Proc. Natl. Acad. Sci. USA
Vol. 74, No. 8, pp. 3617–3620, August 1977
Physiological Sciences

Formation of lung surfactant films from intact lamellar bodies

(organelle/surface activity/spreading pressure)

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Communicated by David W. Talmage, June 9, 1977

ABSTRACT **Lamellar bodies, an intracellular source of lung alveolar surfactant, were isolated from rat lung homogenates and studied in the Langmuir–Adam surface balance. By layering intact lamellar bodies on the surface of a more dense sucrose subphase, we studied the factors affecting film formation from surface tension-vs-time data and determined surface tension–surface area isotherms by compression and expansion of the resulting films. We found that films with properties representative of the alveolar surfactant are formed in the presence of Ca^{2+} or Mg^{2+} alone, or either plus Na^+; that film formation is incomplete with Na^+ alone or on ion-free subphases; and that Ca^{2+}-induced film formation is blocked by chelation with EGTA but is unaffected by diisopropylfluorophosphate. The results suggest that divalent cations induce film formation by interactions at sites within the lamellar bodies and may be responsible for the binding of membrane lipids to membrane proteins in lung surfactant.**

Despite morphological (1, 2) and cytochemical (3, 4) evidence suggesting that lung alveolar surfactant is derived from the lamellar bodies of the type II epithelial cell, it has not been shown that intact lamellar bodies are capable of forming films with the extraordinary surface properties attributed to surfactant. For example, bubbles (5) or films (6) from lung extracts exhibit surface tensions approaching 0 mN/m and large surface tension–surface area hysteresis. And recent results from wettability experiments performed directly on the alveolar surface indicate that surface tensions as low as 9 mN/m may be reached *in situ* (7). Low surface tension in the lung is thought to facilitate opening of small airways and alveoli on inflation (8) and to stabilize lung units during deflation (9), and surfactant abnormalities have been identified in both the infant (10) and the adult (11) respiratory distress syndrome.

Because surface-active lipids float on physiological saline and intact lamellar bodies (d = 1.06–1.07 g/ml) do not, previous observations of surface activity on saline surfaces have been limited to the lamellar body contents obtained by ethanol extraction (12, 13), sonication (13), or dispersion in salt solutions (12–16). Because these procedures resulted in the loss of integrity of the lamellar bodies, we layered intact lamellar bodies on a more dense sucrose subphase and monitored surface activity directly as a function of subphase composition.

In this report, we present evidence that suggests that lamellar bodies require divalent cations in order to produce films having surface tension–surface area relationships characteristic of the extracellular surfactant.

MATERIALS AND METHODS

Lamellar Bodies. Lamellar bodies were obtained from rat lung by a refined homogenization and sucrose density gradient centrifugation procedure (17). Lamellar bodies were recovered from the interphase between 0.45 M and 0.58 M sucrose in purities greater than 90% as demonstrated by electron microscopy. Atomic absorption analysis showed that suspensions of the lamellar bodies in 0.5 M sucrose contained less than 0.05 mM Ca^{2+}. Experiments were performed on lamellar bodies from 16 such preparations. Samples were assayed for lipid P_i by a modification of the Bartlett procedure (18).

Surface Tension Balance. Lamellar bodies were studied for surface tension (γ) vs time (t) and surface tension vs area (A) behavior with an automated Langmuir–Adam surface balance. The trough (200 cm^2) and barrier were Teflon. Surface tension was measured by the change in weight of a Wilhelmy plate (sandblasted platinum, 5 cm perimeter) suspended from a Statham transducing cell. The transducer output was amplified so that a change of 1 mN/m corresponded to a deflection of 3 mm on the Y axis of an X-Y-t recorder (Hewlett-Packard, model 7001AM). Surface area was measured by the change in displacement of a 10-turn potentiometer. Subphase and air temperatures were controlled at 37° ± 0.2° by circulating water through the base of the trough and the walls of an insulated metal cabinet that enclosed the trough. The cabinet door was constructed of plexiglass through which the contact angle between the plate and subphase meniscus could be observed with a ×64 cathetometer telescope with a goniometer eyepiece (Gaertner Scientific Corp., Chicago). The cabinet was mounted on a steel bench below ground level, and vibration control was provided by interposing rubber stoppers and 500 kg of lead bricks and sand, and by placing the cycling motor on a separate bench. An error analysis indicated that the precision of the balance was better than ±0.1 mN/m.

