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Kinetics of phospholipid membrane fusion induced by surfactant apoproteins A and B

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Abstract

Surfactant apoproteins A (SP-A) and B (SP-B) interact with the lipids of surfactant and such protein–lipid interactions may be of importance in several of the steps in the surfactant cycle. We analyzed the kinetics of fusion of dipalmitoylphosphatidylcholine-phosphatidylglycerol (DPPC:PG; 7:3, w/w) phospholipid vesicles induced by SP-B alone, in the presence of 5 mM calcium, and in the presence of calcium and SP-A. Membrane fusion was measured by the method of resonance energy transfer between non-exchangeable fluorophores incorporated in the membrane. Data were analyzed using a mass action kinetic model for membrane fusion between phospholipid vesicles. We found a SP-B dose-dependent increase in lipid mixing within a range of phospholipid concentration of 5 to 100 μM. Calcium caused a small additive increase in lipid mixing, but calcium and SP-A combined markedly increased lipid mixing induced by SP-B. Both aggregation and fusion rate constants increased with an increase in the SP-B/lipid ratio. In the presence of calcium and SP-A, the number of vesicles per fusion product markedly increased, as did the aggregation rate constants, whereas the fusion rate constants remained essentially unchanged.

Keywords: Surfactant; Surfactant apoprotein; Membrane fusion; Kinetics; Tubular myelin; Myelin

1. Introduction

Pulmonary surfactant is the 'anti-atelectasis' factor that lines the alveolar surface, increasing pulmonary compliance and stabilizing lung volumes [1]. Although the phospholipids of surfactant, particularly dipalmitoylphosphatidylcholine (DPPC), form the alveolar surface film, surfactant apoproteins play important, albeit incompletely defined roles in the intracellular assembly of surfactant and in its extracellular metabolism and function (reviewed in [2]).

We previously reported that surfactant apoprotein B (SP-B), a 79-amino-acid-long, cationic, amphipathic, membrane-associated protein [3], enhanced membrane fusion between phospholipid liposomes [4]. We showed that the liposome membranes underwent extensive mixing, but their contents rapidly and completely leaked out. The lipid mixing, measured by energy transfer between fluorescent lipid probes, correlated with a structural rearrangement of the spherical liposomes into large, stacked sheets, as previously reported [5]. These results, coupled with recent studies of the intracellular routing and processing of the SP-B preprotein [6,7], suggested to us that the fusogenic potential of SP-B, released with cleavage of the precursor protein, could be critical for the packaging of surfactant phospholipids in intracellular lamellar bodies [8]. It has also been suggested that SP-B-enhanced fusion of subphase lipids with the surface film may play a role in the adsorption of secreted lamellar body contents to the alveolar surface [9]. Consistent with both hypotheses, newborns with congenital SP-B deficiency have severe respiratory distress [10]. The recent discovery of this lethal familial point mutation in the SP-B gene [11] gives added impetus to establishing the precise functions of SP-B.

In our earlier studies we reported that surfactant apoprotein A (SP-A) aggregated liposomes [12] but, in contrast to SP-B, did not induce lipid mixing [4]. The trafficking of SP-A in type II cells has not been well defined, but there is

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increasing evidence that SP-A may be secreted independent of lamellar bodies [13–15] and then rapidly associate with lamellar body contents in the alveolar lining fluid [15]. Electron microscopy of the alveolus reveals that the membranes released from many different lamellar bodies intermingle in forming a three-dimensional lattice-like arrangement of tubules known as tubular myelin [16]. The precise topology of components in this unusual structure is beyond the resolution of the electron microscope, but the corners of these tubules, where the bilayers cross and merge, are decorated with extramembranous particles [17], probably SP-A [18]. Consistent with these trafficking and morphological studies both SP-A and SP-B are required to form tubular myelin like structures in vitro [5,19]. The morphological studies suggest that membrane aggregation and fusion, both directed with long-range order, must be involved in the extracellular transition from lamellar bodies to tubular myelin. All current results point to critical interdependent roles for SP-A and SP-B, but the precise roles of the two apoproteins in the stepwise processes of aggregation and fusion in the restructuring of lamellar body contents are unclear.

