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Plasma Membrane Trafficking in Alveolar Type II Cells

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Key Words
Alveolar type II cells • ATP • Lamellar bodies • Plasma membrane • Endocytosis

Abstract
Alveolar type II (ATII) cells produce surfactant and release it into the alveolar space via exocytosis of lamellar bodies (LBs). On the other hand, various forms of endocytosis take place, enabling the recycling of surfactant as well as of integral membrane proteins to the LB. Here we investigated the trafficking of protein and lipid components of plasma membrane between the plasma and limiting LB membrane by over-expressing lysosomal associated membrane protein 3 fused to green fluorescence protein (LAMP-3-GFP) and farnesylated DsRed (DsRed-Farn). LAMP-3-GFP was homogenously distributed over the entire limiting LB membrane, whereas DsRed-Farn predominantly accumulated at the plasma membrane. However, in a minor LB fraction, DsRed-Farn was also found in discrete domains at its limiting membrane. Upon stimulation of ATII cells with secretagogues, the area of DsRed-Farn domains on LB surfaces increased 2 to 4 fold within 20 minutes of stimulation. This increase remained unaffected by phenylarsine oxide, an inhibitor of clathrin-dependent endocytosis, but was almost abolished by filipin and indomethacin, blockers of clathrin-independent endocytosis. It was also blocked by bafilomycin A1, wortmannin and LY294002, inhibitors of intra-cellular vesicular transport. We conclude that secretagogues facilitate the transport of plasma membrane components to LBs via a clathrin-independent vesicular transport pathway.

Introduction

The retrieval and recycling of plasma membrane is believed to be an essential step during biogenesis of exocytotic granules in exocrine cells [1]. In ATII cells, exocytosis of lamellar bodies (LBs) accounts for the secretion of surfactant phosholipids and the lipophilic surfactant proteins B and C into the alveolar lumen.

The surfactant lines the alveolar space as a thin surfactant film and reduces surface tension and hence enables inhalation of an appropriate air volume and prevents alveolar collapse. Besides the surfactant release, ATII

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cells also play a major role in clearance of lipids and surfactant from the alveolar space [2]. They were shown to incorporate extracellular lipids and surfactant associated proteins predominantly via clathrin-dependent pathways [3]. More recent studies showed that clathrin-independent endocytotic pathways might also be involved in the re-uptake of surfactant [4, 5].

After endocytosis of surfactant from the alveolar space, lipids and proteins are transported differently. Surfactant protein A (SP-A), for example, recycles via early and recycling endosomal organelles and the major fraction of endocytosed SP-A is re-secreted [6, 7]. On the other hand only a minor fraction of endocytosed lipids are re-secreted directly [6]. The lipids are destined primarily to LBs [4], and therefore surfactant uptake by ATII cells is not only involved in surfactant clearance from the alveolar space, but is also part of surfactant recycling. This recycling pathway covers only surfactant components, which are released by exocytosis from ATII cells.

Recycling not only involves secretory products, but also integral proteins of the limiting LB membrane: For example, surfactant secretagogues stimulate the transfer of the lipid transporter ABCA3 from the LB membrane to the cell surface and *vice versa* [8, 9]. This recycling pathway is clathrin-independent and is therefore different from the one, which was described for surfactant recycling from extracellular space [3-5].

The recycling of lipidic membrane components between the plasma and LB membrane, respectively, is yet unexplored. We used Ds-Red fusion protein with putative motif for farnesylation (DsRed-Farn). Since protein farnesylation is accompanied by the incorporation of protein to the inner leaflet of the plasma membrane [10, 11] the over-expressed fusion protein is a specific marker of the inner plasma membrane leaflet. We show here that DsRed-Farn is internalized upon secretagogue stimulation of ATII cells via clathrin-independent endocytosis. In addition, we demonstrate that incorporated plasma membrane is not internalized into the LB lumen but becomes part of the limiting LB membrane and forms distinct domains.

