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Binding of Annexins to Lung Lamellar Bodies and the PMA-Stimulated Secretion of Annexin V from Alveolar Type II Cells[1]

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To identify lung lamellar body (LB)–binding proteins, the fractions binding to LB-Sepharose 4B in a Ca^{2+}-dependent manner from the lung soluble fractions were analyzed with Mono Q column. Four annexins (annexins III, IV, V, and VIII) were identified by partial amino acid sequence analyses as the LB-binding proteins in the lung soluble fractions. A control experiment using phospholipid (phosphatidylserine/phosphatidylglycerol/phosphtidylcholine) liposome-Sepharose 4B revealed that annexins III, IV and V were the Ca^{2+}-dependent proteins binding to the column in the lung soluble fractions, while annexin VIII was not detected. Thus, annexin VIII might preferentially bind to LB. On the other hand, the only Ca^{2+}-dependent LB-binding protein identified in the bronchoalveolar lavage fluids was annexin V. It was further demonstrated that annexin V was secreted by isolated alveolar type II cells from rats and that the secretion was stimulated by the addition of phorbol ester (PMA), a potent stimulator of surfactant secretion. The PMA-dependent stimulation of annexin V was attenuated by preincubation with surfactant protein-A (SP-A), a potent inhibitor of surfactant secretion. As LB is thought to be an intracellular store of pulmonary surfactant, which is secreted by alveolar type II cells, annexin V is likely to be secreted together with the lamellar body.

Key words: alveolar type II cell, annexin, lamellar body, pulmonary surfactant, secretion.

Pulmonary surfactant, a mixture of lipids and proteins, functions to stabilize alveoli by lowering the surface tension at the air–liquid interface (*1, 2*). The surfactant is synthesized and stored in multilamellar organelles called lamellar bodies (LB) in alveolar type II cells, and secreted into the alveolar space. Agonists inducing elevation of intracellular concentration of Ca^{2+} or cyclic AMP and activation of protein kinase C have been shown to stimulate surfactant secretion from the isolated type II cells (*3*).

We have demonstrated that annexin IV binds to surfactant protein A (SP-A), the major protein component of surfactant apoproteins, in a Ca^{2+}-dependent manner and that annexin IV stimulated Ca^{2+}-dependent aggregation of LB from rat lungs (*4*). These facts led us to speculate that annexin IV might be involved in functions such as the formation of LB and the regulation of secretion by LB (*4, 5*).

Other annexins have been suggested to have a role in surfactant metabolism (*6–8*). It has been reported that the heterotetrameric complex of annexin II-p11 (S100A10) stimulates the fusion between the LB and phospholipid liposomes, and that the secretion of phospholipids by the isolated and permeabilized alveolar type II cells was enhanced by the addition of annexin II tetramer (*6*). It has also been shown that annexin VII (synexin) is a potent stimulator of aggregation of LB and fusion of LB and plasma membranes (*7, 8*).

To examine further the possible involvement of annexins in surfactant metabolism, we attempted to identify the proteins binding to LB in a Ca^{2+}-dependent manner using LB-conjugated affinity chromatography. Here we report that four annexins (annexin III, IV, V, and VIII) were identified as LB-binding proteins, and that annexin V was selectively secreted by isolated alveolar type II cells.

EXPERIMENTAL PROCEDURES

Isolation of Lamellar Bodies (LB) from Lungs—Fractions containing LB were isolated from male Sprague-Dawley rat lungs by the method of Duck-Chong (*9*) with slight modifications (*4*). The isolated LB fractions were resuspended in 0.24 M sucrose, 20 mM Hepes (pH 7.5), and 30 mM NaCl and probe-sonicated with a Sonifier cell disruptor (Heat System-Ultrasonics) for 10 s to obtain a homogeneous suspension, which was stored in ice until use.

Phospholipid (PS/PG/PC) Liposomes—Phospholipids [phosphtidylserine (PS), phosphatidylglycerol (PG), and phosphtidylcholin (PC)] were dissolved in $CHCl_3$ and methanol (2:1) and stored at –40°C. The phospholipid mixture (3 mg; 20% PS, 10% PG, and 70% PC) was dried under N_2 gas and hydrated in buffer containing 10 mM sodium phos-

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Abbreviations: BALF, bronchoalveolar lavage fluids; CAPS, cyclohexylaminopropanesulfonic acid; LB, lamellar body; PTH, phenylthiohydantoin; PMA, phorbol-12-myristate-13-acetate; SP-A, surfactant protein A; TFA, trifluoroacetic acid.

phate (pH 7.4) and 150 mM NaCl by incubating for 45 min at 48°C. The lipid mixture was then vigorously vortexed for 2 min to prepare multilamellar liposomes.