The studies presented here extend these previous qualitative and morphological descriptions of the effects of SP-A and SP-B on the structure of phospholipid liposomes by studying a wide range of lipid-protein ratios in an established assay of liposome fusion. These studies had two aims. First, to determine whether SP-B alone would enhance significant membrane fusion in concentrations consistent with estimates of the physiological concentration in lamellar bodies. Second, to determine whether SP-A enhances the fusion of SP-B containing membranes solely by the well-characterized ability of this protein to cause liposome aggregation.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) and egg phosphatidylglycerol (egg PG) were purchased from Avanti Polar Lipids (Birmingham, AL). The purity of the phospholipids was verified by thin-layer chromatography [20]. Fluorescent lipid probes, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE), and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (RH-PE) were from Avanti Polar Lipids. Fluorescamine, 3(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO), and calcium chloride were from Sigma (St. Louis, MO). Organic solvents of HPLC grade were from Mallinckrodt (Paros, KY).

2.2. Preparation of surfactant apoproteins

Surfactant was isolated from lung lavage of patients who had alveolar proteinosis and from adult dogs, using previously described methods [4]. Surfactant proteins were isolated from the dog and human surfactants using previously described methods [21,22]. The surfactant was first extracted in 1-butanol (1:50, v/v) 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 octyl β-D-glucopyranoside (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. Any insoluble remaining 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 [23] and purity (SDS-PAGE), then stored at −20°C. Stock solutions of SP-A (200 μg/mL) in 5 mM Tris 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; v/v). 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 (M_r = 18 000) pooled accordingly. The pooled fractions were assayed for protein content by fluorescamine analysis in the presence of 0.1% SDS [24] and stored at 4°C. For all experiments, SP-B was dried under N_2 to remove the HCl and resolubilized in methanol (200 μg/mL) before use. Molecular weights of 36 000 and 9000 for SP-A and SP-B, respectively, were used for the calculation of protein molarity.

2.3. Liposome preparation

A phospholipid mixture of DPPC-egg PG (7:3, w/w) was dried under N_2, then resuspended in 50 mM MOPSO/140 mM NaCl/0.1 mM EDTA buffer (pH 6.9 at 37°C). The phospholipid suspension was then passed through an 'Extruder™' (Lipex Biomembranes, Vancouver, Canada) under 50 to 500 lb/inch^2 argon pressure. The liposomes used for all experiments were obtained by series of 6 extrusions through 2 stacked 0.2 μm, then through 0.1 μm polycarbonate filters. Liposome stock suspensions (1.0 M phospholipids) were stored at 4°C.

2.4. Measurement of lipid mixing

Lipid mixing was measured by the method of resonance energy transfer between non-exchangeable fluorophores [25]. The energy transfer of the two fluorophores NBD-PE and Rhodamine-PE used in our experiments remains linearly proportional to their surface density in a lipid bilayer between surface densities of 0.1 and 1 *M*% [25]. In all our experiments, fluorophores were 1 *M*% of the total phospholipids in fluorescently labeled liposomes. Mixing of

labeled with unlabeled bilayers causes dilution of the fluorophores within the membranes and decrease of the energy transfer between them, resulting in an increase of NBD fluorescence. Mixtures of labeled:unlabeled (1:9) liposomes in buffer were continuously stirred in the temperature-regulated 2 mL cuvette of a Fluorolog 2 spectrofluorometer (SPEX Industries, Edison, NJ, USA). A lightproof port allowed for injection of reagents without interruption of the recording. Baseline fluorescence (0%) was that of the mixture before any addition of reagents, and maximum fluorescence (100%) that after addition of the non-ionic detergent $C_{12}E_8$ (Calbiochem, San Diego). NBD fluorescence excitation was set at 450 nm and emission was measured at 520 nm.

2.5. Data analysis

NBD fluorescence measurements for all experiments were sampled every 2 s and stored electronically. Final extents of fluorescence (I) were recorded 10 min after addition of the protein, and just before that of the detergent.

