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Surfactant protein B: effects on lipid domain formation and intermembrane lipid flow

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Abstract

Pulmonary surfactant is a mixture of (phospho)lipids and surfactant specific proteins, lining the alveolar space. During each respiration cycle phospholipids are transferred between the phospholipid monolayer at the air/water interface and a variety of underlying membranes. Surfactant proteins may play a role in facilitating the insertion and removal of phospholipids by affecting the lipid organization of the bilayer and monolayer. The experiments described in this article were carried out in order to investigate the influence of surfactant protein B (SP-B) on the distribution of phospholipids in membranes and on the mixing of lipids between membranes. To determine the distribution of the non-labeled phospholipids in small unilamellar vesicles (SUV), the relative clustering of pyrene-labeled phospholipids was used, by measuring the ratio of excimer-to-monomer (E/M) pyrene fluorescence. In the absence of SP-B it was found that the clustering of the pyrenePC molecules was dependent on the proportion of saturated acyl chains and not on the proportion of negative charges. Addition of the positively charged SP-B to a mixture of DPPC and PG, led to an increase of approximately 20% in E/M ratio, indicating a clustering of the negatively charged PG molecules. This effect was intensified by addition of calcium ions. If pyrenePC-containing SUV were mixed with excess non-labeled SUV in the presence of SP-B and calcium ions, the E/M ratio decreased, corresponding with a flow of the pyrenePC molecules into the acceptor membranes. It is concluded that presence of domains of phospholipids can be detected with the use of pyrene-labeled phospholipids. Furthermore, SP-B showed a concentrating effect on the distribution of the negatively charged phospholipids, a process that could be important in regulating the phospholipid composition of the monolayer.

Keywords: Hydrophobic surfactant protein; Phospholipid domain; Pulmonary surfactant; Dipalmitoylphosphatidylcholine

Abbreviations: SP-A, SP-B and SP-C, surfactant protein A, B and C; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; eggPC, phosphatidylcholine; PG, phosphatidyl-DL-glycerol prepared from eggPC; pyrenePC, phosphatidylcholine 1-palmitoyl-2-(1-pyrenedecanoyl); pyrenePG, phosphatidylglycerol 1-palmitoyl-2-(1-pyrenedecanoyl); E/M ratio, excimer/monomer ratio; SUV, small unilamellar vesicles; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'- 2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis

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1. Introduction

Pulmonary surfactant is a complex mixture of lipids and proteins that lines the alveolar space. Its main function is to reduce the surface tension at the air/liquid interface by regulating the amount of phospholipids in the monolayer. Surfactant consists of 90% lipids and of 8–10% specific proteins [1,2]. The lipids consist mainly of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG), together with other lipids in a lesser amount. The volume of the lung increases with each inspiration, and so does the area of the monolayer lining the alveolar space. To maintain a low surface tension, phospholipids are inserted into the monolayer. During expiration the area decreases, and at end expiration, phospholipids have to be squeezed out of the monolayer. The insertion of phospholipids into the monolayer is slow in the absence of surfactant-specific proteins [3–5].

Four surfactant-specific proteins have been described: surfactant protein A (SP-A) and D (SP-D) are hydrophilic, and surfactant protein B (SP-B) and C (SP-C) are extremely hydrophobic. SP-A is a glycoprotein, assembled from 18 polypeptide chains, which binds phospholipids and carbohydrates [6–8]. It is required for the formation of tubular myelin [9,10], a membrane structure that is thought to be the precursor of the monolayer. SP-A is probably also important in lung defence [11]. SP-B, a protein of 79 amino acid residues with a molecular weight of 8000 under reducing conditions [12], is essential for the reduction of surface tension in the lung. Oosterlaken-Dijksterhuis and co-workers have shown that SP-B induces bilayer contact sites and subsequent lipid mixing between the bilayers [13]. Clinically [14] and in an experimental animal model [15] it has been demonstrated that a (functional) deficiency of this protein is life-threatening. In vitro studies have suggested that the activity of SP-B is synergistically enhanced by SP-A [16–18]. SP-C is the most hydrophobic protein, due to a high content of Val, Ile and Leu. It is a protein of 35 amino acid residues, and it has a highly conserved primary structure. The secondary structure of SP-C is mainly α-helical, both in monolayers and organic solvents [19–21], as well as in bilayer systems [22,23]. SP-C, when present in phospholipid vesicles [3] or a monolayer [24], is able to enhance the adsorption of phospholipids to an air/water interface, and it alters the thermodynamic properties of membranes [22]. Packing rearrangements of DPPC have been described when SP-C is present in a spread monolayer of DPPC [25]. It has been suggested that the hydrophobic surfactant proteins are able to perturb the monolayer, and thereby facilitate insertion or squeeze out phospholipids [26,27].