Materials and Methods

Generation of recombinant adenoviruses

Lamp-3 cDNA from rat were obtained from the resource centre and primary data base (RZPD, Berlin, Germany, clone IRBPp993D126D2). Vectors pDsRed-Monomer-F and pEGFP1-N1 were obtained from Clontech (Saint-Germaine-en-Laye, France). Vectors pDONR221 and pAd/CMV/V5-DEST were purchased from Invitrogen (Karlsruhe, Germany).

cDNA encoding DsRed-Farn were amplified from the vector pDsRed-Monomer-F using the primers DsRed-Farn-F2 (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGT CAG ATC CGC TAG CGC TAC CGG) and DsRed-Farn-R2 (GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC AGC TTG AGC TCG AGA TCT GGA TCC) using standard PCR methods. After isolation of PCR products, amplified cDNA were introduced into pDONR221 by *in-vitro* recombination using BP-clonase II enzyme mix (Invitrogen, Karlsruhe, Germany) and cloned in *E. coli* OmniMax™2-T1^R (Invitrogen, Karlsruhe, Germany). Thereafter, DsRed-Farn cDNA was transferred into adenoviral expression vector pAd/CMV/V5-DEST by *in-vitro* recombination using LR-clonase II enzyme mix (Invitrogen, Karlsruhe, Germany) and recombination products were cloned in *E. coli* OmniMax™2-T1^R (Invitrogen, Karlsruhe, Germany).

Multiple cloning site (MCS) and coding region of EGFP were amplified from plasmid pEGFP-N1 using primers MCSgfp-F1 (GGG ACA AGT TTG TAC AAA AAA GCA GGC TAT GCT GCT AGC GCT ACC GGA CTC AG) and MCSgfp-R1 (GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTT ACT TGT ACA GCT CGT CC) by standard PCR techniques. PCR products were introduced into pDONR221 by *in-vitro* recombination using BP-clonase II enzyme mix (Invitrogen, Karlsruhe, Germany) and cloned in *E. coli* OmniMax™2-T1^R (Invitrogen, Karlsruhe, Germany). Lamp-3 cDNA were amplified by standard PCR protocols using primers lamp-3-R3 (GGC GAC CGG TGG ATC GAT TCT CTG GTA TGC AGA TGA CTG ACG C) and lamp-3-F3 (GGA CTC AGA TCT CGA GAT GCC TGG GCA GAC CTC TGC AGT AG CTG). PCR products and recombinant plasmid pDONR221 containing MCS and EGFP-N1 were cleaved by restriction endo nucleases XhoI and BamHI (Fermentas, St. Leon-Rot, Germany) and recombined thereafter using T4-Ligase (Fermentas, St. Leon-Rot, Germany). Ligation products were cloned into *E. coli* XL1-blue (Stratagene, Waldbronn, Germany). cDNA encoding lamp-3-GFP fusion protein was integrated into adenoviral expression Vector pAd/CMV/V5-DEST by *in-vitro* recombination using LR-clonase II enzyme mix (Invitrogen, Karlsruhe, Germany) and recombination products were cloned in *E. coli* OmniMax™2-T1^R (Invitrogen, Karlsruhe, Germany).

Recombinant adenoviral expression vectors were isolated from *E. coli* cells using QIAGEN plasmid midi kit (Qiagen, Hilden, Germany). Plasmids were linearised using restriction endo nucleases PacI (New England Biolabs, Frankfurt, Germany). Linearised plasmids were transfected into 293A cells (Invitrogen, Karlsruhe, Germany) using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) without further purification directly after heat inactivation of PacI. Transfected cells were cultivated for 5 to 8 days and virus particles were isolated using ViraBind Adenovirus purification kit (Cell Biolabs, Heidelberg, Germany) according to manufacturer's protocol. Virus particles were eluted in elution buffer (Cell Biolabs, Heidelberg, Germany), and stored in elution buffer containing 10% glycerol in aliquots at -80°C.

Isolation and cultivation of ATII cells

Alveolar type II (ATII) cells were isolated from male Sprague-Dawley rats as described previously [12]. Animals of 180 to 200 g weight were anesthetized, treated with heparin, and lungs were cleared by perfusion. After lung lavage with wash solution I (phosphate buffered saline solution (PBS) containing 10 mM HEPES, 0.2 mM EGTA, 5 mM Glucose, pH 7.4) followed by wash solution II (PBS buffer containing 10 mM HEPES, 0.2 mM EGTA, 1.3 mM $MgSO_4$, 5 mM Glucose, pH 7.4), lungs were instilled twice with wash solution II containing elastase (30 U/ml) and trypsin (2 mg/ml) and incubated at 37°C. Protease reaction was stopped by mincing lungs in the presence of DNase and fetal calf serum (FCS) and cell suspension was sequentially filtered through cotton gauze and nylon meshes (150-, 20-, and 10-µm mesh size). Cells were pelleted by centrifugation at 130 x g for 8 min and resuspended in DMEM. Macrophages were removed by panning cell suspension to IgG coated Petri dishes at 37 °C. The unattached cells were removed, pelleted by centrifugation and resuspended in DMEM with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 24 mM $NaHCO_3$ (growth medium) and seeded on µ-dishes (ibidi, Germany) at a density of 40 cells per mm^2. Cells were transfected directly after seeding with recombinant adenoviruses (5x 10^5 PFU) and cultivated for two days.

