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Analysis of binding and membrane destabilization of phospholipid membranes by surfactant apoprotein B

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Abstract

To further elucidate the nature of the molecular interactions of surfactant apoprotein B (SP-B) with phospholipid (PL) membranes, we studied the binding of SP-B to PL membranes and the lipid-dependency of its subsequent effects on leakage and fusion of membranes. SP-B binding to membranes was studied by labeling the protein with the fluorophore 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) and measuring the fluorescence of the labeled protein in the presence of varying amounts of dipalmitoylphosphatidylcholine–egg phosphatidylglycerol (DPPC–eggPG; 7–3). Leakage of contents from liposomes made of DPPC and varying molar fraction of egg phosphatidylcholine (eggPC) or eggPG was assessed by measuring the fluorescence of entrapped water-soluble probes ANTS and DPX. Fusion of membranes was assessed by measuring the fluorescence of membrane-bound NBD–phosphatidylethanolamine (NBD–PE) and rhodamine–PE (RHO–PE). We found that SP-B bound to PL membranes with high affinity and appeared to irreversibly cluster at the membrane surface, leading to graded release of the vesicle contents and eventually fusion of the membranes with increasing protein–lipid ratios. All lipid mixtures tested were susceptible to the membrane disruptive effects of SP-B, but DPPC–eggPG membranes displayed a biphasic response to increasing molar fractions of eggPG, whereas increasing fractions of eggPC elicited a monotonic response.

Keywords: Pulmonary surfactant; Surfactant apoprotein; SP-B; Lipid–protein interaction; Tubular myelius

1. Introduction

Recently, a mutation of the surfactant apoprotein B (SP-B) gene leading to congenital SP-B deficiency was reported in several neonates who died of refractory respiratory distress [1]. Microscopic examination of the lungs of these infants showed misshapen lamellar bodies and aberrant processing of another surfactant apoprotein, SP-C. A model of SP-B deficiency engineered in the mouse [2] has since reproduced both the clinical course and the pathological findings observed in the human, confirming the apparent importance of SP-B to normal lung function.

SP-B is one of four known surfactant apoproteins. The protein monomer is a 79 amino-acid long peptide predicted by primary and secondary structure analysis to form at least four amphipathic helices approximated in a hairpin-like tertiary configuration through

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disulfide bonding [3]. SP-B is found in its secreted form as a 18-kDa dimer, and it is processed from a larger, 42-kDa preprotein in type II cells [4,5]. SP-B processing, its secondary structure and pattern of disulfide bonding are similar to those of other members of the saposin-like family of proteins [6]. This newly described protein family includes, amongst others, the four saposins [7], porcine NK-lysin [3], the amoebapores of *Entamoeba histolytica* [8] and human granulysin [9]. The biological role varies amongst members of this protein family, but they all interact with lipids.

We previously reported that SP-B induces extensive fusion of liposome membranes and complete, rapid leakage of liposome contents [10]. In a later report, we further characterized the interaction of SP-B with lipids using a mass action kinetic model of membrane fusion and showed a dose-dependent effect of SP-B on aggregation and fusion rate constants, an effect that was further increased by calcium and SP-A [11]. We now report experiments investigating the binding of SP-B to phospholipid (PL) membranes and the dependency of the subsequent membrane fusion and leakage of contents on the target PL composition. Our results suggest that SP-B binds to lipids with high affinity and irreversibly aggregates in the membrane, leading first to destabilization of the membrane and a graded release of the liposome contents, and second to fusion between disrupted membranes. Some degree of leakage of liposome contents and fusion of membranes was observed with all lipid mixtures tested, but differences in the protein dose–response curves were observed depending on the charge of the PL headgroups. These results help further our understanding of the role of SP-B in the normal and diseased lung by providing a detailed account of molecular events that may be of importance in lamellar body formation and the structural rearrangements that take place amongst the lipoproteins of secreted surfactant.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), egg phosphatidylcholine (eggPC) and egg phosphatidylglycerol (eggPG) were purchased from Avanti Polar Lipids (Birmingham, AL). PL purity was checked by thin layer chromatography [12]. Fluorescent lipid probes, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)–phosphatidylethanolamine (NBD–PE), and *N*-(lissamine rhodamine B sulfonyl)–PE (RH–PE) were from Avanti Polar Lipids. Fluorescent aqueous probes, 1-aminonaphtalene-3,6,8,-trisulfonic acid (ANTS), *N,N′*-(*p*-phenylenedimethylene)bis(pyridinium bromide) (DPX), and 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole-4yl (fluoro-NBD) were from Molecular Probes (Eugene, OR). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), fluorescamine, 3(*N*-Morpholino)-2-hydroxy-propanesulfonic acid (MOPSO), dimethylformamide (DMF), ethylenediaminetetraacetic acid (EDTA) and calcium chloride were from Sigma (St. Louis, MO). Sephadex LH-60 and G-75 were from Pharmacia (Piscataway, NJ). $C_{12}E_8$, *n*-Octyl-β-D-glucopyranoside (OGP) were from Calbiochem (San Diego, CA). Organic solvents of HPLC grade were from Mallinckrodt (Paros, KY).