Balance Calibration. The transducer was calibrated with weights and with liquids of known surface tension as determined with a du Nouy tensiometer (Central Scientific Co., Chicago). For variable γ-A conditions, the system was tested with L-α-dipalmitoyl lecithin (Fluka, AG., Buchs, Switzerland). After recrystallization from dioxane, the lecithin migrated in a single spot on thin layer chromatographic analysis. When spread from redistilled chloroform on 0.15 M NaCl, it gave a well-defined phase transition and γ-A isotherms in agreement with published data (19).

Subphase Solutions. Triple-distilled water (γ = 72.4 mN/m at 24°; conductivity, 10^{-6} mho) from an all-quartz still was used to prepare the solutions. The subphase (γ = 69.6 mN/m at 37°) was 0.6 M sucrose (Pfanstiehl Laboratories, Waukegan, IL)/10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.4, to which either NaCl and/or $CaCl_2$ or $MgCl_2$ were added prior to or after layering of the lamellar bodies. The Ca^{2+} concentration of the solid sucrose was determined after

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Abbreviations: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid; iPr_2P-F, diisopropylfluorophosphate.

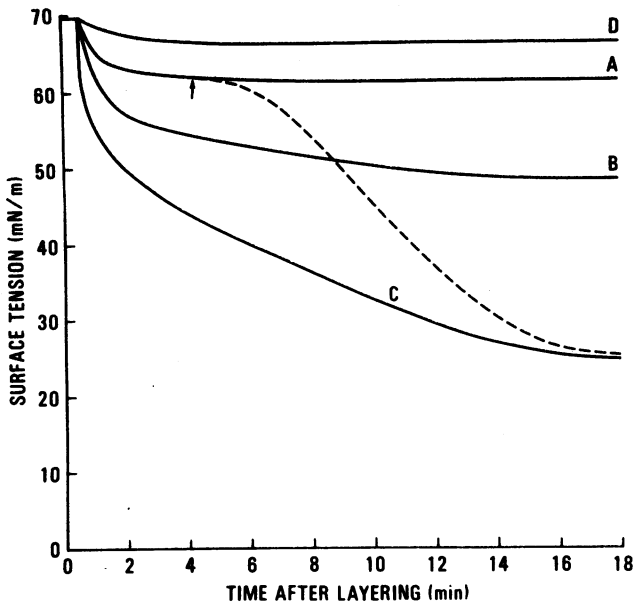

FIG. 1. Film formation (37°) as measured by static surface tension–time curves for intact lamellar bodies layered on: A, 0.6 M sucrose/10 mm Hepes, pH 7.4; B, as A plus 0.154 M NaCl; C, as A plus 3 mM $CaCl_2$ or $MgCl_2$. D, Lamellar bodies + 5.78 ml of 0.1 M ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) layered on subphase A plus 3 mM $CaCl_2$. - - -, Injection of $CaCl_2$ or $MgCl_2$ into subphase A at arrow. In each case 12.5 μg of lipid P_i was layered. The subphase area was 200 cm^2.