Endocytosis assay

For time lapse experiments, ATII cells were washed twice with control solution (c-sol in mM: 140 NaCl, 5 KCl, 10 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, pH 7.4) and were mounted onto confocal microscope (described below). Experiments were performed at room temperature (21°C - 24°C). Images of unstimulated cells were taken at the beginning of each experiment. Afterwards cells were either stimulated by ATP (final concentration 100 µM, Sigma-Aldrich, Steinheim, Germany) or phorbol-12-myristat-13-acetate (PMA, final concentration 0.1 µM, Sigma-Aldrich, Steinheim, Germany) together with ionomycin (final concentration 5 µM, Sigma-Aldrich, Steinheim, Germany) or by PMA (0.1 µM) together with ionomycin (5 µM) and ATP (100 µM). Control cells were treated in a similar way, but remained unstimulated. Images were obtained 5 min, 10 min, 20 min and 30 min after application of drugs.

For testing blockers of endocytosis, cells were pre-incubated 20 min in a humidified incubator at 37°C and 5% CO_2 in growth medium either with 10 µM filipin, 300 µM indomethacine, 0.1 µM bafilomycin A1, 1 µM wortmannin, 70 µM LY294002 or 2 µM phenyl arsine oxide (PAO). Thereafter, cells were stimulated for 30 min by adding ATP (100 µM) to the growth medium and washed twice with control solution directly before investigation.

Immunostaining of EEA1

To combine DsRed-Farn labelling with early endosomal associated protein EEA1 immunostaining, isolated AT II cells were cultivated on 18-well µ-slide (ibidi, Germany) and transfected with DsRed-Farn. Depending upon the experimental setup cells remained untreated, or were pre-incupated at 37°C and 5% CO_2 in a humidified incubator with 1 µM wortmannin in growth medium for 30 min. Afterwards, cells were stimulated with 100 µM ATP in growth medium and were incubated in a humidified incubator. Control cells remained unstimulated. For EEA1 immunostaining the cells were washed with growth medium without FCS, then fixed in phosphate buffered saline (PBS; Biochrom AG, Berlin, Germany) containing 4% paraformaldehyde for 4 min and washed repeatedly with PBS, SLO-buffer (25 mM Hepes-KOH, pH 7.4, 115 mM potassium acetate, 2.5 mM MgCl) and SLO-buffer including 1mM Dithiothreitol (DTT, Plusone® Pharmacia Biotech, Uppsala, Sweden). For permeabilisation, cells were incubated 1 U/ml streptolysin in SLO-buffer with 1 mM DTT for 30 min at 37°C. After several washings with PBS the unspecific binding sites were blocked with 7% FCS in PBS for 1 h, followed by PBS washing. Afterwards, cells were incubated with 1:200 diluted rabbit anti-Early Endosomal Antigen 1 (N-terminal) primary antibody (Sigma-Aldrich, Steinheim, Germany) for 2 h at room temperature. Unbound antibody was washed away with PBS and cells were incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen, Karlsruhe, Germany) for 1 h at room temperature. Finally, cells were washed with PBS and incubated at 4°C over night in PBS.

Confocal Microscopy

A multi-beam confocal microscope (Visitron Systems Puchheim, Germany) was used to image ATII cells. This system included a Zeiss Meta LSM microscope with a 488 nm excitation laser combined with a 500-530 nm emission filter for detecting LAMP-3-GFP and Alexa 488 fluorescence labelled secondary antibody against α-EEA1 and a 543 nm excitation laser combined with a LP 560 nm emission filter for detecting DsRed-Farn. The setup also consisted of a Photometrics® Cascade II 512 camera (Photometrics, Tuscon, USA) and a planapochromate 100x oil immersion objective. A vtInfinity-2D confocal scanner (Visitron Systems GmbH) was used in combination with MetaMorph software (Visitron Systems GmbH, Pucheim, Germany).