Affinity Chromatography—Sepharose 4B was activated with BrCN by the method of Kohn and Wilchek (*10*). Coupling of activated Sepharose 4B (2 ml) with LB (containing 1 mg of protein and 6 μmol of phospholipid) or phospholipid loposomes (PS/PG/PC) was performed by incubating for 10 h at 20°C in 0.24 M sucrose, 10 mM sodium phosphate (pH 7.4), and 150 mM NaCl according to the method for coupling adrenel chromaffin granules to Sepharose 4B (*11*). The column was first washed with 0.1 M Tris (pH 8.0) and 1 mM EGTA, then equilibrated with buffer containing Ca^{2+} before use (see below).

Preparation of LB-Binding Proteins from the Lung and Bronchoalveolar Lavage Fluid (BALF), and PS/PG/PC-Binding Proteins from the Lung—Rabbit lung was perfused through pulmonary arteries with 40 ml of saline, and lung lavage was done with 30 ml of saline containing 1 mM EGTA. Lung lavage was repeated two more times. The combined BALF was centrifuged at 1,000 ×*g* for 10 min and the supernatant was collected. The tissues and the BALF were then stored at –80°C until use. The following steps were carried out at 4°C except where indicated.

To isolate LB- or PS/PG/PC-binding proteins, the frozen lung (20 g) was homogenized three times with 200 ml of homogenizing buffer [10 mM Tris-HCl (pH 7.5), 10% sucrose (w/v), 1 mM EGTA, 2.5 μg/ml soybean trypsin inhibitor, and 0.1 mM phenylmethyl sulfonyl fluoride] for 20 s with a blender with 30-s intervals between each homogenization. The homogenate was centrifuged at 1,000 ×*g* for 5 min, and the supernatant was collected, filtered though two layers of cheesecloth, then centrifuged again at 80,000 ×*g* for 1 h. The supernatant was collected, $CaCl_2$ and $MgCl_2$ were added to final concentrations of 1 and 5 mM, respectively, and the resulting solution was loaded onto LB- or PS/PG/PC-Sepharose 4B that had been equilibrated with 20 mM Hepes (pH 7.5), 0.25 M sucrose, 50 mM NaCl, 1 mM $CaCl_2$, and 5 mM $MgCl_2$. The column was washed with the equilibration buffer and elution was carried out with 20 mM Hepes (pH 7.5), 0.25 M sucrose, 50 mM NaCl, and 1 mM EGTA. The eluted peak fraction was collected (20 ml) and dialyzed against 1 liter of 20 mM Hepes (pH 7.5), and 0.1 mM EGTA for 12 h. After centrifugation at 80,000 ×*g* for 1 h, the dialyzed sample was loaded onto a Mono Q HR5/5 equipped with an FPLC system equilibrated with 20 mM Hepes (pH 7.5) and 0.1 mM EGTA. The column was washed with the equilibration buffer at a flow rate of 0.5 ml/min, then elution was carried out with a linear gradient from 0 to 50% elution buffer [0.5 M NaCl, 10 mM Hepes (pH 7.5), and 0.1 mM EGTA] for over 50 min at 20°C.

To prepare LB-binding proteins from the BALF, the frozen BALF was thawed and $CaCl_2$ and $MgCl_2$ were added to final concentrations of 1 and 5 mM, respectively. Subsequently, the procedure used for the preparation of LB-binding proteins (as described above) was followed.

Identification of LB-Binding Proteins—The eluted peak fractions after Mono Q chromatography were separately dialyzed against 5% acetic acid and lyophilized. The lyophilized samples were solubilized with 2% SDS, subjected to SDS-PAGE, transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA), then stained with Ponceau S. The stained bands were cut out and incubated with 10 mmol of BrCN in 0.3 ml of 70% formic acid (v/v) for 20 h at 25°C. The resulting solutions were lyophilized and dissolved in a small volume of acetic acid (5% v/v), then loaded onto an Inertosil C_{18} column for reverse-phase HPLC (2.1 × 50 mm, GL Science, Tokyo). Elution was performed with a linear gradient of acetonitrile from 0 to 50% in TFA (0.1% v/v) over 100 min at a flow rate of 0.2 ml/min. The eluted peaks were lyophilized, and amino acid sequences were determined using an Applied Biosystems Model 477A protein/peptide sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer (model 120A) (Applied Biosystems, Tokyo).