2.2. Preparation of surfactant apoproteins

Surfactant proteins were isolated from dog and human surfactant using previously described methods [13,14]. Surfactant was first extracted in 1-butanol (1:50; vol:vol) at room temperature and then centrifuged twice at $6000 \times g_{av}$ for 20 min to sediment the butanol-insoluble proteins. The resulting pellet was dried under N_2 and washed twice in 5 mM Tris/150 mM NaCl/20 mM OGP, pH 7.4. The precipitate was then solubilized in 5 mM Tris buffer, pH 7.4, and dialyzed for 48 h against four changes of the same buffer. Remaining insoluble material was removed by centrifugation at $100{,}000 \times g_{av}$ for 30 min. The resulting supernatant containing SP-A was assayed for protein content [15] and purity (SDS-PAGE), then stored at -20°C. Stock solutions of SP-A (500 μg/ml) in 5 mM Tris, pH 7.4 were used in all experiments. The hydrophobic protein SP-B was purified from the butanol supernatant. The supernatant was dried by rotary evaporation and resolubilized in chloroform/methanol/0.1 M HCl (1:1:0.1; vol:vol). Insoluble material was removed by centrifugation and the supernatant was applied to a 3×85 cm Sephadex LH-60 column and eluted at 18 ml/h

using the same solvent at room temperature. The eluted fractions were assayed by SDS-PAGE without reduction of the sample and SP-B containing fractions pooled accordingly. The pooled fractions were assayed for protein content by fluorescamine analysis in the presence of 0.1% SDS [16] and stored at 4°C. For all experiments, SP-B was dried under N_2 to remove the HCl, resolubilized (500 μg/ml) in methanol, and added out of methanol to the vesicle suspension in buffer. Molecular weights of 650,000 and 18,000 for SP-A and SP-B, respectively, were used for the calculation of protein molarity.

2.3. SP-B labeling

Purified SP-B (0.3 mg) was reacted with 0.8 mM fluoro-NBD in 500 μl dry dimethylformamide for 24 h, at room temperature, in the dark [17]. Labeled protein was then dried under nitrogen and resolubilized in 200 μl of dichloromethane/methanol/0.1 N HCl (60:35:5), and separated from free label on a LH-60 HPLC column in methanol/dichloromethane/0.1 N HCl (65:30:5) solvent [18]. Labeling efficiency was about 1.6 mol of NBD per mol of SP-B. Labeled SP-B was resolubilized in methanol before use. The lytic effects of labeled and unlabeled protein on liposomes were indistinguishable, suggesting that labeling did not interfere with the biological properties of SP-B (not shown).

2.4. Liposome preparation

PL mixtures were dried under N_2 and resuspended in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 6.9 at 37°C. PL suspensions were then passed through an 'Extruder™' (Lipex Biomembranes, Vancouver, BC, Canada) under 50 to 500 psi argon pressure. The liposomes used for all experiments were obtained by a series of sequential extrusions through two stacked 0.2 μm, and 0.1 μm polycarbonate filters, which produce vesicles of 100 nm median diameter [10]. PL concentration was measured by phosphorus assay [19]. Liposome stock suspensions (2.0 M PLs) were stored at 4°C.

2.5. SP-B binding to lipids / aggregation in the membrane / reversibility

NBD quantum yield and blue shift of the maximal emission wavelength increase with the hydrophobicity of the fluorophore environment, a property which has been used to study the interaction of NBD-labeled proteins with lipid bilayers [20]. To first determine the wavelength of maximum fluorescence emission of the labeled protein in the presence of lipids, fluorescence excitation was set at 450 nm and emission spectra were recorded at 37°C, under constant stirring, in the temperature-controlled 2 ml cuvette of a Fluorolog 2 spectrofluorometer (SPEX Industries, Edison, NJ), equipped with a light-proof port allowing for injection of reagents without interruption of the recording. Emission at this wavelength (530 nm) was then recorded before and after addition of 0.1 μg NBD-labeled SP-B to increasing amounts of DPPC–eggPG PLs. The maximum fluorescence observed was used for calculation of binding estimates after corrections were made for the intrinsic fluorescence of lipids and of labeled SP-B in buffer alone. Corrected fluorescence in the absence of lipids was taken as a fraction bound of 0 and that in the presence of 2×10^5 molar excess of lipids as a fraction bound of 1.

At high SP-B/PL ratios fluorescence decayed after reaching its maximum. This self-quenching of the fluorophore is consistent with surface aggregation of SP-B in the PL membrane [21]. To quantify the surface aggregation of SP-B in the membrane, we used the absolute decrease in fluorescence over the 5 min following maximum as a numerical correlate of surface aggregation. The values obtained at various SP-B/PL ratios were then linearly transformed using a scale of 0 to 100.

In separate experiments, we studied the reversibility of the surface aggregation of SP-B, using a fixed 20 μg/ml PL concentration and different amounts of labeled SP-B (0.01, 0.1 or 1.0 μg). Fluorescence after addition of labeled SP-B was recorded for 10 min before unlabelled SP-B was injected in 20-fold excess (0.2, 2.0 or 20 μg), and for the following 10 min.

