL fluids samples from patients with various lung diseases [30,31,33] is mostly degraded to smaller molecular weight products, predominately in the range of 33 kDa molecular weight. For example, in the epithelial lining fluids from patients with cystic fibrosis, LAI degradation was shown to be due to the depletion of the N-terminus of LAI mediated by neutrophil elastase, resulting in a complete reduction of LAI functional activity [33]. The change in LAI structure and activity under pathophysiological conditions led to the preparation of the LAI breakdown product (LAI-bp) from rabbit LAI. This rabbit LAI-bp had similar structure and properties to that found in the patients' BAL fluids.

Previous studies in this laboratory have been concerned with the surface phase behavior of PC and PG monolayers in various combinations and the potential for surface phase separation, as measured by fluorescence microscopy, with particular emphasis on the mixing of DPPC, with DPPG and POPG, all major components of lung surfactant [34]. In other studies, we have measured the surface activity of LAI in the absence of phospholipids to learn more about its intrinsic hydrophobicity and surface activity [35]. It was found that LAI adsorbs to the air/water interface

at surface concentrations as high as 1.9 mg/m^2 and that increasing Ca^{2+} up to 10 mM increased this surface activity in the absence of anionic phospholipids at the interface. In this study, we report on the surface activity of LAI in the presence of various PC and PG monolayers as a function of initial surface pressure or surface density and lipid composition. Concurrently, we have measured the effects of LAI on mixed monolayer surface phase behavior and compared such behavior with that of its biologically inactive breakdown product, LAI-bp.

2. Materials

The phospholipids used in this study were: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-rac-glycerol (DPPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-glycerol (POPG), 1,2-dioleoyl-*sn*-glycero-3-phospho-rac-glycerol (DOPG), and 1-palmitoyl-2-[12-[7-nitro-2-1,3-benzoxadiazol-4-yl)amino] dodecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC) (Avanti, Alabaster, AL) all of which had stated purities of 99 + % and were used as received. Chloroform (99.9%, Aldrich) was used as the spreading solvent for all lipids except DPPG, and was used as received. DPPG was dissolved in chloroform : methanol (99.9%, Fisher Scientific) : water (98.9 : 1 : 0.1).

The water used throughout these studies was house-distilled water passed through a Barnstead PCS filtration system that contained an organic removal cartridge, two mixed ion-exchange resin cartridges, a super organic removal cartridge, and a 0.2 μm filter. The water was then distilled twice, once from an alkaline potassium permanganate solution and once from a dilute sulfuric acid solution. The subphase pH was controlled with a 10 mM Tris (hydroxymethyl) aminomethane (Tris) (99.9%, Sigma) buffer that was adjusted to pH 7.4 with 1 N HCl (A.C.S. reagent grade, Aldrich). Selected experiments were performed with added calcium chloride (99.99 + %, Aldrich) or ethylenediaminetetraacetic acid (EDTA) (99.5%, A.C.S. reagent, Aldrich). All experiments were performed at 25°C.

Lung annexin I (LAI) (36 kDa, pI 6.0) was extracted from rabbit lung cytosolic fraction and purified to homogeneity. Details of the isolation have been given elsewhere [29].

The lung annexin I breakdown products (LAI-bp) was obtained by repeated freezing–thawing of purified annexin I (1 μg/μl 0.9% saline) during a 6-month period. The LAI-bp solution was applied to an Amicon microconcentrator-10 (Amicon, Danvers, MA) to allow the passage of fragments with molecular weights < 10 kDa. The fraction remaining in the microconcentrator was washed twice with 0.9% saline and concentrated. The purity of LAI and LAI-bp were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis under denaturing conditions and by thin-layer isoelectric focusing methods [26]. The N-terminal sequence of LAI-bp was determined using an automated Model 477A liquid pulse sequencer and Model 475A gas phase sequencer with on-line Model 120A PTH analyzer and 610A data analysis system (Applied Biosystems, Foster City, CA) at the University of Wisconsin Biotechnology Center. The LAI-bp obtained had an apparent molecular weight of 33 kDa and an isoelectric point (pI) value of 8.5. This sample totally lost annexin I functional activity in now not being able to promote vesicle aggregation and inhibition of phospholipase A_2 activity [27,29]. Partial amino acid sequencing of LAI-bp revealed an amino acid sequence of P-G-S-A-V-S-P-Y-P-A-F-N-P-S-S-D-V-A-A-L. This sequence is idential to the sequence starting at Pro-32 at the N-terminus of the native LAI amino acid sequence, deduced from the rabbit annexin I cDNA nucleotide sequence (accession No. U24656, GenBank/NCBI). The properties of rabbit LAI-bp in terms of molecular weight, pI value, and the N-terminal amino acid sequence were similar to that of the annexin I breakdown product found in the bronchoalveolar lavage fluids from patients with cystic fibrosis [33].