Preparation of Rat Recombinant Annexin V—Total RNA was extracted from the rat liver by the acid guanidinium thiocyanate-phenol-chloroform extraction method (*12*). After synthesis of the first strand cDNA, the annexin V gene was amplified by a PCR with synthesized primers based on the sequence of rat annexin V reported by Pepinsky *et al.* (*13*). *Nco*I and *Bam*HI restriction sites were introduced at 5′ and 3′ termini of the coding sequence of rat annexin V cDNA, respectively. The set of primers used were 5′-agtcgccatggctctcagaggcacc-3′ and 5′-agctggatccgtcagtcatcctcgcc-3′ (restriction sites are underlined). *Nco*I/*Bam*HI-digested wild-type annexin DNAs were subcloned into expression vector (pET-3d) by standard techniques. Expression of protein was induced by adding 0.4 mM isopropyl β-D-thiogalactopyranoside to the transformed *E. coli*. Recombinant annexin V was purified by binding to lipid liposomes in a Ca^{2+}-dependent manner, by a similar method to that described for the purification of annexin IV (*14*). Further purification was performed with DEAE-Toyopearl.

Preparation of Recombinant Bovine Annexin IV and Native Rat Surfactant Protein A (SP-A)—Recombinant bovine annexin IV and native rat SP-A was prepared as described previously (*4, 14*).

Isolation of Rat Alveolar Type II Cells and Analysis of the Secretion of Annexin V and [^{3}H]Phospholipid (PC)—Alveolar type II cells were isolated from adult male Sprague-Dawley rats by tissue dissociation with elastase (Worthington Biochemical) and purified on metrizamide density gradients by the method of Dobbs and Mason (*15*). Type II cells (4×10^6) in 1 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) were incubated with phorbol-12-myristate-13-acetate (PMA) (50 ng/ml) and/or SP-A (1 μg/ml) for 3 h at 37°C with shaking. After incubation, 5 mM EGTA was added to the culture media and incubated for 5 min, then cells were pelleted by centrifugation at 1,500 ×*g* for 5 min. For Western blotting, the supernatant (20 μl) was mixed with 10 μl of 2% SDS, subjected to SDS-PAGE, and proteins were transferred to PVDF membrane.

Assay for secretion of [^{3}H]phospholipid (PC) by alveolar type II cells was performed as described previously (*16*). Briefly, isolated type II cells (2×10^6 cells) were incubated overnight with [^{3}H]choline (0.5 μCi/ml) in DMEM containing 10% FCS, and after addition of PMA (50 μg/ml) and/or SP-A (1 μg/ml), incubated for 3 h at 37°C. After incubation, phospholipids were extracted from both the cells and media, and radioactivity was counted. The phospholipid secretion was expressed as the percentage of radioactivity in medium to total radioactivity.

Antibodies—Polyclonal antibodies against bovine recombinant annexins IV and rat recombinant annexin V were

prepared from rabbits. The bovine annexin IV IgG cross-reacted with rat annexin IV (data not shown). IgG fractions of the antisera were then obtained with protein A-Sepharose 4B (Amersham Pharmacia Biotech., Buckinghamshire, UK).

Western blotting—For Western blotting, the protein samples were resolved by SDS-PAGE, then transferred onto PVDF membrane for 14 h at 12 V in the presence of transfer buffer [10 mM CAPS (pH 11) and 10% methanol (v/v)]. The blots were blocked with 3% skim milk in PBS and then probed with anti-annexin antibodies (1 to 400 dilution). All blots were incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (1 to 3,000 dilution with PBS) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Finally, after washing, antibody visualization was performed using an ECL-Western blotting detection system (Amersham Pharmacia Biotech.).

Other methods—Protein concentrations were determined with a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) or by the method of Bradford (*17*) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of SDS (0.1% w/v) (SDS-PAGE) was performed by the method of Laemmli (*18*).