In a selected number of experiments, we analyzed the kinetics of lipid mixing using the mathematical procedure based on a mass action model [26,27]. The model distinguishes between two steps in the reaction. If V_1 represents primary lipid vesicles, A their aggregate and F the resulting fusion product, the reaction can be described by:

$$V_1 + V_1 \underset{D}{\overset{C}{\rightleftarrows}} A \xrightarrow{f} F \quad (1)$$

The first step, aggregation, is reversible and of second-order with respect to the concentration of the reaction components. The second step includes destabilization and irreversible lipid mixing and is of first-order with respects to the concentration of the reaction components. The current program considers aggregation-fusion products of orders 2 to 8. Numerical estimates were derived for the aggregation rate constant C (M^{-1} s^{-1}), the disaggregation rate constant D (s^{-1}), and the fusion rate constant f (s^{-1}) in Eq. (1). In addition, another parameter K_f, which describes the reduction in the fusion rate constant for liposomes that already fused once, was introduced. K_f, is defined by:

$$f_{ij} = K_f * f_{11} \quad (i,j > 1) \quad (2)$$

where i or j refer to the order of vesicles in a given fusion reaction. When i and j equal 1, then $f_{ij} = f$. The parameter K_f accounts for the apparent slowing of the fusion process at later stages.

The mean estimated number of vesicles per fusion product, N, can be derived from the following equation [28]:

$$I = 100 * (K/(K+L)) * (N-1)/N \quad (3)$$

where I is the final extent of fluorescence obtained (% maximum), K and L are the fractions of non labeled and labeled liposomes, respectively. Since the ratio K/L was 9 in all our experiments:

$$N = 90/(90 - I) \quad (4)$$

for $I \leq 90$(% maximum).

The quality of agreement between estimated and experimental values was evaluated by statistical analysis. R^2 values were in most cases greater than 0.99. The root mean square error (RMSE) was about 1%, where:

$$RMSE = \left[\sum_{i=l}^{i=n} (y_{ci} - y_{ei})^2/(n-4) \right]^{1/2} \quad (5)$$

and n is the number of data points used, and y_{ci} and y_{ei} are calculated and experimental $I(t)$ values, respectively. The factor $(n-4)$ represents the degrees of freedom.

3. Results

Both SP-A and SP-B were added to liposome suspensions out of their stock solutions, 5 mM Tris and methanol, respectively. The reagent volume added varied between 5 and 40 μL. Control experiments showed that identical volumes of Tris buffer and methanol alone did not cause detectable lipid mixing (data not shown). Representative tracings of recorded fluorescence are shown in Fig. 1. Fluorescence after addition of 100 nM SP-B (SP-B/lipid molar ratio = 0.4%) in the presence of 5 mM calcium at time 0 is displayed by the lower line, and in the presence of 5 mM calcium + 100 nM SP-A (SP-A/lipid molar ratio = 0.4%) by the upper line. As reported previously, SP-A enhanced the extent of fusion induced by SP-B [4]. The inset (Fig. 1 inset) depicts example of data that were

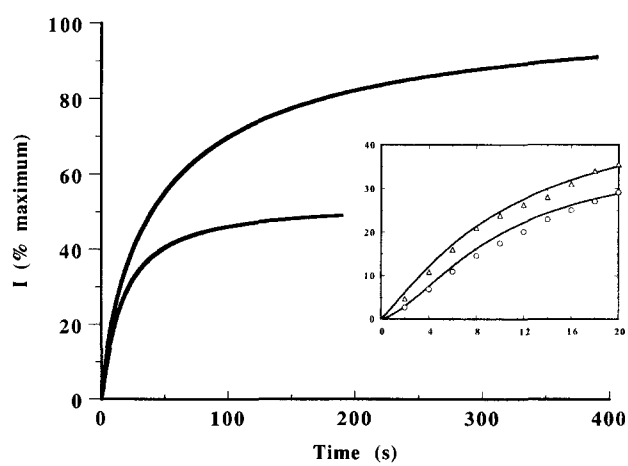

Fig. 1. Examples of fluorescence tracings used to measure final extents (I) and to estimate rate constants for the membrane fusion reaction. 100 nM human SP-B alone (lower tracing), and 100 nM SP-B + dog SP-A (upper tracing) were added to a suspension of 25 μM DPPC-egg PG liposomes in 50 mM MOPSO, 150 mM NaCl, 5 mM $CaCl_2$ buffer, pH 6.9, 37°C. The inset shows the estimated corresponding I values derived from analysis (open symbols) overlaying the experimental tracings.

