LIPID RAFTS AND LUNG SURFACTANT SECRETION

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ABBREVIATIONS

α-SNAP	α-Soluble N-ethylmaleimide-sensitive fusion protein Attachment protein
Type I	Alveolar type I cells
Type II	Alveolar type II cells
ATP	Adenosine triphosphate
BAPTA-AM	Bis-(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester
BSA	Bovine Serum Albumin
CaMKII	Ca^{2+}/Calmodulin-dependent protein kinase II
CMV	Cytomegalovirus
DMEM	Dulbecco's Modified Eagle's Medium
DPPC	Dipalmitoylphosphatidylcholine
ECL	Enhanced Chemiluminescence
EGTA	Ethylene Glycol-bis-(3-aminoethyl ether) N, N'-Tetraacetic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
EHS	Engelbroth-Holm-Swarm
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
FM1-43	N-(3-triethylammonium propyl)-4-

	(dibutilamino)styryl-pyrodinum dibromide
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
EGFP	Enhanced Green Florescent Protein
GPI	Glycosylphosphatidylinositol
HRP	Horse Raddish Peroxidase
HEPES	(2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid)
IEF	Isoelectric focusing
IgG	Immunoglubulin
kDa	kiloDalton
KGF	keratinocyte growth factor
LB-180	Lamellar body membrane protein-180 kDa
MALDI-TOF	matrix-assisted laser desorption/ionization-Time-of-flight
MCD	methyl-β -cyclodextrin
MEM	Minimum Essential Medium
MES	morpholine-ethanesulfonic acid
MBS	MES Buffered Saline
µg	micrograms
mg	milligram
MOI	Multiplicity of Infection
ml	milliliters

MLE	mouse lung epithelial cells
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NP-40	Nonidet P-40
NSF	N-ethylmaleimide sensitive factor
PBS	Phosphate buffered Saline
PC	Phosphatidylcholine
PC12	Pheochromocytoma cells
PKC	Protein Kinase C
PKA	Protein Kinase A
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
R-18	Octadecyl Rhodamine
RDS	Respiratory distress syndrome
RNAi	RNA interference
rRNA	ribosomal RNA
RT-PCR	Reverse transcriptase PCR
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SNARE	soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors
SNAP-23/25	Synaptosome-associated protein of 23/25

SP-C	Surfactant protein C
TTBS	Tween-Tris-Buffered Saline
VAMP	Vesicle associated membrane protein
V-ATPase	Vacuolar ATPase

CHAPTER I

INTRODUCTION

1.1 Alveolar epithelium

The lung alveoli are the basic and functional units involved in gaseous exchange. The lungs undergo constant expansion and compression during the process of respiration. Lung alveoli are lined with lung surfactant that reduces surface tension to prevent the collapse of alveoli at the end of expiration. Lungs are also frequently exposed to harmful microbes, toxins and pollutants. The alveoli are equipped with defense mechanisms to counteract the pathogens. The cell types that line the alveoli are uniquely adapted to withstand the constant mechanical stretch and to function under dynamic conditions. The cells in the lung also communicate by a paracrine or autocrine manner to regulate lung functions (1).

The alveoli are lined by two distinct epithelial cells, the squamous alveolar type I (type I) cells and cuboidal type II cells. They perform distinct and diverse functions. Type II cells constitute two-thirds of alveolar epithelial cell populations whereas type I cells form the other remaining one-third of the population. However type I cells occupy 95% of the surface area owing to their large size. Type I cells are involved in gaseous exchange, fluid homeostasis and act as mechano-sensors for the stretch that is induced during the respiratory process (2).

Type II cells secrete antimicrobial peptides including defensins. They act as progenitors for type I cells and replace the injured type I cell population. Additionally

type II cells are involved in fluid homeostasis (3). Another major function of type II cells is to synthesize, store and secrete lung surfactant. Deficiency of surfactant leads to acute respiratory distress syndrome in infants and is characterized by alveolar collapse and in extreme cases leads to death. The current study investigates the functions of type II cells, in particular surfactant secretion.

1.2 Lung surfactant composition, synthesis and secretion

The lung surfactant is a lipid-rich substance consisting of 90-95% lipids and about 5-10% proteins. Phospholipids constitute 80% and the remaining 20% are contributed by neutral (such as cholesterol) and other lipids. Dipalmitoylphosphatidyl choline is responsible for lowering surface tension.

The protein components of lung surfactant include surfactant protein –A, B, C and D (SP-A, B, C and D). They are synthesized in the endoplasmic reticulum and are further processed in Golgi. The proteins (SP-B and SP-C) are stored in lamellar bodies until released. SP-B and –SP-C are secreted along with surfactant lipids in a regulated manner (4, 5). However, SP-A and –D are secreted in a constitutive fashion and independent of lamellar bodies (6). The secreted SP-A and SP–B aids in the formation of tubular myelin, a precursor of the surfactant monolayer at the air-liquid interface. On the other hand, the secreted SP- B and SP-C help in the adsorption and distribution of surfactant. Apart from surfactant related functions, surfactant proteins are also involved in alveolar defense (7).

Physiologically, type II cells are stimulated by mechanical stretch induced by labor and ventilation. Stretch induces an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), which propagates as a wave to the neighboring type II cells through gap junctions. The net increase in $[Ca^{2+}]_i$ results in surfactant secretion (8, 9). Type II cell exocytosis is

extremely sensitive to fluctuations in $[Ca^{2+}]_i$ (10). However, Ca^{2+} sensors in type II cells are unknown, but, the role of annexin A2 as a possible sensor is proposed (11).

Natural agonists including ATP, UTP, adenosine, platelet activating factor, lipopolysaccharide and IL-1 also stimulate surfactant secretion. A number of compounds including β_2-adrenergic agonists (terbutaline), A_{2B} receptor agonists (adenosine), $P2Y_2$ receptor agonists (ATP and UTP), protein kinase C activators (phorbol esters) and calcium ionophores (A23187) stimulate surfactant secretion (12). Terbutaline and adenosine activate adenyl cyclase which further activates protein kinase A. On the other hand, ATP and phorbol esters activate PKC. The ionophores increase the $[Ca^{2+}]_i$ which activates PKC and Ca^{2+}/calmodulin-dependent kinase II. The activation of various kinases leads to phosphorylation of exocytotic proteins. However, the mechanism of how the phosphorylation induces lung surfactant secretion is poorly understood.

Exocytosis involves the interaction of proteins present on the lamellar bodies and plasma membrane (13). The soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) hypothesis was proposed to explain eukaryotic membrane fusion machinery. According to the SNARE hypothesis, during membrane fusion, the proteins residing on the vesicle or v-SNAREs (on lamellar bodies in type II cells) interact with those on plasma or target membrane or t-SNAREs to form a highly stable coiled-coil ternary SNARE complex. One coil each is contributed by vesicle associated membrane protein (VAMP) and syntaxin and two by SNAP-25. Later, N-ethylmaleimide sensitive factor (NSF), an ATPase along with its adaptor protein, α-souble NSF attachment protein (α –SNAP), binds the SNARE complex, separating the interacting SNARE partners for subsequent rounds of exocytosis. *In vitro* the formation of SNARE complex is sufficient

for close apposition and membrane fusion. The vesicular contents are released through a fusion pore. Evidence for the involvement of SNARE proteins in membrane fusion is strengthened with a recent study which revealed that transmembrane regions of syntaxin protein line the fusion pore (14).

More than 30 SNARE isoforms are reported in mammals, all of which are characterized by conserved aminoacids sequences close to the membrane anchors which form the coiled-coiled structures (15). There are about 15 syntaxin isoforms which inhabit various membranes. Syntaxins 1-4 are involved in exocytosis. Syntaxins associate with membrane by a transmembrane anchor at the C-terminus of the protein. Syntaxins are associated with nSec1/Munc18-1 thus limiting the availability of this protein for interaction. However, following the activation of small GTPases, Rab proteins, the syntaxin is released from nSec1 for subsequent interaction (16). SNAP-25/23 proteins associate with membrane owing to multiple palmitoylations on the cysteine residues. Seven different forms of VAMP proteins are reported. They are anchored to the membrane by a transmembrane domain. The membrane fusion machinery in type II cells contains t-SNAREs (syntaxin-2 and SNAP-23) and v-SNARE, VAMP-2, apart from α-SNAP and the ATPase, NSF (17, 18).

A number of studies from our laboratory had indicated the role of annexin A2 in surfactant secretion (11, 19, 20). Annexins are cytosolic proteins that bind with phospholipid membranes in a Ca^{2+}-dependent manner. Annexin A2 facilitates not only aggregation of liposomes but also promotes the fusion of lamellar bodies with plasma membranes (21-23). Moreover, annexin A2 mediates cortical actin disassembly facilitating surfactant secretion (24). The knockdown of annexin A2 in type II cells

results in reduced secretion, indicating its crucial role in secretion (19, 20). Annexin A7 has also been shown to be involved in surfactant secretion (25, 26).

1.3 Lipid rafts

Cell membranes consist of mixtures of lipids and proteins. According to the raft hypothesis, the lipid bilayer consists of saturated and unsaturated lipids. Saturated lipids confer an ordered (I_o) or gel phase whereas unsaturated lipids confer disordered (I_d) or liquid phase to the membrane. These ordered domains (lipid rafts) are enriched in glycosphingolipids, cholesterol and sphingomyelin. Lipid rafts are defined as "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" (27). The lipid composition of lipid rafts renders them insoluble to ice-cold 1% TX-100 (v/v) solubilization and they float when subjected to sucrose gradient centrifugation. They are hence called detergent resistant or detergent insoluble membrane fractions. Since lipid rafts are highly enriched in cholesterol, its enrichment following isolation and cholesterol dependent association of proteins should be confirmed. The unique lipid composition of lipid rafts enrich them in proteins that are doubly acylated (Src kinases), cholesterol-binding (caveolins), myristoylated and palmitoylated (flotillins) and GPI-anchored. By sequestering proteins and lipids, membrane microdomains exert influence not only on protein-protein interaction but also protein-lipid and lipid-lipid interactions. Thus lipid rafts serve as regulatory centers in various physiological conditions. They are reported to be crucial in a number of metabolic and pathogenic diseases (28). During resting conditions lipid rafts contain few proteins, however, upon stimulation, they cluster and

recruit additional proteins (29). They are thus ideally suited for membrane traffic, signal transduction, and protein sorting in polarized epithelial cells as well as non-polarized cells (30).

SNARE proteins associate with lipid rafts (31-33), although *in vitro* studies revealed that SNARE proteins prefer disordered phases more than ordered (34). In LT3 adipocytes, PC12 and HIT-T15 cells, SNARE proteins are enriched in rafts with SNAP-25/23 to a higher extent (35-37). SNAP-23 has a higher enrichment than its neuronal isoform, SNAP-25, due to its additional cysteine residues (37). The enrichment of v-SNARE is variable with the highest in LT3 cells. However, it was also reported that in PC12 cells, syntaxin was detergent soluble and did not co-localize with the raft markers (38). All the studied SNARE regulatory proteins are efficiently excluded from rafts. Moreover, SNARE complexes are highly enriched in raft fractions, indicating that rafts could be the sites of exocytosis (35). Similarily, Munc18-2/syntaxin3 complexes were excluded from the rafts indicating that a spatial control exists (39). Cholesterol depletion results in altered exocytosis indicating a role of lipid rafts in regulated exocytosis. In summary, raft association of SNARE proteins might pave a way for the interaction of SNARE proteins and provide suitable lipid environment for the membrane fusion. However, there are no reports of SNARE protein organization in type II cells.

Annexin A2 translocates to the Triton X-100 insoluble fractions in bovine chromaffin cells upon stimulation. Annexin A2 is associated with the lipid raft clusters in a Ca^{2+}-dependent manner (40, 41). Cleavage of annexin A2 abolishes its binding with the rafts, indicating that alteration in protein structure may affect its association with rafts (42). Moreover, annexin A2 promotes the formation of lipid microdomains and organizes

the exocytotic machinery (43). There are no reports on raft association of annexin A2 in alveolar type II cells.

Downregulation or knockdown of raft marker proteins have been undertaken to elucidate their roles (44). Caveolin-1 knockdown results in the loss of caveolae, the plasma membrane invaginations. Type II cells lack distinct caveolae in contrast to AT1 cells. Flotillins on the other hand are highly expressed in type II cells and hence can be used as marker proteins to study their roles in lipid rafts formation and surfactant secretion.

Flotillins (or reggies) are highly conserved and ubiquitously expressed. There are two flotillin isoforms, namely -1 (or reggie-2) and -2 (or reggie-1). Different cell types may express both or one of the isoforms. Initially, flotillins were thought to be caveolae-associated proteins. However, it has been shown that its expression is independent of caveolae. Flotillins do not have a distinct transmembrane domain. They associate with rafts owing to the multiple palmitoylation in case of flotillin-1 and additionally myristoylation in case of flotillin-2. Nuclear localization of flotillin-1 but not -2 has also been reported in PC-3 cells (45). The distribution of these proteins thus enable to them to be involved in a number of cellular functions.

Flotillins are implicated in a number of functions including interactions with actin cytoskeleton, formation of filopodia, insulin signaling, axonal regeneration and membrane trafficking (46). Their association with kinases (such as Thy-1) and cell adhesion molecules such as F3 implicates them in signal transduction and the establishment of cell-cell contacts (47). Its role in clathrin-independent endocytosis has been recently reported (48). Silencing or mutation studies in Zebra fish and Drosophila

has indicated the importance of these proteins in embryogenesis. Other studies have indicated that flotillins are involved in neuroprotection following ischemic and reperfusion injury and in progression of Alzheimer pathology (49, 50). The role of flotillins in the formation of membrane rafts has been speculated (51).

Lipid rafts sequester only specific groups of proteins while excluding others. Some proteins specifically translocate into lipid rafts under certain patho-physiogical conditions from non-raft compartments. Similarly, a vice-versa phenomenon was also reported. Such reports indicate the compositional complexity and the need to critically evaluate the lipid raft association of proteins. Proteomic profiling provide a tool to reveal the protein component of these microdomains.

Lipid raft proteomic analysis has been previously reported in various cell types including monocytes (52), sperm cells (53), Jurkat T-cell line (54), HeLa cell line (55), human endothelial cells (56), neutrophils (57) and the rat liver (58). These studies have revealed that raft protein machinery is diverse and specific to each cell type. The identified proteins are important in the functioning of endoplasmic reticulum, early and late endosome trafficking, phagosomes, mitochondria, cytoskeletal assembly and signal transduction. Some of the proteins such as V-ATPases, Ca^{+2}-ATPases, SNARE proteins, annexins, Rab proteins and myelin vesicular protein of 17 kDa (MVP17) are involved in membrane fusion and vesicular trafficking. However, there are no reports on the complete proteomic profile of type II cell lipid rafts.

1.4 V-ATPases

V-ATPases are multi-subunit enzymes which drive the movement of protons against the concentration gradient using the energy of ATP hydrolysis. During this

process they generate a transvesicular proton-electrochemical potential gradient ($\Delta\psi_v$) and a transvesicular pH gradient (ΔpH_v).

Structurally, V-ATPases consists of a peripheral catalytic unit, V1 and a transmembrane proton translocator, VO. The catalytic unit contains 8 subunits (A-H) whereas the proton translocator contains 5 subunits labeled *a, c, c´, c´´* and *d*. V-ATPases are predominantly localized on the intracellular organelles such as lysosomes, endosomes, chromaffin granules, clathrin-coated and synaptic vesicles in a number of cell types including sperm, kidneys and macrophages (59). Plasma membrane localization of V-ATPases is reported in osteoclasts, renal intercalated cells, interdental cells of inner ear, narrow and clear cells in the epididymus and vas deferens (60). The altered plasma membrane V-ATPase activity results in bone resorption, renal acidification, and spermatogenesis (61, 62).

Various isoforms for each of the subunits were reported with tissue specific localization patterns. For example, *a1* and *a2* localised to organelles other than lysosomes whereas *a3* to endosomes and lysosomes in murine macrophages (63). Such expression patterns implicated V-ATPases in diverse tissue specific functions and also inherent differences in regulation of H^+ pumping activity.

Previous studies have indicated the expression of different subunits in bovine, rat and mouse lungs such as *a1, a2, a3, c2-a, e1, d1, d2*, G1 (64-66). In rat lung type II cells, *a* and *e1* mRNA are stably expressed encoding until 4 days after isolation, whereas *c2* expression is drastically reduced by 1 day after isolation (67). The *c2-a* isoform co-localised with the mouse lamellar body membrane protein, LB-180 (68). The differential

expression of protein subunits might indicate either the regulation of enzyme activity or its role in lamellar body functions.

V-ATPases on intracellular organelles are involved in secretory processes such as the uptake and processing of secretory proteins including serotonin, histamine and glutamic acid (69, 70). V-ATPases play a role in exocytosis processes such as the formation of the fusion pore and membrane fusion (71, 72). V-ATPase a1 and c subunits interact with SNARE proteins and are crucial in late stages of vesicular exocytosis (73-76). In *a3* knockout mice, normal acidification and processing of insulin in granules took place in the absence of *a3* protein. However, insulin secretion was decreased, indicating that V-ATPase also functions downstream of the acidification (77).

Lung lamellar bodies maintain acidic pH (78). The acidic pH is essential for the processing of SP-C, aggregation and condensation of surfactant lipids (79, 80) Additionally Ca^{2+} uptake was also dependent on acidification (81). Earlier studies utilized methylamine or NH_4Cl for disrupting the lamellar body pH (82). However, these agents increase cytoplasmic pH along with lamellar body pH. It hence becomes difficult to deduce the effects of disrupting lamellar body pH and its effect of surfactant. There are no studies on the role of V-ATPases in lung surfactant secretion.

1.5 Specific aims and significance

Role of lipid rafts had been studied in PC12, LT3, mast cells, sperm cells, and pancreatic-alpha cells. Type II cells are unique, since the exocytosis is slower when compared to other systems. Thus, it might be possible that lipid rafts might serve as one of the regulatory mechanisms. Moreover, there is no study on the role of lipid rafts in surfactant secretion. Thus, the present study was designed to study lipid raft organization

of type II cell surfactant protein machinery. To this end, we have laid down three specific objectives:

Specific Aim I: Investigate the role of lipid rafts in surfactant secretion, membrane fusion and fusion pore formation.

Specific Aim II: Examine the role of lipid raft marker proteins, flotillin-1 and -2 in the biogenesis of lipid rafts and surfactant secretion.

Specific Aim III: Determine the proteomic profile of type II cell lipid rafts and functions of identified protein, V-ATPase.

The study hence would be the first of its kind to implicate lipid rafts in general and flotillin and V-ATPase proteins specifically in surfactant secretion.

1.6 References

1. Bhaskaran M, Kolliputi N, Wang Y, Gou D, Chintagari NR, Liu L. Trans-differentiation of alveolar epithelial type II cells to type I cells involves autocrine signaling by transforming growth factor beta 1 through the Smad pathway. *J Biol Chem* 2007;282:3968-76.

2. Williams MC. Alveolar type I cells: molecular phenotype and development. *Annu Rev Physiol* 2003;65:669-95.

3. Jin N, Kolliputi N, Gou D, Weng T, Liu L. A novel function of ionotropic gamma-aminobutyric acid receptors involving alveolar fluid homeostasis. *J Biol Chem* 2006;281:36012-20.

4. Clark JC, Wert SE, Bachurski CJ, Stahlman MT, Stripp BR, Weaver TE, Whitsett JA. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc Natl Acad Sci U S A* 1995;92:7794-8.

5. Stahlman MT, Gray MP, Falconieri MW, Whitsett JA, Weaver TE. Lamellar body formation in normal and surfactant protein B-deficient fetal mice. *Lab Invest* 2000;80:395-403.

6. Rooney SA. Regulation of surfactant secretion. *Comp Biochem Physiol A Mol Integr Physiol* 2001;129:233-43.

7. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol* 2005;5:58-68.

8. Ashino Y, Ying X, Dobbs LG, Bhattacharya J. [Ca(2+)](i) oscillations regulate type II cell exocytosis in the pulmonary alveolus. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L5-13.

9. Ichimura H, Parthasarathi K, Lindert J, Bhattacharya J. Lung surfactant secretion by interalveolar Ca2+ signaling. *Am J Physiol Lung Cell Mol Physiol* 2006;291: 596-601.

10. Haller T, Auktor K, Frick M, Mair N, Dietl P. Threshold calcium levels for lamellar body exocytosis in type II pneumocytes. *Am J Physiol* 1999;277:L893-900.

11. Wang P, Chintagari NR, Gou D, Su L, Liu L. Physical and functional interactions of SNAP-23 with annexin A2. *Am J Respir Cell Mol Biol* 2007;37:467-76.

12. Andreeva AV, Kutuzov MA, Voyno-Yasenetskaya TA. Regulation of surfactant secretion in alveolar type II cells. *Am J Physiol Lung Cell Mol Physiol* 2007; 293:L259-71.

13. Dietl P, Haller T. Exocytosis of lung surfactant: from the secretory vesicle to the air-liquid interface. *Annu Rev Physiol* 2005;67:595-621.

14. Han X, Wang CT, Bai J, Chapman ER, Jackson MB. Transmembrane segments of syntaxin line the fusion pore of Ca2+-triggered exocytosis. *Science* 2004;304: 289-92.

15. Chen YA, Scheller RH. SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol 2001;2:98-106.

16. Betz A, Okamoto M, Benseler F, Brose N. Direct interaction of the rat unc-13 homologue Munc13-1 with the N terminus of syntaxin. *J Biol Chem* 1997;272: 2520-6.

17. Abonyo BO, Wang P, Narasaraju TA, Rowan WH 3rd, McMillan DH, Zimmerman UJ, Liu L. Characterization of alpha-soluble N-ethylmaleimide-sensitive fusion attachment protein in alveolar type II cells: implications in lung surfactant secretion. *Am J Respir Cell Mol Biol* 2003;29:273-82.

18. Abonyo BO, Gou D, Wang P, Narasaraju T, Wang Z, Liu L. Syntaxin 2 and SNAP-23 are required for regulated surfactant secretion. *Biochemistry* 2004;43: 3499-506.

19. Gou D, Jin N, Liu L. Gene silencing in mammalian cells by PCR-based short hairpin RNA. *FEBS Lett* 2003;548:113-8.

20. Gou D, Wang P, Jin N, Liu L. Silencing of Annexin II in Primary Culture of Alveolar Epithelial Type II Cells by RNA Interference. *Annexins* 2004;1:31-36

21. Liu L, Fisher AB, Zimmerman UJ. Lung annexin II promotes fusion of isolated lamellar bodies with liposomes. *Biochim Biophys Acta* 1995;1259:166-72.

22. Liu L, Wang M, Fisher AB, Zimmerman UJ. Involvement of annexin II in exocytosis of lamellar bodies from alveolar epithelial type II cells. *Am J Physiol* 1996; 270: L668-76.

23. Chattopadhyay S, Sun P, Wang P, Abonyo B, Cross NL, Liu L. Fusion of lamellar body with plasma membrane is driven by the dual action of annexin II tetramer and arachidonic acid. *J Biol Chem* 2003;278:39675-83.

24. Singh TK, Abonyo B, Narasaraju TA, Liu L. Reorganization of cytoskeleton during surfactant secretion in lung type II cells: a role of annexin II. *Cell Signal* 2004;16:63-70.

25. Chander A, Wu RD. In vitro fusion of lung lamellar bodies and plasma membrane is augmented by lung synexin. *Biochim Biophys Acta* 1991;1086:157-66.

26. Sen N, Spitzer AR, Chander A. Calcium-dependence of synexin binding may determine aggregation and fusion of lamellar bodies. *Biochem J* 1997;322:103-9.

27. Pike LJ. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res* 2006;47:1597-8.

28. Allen JA, Halverson-Tamboli RA, Rasenick MM. Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* 2007;8:128-40.

29. Bini L, Pacini S, Liberatori S, Valensin S, Pellegrini M, Raggiaschi R, Pallini V, Baldari CT. Extensive temporally regulated reorganization of the lipid raft proteome following T-cell antigen receptor triggering. *Biochem J* 2003;369:301-9.

30. Rajendran L, Simons K. Lipid rafts and membrane dynamics. *J Cell Sci* 2005; 118:1099-102.

31. Chamberlain LH, Gould GW. The vesicle- and target-SNARE proteins that mediate Glut4 vesicle fusion are localized in detergent-insoluble lipid rafts present on distinct intracellular membranes. *J Biol Chem* 2002;277:49750-4.

32. Salaun C, James DJ, Chamberlain LH. Lipid rafts and the regulation of exocytosis. *Traffic* 2004;5:255-64.

33. Lang T. SNARE proteins and membrane rafts. *J Physiol* 2007 May 3; [Epub ahead of print]

34. Bacia K, Schuette CG, Kahya N, Jahn R, Schwille P. SNAREs prefer liquid-disordered over "raft" (liquid-ordered) domains when reconstituted into giant unilamellar vesicles. *J Biol Chem* 2004;279:37951-5.

35. Chamberlain LH, Burgoyne RD, Gould GW. SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis. *Proc Natl Acad Sci U S A* 2001;98:5619-24.

36. Salaun C, Gould GW, Chamberlain LH. Lipid raft association of SNARE proteins regulates exocytosis in PC12 cells. *J Biol Chem* 2005;280:19449-53.

37. Salaun C, Gould GW, Chamberlain LH. The SNARE proteins SNAP-25 and SNAP-23 display different affinities for lipid rafts in PC12 cells. Regulation by distinct cysteine-rich domains. *J Biol Chem* 2005;280:1236-40.

38. Lang T, Bruns D, Wenzel D, Riedel D, Holroyd P, Thiele C, Jahn R. SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. *EMBO J* 2001;20:2202-13.

39. Pombo I, Rivera J, Blank U. Munc18-2/syntaxin3 complexes are spatially separated from syntaxin3-containing SNARE complexes. *FEBS Lett* 2003;550: 144-8.

40. Oliferenko S, Paiha K, Harder T, Gerke V, Schwarzler C, Schwarz H, Beug H, Gunthert U, Huber LA. Analysis of CD44-containing lipid rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton. *J Cell Biol* 1999;146: 843-54.

41. Babiychuk EB, Draeger A. Annexins in cell membrane dynamics. Ca(2+)-regulated association of lipid microdomains. *J Cell Biol* 2000;150:1113-24.

42. Babiychuk EB, Monastyrskaya K, Burkhard FC, Wray S, Draeger A. Modulating signaling events in smooth muscle: cleavage of annexin 2 abolishes its binding to lipid rafts. *FASEB J* 2002;16:1177-84.

43. Chasserot-Golaz S, Vitale N, Umbrecht-Jenck E, Knight D, Gerke V, Bader MF. Annexin 2 promotes the formation of lipid microdomains required for calcium-regulated exocytosis of dense-core vesicles. *Mol Biol Cell* 2005;16:1108-19.

44. Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC, Schedl A, Haller H, Kurzchalia TV. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* 2001;293:2449-52.

45. Santamaria A, Castellanos E, Gomez V, Benedit P, Renau-Piqueras J, Morote J, Reventos J, Thomson TM, Paciucci R. PTOV1 enables the nuclear translocation and mitogenic activity of flotillin-1, a major protein of lipid rafts. *Mol Cell Biol* 2005;25:1900-11.

46. Babuke T, Tikkanen R. Dissecting the molecular function of reggie/flotillin proteins. *Eur J Cell Biol* 2007;86:525-32.

47. Stuermer CA, Plattner H. The 'lipid raft' microdomain proteins reggie-1 and reggie-2 (flotillins) are scaffolds for protein interaction and signalling. *Biochem Soc Symp* 2005;72:109-18.

48. Glebov OO, Bright NA, Nichols BJ. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol* 2006;8:46-54.

49. Volonte D, Galbiati F, Li S, Nishiyama K, Okamoto T, Lisanti MP. Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe. *J Biol Chem* 1999; 274:12702-9.

50. Kokubo H, Lemere CA, Yamaguchi H. Localization of flotillins in human brain and their accumulation with the progression of Alzheimer's disease pathology. *Neurosci Lett* 2000;290:93-6.

51. Evans WE 4th, Coyer RL, Sandusky MF, Van Fleet MJ, Moore JG, Nyquist SE. Characterization of membrane rafts isolated from rat sertoli cell cultures: caveolin and flotillin-1 content. *J Androl* 2003;24:812-21.

52. Li N, Mak A, Richards DP, Naber C, Keller BO, Li L, Shaw AR. Monocyte lipid rafts contain proteins implicated in vesicular trafficking and phagosome formation. *Proteomics* 2003;3:536-48.

53. Sleight SB, Miranda PV, Plaskett NW, Maier B, Lysiak J, Scrable H, Herr JC, Visconti PE. Isolation and proteomic analysis of mouse sperm detergent-resistant membrane fractions: evidence for dissociation of lipid rafts during capacitation. *Biol Reprod* 2005;73:721-9.

54. Pompach P, Man P, Novak P, Havlicek V, Fiserova A, Bezouska K. Mass spectrometry is a powerful tool for identification of proteins associated with lipid rafts of Jurkat T-cell line. *Biochem Soc Trans* 2004;32:777-9.

