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Lipid mixing is mediated by the hydrophobic surfactant protein SP-B but not by SP-C

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Pulmonary surfactant contains two families of hydrophobic proteins, SP-B and SP-C. Both proteins are thought to promote the formation of the phospholipid monolayer at the air/fluid interface of the lung. The excimer/monomer ratio of pyrene-labeled PC fluorescence intensities was used to investigate the capacity of the hydrophobic surfactant proteins, SP-B and SP-C, to induce lipid mixing between protein-containing small unilamellar vesicles and pyrene-PC-labeled small unilamellar vesicles. At 37°C SP-B induced lipid mixing between protein-containing vesicles and pyrene-PC-labeled vesicles. In the presence of negatively charged phospholipids (PG or PI) the SP-B-induced lipid mixing was enhanced, and dependent on the presence of (divalent) cations. The extent of lipid mixing was maximal at a protein concentration of 0.2 mol%. SP-C was not capable of inducing lipid mixing at 37°C not even at protein concentrations of 1 mol%. The SP-B-induced lipid mixing may occur during the Ca^{2+}-dependent transformation of lamellar bodies into tubular myelin and the subsequent formation of the phospholipid monolayer.

Introduction

Pulmonary surfactant, a complex lipid-protein mixture synthesized and secreted by alveolar type II cells, reduces surface tension at the air/water interface in the lung [1,2]. It is rich in phospholipid and contains at least three classes of specific proteins. The main phospholipid is dipalmitoylphosphatidylcholine (DPPC), with smaller amounts of PG, unsaturated PC, other phospholipids, and cholesterol [3].

The hydrophobic surfactant proteins, SP-B [4,5] and SP-C [6,7] play an important role in the formation of a phospholipid monolayer at the air/liquid interface. SP-B is also required for the in vitro formation of the highly ordered membrane lattice, tubular myelin [8]. The monomeric form of SP-B has a molecular weight of 8000 and consists of 79 amino acids [9,10]. The monomeric form of SP-C has a molecular weight of 4500 and has one (canine SP-C; [11]) or two palmitoyl group(s) covalently linked to the polypeptide chain [12], which consists of 35 amino acids [9,10]. Both proteins have possible sites for interaction with a phospholipid bilayer [4,9,13].

In a previous study, we showed that SP-B and SP-C, if present in preformed monolayers, induce lipid insertion into the monolayer after the addition of (divalent) cations [14]. The hydrophobic proteins may induce monolayer formation by the following mechanism: (1) induction of close contact between monolayer and vesicles, mediated by (divalent) cations, (2) binding of phospholipid vesicles to SP-B or SP-C present in the monolayer, and (3) phospholipid insertion into the monolayer, facilitated by the surface properties and the hydrophobic nature of the proteins [15].

Pyrene-labeled phospholipids show two kinds of fluorescence emission spectra: a monomer spectrum and an excimer spectrum that originates from excited dimers. The ratio of excimer to monomer fluorescence intensities (E/M) is proportional to the collision frequency of the pyrene moieties, which is dependent on concentration and diffusion [16]. This special property has been used, for instance, to investigate membrane fusion processes [17].

Fusion processes may play a role in the formation of contact sites between membranes during the transformation of lamellar bodies into tubular myelin and in the insertion of (phospho)lipids into the surfactant

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monolayer. In this study, we used the excimer/monomer ratio of pyrene-labeled PC fluorescence intensities to investigate the capacity of the hydrophobic surfactant proteins, SP-B and SP-C, to induce lipid mixing between protein-containing small unilamellar vesicles and pyrene-PC-labeled small unilamellar vesicles.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Roth (Karlsruhe, Germany). Phosphatidylglycerol (PG; sodium salt, prepared from egg yolk PC) and L-α-phosphatidylinositol (PI; ammonium salt, prepared from bovine liver) were purchased from Sigma (St. Louis, MO). Phosphatidylcholine from egg yolk (egg PC) was purified by high performance liquid chromatography and judged chromatographically pure by the use of thin-layer chromatography. 1-Palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrene-PC) was obtained from Molecular Probes (Eugene, OR).