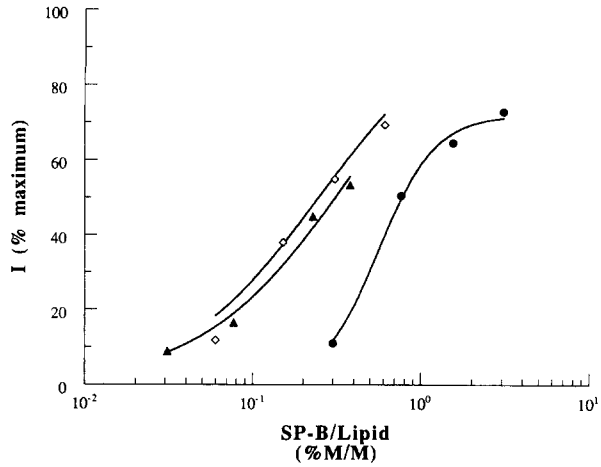

Fig. 2. Effects of lipid concentration on final extents of fusion (10 min). Increasing amounts of human SP-B were added to DPPC-PG liposomes at concentrations of 5 μM (filled circles), 25 μM (open diamonds), and 100 μM (filled triangles) phospholipids in 50 mM MOPSO, 150 mM NaCl, 5 mM $CaCl_2$ buffer, pH 6.9, 37°C.

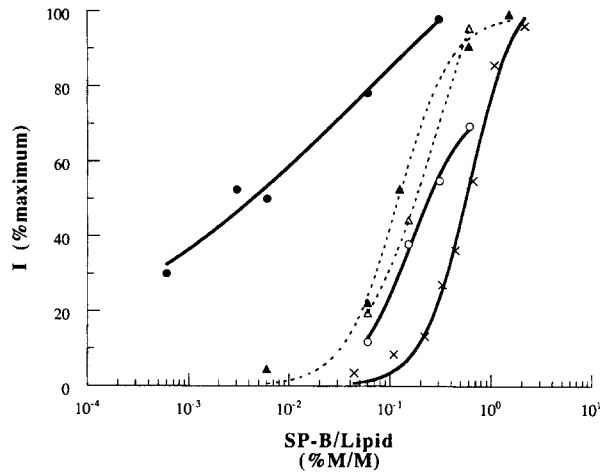

Fig. 3. Effects of temperature, calcium and SP-A addition on final extents of fusion (10 min) induced by SP-B. DPPC-PG liposomes (25 μM) were in 50 mM MOPSO, 150 mM NaCl, 5 mM $CaCl_2$ buffer (pH 6.9). Increasing amounts of human SP-B were added at 20°C (triangles, dashed lines) and at 37°C (circles, solid lines), in the presence (filled symbols) or absence of 50 nM dog SP-A (SP-A/lipid molar ratio = 0.2%; open symbols). At 37°C SP-B was also added in the presence of excess EDTA (crosses).

used in the analysis of lipid mixing kinetics. The values calculated by applying the mass action kinetic model to experimental data and represented here by open symbols overlaying the first 20 s of each experimental tracing, illustrate the close agreement between calculated values and experimental data.

3.1. Effects of lipid concentration

Increasing amounts of SP-B were added to DPPC-egg PG liposome suspensions prepared in three different concentrations: 5, 25, and 100 μM phospholipids. The fluorescence achieved 10 min after addition of the protein(s), I, is a measure of the lipid mixing that took place between vesicles and was plotted as a function of the protein/phospholipid ratios for each of the three liposome concentrations. We found a relationship between the extent of lipid mixing and the SP-B/lipid ratio at all three liposome concentrations (Fig. 2). At low liposome concentration (5 μM phospholipids) higher SP-B/lipid ratios were required to achieve fusion than at higher liposome concentration ($\geq$ 25 μM phospholipids), suggesting that liposome aggregation may be rate-limiting at low lipid concentrations.