RESULTS

Isolation of Lamellar Body-Binding Proteins—To identify the LB-binding proteins, rabbit lung soluble fractions were loaded onto LB-sepharose 4B in the presence of Ca^{2+} and protein peak fractions eluted with EGTA (Ca^{2+}-dependent LB-binding proteins) were next loaded onto a Mono Q column (Fig. 1). SDS-PAGE of the eluted peak fractions showed that peaks 4–7 proteins had apparent molecular masses of about 32–34 kDa and peak 8 protein 68 kDa, and that peaks 1–3 contained only a few proteins (Fig. 1B). A control experiment with unconjugated Sepharose 4B revealed that no Ca^{2+}-dependent binding protein was eluted from the column (data not shown). To identify each protein, the fractions were subjected to SDS-PAGE, transferred to PVDF membrane and then chemically cleaved with BrCN. The fragmented peptides generated were separated with an HPLC reverse-phase column (C_{18}) (data not shown). The N-terminal amino acid sequences of the eluted peptide peak fractions were referred to SWISS-PROT for sequence homology analysis (Table I). The N-terminal amino acid sequences from peak 4 matched with A^{1}–A^{15}, R^{253}–S^{266}, and K^{268}–V^{279} of bovine annexin IV, that from peak 5 matched with V^{85}–S^{102} of human annexin VIII, that from peak 6 matched with V^{85}–S^{102} of human annexin III, and those from peak 7 matched with G^{141}–R^{149} and K^{258}–V^{270} of human annexin V. Although the N-terminus of porcine annexin IV has been demonstrated to be blocked (*19*), it is conceivable that that of rabbit annexin IV is not blocked. We obtained more fragmented peptide peaks for all the peaks, but amino acid sequence analysis revealed the presence of more than two PTH-amino acids in each reaction cycle, indicating that more than two distinct peptide fragments were included. Although we identified only one peptide sequence each for peaks 5 and 6, the sequences were 100% identical with annexins VIII and III, respectively, and no other identical sequence from other proteins was identified by sequence homology analysis. Thus, four annexins (annexins III, IV, V, and VIII) were identified as the Ca^{2+}-

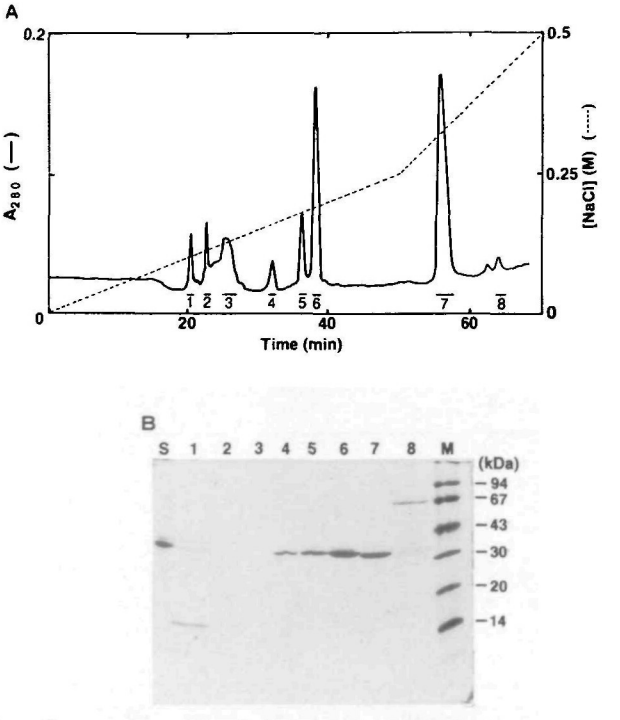

Fig. 1. **Separation of the Ca^{2+}-dependent LB-binding proteins from lung soluble fractions by Mono Q.** A: Elution profile. The Ca^{2+}-dependent LB-binding proteins of the lung soluble fractions after LB-Sepharose 4B affinity chromatography were loaded onto Mono Q HR 5/5 equilibrated with Hepes (20 mM, pH 7.5), and EGTA (0.1 mM). The column was washed with the equilibration buffer, and elution was performed with a linear gradient of NaCl from 0 to 0.25 M. B: SDS-PAGE pattern of the eluted fractions. Lane S, starting material (pooled fraction after LB-Sepharose 4B); lanes 1–8, peak fractions 1–8, respectively, from Mono Q chromatography designated by solid lines under the peaks in A; lane M, molecular weight standards. The numbers are molecular masses in kilodaltons of the marked migration positions.