Junker and Creutz studied lipid dynamics using pyrene-labeled phosphatidylcholine (pyrenePC) or pyrene-labeled phosphatidylglycerol (pyrenePG). Apart from an excited monomeric spectrum, pyrene showed also an excited dimeric (excimeric) spectrum, resulting from energy transfer between pyrene monomers, which is dependent on concentration and lateral mobility of the labeled phospholipids [28]. When pyrenePC is concentrated, the pyrene molecules will interact with each other, forming more excimers. As a result the excimer/monomer ratio (E/M ratio) will increase.

Epifluorescence microscopic observations of monolayers consisting of mixtures of phospholipids with fluorescent reporter molecules suggested the presence of domains of liquid-expanded and liquid-condensed phases [29]. SP-C changed the packing arrangement of DPPC, which resulted in smaller condensed lipid domains [25].

In this paper results are presented of experiments, designed to study the effects of SP-A and SP-B on the formation and the stability of phospholipid domains. Using pyrene-labeled phospholipids, we demonstrated that (1) in a DPPC/PG mixture domains of DPPC and domains of PG are present, and (2) that SP-B, but not SP-A, induced clustering of negatively charged phospholipids.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), and phosphatidylglycerol (PG; prepared from eggPC) were obtained from Avanti Polar Lipids (Alabaster, AL).

EggPC (phosphatidylcholine) was obtained from Sigma (St. Louis, MO). Phosphatidylcholine 1-palmitoyl-2-(1-pyrenedecanoyl) (pyrenePC), and phosphatidylglycerol 1-palmitoyl-2-(1-pyrenedecanoyl) (pyrenePG) were purchased from Molecular Probes (Eugene, OR). Sephadex LH-60 was bought from Pharmacia (Uppsala, Sweden).

SP-B was isolated from canine and pig lung lavage. Fresh lungs were lavaged 3–5 times with a solution of 154 mM NaCl. The proteins were isolated by the method of Hawgood and co-workers [30]. Extraction of pulmonary surfactant was done with n-butanol [7], which was later removed by rotary evaporation. The residue was dissolved in chloroform/methanol/0.1 M HCl (1:1:0.05, v/v). Centrifugation was done to remove the insoluble particles. SP-B was isolated and separated from the lipids by Sephadex LH-60 chromatography [24]. The protein was stored in a mixture of chloroform/methanol (1:1, v/v) at −20°C. The concentration of SP-B was determined by a fluorescamine assay [31] and quantitative amino acid analysis. SP-A was isolated from porcine bronchoalveolar lavage fluid as described before [7] and stored in small portions at −20°C.

2.2. *Methods*

2.2.1. *Preparation of small unilamellar vesicles (SUV)*

For lipid mixing experiments, phospholipids dissolved in chloroform/methanol (1:1, v/v), were dried under a continuous stream of nitrogen at room temperature, and stored overnight in a vacuum exsiccator. The lipid film was hydrated for 10 min in 25 mM Hepes, 0.2 mM EGTA (pH 7.0) at 60°C, while shaken. An MSE Soniprep with a microtip was used for sonication (5 × 20 s, 10 s intervals) at an amplitude of 10 microns, and at a temperature of 55°C. The resulting small unilamellar vesicles (SUV) were kept on ice, and used the same day at which they were prepared.