Image analysis

Z-section reconstruction of images was done by MetaMorph Software. For image analysis the latest version of ImageJ (National Institute of Health, USA, http://rsb.info.nih.gov/ij) was used. The analysis was carried out as described previously [13]. Briefly, images were converted into 8 bit grey scale images. A region of interest (ROI) was manually drawn around the perimeter of clustered LBs. Background was estimated for each image individually as mean grey intensity over a representative area and was subtracted afterwards. Intensity gradients were highlighted by filtering the image with a Laplacian operator. The new gradient image was smoothed in order to filter out any noise speckling using a 3 x 3 mean filter and a binary image was generated by adjusting the threshold to prominent fluorescent particles. This procedure was carried out for each fluorescence channel separately. Areas of overlapping green and red fluorescence were estimated after combining the binarised images for green and red fluorescence channel by the boolean AND-function. The resulting pixels from this operation which are located inside the ROI represent areas on LB's surface of overlapping green and red fluores-

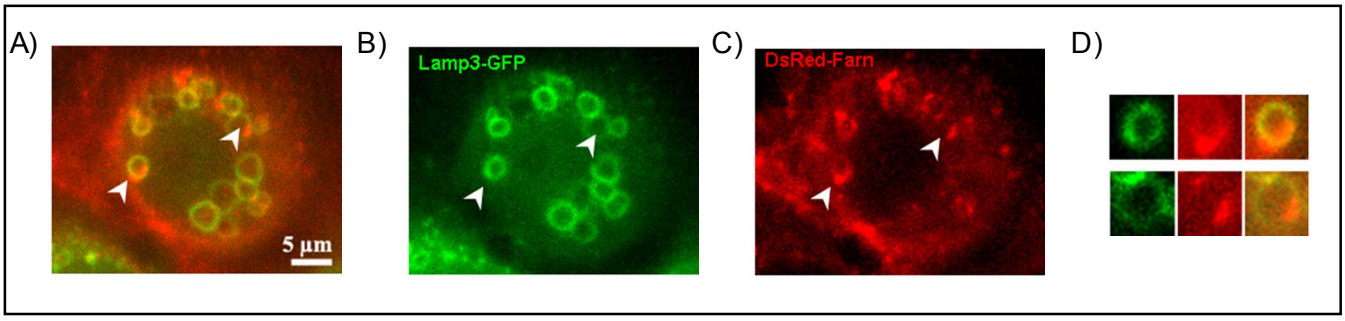

Fig. 1. LAMP-3-GFP and DsRed-Farn over-expression in ATII cells. A) Merged image of a representative ATII cell over-expressing LAMP-3-GFP (green fluorescence) and DsRed-Farn (red fluorescence). DsRed-Farn predominantly localizes at the plasma membrane, whereas LAMP-3-GFP accumulates at the limiting membrane of LBs. Arrowheads indicate LBs, which express DsRed-Farn and LAMP-3-GFP at their surfaces. B, C) Images of green and red fluorescence channel respectively. D) Magnified view of LBs depicted by arrowheads in A to C.