55. Foster LJ, De Hoog CL, Mann M. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci U S A* 2003; 100:5813-8.

56. Sprenger RR, Speijer D, Back JW, De Koster CG, Pannekoek H, Horrevoets AJ. Comparative proteomics of human endothelial cell caveolae and rafts using two-dimensional gel electrophoresis and mass spectrometry. *Electrophoresis* 2004; 25:156-72.

57. Nebl T, Pestonjamasp KN, Leszyk JD, Crowley JL, Oh SW, Luna EJ. Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. *J Biol Chem* 2002;277:43399-409.

58. Bae TJ, Kim MS, Kim JW, Kim BW, Choo HJ, Lee JW, Kim KB, Lee CS, Kim JH, Chang SY, Kang CY, Lee SW, Ko YG. Lipid raft proteome reveals ATP synthase complex in the cell surface. *Proteomics* 2004;4:3536-48. Erratum in: Proteomics. 2004;4: 4032. Lee, Song-Won [corrected to Lee, Sang-Won].

59. Nishi T, Forgac M. The vacuolar (H+)-ATPases--nature's most versatile proton pumps. *Nat Rev Mol Cell Biol* 2002;3:94-103.

60. Pietrement C, Sun-Wada GH, Silva ND, McKee M, Marshansky V, Brown D, Futai M, Breton S. Distinct expression patterns of different subunit isoforms of the V-ATPase in the rat epididymis. *Biol Reprod* 2006;74:185-94.

61. Toyomura T, Murata Y, Yamamoto A, Oka T, Sun-Wada GH, Wada Y, Futai M. From lysosomes to the plasma membrane: localization of vacuolar-type H+ -ATPase with the a3 isoform during osteoclast differentiation. *J Biol Chem* 2003; 278:22023-30.

62. Beyenbach KW, Wieczorek H. The V-type H+ ATPase: molecular structure and function, physiological roles and regulation. *J Exp Biol* 2006;209:577-89.

63. Nishi T, Kawasaki-Nishi S, Forgac M. Expression and function of the mouse V-ATPase d subunit isoforms. *J Biol Chem* 2003;278:46396-402.

64. Smith AN, Jouret F, Bord S, Borthwick KJ, Al-Lamki RS, Wagner CA, Ireland DC, Cormier-Daire V, Frattini A, Villa A, Kornak U, Devuyst O, Karet FE. Vacuolar H+-ATPase d2 subunit: molecular characterization, developmental regulation, and localization to specialized proton pumps in kidney and bone. *J Am Soc Nephrol* 2005;16:1245-56.

65. Murata Y, Sun-Wada GH, Yoshimizu T, Yamamoto A, Wada Y, Futai M. Differential localization of the vacuolar H+ pump with G subunit isoforms (G1 and G2) in mouse neurons. *J Biol Chem* 2002;277:36296-303.

66. Peng SB, Li X, Crider BP, Zhou Z, Andersen P, Tsai SJ, Xie XS, Stone DK. Identification and reconstitution of an isoform of the 116-kDa subunit of the vacuolar proton translocating ATPase. *J Biol Chem* 1999;274:2549-55.

67. Toyomura T, Oka T, Yamaguchi C, Wada Y, Futai M. Three subunit a isoforms of mouse vacuolar H(+)-ATPase. Preferential expression of the a3 isoform during osteoclast differentiation. *J Biol Chem* 2000;275:8760-5.

68. Feng NH, Lin HI, Wang JS, Chou ST, Ma HK, Rooney SA, Lu JF. Differential expression of a V-type ATPase C subunit gene, Atp6v1c2, during culture of rat lung type II pneumocytes. *J Biomed Sci* 2005;12:899-911.

69. Sun-Wada GH, Murata Y, Namba M, Yamamoto A, Wada Y, Futai M. Mouse proton pump ATPase C subunit isoforms (C2-a and C2-b) specifically expressed in kidney and lung. *J Biol Chem* 2003;278:44843-51.

70. Schoonderwoert VT, Martens GJ. Proton pumping in the secretory pathway. *J Membr Biol* 2001;182:159-69.

71. Taupenot L, Harper KL, O'Connor DT. Role of H+-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: evidence for a vesiculogenic function. *J Biol Chem* 2005;280: 3885-97.

72. Morel N, Dunant Y, Israel M. Neurotransmitter release through the V0 sector of V-ATPase. *J Neurochem* 2001;79:485-8.

73. Peters C, Bayer MJ, Buhler S, Andersen JS, Mann M, Mayer A. Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. *Nature* 2001;409:581-8.

74. Morel N, Dedieu JC, Philippe JM. Specific sorting of the a1 isoform of the V-H+ATPase a subunit to nerve terminals where it associates with both synaptic vesicles and the presynaptic plasma membrane. *J Cell Sci* 2003;116:4751-62.

75. Hiesinger PR, Fayyazuddin A, Mehta SQ, Rosenmund T, Schulze KL, Zhai RG, Verstreken P, Cao Y, Zhou Y, Kunz J, Bellen HJ. The v-ATPase V0 subunit a1 is required for a late step in synaptic vesicle exocytosis in Drosophila. *Cell* 2005; 121:607-20.

76. Galli T, McPherson PS, De Camilli P. The V0 sector of the V-ATPase, synaptobrevin, and synaptophysin are associated on synaptic vesicles in a Triton X-100-resistant, freeze-thawing sensitive, complex. *J Biol Chem* 1996;271: 2193-8.

77. Sun-Wada GH, Toyomura T, Murata Y, Yamamoto A, Futai M, Wada Y. The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta-cells. *J Cell Sci* 2006;119:4531-40.

78. Chander A, Johnson RG, Reicherter J, Fisher AB. Lung lamellar bodies maintain an acidic internal pH. *J Biol Chem* 1986;261:6126-31.

79. Beers MF. Inhibition of cellular processing of surfactant protein C by drugs affecting intracellular pH gradients. *J Biol Chem* 1996;271:14361-70.

80. Chander A, Sen N, Wadsworth S, Spitzer AR. Coordinate packaging of newly synthesized phosphatidylcholine and phosphatidylglycerol in lamellar bodies in alveolar type II cells. *Lipids* 2000;35:35-43.

81. Wadsworth SJ, Chander A. H+-and K+-dependence of Ca2+ uptake in lung lamellar bodies. *J Membr Biol* 2000;174:41-51.

82. Chander A, Sen N, Wu AM, Higgins S, Wadsworth S, Spitzer AR. Methylamine decreases trafficking and packaging of newly synthesized phosphatidylcholine in lamellar bodies in alveolar type II cells. *Biochem J* 1996; 318: 271-8. Erratum in: *Biochem J* 1996;318:1079.

CHAPTER II

EFFECT OF CHOLESTEROL DEPLETION ON EXOCYTOSIS OF ALVEOLAR TYPE II CELLS

2.1 Abstract

Alveolar epithelial type II cells secrete lung surfactant via exocytosis. Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) are implicated in this process. Lipid rafts, the cholesterol- and sphingolipid-rich microdomains, may offer a platform for protein organization on the cell membrane. We tested the hypothesis that lipid rafts organize exocytotic proteins in type II cells and are essential for the fusion of lamellar bodies, the secretory granules of type II cells, with the plasma membrane. The lipid rafts, isolated from type II cells using 1% Triton X-100 (v/v) and sucrose gradient centrifugation, contained the lipid raft markers, flotillin-1 and -2 whereas excluded the non-raft marker, Na^+-K^+ ATPase. SNAP-23, syntaxin 2 and VAMP-2 were enriched in lipid rafts. When type II cells were depleted of cholesterol, the association of SNAREs with the lipid rafts was disrupted and the formation of fusion pore was inhibited. Furthermore, the cholesterol-depleted plasma membrane had less ability to fuse with lamellar bodies, a process mediated by annexin A2. The secretagogue-stimulated secretion of lung surfactant from type II cells was also reduced by methyl-beta-cyclodextrin. When the raft-associated cell surface protein, CD44, was cross-linked using anti-CD44 antibodies, the CD44 clusters were observed. Syntaxin 2, SNAP-23, and annexin A2 co-localized with the CD44 clusters. Our results suggested that lipid rafts

may form a functional platform for surfactant secretion in alveolar type II cells, and raft integrity was essential for the fusion between lamellar bodies with the plasma membrane.

Key words: Lipid rafts, SNARE proteins, alveolar type II cells, membrane fusion, surfactant secretion, exocytosis

2.2 Introduction

Lung alveolar epithelium consists of two different types of cells, the cuboidal type II cells and squamous type I cells. Type II cells synthesize, store, and secrete a surface-active lipid-rich substance, the lung surfactant. The released surfactant lines the alveolar epithelium, lowers the surface tension, and thus prevents the collapse of alveoli at end-expiration. Lung surfactant deficiency causes Respiratory Distress Syndrome (RDS) in infants. Type II cells are also involved in defense, injury and repair, and trans-differentiation into type I cells.

Lung surfactant, stored in lamellar bodies, is released upon its fusion with the plasma membrane via exocytosis. The formation of fusion pore precedes the release of lamellar body contents. The SNARE hypothesis was proposed to elucidate the mechanisms of membrane fusion during exocytosis. During fusion, the proteins on the plasma membrane (target or t-SNAREs) and vesicles (vesicular or v-SNARE) form a highly stable, hetero-tetrameric SNARE complex. The two coiled-coil domains are contributed by SNAP-25/23 and one each from syntaxin and VAMP (1). NSF, an ATPase, binds to its ligand, α-SNAP, and then to the SNARE complex to dissociate it and, thus, recycling the interacting components for subsequent cycles. *In vitro* studies have revealed that SNARE complex formation suffices membrane fusion (2), but *in vivo* fusion is a much faster and more complex phenomenon due to the existence of numerous SNARE regulatory proteins. The transmembrane regions of syntaxin appear to line the fusion pore (3). Previous studies from our laboratory have indicated that syntaxin 2, SNAP-23, α-SNAP, and NSF were required for surfactant secretion (4,5).

Annexins are a family of highly conserved proteins known for their Ca^{2+}-dependent association with the negatively charged phospholipids. Some of the members mediated aggregation, as well as the fusion of liposomes. Annexin A2 tetramer had an exceptionally low Ca^{2+} requirement for inducing the membrane fusion. Annexin A2 tetramer has been reported not only to promote the fusion of lamellar bodies with liposomes or the plasma membrane at μM Ca^{2+} concentrations, but also to reconstitute surfactant secretion in permeabilized type II cells (6,7). The silencing of annexin A2 by RNA interference in primary cultures of alveolar type II cells significantly reduced the surfactant secretion (8).

The studies during the past decade have revolutionized the understanding of membrane organization. According to the newly proposed raft hypotheses (9,10), membrane lipids exist in two phases, ordered phase rendered by saturated lipids and disordered phase by unsaturated lipids. The ordered phase lipids form lipid microdomains or lipid rafts, which are rich in cholesterol, sphingolipids, and gangliosides, and are reported to sequester and segregate a number of specific proteins. Thus, they are ideally suited for various processes, including membrane traffic, signal transduction, and apical protein sorting (9). Their involvements in other functions are being reported regularly. The resident raft proteins include GPI-anchored proteins on the exoplasmic leaflet and doubly acylated proteins, and palmitate-anchored proteins on the cytoplasmic leaflet. With their unique property of clustering, these microdomains provide an interacting platform for the various proteins to bring about the ultimate cellular response.

SNARE proteins have been shown to be associated with the lipid rafts in several cell types (11-14). However, when constituted into giant unilamellar vesicles, syntaxin

1A and synaptobrevin 2 preferred disordered domains (15). There are no reports of SNARE protein organization in type II cells. Furthermore, whether lipid rafts participate in the formation of fusion pore and thus membrane fusion was unclear. We hypothesized that lipid rafts organize SNARE proteins on the plasma membrane of alveolar type II cells and are essential for the fusion of lamellar bodies and the plasma membrane during exocytosis. To this end, we isolated lipid rafts from type II cells and determined the association of SNARE proteins with the lipid rafts. We further determined whether the disruption of lipid rafts by the depletion of cholesterol affected fusion pore formation, membrane fusion and surfactant secretion. Additionally, a raft-associated cell surface transmembrane glycoprotein, CD44, was cross-linked to form raft clusters using anti-CD44 antibodies and we determined whether the raft clusters contained exocytotic proteins was determined.

2.3 Materials and Methods

2.3.1 Materials

All chemicals and reagents were of analytical grade and purchased from Sigma (St. Louis, MO), unless otherwise stated. Elastase was purchased from Worthington Chemicals (Lakewood, NJ). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY). Minimal essential medium (MEM) was from ICN Biomedicals (Aurora, OH). Nitrocellulose membrane was from Schleicher & Schuell (Keene, NH). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody, and protein molecular mass markers were from Bio-Rad (Hercules, CA). Cy3-conjugated anti-mouse IgG, HRP-conjugated goat anti-mouse antibodies and bovine serum albumin (BSA) were from Jackson Immunologicals (West Grove, PA). Paraformaldehyde was from Electron Microscopy Services (Ft. Washington, PA). Monoclonal anti-flotillin-1 and -2, anti-α-SNAP, anti-NSF, and anti-CD44 antibodies were from BD Transduction Laboratories (Lexington, KY). Monoclonal anti-Na^+-K^+ATPase antibodies were from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-LB180 antibodies were from Covance Research products (Richmond, CA). Polyclonal rabbit anti-syntaxin 2 and anti-SNAP-23 antibodies were from Synaptic Systems (Gottingen, Germany). Polyclonal rabbit anti-VAMP-2 antibodies were from Stressgen (Victoria, Canada). Polyclonal goat anti-SP-C antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Amphiphilic dye, N-(3-triethylammoniumpropyl)-4-(4-[dibutylamino]styryl) pyridinium dibromide (FM 1-43), Octadecyl rhodamine B chloride (R18), all Alexa-conjugated secondary antibodies and cholesterol assay kit were purchased from Molecular Probes (Eugene, OR). Enhanced chemiluminescence (ECL) detection system was from

Amersham Biotech (Piscataway, NJ). Polyclonal anti-annnexin A2 antibodies were raised in rabbits against isolated bovine annexin A2 and were affinity-purified. TRI reagents were from Molecular Research Center (Cincinnati, OH).

2.3.2 Isolation and culturing of alveolar type II cells

Alveolar type II cells were isolated from male Sprague-Dawley rats (175-200 gms) as previously described (16). The perfused lungs were lavaged, digested with elastase, chopped and filtered. The cells were subjected to IgG panning to remove contaminating macrophages. The unattached cells were pelletted, resuspended in MEM, counted for number, and assayed for viability using trypan-blue exclusion assay. The cell viability ranged from 95-97%. Purity of the cells was greater than 90%, as ascertained by Papanicolou's staining. For the RT-PCR experiment, >96% pure freshly isolated type II cells were obtained by our recently modified protocol (17), in which an additional step, using anti-leukocyte common antigen antibodies and magnetic beads, was included to remove the residual macrophages.

For studying the differential expression of flotillins, isolated type II cells were plated on 35 mm^2 plastic dishes at a density of 1.5×10^6 per dish. The cells were cultured for 3 or 7 days in MEM supplemented with 10% FBS. Media was changed after overnight culture and then every other day. In this culture system, type II cells loose their phenotypes and convert to the type I-like cells with some characteristics of alveolar type I cells.

2.3.3 RT-PCR

Total RNA was isolated from the lung by homogenizing the tissue in TRI reagent or from freshly isolated type II cells by dissolving them in the reagent. The mRNA (1 μg)

was reverse-transcribed to cDNA using M-MLV reverse transcriptase and random hexamer primers and PCR-amplified using gene specific primers against caveolin-1α (Forward: 5'-AAATTGATCTGGTCAACCGC-3'; Reverse: 5'-ATCTCTTCCTGCGTGCTGAT-3'), caveolin-1β (Forward: 5'-ATTGGTTTTACCGCTTGCTG-3'; Reverse: 5'-ATCTCTTCCTGCGTGCTGAT-3'), caveolin-2 (Forward: 5'-CTTCATTGCGGGTATCCTGT-3'; Reverse: 5'-CAGTTGTGGCTCAGTTGCAT-3'), caveolin-3 (Forward: 5'-GGACATTGTGAAGGTGGATTT-3'; Reverse: 5'-GCACTGGATCTCAATCAGGTA-3'), flotillin-1 (Forward: 5'-GCTGAAGAAAGCCACCTACG-3'; Reverse: 5'-CTCAGCTTCAGCTTCTGCCT-3'), flotillin-2 (Forward: 5'-GGGTACAAGGGTTCTGCGTA-3'; Reverse: 5'-TCTGTGCCTCTATGGTGCAG-3'). 18S rRNA was amplified using classic 18S RNA primer pairs (Ambion, Austin, TX). The conditions for PCR amplification were: 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100, 1.6 mM $MgCl_2$, 0.16 mM dNTP mix, 1.6 μM of each primer, 1 unit DNA *Taq* polymerase and 20 ng of cDNA in a final reaction volume of 25 μl. The thermal conditions were 94^0C for 2 min, 35 cycles of 94^0C 30 sec, 55^0C 40 sec, 72^0C 1 min, followed by 72^0C for 8 min. The PCR products were electrophoretically separated on agarose gel for studying the expression pattern of the raft marker proteins.

2.3.4 Western blot

Type II cells, freshly isolated or cultured on plastic dishes for 3 and 7 days, MLE-12 cells, and L2 cells were lysed in the lysis buffer (50 mM Tris-HCl , pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 1 mM PMSF, 10 μg/ml aprotonin, 10 μg/ml leupeptin, and 1 mM benzamidine). Lung tissue was homogenized in the lysis buffer.

Lamellar bodies (LB) and plasma membrane (PM) were directly dissolved in the SDS sample buffer. Proteins were separated on SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted with specific antibodies. The primary antibodies were used at the following dilutions, 1:250 for flotillin-1 and VAMP-2; 1:1000 for syntaxin 2, annexin A2, SNAP-23, Na^+-K^+ ATPase, NSF; and 1:5000 for flotillin-2 and α-SNAP. HRP-conjugated secondary antibodies were used at a 1:2500 dilution in all the cases. The protein bands were visualized with the ECL reagents. The protein bands were quantified using a Bio-Rad densitometric scanner (Hercules, CA).

2.3.5 Immunohistochemistry

The lungs from male Sprague-Dawley rats were perfused with 50 mM PBS (pH 7.4) and then lavaged four times with 5 ml of normal saline. Lungs were fixed by infusing 5 ml of 4% paraformaldehyde into the lungs and kept immersed in the same solution at room temperature for overnight. Paraffin-embedded lungs were sectioned (2 μm) and placed on glass slides (Fisher Scientific, Pittsburgh, PA). The slides were deparaffinized with xylene and rehydrated with graded alcohol and PBS. Antigen retrieval was done by boiling the slides with citrate buffer (10 mM disodium citrate, pH 6.0, and 0.05% Tween-20) for 20 minutes. Immunohistochemistry was performed as previously described (18). Goat anti-SP-C antibodies were used at a dilution of 1:50, whereas mouse anti-flotillin-1 and -2 at 1:100. Alexa 546-conjugated anti-goat and Alexa 488-conjugated anti-mouse antibodies were used at 1:250 dilutions.

2.3.6 Isolation of lipid rafts

Lipid rafts were isolated from alveolar type II cells according to the previously reported method (12). In brief, freshly isolated type II cells ($25 – 30 \times 10^6$) were washed

twice with MEM and once with MBS buffer (25 mM MES and 150 mM NaCl, pH 6.5). The cell pellet was lysed in 700 µl of ice-cold lysis buffer (MBS with 1% Triton X-100, 5 mM EGTA; 1 mM PMSF, 10 µg/ml aprotonin, 10 µg/ml leupeptin, and 1 mM benzamidine) for 45 minutes on ice. Six hundred µl of lysate was carefully mixed with an equal volume of 80% sucrose (w/v) and gently laid at the bottom of an ultracentrifuge tube. Later, 1200 µl, each of 30% and 5% sucrose were laid over the 40% sucrose gradient and centrifuged at 200,000 ×g for 16 hours. Seven fractions were collected from top to bottom as follows: first two fractions of 600 µl each, followed by three fractions of 400 µl each and two fractions of 600 µl each. Pellet was dissolved in 600 µl of lysis buffer and labeled as the eighth fraction. Equal volumes of each fraction were immunoblotted for various raft and non-raft marker proteins. For studying the effects of cholesterol depletion on lipid raft association of SNARE proteins, type II cells were solubilised in MBS containing Saponin (0.5% w/v), 0.5% TX-100, and a cocktail of protease inhibitors. A similar procedure was used in PC12 cells (12).

2.3.7 Fusion pore formation

Alveolar type II cells were grown on coverslips overnight. After removal of the unattached cells and equilibration with fresh MEM for 30 minutes, amphiphilic dye FM1-43 (4 µM) was added and incubated for 10 minutes (19). The cells were stimulated with a combination of secretagogues (0.1 µM phorbol 12-myristate 13-acetate, 100 µM ATP and 20 µM terbutaline) for indicated periods of time in the presence of the dye. The cells were rinsed with 50 mM ice-cold phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde. Under these conditions, the signals were not lost although extensive washing resulted in the loss of the signals. Excess fixative was quenched with 50 mM

ammonium chloride solution in PBS for 5 minutes. The cells were later washed three times with PBS and mounted onto the slides using mounting solution (1.5 % w/v n-propyl gallate and 60% v/v glycerol in PBS). The positively stained lamellar bodies were counted in 50 cells selected at random under oil immersion (100X). Cholesterol depletion from alveolar type II cells was done by incubating the cells with media containing 3 mM methyl β-cyclodextrin (MCD) for about 30 minutes at 37^0C. The control cells were treated the same with MCD-cholesterol complex (40 μg cholesterol and 0.5% (w/v) or 3.78 mM MCD). The cells were washed to remove MCD and incubated with fresh media for further studies. The extent of cholesterol depletion was assessed by cholesterol oxidase method (Amplex Red Cholesterol Assay Kit), as per the instructions of the manufacturer.

To determine if cholesterol depletion affected lamellar body staining, overnight cultured type II cells were used. Following fixation, permeabilization and blocking for non-specific proteins, the cells were incubated with LB-180 antibodies (1:1000) for overnight. Later, they were incubated with Cy3-conjugated anti-mouse antibodies (1:250). Florescence was observed with Nikon Eclipse E600 Microscope.

2.3.8 Membrane fusion

Lamellar bodies and plasma membrane were isolated from perfused rat lungs according to our previous protocols (7). The fusion of lamellar bodies with the plasma membrane was determined by the de-quenching of R18 as previously described (7). R18-labeled plasma membrane (5 μg protein) was incubated with lamellar bodies (10 μg protein) in 1 ml of Ca^{2+}-EGTA buffer (40 mM Hepes, pH 7.0, 100 mM KCl, 1 mM EGTA, 1 mM Ca^{2+} free Ca^{2+}) at 37^oC for 2 min. The purified annexin A2 tetramer was

added to initiate the fusion. Fluorescence was monitored at Ex = 560 nm and Em=590 nm. Fusion was expressed as a percentage of the maximal fluorescence (in the presence of 0.1% Triton X-100).

2.3.9 Surfactant secretion from type II cells

Isolated alveolar type II cells were pre-labeled with 0.6 μCi of [^{3}H] choline and grown in 35 mm^2 cell culture dishes. After overnight culture, unattached cells were removed by washing cells with warm MEM and then incubated in fresh media for 30 minutes. The cells were stimulated with 0.1 μM phorbol 12-myristate 13-acetate, 100 μM ATP and 20 μM terbutaline for 2 hrs. Surfactant secretion assay was done as previously described (6). For cholesterol depletion, cells were incubated with 3 mM MCD for 30 minutes before the stimulation.

2.3.10 Raft Clustering

CD44 cross-linking was done as described (20). In brief, freshly isolated type II cells were washed three times with MEM. The cells were equilibrated in MEM, supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$, at 37^{0}C for 30 minutes. For cross-linking, the cells were incubated with mouse anti-CD44 antibodies (20 μg/ml) for 60 minutes, followed by incubation with Alexa-488-conjugated anti-mouse antibodies (20 μg/ml) for another 60 minutes. The reaction was stopped by adding ice cold PBS. The cells were fixed with 4% paraformaldehyde and cytospun onto glass slides. The control type II cells were incubated with mouse anti-CD44 antibodies and then fixed, followed by the incubation with the secondary antibodies (see below). For cholesterol depletion, the cells were treated with 3 mM MCD for 30 minutes, washed three times with MEM, and cross-linked as described above.

For the double-labeling with CD44 and SNARE or annexin A2 antibodies, the slides above were washed with PBS and then permeabilised with 0.5% (v/v) Triton X-100 for 30 minutes. The fixed cells were incubated with 10% (w/v) FBS and 1% (w/v) BSA for blocking non-specific binding. The cells were incubated with rabbit anti-annexin A2, syntaxin 2, or SNAP-23 antibodies at 1:100, 1:50, and 1:100 dilutions, respectively at 4^0C overnight. The slides were washed and incubated with Alexa-488-conjugated anti-mouse antibodies (20 μg/ml, for control non-cross-linking cells only) and Alexa-546-conjugated anti-rabbit antibodies at 1:250 dilutions. The cells were examined with a Leica confocal laser scanning fluorescent microscope.

2.3.11 Protein Concentration Assay

Total protein concentration in cell lysates was determined by the Dc method (BioRad), and the protein concentration in each fraction of sucrose gradients was measured by the Bradford assay (BioRad).

2.3.12 MTT assay

Overnight grown type II cells (1.5×10^6) in 35 mm dishes were used for the assay. The cells were treated with different concentrations of MCD for 30 minutes at 37^0C. Next, cells were washed with 50 mM PBS and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) for 2 hours at 37^0C. Later, formazan crystals were dissolved in 2 ml of dimethyl sulfoxide by shaking the dishes for 10 minutes. Absorbance was measured at 570 nm. The results were expressed as a percentage of the untreated control cells.

2.3.13 Statistical analysis

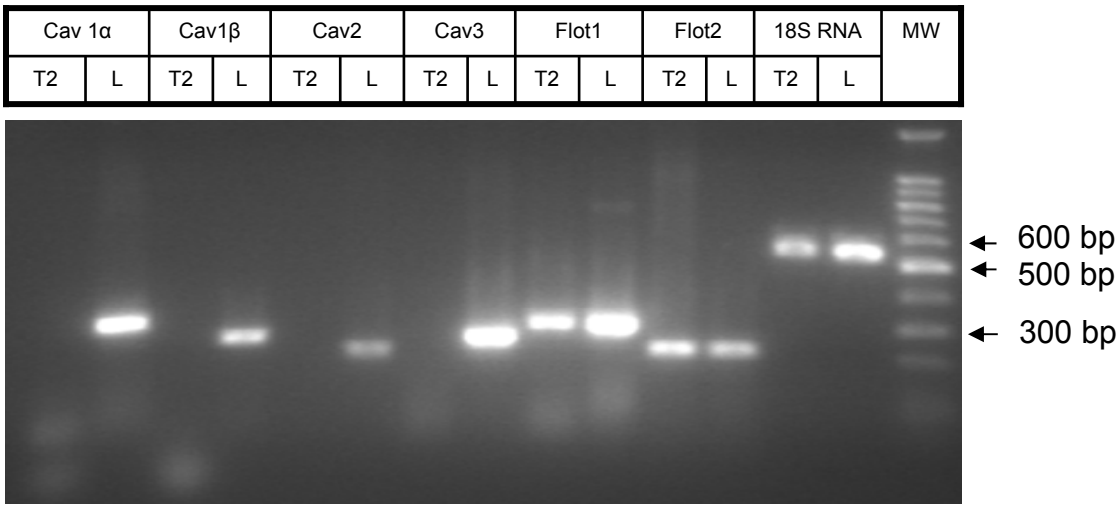
All the experiments were repeated atleast with 3 independent biological samples. Statistical significance was considered only when the $p \leq 0.05$. Student *t* test was used to compare the significant differences between the the control and treatment groups.