Both proteins, LAI and LAI-bp, were stored in concentrated form (typically 5 μg/μl) in silanized centrifuge tubes at −70°C in 0.01 M Tris-HCl buffer containing 5 mM 2-mercaptoethanol, 1 mM EDTA, and 0.15 M NaCl at pH 7.4. The activity of the protein was assayed by measuring the transfer of [^{14}C]DPPC/ [^{3}H]triolein from unilamellar liposomes to multilamellar liposomes made with PC,

dicetylphosphate and cholesterol, as described elsewhere [29].

3. Methods

3.1. Surface pressure measurement and injection of protein beneath lipid monolayers

Experiments measuring the change in surface pressure upon injection of the protein into the subphase beneath the lipid monolayer were carried out using a specially designed small volume (~ 35 ml) trough. The entire instrument was enclosed in a box to reduce drafts and dust and to maintain a high relative humidity ($> 80\%$). The monolayer was spread on one side of a Teflon barrier from lipid solutions using a Hamilton microsyringe (Hamilton, Reno, NV) and was allowed to equilibrate. This could take any where between 30 min to 24 h depending on the lipid used and the state of the monolayer. Mixed monolayers were deposited using a mixed spreading technique [36] where both lipids were dissolved into a single spreading solution. The subphase temperature was maintained using a refrigerated constant temperature circulator (VWR model 1165, VWR, West Chester, PA) which circulates water through channels located in the aluminum base. The initial surface pressure, π_i, of the monolayer at equilibrium was obtained using the Wilhelmy plate technique [37]. The protein was then injected into the subphase from the opposite side of the barrier and the subphase was stirred by placing the trough on a Thermolyne nuova II stirrer (Barnstead/Thermolyne, Dubuque, IA) and placing a stir bar (3 mm in diameter, 10 mm in length) into the well of the trough. The final equilibrium surface pressure was then measured, typically 15–24 h after injection of the protein.

The trough was also designed so that it could be placed onto the stage of a fluorescence microscope (to be discussed below). Surface flow during microscopic observation was controlled using a mask which captured a portion of the monolayer in a small circular cutout in a removable Teflon piece, as previously reported [38,39]. A canal from the surrounding monolayer to the circular cutout ensured equilibration of the monolayer both, inside and outside of the circular area.

3.2. Fluorescence microscopy at the air / water interface

Each of the single-component films and the binary mixed films were examined with the fluorescence microscope to investigate monolayer surface phase behavior. In order to use this technique, 1 mol % of the dye NBD-PC was added to the spreading solution. The monolayer mixture was spread and allowed to equilibrate anywhere from 15 min to several hours. Studies at this level of dye concentration reveal a negligible effect of the dye on the monolayer π-A diagram as described in detail earlier [34].

The fluorescence microscope, constructed from a commercially available microscope (Micromaster, Model E, Fisher Scientific, Pittsburgh, PA) has been described in detail previously [34]. Surface pressure measurement was made using a Nima pressure transducer (Nima, Coventry, UK). The microscope was connected to a video camera (Optronics Engineering, Goleta, CA) via a video relay lens (10x) and the image was viewed on a monitor and stored onto video tape (Mitsubishi HS-U65). Images were captured using a Mac Vision video digitizer and Mac Vision image processing software (Koala Acquisitions), and image analysis was performed using NIH Image software (v. 1.55) available from the National Institutes of Health (Washington, DC).

4. Results

4.1. Surface pressure change in phospholipid monolayers caused by lung annexin I

4.1.1. Effects of lung annexin I concentration

Various amounts of LAI were injected into the subphase containing 5 μM $CaCl_2$ beneath a DPPG monolayer at $\pi_i = 5.0 \pm 0.5$ mN/m with stirring and the resulting change in surface pressure, $\Delta\pi$, was measured. As seen in Fig. 1, the data points follow a sigmoidal curve which appears to begin to level off at ≈ 5 nM. Higher concentrations of LAI were not investigated because of the limited supply of purified protein. As expected from earlier studies [35], no change in surface pressure was measured in the absence of a monolayer, even at a concentration of 4 nM of LAI, indicating that the presence of the

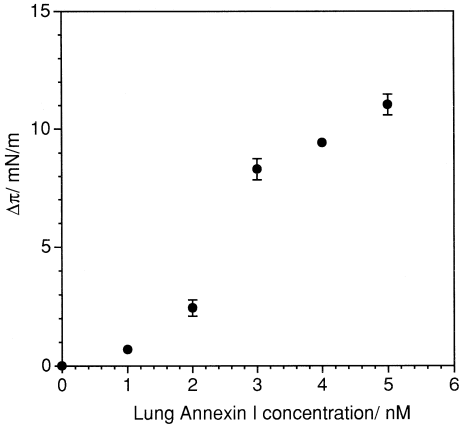

Fig. 1. Change in surface pressure, $\Delta\pi$, after injection of various amounts of lung annexin I below a DPPG monolayer spread on 10 mM Tris-HCl, pH 7.4, 25°C, 5 μM $CaCl_2$, $\pi_i = 5.0$ mN/m.

monolayer significantly enhances the surface activity of LAI.