2.2.2. *E/M ratio measurements*

All experiments were done at 37°C, except when stated otherwise. Fluorescence measurements were done on a Perkin Elmer Luminescence spectrometer LS50, linked to a personal computer, under continuous stirring. Fluorescence emission spectra of SUV (15 nmol lipid; 10 mol% pyrenePC or pyrenePG) dissolved in Hepes (25 mM, pH 7.0) with EGTA (0.2 mM) were recorded. For lipid mixing measurements SUV containing various amounts of protein (15 nmol phospholipids; 10 mol% pyrenePC or pyrenePG) were mixed with SUV (300 nmol phospholipids) [32]. Fluorescence emission spectra were recorded 2 min after the initiation of the experiment. Within that time the process of lipid mixing was completed. Excitation wavelength was 343 nm; emission wavelength 360–550 nm. Two fluorescence maxima were recorded: the monomer fluorescence maximum was found at 377 nm, and the excimer fluorescence maximum at 475 nm. The excimer-monomer ratio (E/M ratio) was calculated and used to express the extent of lipid mixing. Results are expressed as the mean of at least three independent experiments.

3. Results

The fluorescence emission spectrum of pyrenePC-labeled SUV, obtained by the excitation of the vesicle suspension at 343 nm, was characterized by a broad excimer peak at 475 nm and two monomer peaks at 377 and 397 nm. The excimeric peak and the first monomeric peak were used to determine the excimer/monomer ratio (E/M ratio). It was speculated that in a mixture of DPPC, PG and pyrenePC, the DPPC molecules are forming a strongly organised structure, in which the pyrenePC molecules do not fit, mainly because of their less regular acyl chains. Therefore, they should preferentially mix with the PG molecules to be relatively concentrated in this area. It was hypothesized that SP-B could enhance this segregation of lipids. Concentrated pyrene molecules will have a higher collision frequency resulting in an increased excimer/ monomer (E/M) ratio. in order to investigate our hypotheses, E/M ratios of vesicles with 10 mol% pyrenePC, were determined, comparing different combinations of phospholipids and surfactant proteins.

To determine whether the saturation of the phospholipids, or the (negative) charge of the phospholipids was the main factor for the formation of domains, experiments were performed in which one of the two was kept constant. When the saturation was kept constant at 50%, and the negative charge was ranging from 0 to 100%, no clear peak was observed

in E/M ratio (Fig. 1, upper). This was in contrast with the situation in which the negative charge was fixed to 10% and the saturation varied from 25 to 90%, where the E/M ratio rose sharply at 85 to 90% saturation (Fig. 1, lower). Also samples with a negative charge of 0 and 30% were tested, but in all samples the peak in E/M ratio was found at the same position (not shown).

E/M ratios of vesicles with 10 mol% pyrenePC and with 0.2 mol% SP-B incorporated in the vesicles, were determined, comparing different combinations of phospholipids (DPPC/PG, DPPG/PG, DPPC/PC and DPPC/PC). The highest E/M ratio was found when a DPPC/PG mixture (85.5/4.5, wt%) was used (which was interpreted as the highest relative

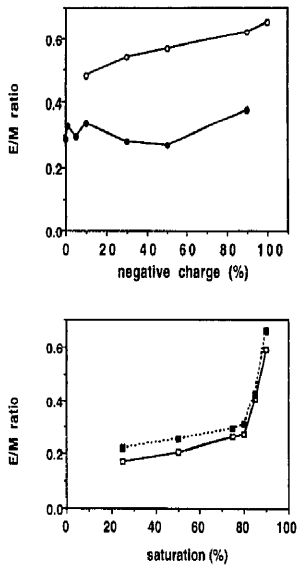

Fig. 1. (Upper) Effect of negative charge on the E/M ratio of SUV. The saturation of the acyl chains was kept constant at 50%, the negative charge was ranging from 0% to 100%. The buffer consisted of 25 mM Hepes, 0.2 mM EGTA, pH 7.0. No calcium ions were added. As a fluorescent molecule was used pyrenePG (○), and pyrenePC (●). (Lower) Effect of saturation on the E/M ratio of SUV. The negative charge was kept constant at 10% (PG), the saturation of the acyl chains was ranging from 25% to 90% (PC). The buffer consisted of 25 mM Hepes, 0.2 mM EGTA, pH 7.0. (□): phospholipid vesicles, (– ■ –): phospholipid vesicles plus 0.05 mol% SP-B.