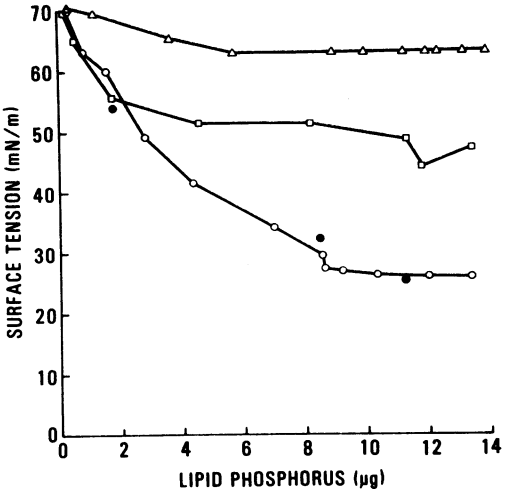

FIG. 2. Equilibrium static surface tension (37°) of lamellar bodies as a function of amount of lipid P_i layered. Subphases were 0.6 M sucrose/10 mM Hepes at pH 7.4 (Δ) with: □, 0.154 M NaCl; ○, 3 mM $CaCl_2$; ●, 3 mM $MgCl_2$. Subphase area was 200 cm^2.

combustion at 600°. The residue after weighing was dissolved in 0.1 M HCl and Ca^{2+} concentration was estimated in a Perkin-Elmer atomic absorption spectrophotometer (model 290) with a $CaCO_3$ solution containing 1.767 g of $LaCl_3$ per liter as a primary standard. The mean residue weight was 0.0109% (as CaO) and the Ca^{2+} concentration was 0.93 μmol/mol of sucrose.

Procedure. Lamellar bodies were kept at 0° prior to layering and were applied to the trough within 10 min after removal from the gradient. An experiment consisted of two parts: (*i*) an aliquot of the lamellar bodies was carefully layered with a glass micropipette onto the subphase and the γ–t curve, at constant area, was recorded until the surface tension drop was less than 0.1 mN/m per min; (*ii*) the film was then compressed and reexpanded several times between 200 and 40 cm^2 at a rate of 0.3 cm^2/s and the γ–A isotherms were recorded.

RESULTS

Film Formation. Film formation was both more rapid (greatest $-d\gamma/dt$) and more complete (lowest final static surface tension) with 3 mM Ca^{2+} or Mg^{2+} than with 150 mM Na^+ alone or no added ions (Fig. 1). When subphase Ca^{2+} or Mg^{2+} concentration was increased to 10 or 20 mM, the drop in γ was accelerated but the final surface tension was never less than 24–25 mN/m. When Ca^{2+} or Mg^{2+} was also injected underneath the surface of the latter films at $d\gamma/dt < 0.1$ (dashed line, Fig. 1), the final surface tension and γ–A curve on compression were the same as those found when Ca^{2+} or Mg^{2+} was present prior to layering. Conversely, injection of Na^+ underneath Ca^{2+} or Mg^{2+} films at equilibrium (γ = 24–25 mN/m) failed to decrease the surface tension further. Each curve of Fig. 1 resulted from the application of lamellar bodies containing 12.5 μg of lipid P_i. If all of the lipid P_i was dipalmitoyl lecithin, this amount of lipid is equivalent to about 4.5 monolayers at 200 cm^2.

Amounts of lipid considerably in excess of one monolayer were required to achieve maximal surface tension lowering (Fig. 2). We layered from 0.28 to 14 μg of lipid P_i (about 0.1 to about 5.0 monolayers of dipalmitoyl lecithin) in the various experiments. The most interesting feature of these data is that surface tension reached a nearly constant value with increasing lipid. More lipid appeared to be required to reach maximal surface tension lowering with Ca^{2+} or Mg^{2+} than with either Na^+ or no added ions. The shape of the curves in Fig. 2 is similar to γ–bulk concentration curves for surfactants that undergo micellar aggregation at the onset of the region with essentially zero slope.

Surface Tension–Surface Area Behavior. Fig. 3 shows the compression–expansion isotherms of the films formed in the experiments of Fig. 1. In order to show closed γ–A loops, second complete cycles are presented. Ionic effects on minimal surface tension at 40 cm^2 were again in the order Ca^{2+} or Mg^{2+} < Na^+ < no ions. A distinguishing feature of the films induced by divalent ions was the appearance of a liquid condensed phase (Fig. 3*C*) of low surface compressibility [$C_s = (1/A)\, dA/d\gamma$]. Values of C_s ranged from 0.015 to 0.03 cm/dyne for the Ca^{2+} and Mg^{2+} films. Ion-free films never showed a condensed phase (Fig. 3*A*); Na^+ films occasionally showed the onset of condensation (Fig. 3*B*) with C_s = 0.02–0.04 cm/dyne.