4.1.2. Effects of calcium ion concentration

Fig. 2 shows the changes in surface pressure measured after injection of LAI (5 μg) into the subphase below a DPPG monolayer which contained various concentrations of calcium ions. The error bars represent the range of data points based on at least two independent experiments. In the presence of sufficient amounts of EDTA to bind the Ca^{2+}, $\Delta\pi$ was ~ 2–3 mN/m, indicating measurement of some non-calcium-dependent effects on surface pressure. Increasing the calcium concentration to 0.5 μM significantly enhanced LAI-phospholipid interactions; however, as the concentration of calcium was further increased, $\Delta\pi$ decreased, indicating an additional effect of calcium on the monolayer (discussed below). The rate of lung annexin I accumulation at the interface, as reflected in the rate of change of surface pressure (data not shown), was also found to increase upon addition of calcium ions to the subphase. All subsequent studies were conducted at a Ca^{2+} concentration of 5 μM, where $\Delta\pi$ values with DPPG and POPG were very significant, under the conditions of the various experiments conducted. The change in surface pressure upon addition of LAI (5 μg) below a DPPC monolayer spread on buffer containing 1 mM EDTA was also measured and found to be 2.6 ± 0.3 mN/m, close to the value of 1.8 ± 0.4 mN/m obtained in the presence of 5 μM $CaCl_2$, thus indicating only non-specific monolayer penetration of DPPC.

The relative specificity of the LAI-DPPG interactions for Ca^{2+} was further tested by performing experiments on subphases that contained the divalent cations, magnesium or barium, instead of calcium. As seen with the data presented in Table 1, at a divalent cation concentration of 5 μM, the interaction of LAI (5 μg) increases in the following order: $Ca^{2+} > Ba^{2+} > Mg^{2+} >$ EDTA, with Ca^{2+} mediating the interactions to a greater extent than the other divalent cations tested.

The reversibility of the calcium-dependent interaction of LAI with DPPG monolayers was examined by first spreading the DPPG monolayer on buffer containing 5 μM $CaCl_2$ to a given π_i. After allowing the monolayer to equilibrate, LAI was injected below the monolayer and the system was again allowed to equilibrate. As reported above, the surface pressure increased upon addition of LAI to monolayers at a low π_i (~ 5 mN/m), and decreased when added to monolayers equilibrated at high π_i (~ 30 mN/m). A concentrated EDTA solution (0.5 M) was injected below the monolayer to form a subphase that contained a 1 mM EDTA solution. No significant change in surface pressure was observed at either π_i value, indicating considerable irreversibility of LAI calcium-mediated interactions with DPPG monolayers

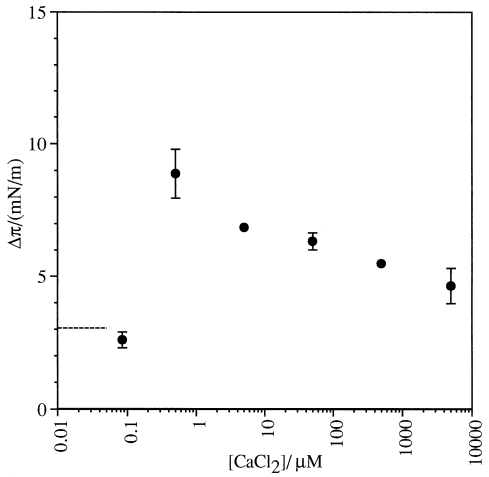

Fig. 2. Change in surface pressure, $\Delta\pi$, after injection of 5 μg of lung annexin I below a DPPG monolayer spread on 10 mM Tris-HCl, pH 7.4, 25°C, and various concentrations of $CaCl_2$. The dashed line represents the $\Delta\pi$ obtained at zero concentration of $CaCl_2$ in the presence of 1 mM EDTA.

Table 1
Change in surface pressure upon injection of LAI (5 μg) below a DPPG monolayer spread on Tris-HCl, pH 7.4, with added salts at an initial surface pressure of 5.0 ± 0.5 mN/m

Subphase ion	$\Delta\pi$ (mN/m)
5 μM magnesium chloride	5.0 ± 0.5
5 μM calcium chloride	9.4 ± 0.0
5 μM barium chloride	6.4 ± 0.3
1 mM EDTA	3.1 ± 0.3

under these conditions. The order of the addition of the calcium and EDTA solutions was then reversed, wherein the experiment was conducted in a similar fashion except that the monolayer was initially equilibrated at a π_i of ~8 mN/m on a subphase that contained 1 mM EDTA. LAI was then injected below the monolayer and only a slight increase in surface pressure (2–3 mN/m) was measured, as already observed, for the non-calcium-dependent interactions. Addition of $CaCl_2$ (a sufficient amount to increase the subphase $CaCl_2$ concentration to 5 μM in the absence of EDTA) did not enhance the LAI interaction as measured by $\Delta\pi$. Thus, it is concluded that the $\Delta\pi$ values measured in the presence of Ca^{2+} with DPPG represent true Ca^{2+}-dependent phospholipid binding of LAI.

