Inhibition of Pulmonary Surfactant Biophysical Activity by Cationic Polyamino Acids

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Purpose. The purpose of this study is to investigate the interaction of cationic polyamino acids, polylysine and polyarginine, with rat pulmonary surfactant at the air/water interface.

Methods. Surface pressure measurements of rat pulmonary surfactant in the presence and absence of polyamino acids were carried out in both dynamic and static modes.

Results. In dynamic cycle studies, compression and expansion of adsorbed surfactant films in the presence of the cationic polyamino acids resulted in a delayed attainment of the plateau surface pressure. In area studies of spread surfactant films at constant surface pressure, cationic polyamino acids in the subphase resulted in an increase in film area. Increased film area was also observed when a polyamino acid was injected beneath films of dipalmitoylphosphatidylcholine/phosphatidylglycerol. In the presence of the cationic polyamino acids, the equilibrium surface pressure (at constant film area) of pulmonary surfactant was elevated in a concentration- and molecular weight-dependent manner.

Conclusions. These data indicate that the model cationic peptides interact with surfactant lipid, possibly electrostatically with phosphatidylglycerol. It is concluded that the surface activity of pulmonary surfactant is significantly inhibited by the presence of the polycations, possibly by the formation of a mixed lipid/polyamino acid film.

KEY WORDS: pulmonary surfactant; monolayer; surface pressure; polyamino acid.

INTRODUCTION

Delivery of drugs directly to the respiratory tract by inhalation is becoming increasingly more popular (1,2). This route holds distinct advantages such as ease of accessibility and rapid onset of either local or systemic activity (1–3). As with any route of administration, great care must be exercised to insure that inadvertent toxic effects do not result from the delivery of drug or adjuvant to the site of absorption (4).

Pulmonary surfactant is a mixture of phospholipids, other assorted lipids, and associated proteins secreted by type II pneumocytes (5–7). In the alveolus and small airways, pulmonary surfactant decreases markedly the surface tension of the air/water interface, thereby maintaining morphology and function critical for respiration (5,6). Pathologic leakage of plasma proteins into the lung inhibits the surface activity of the pulmonary surfactant, resulting in alveolar collapse (Figure 1), small airway closure (5), and ultimately, hypoxia and multiple organ failure (6–8). The ability of plasma proteins to inhibit pulmonary surfactant activity suggests that other proteins or polypeptides delivered to the lung may also have the potential to induce an adverse biological response. Molecular details of the interactions of proteins with pulmonary surfactant lipids are lacking (8), making it impossible to predict the extent to which other proteins or peptides might inhibit surfactant activity.

The most prevalent component of pulmonary surfactant is dipalmitoylphosphatidylcholine (DPPC). This disaturated, zwitterionic lipid is responsible for the formation of a rigid film that reduces dramatically the surface tension of the interface upon exhalation (9,10). Anionic lipids such as phosphatidylglycerol also play a role in the surface behavior of pulmonary surfactant, possibly aiding in the respreading of the DPPC over the expanding interface during inhalation (9,11). Electrostatic interactions of these anionic lipids with cationic substances could be one mechanism of influencing surface activity. Polycations are known to influence the surface properties of pure films of anionic lipids at the air/water interface (12,13) and to alter the physical properties of anionic lipid vesicle systems (14–16). The present study was designed to investigate the extent to which molecular interactions with cationic polyamino acids polylysine and polyarginine could influence *in vitro* the surface tension lowering properties of rat pulmonary surfactant critical for respiration. These cationic polyamino acids were chosen as models to maximize electrostatic interactions with surfactant lipids (15,16). Polyamino acids have long been employed as models of selective protein characteristics (17), particularly that of binding to acidic phospholipid membranes (12,13,15,16,18).