The order of minimal surface tension (Ca^{2+} or Mg^{2+} < Na^+ < no ions) persisted throughout the range of lipid P_i layered (Fig. 4). The concentrations of lipid at 40 cm^2 at which the surface tensions are plotted in Fig. 4 are theoretically 5 times higher than the concentrations at 200 cm^2 in Fig. 2. Thus, most of the surface tension reduction on compression occurs at less than one monolayer dipalmitoyl lecithin equivalent. Again, Ca^{2+} and Mg^{2+} films appeared to require more lipid to achieve maximal reduction in surface tension.

The Ca^{2+} γ–lipid P_i curve appeared to undergo a minimum but this may be an artifact due to instability of the films below 10 mN/m. For example, during compression of Ca^{2+} or Mg^{2+} films, we observed finite contact angles (ϕ) below 10 mN/m and particularly at 5–7 mN/m. Angles as large as 30° accompanied by a fall of the meniscus on the plate were occasionally observed at $\gamma \sim 5$ mN/m. Films were unstable in this totally collapsed region (Fig. 3*C*) and spillage of material over the trough edges was nearly always observed. Because the surface

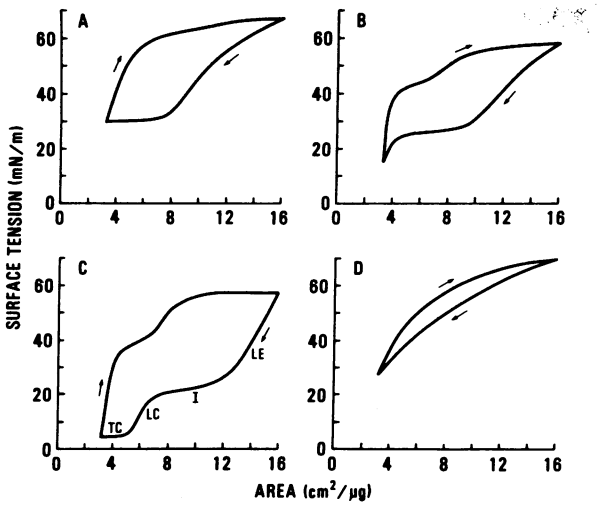

FIG. 3. Surface tension–surface area isotherms (37°) of the films formed under the conditions described in Fig. 1. Films were compressed to 40 cm^2 and reexpanded to 200 cm^2 at a rate of 0.3 cm^2/s. Second complete cycles are shown. In each case 12.5 μg of lipid P_i was layered. Subphases: *A*, 0.6 M sucrose/10 mM Hepes, pH 7.4; *B*, *A* plus 0.154 M NaCl; *C*, *A* plus 3 mM $CaCl_2$ or $MgCl_2$. *D*, Lamellar bodies + 5.78 ml of 0.1 M EGTA layered on subphase *A* plus 3 mM $CaCl_2$. *C* shows liquid expanded (LE), intermediate (I), liquid condensed (LC), and totally collapsed (TC) regions along the compression isotherm.

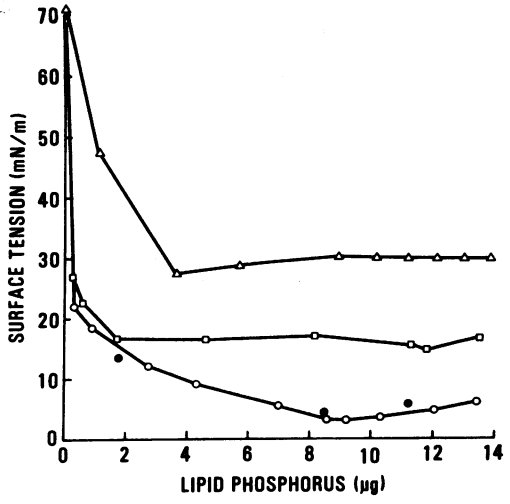

FIG. 4. Surface tension (37°) of lamellar bodies at 40 cm^2 (maximal compression) as a function of the amount of lipid P_i layered at 200 cm^2. Subphases were 0.6 M sucrose/10 mM Hepes, pH 7.4 (Δ) with: □, 0.154 M NaCl; ○, 3 mM $CaCl_2$; ●, 3 mM $MgCl_2$.