4.1.3. Effects of phospholipid structure and initial surface pressure

The change in surface pressure upon addition of LAI (5 μg) as a function of the initial surface pressure was measured for lipid monolayers of DPPC, DPPG, and POPG (Fig. 3) spread on buffer containing 5 μM $CaCl_2$. The values of $\Delta\pi$ for the DPPC monolayer were all small, 2–3 mN/m or less, similar to the value observed when EDTA was used as a subphase in the experiments performed with DPPG. This, again, would appear to indicate only nonspecific interaction between LAI and DPPC. For the PG lipids, as π_i increased the corresponding values of $\Delta\pi$ decreased. In all cases, the $\Delta\pi$ values reached zero somewhere in the region $20 < \pi_i < 30$ mN/m. Interestingly, at $25 < \pi_i < 35$ mN/m, negative $\Delta\pi$ values were measured with the POPG monolayer but not with DPPG (to be discussed below). Finally, at π_i of ~40 mN/m, $\Delta\pi$ was zero for all lipids. Either the protein was no longer interacting with the

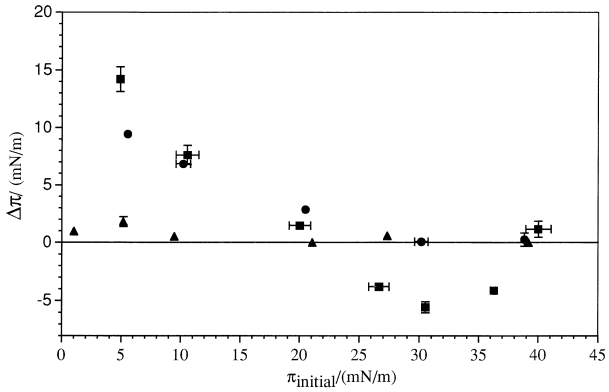

Fig. 3. Change in surface pressure, $\Delta\pi$, after injection of 5 μg of lung annexin I below phospholipid monolayers spread on a 10 mM Tris-HCl, pH 7.4, 25°C, 5 μM $CaCl_2$ at various initial surface pressures, π_i: (▲) DPPC; (●) DPPG; and (■) POPG.

monolayer or, more likely, $\Delta\pi$ measurements were no longer sensitive to the perturbations caused by the interaction of the protein with the monolayer at this surface pressure. A similar $\Delta\pi$–π_i profile was obtained for a DPPG monolayer spread on buffer containing 5 mM $CaCl_2$ rather than 5 μM $CaCl_2$ (data not shown).

The changes in surface pressure upon injection of LAI (5 μg) below mixed DPPC/PG monolayers spread on buffer containing 5 μM $CaCl_2$ at π_i ~ 5 mN/m were measured at various surface compositions as shown in Fig. 4. A small $\Delta\pi$, 2–3 mN/m, was measured in mixtures that only contained

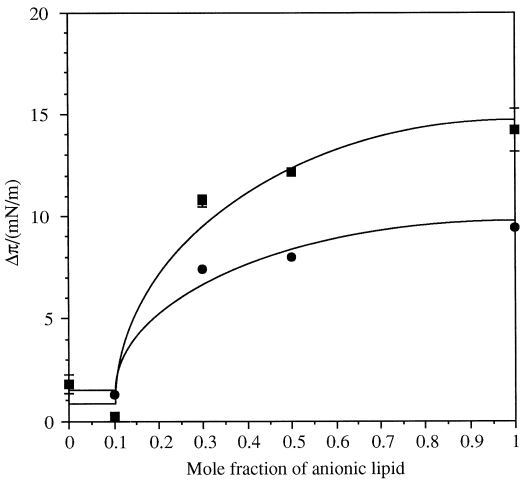

Fig. 4. Change in surface pressure, $\Delta\pi$, after injection of 5 μg of lung annexin I below different compositions of: (●) DPPC/DPPG; (■) DPPC/POPG, spread on 10 mM Tris-HCl, pH 7.4, 25°C, 5 μM $CaCl_2$, and $\pi_i = 5.0$ mN/m.

Table 2
Change in surface pressure upon injection of protein (5 μg) below a mixed DPPC/POPG (1:1) monolayer spread on 10 mM Tris-HCl, pH 7.4, 25°C, and 5 μM $CaCl_2$ at indicated initial surface pressure

π_i (mN/m)	$\Delta\pi$ (mN/m)	
	LAI	LAI bp
1.2 ± 0.2	8.8	3.3
5.8 ± 1.0	11.4	2.3
16.0 ± 0.5	−2.8	−2.3

10 mol% of anionic lipid, close to the value obtained for DPPC monolayers alone. Increasing the anionic lipid content to 30 mol%, however, significantly increased $\Delta\pi$, with these increasing values further as the anionic lipid content of the mixed monolayer was increased. The $\Delta\pi$ values for the mixed DPPC/POPG monolayer were consistently higher than those obtained for the mixed DPPC/DPPG monolayers.