2.4 Results

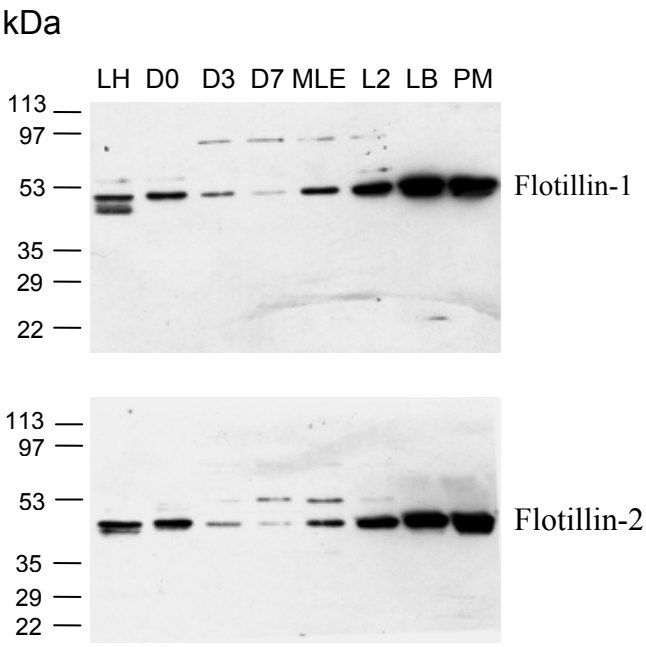
2.4.1 Identification of raft marker proteins

Previous studies have reported the presence of lipid raft marker proteins in lung tissue (21,22). However, it is unclear whether these markers exist in alveolar type II cells. RT-PCR was performed to assess the presence of mRNA of various lipid raft markers in these cells, including caveolin-1α, -1β, -2, and -3, and flotillin-1 and -2. Our results indicated that flotillin-1 and -2 mRNAs were expressed in both type II cells and lung tissue, whereas caveolin-1α, -1β, -2, and -3 mRNAs were only expressed in lung tissue, but not in type II cells (Fig. II. 1A). Western blotting revealed that flotillin-1 and -2 proteins were expressed in freshly isolated type II cells (Fig. II. 1B). However, flotillin-1 and -2 protein expression decreased when type II cells were cultured on plastic dishes, in which type II cells were known to trans-differentiate into the cells with some type I cell characteristics. Furthermore, both flotillin-1 and -2 were highly enriched in lamellar bodies fractions and plasma membrane fractions. They were also expressed in two lung epithelial cell lines: MLE-12 (mouse) and L2 (rat). Double-labeling with anti-SP-C (alveolar type II cell marker) and anti-flotillin-1 and -2 on rat lung tissue revealed that both flotillins were specifically localized in alveolar type II cells, but expression was minimal or low in type I cells (Fig. II. 1C). The controls without primary antibodies did not show labeling (data not shown).

A


Cav 1α
Cav1β
Cav2
Cav3
Flot1
Flot2
18S RNA
MW
T2
L
600 bp
500 bp
300 bp

B


kDa
LH D0 D3 D7 MLE L2 LB PM
113
97
53
35
29
22
Flotillin-1
Flotillin-2

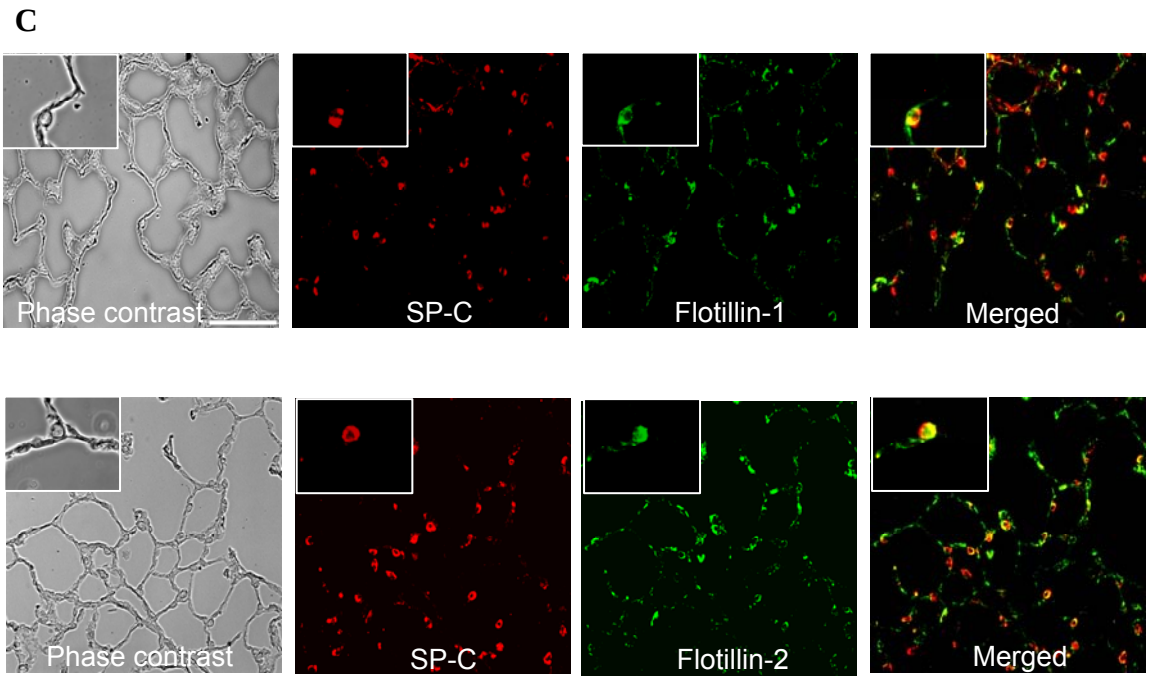


Fig. II. 1. Identification of lipid raft markers in alveolar type II cells. (A) RT-PCR: The mRNAs extracted from freshly isolated alveolar type II cells (> 96% purity, T2) and whole lung tissue (L) were reverse-transcribed and amplified for 35 cycles with gene specific primers against caveolin (Cav) 1α, 1β, 2 and 3, flotillin (Flot) 1 and 2, and 18S rRNA. PCR products were electrophoretically separated on an agarose gel. MW: 100 bp DNA ladder. (B) Western blot: Freshly isolated type II cells (D0), 3 or 7 day-cultured type II cells on plastic dishes (D3 and D7), lung epithelial cell lines, MLE-12 and L2 cells, along with lung homogenate (LH), purified plasma membrane (PM) and lamellar bodies (LB), were either lysed and/or solubilised in SDS sample buffer. Equal amounts of total protein (20 μg) were separated by SDS-PAGE and immunoblotted using anti-flotillin-1 and -2 antibodies. (C) Immunohistochemistry: Lung tissue sections were permeabilised and blocked before being incubated with goat-anti-SP-C and mouse anti-flotillin- 1 and -2 antibodies. They were then incubated with donkey anti-goat Alexa-546-conjugated and rabbit anti-mouse Alexa-488-conjugated antibodies. The slides were

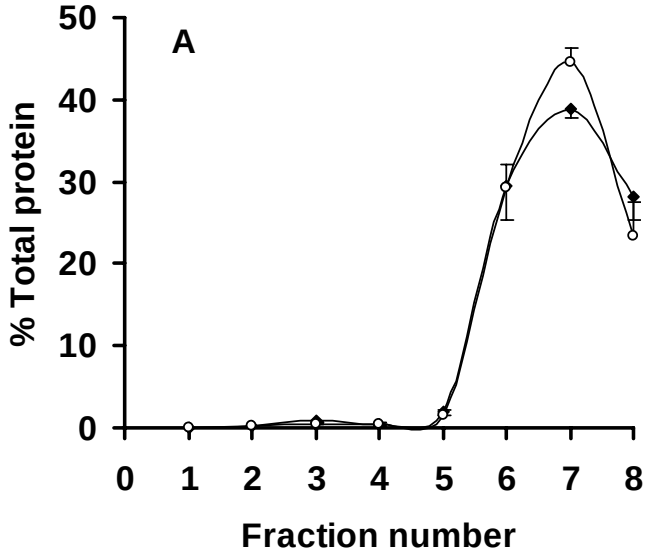
observed for immunoflorescence. Upper panels: double labeling with anti-flotillin-1 and SP-C antibodies; Lower panels: double labeling with anti-flotillin-2 and SP-C antibodies. Shown in the inset is the magnified image (100 ×) of a single type II cell. Scale bar: 20 μm.

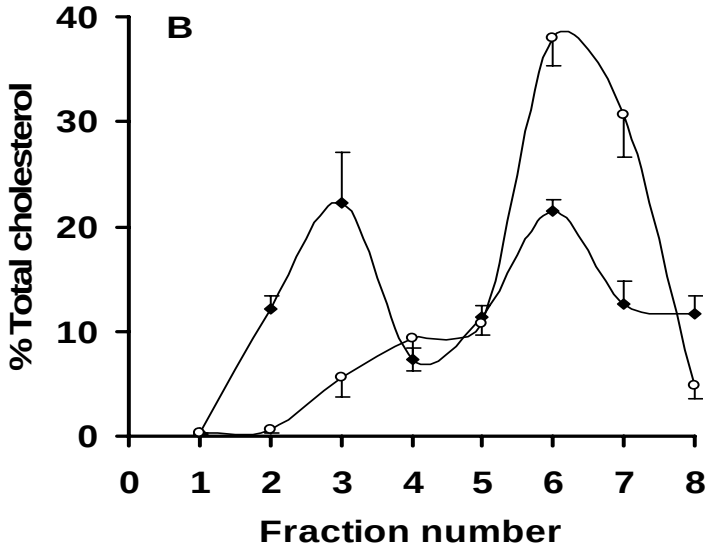
2.4.2 SNARE protein association with lipid rafts

Lipid rafts are characterized by their resistance to non-ionic detergent solubilization and flotation when subjected to a discontinuous sucrose gradient centrifugation. Additionally, they are highly enriched in cholesterol. We solubilized type II cells in ice-cold 1% (w/v) Triton X-100 and subjected the lysate to a gradient centrifugation. A white flocculent band was visible at the interface of 5 and 30% sucrose gradients. Seven fractions, collected from top to bottom (fractions 1 through 7), and the pellet (fraction 8), dissolved in lysis buffer, were analyzed for the contents of total proteins and cholesterol. Fraction 3 contained only 0.79 ± 0.15% of total protein, but 22.30 ± 4.76% of total cholesterol (Fig. II. 2A and B). In contrast, soluble fractions (fractions 6 and 7) contained significant amount of total proteins (29.44 ± 2.74 and 38.94 ± 1.79% respectively), but a relatively small amount of cholesterol (21.43 ± 1.1 and 12.56 ± 2.23% of total cholesterol, respectively). Each of the fractions was also screened for the presence of the lipid raft markers, flotillin-1 and –2, and the non-lipid raft marker, Na^+-K^+ ATPase. The results showed that fraction 3 was enriched in flotillin-1 and -2, whereas Na^+-K^+ ATPase was confined to the soluble fractions 6 and 7 (Fig. II. 2C). Thus, fraction 3 was confirmed to be the detergent insoluble and cholesterol-rich raft fraction. We further quantified the data by calculating the enrichment. A ratio of the flotillin band density and total protein in fraction 3 was first obtained. Similarly, a ratio of flotillin band densities in all the fractions and total protein in all the fractions was obtained. The first value was divided with the second to arrive at enrichment for flotillin. The data revealed that flotillin-1 and -2 were enriched by 42.66 ± 2.00 and 28.76 ± 9.09 fold (n=3) in the raft fraction. To study the effect of cholesterol sequestration on the lipid raft integrity, we

depleted cholesterol from type II cells using saponin (0.5% w/v) and 0.5% TX-100 in lysis buffer according to a reported procedure (12). In this case, cholesterol content in fraction 3 was reduced by approximately 75%. A major portion of flotillin-1 and -2 shifted from raft (fraction 3) to non-raft fraction (Fig. II. 2C).

To examine the association of SNARE proteins with the lipid rafts of type II cells, an equal volume of each fraction was immunoblotted for the presence of major SNARE isoforms in type II cells: SNAP-23, syntaxin 2, and VAMP-2. Both t-SNAREs were present in the rafts, but SNAP-23 was highly enriched (24.12 ± 4.80 fold, n=3) in lipid rafts in comparison with syntaxin 2 (11.80 ± 3.73 fold, n=3) (Fig. II. 2C). On the other hand, VAMP-2, a v-SNARE, was moderately enriched (17.73 ± 4.45 fold, n=3) in lipid rafts. Other two SNARE regulatory proteins: α-SNAP and NSF were not found in the raft fraction of type II cells (data not shown). When type II cells were solubilized in 0.5% saponin and 0.5% TX-100, the amount of SNARE proteins associated with the raft fraction was markedly decreased. The results indicated a genuine association of SNARE proteins with the lipid rafts.


A
% Total protein
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8
Fraction number


B
% Total cholesterol
40
30
20
10
0
0 1 2 3 4 5 6 7 8
Fraction number

C

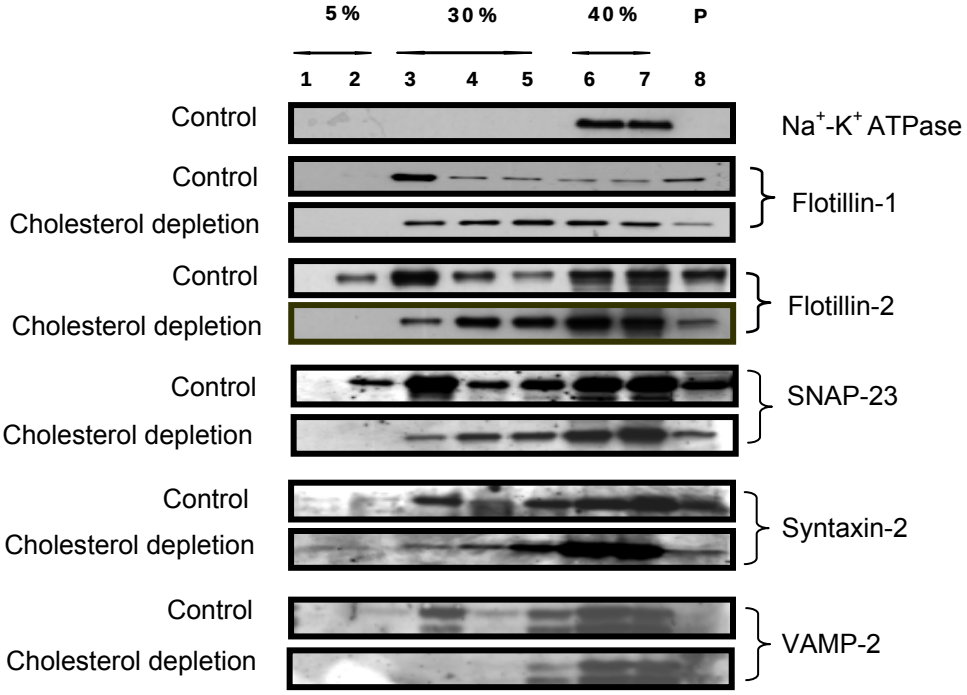


Fig. II. 2. Effect of cholesterol depletion on SNARE protein association with lipid rafts. (A, B) Isolation of lipid rafts from alveolar type II cells**:** Lipid rafts were isolated from freshly isolated type II cells based on their detergent insolubility in 1% (w/v) Triton X-100 flotation on sucrose gradients. Seven fractions (1-7) were collected from top to bottom and the pellet solubilized in lysis buffer (fraction 8). Protein (A) and cholesterol (B) were measured in each of the fractions before and after cholesterol depletion (0.5% Triton X-100 and 0.5%, w/v Saponin). The results were expressed as a percent of total protein and cholesterol, respectively. Data shown are means ± S.E. (n=3). Diamonds: 1% Triton X-100, circles: 0.5% Triton X-100 and 0.5% Saponin. (C) SNARE association with lipid rafts: Type II cells were lysed in either 1% Triton X-100 (control) or 0.5% Triton X-100 and 0.5% saponin (cholesterol depletion) and subjected to raft isolation. Seven fractions were collected from top to bottoms. P: pellet. 5%, 30% and 40%

represent sucrose gradient. Each fraction was examined, by western blot, for the presence of the lipid raft marker proteins, flotillin-1 and -2, the non-lipid raft protein marker, Na^+-K^+ ATPase, various SNARE proteins, SNAP-23, syntaxin 2 and VAMP-2. The immunublots shown are representatives of 3 independent experiments.

Table. II. 1. Distrubution of in fractions following differential centrifugation (per cent of total)

Fraction #	Flotillin-1	Flotillin-2	Syntaxin-2	SNAP-23	VAMP-2
1	3.42 ± 1.22	0.91 ± 0.33	1.90 ± 0.71	0.74 ± 0.39	6.29 ± 1.15
2	4.00 ± 0.91	4.33 ± 2.28	2.58 ± 0.80	5.86 ± 1.98	7.31 ± 1.41
3	32.87 ± 3.47	20.06 ± 2.06	9.10 ± 1.41	26.76 ± 2.76	14.13 ± 1.51
4	7.96 ± 1.74	7.21 ± 2.07	3.87 ± 0.80	10.51 ± 1.28	9.67 ± 1.46
5	6.95 ± 1.33	7.85 ± 1.45	14.83 ± 2.75	14.52 ± 0.80	15.06 ± 0.88
6	9.96 ± 2.37	22.06 ± 2.68	25.81 ± 2.87	19.35 ± 0.40	20.35 ± 1.11
7	11.96 ± 1.46	21.23 ± 2.02	27.27 ± 3.25	19.96 ± 0.81	17.56 ± 1.48
8	22.45 ± 4.95	16.33 ± 1.70	15.34 ± 2.86	11.21 ± 2.09	9.32 ± 0.85

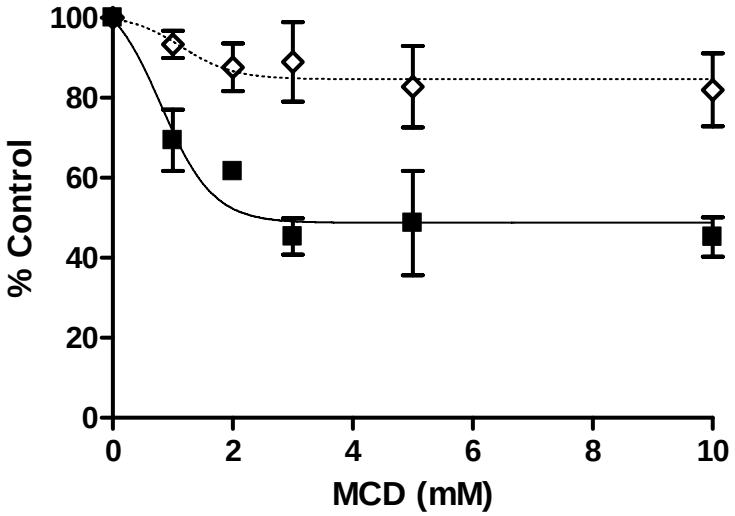
2.4.3 Effect of cholesterol depletion on fusion pore formation

Having confirmed the association of SNARE proteins with the lipid rafts, we then questioned whether it has functional relevance to surfactant exocytosis in type II cells. To maintain the integrity of cell membrane and viability of the cells, MCD was used to deplete membrane cholesterol from type II cells. Cyclodextrins have been reported to specifically deplete membrane cholesterol (23). Incubation of type II cells with MCD for 30 minutes at 37°C resulted in a dose-dependent decrease of cholesterol in type II cells (Fig. II. 3A). 3 mM MCD depleted 57 ± 6% of cholesterol without significantly affecting the cell viability (>90 %).

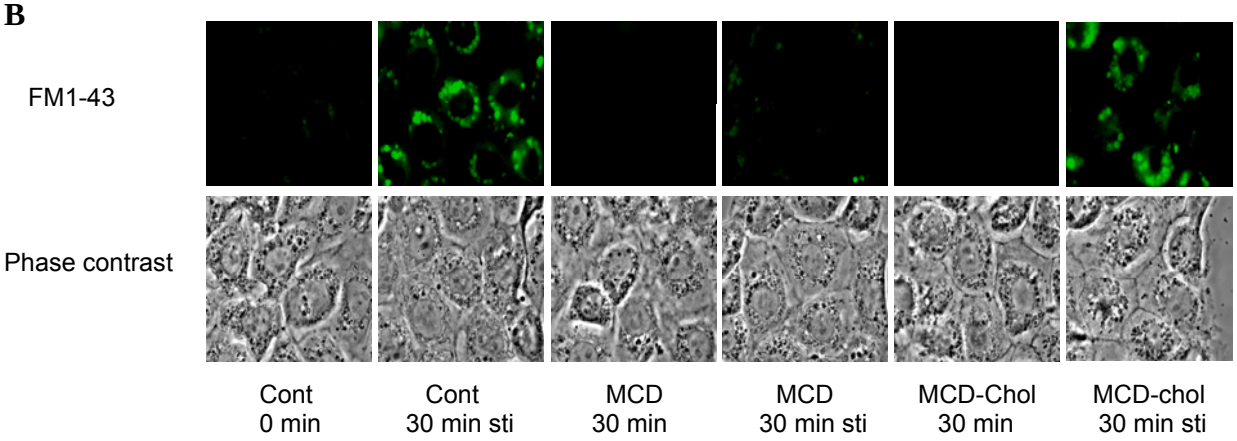
During exocytosis, surfactant laden lamellar bodies fuse with the plasma membrane and release their contents via the fusion pore. The dynamics of surfactant release can be monitored for the formation of fusion pore using an amphiphilic dye, FM 1-43 (19,24). The amphiphilic dye gains access to the lipids in the lamellar bodies through the fusion pore and stains the vesicular contents green. We sought to determine whether cholesterol depletion affects the fusion pore formation. Type II cells were pre-loaded for 10 minutes with FM1-43 (4 μM) and stimulated with a mixture of secretagogues. The formation of fusion pore was monitored by counting positively stained lamellar bodies. When the cells were cholesterol-depleted with 3 mM MCD, the fusion pore formation was completely inhibited. When the cells were treated with cholesterol-MCD complex, the fusion pore formation was unaffected (Fig. II. 3B, C). The effect of cholesterol depletion on the fusion pore formation is not due to the decrease in the number of lamellar bodies caused by MCD, because MCD does not affect the number of lamellar bodies as determined by staining type II cells with antibodies against a

lamellar body membrane protein, LB-180 (Fig. II. 3D). The number of positively stained lamellar bodies in the control and cholesterol-depleted cells was 25.92 ±1.42 and 24.56 ±1.37 per cell, respectively.

A


100
80
60
40
20
0
% Control
0
2
4
6
8
10
MCD (mM)

B


FM1-43
Phase contrast
Cont
0 min
Cont
30 min sti
MCD
30 min
MCD
30 min sti
MCD-Chol
30 min
MCD-chol
30 min sti

C. Quantification of fusion pore formation

Condition	Number of FM1-43 positive lamellar bodies/50 cells
0-time	0.67 ± 0.67
30 minute control	6.00 ± 6.00
30 minute stimulation	346 ± 17.35
MCD-0-time	0.00
MCD-30 minutes	0.00
MCD-30 minutes-stimulation	6.08 ± 6.00
MCD-Cholesterol-0-time	0.00
MCD-Cholesterol-30 minutes	0.00
MCD-Cholesterol-30 minutes-stimulation	362 ± 13.57

D

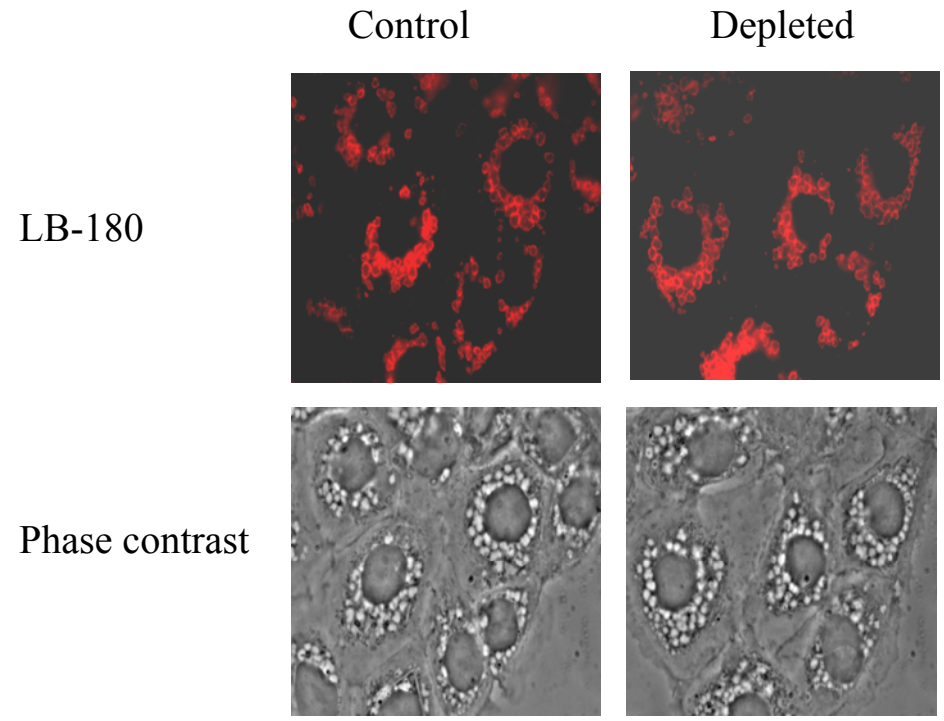
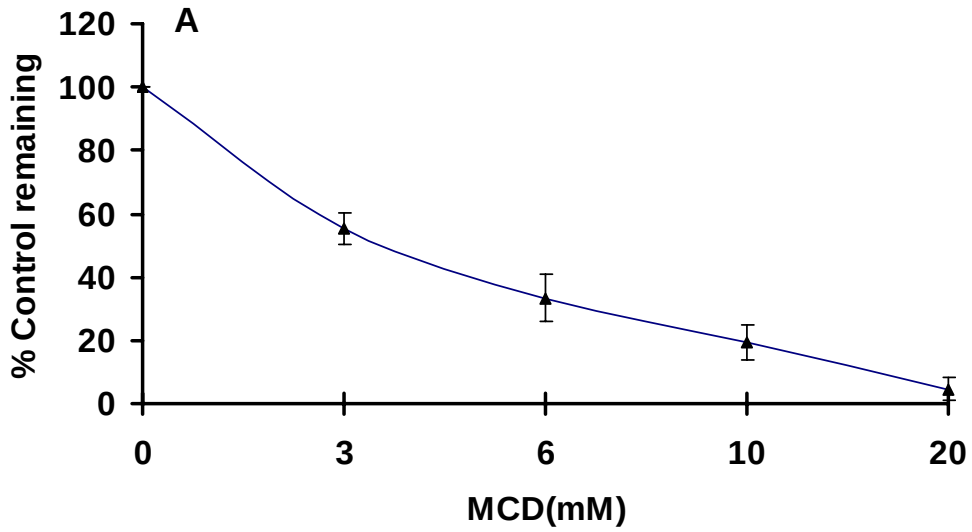
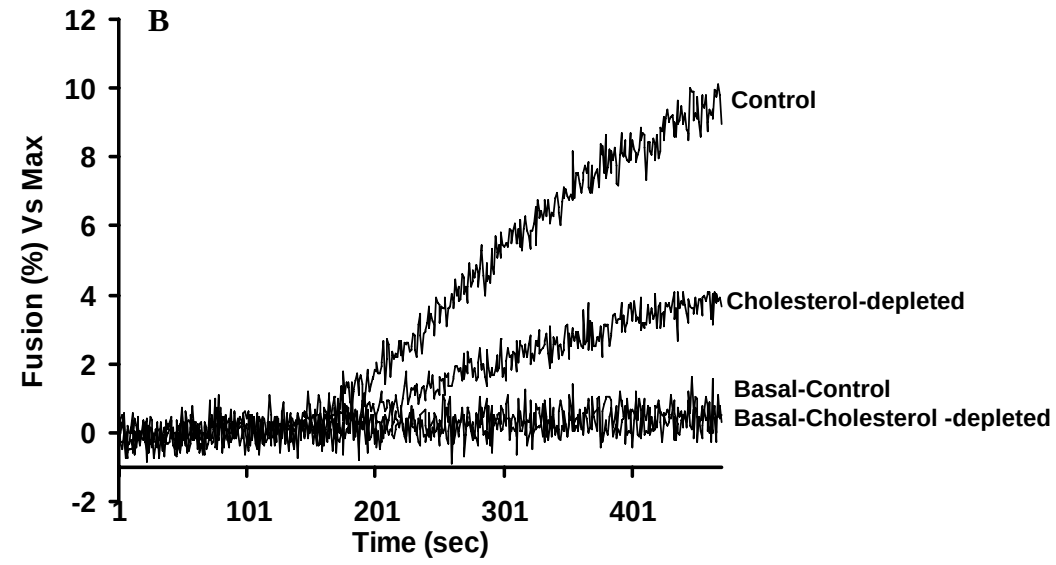


Fig. II. 3. Cholesterol depletion inhibits the fusion pore formation in type II cells. (A) Cholesterol concentration and viability: Overnight cultured type II cells were treated with different concentrations of methyl-β-cyclodextrin (MCD) and the total cholesterol concentration and cell viability were measured using the Amplex red cholesterol assay kit and MTT assay, respectively. The results were expressed as percentage of control. Data shown are means ± SE from 3 independent cell preparations. Square: cholesterol, Diamond: cell viability. (B) Fusion pore formation: The overnight cultured cells were treated with 3 mM MCD or MCD-cholesterol complex (MCD-chol) and stimulated (sti) with secretagogues (0.1 mM ATP, 0.1 μM PMA and 20 μM terbutaline) for 30 min. The fusion pore formation was monitored by FM1-43 fluorescence. Upper panel, FM1-43 staining; lower panel, phase contrast. (C) Quantification. The fusion pore formation was expressed as the number of the FM1-43-positive lamellar bodies per 50 cells. The results shown are means ± SE (n=3 independent cell preparations). (D) Effect of cholesterol depletion on the quality of lamellar bodies. Overnight cultured type II cells were treated with 3 mM MCD. The number of lamellar bodies was monitored by staining the cells with antibodies against a lamellar body membrane protein, LB-180.