2.6. Measurement of lipid mixing

Lipid mixing was measured by the method of resonance energy transfer between the non exchangeable fluorophores NBD–PE and Rhodamine–PE [22]. In all experiments, the molar ratio of fluorophores to

PLs in labeled liposomes was 0.01 and 1:9 mixtures of labeled:unlabeled liposomes were used. Excitation and emission were set at 450 and 520 nm, respectively. Baseline fluorescence (0%) was that before any addition of reagents, and maximum fluorescence (100%) that after addition of the non-ionic detergent $C_{12}E_8$.

2.7. Measurement of leakage of liposome contents

Leakage of liposome contents was measured using the water-soluble fluorescent probe ANTS and its collisional quencher DPX [23]. Liposomes were prepared in buffer containing 12.5 mM ANTS and 45 mM DPX and free probes were removed on a Sephadex G-75 column (1.5 cm × 20 cm). Excitation was set at 360 nm and emission was recorded at 520 nm through a Corning 470 nm cut-off filter, while other experimental conditions were identical to those used for the measurement of lipid mixing. Baseline fluorescence was that before any addition and maximum fluorescence that after $C_{12}E_8$ detergent lysis.

To determine whether leakage of contents occurred as an 'all or none' (i.e., some of the liposomes release all of their contents) vs. a 'graded' release (i.e., all of liposomes release some of their contents), we performed the following experiments. Liposome suspensions were prepared that contained decreasing fraction amounts of the fluorophores used in the leakage assay (12.5 mM ANTS and 45 mM DPX). The fluorescence of each liposome suspension was measured before (baseline fluorescence) and after detergent lysis (final fluorescence). Baseline to final fluorescence ratios were plotted against the amount of fluorophores present in the liposomes before lysis, allowing a DPX quenching curve to be generated. Then, separate DPPC–eggPG (7–3) liposome suspensions containing 12.5 mM ANTS and 45 mM

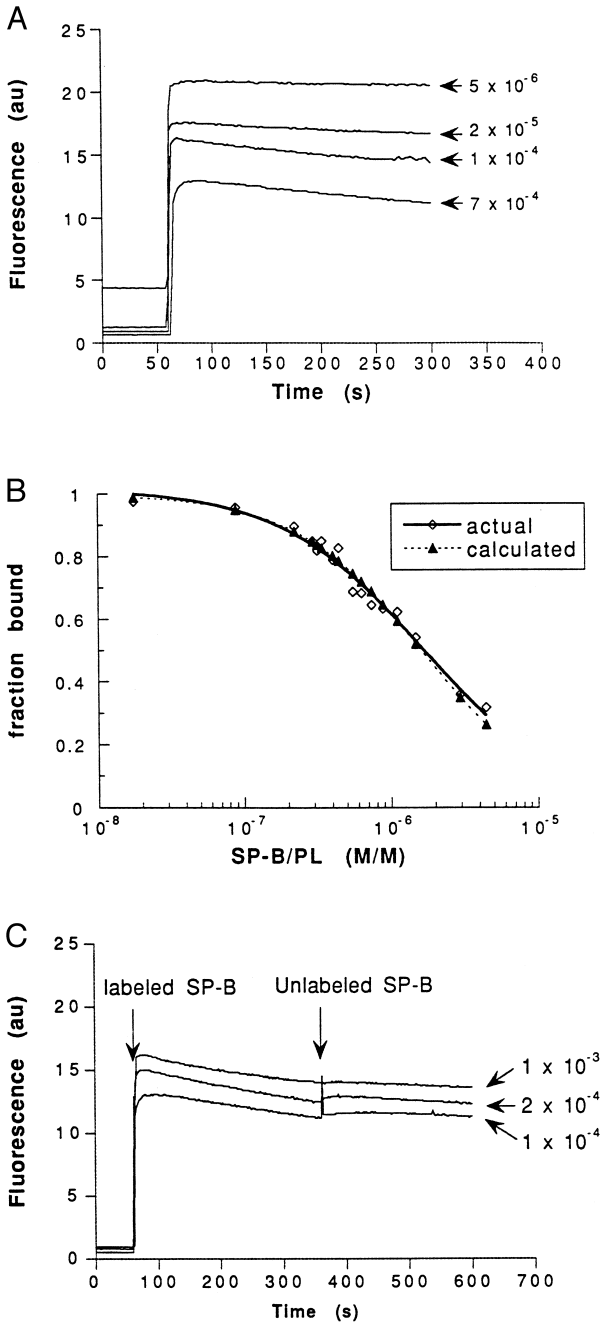

Fig. 1. Binding of SP-B to PLs. (A) 0.1 μg of NBD-labeled SP-B was added at 60 s, out of methanol, to increasing amounts of DPPC-eggPG (7–3;wt–wt) in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 6.9, 37°C. Fluorescence was recorded at 530 nm. Four representative tracings are shown, corresponding to SP-B/PL molar ratios of 7×10^{-4}, 1×10^{-4}, 2×10^{-5} and 5×10^{-6}. (B) Peak fluorescence after addition of the labeled SP-B was corrected for fluorescence of the lipids alone and of the labeled protein in buffer and plotted against the SP-B/PL molar ratios. The calculated values derived from the analytical model are also shown for comparison. (C) Unlabeled SP-B (2 mg) was added in 20-fold excess of labeled SP-B (0.1 μg) to varying amounts of DPPC-eggPG (7–3;wt–wt) in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 6.9, 37°C. Both proteins were added out of methanol, labeled SP-B at 60 s, unlabeled SP-B at 360 s. Fluorescence was recorded at 530 nm. Three representative tracings are shown, for SP-B/PL molar ratios of 1×10^{-4}, 2×10^{-4} and 1×10^{-3}.