The possible interaction of LAI-bp with the DPPC/POPG binary monolayers at three values of π_i was also studied. Table 2 compares the changes in the surface pressure for each protein at approximately the same π_i. LAI-bp appeared to interact with the binary monolayers only in a non-specific fashion, yielding very small $\Delta\pi$ values even at π_i as low as 1 mN/m.

4.2. Effects of lung annexin I on phase equilibria in mixed DPPC / POPG mixtures as measured by fluorescence microscopy

Mixtures of DPPC and POPG, under the current experimental conditions, were found in previous studies to phase separate at the air/water interface at a π as low as 1 mN/m [34]. Furthermore, the amount of phase separation was found to increase as surface

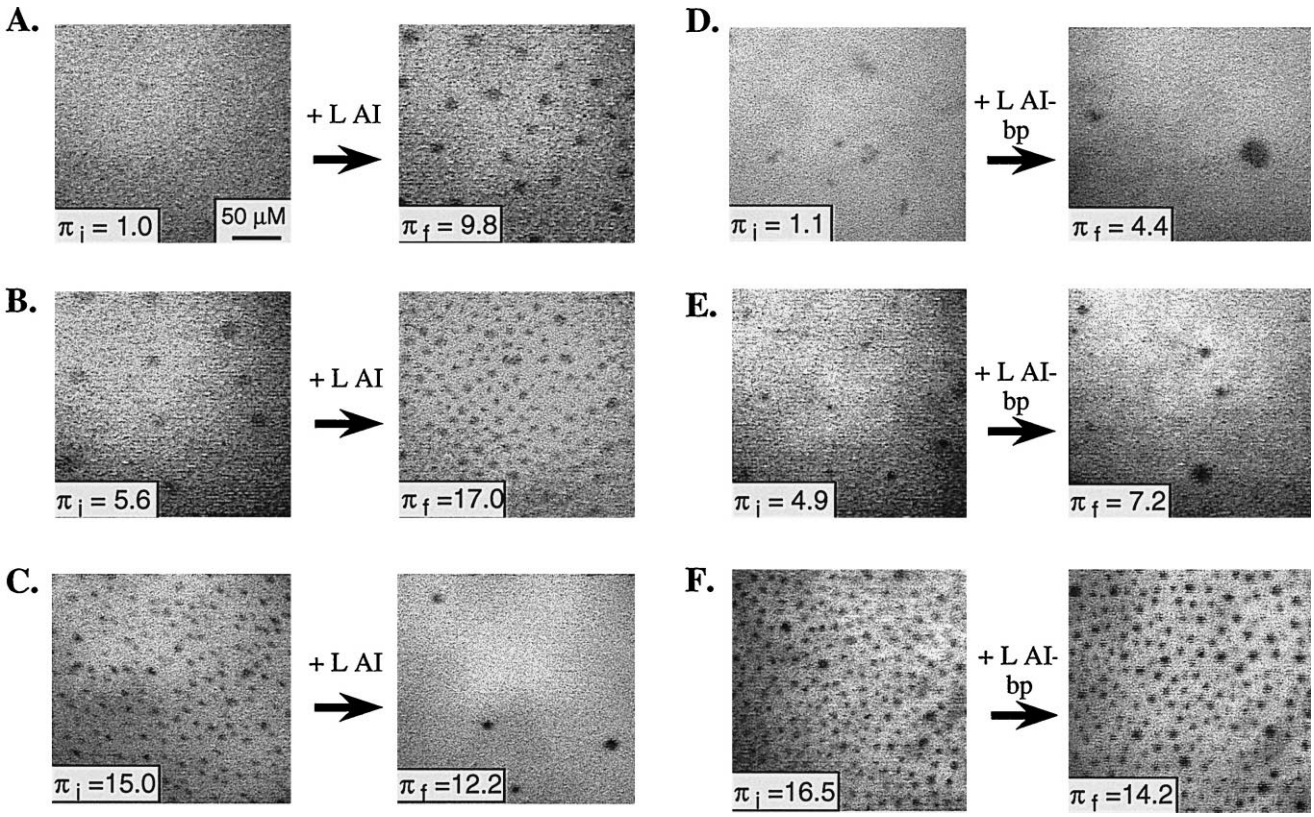

Fig. 5. Fluorescence microscope images of binary DPPC/POPG (1:1) monolayers spread on 10 mM Tris-HCl, pH 7.4, 25°C, 5 μM $CaCl_2$ before, and after the addition of LAI (A, B, and C) or LAI-bp (D, E, and F) to the subphase. Initial surface pressure (π_i in mN/m) of the monolayer and final surface pressure (π_f in mN/m), i.e. after addition of the protein, are given in each respective image.

Table 3
The percentage of dark domains before, and after the injection of protein (5 μg) below a DPPC/POPG (1:1) binary monolayer spread on 10 mM Tris-HCl, pH 7.4, 25°C, and 5 μM $CaCl_2$, at various initial surface pressures (see Figs. 5–7).