EXPERIMENTAL SECTION

A. Isolation and Characterization of Rat Surfactant

Rat surfactant was isolated according to the method of Holm et al. (19). Rats were sacrificed by injection of sodium pentothal and the lungs were lavaged *in situ* with 2 × 10 ml of sterile saline (0.9%). The lavages were pooled and centrifuged at 300 × g for 10 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged at 15,000 × g for 30 minutes at 4°C. The pellet was resuspended and washed twice with 10 ml of sterile saline centrifuging at 15,000 × g after each wash. The final pellet was resuspended in 5 ml of sterile saline. The constituent phospholipids of surfactant were determined by a thin-layer chromatography approach outlined previously (20). All surfactant concentrations are expressed in terms of dry weight (excluding buffer salts).

B. Air/Water Interface Experiments

Cationic polyamino acids polylysine, average Mol. Wt. 3000 (polyLys 3K) and 100,000 (polyLys 100K), and polyarginine, average Mol. Wt. 139,000 (polyArg 139K), were employed. All polyamino acids were used as received (Sigma Chemical).

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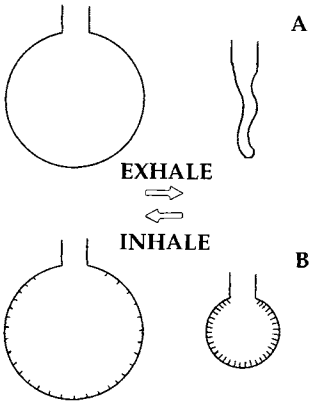

Fig. 1. Schematic representation of alveolus during the respiration cycle in the absence (A) and presence (B) of functional pulmonary surfactant.

a. Dynamic Compression-Expansion Cycling. All monolayer experiments were carried out in a Teflon trough held at 37°C (KSV-3000, Finland). Surface pressure was measured by the Wilhelmy plate method (21). Surface pressure is defined as the surface tension of the buffer alone (0.15 M NaCl, 0.01 M phosphate, pH 7.4) minus the surface tension of the surfactant film either with or without added polyamino acid. By this definition, the lower the surface tension, the greater the surface pressure. Initially, 8.4×10^{-4} grams of surfactant (in aqueous suspension) were injected beneath the surface of 900 ml of buffer in the trough. After 20 minutes, 1×10^{-6} moles of polyamino acid was injected into the subphase. The trough has a maximum interfacial area of 675 cm^2 and is outfitted with four magnetic stirrers. After steady state surface pressure was attained (generally, within 30 minutes), the surface film was compressed laterally at a rate of 100 cm^2/min until the total film area was reduced by half. The film was immediately expanded at the same rate until full area was regained, the entire cycle taking approximately 6 minutes. Upon completion of one cycle, another cycle was immediately started until 5 total cycles were completed. Each experiment was performed four times. Due to the complex composition of pulmonary surfactant, expression of film area on a per molecule basis is not practical and total interfacial area is employed instead.

b. Film Area at Constant Surface Pressure. Employing the methods of Keough et al. (21), 9×10^{-5} grams of surfactant (in suspension) were carefully spread from a glass microsyringe directly onto the surface of 900 ml of the aqueous buffer (in the teflon trough) to yield a surface pressure of 18 dynes/cm. To maintain as closely as possible the distribution of components found in vivo, surfactant films are commonly formed by spreading directly from aqueous suspension (6,7,19,21). After 20 minutes, the film was slowly compressed (10 dynes/min) until the desired surface pressure (between 25 and 45 dynes/cm) was attained. The instrument was then switched to the constant pressure mode to automatically compress or expand the film, maintaining the surface pressure at the preset value. Polyamino acids were injected into the subphase to a final concentration of 1×10^{-6} molar and the surface area of the compressed film was recorded as a function of time. Each experiment was repeated a total of three times. Film area changes were also examined for polyArg 139K injected beneath films of DPPC and 9:1 (molar ratio) DPPC-phosphatidylglycerol (spread from chloroform) held at a surface pressure of 25 dynes/cm. All phosphatidylglycerol was obtained from egg yolk (Sigma Chemical Co., St. Louis)

c. Equilibrium Surface Pressure at Constant Area. Sufficient surfactant suspension was injected into 55 ml of buffer in a water-jacketed beaker to attain a concentration of 9×10^{-7} g/ml. The beaker provided an air/buffer interfacial area of 33 cm^2. After 15 minutes, 0.05 ml of polyamino acid solution of an appropriate concentration was injected into the gently stirred subphase. Surface activity was followed as a function of time until equilibrium was reached (generally, 20 to 40 minutes). Each experiment was repeated a total of 3 to 4 times.