DPX were reacted with varying amounts of SP-B and fluorophores released in each sample were removed by G-75 Sephadex chromatography at the end of each experiment. Following chromatography, sample fluorescence was measured before and after detergent lysis to generate sample baseline to final fluorescence ratios. Baseline to final fluorescence ratios are predicted to remain constant in 'all or none' release or to match values of the quenching curve in a 'graded' release [24].

3. Results

3.1. SP-B binding to lipids

To characterize SP-B binding to lipids, NBD-labeled SP-B was reacted with varying amounts of DPPC–eggPG (7–3). Upon interaction with lipids the fluorescence emitted by the labeled protein underwent a blue shift (not shown). The magnitude of the blue shift (10–12 nm) was consistent with the label

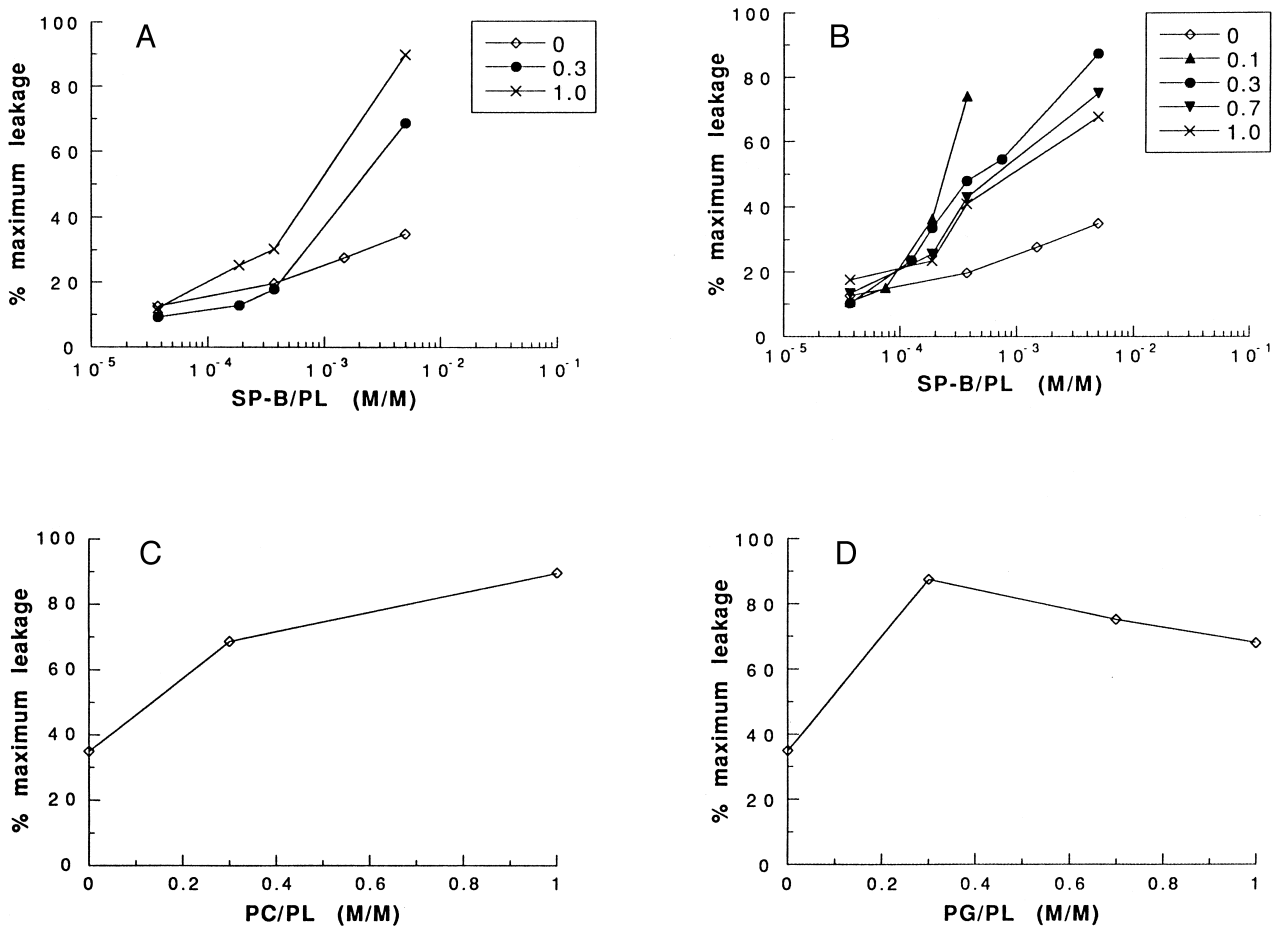

Fig. 2. Leakage of liposome contents induced by SP-B. (A) 20 μg/ml DPPC-eggPC liposomes containing the water-soluble fluorescent probes ANTS and DPX were incubated in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 6.9, 37°C. EggPC/PL molar ratios were 0.0, 0.3 and 1.0, respectively. Varying amounts of SP-B were added to the suspension out of methanol at 1 min. The fluorescence values reached 10 min after injection of SP-B were plotted against SP-B/PL molar ratios. (B) Liposomes eggPG molar ratios were 0.0, 0.1, 0.3, 0.7 and 1.0, respectively. The remainder of the experimental procedure was as in (A). (C) Fluorescence emitted by 20 μg/ml DPPC-eggPC liposomes of varying eggPC molar ratios (0.0; 0.3; 1.0), 10 min after injection of human SP-B at a SP-B/PL molar ratio of 5×10^{-3}. The remainder of the experimental procedure was as in (A). (D) Fluorescence emitted by 20 μg/ml DPPC-eggPG liposomes of varying eggPG molar ratios (0.0; 0.3; 0.7; 1.0), 10 min after injection of human SP-B at a SP-B/PL molar ratio of 5×10^{-3}. The remainder of the experimental procedure was as in (A).

residing at the surface of the bilayer rather than buried within [17]. The fraction of SP-B bound was derived from the maximum fluorescence observed after correction for intrinsic fluorescence of the lipids and of the labeled protein (Fig. 1B). Data were analyzed after a procedure outlined by Nir et al. [25]. SP-B bound to DPPC–eggPG with an apparent surface partition coefficient of 2.7×10^7 M^{-1}. An estimated 600 ± 300 sites per liposome were present for SP-B binding. There was no evidence of positive or negative cooperativity. Estimates of the number of SP-B molecules bound per liposome at various SP-B/PL molar ratios, shown in Fig. 4, indicate that fewer than 50 bound molecules per liposome appeared necessary to elicit significant leakage of liposome contents. The apparent binding affinity of SP-B for DPPC–eggPG increased linearly with the amount of eggPG present in the membrane (not shown).