Protein	π_i (mN/m)	% dark (before)	% dark (after)
LAI	1.0	0.9	10.3
	5.5	4.5	9.3
	15.0	6.8	1.8
LAI-bp	1.1	2.0	4.2
	4.9	1.1	1.7
	16.5	8.4	10.9

pressure increased. In the present study, the changes in phase equilibria of mixed DPPC/POPG monolayers (with 1 mol% NBD-PC) spread on buffer containing 5 μM $CaCl_2$, after injection of protein (5 μg) into the underlying subphase, were examined using fluorescence microscopy at various π_i for LAI and LAI-bp (Fig. 5(A–F)). The images both, before and after introduction of the protein were recorded and the percentage of dark domains in each field were quantified using the NIH Image software (Table 3). At low π_i (up to $\pi_i \sim 6$ mN/m), the addition of LAI increased surface pressure as was described above. As expected, based on previous observations, an increase in the amount of phase separated material from 10- to 20-fold. At higher π_i, however, a decrease in surface pressure was measured and an unusually large loss (i.e. the presence of a lesser amount of domains at the same surface pressure in the absence of protein) in the amount of phase separated material was observed. The breakdown protein, in contrast, only changed the surface pressure by small amounts, $\sim 2–3$ mN/m, regardless of π_i. Correspondingly, no significant change in the appearance of dark domains was observed for any π_i examined (Fig. 5 and Table 3).

5. Discussion

The affinity and specificity of LAI for phospholipids spread as monolayers has been investigated by measuring the change in surface pressure, $\Delta\pi$, upon injection of LAI beneath a variety of single-component and binary monolayers at a low initial surface pressure of 5 mN/m. In the absence of a monolayer, at this LAI concentration, no $\Delta\pi$, and hence adsorption, can be measured, while in the presence of a lipid monolayer the addition of LAI appears to have significant affinity for the interface (Fig. 1). The $\Delta\pi$ value for zwitterionic DPPC was found to be significantly smaller than for the anionic phospholipids, indicating specificity of the protein for the anionic phospholipids.

Further evidence showing that LAI interacts specifically with anionic lipids in these monolayer systems was gathered from the experiments performed with the mixed DPPC/PG monolayers. Fig. 4 shows that low $\Delta\pi$ values (~ 2 mN/m) were obtained in the presence of only 10 mol% anionic lipid, the same value expected when a pure DPPC monolayer is spread, thus indicative of the dominance of nonspecific interactions. The interactions, as measured by $\Delta\pi$, however, are significantly increased by increasing the amount of anionic lipid to 30 mol%. Addition of 50 mol%, or higher, of anionic lipid did not increase $\Delta\pi$ significantly, suggesting that there may be a specific stoichiometry that exists between LAI and the number of anionic lipids necessary to maximize LAI-anionic phospholipid interactions.

Beyond any specific interactions that might be occurring between LAI and the anionic phospholipids, it is of interest to examine the role played by the phospholipid monolayer at a particular value of π_i in influencing $\Delta\pi$. To do this, let us assume that there must be sufficient void space in the monolayer to accommodate the protein so as to produce a $\Delta\pi$. Furthermore, let us define this void space in terms of free area, A_f, where

$$A_f = A - A_0 \quad (1)$$

and where A is the area/molecule at a particular π_i and A_0 is the area actually occupied per molecule, obtained from the π–A isotherm at the point of closest packing and collapse. In Fig. 6, we present values of $\Delta\pi$ obtained for LAI and various phospholipids as a function of the free area, A_f at a particular π_i. To expand our ability to examine this effect, we have included one additional set of data using DOPG, a much more expanded monolayer relative to DPPC, DPPG, and POPG [34] and, hence, very different expected values of A_f at the same π_i. From Fig. 6 it is apparent that the significant differences noted in $\Delta\pi$ for DPPC and the anionic phospholipids are not controlled by free area alone. However, it appears

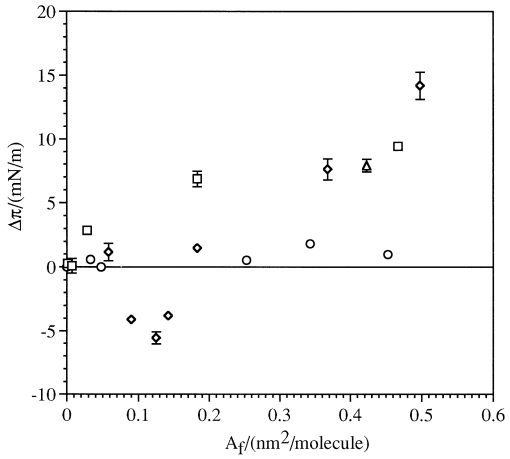

Fig. 6. Change in surface pressure, $\Delta\pi$, after injection of 5 μg of lung annexin I below phospholipid monolayers spread on 10 mM Tris-HCl, pH 7.4, 25°C, 5 μM $CaCl_2$ as a function of free area, A_f, of the monolayer: (□) DPPG; (○) DPPC; (◇) POPG; and (△) DOPG.