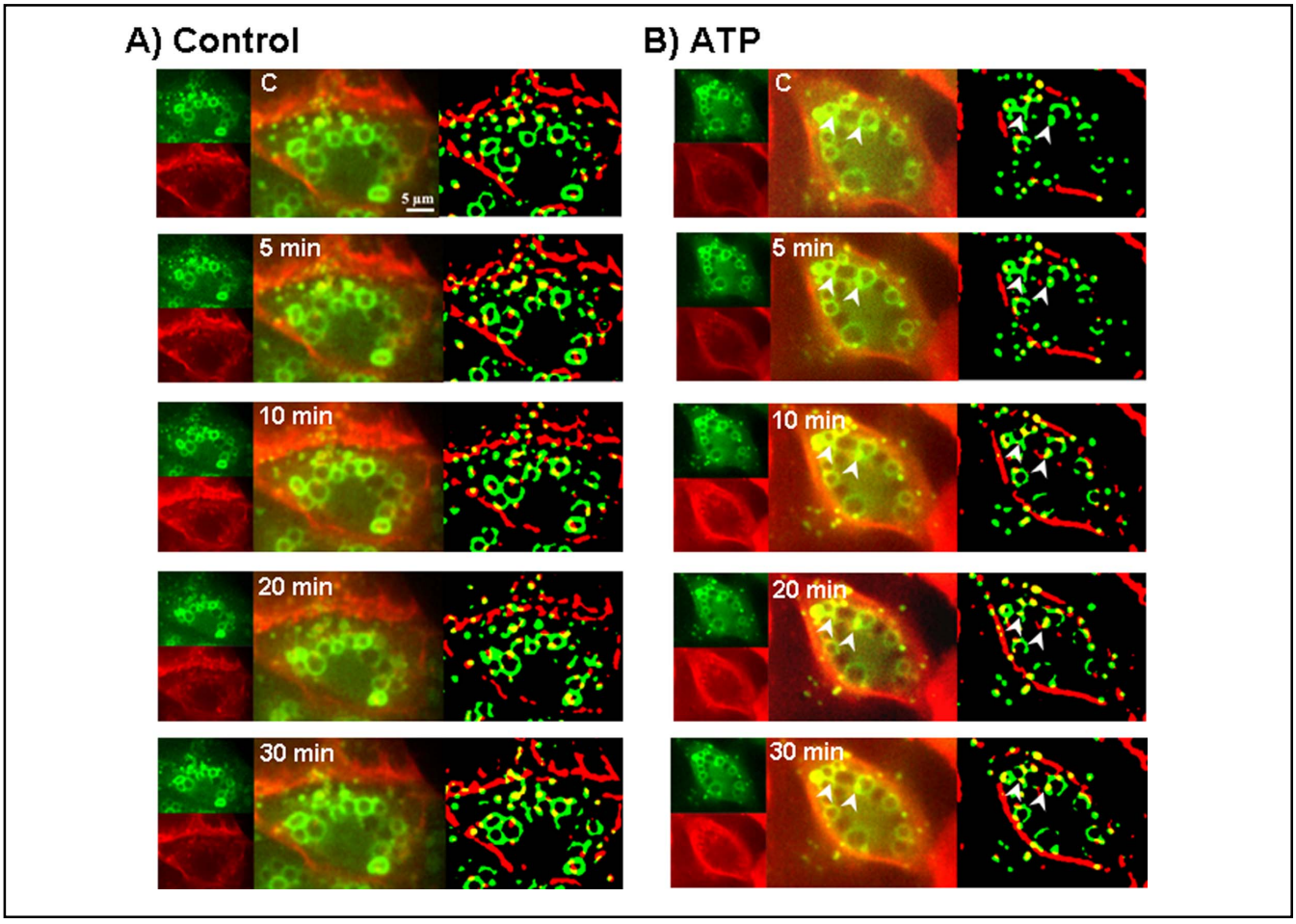

Fig. 2. Representative time lapse experiments of single ATII cells expressing DsRed-Farn and LAMP-3-GFP. Merged images are shown together with the images for each fluorescence channel (small insets: green = LAMP-3-GFP, red = DsRed-Farn). Binarised images for both red and green fluorescence channels were merged and shown as RGB images on the right hand side. A) Control cells (C = control solution at the beginning of the experiment, time points = time after adding additional control solution to the extracellular solution) B) Cells stimulated simultaneously with ATP (C = control solution at the beginning of the experiment, time points = time after adding ATP to the extracellular solution). Arrowheads highlight representative regions of increasing red fluorescence on LB's surface.

cence. Since the number of LBs per cell as well as the size of LBs vary, the red and green fluorescent area at the LB perimeter were normalised to the green fluorescent area at LB's outline according to the following equation RPA = $N_{(red+green)}/N_{(green)}$