2.4.4 Effect of cholesterol depletion on membrane fusion

We have previously shown that annexin A2 promotes the fusion of lamellar bodies with the plasma membrane using an *in vitro* assay (7). Using this assay, we determined whether the depletion of cholesterol affects the membrane fusion. The incubation of the isolated plasma membrane with MCD resulted in a dose-dependent reduction of cholesterol in the plasma membrane preparation (Fig. II. 4A). Three mM MCD depleted ~ 50% of cholesterol from the plasma membrane. Under this condition, annexin A2-mediated fusion of lamellar bodies with the cholesterol-depleted plasma membrane was significantly decreased (Fig. II. 4B and C). The controls without the addition of annexin A2 did not show fusion for the untreated or cholesterol-depleted plasma membrane.





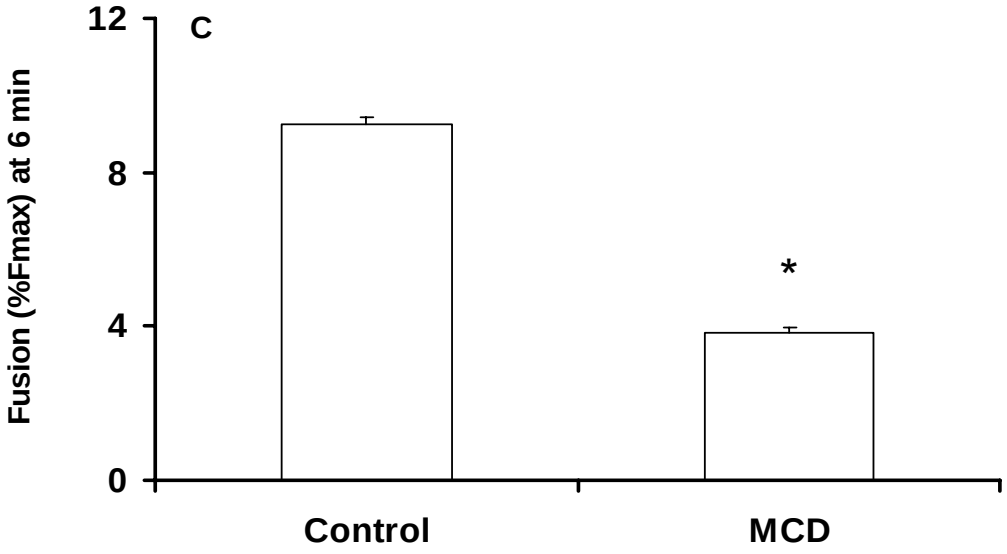


Fig. II. 4. Cholesterol depletion alters membrane fusion activity. (A) Plasma membranes isolated from lung tissue were treated with different concentrations of MCD for 30 min at 37^{o}C. Cholesterol content was assayed and the results expressed as a percentage of control. Data shown are means ± SE (n=3). (B, C) 5 µg/ml of control or

cholesterol-depleted plasma membranes (3 mM MCD) were mixed with 10 µg/ml of lamellar bodies in 1 ml of 1 mM Ca^{2+}-EGTA buffer and membrane fusion was initiated by addition of 10 µg annexin A2 tetramer. The basal fusion was without the addition of annexin A2 teramer. *B* illustrates fusion curve as a function of time and C the fusion content at 6 minutes after the addition of annexin A2 (means ± SE, n=3). *P<0.05 v.s control.

2.4.5 Effect of cholesterol depletion on surfactant secretion by type II cells

We also examined the effects of cholesterol depletion on surfactant secretion. As shown in Fig. II. 5, the secretion of lung surfactant was stimulated 4 fold by secretagogues. 3 mM MCD reduced the stimulated surfactant secretion by 69%. MCD also reduced the basal secretion, but did not reach a significant level.

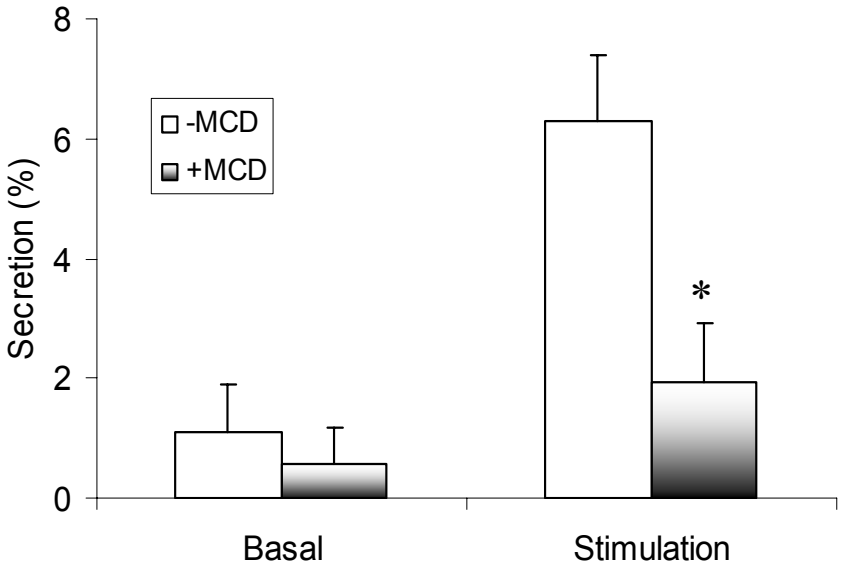
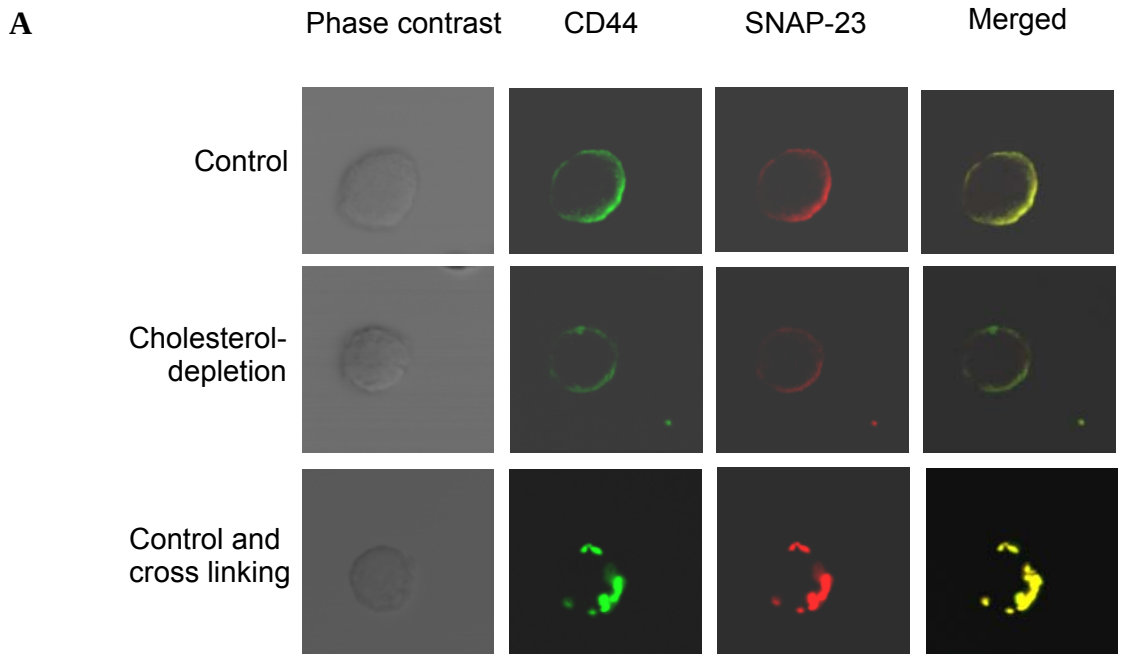


Fig. II. 5. Effect of cholesterol depletion on surfactant secretion. Overnight cultured type II cells were treated with 3 mM MCD for 30 min and then incubated without (basal) and with 0.1 mM ATP, 0.1 μM PMA, and 20 μM terbutaline for 2 hours (stimulated). Surfactant secretion was assayed. *P <0.05 vs no MCD treatment (Student *t* test, n=3) *White bars*, -MCD, *Shaded bars*, +MCD.

2.4.6 Co-localization of SNARE proteins with raft clusters

The characteristic feature of rafts is their clustering upon cross-linking the raft-associated proteins. The clusters, thus, aggregate the different protein associated with them and might represent the sites for protein interactions. CD44 is a transmembrane cell surface glycoprotein expressed by type II cells. We cross-linked CD44 proteins using anti-CD44 and secondary antibodies to determine whether SNARE proteins were recruited to rafts clusters. Both control and cross-linked cells were counterstained with anti-syntaxin 2 or SNAP-23. In the control cells, CD44, syntaxin 2, and SNAP-23 stained the plasma membrane, and cholesterol depletion did not change the distribution of these proteins (Fig. II. 6A and B). However, in the cross-linking cells, CD44 formed clusters or patches. Syntaxin 2 and SNAP-23 co-localized with the CD44 clusters, suggesting that syntaxin 2 and SNAP-23 may reside in the same lipid rafts. Efforts to further test this idea by disrupting rafts using 3 mM MCD were equivocal. Incomplete reduction in cluster formation and loss of CD44 florescence was observed owing to a 50-60% decrease in cholesterol content under our conditions. However, reasons for the loss of CD44 staining are unknown.


A
Phase contrast
CD44
SNAP-23
Merged
Control
Cholesterol-depletion
Control and cross linking

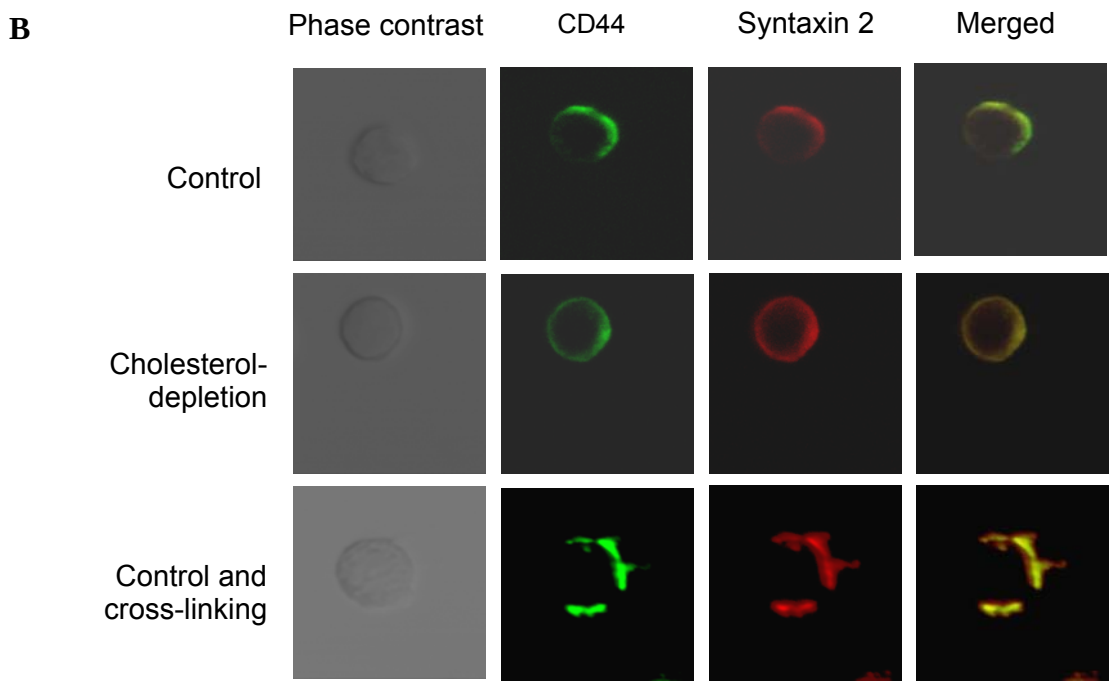
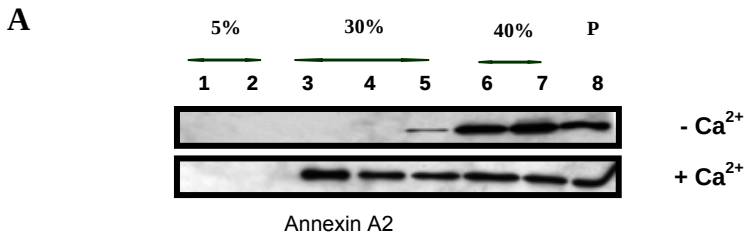


Fig. II. 6. SNARE proteins co-localize with raft clusters: Cells shown in the top two rows were fixed with 4% (w/v) paraformadehyde after exposure to mouse anti-CD44 but before exposure to anti-mouse IgG antibody. Cells shown in the bottom row were not fixed until after exposure to ant-mouse IgG antibody. Cells were double-labeled with anti-CD44 and anti-syntaxin 2 (A) or anti-SNAP-23 (B) antibodies. Images were taken with a confocal fluorescence microscope.

2.4.7 Annexin A2 and raft clustering

Our previous reports have indicated that annexin A2 plays an important role in surfactant secretion (6,7). We investigated whether annexin A2 is associated with the lipid rafts of type II cells. Since annexin A2 binds to phospholipids in a Ca^{2+}-dependent manner, we included 200 μM Ca^{2+} in the lysis buffer and subjected the type II cell lystate to the raft isolation. We found that annexin A2 was enriched in the raft fraction by 38-fold in the presence of Ca^{2+} (Fig. II. 7A). There was no association of annexin A2 with lipid raft in the absence of Ca^{2+}. We further cross-linked CD44 proteins to see whether annexin A2 was recruited to rafts clusters. In the control cells, annexin A2 stained the plasma membrane (Fig. II. 7B). In the cross-linking cells, a co-localization of annexin A2 with CD44 in the patches was evident, suggesting that annexin A2 was recruited to the raft clusters at the cytosolic side.



B

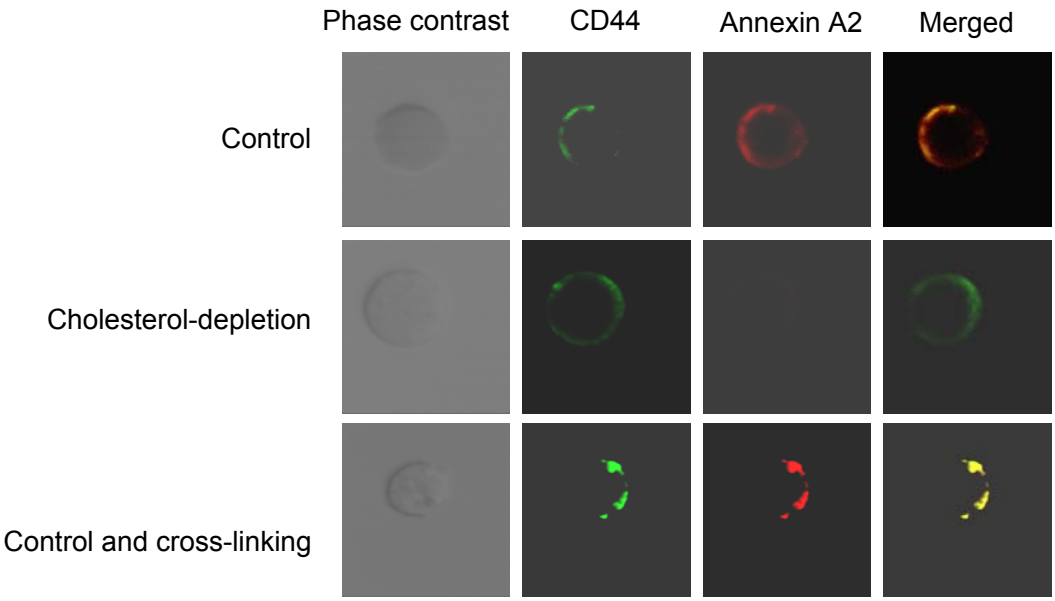

Phase contrast
CD44
Annexin A2
Merged
Control
Cholesterol-depletion
Control and cross-linking

Fig. II. 7. Annexin A2 co-localizes with the lipid raft clusters. (A) Association of annexin A2 with the lipid rafts: Freshly isolated type II cells were lysed with 1% Triton X-100 in the presence or absence of 200 μM Ca^{2+}, and separated on sucrose gradients. Seven fractions were collected from top to bottoms. P: pellet. 5%, 30% and 40% represent sucrose gradients. Each fraction was immunoblotted for annexin A2. (B) Raft clustering: CD44 clustering was done as described earlier. Cells were double-labeled with anti-CD44 and anti-annexin A2 antibodies. Images were taken with a confocal fluorescence microscope.

2.5 Discussion

Exocytosis is an orderly process involving the interaction of highly conserved and diverse classes of proteins, resulting in the fusion between two opposing membranes. Lung surfactant secretion by type II cells is a highly regulated process. SNAREs and annexin A2 have been implicated in surfactant secretion. Our present studies demonstrated that SNARE proteins were associated with the lipid rafts of type II cells and that lipid raft integrity was essential for this association as well as fusion pore formation, membrane fusion, and lung surfactant secretion. The cross-linking of a transmembrane glycoprotein, CD44, using antibodies caused raft clustering and the recruitment of SNAREs and annexin A2 into these raft clusters. These results suggested that lipid rafts may organize the exocytotic proteins on the membrane, and raft clusters may provide the docking and fusion sites for exocytosis.

Earlier studies have shown that caveolin and flotillins were highly expressed in lung tissue (21,22,25,26). Caveolin-1 protein was detected in both alveolar epithelial type I and type II cells, but distinct plasma membrane invaginations were not observed in type II cells (25-27). However, we were not able to detect caveolins 1-4 in the freshly isolated type II cells by RT-PCR although their mRNAs expression were observed in lung tissue under the same conditions. The reasons for the contrary results are not clear. Both mRNA and protein expression of flotillin-1 and –2 were present in type II cells. Immunohistochemistry confirmed their specific localization in type II cells, but not type I cells. Flotillin-1 and -2 protein expression in the type II cells cultured on plastic dishes was decreased. A similar expression pattern for flotillin was reported when PC12 cells were cultured *in vitro* (28). Expression pattern of flotillins and caveolins in type I and II

cells might reflect the specific roles of these proteins in different cells, although there are a number of cells expressing both caveolins and flotillins, such as skate erythrocytes (29) and neonatal rat cardiomyocytes (30). Additionally, flotillin-1 and -2 were enriched on the plasma membrane and lamellar bodies, indicating their possible roles in membrane trafficking.

Lipid rafts offer an interacting platform for many proteins involved in signal transduction and membrane trafficking, apart from being involved in protein sorting in a number of cells. Based on cellular fractionation and biochemical analysis, SNARE proteins were observed to be enriched in the lipid rafts of alveolar type II cells. This is also supported by the co-localization of SNAP-23 and syntaxin 2 with raft clusters upon cross-linking of a raft-associated cell surface protein CD44. The extent of enrichment for SNAP-23 was higher than its cognate t-SNARE partner, syntaxin 2, and v-SNARE protein, VAMP-2. Similar results were observed in other cells (12,14). The enrichment of SNAP-23 might be attributed to the palmitoylation at multiple cysteine residues. Most recently, it has been reported that SNAP-23 was enriched to a higher extent than SNAP-25 in PC12 cells, due to the substitution of phenyl alanine residue in SNAP-25 with a cysteine residue in SNAP-23 (31). However, the mechanism for the association of syntaxin 2/VAMP-2 with lipid rafts of type II cells is unknown. Most syntaxin isoforms were associated with lipid rafts to various degrees depending on cell type and isoform (12,32,33). However, one report showed that syntaxin 2 was excluded from the lipid rafts in mast cells (14). When reconstituted into simple artificial membrane (liposome) containing phospholipids, cholesterol and spingomyelin, syntaxin 1A and VAMP-2 preferred liquid-disordered domains (non-raft phase) (15), even though syntaxin 1A was

able to bind to cholesterol (13). Although the role of other lipid factors, such as phosphatidylinositol-4,5-bisphosphate in the targeting of syntaxin to lipid rafts can not be ruled out (34), it appeared that additional intrinsic factors and/or protein-protein interactions were required for the association of syntaxin and VAMP with lipid rafts in biological membranes. For example, SNAP-23 might bring syntaxin 2 into the rafts via the formation of a heterodimer since syntaxin and SNAP-23/25 were observed to form dimers, which may act as a docking site for VAMP-2, ultimately leading to the formation of a ternary SNARE complex (35). Also, the cleavage of SNAP-25 by botulinum neurotoxin E disrupted syntaxin 1-SNAP-25 dimer and its co-localization in defined clusters (36). There was a significant amount of SNAREs present in non-raft fraction. Since lipid rafts are dynamic structures, it is likely that SNAREs can move in and out of lipid rafts, depending on the cell status and the physiological response.

When type II cells were treated with MCD, cellular cholesterol content was reduced to ~50-60%. Under the depleted conditions, the secretagogue-stimulated surfactant secretion decreased by ~70%. Since MCD can not enter the cells (23), it might deplete cholesterol on the plasma membrane without affecting intracellular membrane cholesterol. Thus, the actual reduction of cholesterol on the plasma membrane may be higher than 50%, which may account for a higher inhibition of surfactant secretion. The reduced secretion was probably not due to the decreased surfactant synthesis owing to the inability of MCD to permeate the cells or due to the depletion of lamellar bodies because MCD does not affect the number of lamellar bodies. Since SNARE proteins are enriched in lipid rafts and the lipid raft integrity depends on cholesterol, the reduced surfactant secretion by MCD is likely due to the disruption of lipid raft integrity and thus

disassembly of interacting platforms, provided by lipid rafts that are necessary for the SNARE complex formation.

The size of lipid rafts is small (< 70 nm). Although cross-linking and fluorescence energy transfer (FRET) revealed their existence in living cells (37,38), lipid rafts are too small to function as docking and fusion sites. Individual rafts contain only a few numbers of proteins for protein-protein interactions to occur. However, lipid rafts might aggregate or form clusters and act as the possible sites of exocytosis. Indeed, we observed that syntaxin 2 and SNAP-23 were co-localized with CD44 clusters. The mechanisms of lipid raft cluster formation in the cells are however unclear. One of the candidates that might play a role in this process is annexin A2. It was associated with lipid rafts in type II cells (Fig. 7) and other cells (39) in a Ca^{2+}-dependent manner. The N-terminus of annexin A2 is required for the association (40). The Ca^{2+}-independent binding of annexin A2 to the membrane has also been reported (41), and this binding appears to depend on cholesterol (42,43). Similar to syntaxin 2 and SNAP-23, annexin A2 was recruited to raft clusters upon the cross-linking of CD44 (Fig. 7), suggesting role of annexin A2 in raft clustering and, thus, in the creation of docking and fusion sites in secretory cells. This conclusion is further supported by the following observations: (i) the infection of HeLa cells with enteropathogenic *E. Coli* induced actin-rich pedestals, clustering of raft clusters and recruitment of annexin A2 to such clusters (44); (ii) a trans-dominant mutant of annexin A2 comprising the N-terminal domain of annexin A2 and the N-terminus of p11 lead to the formation of large annexin A2 aggregates and co-clustering of the raft-associated protein, CD44 with the aggregates (20); and (iii) annexin A2 was found to be associated with nicotine-induced lipid rafts in chromaffin cells (45).

In summary, lipid rafts in type II cells may organize the SNARE protein machinery and their integrity is crucial for the fusion of lamellar bodies with the plasma membrane.

2.6 Acknowledgements

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2.7 References

1. Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* 1998;395:347-353.

2. Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE. SNAREpins: minimal machinery for membrane fusion. *Cell* 1998; 92:759-772.

3. Han X, Wang CT, Bai J, Chapman ER, Jackson MB. Transmembrane segments of syntaxin line the fusion pore of Ca2+-triggered exocytosis. *Science* 2004;304:289-292.

4. Abonyo BO, Wang P, Narasaraju TA, Rowan WH, III, McMillan DH, Zimmerman UJ, Liu L. Characterization of alpha-Soluble N-Ethylmaleimide-Sensitive Fusion Attachment Protein in Alveolar Type II Cells: Implications in Lung Surfactant Secretion. *Am.J.Respir.Cell Mol.Biol* 2003;29:273-282.

5. Abonyo BO, Gou D, Wang P, Narasaraju T, Wang Z, Liu L. Syntaxin 2 and SNAP-23 are required for regulated surfactant secretion. *Biochemistry* 2004;43:3499-3506.

6. Liu L, Wang M, Fisher AB, Zimmerman UJP. Involvement of annexin II in exocytosis of lamellar bodies from alveolar epithelial type II cells. *Am.J.Physiol* 1996;270:L668-L676.

7. Chattopadhyay S, Sun P, Wang P, Abonyo B, Cross NL, Liu L. Fusion of lamellar body with plasma membrane is driven by the dual action of annexin II tetramer and arachidonic acid. *J.Biol.Chem* 2003;278:39675-39683.

8. Gou D, Wang P, Jin N, Liu L. Silencing of annexin II in primary culture of alveolar epithelial type II cells. *Annexins* 2004;1:31-36.

9. Rajendran L, Simons K. Lipid rafts and membrane dynamics. *J.Cell Sci* 2005;118:1099-1102.

10. Brown DA, London E. Functions of lipid rafts in biological membranes. *Annu.Rev.Cell Dev.Biol* 1998;14:111-136.

11. Lafont F, Verkade P, Galli T, Wimmer C, Louvard D, Simons K. Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells. *Proc.Natl.Acad.Sci.U.S.A* 1999;96:3734-3738.

12. Chamberlain LH, Burgoyne RD, Gould GW. SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis. *Proc.Natl.Acad.Sci.U.S.A* 2001;98:5619-5624.

13. Lang T, Bruns D, Wenzel D, Riedel D, Holroyd P, Thiele C, Jahn R. SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. *EMBO J* 2001;20:2202-2213.

14. Pombo I, Rivera J, Blank U. Munc18-2/syntaxin3 complexes are spatially separated from syntaxin3-containing SNARE complexes. *FEBS Lett* 2003;550:144-148.

15. Bacia K, Schuette CG, Kahya N, Jahn R, Schwille P. SNAREs prefer liquid-disordered over "raft" (liquid-ordered) domains when reconstituted into giant unilamellar vesicles. *J.Biol.Chem* 2004;279:37951-37955.

16. Dobbs LG, Gonzalez R, Williams MC. An improved method for isolating type II cells in high yield and purity. *Am.Rev.Respir.Dis* 1986;134:141-145.

17. Chen JW, Chen Z, Narasaraju T, Jin N, Liu L. Isolation of hightly pure alveolar epithelial type I and type II cells from rat lungs. *Lab Invest* 2004;84:727-735.

18. Narasaraju TA, Jin N, Narendranath CR, Chen Z, Gou D, Liu L. Protein nitration in rat lungs during hyperoxia exposure: a possible role of myeloperoxidase. *Am.J.Physiol Lung Cell Mol.Physiol* 2003;285:L1037-L1045.

19. Bates SR, Tao JQ, Notarfrancesco K, DeBolt K, Shuman H, Fisher AB. Effect of Surfactant Protein-A on Granular Pneumocyte Surfactant Secretion in Vitro. *Am.J.Physiol Lung Cell Mol.Physiol* 2003;285:L1055-L1065.

20. Oliferenko S, Paiha K, Harder T, Gerke V, Schwarzler C, Schwarz H, Beug H, Gunthert U, Huber LA. Analysis of CD44-containing lipid rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton. *J.Cell Biol* 1999;146:843-854.

21. Nanjundan M, Possmayer F. Pulmonary lipid phosphate phosphohydrolase in plasma membrane signalling platforms. *Biochem.J* 2001;358:637-646.

22. Palestini P, Calvi C, Conforti E, Daffara R, Botto L, Miserocchi G. Compositional changes in lipid microdomains of air-blood barrier plasma membranes in pulmonary interstitial edema. *J.Appl.Physiol* 2003;95:1446-1452.

23. Neufeld EB, Cooney AM, Pitha J, Dawidowicz EA, Dwyer NK, Pentchev PG, Blanchette-Mackie EJ. Intracellular trafficking of cholesterol monitored with a cyclodextrin. *J.Biol.Chem* 1996;271:21604-21613.

24. Haller T, Ortmayr J, Friedrich F, Volkl H, Dietl P. Dynamics of surfactant release in alveolar type II cells. *Proc.Natl.Acad.Sci.U.S.A* 1998;95:1579-1584.

25. Campbell L, Hollins AJ, Al-Eid A, Newman GR, von Ruhland C, Gumbleton M. Caveolin-1 expression and caveolae biogenesis during cell transdifferentiation in lung alveolar epithelial primary cultures. *Biochem Biophys Res Commun* 1999;262:744-751.

26. Newman GR, Campbell L, von Ruhland C, Jasani B, Gumbleton M. Caveolin and its cellular and subcellular immunolocalisation in lung alveolar epithelium: implications for alveolar epithelial type I cell function. *Cell Tissue Res* 1999;295:111-120.

27. Kolleck I, Guthmann F, Ladhoff AM, Tandon NN, Schlame M, Rustow B. Cellular cholesterol stimulates acute uptake of palmitate by redistribution of fatty acid translocase in type II pneumocytes. *Biochemistry* 2002;41:6369-6375.