that differences between the three molecules in the PG group may be correlated with A_f. This seems to indicate that, at low π_i, the ability of LAI to penetrate and create an increase in surface pressure, $\Delta\pi$, arises because of the specific Ca^{2+}-dependent interaction coupled with the free area available at the interface. Thus, as the free area decreases, $\Delta\pi$ decreases despite the strong tendencies for the interaction between LAI and the various anionic phosphatidylglycerols. Indeed, from the data shown in Fig. 3, we can conclude that any LAI that had penetrated the monolayer at lower π_i and higher A_f, had been excluded or ''squeezed out'' at π_i equaling $\sim$20–25 mN/m and higher. Based on earlier studies with other annexins and certain anionic phospholipid monolayers that reported ordered arrays of annexin beneath the monolayers at higher π_i [14,15,18], we would conclude that in the present case beyond π_i values of 20–25 mN/m, LAI remains interacted with the anionic phospholipids, but it cannot penetrate into the monolayer. One indication of this comes from the results for LAI and POPG at $25\,\text{mN/m} \leq \pi_i \leq 35\,\text{mN/m}$, where we observe negative values of $\Delta\pi$. Such a decrease in π at constant area appears to indicate that the underlying LAI has caused a condensation of the POPG monolayer through the specific interaction, thus giving rise to an overall reduction in surface pressure. Such condensation, however, does not seem to occur with DPPG. To test this hypothesis, we can estimate the compressibility of each monolayer, C, where

$$C = -\frac{1}{A}\frac{dA}{d\pi} \quad (2)$$

under the conditions of these experiments, and observe the extent to which DPPG is less compressible than POPG. Table 4 lists the compressibilities of DPPG and POPG at π_i equal to 30 mN/m and, indeed, C is much less for DPPG than for POPG. Therefore, we might expect the interaction of LAI not to cause any significant change in the area/molecule within the DPPG monolayer and, thus, not affect surface pressure. The POPG monolayer, in contrast, is relatively compressible and, in this case, we might expect the interaction of the protein to reduce the area/molecule of the lipid film causing a drop in surface pressure. For all of the monolayers studied, no interactions of the protein with the lipid monolayer could be detected at π_i of $\sim$40 mN/m or greater, using $\Delta\pi$ experiments.

The calcium-dependent nature of annexin–phospholipid interactions has been well documented in the literature [6]. Therefore, it would be expected that calcium would play an important role in LAI–phospholipid interactions as measured in this study. Experiments with PG monolayers in the absence, and presence of calcium have shown that some non-calcium-dependent interaction of LAI with anionic monolayers occurs. However, small amounts of calcium (0.5 μM) significantly enhance these interactions (Fig. 2). The decrease in surface pressure observed at higher levels of calcium (> 5 μM) in Fig. 2 most likely occurs because of the ability of calcium to simultaneously affect the DPPG monolayer, causing it to become more condensed. To understand this more clearly, it is helpful to refer to the Gibbs

Table 4
Compressibility of PG monolayers spread on 10 mM Tris-HCl, pH 7.4, 25°C, and 5 μM $CaCl_2$, at a surface pressure of 30 mN/m

Lipid	Area (nm^2/molecule)	Compressibility (mN^{-1})
DPPG	0.402	7.11×10^{-4}
POPG	0.618	7.19×10^{-3}

equation for two components, the spread monolayer, and an adsorbing solute,

$$d\pi = RT\Gamma_I d\log a_I + RT\Gamma_S d\log a_S \quad (3)$$

where Γ is the surface concentration, a the activity of the component, R the gas constant, and T the temperature. The symbols I and S refer to the spread phospholipid monolayer component and the adsorbed protein, respectively. Calcium has been shown to increase the intrinsic non-specific surface activity of LAI at the air/water interface [35], so we might expect $\Delta\pi$ to be dominated by the effect of Ca^{2+} on the second term in Eq. (1) at low calcium concentrations, with little effect on the first term in Eq. (1); therefore, an increase in surface pressure would be expected as the surface concentration of the species being adsorbed, Γ_S, increases upon being introduced to the sub-solution. At higher calcium concentrations, however, Ca^{2+} also has a significant effect on the state of the monolayer. It has been previously observed, for example, that 5 mM $CaCl_2$ significantly condenses both DPPG and POPG monolayers [34]. This would have a large effect on the first term in Eq. (1), causing a loss in π as the monolayer is condensed; thus the initial increases in $\Delta\pi$ shown in Fig. 2 are most likely due to an increase in the surface activity of LAI while the decreases in $\Delta\pi$ are most likely due to the condensation of the monolayer caused by the higher calcium concentration. This condensation would be expected to decrease the protein's ability to penetrate the monolayer.