TABLE I. **Amino acid sequences of peptide fragments of LB-binding proteins generated by BrCN.**

Peak number	Amino acid sequence of the peptides	Corresponding sequence
4:	ASKGGTIKAASGFNA	A^{1}SKGGTIKAASGFNA^{15} (bovine annexin IV)
4:	ANKPAYFAERLYKS	R^{253}NKSAYFAERLYKS^{266} (bovine annexin IV)
4:	KGLGTDDDTLIRV	K^{268}GLGTDDDTLIRV^{279} (bovine annexin IV)
5:	YPPYRYEAKEDHDA	Y^{92}PPYRYEAKEDHDA^{105} (human annexin VIII)
6:	VALVTPPAVFDA#QLKKS	V^{85}ALVTPPAVFDACQLKKS^{102} (human annexin III)
7:	GDTSGYYQR	G^{141}DTSGYYQR^{149} (human annexin V)
7:	KGAGTDDHTLIRV	K^{258}GAGTDDHTLIRV^{270} (human annexin V)

Each peptide peak in Fig. 1 was lyophilized and the amino acid sequence was determined. The amino acid sequences determined were referred to the protein data bank (SWISS-PROT) for sequence homology analysis. #, not determined.

dependent LB-binding proteins. Since the content of LB has been demonstrated to be about 90% lipid, in which acidic phospholipid (PG) is included (*20*), and since annexins preferentially bind acidic phospholipid–containing lipids (*21, 22*), the annexins might have merely bound to the lipid fractions of the LB column. To check this possibility we next used a PS/PG/PC-Sepharose 4B column. The proteins from lung soluble fractions binding to this column in a Ca^{2+}-dependent manner were then analyzed by Mono Q chromatography (Fig. 2A). The elution volumes and SDS-PAGE pattern of the peaks 5′, 6′, and 7′ in Fig. 2 were quite similar to those of the peaks 4, 6, and 7 in Fig. 1, respectively, but the protein peak corresponding to peak 5 in Fig. 1A was missing in Fig. 2A. The mobilities of the bands of peaks 5′–7′ (Fig. 2) differed somewhat from those of peaks 4, 6, and 7 (Fig. 1), respectively, because the peaks in Figs. 1 and 2 were resolved on different SDS-gels with different running times. The HPLC patterns of the fragmented peptides from peaks 4 and 5′, 6 and 6′, and 7 and 7′, respectively, were quite similar to each other (data not shown). Amino acid sequence analysis of the BrCN-cleaved peptide fragments revealed that peaks 5′, 6′ and 7′ were identical with peaks 4, 6 and 7 in Fig. 1A, respectively (Table I) (data not shown). Therefore, annexin VIII, which is absent in the eluate of the PS/PG/PC-column chromatography, may be a specific LB-binding protein.

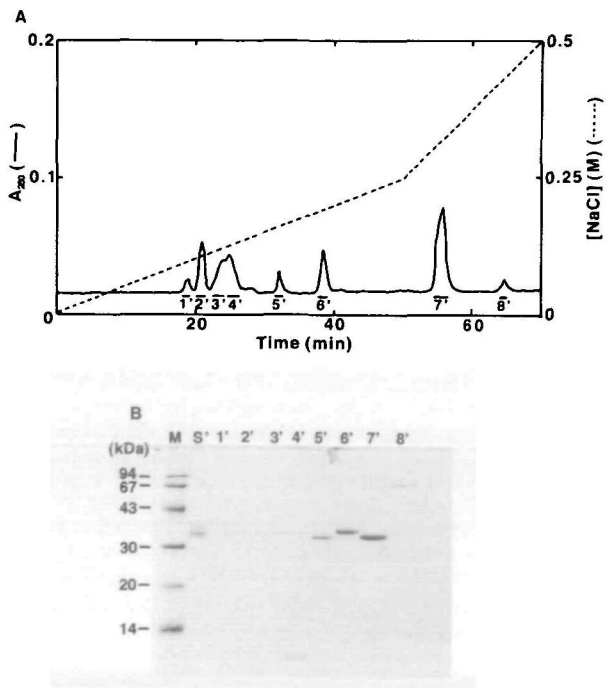

Fig. 2. **Separation of the acidic phospholipid-binding proteins from lung soluble fractions by Mono Q.** A: Elution profile. The Ca^{2+}-dependent phospholipid-binding proteins of the lung soluble fractions after PS/PG/PC-Sepharose 4B affinity chromatography were loaded onto Mono Q HR 5/5 equilibrated with Hepes (20 mM, pH 7.5), and EGTA (0.1 mM). The column was washed with the equilibration buffer, and elution was performed with linear gradient of NaCl from 0 to 0.25 M. B: SDS-PAGE pattern of the eluted fractions. Lanes 1′–8′, fractions 1′–8′, respectively, designated by solid lines under the peaks in A; lane M, molecular mass standards. The numbers are molecular masses in kilodaltons of the marked migration positions.