3.2. Effects of calcium and SP-A addition

As noted in our previous studies [4], the addition of 5 mM calcium by itself had a small effect on lipid mixing induced by SP-B (Fig. 3). In contrast, addition of SP-A and calcium markedly enhanced SP-B-induced lipid mixing, and this effect was dose-dependent (Fig. 4). Even at SP-B doses which by themselves caused undetectable lipid mixing ($\leq$ 0.2% SP-B/lipid molar ratio), SP-A greatly augmented SP-B-induced lipid mixing (Fig. 3).

Estimates for the aggregation (C), disaggregation (D), and fusion (f) rate constants that best simulated the experimental data are shown in Table 1. The starting lipid concentration was 25 μM in all cases analyzed. The calculated aggregation and fusion rate constants at various SP-B and SP-A/lipid ratios are most consistent with the effects of SP-A on SP-B induced fusion being attributable to its aggregative properties. At the starting 0.4 M% SP-B/lipid ratio, the aggregation rate constant, C, doubled in the presence of 100 nM SP-A (0.4 M% SP-A/lipid ratio), but the fusion rate constant, f, remained unchanged (Table 1). The addition of SP-A also resulted in a dramatic increase in the average number of vesicles in a fusion product from 2.9 to > 100 (Table 1).

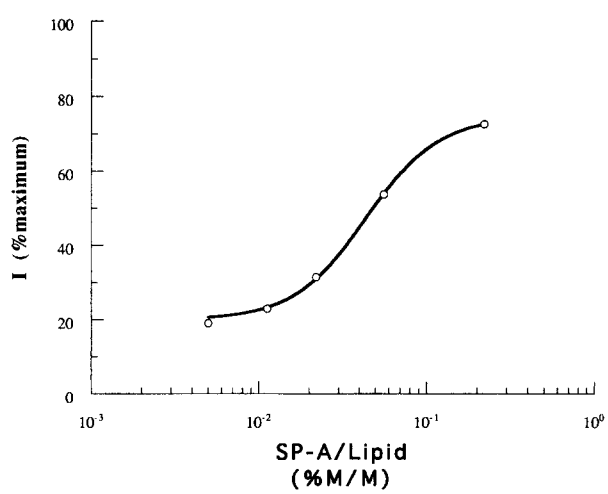

Fig. 4. Effects of SP-A concentration on final extents of fusion (10 min) induced by SP-B. DPPC-PG liposomes (25 μM) were in 50 mM MOPSO, 150 mM NaCl, 5 mM $CaCl_2$ buffer, pH 6.9, 37°C. Fluorescence was measured (open circles) after increasing amounts of dog SP-A were added to 20 nM human SP-B (SP-B/phospholipid molar ratio = 0.08%).

Table 1
Estimates of number of vesicles per fusion products and rate constants for the lipid mixing reaction

Temperature (C°)	SP-B/lipid (% *M/M*)	SP-A/lipid (% *M/M*)	*N* (Eq. (3))	Rate constants			K_f
				C ($\times 10^8$ M^{-1} s^{-1})	D (s^{-1})	f (s^{-1})	
37	0.02	0.02	1.2	0.05–0.1	0.2	0.05–0.1	1/6
37	0.02	0.4	1.9	1.3	0.2	0.1	1/6
37	0.08	0.02	2.0	1.8	0.2	0.3	1/6
37	0.08	0.4	100.0	3.2	0.2	0.3	1 (> 1/2)
37	0.4	0.02	6.4	3.8	0.2	0.4	1 (> 1/2)
37	0.4	0.4	> 100	6.0	0.2	0.6	1/6
20	0.08	0.02	2.7	1.0	0.1	0.1	1/5
20	0.08	0.4	2.1	4.0	0.1	0.02	1/5
20	0.4	0.02	13.4	4.0	0.1	0.27	1/4
20	0.4	0.4	11.9	6.0	0.1	0.17	1/4
20	0.4	0	9.3	2.0	0.1	0.23	1/3
37	0.4	0	2.9	3.0	0.2	0.6	1/3
42	0.4	0	1.7	3.5	0.5	0.4	< 1/10

Conditions are as explained in the methods. Phospholipid concentration is 25 μM. Rate constants estimates for the aggregation (C), disaggregation (D) and fusion (f) steps of the lipid mixing process were calculated using the mass action kinetic model [26,27]. K_f is a correction factor for fusion rate constant for vesicles that already fused once. The estimated uncertainties in C, f, D and K_f are 20%, 30%, 50% and 50%, respectively. In certain cases, larger uncertainties are indicated. N is the estimated mean number of vesicles per aggregation-fusion product after 10 min of incubation.