The ability of LAI to mediate fusion of phospholipid vesicles is most likely related to the protein's ability to interact with the lipids (as was shown above) and to perturb the lipid organization, in some way. The interaction of LAI with phase separated DPPC/POPG (1:1) binary monolayers was found to be significantly different from the observations made above for single component monolayers. In both systems, the interaction of LAI caused an increase in surface pressure at relatively small π_i and $\Delta\pi$ decreased with increasing surface pressure. However, ''squeeze-out'' or expulsion of LAI occurred at π_i of only 15 mN/m (data not shown) and a condensation of the monolayer was measured at pressures as low as 20 mN/m. A likely cause for these differences is the nature of single vs. two-component monolayer homogeneity. While the single component anionic monolayers are homogeneous in charge distribution throughout the monolayer, the binary monolayer is highly phase separated and would necessarily have an inhomogeneous distribution of charges which could quantitatively affect $\Delta\pi$ caused by the interaction of the protein with the monolayer (to be discussed below).

Fluorescence microscopy of the mixed monolayers, in this study, was performed to detect changes in phase equilibria that may occur upon introduction of the protein. For LAI at low surface pressures, when the monolayer is ''loosely'' packed, the protein appears to interact specifically with the anionic PG lipids in a Ca^{2+}-dependent manner by penetrating into the monolayer, causing it to expand and increasing π. The resulting increase in surface pressure shifts the phase equilibria of the monolayer so that it now exhibits properties characteristic of the new surface pressure, i.e., increased phase separation in the DPPC/POPG mixed monolayers (Fig. 5). This is schematically illustrated in Fig. 7(A). At high surface pressure, when the monolayer is relatively ''tightly'' packed, the protein is no longer able to penetrate the monolayer but it is able to interact with the monolayer from the subphase side and condense the anionic PG lipids. This allows the expansion of the other species within the film, thus reducing the surface pressure at the constant area and decreasing the phase separation in the DPPC/POPG mixed monolayers (Fig. 5). This is schematically illustrated in Fig. 7B.

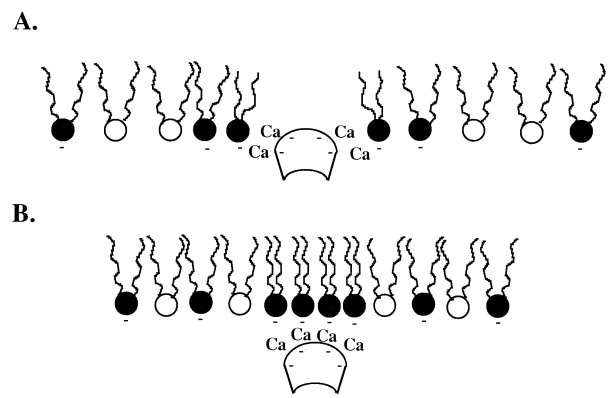

Fig. 7. A schematic illustration of how lung annexin I might interact with anionic phospholipids (●) spread as monolayers with DPPC (○) at an air/water interface: (A) at low initial surface pressure; and (B) at high initial surface pressure.

Finally, the interaction of LAI-bp was investigated and compared to that of LAI under a few conditions using the binary DPPC/POPG monolayer. The LAI-bp interactions resulted in $\Delta\pi$ values that were indicative of nonspecific interactions, i.e. 2 mN/m. This observation strongly suggests that the N-terminus, although it may not be directly involved with phospholipid-binding, does play a significant role in determining the specificity of LAI-anionic phospholipid interactions. In contrast to LAI, LAI-bp did not change the phase equilibria in the monolayer to any extent. The nonspecific interaction of LAI-bp with DPPC/POPG monolayers and its inability to elicit any structural changes in the monolayer may explain the lack of aggregation, fusion activity, and phospholipase A_2 inhibition of this protein as observed by Tsao et al. [33].

6. Conclusions

Using a relatively simple in vitro model system, containing phospholipids representative of those found in lung surfactant, we have been able to demonstrate the ability of lung annexin I, LAI, to penetrate into phospholipid monolayers and to differentiate its Ca^{2+}-dependent binding to anionic phosphatidylglycerols from its nonspecific penetration into the zwitterionic DPPC. For the Ca^{2+}-dependent binding, we have shown that LAI penetrates the phospholipid monolayer to an extent related to the available free area, A_f, and that as the free area is decreased beyond a certain level of close packing LAI is excluded from the monolayer. Thus fluctuations in the packing of anionic phospholipid molecules at an interface can determine the extent to which LAI is inserted after the specific interaction has occurred. We provide further evidence to show that LAI, either in the monolayer or excluded, exerts a significant Ca^{2+}-dependent effect on the structure and properties of the phospholipid monolayer. In particular, we have shown with mixed monolayers, such as DPPC/POPG, that LAI can produce both enhanced and decreased surface phase separation, depending on the degree of molecular packing and the compressibility of the lipids involved. These effects of surface phase behavior could play an important role in the ability of LAI to promote bilayer fusion and aggregation in vitro and in vivo.

Preliminary experiments with the LAI breakdown protein, which does not have the aggregation and fusion activity possessed by LAI, showed that this protein did not have any specific affinity for the binary DPPC/POPG monolayers and did not have the ability to alter the phase equilibria. This suggests that the amino-terminus is involved in regulating the protein's ability to mediate specific interactions with anionic phospholipid monolayers.

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