Isolation of SP-B and SP-C

SP-B and SP-C were isolated from porcine lung lavage. Porcine lungs were obtained from the slaughterhouse and lavaged 3–5 times with a solution of 154 mM NaCl. Pulmonary surfactant was prepared from the bronchoalveolar lavage by the method of Hawgood et al. [18]. Lung surfactant was extracted with 1-butanol [19]. Butanol was dried by rotary evaporation, and the residue was dissolved in chloroform/methanol/0.1 M HCl (1:1:0.05, by vol.). Insoluble material was removed by centrifugation. SP-B and SP-C were separated from lipids and purified to homogeneity by Sephadex H-60 chromatography as described before [20]. Monolayer studies indicated that the isolated proteins were surface active [14,15].

Small unilamellar vesicles (SUV)

Lipids with or without SP-B or SP-C dissolved in chloroform/methanol (1:1, v/v) were dried under a stream of nitrogen at 37°C. The lipid films were hydrated in 25 mM Hepes (pH 7.0) supplemented with 0.2 mM EGTA at 50°C. The suspensions were sonicated with a 0.5 inch flat-top disrupter tip for 1 min at 50 W. All vesicles were prepared freshly each day and kept on ice until use. Differential scanning calorimetry measurements showed that at 37°C phospholipids of all types of vesicles used were in the liquid crystalline phase (L_α).

Measurements of lipid mixing

Lipid mixing experiments were performed at 37°C unless otherwise stated. Phospholipid SUV (300 nmol of lipid) containing different amounts of SP-B or SP-C were mixed with pyrene-PC-labeled SUV (15 nmol of lipid containing 10 mol% of pyrene-PC) in a buffer of 25 mM Hepes (pH 7.0) supplemented with 0.2 mM EGTA at a final volume of 2 ml. Fluorescence emission spectra (excitation wavelength = 343 nm, emission wavelength = 360–550 nm) were recorded under continuously stirring of the sample on an SLM-Aminco SPF-500 C fluorescence spectrophotometer equipped with a thermostatically controlled cuvette holder. The excimer to monomer fluorescent ratio (E/M) was calculated by dividing the fluorescent intensity at 475 nm by the intensity at 377 nm. At constant temperature and viscosity the E/M ratio is proportional to the local probe concentration. The validity of this relation was confirmed for the pyrene-PC containing vesicles that were used (Fig. 1). Results are given as a representative of at least two separate experiments. The variation in the decrease of the E/M ratio between individual experiments was less than 0.2.

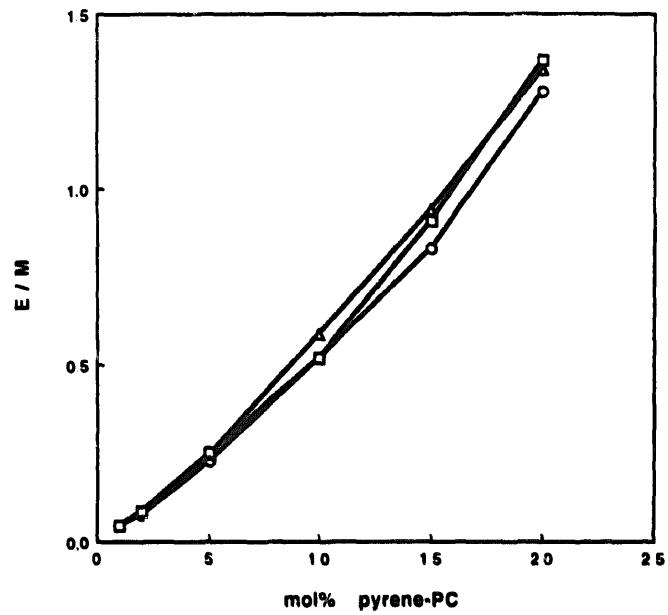

Fig. 1. Relation between percentage of pyrene-PC in used vesicles and recorded E/M ratio. (○), DPPC/PG (30 mol% PG); (□), DPPC/PG/egg PC (5 mol% PG; 25 mol% egg PC). (△), DPPC/egg PC (30 mol% egg PC). Addition of $CaCl_2$ (3 mM) did not alter E/M ratios significantly.