3.3. Effect of temperature

Because temperature affects the physical state of lipid bilayers and influences the binding of proteins to the bilayer as well as the diffusion rate of bound proteins in the plane of the membrane, we studied the effect of temperature on lipid mixing at various SP-B/lipid ratios in the presence and absence of SP-A. We found that in the absence of added SP-A, final extents of lipid mixing after 10 min increased slightly at 20°C vs. 37°C (Fig. 3). In contrast, lower temperature blunted the synergistic effect of SP-A (Fig. 3).

We also analyzed the effect of temperature on the kinetics of membrane fusion in a limited number of cases (Table 1). In the presence of SP-B alone (0.4 *M%* SP-B/lipid ratio), aggregation and fusion rate constants increased with temperature increasing from 20°C to 37°C. In the presence of both SP-A and SP-B, however, the aggregation rate constant remained unchanged when the temperature increased from 20°C to 37°C, again suggesting that SP-A mainly promotes the fusion reaction through its aggregative properties.

4. Discussion

This study was limited to liposomes with a fixed binary phospholipid composition to limit the matrix of variables investigated and allow us to focus primarily on the effect of protein-lipid stoichiometry on liposome fusion. The choice of 70% DPPC and 30% PG was based on the composition of surfactant and also to allow us to compare our results with those from previous studies [4,38]. Additionally, the results from studies with both SP-A [29] and SP-B [30,31] suggest specific interactions with PG may be important in surfactant function. Our conclusions, however, cannot be immediately extended to the physiological state because the lipid composition clearly does not reproduce the complexity of natural surfactant [32]. Further experiments will be required to assess such potentially important variables as cholesterol and unsaturated phosphatidylcholines. Also, the method of adding SP-B to the pre-formed liposomes from a methanol solution may have influenced the results by protein losses to precipitation or the introduction of significant micro-heterogeneity in the stoichiometry of the lipoproteins formed. We did not attempt to compare the bulk phase SP-B content with the composition of the final lipoprotein products.

With these reservations stated, our results show that SP-B is a very potent membrane fusogen. Fusion occurs even at bulk protein contents as low as 0.02 *M%* and is maximal between 0.5 and 1 *M%*, depending on the lipid concentration (Fig. 2). At the higher SP-B/lipid ratios the rates and extent of fusion we report are amongst the highest observed in similar systems with other proteins or peptides [27,33,34]. Precise quantitation of SP-B in lamellar bodies or secreted surfactant is difficult. The SP-B content of lamellar bodies is reported to range from 0.01–0.05 *M%* [35]. This estimate is based on protein recovered from a chromatography column and is likely to represent an underestimate of the true protein content. Provided other factors such as pH, ionic strength and lipid composition do not significantly alter the fusogenic potency of SP-B, these estimates suggest the amount of SP-B present in lamellar bodies is sufficient to play a major role in the formation of the closely-packed sheets of lipids in these organelles. Temperature and Ca^{2+} both affect the aggregation of PG containing liposomes, but very little effect of

calcium or temperature on the SP-B effect was observed in our studies. Our kinetic analysis, using a model and assumptions previously presented in detail [27], suggests the fusion rate constant was directly related to the SP-B/lipid ratio. This suggests the formation of lamellar bodies, assuming the hypothesis giving SP-B a role in this process is correct, would be very dependent on the lamellar body SP-B content. The abnormal lamellar body morphology in infants with congenital absence of SP-B is consistent with, but does not prove, this idea [36].