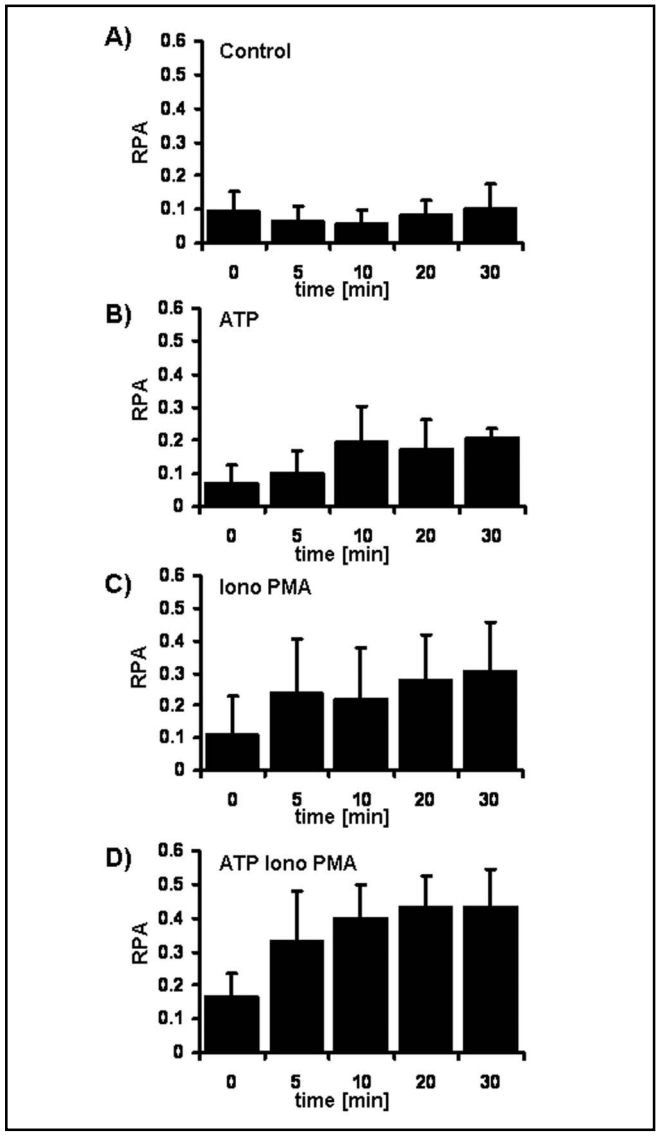

Fig. 3. Bar diagram represents relative pixel areas versus time from time lapse experiments. Relative pixel areas were measured as described in image analysis. Data are given as mean values ± SD. A) Control B) ATP C) ionomycin and PMA, D) ATP, ionomycin and PMA.

with RPA as the relative pixel area, $N_{(red+green)}$ as pixel number of red and green fluorescence and $N_{(green)}$ as number of pixels with green fluorescence.

Results

Heterologous over-expression of LAMP-3-GFP and DsRed-Farn

ATII cells over-expressing fusion protein DsRed-Farn showed an accumulation of red fluorescence signal at the plasma membrane, whereas LAMP-3-GFP over-expression resulted in a green fluorescence signal local-