Annexin in Alveolar Space—We next analyzed the LB-binding protein(s) in the outer space of rabbit lung [bronchoalveolar lavage fluid (BALF)]. BALF was first loaded onto LB-Sepharose 4B in the presence of Ca^{2+} and eluted with EGTA (data not shown). The eluted fractions were analyzed by Mono Q chromatography (Fig. 3A). The SDS-PAGE pattern of the eluted peak fractions showed that the only detectable band, with molecular mass of 33 kDa, was in fraction 4″ (Fig. 3B). The other fractions apparently contain little protein on the Coomassie blue-stained gel (Fig. 3B). The fraction volume agreed well with the elution volume of rabbit annexin V (Fig. 1), and amino acid sequence analysis of the BrCN-cleaved peptide fragments revealed that peaks 4″ are identical with annexin V (Table I) (data not shown). Western blot analysis also demonstrated that the band was immunostained with anti-annexin V but not with anti-annexin IV IgG (Fig. 3C).

Secretion of Annexin V by Alveolar Type II Cells—Pulmonary surfactant is synthesized and secreted by alveolar type II cells. In isolated type II cells, phospholipid secretion is stimulated by phorbol ester, and the stimulation is attenuated by surfactant protein A (SP-A) (Fig. 4C) (*23, 24*). To examine whether type II cells secrete annexin V, culture

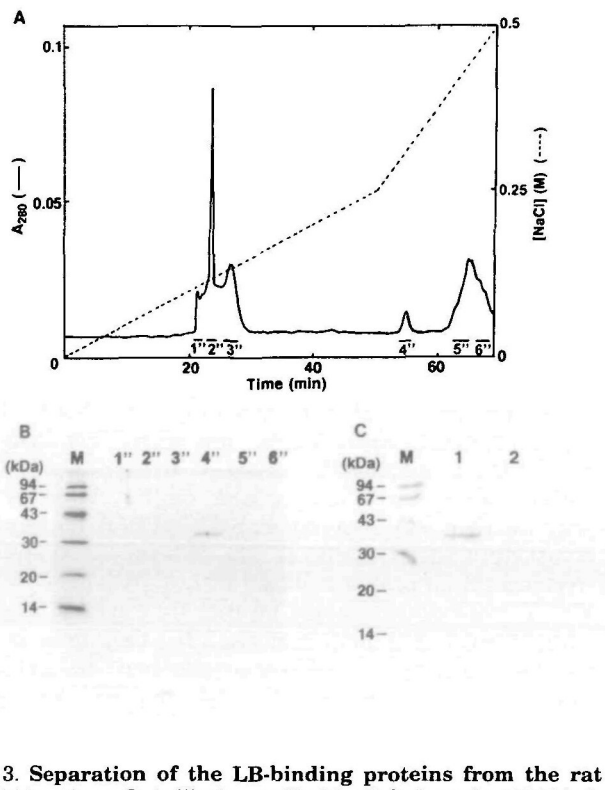

Fig. 3. **Separation of the LB-binding proteins from the rat BALF by Mono Q.** A: Elution profile. The Ca^{2+}-dependent LB-binding proteins of BALF after LB-Sepharose 4B affinity chromatography were loaded onto Mono Q HR 5/5 equilibrated with Hepes (20 mM, pH 7.5), and EGTA (0.1 mM). The column was washed with the equilibration buffer, and elution was performed with linear gradient of NaCl from 0 to 0.25 M. B: SDS-PAGE pattern of the eluted fractions. Lanes 1″–6″, fractions 1″–6″, respectively, designated by solid lines under the peaks in A. C: Western blot analysis of fraction 4 in A. Lane 1, probed with anti-rat annexin V; lane 2, probed with polyclonal anti-bovine annexin IV IgG. Antibody visualization was performed with diaminobenzidine. Lane M, molecular mass standards stained with Coomassie Blue, marked in kilodaltons.

media of the isolated type II cells were analyzed by Western blotting with anti-annexin V antibody (Fig. 4A). An immunostained band with a molecular mass of 33 kDa was visualized and the intensity of the band was augmented by the addition of PMA (Fig. 4A). Densitometric analysis showed that the intensity of the immunoreactive 33-kDa protein was about four times greater than that of the control (Fig. 4B). The intensity of the PMA-stimulated immunoreactive band was attenuated to the control level when the cells were preincubated with SP-A before the addition of PMA (Fig. 4B). Although the exact amount of annexin V cannot be measured by Western blotting, the amount of annexin V secreted by the cells was apparently altered by PMA and SP-A. ^{3}H-labeled phospholipid secretion by the cells was stimulated by PMA and was attenuated to the control level in the presence of SP-A (Fig. 4C). Thus, the secretion of annexin V responded to PMA and SP-A in a similar manner to phosphlipid secretion by type II cells.