Results

The excimer/monomer ratio of pyrene-PC fluorescence intensities (E/M) was used to investigate the capacity of SP-B and SP-C to induce lipid mixing between protein-containing vesicles and pyrene-PC-labeled vesicles. The fluorescence emission spectrum of pyrene-PC-labeled vesicles (DPPC/PG, 30 mol% PG; containing 10 mol% of pyrene-PC) obtained by the excitation of the vesicle suspension at 343 nm exhibited

a broad excimer fluorescence at 475 nm and two sharp peaks at 377 nm and 410 nm due to monomer fluorescence. In the presence of Triton X-100 (0.1% v/v) only monomer fluorescence was observed. When vesicles (DPPC/PG, 30 mol% PG) containing different amounts of SP-B were mixed with pyrene-PC-labeled vesicles, the emission spectra were very similar to that of pyrene-PC-labeled vesicles (data not shown). Fig. 2 shows that after the addition of $CaCl_2$ to a final concentration of 3 mM, lipid mixing between DPPC/PG (30 mol% PG) vesicles and pyrene-PC-labeled vesicles occurred as became apparent by a small decrease of the *E/M* ratio (decrease of the *E/M* ratio from 1.2 to 1.0). This observation can be largely attributed to the fusion capacity of PG, which is mediated by Ca^{2+}. When SP-B was present in the DPPC/PG (30 mol% PG) vesicles, the extent of lipid mixing was enhanced. The extent of lipid mixing was dependent on the protein concentration in the vesicles and was maximal at a protein concentration of 0.2–0.4 mol% (decrease of the *E/M* ratio from 1.2 to 0.5; Fig. 2). At this protein concentration the *E/M* ratio reached a plateau after 2 min indicating that the kinetics of lipid mixing were very fast. At lower protein concentrations the *E/M* ratio reached a plateau after 4–8 min (data not shown). When SP-C was present in the DPPC/PG (30 mol% PG) vesicles, apart from the PG-induced lipid mixing no protein-induced lipid mixing was observed at protein concentrations up to 1 mol% (Fig. 2).

It is generally believed that anionic lipid–Ca^{2+} interactions play an important role in fusion reactions. Therefore, we investigated whether apart from the PG-induced lipid mixing, PG–Ca^{2+} interactions were required for the SP-B-induced lipid mixing. Vesicles of

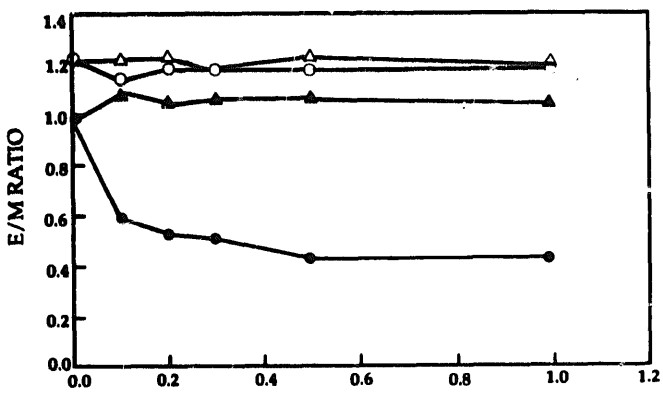

Fig. 2. Effect of protein concentration on lipid mixing. Pyrene-PC-labeled vesicles of DPPC/PG (30 mol% PG) were mixed with protein-containing vesicles of DPPC/PG (30 mol% PG) at 37°C. (○), SP-B-containing vesicles in the absence of $CaCl_2$. (●), SP-B-containing vesicles in the presence of 3 mM $CaCl_2$. (△), SP-C-containing vesicles in the absence of $CaCl_2$. (▲), SP-C-containing vesicles in the presence of 3 mM $CaCl_2$.