Several investigators have studied the effects of SP-A, a large multivalent protein, on liposome aggregation. SP-A binds reversibly to liposomes of different compositions provided binding is carried out below the phase transition temperature of the lipids [29,37]. The binding is not dependent on calcium [29], but in the presence of calcium [12] and certain other divalent cations [38] SP-A induces massive liposome aggregation. The calcium-induced aggregation of liposomes correlates with calcium binding to specific sites in SP-A and an induced aggregation of the protein even in the absence of lipids [39]. These results suggest SP-A aggregates liposomes by protein-protein cross-bridges. Although SP-A alone produces no evidence of fusion even when present in high amounts, a striking enhancement of SP-B induced fusion has been reported [4] and seen again in this study. This synergy was present even at concentrations of SP-B too low ($< 0.02\%$ molar ratio) to cause detectable fusion.

By modeling the reaction in terms of a two step process of reversible aggregation leading to irreversible fusion products, we hoped to understand this synergy further. According to this model, SP-A enhances the aggregation rate of liposomes containing SP-B two to three fold, result which is consistent with previous qualitative studies with SP-A [12,38]. The model also predicts SP-A has little, if any, effect on the fusion rate constant. The almost total loss of protein synergy at 20°C also suggests SP-A-induced aggregation alone is sufficient to explain the enhanced fusion at 37°C, when desaggregation rates are increased relative to those at lower temperature [40]. Our results with substitution of mellitin for SP-B, however, which showed no enhancement of fusion by SP-A [4], may be more consistent with more specific interactions between SP-A and SP-B than a simple two step model of SP-A induced aggregation followed by SP-B induced fusion allows. Whether the two proteins interact directly or through effects on the lipids, such as modulation of phase transitions or domain boundaries, remains to be established. By whatever mechanism, the net impact of protein synergy is dramatic and is quantitatively reflected in a dramatic increase in the estimated mean number of liposomes contributing to a single fusion product 10 min after the reaction begins. At certain protein stoichiometries the combined effects on aggregation and fusion result in a 100-fold increase in this parameter. A better understanding of the mechanisms underlying protein synergy in surfactant structure will be necessary to progress on the important question of how these complex structures mediate the remarkable surface properties of this material.

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References

[1] Clements, J.A. (1957) Proc. Soc. Exp. Biol. Med. 95, 170–172.
[2] Johansson, J., Curstedt, T. and Robertson, B. (1994) Eur. Respir. J. 7, 372–391.
[3] Vandenbussche, G., Clerex, A., Curstedt, T., Johansson, J., Jornvall, H. and Ruysschaert, J.M. (1992) J. Am. Chem. Soc. 31, 9169–9176.
[4] Poulain, F.R., Allen, L., Williams, M.C., Hamilton, R.L. and Hawgood, S. (1992) Am. J. Physiol. 262, L730–L739.
[5] Williams, M.C., Hawgood, S. and Hamilton, R.L. (1991) Am. J. Respir. Cell Mol. Biol. 5, 41–50.
[6] Weaver, T.E. and Whitsett, J.A. (1989) Am. J. Physiol. 257, L100–L108.
[7] Voorhout, W.F., Veenendaal, T., Haagsman, H.P., Weaver, T.E., Whitsett, J.A., Van Golde, L.M.G. and Geuze, H.J. (1992) Am. J. Physiol. 263, L479–L486.
[8] Hawgood, S., Latham, D., Borchelt, J., Damm, D., White, T., Benson, B. and Wright, J.R. (1993) Am. J. Physiol. 264, L290–L299.
[9] Oosterlaken-Dijksterhuis, M.A., Haagsman, H.P., Van Golde, L.M.G. and Demel, R.A. (1991) Biochemistry 30, 8276–8281.
[10] Nogee, M.L., DeMello, D.E., Dehner, L.P. and Colten, H.R. (1993) N. Engl. J. Med. 328, 406–410.
[11] Nogee, L.M., Garnier, G., Dietz, H.C., Singer, L., Murphy, A.M., deMello, D.E. and Colten, H.R. (1994) J. Clin. Invest. 93, 1860–1863.
[12] Hawgood, S., Benson, B.J. and Hamilton, R.J. (1985) Biochemistry 24, 184–190.
[13] Froh, D., Ballard, P.L., Williams, M.C., Gonzales, J., Goerke, J., Odom, M.W. and Gonzales, L.W. (1990) Biochim. Biophys. Acta 1052, 78–89.
[14] Ikegami, M., Ueda, T., Purtell, J., Woods, E. and Jobe, A. (1994) Am. J. Respir. Cell Mol. Biol. 10, 413–418.
[15] Doyle, I.R., Barr, H.A. and Nicholas, T.E. (1994) Am. J. Respir. Cell Mol. Biol. 11, 405–415.
[16] Young, S.L., Fram, E.K. and Larson, E.W. (1992) Exp. Lung Res. 18, 497–504.
[17] Hassett, R.J., Engleman, W. and Kuhn III, C. (1980) J. Ultrastruct. Res. 71, 60–67.
[18] Voorhout, W.F., Veenendaal, T., Haagsman, H.P., Verkleij, A.J., Van Golde, L.M.G. and Geuze, H.J. (1991) J. Histochem. Cytochem. 39, 1331–1336.
[19] Suzuki, Y., Fujita, Y. and Kogishi, K. (1989) Am. Rev. Respir. Dis. 140, 75–81.
[20] Touchstone, J.C., Chen, J.C. and Beaver, K.M. (1979) Lipids 15, 61–62.
[21] Warr, R.G., Hawgood, S., Buckley, D.I., Crisp, T.M., Schilling, J., Benson, B.J., Ballard, P.L., Clements, J.A. and White, R.T. (1987) Proc. Natl. Acad. Sci. USA 84, 7915–7919.