28. Volonte D, Galbiati F, Li S, Nishiyama K, Okamoto T, Lisanti MP. Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe. *J.Biol.Chem* 1999;274:12702-12709.

29. Musch MW, Koomoa DL, Goldstein L. Hypotonicity-induced exocytosis of the skate anion exchanger skAE1: role of lipid raft regions. *J.Biol.Chem* 2004;279:39447-39453.

30. Rybin VO, Grabham PW, Elouardighi H, Steinberg SF. Caveolae-associated proteins in cardiomyocytes: caveolin-2 expression and interactions with caveolin-3. *Am.J.Physiol Heart Circ.Physiol* 2003;285:H325-H332.

31. Salaun C, Gould GW, Chamberlain LH. The SNARE proteins SNAP-25 and SNAP-23 display different affinities for lipid rafts in PC12 cells. Regulation by distinct cysteine-rich domains. *J.Biol.Chem* 2005;280:1236-1240.

32. Gil C, Soler-Jover A, Blasi J, Aguilera J. Synaptic proteins and SNARE complexes are localized in lipid rafts from rat brain synaptosomes. *Biochem.Biophys.Res.Commun* 2005;329:117-124.

33. Xia F, Gao X, Kwan E, Lam PP, Chan L, Sy K, Sheu L, Wheeler MB, Gaisano HY, Tsushima RG. Disruption of pancreatic beta-cell lipid rafts modifies Kv2.1 channel gating and insulin exocytosis. *J.Biol.Chem* 2004;279:24685-24691.

34. Aoyagi K, Sugaya T, !Lost Data, Yamamoto S, Terakawa S, Takahashi M. The activation of exocytotic sites by the formation of phosphatidylinositol-4,5-bisphosphate microdomains at syntaxin clusters. *J Biol.Chem* 2005;280:17346-17352.

35. Hu K, Carroll J, Fedorovich S, Rickman C, Sukhodub A, Davletov B. Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. *Nature* 2002;415:646-650.

36. Rickman C, Meunier FA, Binz T, Davletov B. High affinity interaction of syntaxin and SNAP-25 on the plasma membrane is abolished by botulinum toxin E. *J Biol.Chem* 2004;279:644-651.

37. Friedrichson T, Kurzchalia TV. Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. *Nature* 1998;394:802-805.

38. Varma R, Mayor S. GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* 1998;394:798-801.

39. Babiychuk EB, Draeger A. Annexins in cell membrane dynamics. Ca(2+)-regulated association of lipid microdomains. *J.Cell Biol* 2000;150:1113-1124.

40. Babiychuk EB, Monastyrskaya K, Burkhard FC, Wray S, Draeger A. Modulating signaling events in smooth muscle: cleavage of annexin 2 abolishes its binding to lipid rafts. *FASEB J* 2002;16:1177-1184.

41. Liu L, Tao JQ, Zimmerman UJP. Annexin II binds to the membrane of A549 cells in a calcium-dependent and calcium-independent manner. *Cell.Signal* 1997;9:299-364.

42. Harder T, Kellner R, Parton RG, Gruenberg J. Specific release of membrane-bound annexin II and cortical cytoskeletal elements by sequestration of membrane cholesterol. *Mol.Biol.Cell* 1997;8:533-545.

43. Ayala-Sanmartin J, Henry JP, Pradel LA. Cholesterol regulates membrane binding and aggregation by annexin 2 at submicromolar Ca(2+) concentration. *Biochim.Biophys.Acta* 2001;1510:18-28.

44. Zobiack N, Rescher U, Laarmann S, Michgehl S, Schmidt MA, Gerke V. Cell-surface attachment of pedestal-forming enteropathogenic E. coli induces a clustering of raft components and a recruitment of annexin 2. *J.Cell Sci* 2002;115:91-98.

45. Chasserot-Golaz S, Vitale N, Umbrecht-Jenck E, Knight D, Gerke V, Bader MF. Annexin 2 Promotes the Formation of Lipid Microdomains Required for Calcium-regulated Exocytosis of Dense-Core Vesicles. *Mol.Biol.Cell* 2005;16:1108-1119.

CHAPTER III

KNOCKDOWN OF FLOTILLIN-2 INHIBITS LUNG SURFACTANT SECRETION BY ALVEOLAR TYPE II CELLS

3.1 Abstract

Lung surfactant secretion involves the fusion of lamellar bodies with the plasma membrane. Membrane rafts enriched with SNARE proteins are crucial for this process. We investigated the role of flotillins, membrane raft proteins, in this process by decreasing the expression of flotillin-1 and -2 by RNA interference. The reduction of flotillin-2 inhibited lung surfactant secretion, but it did not disrupt membrane rafts or the association of SNAREs with the rafts nor did it alter the cellular cholesterol level. We conclude that flotillin-2 is required for surfactant secretion but not via the association of SNAREs with the rafts or cholesterol homeostasis.

Keywords: flotillin-2; lung surfactant; alveolar type II cells; lung; membrane fusion; exocytosis

3.2 Introduction

Lung surfactant is stored in lamellar bodies and exocytosed following fusion of the lamellar bodies with the plasma membrane of alveolar type 2 (type II) cells [1]. However, the secretion of surfactant is a much slower process when compared to neurotransmitter release, indicating that additional control mechanisms are needed for surfactant secretion. A number of proteins have been shown to be involved in surfactant secretion including SNAREs, NSF, α-SNAP and annexin A2 [2-5]. In addition, SNAP-23, syntaxin-2 and VAMP-2 are enriched in type II cell membrane rafts. Cholesterol depletion has been shown to drastically reduce membrane fusion and surfactant secretion [6], indicating a role for membrane rafts in surfactant secretion.

Flotillins (or reggies) are evolutionarily conserved and ubiquitously expressed proteins in bacteria, yeast and eukaryotes. There are two isoforms, flotillin -1 (or reggie-2) and flotillin-2 (or reggie-1). They are expressed in many cell types including type II cells [6]. Flotillins associate with membrane rafts owing to multiple palmitoylation at Cys-34 in the case of flotillin-1 and additional myristoylation at Gly-2 in the case of flotillin-2. Flotillin-1, but not flotillin-2, is also located in the nuclei of PC-3 cells, a human prostate cancer cell line [7]. The distribution of these proteins indicates that flotillin-1 and flotillin-2 may differ in their cellular functions.

Several functions of flotillins have been reported including interaction with actin, kinases and the cytoskeleton; formation of filopodia; insulin signaling; axonal regeneration and cell-cell contacts and membrane trafficking [8-10]. Overexpression of flotillins results in defects in the imaginal discs in the eyes and wings of *Drosophila* [11]. Other evidence shows that flotillin overexpression leads to the emergence of filopodia in

various epithelial cell lines [12]. Flotillins are highly expressed during certain stages of zebrafish embryonic development, and knockdown of flotillins by morpholino antisense oligonucleotides has been shown to cause brain defects in zebrafish embryos [11-13].

The evidence for a role of flotillins in exocytosis is lacking except for a recent study which indicates the participation of flotillin-1 in mast cell exocytosis [14]. In this study, we investigated the functional role of flotillins in relation to surfactant secretion in type II cells.

3.3 Materials and Methods

3.3.1 Construction of adenoviral vectors:

The mouse U6 promoter was PCR-amplified with *Pfu* DNA polymerase from pSilencer 1.0 vector with upstream primer 1 (5'-CACCGCGGATCGATCCGACGCCGCCATCTCTA-3') and downstream primer 2 (5'-GCTTCGAAGAATTCCCGGGTCTCTCAAACAAGGCTTTTCTCCAA-3'). Five restriction sites (*Eco31*I, *Sma*I, *Eco*RI, *BstB*I and *Bgl*II) were introduced at the 3-end of the mU6 promoter. After the double digestion with *Sac*II and *Bgl*II restriction enzymes, the mU6 promoter was inserted into the pENTR/CMV-EGFP vector (Invitrogen, Carlsbad, CA) through corresponding sites as described earlier [15]. Later, the pENTR/mU6-CMV-EGFP vector was linearized with *Bsa1* and *BstB*I restriction enzymes to facilitate directional cloning of the annealed shRNAs oligonucleotides with 5`-TTTG and 5'-GC overhangs. We designed three siRNA sequences targeted to different regions of each gene using Web-based SiRNA Design Software (http://i.cs.hku.hk/~sirna/software/sirna.php). These siRNAs include 118-137, 522-540 and 584-603 of flotillin-1 mRNA which were designated as F-1 shRNA (A), (B) and (C), respectively. Similarly, shRNAs, 53-73, 602-620 and 1163-1182 of flotillin-2 mRNA were named F-2 shRNA (A), (B) and (C), respectively. The annealed oligonucleotides with 5`-TTTG and 5'-GC overhangs were composed of a sense strand containing 19-21 nucleotides, followed by a short spacer (5'-TTCAAGAGA-3'), an antisense strand and five thymidines. All the shRNA inserts subcloned into the pENTR/mU6-CMV-EGFP vector were verified by DNA sequencing. The shRNA and EGFP expression cassette in the pENTR vector were then switched into the adenoviral vector, pAd/PL-DEST, through

the Gateway technique, exactly as described by the vendor. The resulting adenoviral plasmids were linearized by *Pac*I, purified by Phenol-chloroform, and then transfected into 293A cells with Lipofectamine 2000 for adenovirus generation. Later the viruses were further amplified in 293A cells. The titers of adenovirus were determined through the EGFP signal in 293A cells after one day post-infection with serial dilutions of the virus.

3.3.2 Knockdown of flotillins in L2 cells

L2 cells, derived from rat lung epithelial cells, were used at 18-25 passages. The cells (1×10^5) were subcultured in 0.5 ml of F12K (Ham's modification) supplemented with 10% FBS, non-essential amino acids and penicillin and streptomycin in 12-well cell culture plates. The cells were infected with various doses of adenoviruses with multiplicity of infection (MOI) units from 0-150 on the day of subculture. The media was changed after day 1 and day 3 of culture. At day 5, the cells were washed with ice-cold 50 mM phosphate buffered saline (PBS) and lysed with lysis buffer [50 mM Tris (pH 7.4); 150 mM NaCl; 1% (v/v) Triton X-100 and protease inhibitor cocktail]. Protein concentrations were determined using the Dc method (BioRad, Hercules, CA). The lysates were used for immunoblotting.

3.3.3 Surfactant secretion

Type II cells were isolated as previously described [6]. The purity and viability of the cells were greater than 90% and 95%, respectively. Type II cells were cultured on collagen-matrigel matrix with air-liquid interface as reported earlier [16]. Surfactant secretion was assayed as previously described [4].

3.3.4 Knockdown of flotillins in type II cells:

The cells were cultured and infected (100 MOI) as described earlier [17]. Following infection on day 2, the cells were further cultured for 5 more days on an air-liquid interface. Cells were lysed at the end of 5 days of culture. Cell lysates were later probed for flotillin-1 and -2 to assess the level of knockdown. The blots were re-probed with anti-β-actin as a loading control.

3.3.4 Isolation of membrane rafts

The protocol for isolation of L2 cell membrane rafts was similar to that from type II cells as previously described [6]. Adenovirus (100 MOI) was added at the day of subculture, and culture continued for 5 days. Later the cells were solubilized in ice-cold lysis buffer (MBS with 1% Triton X-100 and protease inhibitor cocktail) for 45 min. Equal amounts of total protein from control and siRNA-treated cells were then subjected to raft isolation using a discontinuous sucrose gradient centrifugation. Fractions 1, 2, 3 and 4 were collected from top to bottom of the gradients. The membrane pellet (fraction 5) was re-suspended in lysis buffer. Membrane rafts (fraction 2) were enriched at the interface between the 5% and 30 % sucrose gradients. Equal volumes of fractions were used for detecting flotillin-1, -2 and SNAREs by Western blot.

3.3.5 Cholesterol content

Cholesterol content was determined using the Amplex Red Cholesterol assay kit (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer.

2.3.6 Immunoblotting

Immunoblotting was done exactly as described earlier [6]. Proteins were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes. The

membranes were blocked with 5% non-fat skim milk and then incubated overnight with either anti-flotillin-1, anti-syntaxin-2, anti-SNAP-23 or anti-β-actin at a 1:1000 dilution or with anti-flotillin-2 or anti-GAPDH at a 1:5000 dilution. After washing, the membranes were incubated with HRP-conjugated goat anti-rabbit or goat anti-mouse antibodies at a 1:2500 dilution. Later, the proteins were visualized using ECL reagents and X-film.

3.3.7 Cell Viability

Cell viability following adenovirus infection was ascertained by the MTT assay as described previously [6].

3.3.8 Statistical analysis

All the experiments were repeated atleast with 3 independent biological samples. Statistical significance was considered only when the $p \leq 0.05$. Student *t* test was used to compare the significant differences between the the control and treatment groups.

3.4 Results

3.4.1 Knockdown of flotillins in L2 cells

Adenoviral vectors containing shRNA sequences are being extensively used for knockdown of protein owing to their high infection efficiency in cells, particularly primary cells. The adenoviral vectors used in the present study contained shRNA sequences under the control of the mouse U6 promoter and EGFP expression under the control of the CMV promoter. The siRNA sequences were targeted to different regions of flotillin-1 and -2. The construct with irrelevant sequences served as a virus control (VC). The cells which were not infected with any virus were used as blank controls (BC). The use of VC and BC enabled us to monitor any virus effects.

L2 cells were used for screening the effective constructs. Our earlier studies have demonstrated the expression of flotillin-1 and -2 in L2 cells [6]. Our preliminary studies indicated that shRNA sequences targeted to flotillin-1 nucleotides 118-137 [F-1 shRNA (A)], but not to nucleotides 522-540 [F-1 shRNA (B)] or 584-603 [F-1 shRNA (C)] was effective in silencing the expression of flotillin-1 (data not shown). Therefore, this sequence was used for subsequent experiments. Infection efficiency was increased as the MOI of the virus increased as observed by the EGFP florescence (Fig. III. 1A). When the cells were infected with F-1 shRNA (A), a significant decrease in protein expression of flotillin-1 was observed. This effect was virus-dose dependent. A maximal depression of flotillin expression was seen at MOI $\geq$ 70 (Fig. III. 1B). The control virus had no effects on flotillin-1 expression. There was no statistically significant difference in cell viability in all the treatments (data not shown).

Similar analysis was performed for the flotillin-2 knockdown. Preliminary results indicated that the siRNA sequences targeted to 1163-1182 [F-2 shRNA (C)] of flotillin-2 were the most effective in silencing the flotillin-2 protein expression in comparison with 53-73 [F-2 shRNA (A)] and 602-620 [F-2 shRNA (B)] (data not shown). Once again, virus dose-dependent infection efficiency was observed (Fig. III. 1D). The F-2 shRNA (C) decreased flotillin-2 protein expression and the control virus had no effects (Fig. III. 1D). The F-2 shRNA (C) did not affect the cell viability (data not shown).

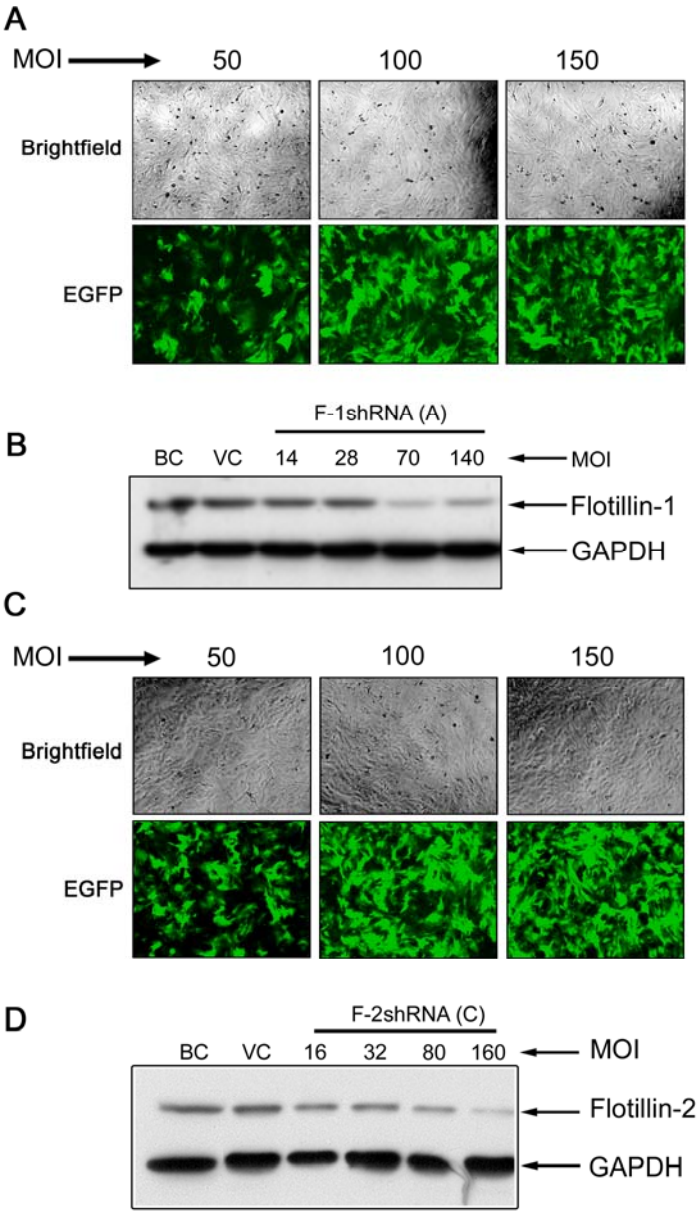

Figure III. 1. Knockdown of flotillin-1 and flotillin-2. L2 cells were infected with different doses (MOIs) of F-1 shRNA (A) adenovirus and cultured for 5 days. A) Infection efficiency. Shown are the representative EGFP fluorescence images, indicating the infection efficiency. B) Dose-dependent knockdown of flotillin-1. The flotillin-1 protein level was determined by Western blot. Equal loading was confirmed by re-

probing the membranes with anti-GAPDH antibodies. L2 cells were also infected with various doses MOIs of F-2 shRNA (C) adenovirus and cultured for 5 days. C) Infection efficiency. Shown are the representative EGFP florescence images. B) Dose-dependent knockdown of flotillin-2. The flotillin-2 protein level was determined by Western blot. BC: blank control, VC: virus control (MOI= 140).

3.4.2 Effect of flotillin silencing on surfactant secretion

The role of flotillins in surfactant secretion by type II cells was subsequently studied. To this end, type II cells were cultured on an air-liquid model and infected with adenovirus containing F-1 shRNAs and F-2 shRNAs. F-1 shRNA (A) almost completely silenced flotillin-1 expression (Fig. III. 2A). However, F-1 shRNA (A) also decreased flotillin-2 expression although a significant amount of flotillin-2 remained in the F-1 shRNA (A)-treated type II cells (Fig. III. 2B). Among F-2 shRNAs, F-2 shRNA (C) was the most effective in silencing flotillin-2 expression. This shRNA also reduced flotillin-1 expression (Fig. III. 2A and B), probably because the stability of flotillin-1 is dependent on flotillin-1 [11, 18]. Using F-1 shRNA (A) and F-2 shRNA (C), we determined the effect of flotillin-1 and -2 silencing on surfactant secretion. There were no statistically significant differences in the basal and stimulated secretions between control and F-1 shRNA (A) - treated cells (Fig. III. 2C). However, F-2 shRNA (C) reduced stimulated surfactant secretion. The basal secretion appears to be decreased in the F-2 shRNA (C)-treated cells but did not reach a significant level. The control virus had no effect on surfactant secretion.

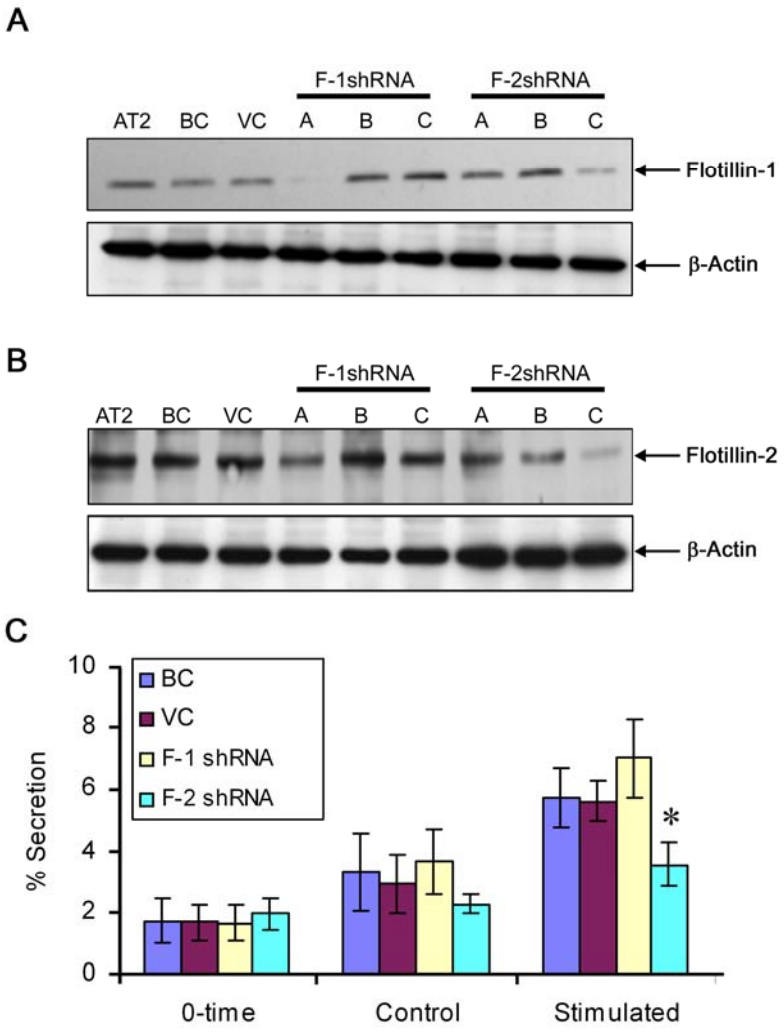

Figure III. 2. Effect of flotillin silencing on surfactant secretion. Type II cells were infected with different shRNA adenovirus at an equal dose of 100 MOI and cultured for 5 days. Equal amounts of proteins were immunoblotted for *A)* flotillin-1 and *B)* flotillin-2. The same membranes were re-probed for β-actin to confirm equal loading. *C)* Surfactant secretion. The control and flotillin knockdown cells were washed and equilibrated 30 min in serum-free medium (0-time). The cells were incubated 2.5 h in the absence (control) or presence of 100 μM ATP, 0.1 μM PMA and 10 μM terbutaline (stimulated). The results were expressed as means ± S.E. **P* < 0.05 versus VC (Student *t* test n ≥ 3). BC: blank control, VC: virus control (MOI= 140).

3.4.3 Effect of flotillin silencing on raft formation

Earlier studies speculated a role of flotillins in the formation of membrane rafts [19]. We examined whether the loss of flotillin affects raft formation in lung cells. Because large amounts of cells were needed for this experiment, we used L2 cells rather than type II cells for this experiment. The control and flotillin knockdown L2 cells were subjected to raft isolation. Following raft isolation, various fractions were collected. Fraction 2 represents the raft fraction (the interface between 5% and 30% sucrose). Each fraction was analyzed for protein and cholesterol content. When compared with blank and virus controls, there were no obvious differences in the amounts of protein and cholesterol of the raft fractions between control and flotillin-1 or -2 knockdown cells (Fig. III. 3A and B). When cholesterol concentration was expressed as μg per μg protein, cholesterol content in the raft fraction (fraction 2) was enriched 3-, 10-, and 20- fold in comparison with fractions 3, 4, and 5 (data not shown).

3.3.4 Effect of flotillin silencing on SNARE protein association with membrane rafts

Membrane rafts have been implicated in exocytotic processes. SNARE proteins were differentially enriched in rafts in a number of cell types including type II cells [6, 20]. We therefore examined whether knockdown of flotillin leads to dissociation of SNAREs with membrane rafts. Following silencing, little flotillin-1 and -2 proteins were detected in any of the fractions, indicating an effective silencing. When raft fractions (fraction 2) were probed for SNARE proteins following silencing, there was no change in the association of these proteins with membrane rafts (Fig. III. 3C and D). The results indicate that flotillin-1 and -2 were not involved in the association of syntaxin-2 and SNAP-23 in L2 cells.

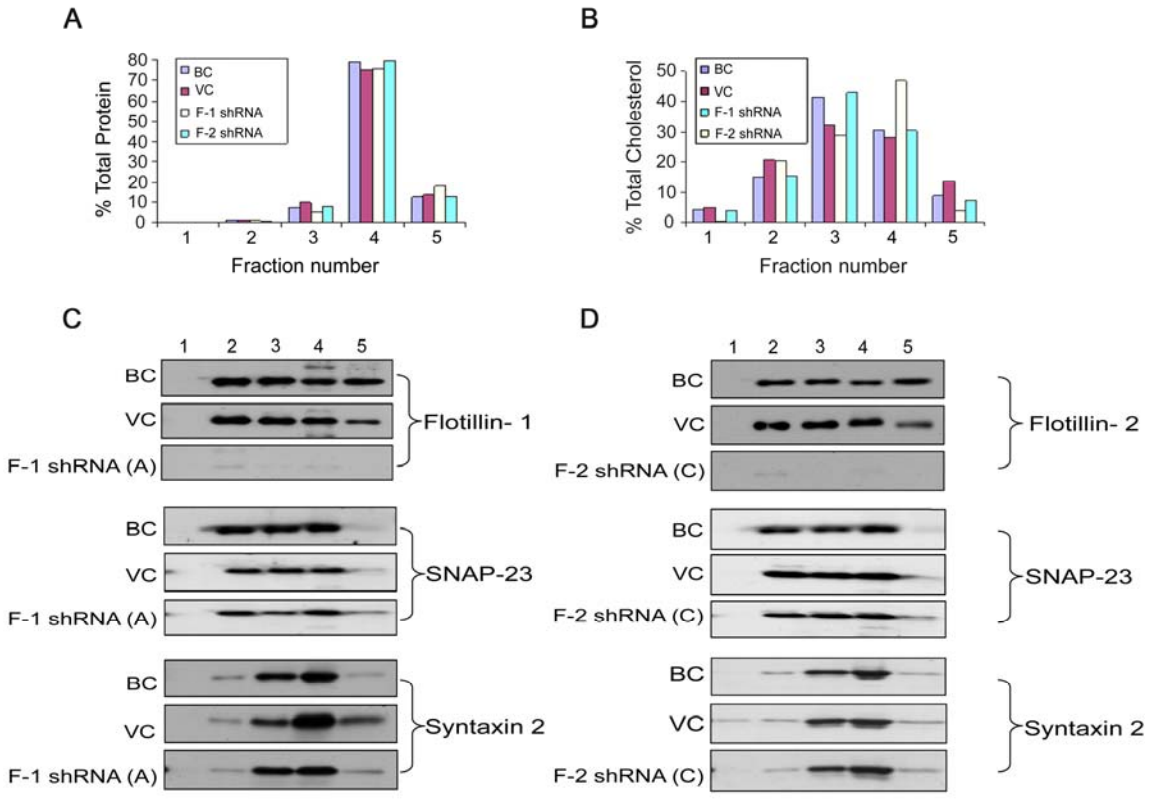

Figure III. 3. Effect of flotillin silencing on membrane raft formation. *A and B) Total protein content and total cholesterol content.* Following flotillin-1 and -2 knock down with F-1shRNA (A) or F-2 shRNA (C) adenovirus (100 MOI), L2 cell lysates were subjected to raft isolation. Total protein *A)* and cholesterol content *B)* in each fraction were measured and expressed as a percentage of total protein or cholesterol in all fractions. Following knockdown of flotillin-1 *C)* or -2 *D)* with F-1shRNA (A) or F-2 shRNA (C) adenovirus (100 MOI), membrane rafts were isolated. Equal volumes of various fractions were probed for flotillin-1 or flotillin-2, SNAP-23 and syntaxin 2. BC: blank control, VC: virus control (MOI= 140). Lanes 1-5 represent the respective fractions.

3.4.5 Effect of flotillin silencing on cholesterol content

Membrane rafts are also reported to be involved in cholesterol homeostasis. We further investigated the effects of flotillin-1 and -2 silencing on cholesterol levels in L2 cells. Following silencing of flotillin-1 and -2, cell lysates were analyzed for total cholesterol levels. Our results indicated that there were no statistical significant differences in cholesterol levels under silencing conditions when compared with controls (Fig. III. 4).