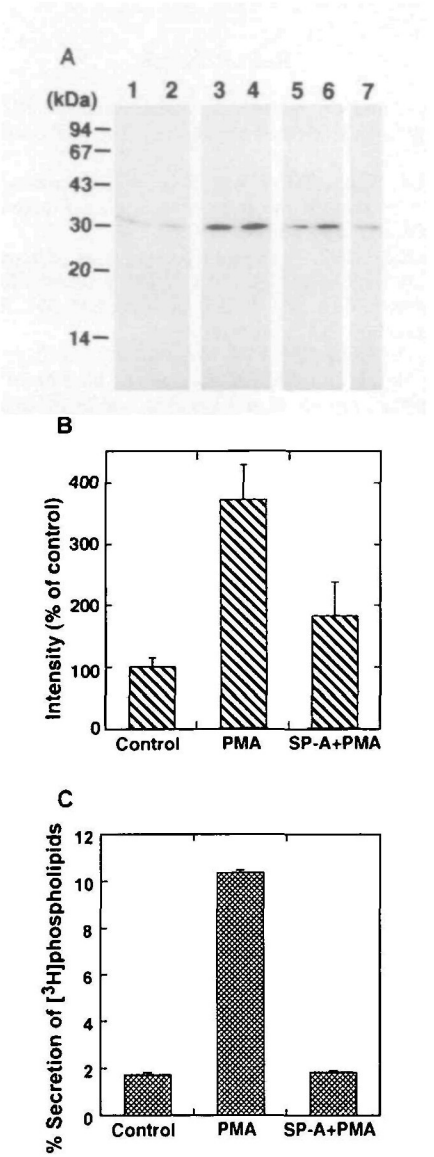

Fig. 4. **Secretion of annexin V and phospholipid (PC) by isolated alveolar type II cells.** A: Isolated rat alveolar type II cells (4×10^6 in 1 ml of DMEM containing 10% FCS) were incubated in the presence or absence of PMA (50 μg/ml) and/or rat SP-A (1 μg/ml) for 3 h at 37°C, and then 5 mM EGTA was added. After centrifugation, the supernatant (20 μl) was subjected to SDS-PAGE and transferred to PVDF membrane. Immunoblotting was performed with anti–rat annexin V antibody, and the antibody visualization was performed using ECL-Western blotting detection system (Amersham). Molecular masses marked in kilodaltons are indicated on the left. Lanes 1 and 2, control; lanes 3 and 4, PMA; lanes 5 and 6, SP-A and PMA; lane 7, SP-A. Two separate experiments under the same conditions are shown in lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6, respectively. B: Densitometric analysis after immunoblotting. Data represent mean ± SE of four experiments. C: Effect of PMA and/or SP-A on phospholipid (PC) secretion by type II cells. Isolated type II cells (2×10^6 cells) were incubated overnight with [^{3}H]choline, and PMA (50 μg/ml) and/or SP-A (1 μg/ml) were added, and then incubated for 3 h at 37°C. After the final incubation, phospholipids were extracted from both cells and medium, and the phospholipid secretion was expressed as the percentage of radioactivity in medium to total radioactivity.

DISCUSSION

Since lipids make up about 90% of pulmonary surfactant, and PG, which is found in very low concentrations in most mammalian membranes, is enriched in surfactant (5–15% of the phospholipids) (*20*), it is possible that the annexins identified using LB-Sepharose 4B had bound to the acidic lipid fractions of LB. Indeed, several LB-binding proteins (annexins) were identified with the control column (PS/PG/PC-Sepharose 4B) (Figs. 1 and 2). However, a detectable amount of annexin VIII was found with LB-Sepharose 4B, but not with PS/PG/PC-Sepharose 4B (Figs. 1 and 2), suggesting that annexin VIII might be a specific LB-binding protein. Annexin VIII might have bound to LB-specific lipid species and/or protein(s), although a specific component other than surfactant proteins of LB has not yet been identified. Annexin VIII was first identified as a vascular anticoagulant protein in placenta (*25*) and shown to be expressed predominantly in lung and placenta (*26*). It was also shown to be a specific marker in acute promyelotic leukemia (*26, 27*). Although annexin VIII inhibits PLA_2 activity and possesses anti-coagulation activity *in vitro*, the functional role of annexin VIII *in vivo* is still obscure, since it is not likely to be secreted from the cells (*26*). Whether such *in vitro* activity of annexin VIII is involved in the process of LB secretion in the lung is an intriguing question that should be addressed by investigation of the functional relevance of the annexin isoforms in the lung.