RESULTS AND DISCUSSION

The data in Table 1 show the distribution of phospholipids found in rat pulmonary surfactant by thin-layer chromatography. As expected, the primary phospholipid found is phosphatidylcholine, followed by a relatively high concentration of phosphatidylglycerol (22).

To maintain optimal alveolar morphology and to decrease the work of breathing requires pulmonary surfactant that will 1), upon exhalation, rapidly achieve and maintain, a compressed monolayer over the contracting interface, exhibiting a stable plateau surface pressure of 60–70 dynes/cm, and 2) upon inhalation, rapidly respread over the expanding interface (6,7,21–24). The surface tension lowering behavior of pulmonary surfactant may be effectively modeled *in vitro* by dynamic surface pressure-area isotherms (6,7,21–24). Typical surface pressure-area isotherms for adsorbed films of pulmonary surfactant in the presence and absence of polyArg 139K in the buffer are shown in Figure 2 and film areas at the initial attainment of plateau surface pressure are listed in Table 2. In the absence of polyArg 139K, compression of the surfactant film results in the attainment of a plateau surface pressure of 60 dynes/cm at a film area of 500 cm^2. Upon further compression, this surface pressure is maintained until the expansion phase of the cycle is begun. In the presence of polyArg 139K, the surfactant film fails to achieve the plateau surface pressure at the same film area; it must be compressed to a much smaller area (430 cm^2) before 60 dynes/cm is attained. Reducing the rate of compression to 25 cm^2/min. had no influence upon the results. The greater compression necessary to attain plateau surface pressure is also observed in the presence of the polylysines (Table 2).

Table 1. Phospholipid Composition of Rat Pulmonary Surfactant

Phospholipid	Percent of total phospholipid[a]
Phosphatidylcholines	80 ± 4%
Phosphatidyldylglycerol	8 ± 2%
Phosphatidylethanolamine Phosphatidylinositol Phosphatidylserine[b]	9 ± 1%
Unknown	3 ± 5%

[a] Average value ± standard deviation.
[b] These three phospholipids ran as a single spot on TLC.

Table 2. Film Areas of Pulmonary Surfactant at the Initial Attainment of Plateau Surface Pressure

Film	Film area (cm^2)[a]
Control	500 ± 20
Surfactant + polyArg 139K	430 ± 18
Surfactant + polyArg 139K[b]	440 ± 10
Surfactant + polyLys 100K	442 ± 21
Surfactant + polyLys 3K	440 ± 28

[a] Average ± standard deviation.
[b] Observed at a film compression rate of 25 cm^2/min.

These data clearly indicate that the presence of cationic polyamino acids in the subphase inhibits the ability of the surfactant film to attain rapidly the plateau surface pressure considered necessary to maintain alveolar morphology.