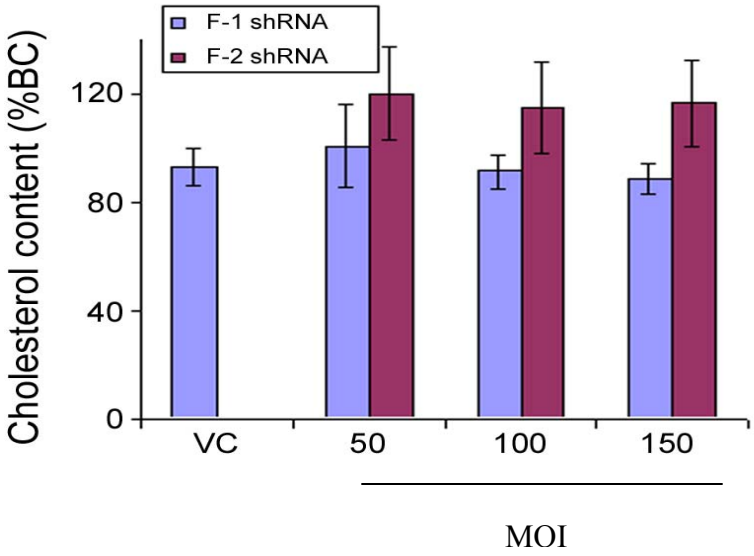

Figure 4: Effect of flotillin silencing on cholesterol content. L2 cells were infected with F-1shRNA (A) or F-2 shRNA (C) adenovirus (100 MOI). Cholesterol levels were assayed and expressed as a percentage of BC. *Blue bars*, F-1 shRNA (A); *Purple bars*, F-2 shRNA (C). Shown are means ± S.E. ($n \geq 3$). BC: blank control. VC: virus control (MOI= 140).

3.5 Discussion

Flotillins are membrane-bound raft marker proteins involved in a number of functions including membrane trafficking. The present study investigated the role of these proteins in surfactant secretion. Our results indicated that silencing of one of the isoforms, flotillin-2, decreased surfactant secretion.

Our results indicated that the flotillin-2 knockdown led to reduced expression of flotillin-1. Previous studies have demonstrated that the stability of flotillin-1 protein depends on the presence of flotillin-2 as the former undergoes proteosomal degradation in the absence of the latter [11, 18]. Similar results have been reported for other SPFH family proteins such as prohibitins, HflK and HflC [21, 22]. In the caveolin-1 knockout mice, the caveolin-2 level is decreased [23].

Flotillin-2, but not flotillin-1 knockdown, resulted in a reduced surfactant secretion. Similar to our results, flotillin-1 knockdown did not affect the mast cell degranulation following simulation with phorbol esters or the calcium ionophore, A23187. However, a reduction of flotillin-1 level decreased degranulation induced by FcεR cross-linking, indicating its role in IgE-mediated signaling [14].

Surfactant secretion by type II cells is a SNARE protein-dependent process and SNAREs are associated with membrane rafts [2, 3, 5, 6]. We hypothesized that flotillins may play a role in the SNARE association with membrane rafts. However, our results indicated that there were no changes in the pattern of SNARE association with membrane rafts in the absence of flotillin-1 or -2. Thus, SNARE protein association with rafts appears to be independent of flotillins. Previous studies reported that flotillin-2 did not

interfere with the targeting of Gαq, Cbp and Lyn kinase proteins to the membrane rafts, but regulated their activities [14, 24].

In the present study, we found that the formation of membrane rafts was not altered by the knockdown of flotillins as indicated by total cholesterol content and the amount of total protein in the raft fractions. This is consistent with the finding in RBL-2H3 cells [14]. The loss of caveolin-1 disrupts the distinct plasma membrane invaginations, but does not affect the raft formation [23]. The detergent-resistant membrane rafts isolated by sucrose gradient centrifugation are extremely diverse and heterogeneous and are composed of several rafts with distinct raft markers [25, 26]. It is possible that the changes in a particular type of raft on lamellar bodies or plasma membrane due to the loss of flotillins can not be detected by the chosen biochemical analyses.

In 3T3-L1 cells, flotillin-1 co-localized with LAMP-1 positive vesicles and in macrophages, thus implicating the protein in phagosome maturation [27-29]. Moreover, flotillin-1 was shown to interact with ABCA1, the main protein involved in cholesterol transport in human macrophages [30]. ABCA1 deficiency in mice resulted in altered surfactant metabolism [31]. Our previous study indicated an abundance of flotillins on lamellar bodies [6]. These findings prompted us to investigate cholesterol homeostasis following flotillin-1 and -2 knock down. However, we did not find any changes in total cellular cholesterol after flotillin-1 or -2 silencing.

In summary, flotillin-2 is required for surfactant secretion independent of SNARE association of membrane rafts. The mechanisms for flotillin-2-mediated surfactant secretion require further investigation.

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Contribution of co-cuthors: Dr. Deming Gou designed the adenoviral vectors.

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3.6 References

1. Dietl P, Haller T. Exocytosis of lung surfactant: from the secretory vesicle to the air-liquid interface. *Annu Rev Physiol* 2005;67:595-621.

2. Abonyo BO, Wang P, Narasaraju TA, Rowan WH 3rd, McMillan DH, Zimmerman UJ, Liu L. Characterization of alpha-soluble N-ethylmaleimide-sensitive fusion attachment protein in alveolar type II cells: implications in lung surfactant secretion. *Am J Respir Cell Mol Biol* 2003;29:273-82.

3. Abonyo BO, Gou D, Wang P, Narasaraju T, Wang Z, Liu L. Syntaxin 2 and SNAP-23 are required for regulated surfactant secretion. *Biochemistry*. 2004;43: 3499-506.

4. Gou D, Wang P, Jin N, Liu L. Silencing of annexin II in primary culture of alveolar epithelial type II cells. *Annexins* 2004;1:31-36.

5. Wang P, Chintagari NR, Gou D, Su L, Liu L. Physical and functional interactions of SNAP-23 with annexin A2. *Am J Respir Cell Mol Biol* 2007;37:467-76.

6. Chintagari NR, Jin N, Wang P, Narasaraju TA, Chen J, Liu L. Effect of cholesterol depletion on exocytosis of alveolar type II cells. *Am J Respir Cell Mol Biol* 2006;34:677-87.

7. Santamaria A, Castellanos E, Gomez V, Benedit P, Renau-Piqueras J, Morote J, Reventos J, Thomson TM, Paciucci R. PTOV1 enables the nuclear translocation and mitogenic activity of flotillin-1, a major protein of lipid rafts. *Mol Cell Biol* 2005;25:1900-11.

8. Stuermer CA, Langhorst MF, Wiechers MF, Legler DF, Von Hanwehr SH, Guse AH, Plattner H. PrPc capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. *FASEB J* 2004; 18:1731-3.

9. Glebov OO, Bright NA, Nichols BJ. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol* 2006;8:46-54.

10. Babuke T, Tikkanen R. Dissecting the molecular function of reggie/flotillin proteins. *Eur J Cell Biol* 2007;86:525-32.

11. Hoehne M, de Couet HG, Stuermer CA, Fischbach KF. Loss- and gain-of-function analysis of the lipid raft proteins Reggie/Flotillin in Drosophila: they are posttranslationally regulated, and misexpression interferes with wing and eye development. *Mol Cell Neurosci* 2005;30:326-38.

12. Neumann-Giesen C, Falkenbach B, Beicht P, Claasen S, Luers G, Stuermer CA, Herzog V, Tikkanen R. Membrane and raft association of reggie-1/flotillin-2: role

of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. *Biochem J* 2004;378:509-18.

13. von Philipsborn AC, Ferrer-Vaquer A, Rivera-Milla E, Stuermer CA, Malaga-Trillo E. Restricted expression of reggie genes and proteins during early zebrafish development. *J Comp Neurol* 2005;482:257-72.

14. Kato N, Nakanishi M, Hirashima N. Flotillin-1 regulates IgE receptor-mediated signaling in rat basophilic leukemia (RBL-2H3) cells. *J Immunol* 2006;177:147-54.

15. Gou D, Narasaraju T, Chintagari NR, Jin N, Wang P, Liu L. Gene silencing in alveolar type II cells using cell-specific promoter in vitro and in vivo. *Nucleic Acids Res* 2004;32:e134.

16. Mason RJ, Lewis MC, Edeen KE, McCormick-Shannon K, Nielsen LD, Shannon JM. Maintenance of surfactant protein A and D secretion by rat alveolar type II cells in vitro. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L249-58.

17. Jin N, Kolliputi N, Gou D, Weng T, Liu L. A novel function of ionotropic gamma-aminobutyric acid receptors involving alveolar fluid homeostasis. *J Biol Chem* 2006;281:36012-20.

18. Solis GP, Hoegg M, Munderloh C, Schrock Y, Malaga-Trillo E, Rivera-Milla E, Stuermer CA. Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. *Biochem J* 2007;403:313-22.

19. Evans WE 4th, Coyer RL, Sandusky MF, Van Fleet MJ, Moore JG, Nyquist SE. Characterization of membrane rafts isolated from rat sertoli cell cultures: caveolin and flotillin-1 content. J Androl 2003;24:812-21.

20. Chamberlain LH, Gould GW. The vesicle- and target-SNARE proteins that mediate Glut4 vesicle fusion are localized in detergent-insoluble lipid rafts present on distinct intracellular membranes. *J Biol Chem* 2002;277:49750-4.

21. Berger KH, Yaffe MP. Prohibitin family members interact genetically with mitochondrial inheritance components in Saccharomyces cerevisiae. *Mol Cell Biol* 1998;18:4043-52.

22. Nijtmans LG, Artal SM, Grivell LA, Coates PJ. The mitochondrial PHB complex: roles in mitochondrial respiratory complex assembly, ageing and degenerative disease. *Cell Mol Life Sci* 2002;59:143-55.

23. Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC, Schedl A, Haller H, Kurzchalia TV. Loss of

caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* 2001;293:2449-52.

24. Sugawara Y, Nishii H, Takahashi T, Yamauchi J, Mizuno N, Tago K, Itoh H. The lipid raft proteins flotillins/reggies interact with Galphaq and are involved in Gq-mediated p38 mitogen-activated protein kinase activation through tyrosine kinase. *Cell Signal* 2007;19:1301-8.

25. George S, Nelson MD, Dollahon N, Bamezai A. A novel approach to examining compositional heterogeneity of detergent-resistant lipid rafts. *Immunol Cell Biol* 2006;84:192-202.

26. Pike LJ. Lipid rafts: heterogeneity on the high seas. *Biochem J* 2004;378:281-92.

27. Dermine JF, Duclos S, Garin J, St-Louis F, Rea S, Parton RG, Desjardins M. Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. *J Biol Chem* 2001;276:18507-12.

28. Huynh KK, Eskelinen EL, Scott CC, Malevanets A, Saftig P, Grinstein S. LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO J* 2007; 26:313-24.

29. Liu J, Deyoung SM, Zhang M, Dold LH, Saltiel AR. The stomatin/prohibitin/flotillin/HflK/C domain of flotillin-1 contains distinct sequences that direct plasma membrane localization and protein interactions in 3T3-L1 adipocytes. *J Biol Chem* 2005;280:16125-34.

30. Bared SM, Buechler C, Boettcher A, Dayoub R, Sigruener A, Grandl M, Rudolph C, Dada A, Schmitz G. Association of ABCA1 with syntaxin 13 and flotillin-1 and enhanced phagocytosis in tangier cells. *Mol Biol Cell* 2004;15:5399-407.

31. Bates SR, Tao JQ, Collins HL, Francone OL, Rothblat GH. Pulmonary abnormalities due to ABCA1 deficiency in mice. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L980-9.

CHAPTER IV

VACUOLAR ATPases: POTENTIAL ROLES IN LUNG SURFACTANT SECRETION BY ALVEOLAR TYPE II CELLS

4. 1 Abstract

Lipid rafts organize protein and lipid machinery involved in many biological functions. The proteomic profile of alveolar type II cell lipid rafts revealed a small, but diverse group of proteins involved in the regulation of pH, cell cycle and proliferation, metabolism, oxygen transport, and exocytosis. Vacuolar ATPases (V-ATPase) dominated lipid raft proteome and was examined for its role in surfactant secretion. The *a1* and B subunits were enriched in lipid rafts and lamellar bodies. Bafilomycin A1 (Baf A1), an inhibitor of V-ATPase dissipated the lamellar body pH gradient as indicated by the loss of quinacrine accumulation. V-ATPase inhibition increased surfactant secretion. Baf A1-stimulated secretion was effectively blocked by intracellular Ca^{2+}-chelator, BAPTA-AM. This increase in secretion was inhibited by staurosporine and KN-62, the inhibitors of protein kinase C and calcium/calmodulin-dependent kinase II, respectively. Moreover, thapsigargin reduced the Baf A1-induced surfactant secretion, indicating the role of endoplasmic reticulum Ca^{2+} pool in Baf A1-mediated secretion. Further, the stimulation of alveolar type II cells with lung surfactant secretagogues increased pH in lamellar bodies. In summary, we conclude that V-ATPase regulates surfactant secretion via an increased Ca^{2+} mobilization and the activation of protein kinase C and Ca^{2+}/calmodulin-dependent kinase II.

Key words: Lipid rafts, alveolar type II cells, V-ATPases, lamellar bodies, surfactant secretion.

4. 2 Introduction

Lipid rafts are specialized microdomains on the plasma membrane or subcellular membranes. Lipid rafts are enriched in saturated lipids including sphingolipids and cholesterol and specialized groups of proteins such as those which are acylated (Src kinases), post-translationally modified (flotillins) and cholesterol bound (caveolins). Lipid rafts can be isolated based on their Triton X-100 insolubility at 4^{o}C. Cholesterol depletion resulted in decreased association of raft proteins and ultimately their associated functions.

Lipid rafts are implicated in exocytosis (1-7), endocytosis (8), signal transduction (9), membrane trafficking (10), bacterial entry (11) and virus budding (12). They are associated with a number of metabolic diseases including Alzheimer's (13).

The cuboidal alveolar type II cells synthesize, store and secrete lung surfactant, a lipid-rich surface active substance. The lung surfactant lowers the surface tension preventing the collapse of lungs. The secretion of surfactant is a relatively slow process when compared neurotransmitter release. Lung surfactant secretion is a highly regulated process. Our laboratory had earlier reported that SNAP-23 and syntaxin 2 were critical for lung surfactant secretion. Additionally, NSF and α-SNAP were implicated in surfactant secretion (14, 15). Our recent study indicated lipid rafts existed both on the lamellar body and plasma membranes. SNAP-23 associated with lipid rafts to a greater extent when compared syntaxin 2 and VAMP-2. Cholesterol depletion not only drastically reduced surfactant secretion but also fusion of lamellar bodies with plasma membrane. Syntaxin 2 and SNAP-23 co-localised with CD44 clusters.

Lipid rafts contain only distinct proteins. The lipid raft proteomic profile would help to uncover the protein machinery contained in them and enable to further study these proteins in different functions. Lipid rafts proteomic studies have been undertaken in T-cells (16, 17), human endothelial cells (18), mouse sperms (19), human smooth muscle cells (20) rat inestitinal mucosal cells (21), exocrine pancreatic cells (22) and HL-60 cells (23). These studies indicated that lipid rafts contained proteins involved in phosphorylation, cytoskeletal rearrangements, exocytosis, cell cycle and signal transduction (24).

Vacuolar ATPases are multi-subunit enzymes that drive the movement of protons using the energy of the ATP hydrolysis. They are present on intracellular organelles including endosomes, lysosomes, secretory granules and synaptic vesicles and mediate the acification of these organelles. V-ATPases also exist on plasma membranes in some types of cells such as macrophages, neutrophils, kidney intercalated cells and osteoclasts. Organellar acidification is crucial for the dissociation of ligand-receptor complexes, processing of secretory proteins and accumulation of neurotransmitters. Extracellular acidification is crucial for bone resorption, urinary acidification or alkalinization and maintenance of intracellular pH. The mutations in genes coding for V-ATPase subunits contribute to a number of diseases (24-27).

V-ATPases has been implicated in exocytosis. The secretion of insulin in *a3*-subunit knock-out mice was reduced. Several subunits interact with SNARE proteins in a number of cells systems (29-31). In yeast, integral membrane VO subunits a form a fusion pore during membrane fusion. (32, 33).

Lung lamellar bodies have lysosomal properties. They maintain an internal acidic milieu owing to the presence of V-ATPases (34). The acidic lamellar body pH is crucial for surfactant protein B and C processing (35). NH_4Cl or methylamine increased surfactant secretion. However, it should be noted that these agents not only increase lamellar body pH but also cytosolic pH (42).

In this study, we attempted to identify new components in type II cell lipid rafts using mass spectrometry. Our results revealed a number of novel proteins involved in energy metabolism, cytoskeletal re-arrangement, cell proliferation and pH regulation. We further studied the role of one of the identified proteins, V-ATPases in lung surfactant secretion and underlying mechanisms.

4.3 Materials and methods

4.3.1 Reagents

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody, and protein molecular mass markers were from Bio-Rad (Hercules, CA). HRP-conjugated goat anti-mouse antibody was from Jackson Immunologicals (West Grove, PA). Porcine pancreatic elastase was obtained from Worthington (Lakewood, NJ), mouse anti-flotillin-1 was from BD Biosciences (San Jose, CA), rabbit anti-v-ATPase a1 (H-140) and mouse monoclonal B1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-Na^+-K^+ATPase α1-subunit antibodies were from Upstate Biotechnology (Lake Placid, NY). Bafilomycin A1 was from LC Laboratories (Woburn, MA). Enhanced Chemiluminiscence detection system was from GE Health Care (Piscataway, NJ). Methyl-^{3}H-Choline chloride was from PerkinElmer (Waltham, MA). All the reagents and chemicals used for 2-D gel electrophoresis were from Bio-Rad.

4.3.2 Alveolar type II cell Isolation

Type II cells were isolated from Sprague-Dawley male rat lungs as described earlier (7). Purity and viability of the cells were greater than 90 and 95%, respectively.

4.3.3 Isolation of lipid rafts

Following isolation, the type II cells were washed once with MEM and then with MBS (25 mM MES and 150 mM NaCl, pH 6.5) buffer twice. Lipid rafts were isolated exactly as described earlier (7). In brief, the cell were lysed in ice-cold 1% Triton X-100 and incubated on ice for 45 min. For cholesterol depletion, type II cells were lysed with 0.5% Triton X-100 and 0.5% (w/v) saponin. Equal amounts of proteins in 600 µl were used for isolation of lipid rafts. Ther lysates (in 600 µl) were mixed with equal volume of

80% sucrose and laid at bottom of the ultracentrifuge tube. Similarly, 1200 µl of 30% and 5% sucrose were laid on top of 40% sucrose. The gradients were then subjected for raft isolation. Following rafts isolation, different fractions were collected from top to bottom of the gradients. First two fractions (1 and 2) of 600 µl each were drawn from top of the gradients. Three fractions (3, 4, and 5) of 400 µl each were collected. Subsequently, two fractions (6 and 7) of 600 µl were drawn. The pellet was dissolved in 600 µl of lysis buffer. The fractions were probed for the raft marker protein, flotillin-1. Lipid rafts were consistently isolated from the interface between 5 and 30% sucrose gradients *i.e.,* fraction 3. The raft fractions were diluted four times with MBS buffer, followed by centrifugation at 25,000 rpm. The pellet was resuspended in MBS buffer and stored at -80^{0}C until used. We consistently obtained ~125 µg of raft protein from 25 million type II cells.

4.3.4 2-dimensional (2-D) Gel Electrophoresis

Lipid rafts fractions were first cleaned up using the ReadyPrep 2-D cleanup kit and the resuspended in 2-D rehydration/sample buffer (8 M Urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampolyte, 0.002% bromophenol blue). Precast immobilized pH gradient (IPG) strips (pH 3-10) were rehydrated and loaded with the protein samples for overnight at room temperature. The proteins were separated on the first dimension by isoelectric focusing (IEF) at room temperature until 40-60,000 Vhrs were reached. It took 12-16 hours for IEF. Following IEF, the IPG strips were equilibrated in SDS-PAGE equilibration buffer I [6 M Urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT] for 10 min and then with buffer II (equilibration buffer I plus 2.5% (w/v) iodoacetamide] for additional 10 min. The proteins were further separated on precast 8-

16% polyacrylamide gels based on their molecular masses. The gels fixed with 40% (v/v) methanol and 10% (v/v) acetic acid for 1 hour, washed with sterile water for two times (10 min each) and stained for overnight in staining buffer [0.08% (w/v) coomassie brilliant blue G250; 1.6% (w/v) ortho-phosphoric acid; 8% (w/v) ammonium sulphate; 20% (v/v) methanol]. The gels were then destained with 1% acetic acid several times until coomassie particles were removed. The gel was later scanned and proteins spots excised for trypsinolysis and mass spectrometry.

4.3.5 Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight (MALDI-TOF) mass spectrometry

The gel spots were first destained with 50% acetonitrile and then with 100% acetonitrile and 100 mM ammonium bicarbonate (ABC). The gels were subsequently rehydrated with freshly made reducing buffer (10 mM DTT, 25 mM ABC) for 1 hour at 56^0C. The proteins were subjected for alkylation for 1 hour (55 mM iodoacetamide in 25 mM ABC) and then to dehydration and rehydration with 100% and 50% acetonitrile for additional 1.5 hours. Subsequently the proteins were subjected to trypsin digestion (8.3 µg/ml in 25 mM ABC) for 4 hours at 37^0C. The proteins were then precipitated with 0.1% trifluoroacetic acid. The trypsinized proteins were spotted onto MALDI plates and overlaid with freshly made matrix (5 mg/ml of alpha-cyano-4-hydroxycinnamic acid). For better mass accuracy, the calibration standards were spotted in neighboring wells of the plate. Peptide mass fingerprints were obtained on a mass spectrometer based on MALDI-TOF data (Voyager DE-PRO, Applied Biosystems) in the reflector mode. The post-acquisition processing was done using the Explorer software. The spectra were smoothed by Gaussian method.

4.3.6 Database searching

The peptide mass spectra generated following mass spectrometry was searched against mass spectrometry protein sequence database using MASCOT software (Matrix Science Ltd., London). Peptide masses were searched against both mammalian and rodent databases. Peaks obtained due to keratin contamination were excluded from database searching. The proteins were identified according to their probability based MOlecular WEight SEarch (MOWSE) score. Only those proteins with high scores are included. The higher the score, the more likely is the confidence in the identification ($p<0.05$). The following parameters were set in the search process: only monoisotopic masses were included in the search, only one missed cleavage per peptide, variable modifications such as carbamidomethyl and propionamide of cysteines, oxidation of methionines, peptide mass tolerance ± 100 ppm, peptide charge state of 1+. All matching spectra were manually reviewed.

4.3.7 Phosphatidylcholine (PC) secretion Assay

Overnight cultured cells were assayed for PC secretion exactly as described earlier (7). In brief, overnight cultured were washed to remove unattached cells. The cells were treated with Baf A1 for 1 hour. A set of dishes were taken out for estimation of lipid secreted during this time (zero time). The cells were stimulated with a combination of secretagogues (100 μM ATP, 0.1 μM PMA and 10 μM terbutaline) for additional 2 hours in the presence of inhibitor. Media and cell were collected and lipid extraction was exactly as described before (7). The amounts of lipids secreted were expressed as a percent of total lipids. The zero time secretion values were subtracted from control and stimulated values to arrive at a net basal and stimulated secretion.

To study the role of thapsigargin on the Baf A1-induced PC secretion, the cells were pre-treated with 100 μM thapsigargin for 15 min and then with Baf A1.

To determine the activation of protein kinase C (PKC) and Ca^{2+}/Calmodulin-dependent Kinase II (CaMKII) following V-ATPase inhibition, overnight cultured type II cells were treated with 100 nM of staurosporine and 10 μM KN-62 to inhibit PKC and CaMKII, respectively in the presence of 20 nM Baf A1. The cells were then assayed for their secretory capabilities as described above.

4.3.8 Quinacrine staining

Type II cells were plated at a density of 0.5-1x10^6 cells in 35 mm^2 dishes. Following overnight culture in DMEM (supplemented with 10% FBS, non-essential amino acids, penicillin and streptomycin), the cells were washed to remove unattached cells. Freshly made quinacrine was added to media at a final concentration of 10 μM. Lamellar body staining was examined within 1-2 minutes upon addition of the dye using florescence microscope.

For detecting the changes in lamellar body, overnight cultured type II cells were stimulated with lung surfactant secretagogues (200 μM ATP, 0.1 μM PMA and 10 μM terbutaline) for various times. At the end of incubation, the cells were loaded with 10 μM quinacrine for about one minute. The media was immediately decanted and the cells fixed with 4% paraformaldehyde. Fluorescence was monitored by florescence microscopy (Nikon). We included unstimulated control cells for each of the time points to prevent bias with respect to loading and quenching of the dyes. The microscopic fields were chosen at random and at least 2-3 fields with about 8-10 cells in each field were selected for further analysis. The time lapse between capturing the images under both the

conditions (stimulated and unstimulated) for that time point was ≤ 5 min. During this time, we did not observe any quenching of the dye. Identical exposure settings were used for capturing images for each time-point. The images were captured with a 40X objective lens. Sometimes the order of capturing images between control and treated was reversed to check if there were any differences due to the time of capturing images. In our hands, the order of capturing images did not reveal any differences when captured within this time.

For quantitation, the intensity of quinacrine was studied using the Metavue software. In brief, the stained regions (in this case lamellar bodies) were carefully selected and integrated intensity of the quinacrine stained regions was measured and expressed as arbitrary units per cell. The intensity of all the cells was thus measured and the average intensity was calculated. Later, the intensity was expressed as per cent of control.

4.3.9 MTT assay

The viability of the cells following V-ATPase inhibition was monitored using MTT assay exactly as described earlier (7).

4.3.10 Statistical analysis

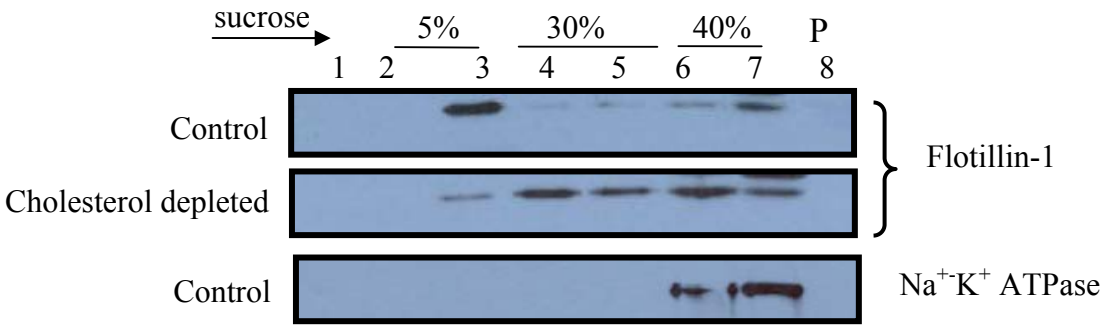
All the experiments were repeated atleast with 3 independent biological samples. Statistical significance was considered only when the $p \leq 0.05$. Student *t* test was used to compare the significant differences between the the control and treatment groups.

4.4 Results

4.4.1 2-D gel electrphoresis

We isolated lipid rafts from alveolar type II cells and performed proteomic analysis to identify proteins present in lipid rafts. Alveolar type II cells were solubilized with 1% Triton X-100 and subjected to a sucrose gradient centrifugation as previously described (7). The isolated rafts were enriched in flotillin-1, a lipid raft marker but excluded Na^{+}-K^{+}-ATPase, a non-raft marker protein. (Fig. IV. 1A, fraction 3). Additionally, the association of flotillin-1 was cholesterol dependent which indicated the standardized isolation protocol (7). Raft proteins (500-600 μg) were separated on first dimension by IEF using a IPG strip (pH 3-10) and then by SDS-PAGE using a 8-16% gradient gel. The gels were stained with colloidal coomassie blue (Fig. IV. 1B). In addition to rodent database, which is a relatively small, we also searched human and mouse databases for the identification of proteins. Some of the identified proteins are shown in 2-D gel (Fig. IV. 1B). The proteins were identified using probability based MOWSE scores**.** The results of the peptide mass fingerprinting are summarized in Table 1. The list included cytoskeletal proteins, V-ATPases, phospholipid binding proteins and some mitochondrial enzymes. Because of their importance in lamellar body acidification, we chose V-ATPase for further studies.