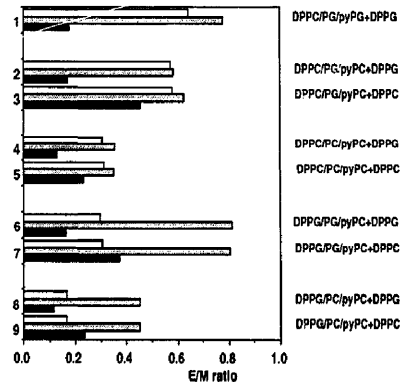

Fig. 2. E/M ratios of different phospholipid mixtures in SUV. To 15 nmol of SUV (85.5/4.5/10, saturated phospholipid/non-saturated phospholipid/pyrene-labeled phospholipid; mol%) with 0.2 mol% SP-B incorporated (a: white), 3 mM calcium ions were added (b: light gray), followed by excess (300 nmol) phospholipid SUV (c: dark gray). The buffer consisted of 25 mM Hepes, 0.2 mM EGTA, 10 mM NaCl, pH 7.0. The experiment was repeated twice with similar results (1) DPPC/PG/pyrenePG plus DPPG, (2) DPPC/PG/pyrenePC plus DPPG, (3) DPPC/PG/pyrenePC plus DPPC, (4) DPPC/PC/pyrenePC plus DPPG, (5) DPPC/PC/pyrenePC plus DPPC, (6) DPPG/PG/pyrenePC plus DPPG, (7) DPPG/PG/pyrenePC plus DPPC, (8) DPPG/PC/pyrenePC plus DPPG, (9) DPPG/PC/pyrenePC plus DPPC.

concentration of pyrene-labeled molecules in a PG-containing area, Fig. 2, 2a and 3a). Mixtures of DPPC/PC (Fig. 2, 4a and 5a), DPPG/PG (Fig. 2, 6a and 7a) and DPPG/PC (Fig. 2, 8a and 9a) showed lower E/M ratios. If, in combination with DPPC/PG, pyrenePG was used instead of pyrenePC, there was also a strong clustering of pyrene-labeled molecules (Fig. 2, 1a). A mixture with 85.5% DPPG (and 4.5% PC or PG), to which 3 mM calcium ions were added, showed a considerable increase of the E/M ratio (correlating with a relative concentration of the pyrenePC molecules, Fig. 2, 6b–9b). No major increase of E/M ratio was observed when calcium ions were added to 85.5% DPPC (and 4.5% PC or PG) (Fig. 2, 2b–5b).

After the addition of excess vesicles (pure DPPG or DPPC) to the SP-B containing mixtures, lipid mixing was started, and pyrenePC molecules were able to spread in a larger surface. This resulted,

depending on the lipid mixture, in a considerable decrease of the E/M ratio. When vesicles of DPPC/PG were mixed with pure DPPG, a vast decrease of the E/M ratio was observed (Fig. 2, 2c), in contrast to addition of excess DPPC, which gave only a minor decrease in E/M ratio (Fig. 2, 3c). The same pattern was found with the combination of DPPC/PC and DPPG vesicles which gave a decrease of E/M ratio (Fig. 2, 4c), and the combination of DPPC/PC and DPPC vesicles where the decrease of E/M ratio was lower (Fig. 2, 5c).