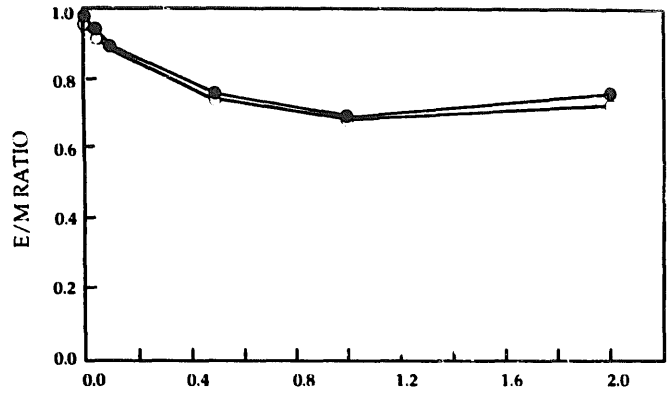

Fig. 3. Effect of the SP-B concentration on lipid mixing in the absence of PG. Pyrene-PC-labeled vesicles of DPPC/egg PC (30 mol% egg PC) were mixed at 37°C with vesicles of DPPC/egg PC (30 mol% egg PC) containing different concentrations of SP-B. (○), In the absence of $CaCl_2$. (●), In the presence of 3 mM $CaCl_2$.

DPPC/egg PC (30 mol% egg PC) containing different amounts of SP-B were mixed with pyrene-PC-labeled DPPC/egg PC (30 mol% egg PC) vesicles. Fig. 3 shows that no Ca^{2+}-mediated SP-B-induced lipid mixing occurred. At higher protein concentrations a slightly decreased *E/M* ratio was observed, both in the absence and in the presence of Ca^{2+}. To study the effect of PG on the SP-B-induced lipid mixing in more detail, the amounts of PG in the protein-containing vesicles and in the pyrene-PC-containing vesicles were varied. In protein-containing vesicles with a fixed amount of SP-B (0.2 mol%) the PG concentration was varied from 0 to 30 mol% of phospholipid. The percentage of unsaturated phospholipid in these vesicles was kept constant by using different molar percentages of egg PC (0 to 30 mol%). These types of vesicles were mixed with pyrene-PC-labeled vesicles of DPPC containing 0, 5, and 30 mol% of PG. Also in these vesicles the amount of total unsaturated phospholipid was kept constant. $CaCl_2$ was added to a final concentration of 3 mM. Figs. 4A and 4B show that with pyrene-PC-labeled vesicles of DPPC/PG (30 and 5 mol% PG, respectively) no strong correlation was found between the SP-B-induced lipid mixing and the PG concentration in the protein-containing vesicles. Interestingly, when PG was absent from the protein-containing vesicles, lipid mixing proceeded in the absence of $CaCl_2$ (Figs. 4A and 4B). This suggests that under these experimental conditions the net positive charge of SP-B is sufficient to induce lipid mixing. If PG was absent from the pyrene-PC-labeled vesicles and the concentration of PG was varied in the protein-containing vesicles the *E/M* ratio did not change much. A small decrease was observed in the absence of PG (Ca^{2+}-independent) and at high PG concentrations (Ca^{2+}-dependent) (Fig. 4C).

Vesicles of DPPC/PI were used to investigate whether PG can be replaced by PI in mediating SP-B-

induced lipid mixing. Fig. 5 shows that apart from a PI-induced lipid mixing (decrease of the E/M ratio from 0.7 to 0.5) also SP-B-induced lipid mixing is observed. The extent of lipid mixing was dependent on the protein concentration in the protein-containing vesicles and was maximal at a protein concentration of 0.2–0.4 mol% (decrease of the E/M ratio from 0.7 to 0.2). This would imply that charge rather than molecular structure of PG is important in enhancing the SP-B-induced lipid mixing between protein-containing vesicles and pyrene-PC-labeled vesicles.

In the presence of PG, the protein-induced lipid mixing was mediated by Ca^{2+} ions. To investigate whether Ca^{2+} is specific for this protein-induced lipid mixing, other (divalent) cations were studied for their capacity to mediate the protein-induced lipid mixing. Fig. 6 shows that the extent of the PG-induced lipid mixing was almost the same with all the different (divalent) cations used. The extent of the SP-B-induced lipid mixing in the presence of divalent cations like $MgCl_2$ or $MnCl_2$ was very similar to that in the presence of $CaCl_2$ (final concentrations: 3 mM). The addition of NaCl (150 mM) or HCl (lowering the pH from pH 7.0 to pH 4.0) also induced protein-induced lipid mixing, albeit in case of NaCl at some lower extent than divalent cations.