A


sucrose
5%
30%
40%
P
1
2
3
4
5
6
7
8
Control
Cholesterol depleted
Flotillin-1
Control
Na+-K+ ATPase

B

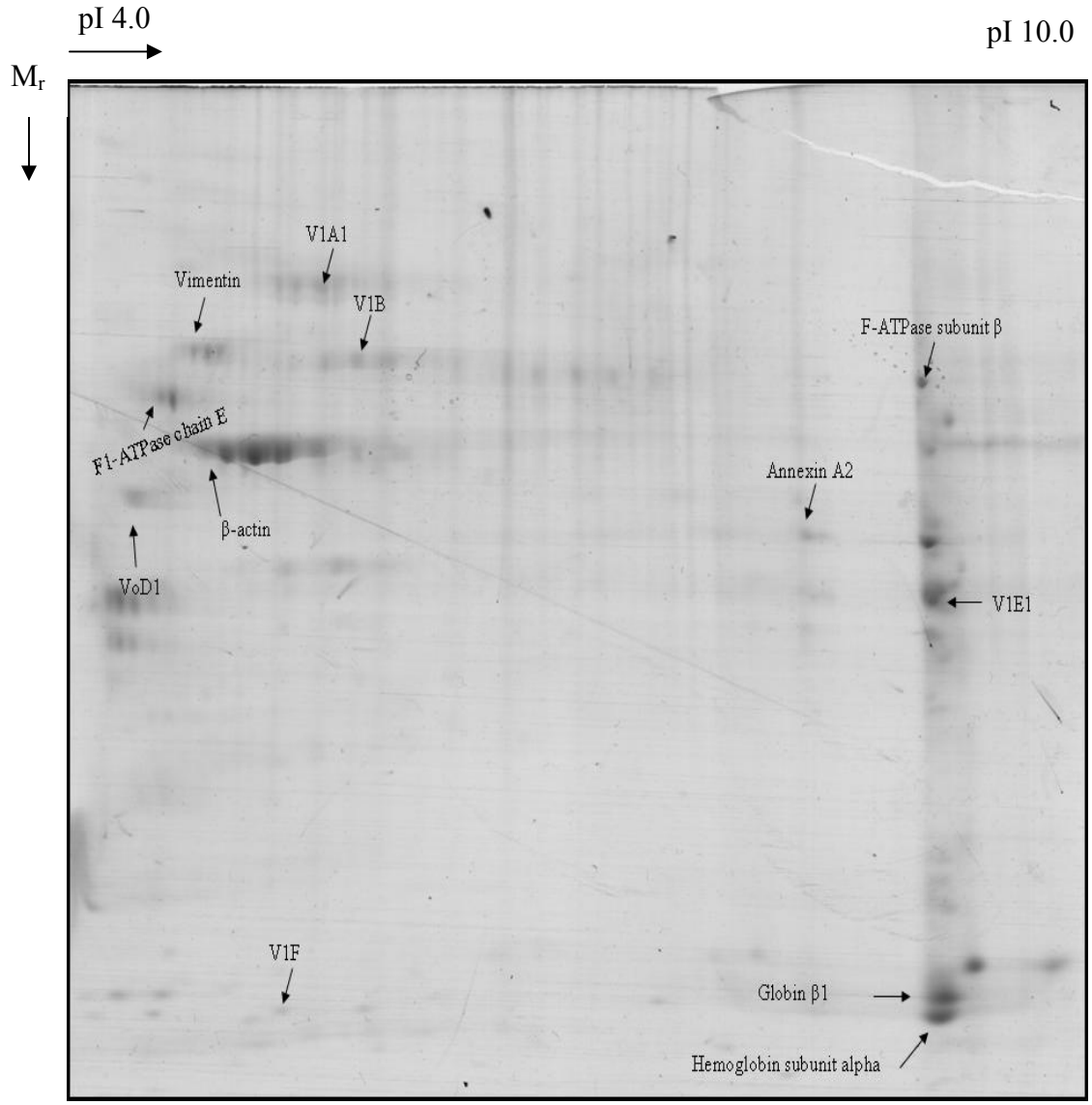


Fig. IV. 1. 2-*D gel electrophoresis* of *type II cell lipid rafts:* A) Confirmation of lipid rafts isolated from type II cells: Freshly isolated type II cells were lysed in the presence of 1% Triton X-100 (control) or 0.5% Triton X-100 and 0.5% (w/v) saponin (cholesterol depleted) at 4^0C for 45 minutes. Later, the lysate was subjected for sucrose gradient centrifugation. Seven Fractions were collected from the top. The pellet was dissolved in lysis buffer (fraction P). The fractions were immunoblotted for flotillin-1, a raft marker protein and Na^+-K^+ ATPase, a non-raft marker protein. Shown are the representative

immunoblots. B) 2-D gel electrophoresis: Lipid rafts proteins were subjected 2-D gel electrophoresis. Later, the gels were stained with coomassie blue stain. Shown is a representative of 3 runs.

Table. IV. 1: Proteomic profile of lipid rafts isolated from alveolar type II cells

Protein	NCBI Accesion #	M_r (kDa)	pI	Peptides matched / Searched	Sequence covered	Mascot score / Mascot threshold
Vacuolar Acidification						
V-ATPase, V1 subunit A	AAC52410	68	5.46	23/103	36	97/67
V-ATPase, V1 subunit B	AAC52411	57	5.57	14/79	27	155/55
V-ATPase, VO subunit D1	Q5M7T6	40	4.89	8/23	26	79/55
V-ATPase, V1 subunit E1	Q6PCU2	26	8.44	12/52	43	84/55
V-ATPase, V1 subunit F	BAB24692	13	5.52	8/35	63	97/55
ATP synthesis						
F-ATPase subunit β	1MABA	53	8.73	19/48	37	151/55
F-ATPase, subunit E	1MABB	49	5.11	11/26	34	97/63
Cytoskeleton-associated						
β -actin	ATRTC	42	5.29	10/14	28	113/55
Vimentin	P31000	54	5.06	22/96	45	126/55
SPFH domain family, member 2	Q8BFZ9	38	5.37	10/10	25	157/63
Cell Cycle and repair						
Prohibitin	AAH72518	30	5.57	8/13	24	84/63
Ca^{2+}-dependent membrane binding						
Annexin A2	NP_06370	39	7.53	19/50	52	155/55
Signal transduction						
Caveolin 1β	AAL33580	17	4.97	7/19	31	76/55
Miscellaneous						
Globin β1	AAB30298	16	7.98	8/46	58	81/55
Hemoglobin subunit α-1/2	HBA_RAT	15	7.93	5/15	50	70/55

4.4.2 Association of V-ATPase subunits with lipid rafts

We first used western blot analysis to confirm the association of V-ATPase with lipid rafts. Each fraction from raft isolation was examined for V-ATPase a1 and B1/2 subunits. As shown in Fig. IV. 2, B1/2 and a1 subunits were present mainly in raft fraction (fraction 3). Furthermore, depletion of cholesterol resulted in the dissociation of both subunits with lipid rafts. Our results indicated a genuine association of *a1* and B1/2 with lipid rafts in a cholesterol dependent manner.

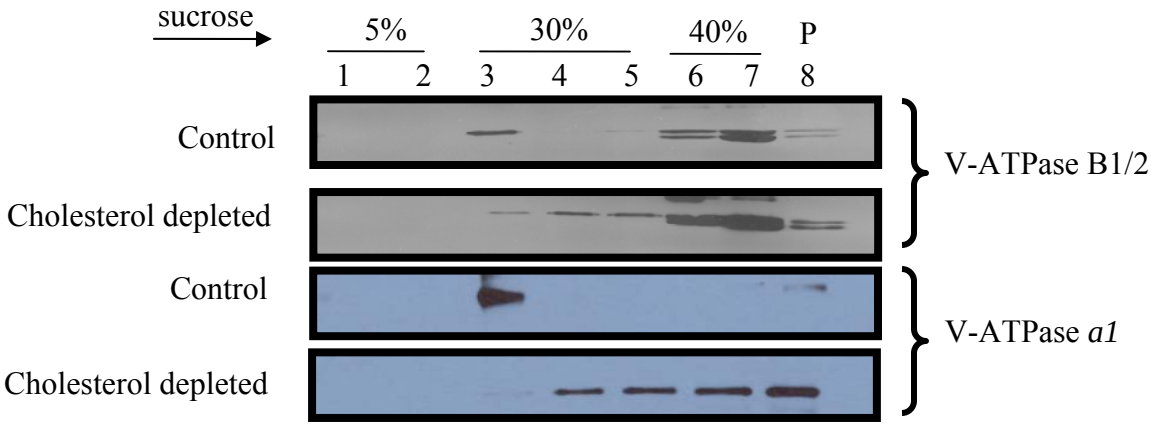


Fig. IV. 2. Association of V-ATPase subunits with lipid rafts: Type II cells were lysed in 1% Triton X-100 (control) or 0.5% Triton X-100 + 0.5% saponin (cholesterol depleted). Later, the lysates were subjected for raft isolation and various fractions were collected. Equal volumes of fractions were immunoblotted for v-ATPase *a1* and B1/2 subunits.

4.4.3 Differential expression of V-ATPase subunits

We then studied the subcellular localization of V-ATPase subunits. Immunoblotting revealed a much higher amount of *a1* and B1/2 subunits in type II cell lysates when compared to lung homogenate (Fig. IV. 3). While plasma membrane fraction had a small amount of both subunits, lamellar body fractions contain abundant *a1* and B1/2 subunits.

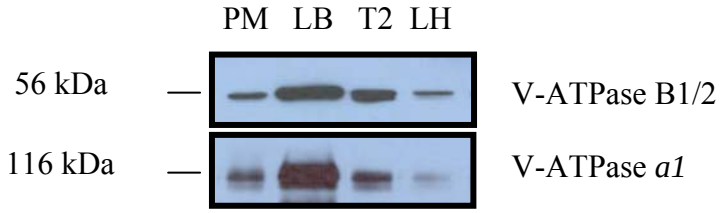
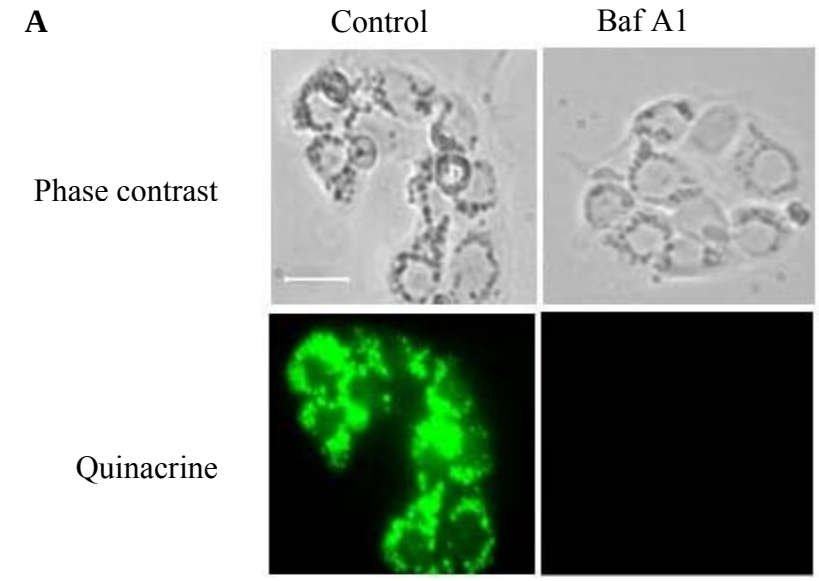


Fig. IV. 3. Western blots of V-ATPase subunits: Equal amount of total protein isolated from plasma membrane (PM), Lamellar bodies (LB), type II cells (T2), and lung tissue (LH) were immunoblotted for V-ATPase *a1* and B1/2 subunits.

4.4.4 Effect of V-ATPase inhibition on lamellar body pH gradient and surfactant secretion

The acidic milieu in the lamellar bodies is required for processing of surfactant proteins and packaging of surfactant lipids (35, 42). Since V-ATPases were involved in the acidification or generation of the pH gradient across the lamellar body membrane, we studied if V-participate in surfactant secretion (34). We inhibited V-ATPase with Baf A1, a macrolide antibiotic isolated from *Streptomyces griseus.* Baf A1 is a specific and potent inhibitor of V-ATPase at nanomole concentrations. We ascertained the inhibition of V-ATPase by monitoring the accumulation of quinacrine (34, 35). Type II cells were treated with 20 nM Baf A1 for various times. The pH gradient was effectively dissipated within an hour. As shown in Fig. IV. 4A, in untreated type II cells, quinacrine accumulates in the acidic lamellar bodies. Fluorescence in the cells treated with Baf A1 was lost, indicating an increase in lamellar body pH.

To investigate the effect of V-ATPase inhibition on surfactant secretion, type II cells were treated with Baf A1 and surfactant secretion measured. Baf A1 increased surfactant secretion to 4.30 ± 0.29 when compared to 1.75 ± 0.21 percent in untreated cells (Fig. IV. 4B). The increase in secretion was not due to deleterious effects as cell viability was unchanged in the Baf A1-treated cells (data not shown). Additionally, stimulated surfactant secretion was also examined. Lung secretagogues were added following 1 hour of treatment with Baf A1 since by that time the pH gradient was lost. When type II cells were stimulated with a combination of secretagogues, Baf A1 slightly affected surfactant secretion. This probably is because the secretion had reached a saturation level under stimulated conditions.



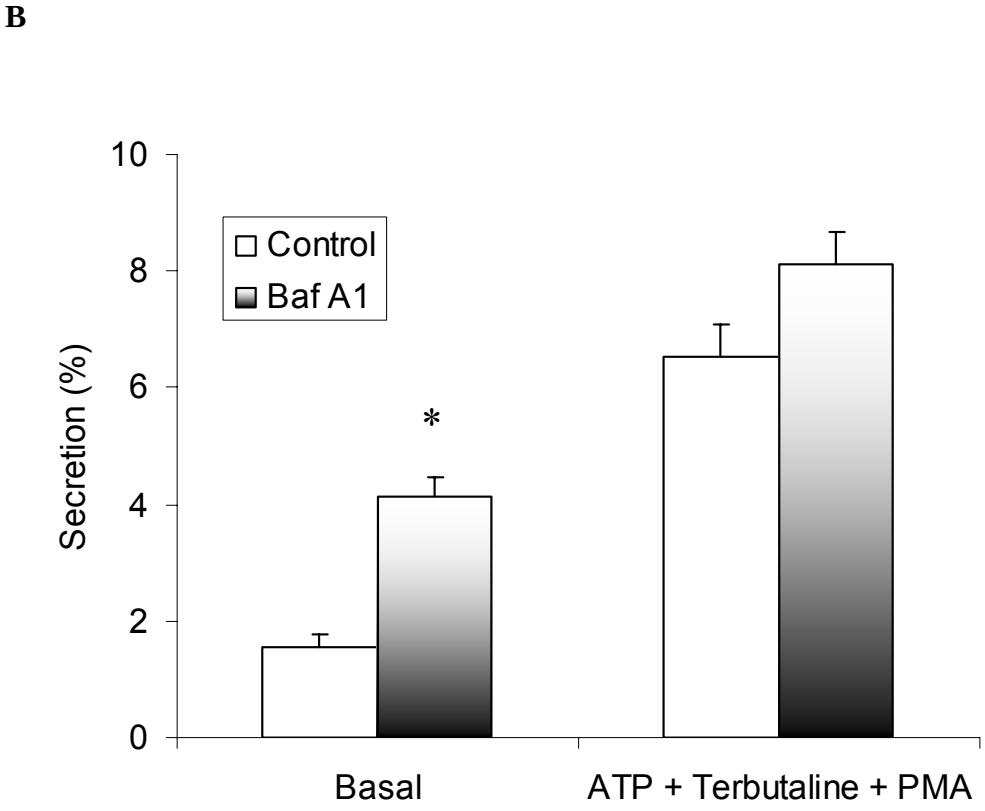


Fig. IV.4. Effect of Baf A1 on the pH gradient of lamellar bodies and surfactant secretion: A) Overnight cultured cells were treated without or with 20 nM Baf A1 for 1

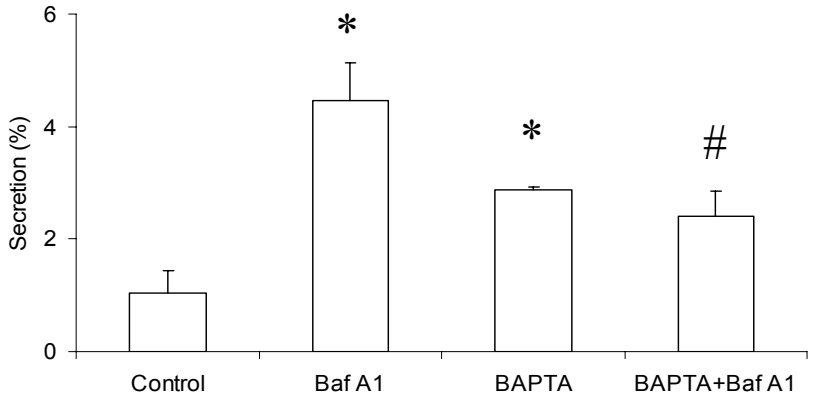
hour. Later the quinacrine was added at a final concentration of 10 μM. Fluorescence microscopy was undertaken to monitor quinacrine staining. Shown are the representative images of 3 biological independent cell preparations. Scale bar: 40 μm B) PC secretion: Freshly isolated type II cells were labeled with [^{3}H]-choline overnight. Later, cells were treated with 20 nM Baf A1 for 1 hour. The cells were then stimulated with a combination of 100 μM ATP; 10 μM terbutaline and 0.1μM PMA for 2 additional hours. Secreted lipids were assayed and expressed as per cent of total lipids. Shown is means ± SE. $*P \leq 0.05$ *v.s* control (Student *t* test $n \geq 3$). *Open bars*, control; *shaded bars*, Baf A1.

4.4.5 Effect of Ca^{2+} chelators and thapsigargin on V-ATPase-mediated surfactant secretion

In a quest to understand the mechanisms of Baf A1-induced increase in surrfactant secretion, we first explored the possibility of an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) as one of the reasons. To this end, type II cells were pre-treated with BAPTA-AM to chelate intracellular Ca^{2+} and then treated with Baf A1. BAPTA-AM slightly increased secretion consistent with an earlier report (36). BAPTA-AM significantly decreased Baf A1-stimulated secretion, indicating mobilization of intracellular Ca^{2+} following V-ATPase inhibition (Fig. IV. 5A).

Endoplasmic reticulum (ER) is one of the Ca^{2+} stores in cells. Ca^{2+}-ATPase is a Ca^{2+} channel on ER membrane responsible for the accumulation of Ca^{2+} in ER. We depleted the ER Ca^{2+} pool by inhibiting the Ca^{2+}-ATPase using thapsigargin. There were no differences in secretion following treatment with thapsigargin when compared with the control (Fig. IV. 5). However, Baf A1-mediated secretion was effectively inhibited by thapsigargin.

A



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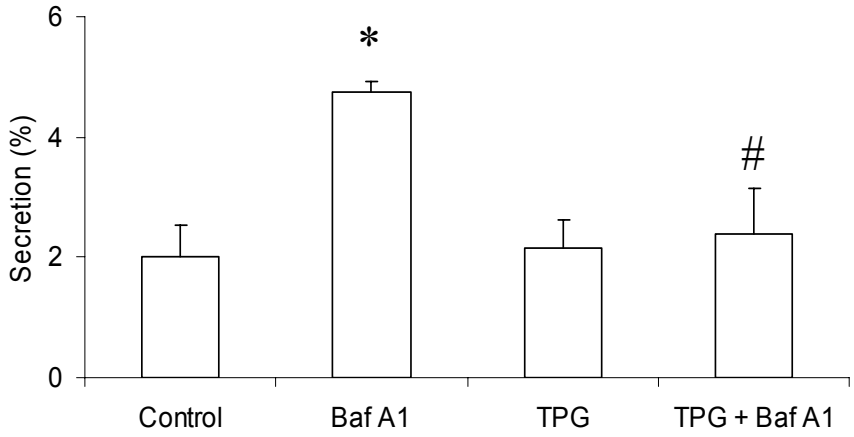


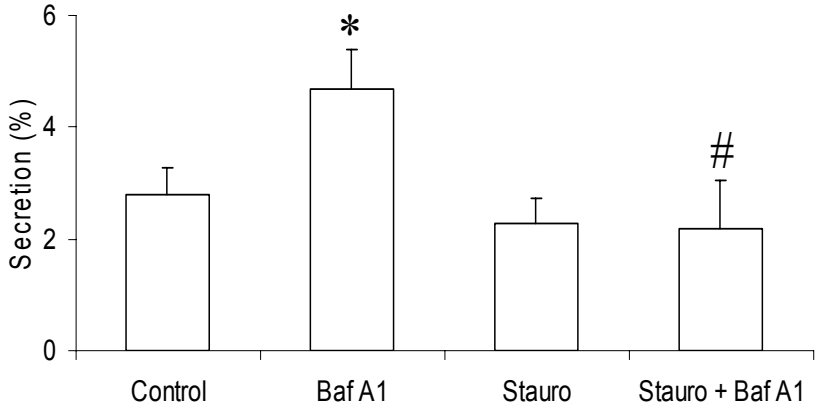
Fig. IV. 5. ***Effect of Ca^{2+} chelators and thapsigargin on V-ATPase mediated-surfactant secretion:*** Overnight cultured type II cells were treated with Baf A1 in the presence or absence of intracellular Ca^{2+}-chelator, A) BAPTA-AM (50 μM) and B) ER Ca^{2+}-ATPase inhibitor, thapsigargin (TPG; 100 μM). Data shown are means ± SE. *P<0.05 *v.s* control; #P<0.05 *v.s* Baf A1 (Student *t* test n ≥ 3). Numbers shown in the bars indicated the number of independent cell preparations.

4.4.6 Effect of PKC and CaMKII inhibition on V-ATPase-mediated surfactant secretion

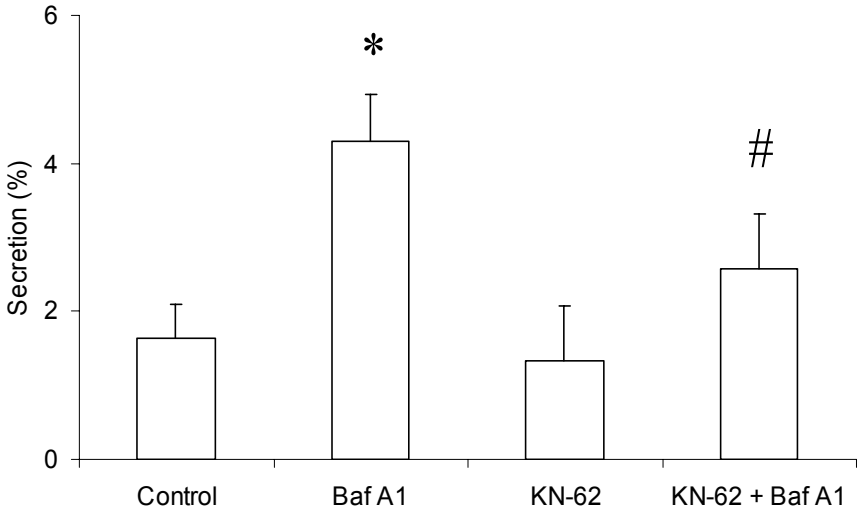
An increase in intracellular Ca^{2+} may activate protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII), which have been shown to be involved on surfactant secretion. We thus examined whether the inhibition of PKC and CaMKII would block the Baf A1-mediated secretion. Staurosporine and KN-62 were used to inhibit PKC and CaMKII, respectively. Both agents effectively inhibited the Baf A1-mediated increase in surfactant secretion (Fig. IV. 6). The inhibitors alone had no effect.

Surfactant secretion can be stimulated through β2-adrenergic receptors and the activation of protein kinase A. If Baf A1 elicits its effect by Ca^{2+}, PKC/CaMKII pathways, an additive effect should be observed when Baf A1 and terbutaline (β2 agonist) were used together. Indeed, the addition of Baf A1 and terbutaline resulted in an additive increase in secretion (Fig. IV. 6).

A



B


6
4
2
0
Secretion (%)
*
#
Control
Baf A1
KN-62
KN-62 + Baf A1

C

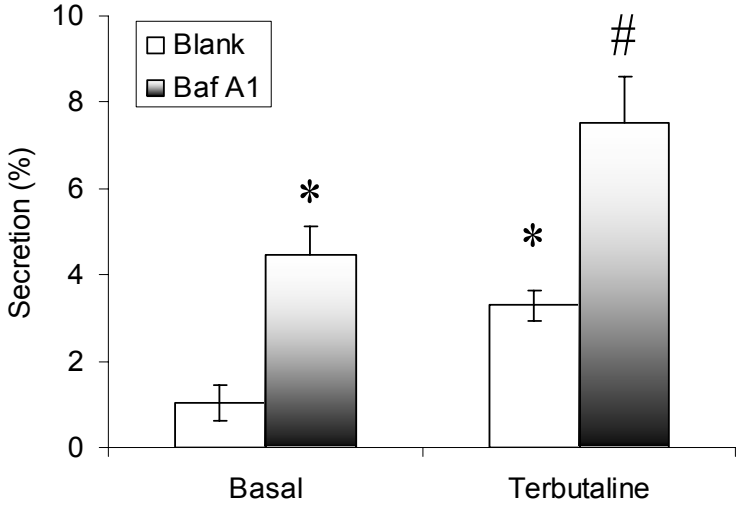
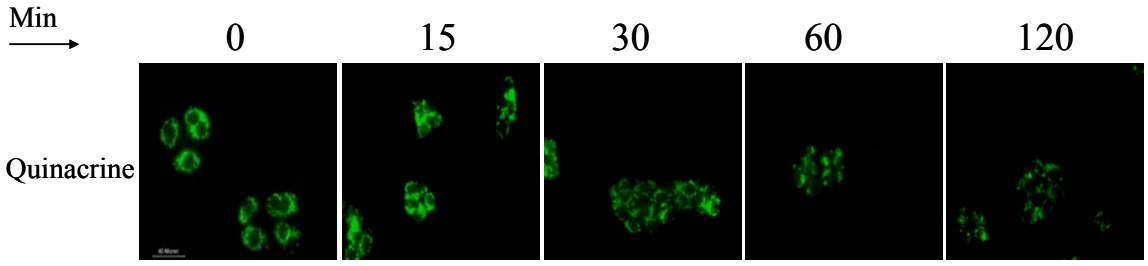

10
8
6
4
2
0
Secretion (%)
Blank
Baf A1
*
*
#
Basal
Terbutaline

Fig. IV. 6 Effect of PKC and CaMKII inhibitors on V-ATPase-mediated secretion: Overnight cultured type II cells were treated with Baf A1 in the presence or absence of A) Staurosporine (100 nM) or B) KN-62 (10 μM) and C) Terbutaline. Later PC secretion assay was done. Data shown are Means ± SE. *P<0.05 *v.s* control; #P<0.05 *v.s* Baf A1 (Student *t* test $n \geq 3$). Numbers shown in the bars indicated the number of independent cell preparations.

4.9 V-ATPase is inhibited during lamellar body exocytosis

Finally we asked if there were a physiological relevance of V-ATPase inhibition in surfactant secretion. We examined the effect of type II cell stimulation on the pH gradient across lamellar body membrane. We stimulated type II cells with a combination of lung surfactant secretagogues for various times and monitored the lamellar body pH by the accumulation of quinacrine. A gradual decrease in accumulation of the dye was observed in the lamellar bodies following stimulation with secretagogues (Fig. IV.7A). The quantitation of the intensity of fluorescence revealed a ~ 50% decrease at 60 min (Fig. IV. 7B).

A



B

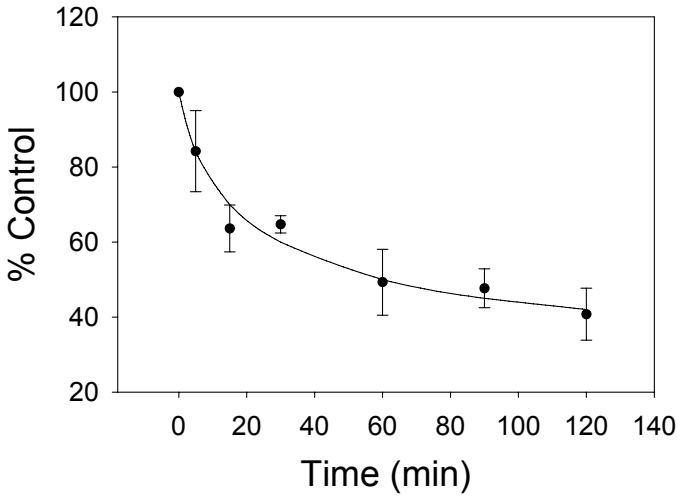


Fig. IV. 7. Monitoring of lamellar body pH following stimulation of type II cells: Overnight cultured type II cells were stimulated with a combination of ATP (200 μM) and terbutaline (20 μM) for various times as indicated in the figure. At the end of incubation time, the cells were loaded with 10 μM quinacrine for about one minute. The cells were then immediately fixed before microscopy. A) Shown are the representative images indicating the changes in quinacrine accumulation patterns as a function of time. Scale bar: 40 μM. B) The intensity of quinacrine staining was quantified and expressed as percent of control. Shown are means ± SEM (n = 3 independent cell preparations).

4. 5 Discussion

Type II cells are involved in synthesis, storage and secretion of lung surfactant. Our previous study indicated the role of lipid rafts in surfactant secretion (7). However, data is lacking on the entire protein components of lipid rafts in many cell types including type II cells. The results uncovered the proteomic profile of lipid rafts isolated from type II cells. The proteomic profile indicated proteins involved in diverse processes. V-ATPases subunits were present in the lipid rafts and hence this protein was studied in detail for its role in surfactant secretion. Our results indicated that inhibition of V-ATPase triggers surfactant secretion by mobilizing $[Ca^{2+}]_i$ which activate PKC and CaMKII.

Type II cell lipid raft proteins included those involved in acidification (V-ATPases), metabolism (ATP synthases) and cellular proliferation and differentiation (prohibitin), phospholipids-binding proteins (annexin A2), cytoskeletal proteins (actin), and oxygen exchange (globin beta1). Many of these proteins were also found in lipid rafts of other cells (16-24). Our studies indicated that type II cell lipid rafts contained a small set of proteins. However, the numbers of proteins identified in other studies were higher than this study mainly due to use of tandem mass spectrometry in those studies.