Addition of phospholipids to DPPG/PG- or DPPG/PC-vesicles (in the presence of calcium ions), resulted in a considerable decrease of the E/M ratio (Fig. 2, 6c–9c). The addition of DPPG led to a decrease of the E/M ratio to a level lower than the initial E/M ratio (6c and 8c), whereas after DPPC addition, an E/M ratio was found which was slightly higher than the E/M ratio found before the addition of calcium ions (Fig. 2, 7c and 9c). Addition of excess DPPG to a DPPC/PG/pyrenePG mixture resulted in a vast decrease of the E/M ratio.

These results suggested domain formation in a mixture of different phospholipids. In the presence of SP-B pyrenePC molecules preferred a PC domain over a (DP)PG domain; and a (DP)PG domain over a DPPC domain. PyrenePG had a preference for DPPG over DPPC.

In order to determine the distribution of pyrenePC molecules in different mixtures, the E/M ratio was measured of SUV with different phospholipid compositions. In all mixtures (DPPC/PG or PG/eggPC), 10% pyrenePC was present. The highest E/M ratio was found when a high DPPC concentration was

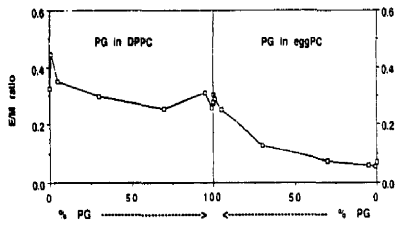

Fig. 3. Effect of different phospholipid mixtures on E/M ratio. In SUV, including 10 mol% pyrenePC, the composition was ranging from 100% DPPC via 100% PG to 100% eggPC. The buffer consisted of 25 mM Hepes, 0.2 mM EGTA, 10 mM NaCl, pH 7.0.

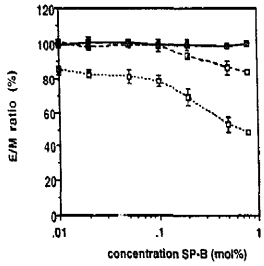

Fig. 4. Effect of the addition of different concentrations of SP-B on lipid mixing of SUV made of DPPC/PG (7:3, mol/mol). To a mixture of 15 nmol SUV and 300 nmol SUV (————), SP-B was added (- - -), followed by 3 mM calcium ions (- - -). The buffer consisted of 25 mM Hepes, 0.2 mM EGTA, pH 7.0.

used (Fig. 3). When the PG concentration was higher than 5%, the E/M ratio was significantly decreased, indicating that all pyrenePC molecules could mix relatively well in this domain. The combination PG/eggPC showed a similar pattern. When the eggPC concentration was higher than 5%, the E/M ratio was lower. PyrenePC was preferentially expelled out of the PG domein, to be concentrated in a domain consisting of eggPC and pyrenePC.

SP-B dissolved in methanol was added to mixtures of SUV of DPPC/PG/pyrenePC. SP-B was added in concentrations ranging from 0.005 to 0.8 mol%, followed by 3 mM calcium ions (Fig. 4). At an SP-B concentration of 0.2 mol%, SP-B-induced lipid mixing is apparent. To obtain a maximal lipid mixing induced by SP-B, minimally 0.8 mol% SP-B was required. In all the samples a calcium-dependent lipid mixing was observed. Interestingly, also a slight but significant decrease in E/M ratio was found when no calcium is available, beginning at an SP-B concentration of 0.2 mol%.

To study the influence of SP-B and SP-A on the distribution of the phospholipids, SUV were made of DPPC/PG/pyrenePC (63:27:10, mol%). After measuring the E/M ratio, calcium ions, SP-B (dissolved in methanol), or SP-A were added to the SUV. When the E/M ratio of SUV was set to 100%, addition of SP-B to SUV caused an increase of E/M ratio of approximately 8%, and extra addition of calcium ions increased the E/M ratio to 33% (Fig. 5). Addition of calcium ions without SP-B showed an increase of