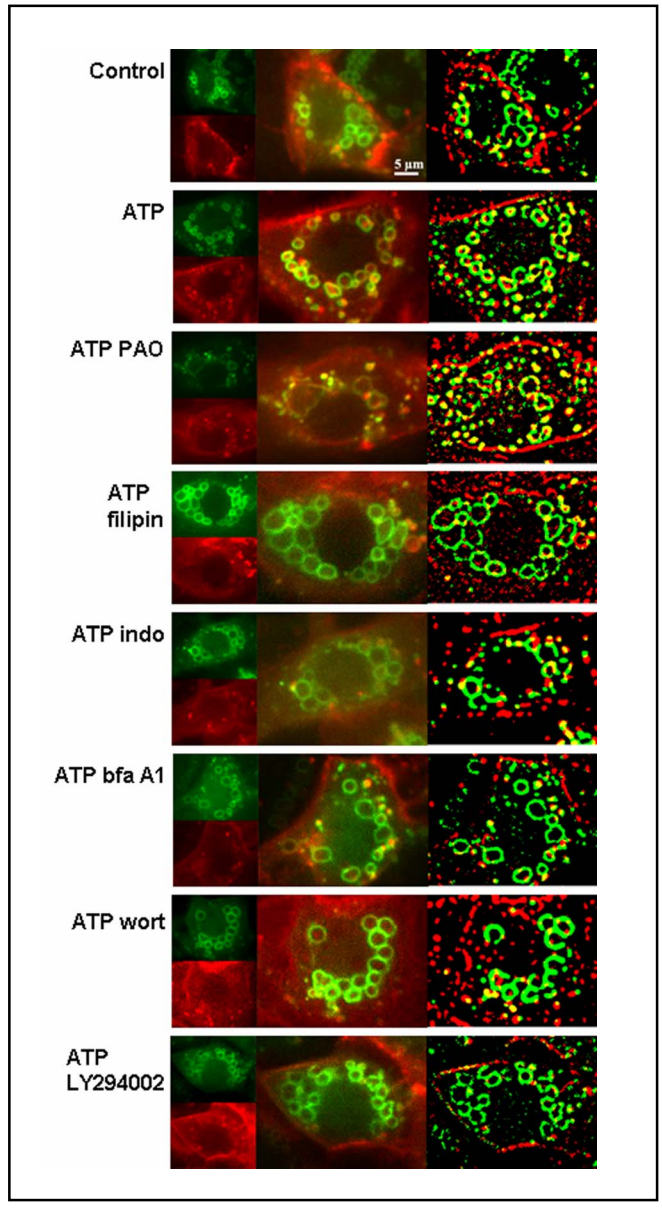

Fig. 4. Effect of blockers of endocytosis on ATP-induced DsRed-Farn uptake. For representative experiments merged images are shown together with the images for each single fluorescence channel (small insets: green = LAMP-3-GFP, red = DsRed-Farn). Binarised images for both red and green fluorescence channels were merged and shown as RGB images on the right hand side. Control = unstimulated control cells, ATP = cells after ATP stimulation for 30 min, ATP PAO = cells treated with phenyl arsine oxide for 20 min prior to ATP stimulation in the presence of PAO, ATP filipin = cells treated with filipin 20 min prior to ATP stimulation in the presence of filipin, ATP indo = cells treated with indomethacin 20 min prior to ATP stimulation in the presence of indomethacin, ATP bfa A1 = cells treated with bafilomycin A1 20 min prior to ATP stimulation in the presence of Bafilomycin A1, ATP wort = cells treated with wortmannin 20 min prior to ATP stimumlation in the presence of wortmannin, ATP LY = cells treated with LY294002 20 min prior to ATP stimulation in the presence of LY294002.

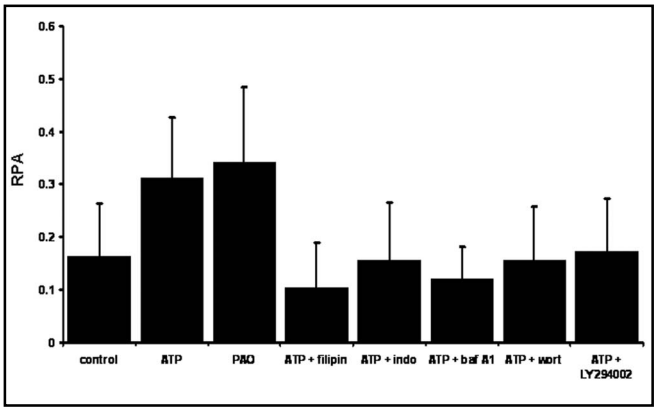

Fig. 5. Bar diagram represents relative pixel area RPA from experiments shown in Fig. 4. Data points are given as mean ± SD.

ised to the limiting membrane of LBs (Fig. 1). This staining pattern is consistent with the known targeted protein localization to LB and plasma membrane, respectively [14]. Both membrane compartments could be distinguished by their different fluorescent signals. However, in most cells a minor fraction of LBs exhibited both fluorescence fusion proteins at their limiting membrane. In these LBs the red fluorescence signal appears to localise at discrete areas at LB's surface rather than being equally distributed over the entire LB's membrane (Fig. 1D).

Secretagogues increase the amount of LBs labelled with DsRed-Farn

In order to test, if the accumulation of DsRed-Farn to LB's membrane is due to a regulated internalization of the red fluorescent fusion protein, we performed time lapse experiments using multi-beam confocal microscopy. In these experiments, ATII cells over expressing both LAMP-3-GFP and DsRed-Farn were stimulated with the secretagogues ATP or PMA + ionomycin or ATP + ionomycin + PMA. There was no significant increase of red fluorescence in control cells (Fig. 2A), whereas stimulation with secretagouges resulted in an increase of red fluorescence on the surface of LBs (example for ATP stimulation is given in Fig. 2B). Stimulation with ATP + ionomycin + PMA resulted in the fastest and strongest increase, consistent with the observation that this secretagogue is the most potent stimulation for LB exocytosis [15].