Discussion

The hydrophobic surfactant proteins, SP-B and SP-C, play an important role in monolayer formation by

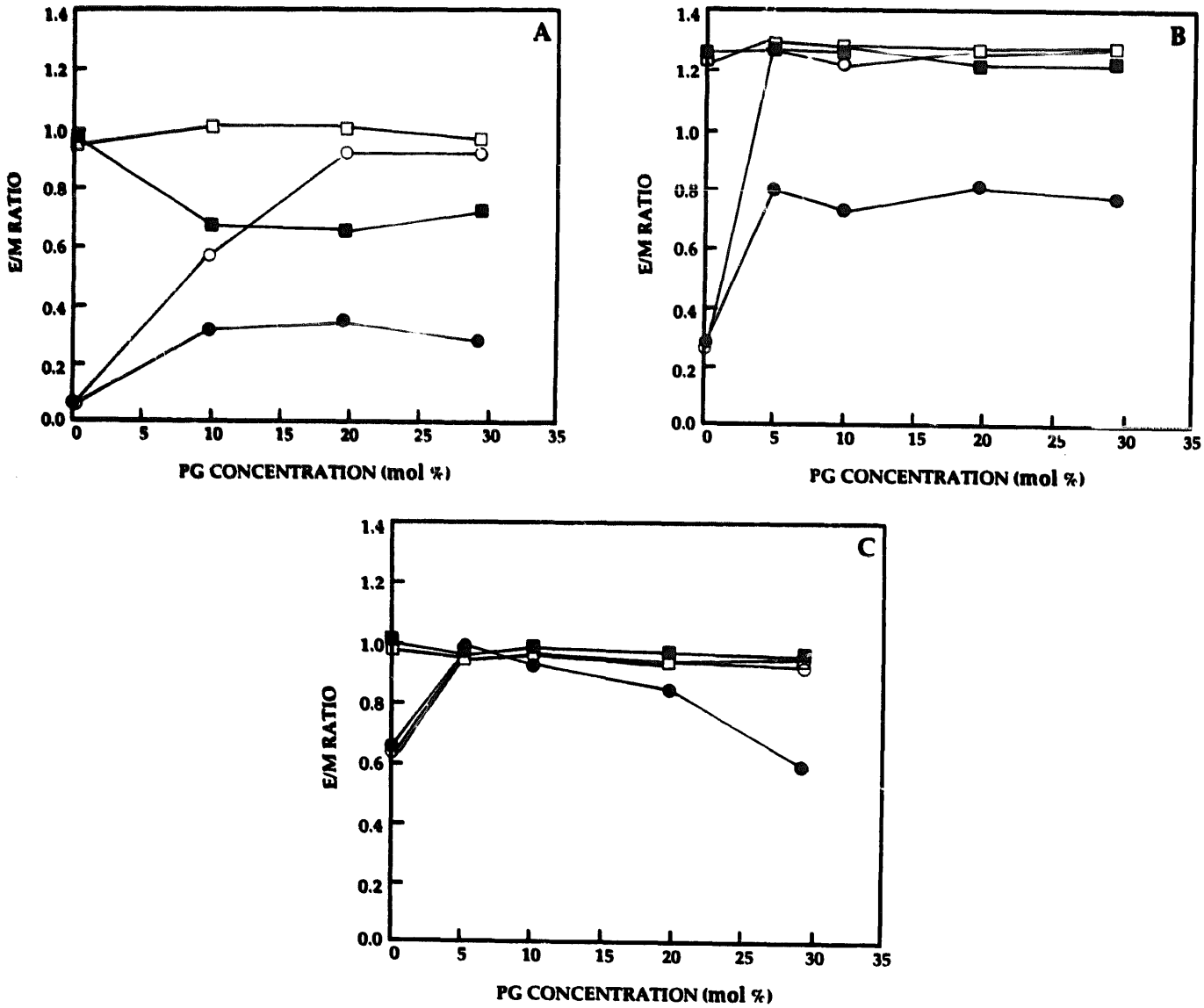

Fig. 4. Effect of the total amount of PG on SP-B-induced lipid mixing. Pyrene-PC-labeled vesicles of (A) DPPC/PG (30 mol% PG); (B) DPPC/PG/egg PC (5 mol% PG; 25 mol% egg PC); or (C) DPPC/egg PC (30 mol% egg PC), were mixed at 37°C with vesicles of DPPC and different concentrations of PG. The percentage unsaturated phospholipid was kept constant by using different molar percentages of egg PC. (□), In the absence of protein and $CaCl_2$. (■), In the absence of protein and in the presence of 3 mM $CaCl_2$. (○), In the presence of 0.2 mol% SP-B and in the absence of $CaCl_2$. (●), In the presence of 0.2 mol% SP-B and 3 mM $CaCl_2$.

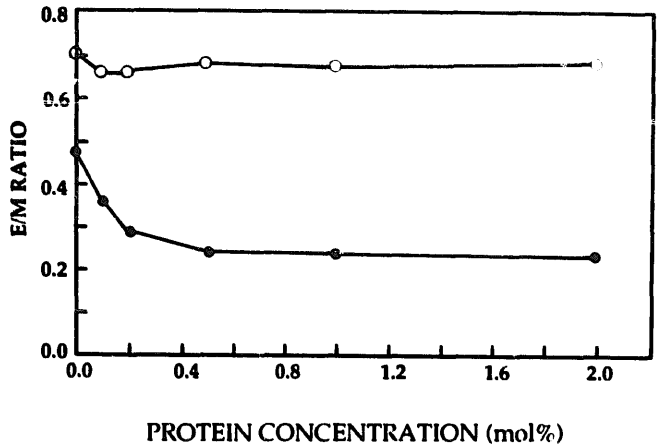

Fig. 5. Effect of the SP-B concentration on lipid mixing in the presence of PI. Pyrene-PC-labeled vesicles of DPPC/PI (30 mol% PI) were mixed at 37°C with vesicles of DPPC/PI (30 mol% PI) containing different concentrations of SP-B. (○), In the absence of $CaCl_2$. (●), In the presence of 3 mM $CaCl_2$.

enhancing the adsorption of phospholipids from protein containing vesicles to an air/water interface [4–7]. Both proteins, if present in a preformed monolayer, induce insertion of phospholipids from pure phospholipid vesicles into the monolayer after the addition of (divalent) cations [14]. In both processes, SP-B is more efficient than SP-C. In this study, the excimer/monomer ratio of pyrene-PC fluorescence intensities was used to investigate the capacity of both SP-B and SP-C to induce lipid mixing between protein-containing vesicles and pyrene-PC-labeled vesicles. To facilitate direct comparison with the studies on protein-induced insertion of phospholipids from SUV into the monolayer [14,15], the experiments were carried out in the same (low ionic strength) buffer. It is shown that SP-B can induce lipid mixing between protein-containing vesicles and pyrene-PC-labeled vesicles. In the presence of negatively charged phospholipids the extent of lipid mixing was enhanced, and in this situation lipid mixing was dependent on (divalent) cations. The kinetics of this lipid mixing process are very fast, with the E/M ratio reaching a plateau within 2–8 min. Under these conditions, SP-C was not capable of inducing lipid mixing between protein-containing vesicles and pyrene-PC-labeled vesicles.