The extent to which polyamino acids interact with surfactant components in a static lipid film was examined. Figure 3 shows the area of a spread film of pulmonary surfactant held at a surface pressure of 30 dynes/cm. Upon injection of polyArg 139K into the subphase, the film expands by about 40 +/− 5 cm^2 indicating that the polyamino acid can insert into an area-determining region of the lipid film. In the absence of any lipid film at the air/water interface, all polyamino acids examined exhibited no surface activity, consistent with the hypothesis that interaction is with some component of the film. Figure 4 shows that an inverse relationship exists between the relative area increase of the surfactant film after injection of polyArg 139K and the surface pressure. Extrapolation of the line to the x-axis indicates that surfactant films held at 45 dynes/cm or higher will show no area increase upon injection of polyArg 139K. The behavior of the film at higher surface pressures could occur either by preventing polyArg 139K from interacting with area-determining regions of the interfacial film (25,26), or by removing anionic phospholipids that supply the putative binding site(s) for the polycation in the surfactant film (9–11). In the absence of any polyelectrolytes, Chung et al (9) have shown that phosphatidylglycerol is removed from the interface upon compression of a mixed film (DPPC/phosphatidylglycerol) beyond 45 dynes/cm. In the present study, analysis of Langmuir-Blodgett samples of the surfactant film were inconclusive in resolving the possibility of preventing polyArg 139 interaction verses that of phosphatidylglycerol removal. The exact location of the cationic polyamino acid in the interfacial region at surface pressures less than 45 dynes/cm is not clear. Born energy of transfer considerations make it unlikely that the ionized amine groups of lysine or arginine partition to a large extent into the hydrocarbon chain region of the phospholipids. Even unionized lysine and arginine residues are thought not to partition strongly into hydrophobic media (27). More likely, the cationic residues probably reside in the vicinity of the ionized headgroups of the anionic lipids (13,25).

In order to verify that a minor fraction of an anionic phospholipid in the midst of zwitterionic phospholipid is capable of acting as a binding site for cationic polyamino acids, studies were carried on model monolayers of DPPC and phosphatidylglycerol of well-defined composition. Injecting polyArg 139K beneath a spread film of DPPC and phosphatidylglycerol (9:1 molar ratio, the approximate ratio in pulmonary surfactant, ref. 28) held at a surface pressure of 25 dynes/cm resulted in an increase in area of about 12% (Figure 3). Similar to the results observed with surfactant films, injecting polyArg 139K beneath DPPC/phosphatidylglycerol films held at 45 dynes/cm were resistant to area expansion. In addition, injection of the polyamino acids beneath films of pure DPPC (even as low as 10 dynes/cm) did not result in any film expansion (data not shown). The fact that DPPC/phosphatidylglycerol mixtures, and not DPPC alone, show film expansion in the presence of the polyamino acids supports the conclusion that the interaction is indeed between the anionic phosphatidylglycerol and the cationic polyamino acid. The results do not go so far as to prove that the only binding site for cationic polyamino acids in pulmonary sur-