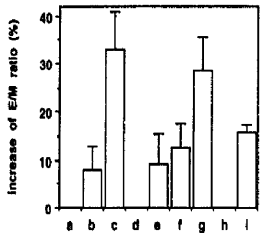

Fig. 5. Relative increase of E/M ratio after the addition of proteins to SUV made of DPPC/PG/pyrenePC (63:27:10, mol%). The buffer consisted of 25 mM Hepes, 0.2 mM EGTA, pH 7.0. (a,b,c) SUV, addition of 0.2 mol% SP-B, followed by addition of 3 mM calcium ions, (d,e,f,g) SUV, addition of 0.2 mol% SP-B, followed by 10 μg/ml SP-A, followed by 3 mM calcium ions, (h,i) SUV, addition of 3 mM calcium ions.

E/M ratio of 15%, demonstrating a condensing influence of SP-B on the distribution of the pyrenePC molecules. SP-A had no significant influence on the E/M ratio. SUV, with or without SP-B incorporated in the SUV, showed no significant difference in E/M ratio (not shown).

Vesicles (DPPC/PG/pyrenePC (63:27:10; mol%), containing various amounts of SP-B, were mixed with DPPC/PG vesicles (7:3, mol%), and the E/M ratio was measured as a parameter of lipid mixing. Calcium-dependent lipid mixing of PG-containing vesicles was induced by SP-B, and a protein concentration of 0.2 mol% gave a maximal lipid mixing. The hydrophilic SP-A induced calcium-independent lipid mixing only at high SP-B concentrations, but no synergistic effect on lipid mixing was observed (Fig. 6). With albumin, no effect on the E/M ratio was obtained.

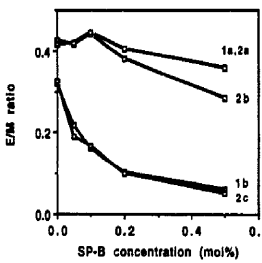

Fig. 6. Lipid mixing as a function of SP-B concentration. The E/M ratio is a parameter of lipid mixing. (1a, 2a): SUV of DPPC/PG/pyrenePC (63:27:10, mol%) were mixed with SUV of DPPC/PG (7:3; mol%) with SP-B incorporated, (1b): addition of 3 mM $CaCl_2$ to mix 1a; (2b): addition of 10 μg SP-A to mix 2a, (2c): addition of 3 mM $CaCl_2$ to mix 2b.

4. Discussion

During inspiration, phospholipids are inserted into the monolayer lining the alveolar space. The phospholipids are secreted by alveolar type II cells, and stored as tubular myelin. To insert the phospholipids into the monolayer it is necessary to perturb the monolayer. The hydrophobic surfactant proteins are good candidates for this task: they are part of the surfactant, and it has been demonstrated that SP-B and SP-C are necessary for the enhancement of the insertion of phospholipids into the monolayer. In this paper results of experiments are presented that were designed to obtain more information on the distribution of the phospholipid molecules, and the influence of SP-B on this distribution.

It is hypothesized that in a mixture of DPPC and PG, these lipids tend to form clusters preferentially made of either PG molecules or DPPC molecules. The DPPC molecules are forming a strongly organised structure. Unsaturated phospholipids and therefore the pyrenePC molecules do not fit in this structure, mainly because of their less regular acyl chains. With pyrenePC as the fluorescent molecule, the highest E/M ratio was found in the DPPC/PG mixture (Fig. 2). This could be explained by the assumption that under these conditions pyrenePC molecules do not distribute in the DPPC area. This results in a relative clustering of pyrenePC molecules together with the PG molecules in a mixed domain, a process that is enhanced by the positively charged SP-B. The formation of DPPC-rich domains may be an important physiological function of this protein. Previously, SP-B was found to interact specifically with PG [13,33]. The rise in E/M ratio, observed when calcium ions were added to the vesicles which consisted mainly of DPPG, was a result of the interaction between the negatively charged vesicles and calcium ions.