The presence of V-ATPases in the type II cell lipid rafts appears intriguing. The preponderance of V-ATPase in the lipid rafts of other cells have been reported (37-39). These proteins have specialized functions on the plasma membranes apart from intracellular membranes. Our previous studies have shown that the lipid raft marker proteins, flotillin-1 and -2 were present on lamellar bodies, indicating that lipid rafts exist in intracellular compartments (7).

Lamellar bodies in type II cells maintain acidic milieu (pH 5.5-6.0) due to the V-ATPase activity (34, 40). The acidic pH was confirmed by accumulation of dyes such as quinacrine and acridine orange (34, 42). Acidic pH in the lamellar body is essential for packaging of surfactant lipids, surfactant protein-A dependent aggregation of lipids, proteolytic processing of surfactant protein-B and C and Ca^{2+} uptake (34, 41-43). V-ATPases on the organelles generate transvesicular proton-electrochemical potential gradient ($\Delta\psi_v$) and pH gradient (ΔpH_v). The treatment of type II cells with methylamine and ammonium chloride increases lamellar body pH and enhances surfactant secretion (42). However, methylamine and ammonium chloride also increase cytosolic pH transiently. Our results indicated that the dissipation of lamellar body pH by Baf A1 increases surfactant secretion suggesting that the increase of surfactant secretion is due to an increase in lamellar body pH but not due to cytosolic pH. Inhibition of V-ATPase resulted in decreased exocytosis in PC12, GH3, pancreatic alpha and beta cells (45-49) whereas increased secretion in pituitary, glial, macrophages and neutrophils (50-52). Interestingly in some cases, there was no effect of V-ATPase inhibition on secretion (49, 53). It thus appears different cell type respond differently to V-ATPase inhibition.

Intracellular Ca^{2+} can be altered by influx of Ca^{2+} from extracellular mileu and mobilization of Ca^{2+} from intracellular pools. Lamellar bodies contain high levels of Ca^{2+} and could be one of the potential intracellular sources. Our results have shown that intracellular Ca^{2+} chelation abrogated the Baf A1-induced secretion. Lysosomal pH dissipation in macrophages led to gradual loss of lysosomal Ca^{2+} with a simultaneous increase in cytosolic levels (54). A similar process may occur in lamellar bodies since V-ATPase were predominantly expressed on lamellar body membrane and its inhibition led

to the dissipation of pH gradient. It is possible that released Ca^{2+} from lamellar bodies increases localized Ca^{2+} concentration and thus increase surfactant secretion. In fact, it has been reported that the Ca^{2+} concentration in exocytotic lamellar bodies is higher than that in perinuclear lamellar bodies (55). Similarly, in adrenal chromaffin cells, increase in vesicular pH induced release of Ca^{2+} and increased secretion (56).

Depletion of ER pool inhibited the V-ATPase-mediated increase in secretion indicating the cross-talk between ER and lamellar body Ca^{2+} pools. Our study is supported by previous reports which indicated that small, localized changes in Ca^{2+} induce global Ca^{2+} waves due to interplay between different Ca^{2+} pools. In arterial smooth muscle cells, NAADP increases Ca^{2+} release from Baf A1-sensitive compartment, which in turn further induces Ca^{2+} release from sarcoplasmic reticulum by calcium induced calcium release owing to the close apppsition of these two pools (57). It is possible that such an apposition exist in type II cells.

When type II cells were treated with PKC and CaMKII inhibitors, we observed an inhibition in Baf A1-induced surfactant secretion. Similar results were reported for lysosomal secretion in macrophages. (58). Our results thus supported the idea that the V-ATPase mediated surfactant secretion is mediated by PKC and CaMKII and that the activation was due to increase in $[Ca^{2+}]_i$.

We then asked if the V-ATPase activity was modulated during normal physiological conditions. The accumulation of quinacrine dye gradually decreased when the cells were stimulated with lung surfactant secretagogues. When quinacrine accumulation was quantified, we found ~ 50% reduction in intensity. It should be noted

that this is an average intensity of all the lamellar bodies in type II cells irrespective of whether there were undergoing exocytosis.

In summary, we propose the following model for V-ATPase mediated-surfactant secretion. V-ATPase mediates the acidification of lamellar bodies. Inhibition of V-ATPase results dissipation of lamellar body pH gradient. The increase in lamellar body pH results in mobilization of Ca^{2+} from lamellar bodies, leading to an increase in cytosolic free Ca^{2+}. Such an increase further releases Ca^{2+} from the ER pool. The net surge on Ca^{2+} concentration activates PKC and CaMKII. The activation of these enzymes finally results in increased surfactant secretion.

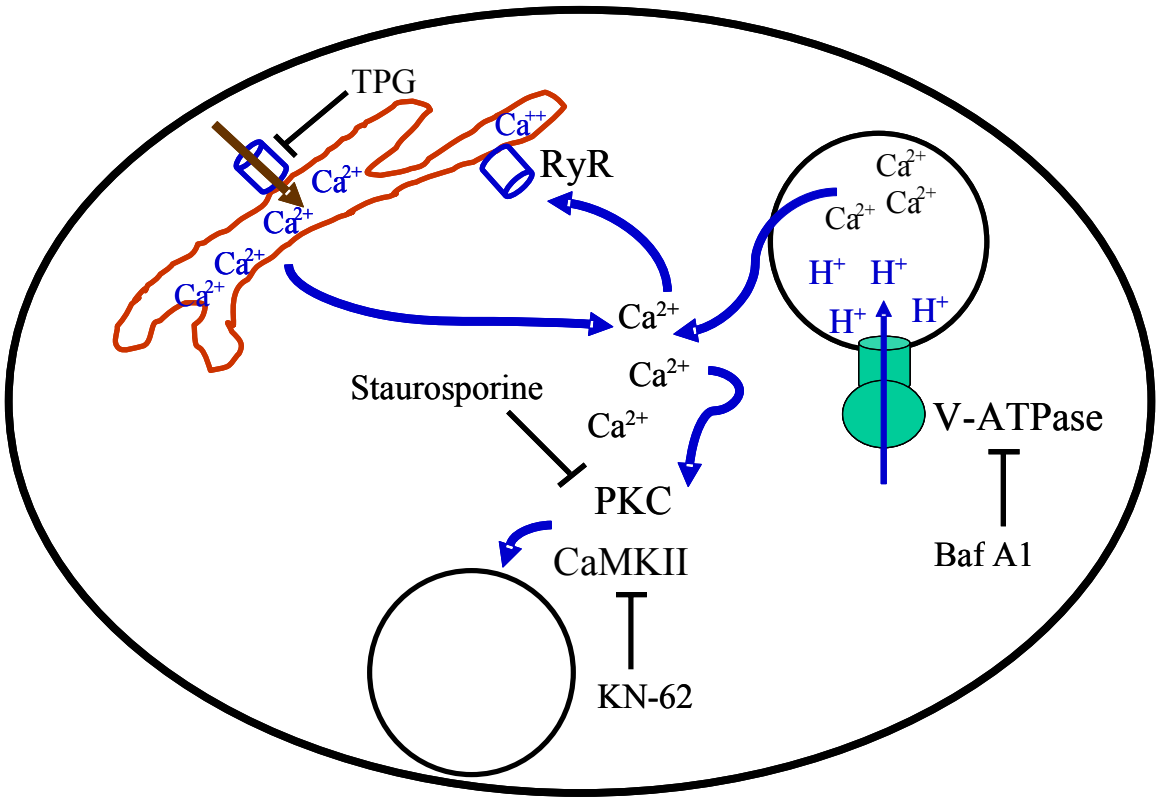


Fig. IV. 8. Schematic representation of proposed events following V-ATPase inhibition:

In this model, following V-ATPase inhibition by Baf A1, we propose that there is mobilization of Ca^{2+} from lamellar bodies. The small localized changes in Ca^{2+} concentration leads to further release of Ca^{2+} from ER store (probably by activation of RyR). The global increase in Ca^{2+} concentration leads to activation of PKC and CaMKII leading to their activation and finally increased surfactant secretion.

Acknowledgments

Contribution of co-cuthors: Dr. Amarjit Mishra participated in PC secretion experiments; Lijing Su had done western blotting; Dr. Ayalew Sahlu helped in 2-D gel electrophoresis and Dr. Steve Hartson helped with mass spectrometry.

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4.6 References

1. Chamberlain LH, Burgoyne RD, Gould GW. SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis. *Proc Natl Acad Sci U S A* 2001;98:5619-24

2. Chamberlain LH, Gould GW. The vesicle- and target-SNARE proteins that mediate Glut4 vesicle fusion are localized in detergent-insoluble lipid rafts present on distinct intracellular membranes. *J Biol Chem* 2002;277: 49750-4.

3. Salaun C, James DJ, Chamberlain LH. Lipid rafts and the regulation of exocytosis. *Traffic* 2004;5: 255-64.

4. Salaun C, Gould GW, Chamberlain LH. Lipid raft association of SNARE proteins regulates exocytosis in PC12 cells. *J Biol Chem* 2005;280:19449-53.

5. Salaun C, Gould GW, Chamberlain LH. The SNARE proteins SNAP-25 and SNAP-23 display different affinities for lipid rafts in PC12 cells. Regulation by distinct cysteine-rich domains. *J Biol Chem* 2005;280:1236-40.

6. Puri N, Roche PA. Ternary SNARE complexes are enriched in lipid rafts during mast cell exocytosis. *Traffic* 2006;7:1482-94.

7. Chintagari NR, Jin N, Wang P, Narasaraju TA, Chen J, Liu L. Effect of cholesterol depletion on exocytosis of alveolar type II cells. *Am J Respir Cell Mol Biol* 2006; 34:677-87.

8. Pelkmans, L. and Helenius, A. Endocytosis via caveolae. *Traffic* 2002;3:311-320.

9. Allen JA, Halverson-Tamboli RA, Rasenick MM. Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci* 2007; 8:128-40.

10. Schuck S, Simons K. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. *J Cell Sci* 2004; 117: 5955-64.

11. Zaas DW, Duncan M, Rae Wright J, Abraham SN. The role of lipid rafts in the pathogenesis of bacterial infections. *Biochim Biophys Acta* 2005;1746:305-13.

12. Takeda, M., Leser, G. P., Russell, C. J. and Lamb, R. A. Influenza virus hemagglutinin concentrates in lipid raft microdomains for efficient viral fusion. *Proc. Natl. Acad. Sci. USA* 2003;100:14610-14617.

13. Cordy JM, Hooper NM, Turner AJ. The involvement of lipid rafts in Alzheimer's disease. *Mol Membr Biol* 2006;23:111-22.

14. Abonyo BO, Wang P, Narasaraju TA, Rowan WH 3rd, McMillan DH, Zimmerman UJ, Liu L. Characterization of alpha-soluble N-ethylmaleimide-sensitive fusion attachment protein in alveolar type II cells: implications in lung surfactant secretion. *Am J Respir Cell Mol Biol* 2003;29:273-82.

15. Abonyo BO, Gou D, Wang P, Narasaraju T, Wang Z, Liu L. Syntaxin 2 and SNAP-23 are required for regulated surfactant secretion. *Biochemistry* 2004;43:3499-506.

16. Bini L, Pacini S, Liberatori S, Valensin S, Pellegrini M, Raggiaschi R, Pallini V, Baldari CT. Extensive temporally regulated reorganization of the lipid raft proteome following T-cell antigen receptor triggering. *Biochem J* 2003;369:301-9.

17. Razzaq TM, Ozegbe P, Jury EC, Sembi P, Blackwell NM, Kabouridis PS. Regulation of T-cell receptor signalling by membrane microdomains. *Immunology* 2004;113: 413-26.

18. Sprenger RR, Horrevoets AJ. Proteomic study of caveolae and rafts isolated from human endothelial cells. *Methods Mol Biol* 2007;357:199-213.

19. Sleight SB, Miranda PV, Plaskett NW, Maier B, Lysiak J, Scrable H, Herr JC, Visconti PE. Isolation and proteomic analysis of mouse sperm detergent-resistant membrane fractions: evidence for dissociation of lipid rafts during capacitation. *Biol Reprod* 2005;73:721-9.

20. MacLellan DL, Steen H, Adam RM, Garlick M, Zurakowski D, Gygi SP, Freeman MR, Solomon KR. A quantitative proteomic analysis of growth factor-induced compositional changes in lipid rafts of human smooth muscle cells. *Proteomics* 2005; 5:4733-42.

21. Nguyen HT, Amine AB, Lafitte D, Waheed AA, Nicoletti C, Villard C, Letisse M, Deyris V, Roziere M, Tchiakpe L, Danielle CD, Comeau L, Hiol A. Proteomic characterization of lipid rafts markers from the rat intestinal brush border. *Biochem Biophys Res Commun* 2006;342:236-44.

22. Berkane AA, Nguyen HT, Tranchida F, Waheed AA, Deyris V, Tchiakpe L, Fasano C, Nicoletti C, Desseaux V, Ajandouz el H, Comeau D, Comeau L, Hiol A. Proteomic of lipid rafts in the exocrine pancreas from diet-induced obese rats. *Biochem Biophys Res Commun* 2007;355:813-9.

23. Yanagida M, Nakayama H, Yoshizaki F, Fujimura T, Takamori K, Ogawa H, Iwabuchi K. Proteomic analysis of plasma membrane lipid rafts of HL-60 cells. *Proteomics* 2007;7:2398-409.

24. Foster LJ, De Hoog CL, Mann M. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci U S A* 2003; 100:5813-8.

25. Karet FE, Finberg KE, Nelson RD, Nayir A, Mocan H, Sanjad SA, Rodriguez-Soriano J, Santos F, Cremers CW, Di Pietro A, Hoffbrand BI, Winiarski J, Bakkaloglu A, Ozen S, Dusunsel R, Goodyer P, Hulton SA, Wu DK, Skvorak AB, Morton CC, Cunningham MJ, Jha V, Lifton RP. Mutations in the gene encoding B1 subunit of H+-ATPase cause renal tubular acidosis with sensorineural deafness. *Nat Genet* 1999;21:84-90.

26. Frattini A, Orchard PJ, Sobacchi C, Giliani S, Abinun M, Mattsson JP, Keeling DJ, Andersson AK, Wallbrandt P, Zecca L, Notarangelo LD, Vezzoni P, Villa A. Defects in TCIRG1 subunit of the vacuolar proton pump are responsible for a subset of human autosomal recessive osteopetrosis. *Nat Genet* 2000;25:343-6.

27. Smith AN, Skaug J, Choate KA, Nayir A, Bakkaloglu A, Ozen S, Hulton SA, Sanjad SA, Al-Sabban EA, Lifton RP, Scherer SW, Karet FE. Mutations in ATP6N1B, encoding a new kidney vacuolar proton pump 116-kD subunit, cause recessive distal renal tubular acidosis with preserved hearing. *Nat Genet* 2000;26:71-5.

28. Vargas-Poussou R, Houillier P, Le Pottier N, Strompf L, Loirat C, Baudouin V, Macher MA, Dechaux M, Ulinski T, Nobili F, Eckart P, Novo R, Cailliez M, Salomon R, Nivet H, Cochat P, Tack I, Fargeot A, Bouissou F, Kesler GR, Lorotte S, Godefroid N, Layet V, Morin G, Jeunemaitre X, Blanchard A. Genetic investigation of autosomal recessive distal renal tubular acidosis: evidence for early sensorineural hearing loss associated with mutations in the ATP6V0A4 gene. *J Am Soc Nephrol* 2006;17:1437-43.

29. Galli T, McPherson PS, De Camilli P. The V0 sector of the V-ATPase, synaptobrevin, and synaptophysin are associated on synaptic vesicles in a Triton X-100-resistant, freeze-thawing sensitive, complex. *J Biol Chem* 1996;271:2193-8.

30. Hiesinger PR, Fayyazuddin A, Mehta SQ, Rosenmund T, Schulze KL, Zhai RG, Verstreken P, Cao Y, Zhou Y, Kunz J, Bellen HJ. The v-ATPase V0 subunit a1 is required for a late step in synaptic vesicle exocytosis in Drosophila. *Cell* 2005;121:607-20.

31. Sun-Wada GH, Toyomura T, Murata Y, Yamamoto A, Futai M, Wada Y. The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta-cells. *J Cell Sci* 2006;119:4531-40.

32. Morel N, Dunant Y, Israel M. Neurotransmitter release through the V0 sector of V-ATPase. J *Neurochem* 2001;79:485-8.

33. Peters C, Bayer MJ, Buhler S, Andersen JS, Mann M, Mayer A. Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. *Nature* 2001;409:581-8.

34. Chander A, Johnson RG, Reicherter J, Fisher AB. Lung lamellar bodies maintain an acidic internal pH. *J Biol Chem* 1986;261:6126-31.

35. Beers MF. Inhibition of cellular processing of surfactant protein C by drugs affecting intracellular pH gradients. *J Biol Chem* 1996;271:14361-70.

36. Strayer DS, Hoek JB, Thomas AP, White MK. Cellular activation by Ca^{2+} release from stores in the endoplasmic reticulum but not by increased free Ca^{2+} in the cytosol. *Biochem J* 1999;344:39-46.

37. Li N, Shaw AR, Zhang N, Mak A, Li L. Lipid raft proteomics: analysis of in-solution digest of sodium dodecyl sulfate-solubilized lipid raft proteins by liquid chromatography-matrix-assisted laser desorption/ionization tandem mass spectrometry. *Proteomics* 004;4:3156-66.

38. Bae TJ, Kim MS, Kim JW, Kim BW, Choo HJ, Lee JW, Kim KB, Lee CS, Kim JH, Chang SY, Kang CY, Lee SW, Ko YG. Lipid raft proteome reveals ATP synthase complex in the cell surface. *Proteomics* 2004;4:3536-48. Erratum in: *Proteomics* 4: 4032. Lee, Song-Won [corrected to Lee, Sang-Won].

39. Nguyen HT, Amine AB, Lafitte D, Waheed AA, Nicoletti C, Villard C, Letisse M, Deyris V, Roziere M, Tchiakpe L, Danielle CD, Comeau L, Hiol A. Proteomic characterization of lipid rafts markers from the rat intestinal brush border. *Biochem Biophys Res Commun* 2006;342:236-44.

40. Wadsworth SJ, Spitzer AR, Chander A. Ionic regulation of proton chemical (pH) and electrical gradients in lung lamellar bodies. *Am J Physiol* 1997;273:L427-36.

41. Chander A, Fisher AB, Strauss JF 3rd. Role of an acidic compartment in synthesis of disaturated phosphatidylcholine by rat granular pneumocytes. *Biochem J* 1982;208:651-8.

42. Chander A, Sen N, Wu AM, Higgins S, Wadsworth S, Spitzer AR. Methylamine decreases trafficking and packaging of newly synthesized phosphatidylcholine in LBs in alveolar type II cells. *Biochem J* 1996;318:271-8. Erratum in: *Biochem J* 318: 1079.

43. Ruano ML, Perez-Gil J, Casals C. Effect of acidic pH on the structure and lipid binding properties of porcine surfactant protein A. Potential role of acidification along its exocytic pathway. *J Biol Chem* 1998;273:15183-91.

44. Wadsworth SJ, Chander A. H+-and K+-dependence of Ca^{2+} uptake in lung LBs. *J Membr Biol* 2000;174:41-51.

45. Kasai Y, Ohta T, Nakazato Y, Ito S. Release of dopamine and ATP from PC12 cells treated with dexamethasone, reserpine and Baf A1. *J Vet Med Sci* 2001;63:367-72.

46. Varro A, Nemeth J, Dickinson CJ, Yamada T, Dockray GJ. Discrimination between constitutive secretion and basal secretion from the regulated secretory pathway in GH3 cells. *Biochim Biophys Acta* 1996;1313:101-5.

47. Yamada H, Otsuka M, Hayashi M, Nakatsuka S, Hamaguchi K, Yamamoto A, Moriyama Y. Ca2+-dependent exocytosis of L-glutamate by alphaTC6, clonal mouse pancreatic alpha-cells. *Diabetes* 2001;50:1012-20.

48. Barg S, Huang P, Eliasson L, Nelson DJ, Obermuller S, Rorsman P, Thevenod F, Renstrom E. Priming of insulin granules for exocytosis by granular Cl(-) uptake and acidification. *J Cell Sci* 2001;114:2145-54.

49. Taupenot L, Harper KL, O'Connor DT. Role of H+-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: evidence for a vesiculogenic function. *J Biol Chem* 2005;280:3885-97.

50. Schoonderwoert VT, Holthuis JC, Tanaka S, Tooze SA, Martens GJ. Inhibition of the vacuolar H+-ATPase perturbs the transport, sorting, processing and release of regulated secretory proteins. *Eur J Biochem* 2000;267:5646-54.

51. Nishiguchi M, Tokugawa K, Yamamoto K, Akama T, Nozawa Y, Chaki S, Ueki T, Kameo K, Okuyama S. Increase in secretion of glial cell line-derived neurotrophic factor from glial cell lines by inhibitors of vacuolar ATPase. *Neurochem Int* 2003;42: 93-8.

52. Tapper H. The secretion of preformed granules by macrophages and neutrophils. *J Leukoc Biol* 1996;59:613-22.

53. Waterford SD, Kolodecik TR, Thrower EC, Gorelick FS. Vacuolar ATPase regulates zymogen activation in pancreatic acini. *J Biol Chem* 2005;280:5430-4.

54. Christensen KA, Myers JT, Swanson JA. pH-dependent regulation of lysosomal calcium in macrophages. *J Cell Sci* 2002;115:599-607.

55. Eckenhoff RG, Somlyo AP. Rat lung type II cell and lamellar body: elemental composition in situ. *Am J Physiol* 1988;254:C614-20.

56. Haynes CL, Buhler LA, Wightman RM. Vesicular Ca(2+) -induced secretion promoted by intracellular pH-gradient disruption. *Biophys Chem* 2006;23:20-4.

57. Kinnear NP, Boittin FX, Thomas JM, Galione A, Evans AM. Lysosome-sarcoplasmic reticulum junctions. A trigger zone for calcium signaling by nicotinic acid adenine dinucleotide phosphate and endothelin-1. *J Biol Chem* 2004;279:54319-26.

58. Sundler R. Lysosomal and cytosolic pH as regulators of exocytosis in mouse macrophages. *Acta Physiol Scand* 1997;161:553-6.

CHAPTER V

SUMMARY AND CONCLUSIONS

Type II cells synthesize, store and secrete lung surfactant. Earlier studies indicated that annexin A2, syntaxin 2, SNAP-23, VAMP-2, NSF and α-SNAP were involved in surfactant secretion. However, their organization on the cell membrane was not studied. The present study was designed to investigate the role of lipid rafts in the organization of surfactant secretion protein machinery. In the second part of work, flotillins, the lipid rafts marker proteins were selected for investigating their roles in surfactant secretion. Finally, the proteomic profiling of lipid rafts was performed to unravel the protein components of lipid rafts. Various subunits of V-ATPase were found in the proteomic profile along with other proteins. We have studied in detail the role of V-ATPases in regulating lung surfactant secretion.

We studied the expression profiles of flotillins and caveolins, the lipd raft marker proteins in isolated type II cells and rat lung tissue sections. Our results indicated that type II cells express flotillins but not caveolins and hence these proteins were used for confirming lipid rafts isolation. Lipid rafts were isolated based on their ability to resist 1% Triton X-100 solubilization and floatation on discontinous sucrose gradients. Isolated lipid rafts were characterized by enrichment of flotillin-1 and -2 and high cholesterol content. Furthermore, Na^+-K^+ATPase, a non-raft marker protein was completely excluded from lipid raft fractions. SNARE proteins were enriched in lipid rafts in a cholesterol dependent manner. Cholesterol depletion reduced surfactant secretion, fusion

pore formation and membrane fusion. Hence, lipid rafts integrity was essential for all these processes. Additionally, SNARE proteins co-localised with CD44 clusters. Annexin A2 was associated with lipid rafts in a Ca^{2+}-dependent manner and also with CD44 clusters. The results suggest that lipid rafts might provide an amicable atmosphere for SNARE protein interactions and organize membrane fusion machinery.

Flotillin-1 and -2 were highly enriched in type II cells when compared with type I cells. Additionally, their expression was abundant on plasma membranes and lung lamellar bodies, indicating that lipid rafts existed in the intracellular compartments also. We further studied the role of these proteins in surfactant secretion. To this end, the expression of flotillins was silenced using adenovirus-mediated RNAi. We successfully silenced the expression of flotillins in type II cells and the lung cell line, L2. Flotillin-2 but not -1 silencing reduced surfactant secretion by type II cells. To further undertand the mechanism of the decrease, lipid rafts were isolated following knockdown of flotillins in L2 cells. When analysed for SNARE protein association with lipid rafts, we did not find any significant differences following flotillin-1 and -2 silencing, indicating that SNARE protein association with lipid rafts was independent of flotillins. Since flotillins were involved with cholesterol homeostasis, we have assayed for cholesterol contents following silencing of flotillins. Cholesterol levels were unchanged after silencing. Thus it appears that flotillin-2 affects surfactant secretion independent of SNARE protein association and raft formation.

Lipid rafts contain only specific proteins, which are involved in diverse functions. We hence studied the proteomic profile of lipid rafts by mass spectrometry. Our results revealed that lipid rafts proteins are associated with vacuolar acidification, energy

metabolism, oxygen exchange and cell cycling. We selected V-ATPases, to study their roles in surfactant secretion. Following inhibition of V-ATPase by Baf A1, the lamellar body pH gradient was dissipated as indicated by the loss of quinacrine statining. V-ATPase inhibition resulted in increased surfactant secretion when compared with controls. The V-ATPase mediated increase in secretion was effectively inhibited by intracellular Ca^{2+}-chelator suggesting the mobilization of intracellular Ca^{2+} following V-ATPase inhibition. Additionally, staurosporine, and KN-62, inhibitors of PKC and CaMKII, respectively inhibited the V-ATPase mediated increase in secretion, indicating the activation of these enzymes following V-ATPase inhibition. When lamellar body pH was monitored following stimulation with a combination of secretagogues, we found a gradual increase in lamellar body pH, indicating that V-ATPase activity might be reduced during surfacatant secretion. Thus, we conclude that V-ATPase regulates surfactant secretion by type II cells. We proposed a mechanism of V-ATPase mediated surfactant secretion.

In summary, we for the first time implicated the role of lipid rafts in the organization of surfactant protein secretion machinery. Further, flotillin-2 had been found to be involved in surfactant secretion independent of lipid raft formation. Finally, V-ATPase, one of the proteins identified in the proteomic profile of lipid rafts has been shown to regulate surfactant secretion.

APPENDIX

PERMISSION GRANTED FOR THE PURPOSE INDICATED.

Chintagari NR, Jin N, Wang P, Narasaraju TA, Chen J, Liu L. 2006. Effect of cholesterol depletion on exocytosis of alveolar type II cells. American Journal of Respiratory Cell and Molecular Biology. 34(6):677-87.

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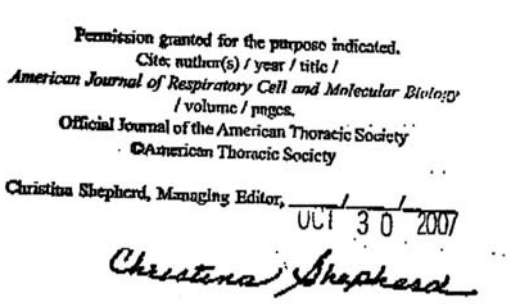

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The present study was initiated to elucidate the role of lipid rafts in lung surfactant secretion. Lipid rafts were isolated and probed for the association of SNARE proteins. Cholesterol depletion was done to study its role in modulating the surfactant secretion and membrane fusion. Flotillin proteins were silenced to examine their role in surfactant secretion. Finally, we determined the proteomic profile of type II cell lipid rafts to identify novel proteins in this unique subproteome and study the roles of one of the identified proteins, namely, v-ATPases, in surfactant secretion. We have used a number of techniques including RT-PCR, immunoblotting, immunoflorescence, secretion assay, membrane fusion assay, isolation of plasma membrane and lamellar bodies, and RNA interference to address the biological phenomenon.

Findings and Conclusions:

1. SNARE proteins were differentially associated with lipid rafts in a cholesterol-dependent manner.
2. Cholesterol depletion drastically reduced surfactant secretion, membrane fusion and fusion pore formation.
3. SNARE proteins were associated with CD44 clusters.
4. Flotillin-2 but not -1 reduced surfactant secretion.
5. Flotillin (s) did not affect the association of SNARE proteins with rafts.
6. Lipid raft formation was not altered by knockdown of flotillin-1 and -2.
7. Type II cell lipid raft proteome contained proteins involved in cell proliferation, pH regulation and energy metabolism.
8. V-ATPases mediate the acidification of lamellar bodies and V-ATPase inhibition leads to increased surfactant secretion.
9. V-ATPase inhibition leads to the mobilization of Ca^{2+} from lamellar bodies, which in turn cause further release of Ca^{2+} from ER. The net surge activates protein kinase C and Ca^{2+}/calmodulin-dependent kinase II leading to increased secretion.

ADVISER'S APPROVAL: Dr. LIN LIU