Following injection of the labeled protein in the cuvette containing the liposome suspension, fluorescence intensity reached a maximum and decayed with time due to NBD self-quenching (Fig. 1A and C). Although most of the measurements were made using concentrations of labeled SP-B of 0.1 μg/ml, we found that at lower (0.01 g/ml) or higher (1.0 μg/ml) concentrations of labeled SP-B, the fluorescence intensity decay exhibited similar dependency on SP-B/lipid molar ratios (data not shown). The fluorescence intensity decay increased with SP-B/lipid molar ratios, suggesting that SP-B molecules aggregated in the membrane. No dequenching was seen following addition of large excess unlabelled SP-B (Fig. 1C), indicating that the surface aggregation of SP-B molecules did not reverse rapidly. The lack of reversal seemed independent of the absolute amount of SP-B added (not shown).

3.2. Leakage of liposome contents

To determine the influence of lipid composition on leakage of liposome contents by SP-B, DPPC–eggPG and DPPC–eggPC liposomes of increasing molar fractions (0 to 1) of eggPG and eggPC were reacted with varying amounts of SP-B. Irrespective of lipid composition, leakage increased with the SP-B/PL ratio (Fig. 2A and B). However, whereas leakage proportionately increased with the fraction of eggPC present (Fig. 2C), it displayed a biphasic pattern when the unsaturated lipid was eggPG (Fig. 2D). At the SP-B/PL molar ratio displayed in Fig. 2D (5×10^{-3}), induced leakage was highest at 0.3 eggPG mole fraction and decreased slightly with increasing eggPG mole fractions. However, at lower SP-B/PL molar ratios (5×10^{-4}), leakage was maximum at an eggPG mole fraction as small as 0.1. Substituting DPPG for eggPG did not alter the extent of leakage (not shown).

Table 1
Analysis of mechanism of leakage of liposome contents by SP-B

SP-B/PL ($\times 10^{-3}$)	Baseline/final fluorescence ratios		
	Experimental	Predicted	
		'Graded'	'All or none'
0.25	0.26	0.28	0.21
0.50	0.27	0.30	0.21
1.25	0.33	0.35	0.21
2.50	0.46	0.43	0.21
3.75	0.56	0.52	0.21

See Section 2 for experimental conditions.

We also determined whether liposome contents were released in an 'all or none' or 'graded' fashion. Only DPPC–eggPG (7–3) liposomes were used for these experiments. The results shown in Table 1 indicate that the pattern of leakage was most consistent with graded release of the liposome contents, as the measured values followed the predicted values for graded release closely, even at SP-B/PL molar ratios (3.75×10^{-3}) that promoted over 80% release of the liposome contents.