It has been suggested that the heterotetrameric complex of annexin II-p11 (S100A10) and annexin VII (synexin) are involved in regulating surfactant secretion (*6–8*). However, annexins II and VII were both undetectable in the present study, although they were found in detectable amounts in isolated alveolar type II cells (*6*). Further study will be required to clarify the involvement of annexins in the metabolism of LB.

In the present study, annexin V was identified in BALF from the rabbit (Fig. 3) and shown to be secreted from the isolated alveolar type II cells (Fig. 4). The secretion of annexin V was enhanced by PMA, a potent stimulator of surfactant lipid secretion (*28*). The PMA-enhanced secretion of annexin V was attenuated by the addition of SP-A, a potent inhibitor of surfactant lipid secretion (*23*). These results clearly indicate that annexin V might be secreted together with surfactant. On the other hand, it is plausible that the augmented secretion of annexin V was caused by injury of

the cells during culture under various conditions. However, since the cell viability analyzed with Trypan Blue was over 90% under all the different conditions (data not shown), it is unlikely that the increased amount of annexin V in the culture media was due to the lysis of the type II cells. Annexin V has been shown to relocate from cytosol to membranes in conjunction with an increase in intracellular Ca^{2+} concentration (*29, 30*). It was further shown that relocation of annexin V was enhanced by okadaic acid in platelets, where annexin V–binding phosphorylated proteins were likely to be involved, and the relocation was reversed by alkaline phosphatase (*31*). The PMA-stimulated augmentation of annexin V secretion in type II cells might be mediated by a phosphorylation-dependent process, although we have not yet identified annexin V–binding phosphorylated protein in type II cells.

Several lines of evidence suggest that some annexins are secreted from cells, although annexins have no signal sequences. Secretion of annexin V was found in both chick-embryo chondrocytes and fibloblasts (*32, 33*). As annexin V has been shown to bind collagens types II and X, it has been suggested that annexin V might be involved in cartilage mineralization (*34*). In addition, the reduced expression of annexin V in placenta might be one of the causes of the thromboembolic conditions in antiphospholipid antibody syndrome (*35*). We tested whether annexin V alters phospholipid secretion or reuptake of phospholipids by type II cells. However, no significant change was observed with 10 μg/ml exogenous annexin V (data not shown). It was also shown that annexin V is localized not only in cytoplasm but also nuclei (*36, 37*). These results thus make it difficult to understand the functional properties of annexin V.

Annexin I was detected in peritoneal exudates, prostate gland, and pulmonary outer space (*13, 38, 39*). Elevated expression and secretion of annexin in a dose-dependent manner I were detected in both alveolar macrophages and alveolar type II cells on administration of glucocorticoids (*38, 40*). The amounts of annexin I in the BALF of patients with idiopathic pulmonary fibrosis were increased by the administration of corticosteroids (*41*). Detectable amounts of annexin I were found in both control and PMA-stimulated culture media of isolated type II cells by Western blot analysis, but the amounts were unchanged in the presence or absence of PMA (data not shown), suggesting that annexins I and V are secreted by different mechanism. It has been demonstrated that exogenous annexin I inhibits cell growth of A549 cells by suppressing the release of PGE_2, which is necessary for their proliferation (*42, 43*), modulates migration of neutrophils (*44, 45*) and downregulates adhesion of U937 cells to endothelium (*46*). Although annexin I was not identified as an LB-binding protein in the present study, it is possible that annexin I present in lung outer space might have a role in the regulation of surfactant metabolism.

In summary, we have demonstrated that several annexins bind to the lung LB in a Ca^{2+}-dependent manner, and that annexin VIII is probably a specific binding protein for LB. Annexin V was secreted by isolated alveolar type II cells and the secretion was stimulated by PMA and inhibited by SP-A, which seems to suggest that both LB and annexin V secretions are governed by the same mechanism.

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