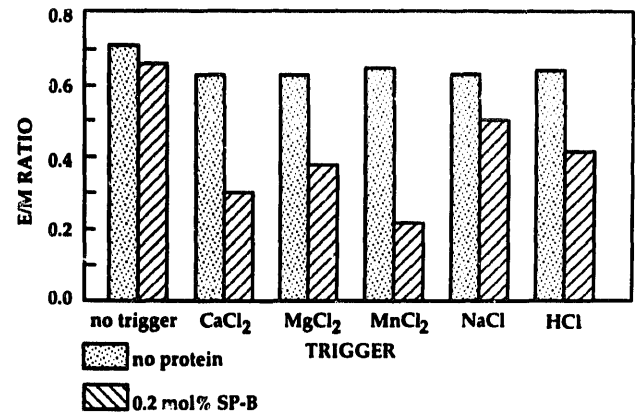

Fig. 6. Effect of cations on SP-B-induced lipid mixing. Pyrene-PC-labeled vesicles of DPPC/PG (30 mol% PG) were mixed at 37°C with vesicles of DPPC/PG (30 mol% PG) containing either no protein or 0.2 mol% SP-B. $CaCl_2$, $MgCl_2$, and $MnCl_2$ were added to a final concentration of 3 mM. NaCl was added to a final concentration of 150 mM and HCl was added lowering the pH from pH 7.0 to 4.0.

Shiffer et al. [21] described that the external addition of a mixture of SP-B and SP-C to large unilamellar vesicles containing either PG or DPPG induced the release of fluorescent probe entrapped in part of the vesicles. In the presence of 3 mM $CaCl_2$ the external addition of a mixture of SP-B and SP-C not only affected the permeability of the vesicles but also induced lipid mixing as was monitored by resonance energy transfer between two different fluorescent lipid probes [21].

The addition of Ca^{2+} caused a decrease of the E/M ratio in the absence of SP-B if pyrene-PC-labeled DPPC/PG vesicles (30 mol% PG) were mixed with PG-containing vesicles (Fig. 4A). It has been reported that unilamellar vesicles of PG aggregate and fuse after addition of divalent cations, the threshold concentration of cations inducing aggregation being lower than the threshold concentration inducing fusion [22,23]. It is not clear whether the observed PG-induced lipid mixing in the absence of protein is a result of aggregation or fusion because aggregation alone may also result in lipid mixing [24].

If SP-B was present in DPPC/egg PC (30 mol% egg PC) vesicles and PG was present only in the pyrene-PC-labeled vesicles, lipid mixing proceeded in the absence of Ca^{2+} (Fig. 4). This may indicate that the net positive charge of SP-B may be sufficient to overcome repulsive forces between protein-containing vesicles and pyrene-PC-labeled vesicles. The SP-B-induced lipid mixing was dependent on the total amount of PG present in the sample (Fig. 4), indicating that the overall charge distribution (PG/Ca^{2+} ratio), which is related to repulsive forces between vesicles, is important for this process.

When PG was replaced by another negatively charged phospholipid like PI, Ca^{2+}-mediated PI-induced lipid mixing was observed. The extent of the PI-induced lipid mixing was comparable to that of the PG-induced lipid mixing. In the presence of SP-B apart from PI-induced lipid mixing notable SP-B-induced lipid mixing was observed, indicating that charge rather than molecular structure of PG is important in enhancing the SP-B-induced lipid mixing. This is in agreement with the results of Beppu et al. [25] and Hallman et al. [26] that PI can replace PG without affecting the most important physiologic and physicochemical properties of surfactant. The negatively charged phospholipids may enhance the SP-B-induced lipid mixing by (1)

affecting the structure of the vesicles, (2) interacting with the net positively charged protein, or (3) inducing lipid mixing themselves.

(Divalent) cations other than Ca^{2+} were investigated for their capacity to mediate the SP-B-induced lipid mixing. At the concentrations used, the extent of the PG-induced lipid mixing was very similar with the different (divalent) cations (Fig. 6). It is shown that Ca^{2+} ions can be replaced by other divalent cations like Mn^{2+} and Mg^{2+} (3 mM), by monovalent cations like Na^{+} (150 mM), and by lowering the pH from pH 7.0 to pH 4.0 (Fig. 6). This observation indicates that Ca^{2+} is not specific for the SP-B-induced lipid mixing and supports the idea that apart from interaction with negatively charged phospholipids, (divalent) cations play a role in reducing repulsive forces between the protein-containing vesicles and pyrene-PC-labeled vesicles.

The capacity of SP-B to induce lipid mixing may be important for the formation of contact sites between membranes during the transformation of lamellar bodies into tubular myelin and the subsequent formation of the phospholipid monolayer at the air/liquid interface in the lung.

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