tension is proportional to $1/\cos\phi$, the values reported for $\gamma \sim 5$ mN/m are low by not more than 15%. An error of this magnitude does not alter the order of minimal tensions among the various ionic environments. Contact angle hysteresis is thought to be responsible for part of the γ–*A* hysteresis observed with lung surfactant films (20) but we have not made a detailed study of this phenomenon.

Surfactant Activation. To clarify the mechanism by which surfactant is activated, the effects of EGTA and diisopropylfluorophosphate (iPr_2P-F) on film formation by Ca^{2+} were examined. Because enough EGTA had to be supplied to chelate the subphase Ca^{2+}, we first ran controls with EGTA alone and with EGTA plus lamellar bodies on ion-free sucrose. The effects of EGTA alone on the surface activity of buffered sucrose were minor. Solutions of 0.6 M sucrose/10 mM Hepes, pH 7.4/1.5 mM EGTA gave film pressures (surface tension lowerings) of 1.8 mN/m and 3.9 mN/m for static and compression to 40 cm^2 conditions, respectively. Incubation of lamellar bodies with EGTA (30 min, 0°) gave surface tensions on ion-free sucrose 3–5 mN/m (static) and 6–8 mN/m (compression) lower than with lamellar bodies alone. Figs. 1*D* and 3*D* show the results of layering lamellar bodies plus EGTA on a Ca^{2+} subphase. It is clear that EGTA not only blocks the effects of Ca^{2+} but also produces a film that is less surface active in two respects: lower $-d\gamma/dt$ and γ–*A* hysteresis than the ion-free control (Figs. 1*A* and 3*A*).

iPr_2P-F inhibits the activity of phospholipases A_1 (EC 3.1.1.32) and A_2 (EC 3.1.1.4) from brain (21). Because both enzymes have been identified in lamellar bodies from rabbit lung (16), we studied the effect of iPr_2P-F on film formation. Lamellar bodies were incubated (30 min, 24°) in 3 mM iPr_2P-F prior to layering. iPr_2P-F failed either to prevent film formation or to alter γ–*A* behavior on a Ca^{2+} subphase. Controls with the same amount of iPr_2P-F alone (subphase concentration, about 0.1 μM) showed no detectable film pressure.

More revealing experiments were those in which lamellar bodies were layered on ion-free sucrose and allowed to stand for 18 hr prior to compression. The final static surface tensions and the γ–*A* curves were essentially the same as observed with lamellar bodies on ion-free sucrose when compression was initiated only 5–10 min after layering (Figs. 1*A* and 3*A*). Subsequent injection of Ca^{2+} into the subphase gave results similar to those shown in the dashed curve of Fig. 1 and in Fig. 3*C*. Because electron microscopy showed that virtually 100% of the lamellar bodies were broken after only 1 hr at 37° (17), it is apparent that lamellar bodies do not spread as a complete film on ion-free media even though the limiting membrane is disrupted.

DISCUSSION

During film formation from lamellar bodies layered on Ca^{2+} or Mg^{2+} subphases, the maximal surface tension lowering, to about 24–25 mN/m, probably represents the "equilibrium spreading pressure" for lung surfactant. The equilibrium spreading pressure is the pressure that occurs at thermodynamic equilibrium between the outer or monolayer and a bulk phase of the same material (22). Because long-chain hydrocarbons in bulk have surface tensions around 25–30 mN/m, the Ca^{2+} or Mg^{2+} films from lamellar bodies are probably composed of nearly pure lipid with hydrocarbon chains exposed to air and, in this sense, represent complete release of lamellar body contents. On the other hand, Na^+-induced films never exhibited static surface tensions less than 40 mN/m during formation and may be classified as incomplete.