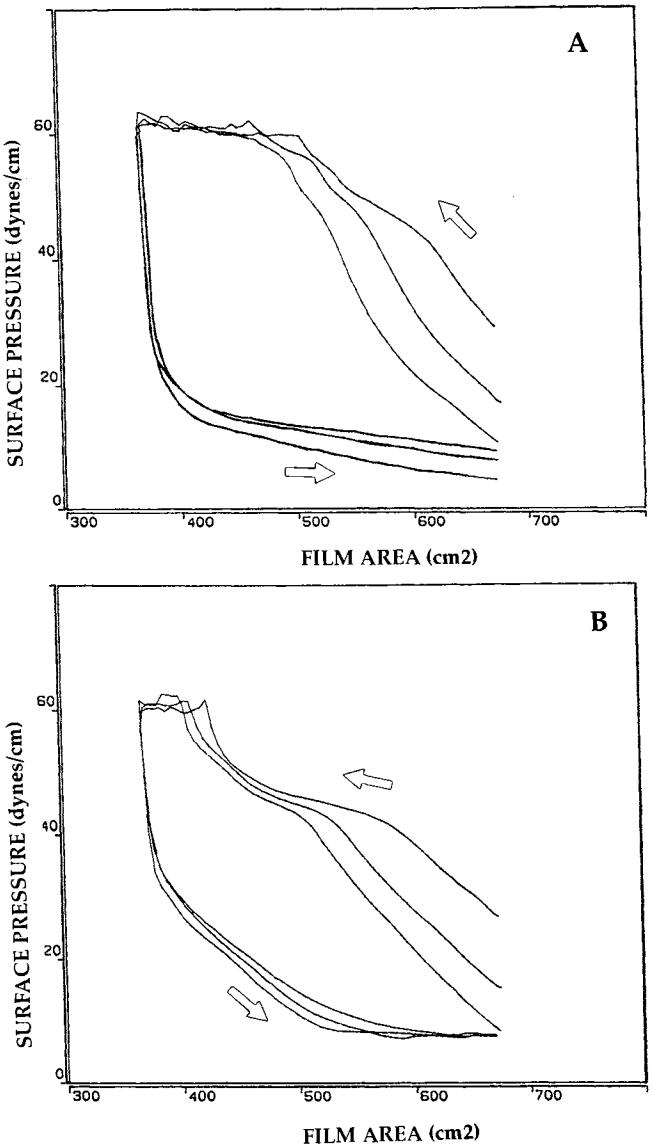

Fig. 2. Typical surface pressure-film area isotherms for rat pulmonary surfactant films in the absence (A) and presence (B) of 10^{-6} M polyArg 139K. In both cases, the fifth, tenth, and twentieth compressions are shown. The stars mark the initial attainment of the plateau surface pressure. The compression-expansion cycle begins in the lower right corner and proceeds counter-clockwise.

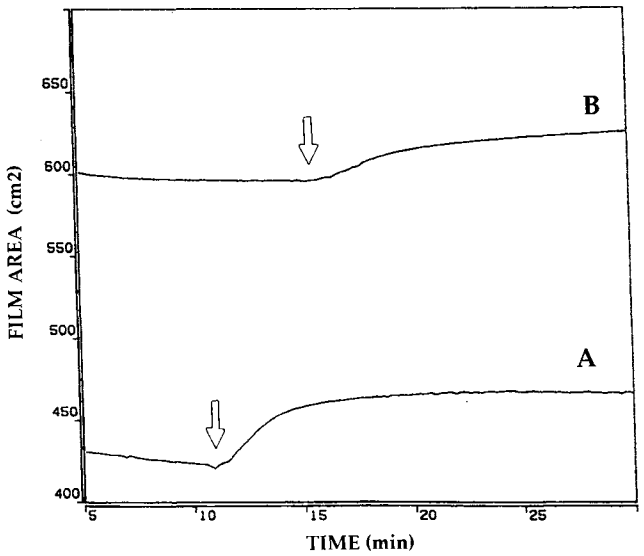

Fig. 3. Surface area of a spread film of rat pulmonary surfactant held at a constant surface pressure of 30 dynes/cm (A) and of a spread film of 9:1 mixture of dipalmitoylphosphatidyl- choline/ phosphatidylglycerol held at a constant pressure of 25 dynes/cm (B). The arrows mark the points at which polyArg 139K was injected into the subphase.

factant is phosphatidylglycerol. Rather, the results do indicate that such interactions with phosphatidylglycerol may be relevant in pulmonary surfactant.