3.3. Liposome fusion

Several PL mixtures of various composition were tested in a membrane fusion assay to determine the influence of PL composition on the degree of membrane fusion by SP-B. In all combinations tested, the degree of lipid mixing increased with the SP-B molar ratio (Fig. 3A and B), as reported before for eggPG/PL ratios of 0.3 [11]. Also, similar to what was seen with leakage of contents, the extent of fusion increased monotonically with eggPC (Fig. 3C), but not with eggPG molar ratios (Fig. 3D). Fusion of eggPG-containing liposomes was minimal with pure eggPG and reached its maximum at eggPG molar ratios of 0.1 to 0.3. Substituting DPPG for eggPG did not alter the extent of fusion observed (not shown).

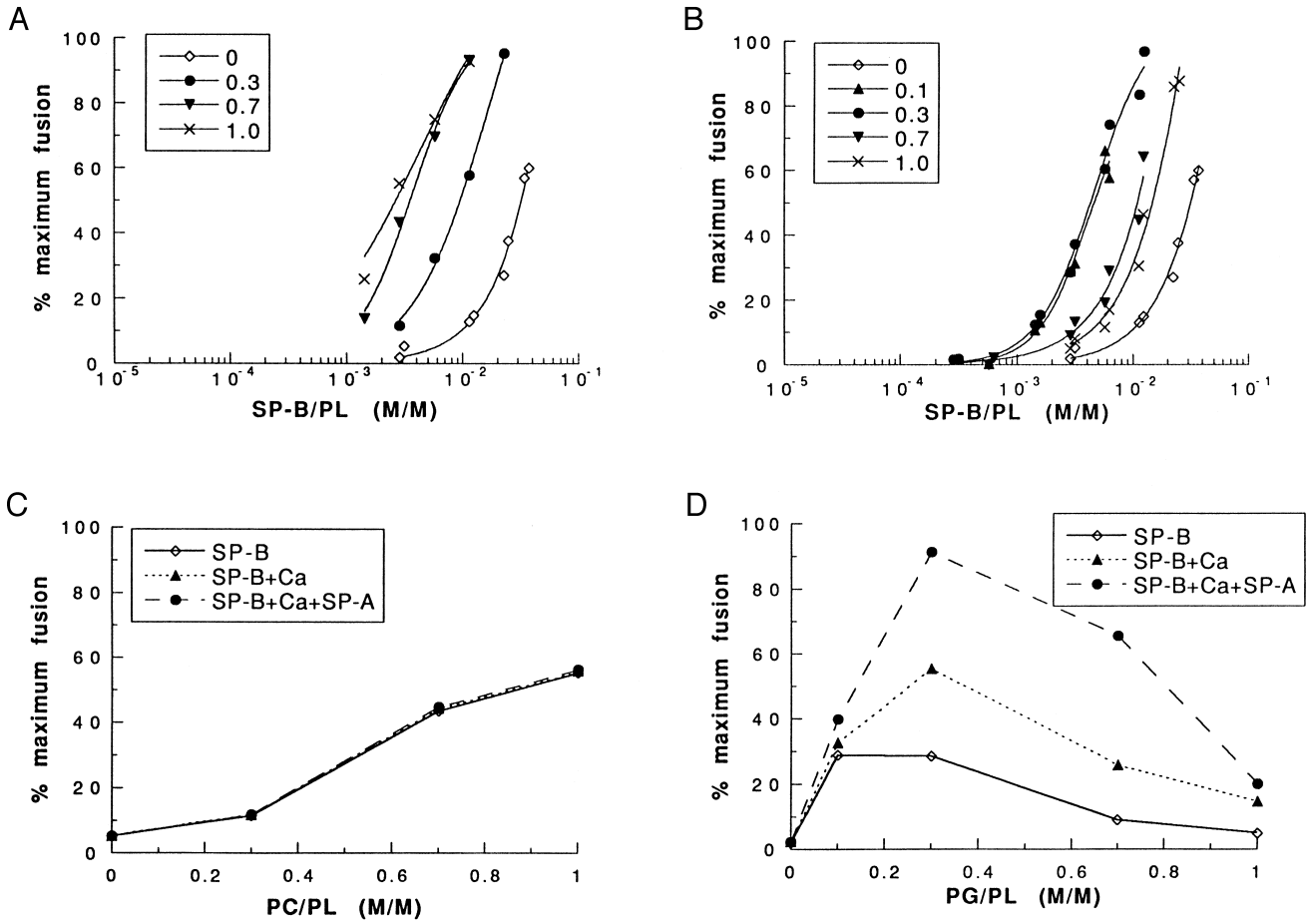

Fig. 3. Liposome membrane fusion induced by SP-B. (A) A mixture of labeled (with NBD-PE and Rhodamine-PE) and unlabeled DPPC-eggPC liposomes (20 μg/ml) was incubated in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer, pH 6.9, 37°C. EggPC/PL molar ratios were 0.0, 0.3, 0.7 and 1.0, respectively. Varying amounts of SP-B were added to the suspension out of methanol at 1 min. The fluorescence values reached 10 min after injection of SP-B were plotted against SP-B/PL molar ratios. (B) Liposomes eggPG molar ratios were 0.0, 0.1, 0.3, 0.7 and 1.0, respectively. The remainder of the experimental procedure was as in (A). (C) Fluorescence emitted by 20 μg/ml DPPC-eggPC liposomes of varying eggPC molar ratios (0.0; 0.3; 0.7; 1.0), 10 min after injection of human SP-B at a SP-B/PL molar ratio of 3×10^{-3} (solid line), under experimental procedure as that in (A). Similar experiments were carried out in the presence of 5 mM calcium (short dashed line) and 5 mM calcium and 50 nM dog SP-A (long dashed line). (D) Fluorescence emitted by 20 μg/ml DPPC-eggPG liposomes of varying eggPG molar ratios (0.0; 0.1; 0.3; 0.7; 1.0), 10 min after injection of human SP-B at a SP-B/PL molar ratio of 3×10^{-3} (solid line), under experimental procedure as that in (A). Similar experiments were carried out in the presence of 5 mM calcium (short dashed line) and 5 mM calcium and 50 nM dog SP-A (long dashed line).

Calcium and/or SP-A had no effect on eggPC-containing mixtures (Fig. 3C) but enhanced the fusion of eggPG-containing liposomes, with maximum enhancement seen between molar ratios of 0.3 and 0.7 of eggPG (Fig. 3D). In separate experiments using cholesterol, we found that as much as 10% cholesterol by weight did not measurably alter the final extents of fusion observed with eggPG-containing mixtures (not shown).

Fig. 4 shows estimates of membrane binding and surface aggregation of SP-B and measured final extents of leakage and fusion plotted against SP-B/PL molar ratios. The figure also displays the estimated number of SP-B molecules bound per liposome at each SP-B/PL molar ratio tested in the binding assay (upper $\times$-axis). Aggregation of SP-B was detectable at SP-B molar ratios ($< 3 \times 10^{-5}$) that caused no measurable leakage or fusion, and reached its maxi-