On compression, the area at which the expanded-intermediate phase transition occurred (Fig. 3) was in the order Ca^{2+} or $Mg^{2+} > Na^+ >$ no ions, showing that the Ca^{2+} or Mg^{2+} films were the most expanded. The Ca^{2+} or Mg^{2+} films could also be compressed beyond the intermediate region to collapse. Although films formed with divalent ions exhibited the lowest surface tensions, films on Na^+ gave surface compressibilities, in the condensed region, of low magnitude similar to those with Ca^{2+} or Mg^{2+}. Based on the compressibility data alone, films formed by Na^+ ions might be adequate to support normal respiratory function. For example, in one case of the adult respiratory distress syndrome, surface compressibility was found to be the surfactant film property that changed significantly in relation to altered lung mechanics (11). Low surface compressibility is thought to be important to lung function because

it allows the attainment of low γ with only minimal dimensional changes (23).

The results with lamellar bodies plus EGTA on Ca^{2+} subphases represent an interesting case of retrograde film behavior. Because Ca^{2+} contamination of the sucrose probably was not a factor, we might postulate that EGTA caused a structural change in the lamellar body lipids such as micelle formation, the result of which was to increase greatly the lipid solubility in the subphase. An increase in the relative concentration of protein at the surface would be expected to accompany lipid solubilization. Either lipid-depleted (24) or protein-enriched (25) films give featureless γ–A hysteresis loops similar to that shown in Fig. 3*D*. The further implication of the results in Figs. 1*D* and 3*D* is that metallic cations chelated by EGTA are in some way responsible for the binding of membrane proteins to membrane lipids in lung surfactant.

Divalent ions act to form films primarily from within lamellar bodies rather than by a mere disruption of the limiting membrane. The site of interaction is suggested by the experiments in which lamellar bodies were allowed to stand on ion-free sucrose for up to 18 hr and were found not to spread. This conclusion is in agreement with the observation that the limiting membrane of the organelle is integrated with the plasmalemma at the time of secretion (1, 2).

Our data also raise interesting questions about the morphology of the extracellular "lung surfactant." One form of surfactant is believed to be tubular myelin (15, 26), a highly ordered lattice composed of bilayers. The isolation and chemical composition of tubular myelin has been reported only once (15), and its role in lung function is unknown. Recently it has been shown that, when incubated in test tubes with Ca^{2+}, lamellar bodies undergo conversion to tubular myelin (17). Preliminary data from our laboratory (27) suggest that tubular myelin does not spread on 0.8 M sucrose and, in fact, shows γ–A behavior similar to that in Fig. 3*D*. But the data reported herein show that lamellar bodies are highly surface active when spread on Ca^{2+} subphases. One explanation of this apparent paradox is that, when layered on Ca^{2+}, lamellar bodies undergo simultaneous conversion to tubular myelin with concomitant release of lipid to form a film of low surface tension. However, because of its density (0.8 M sucrose is required to hold it on the surface) and the low surface tension, tubular myelin sinks into the subphase. The greater density of tubular myelin relative to lamellar bodies reflects phospholipid-to-protein ratios (g/g) of 2.0 and 4.7, respectively (15). The lipid, which would be removed from the surface, in the tubular myelin may partly explain why Ca^{2+} films gave compression isotherms with features representative of classical insoluble monolayers even when the lamellar bodies contained up to 5 monolayers of dipalmitoyl lecithin at maximal area or nearly 25 monolayers at minimal area. The elucidation of the role of tubular myelin in surfactant transport and film formation will require the isolation of a highly pure tubular myelin fraction.

This work was supported by U.S. Public Health Service Research Grants HL17544 and HL17444 and Training Grant HL07085.

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