In the film experiments outlined above, it was observed that interaction of the polycation with surfactant resulted in film expansion at a constant surface pressure. This was explained as the result of polycation interacting with anionic components of surfactant in area-determining positions of the film. If the experiment is modified such that the film area is held constant, movement of polycation into an area-determining position will compress the film and result in an elevated surface pressure (25). Surface pressure elevation as a function of the concentration and molecular weight of the cationic polyamino acid in the buffer were examined. Figure 5 presents a typical plot of the surface pressure of the fixed area film as a function of time. In the absence of polyamino acids, lung surfactant preparations show an equilibrium surface pressure of 18 +/− 2 dynes/cm. Injection of cationic polyamino acids in the subphase results in an increase in the surface pressure compared to control, the increase being dependent upon the molecular weight of the polyamino acid. Injection of the anionic polyglutamate (ave. mol. wt. 100,000) under the pulmonary surfactant film resulted in no effect on the equilibrium surface pressure (data not shown). Figure 6-A shows the percent increase in equilibrium surface pressure, plotted as a function of cationic polyamino acid concentration. For the high molecular weight polycations, polyArg 139K and polyLys 100K, the percent increase in surface pressure is substantial and occurs at low concentrations. For polyLys 3K, the extent of surface pressure increase is more modest. If it is assumed that each lysine or arginine residue is ionized and interacts with the lipid film, the polyamino acids would be expected to be adsorbed in an extended, all train configuration (29). Under these conditions, the mass adsorbed to the interface, and therefore, the surface pressure increase, would be independent of the molecular weight. It would then be expected that polyLys 3K and polyLys 100K should result in identical film expansions. The molecular weight effects observed in Figure 6A clearly do not support such a mechanism. The interaction with the surface may be examined more closely by plotting the surface pressure change as a function of total monomer concentration (concentration of polymer x average number of monomers per polymer, ref. #25). If all monomers in the polymer chain are interacting identically and independently with the surfactant layer, then the curves for polyLys 3K and polyLys 100K should overlap. In Figure 6B, polyLys 3K and polyLys 100K do not converge to a single line. Rather, the higher molecular weight polymer exhibits a greater surface pressure change than the lower molecular weight for monomer concentrations above 10^{-4} molar. By 10^{-2} molar mono-

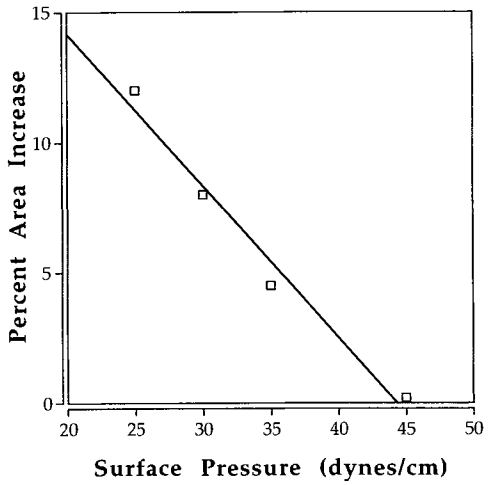

Fig. 4. Relative percent surface area increase of rat pulmonary surfactant films as a function of the film pressure after the injection of polyArg 139K into the subphase.

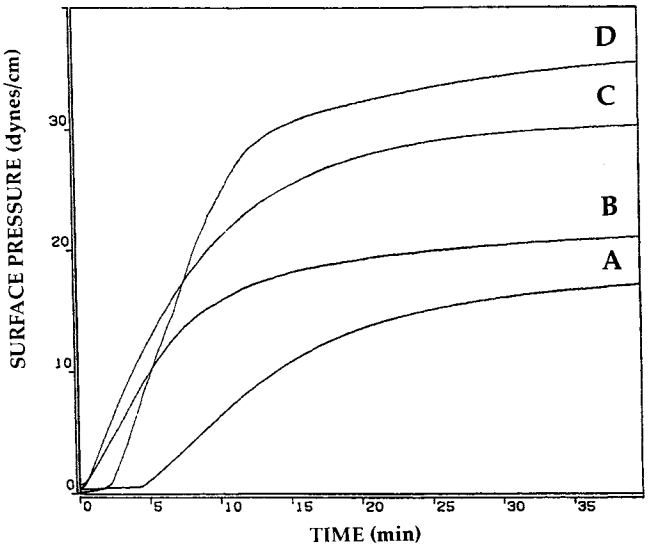

Fig. 5. Surface Pressure as a function of time for surfactant alone, (A); surfactant plus 10^{-3} molar polyLys 3K, (B); surfactant plus 10^{-3} molar polyLys 100K, (C); surfactant plus 10^{-3} molar polyArg 139K, (D).