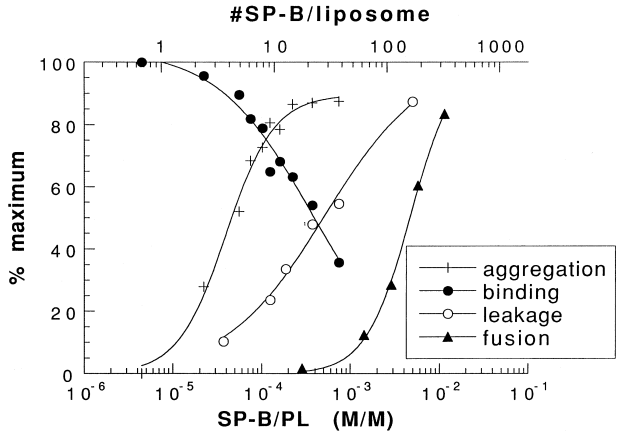

Fig. 4. Protein binding and surface aggregation, leakage of contents and fusion of liposomes by SP-B. Binding of SP-B (filled circles), surface aggregation of SP-B at the membrane (crosses), extent of leakage of contents (circles) and membrane fusion (triangles) after 10 min were plotted against SP-B/PL molar ratios for a DPPC-eggPG (7–3) liposome mixture. The upper *x*-axis displays the estimated number of SP-B molecules bound at various SP-B/PL molar ratios and corresponding mass fractions of bound SP-B. Experimental procedures for binding, leakage and fusion are outlined in the legends of Fig. 1B, Fig. 2A, and Fig. 3A, respectively. Surface aggregation of SP-B at the membrane was expressed as the amount (linearly transformed to a scale of 0 to 100) of NBD fluorescence quenching observed in the 5 min following injection of labeled SP-B.

mum values after about 15–20 molecules of SP-B were bound. Leakage was detectable at SP-B molar ratios greater than 5×10^{-5}, when surface aggregation of the protein had reached half its maximum, and fusion at ratios greater than 5×10^{-4}, when surface aggregation was maximum and leakage half-maximum.

4. Discussion

The lipid composition of surfactant is complex and the importance of its various components to its functions is only partially understood. The recent characterization of human [1] and mouse [2] SP-B deficiency emphasizes the importance of SP-B biological role, and strongly suggests that it is at least in part mediated through an interaction with lipids, those in lamellar bodies and in secreted surfactant.

The first goal of our study was to investigate in detail the influence of lipid composition on the interaction of SP-B with lipid membranes using well-characterized in vitro assays of liposome lysis and membrane fusion [10]. SP-B is an hydrophobic, cationic dimer, and its affinity for negatively charged PLs has been well documented [26,27]. We and others have previously shown that SP-B is a potent fusogen of eggPG-containing PL membranes [11,28], a function which may be relevant to the formation of tubular myelin and lamellar bodies [10,29]. As was the case in our previously reported experiments [10,11], SP-B was added to pre-formed liposomes from a methanol solution. This method of addition may have influenced our results by protein loss to precipitation. We did not attempt to compare bulk phase SP-B content with that of the final lipoprotein products. Our present results indicate that maximum lysis and fusion of DPPC–eggPG membranes were obtained with eggPG/PL molar ratios of 0.1 or 0.3. At eggPG/PL molar ratios equal to or greater than 0.5, the magnitude of the measured effects rapidly decreased, even though the binding of SP-B to the lipid membranes kept increasing.