SP-B is able to induce lipid mixing in the presence of calcium ions, thereby exchanging phospholipids

[13]. When vesicles of DPPC/PG and DPPG were brought together, the vast decrease of E/M ratio was due to the distribution of pyrenePC into the DPPG area, because all pyrenePC molecules could spread relatively well in this domain. Addition of excess DPPC to DPPC/PG vesicles did not induce pyrenePC redistribution, and the pyrenePC stayed concentrated in the mixed pyrenePC/PG domain. The results of the addition of DPPC and DPPG to mixtures of DPPC/PC was based on the same mechanism. The increase of E/M ratio of DPPG/PG- and DPPG/PC-vesicles, which occurred after exposure to calcium ions, was opposed by the addition of excess phospholipids. Addition of excess DPPG resulted in a more pronounced decrease in E/M ratio than addition of excess DPPC. This is explained by the redistribution of the pyrenePC molecules in the newly added DPPG. The vast decrease in E/M ratio, observed when excess DPPG was added to a DPPC/PG/pyrenePG mixture, was interpreted by a dilution of pyrenePG in (DP)PG domains.

Taken collectively, these results suggest the formation of domains in mixtures of phospholipids present in pulmonary surfactant. The pyrenePC molecules, which were used for detection, had a preference for the eggPC- or PG-rich domains (Fig. 3). This could be due to the strong organisation of the DPPC molecules in the D 'PC domain, where pyrenePC was preferentially expelled out of the DPPC domain, to be concentrated in the PG domain. In a combination of pyrenePC, eggPC and PG, the pyrenePC was preferentially expelled out of the PG domain, to be concentrated in the eggPC domain.

Addition of SP-B to SUV (DPPC/PG/pyrenePC; 63:27:10, mol%) resulted in an increase of E/M ratio of 9% (Fig. 5). SP-A, alone or in combination with SP-B, had no significant influence on the E/M ratio, whereas calcium ions greatly increased the E/M ratio, indicating a relative clustering of the pyrene molecules. The poor effect of SP-A is in line with the results of Poulain and co-workers, who used a different fluorescent probe [34].

The experiments done with SUV with different compositions, but in which the negative charge or in which the saturation stayed constant, demonstrated that not the negative charge, but the saturation was responsible for the concentration of pyrenePC or pyrenePG (Fig. 1).

We chose to use 15 nmol donor SUV and 300 nmol acceptor SUV for the lipid mixing experiments. SP-B can be added to the system in different ways: (1) incorporated in the vesicles with the fluorescent lipid; (2) incorporated in the vesicles without the fluorescent lipid; (3) addition of SP-B dissolved in methanol. When SP-B was present in 15 nmol SUV, 0.2 mol% of this protein was necessary to obtain a maximal lipid mixing (not shown). This corresponds with a total amount of 0.03 nmol SP-B. With the protein present in the 300 nmol fraction, also 0.2 mol% SP-B was needed [13], but this corresponded with 0.6 nmol SP-B. Addition of SP-B from methanol was the least efficient (Fig. 4). Minimally 0.5 mol% SP-B was required to obtain a maximal lipid mixing (0.5 mol% of 315 nmol = 1.575 nmol). This could be explained by a different availability of SP-B in the different situations. In the case of SP-B added to the medium from methanol, part of the SP-B may not be distributed evenly between the vesicles, part of the SP-B is dissolved in the subphase and part of the SP-B may be precipitated. Interestingly, some lipid mixing is obtained in the absence of calcium ions, starting at an SP-B concentration of 0.2 mol%. This suggests that the positive charges of SP-B are sufficient to overcome the negative charges of the phospholipids, resulting in lipid molecules flowing from one vesicle into the other. Calcium ions enhance this process.

It is concluded that in membranes prepared from important surfactant phospholipids, these phospholipids are not evenly distributed, but can form clusters (or domains). In the absence of calcium ions the saturation of acyl chains was responsible for this separation. Addition of SP-B to SUV causes clustering of the negatively charged PG molecules. Lipid mixing was induced by SP-B, and enhanced by SP-A if minimally 0.2 mol% SP-B was present.

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