[22] Hawgood, S., Benson, B.J., Schilling, J., Damm, D., Clements, J.A. and White, R.T. (1987) Proc. Natl. Acad. Sci. USA 84, 66–70.
[23] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[24] Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213–220.
[25] Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099.
[26] Nir, S., Stegmann, T. and Wilschut, J. (1986) Biochemistry 25, 257–266.
[27] Nir, S. (1991) in Membrane Fusion (Wilschut, J. and Hoekstra, D., eds.), pp. 127–153, Dekker, New York.
[28] Nir, S., Stutzin, A. and Pollard, H.B. (1987) Biochim. Biophys. Acta 903, 309–318.
[29] King, R.J., Carmichael, M.C. and Horowitz, P.M. (1983) J. Biol. Chem. 258, 10672–10680.
[30] Baatz, J.E., Elledge, B. and Whitsett, J.A. (1990) Biochemistry 29, 6714–6720.
[31] Shiffer, K., Hawgood, S., Düzgünes, N. and Goerke, J. (1988) Biochemistry 27, 2689–2695.
[32] King, R. (1982) J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 53, 1–8.
[33] Parente, R.A., Nir, S. and Szoka, F.J. (1988) J. Biol. Chem. 263, 4724–4730.
[34] Nieva, J.L., Nir, S., Muga, A., Goni, F.M. and Wilschut, J. (1994) Biochemistry 33, 3201–3209.
[35] Oosterlaken-Dijksterhuis, M.A., Van Eijk, M., Van Buel, B.L., Van Golde, L.M.G. and Haagsman, H.P. (1991) Biochem. J. 274, 115–119.
[36] deMello, D.E., Heyman, S., Phelps, D.S., Hamvas, A., Nogee, L., Cole, S. and Colten, H.R. (1994) Am. J. Respir. Cell Mol. Biol. 11, 230–239.
[37] King, R.J. and MacBeth, M.C. (1981) Biochim. Biophys. Acta 647, 159–168.
[38] Efrati, H., Hawgood, S., Williams, M.C., Hong, K. and Benson, B.J. (1987) Biochemistry 26, 7986–7993.
[39] Haagsman, H.P., Sargeant, T., Hauschka, P.V., Benson, B.J. and Hawgood, S. (1990) Biochemistry 29, 8894–8900.
[40] McCormack, F.X., Calvert, H.M., Watson, P.A., Smith, D.L., Mason, R.J. and Voelker, D.R. (1994) J. Biol. Chem. 269, 5833–5841.