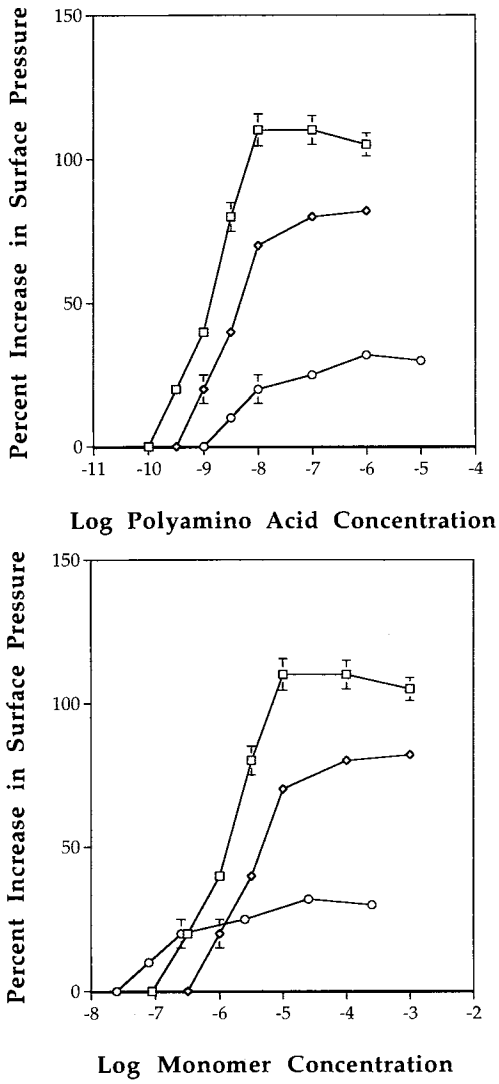

Fig. 6. Percent increase in surface pressure of rat pulmonary surfactant as a function of the concentration of the polymeric cation; (A), plotted as a function of the log molar concentration of polyamino acid, and (B), plotted as a function of the log molar concentration of the amino acid residues. (○) polyLys 3K; (◇) polyLys 100K; (□) polyArg 139K. In all three cases, standard error of the mean was less than ±8%.

mer concentration, polyLys 100K exhibits an almost 2-fold greater increase in surface pressure than does polyLys 3K. These results suggest that the higher molecular weight polymers may have a higher affinity for the surfactant-covered interface, in agreement with a cooperativity model of Kim et al (15). Under this model, each basic residue added to a polyamino acid increased the binding affinity of the polymer for an anionic membrane tenfold (15). In the present study, an increased affinity of a polyamino acid as a function of molecular weight could come about by a change in the extent of ionization of the polymer (29) or by a change in the conformation, altering the number-density of residues at the interface (13,15,16). Solution conformation of polylysines are known to be pH dependent (29) in the region of the pKa (lysine pKa = 10.9, ref. 17). The effects of the surface on molecular conformation or on the extent of ionization are complex and not easily predicted (13,15,26,27,29). These same variables may be, in part, responsible for the experimental difference between polyLys 100K and polyArg 139K.

In summary, the results of the present *in vitro* study indicate that the surface properties of pulmonary surfactant are inhibited in the presence of cationic polyamino acids. Specifically, cationic polyamino acids delay the attainment of plateau surface pressure during the dynamic compression and expansion of the surfactant film. The cationic polyamino acids appear to be attracted to the anionic lipids in the interface and insert into the phospholipid film. The interaction is dependent upon the concentration and molecular weight of the polyamino acid. While cationic polyamino acids may interact strongly with anionic lipids, the stoichiometry of the interaction and the exact location of the polycation in the interfacial region are not yet known.

The results suggest that caution should be exercised when formulating drug delivery systems for inhalation. Cationic peptides, protein drugs and formulation adjuvants could potentially interact with pulmonary surfactant resulting in altered surface activity. Considerable work remains to be done in elucidating all of the forces of interaction between proteins and surfactant lipids before accurate predictions of inhibitory potential can be made.

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