The synergistic effects of SP-A and calcium on SP-B-induced leakage and fusion were maximum at an eggPG/PL molar ratio of 0.3, indicating that certain PL stoichiometries might optimize lysis and membrane fusion by SP-B. The molecular basis for our observation remains unclear, but may be related to levels of membrane destabilization, pattern of domain formation or other similar mechanisms. As analyzed in detail previously [30,31], calcium promotes fusion of liposomes that contain acidic PLs in at least two ways: by promoting membrane aggregation and by rendering the membranes more fusogenic. In a system of mixed PL liposomes, the effective concentration of calcium at the surface of liposomes will increase with the magnitude of the negative charge density, i.e., the content of anionic lipids. Consistent with this observation, we found that the enhancing effect of calcium on SP-B-induced membrane fusion increased with the eggPG content (Fig. 3D). As the eggPG mole fraction was increased from 0.1 to 1.0, the enhancing effect of calcium increased by 10% ($\times$1.1) to 300% ($\times$3.0) over baseline. The enhancement of fusion by calcium and SP-A in combination followed a similar pattern of increasing effect with the eggPG mole fraction. However, the extent of the fusion reaction ultimately depends on

the rate limiting step in the reaction. As Fig. 3D indicates, the maximal extent of fusion occurred at an eggPG mole fraction of 0.3. At that fraction, the membrane destabilizing effect of SP-B by itself or in combination with calcium and SP-A was maximum. Similar mole fractions of eggPG were found to promote optimal formation of tubular myelin in vitro by Suzuki et al. [32].

Unsaturated PC also promoted extensive leakage and membrane fusion by SP-B, but the effects observed were monotonically related to the amount of eggPC present, unlike the effects of SP-B on eggPG. Native surfactant contains about 25% unsaturated PC [33], possibly more in certain species, and yet its role in surfactant remains unclear. Although SP-B clearly interacted with eggPC-containing membranes and induced leakage and fusion to extents similar to those observed with eggPG-containing membranes, calcium and SP-A had no enhancing effect in the absence of negatively charged PLs. This added property of eggPG more likely relates to the charge of the headgroup than to differences in the phase transition temperatures between the respective mixtures, as DPPG and egg PG behaved similarly in our assays. On the other hand, the role of unsaturated PC in surfactant may be more complex than its broadening effects on the phase transition of PL mixtures [34]. Cholesterol had no measurable impact on lysis or fusion by SP-B on DPPC–eggPG membranes, even when present at cholesterol/PL molar ratios of 0.2 (10% by weight), in contrast to its effects on the surface properties of mixtures of DPPC and SP-B measured by the Wilhelmy plate method [35].

The second goal of our study was to characterize the binding of SP-B to eggPG-containing membranes by labeling the protein with NBD, a fluorescent molecule which increases its quantum yield in hydrophobic environments. Our results show that, within the conditions of our experiments, SP-B bound to DPPC–eggPG membranes with high affinity (surface partition coefficient $= 2.7 \times 10^7$ M^{-1}). The exact affinity of SP-B for lipids in vivo may be quite different however, and cannot be safely extrapolated from our results. SP-B appeared to aggregate in the membrane at high protein-to-lipid molar ratios, as indicated by the NBD self-quenching we noted. This surface aggregation of SP-B was also suggested by others using deuterium nuclear magnetic resonance in the study of PL/SP-B mixtures [36]. The phenomenon of fluorescence quenching was not reversed by the addition of non-labeled protein, suggesting that protein aggregates dissociated slowly under these conditions. Combined with our observation that SP-B induced a 'graded' release of liposomes contents, the irreversibility of the protein surface aggregation suggests that the protein clusters were not pore-forming.

The actual membrane topography and size of the putative SP-B protein clusters remain uncertain. Although as many as 600 SP-B binding sites may exist on the membrane of a liposome 100 nm in diameter, our results suggest that surface aggregation of the protein was already maximum after as few as 15 SP-B molecules had bound (Fig. 4). For a given amount of PLs, fusion required about 10-fold more SP-B than did leakage and 100-fold more than did maximal surface aggregation of SP-B to occur. No significant destabilization of the membrane was detected until surface aggregation of the protein appeared to be maximal. Our results point to a dose-related effect of SP-B on membrane integrity, from irreversible protein aggregation at the surface of the liposome membrane to graded release of liposome contents and eventually fusion of the membranes. However, the precise stoichiometries of protein and lipids interacting at various bulk ratios remain uncertain and may be altered by the structural rearrangements of membranes occurring with each process [10].

Our present studies show that SP-B binds to lipids with high affinity. Following its aggregation at the membrane surface, SP-B causes membrane destabilization leading to membrane fusion. The destabilizing effects of SP-B on membranes showed a definite dependency on lipid composition, with a biphasic response to eggPG and a monotonic response to eggPC content. These observations may be of relevance to the role of SP-B in vivo and influence the choice of composition and stoichiometry in new formulations of exogenous surfactant preparations for replacement therapy.

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