The accumulation of DsRed-Farn at the LB surface was measured in representative z-sections as pixels of overlapping red and green fluorescence at the limiting membrane of LBs and quantified as relative pixel area, RPA (Fig. 3). RPA was found to be 0.10 ± 0.05 (N = 9) in control cells at the beginning of experiment and did not change significantly even after 30 min (0.11 ± 0.07, N = 9). However, when cells were stimulated with secretagogues, RPA increased steadily over time and reached a steady state after 10 min to 20 min of stimulation. ATP led to an RPA increase from 0.07 ± 0.05 before ATP application to 0.21 ± 0.02 after 30 min of ATP stimulation (N = 9). RPA increased from 0.11 ± 0.1 to 0.31 ± 0.1, N = 6 after 30 min stimulation with ionomycin + PMA and from 0.16 ± 0.07 to 0.43 ± 0.1, N = 7 after 30 min exposure to ATP + ionomycin + PMA. In the investigated cell populations, RPA did not differ at time point 0 (before secretagogue application) when compared with each other or to control cells. After 30 min of stimulation, the difference in RPA was found to be highly significant when the investigated populations of stimulated cells were compared to control cells (students' t-test: 1.6 exp-4 for ATP vs. control, 0.017 for PMA + ionomycin vs. control and 5.1 exp-5 for ATP + ionomycin + PMA vs. control). These results indicate that secretagogue stimulation leads to DsRed-Farn accumulation on LBs.

Pathways of DsRed-Farn uptake by LBs

The translocation of the transporter ABCA3 from the cell surface to LBs was recently shown to depend on clathrin-independent endocytosis [9] and suggests that the uptake of the fluorescent fusion protein DsRed-Farn by LBs follows a similar mechanism. Since ATP is physiologically the most relevant secretory stimulus, we investigated the effect of blockers of endocytosis on DsRed-Farn uptake upon ATP stimulation. Cells were incubated in the presence of ATP with several blockers of clathrin-dependent as well as -independent endocytosis (Fig. 4), and the DsRed-Farn uptake was quantified by determination of the RPA (Fig. 5) as described above. RPA was 0.16 ± 0.1 (N = 73) under control conditions and 0.31 ± 0.11 (N = 69) after 30 min of ATP stimulation. In order to test, if clathrin-dependent endocytotic pathways are involved in DsRed-Farn uptake by LBs, ATII cells were stimulated in the presence of 2 μM phenylarsine oxide (PAO), an inhibitor of clathrin-dependent endocytosis [16-18]. In these cells RPA was 0.34 ± 0.14 (N = 54), which was not different from the RPA observed in ATP stimulated cells.

In contrast to PAO, filipin and indomethacin, both blockers of clathrin-independent endocytosis [19-21], abolished ATP induced uptake of DsRed-Farn by LBs almost completely (RPA 0.1 ± 0.09, N = 24 for filipin and 0.16 ± 0.1, N = 18 for indomethacin). Thus the uptake of DsRed-Farn depends on clathrin-independent endocyto-

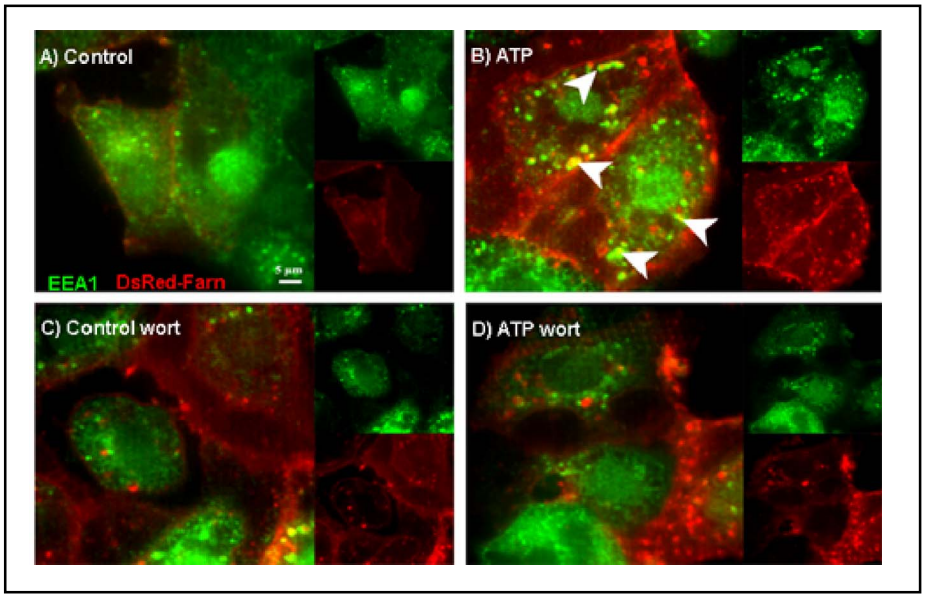

Fig. 6. Immunostaining of EEA1 in DsRed-Farn expressing ATII cells. Green fluorescence indicates the distribution of early endosomal antigen 1 (EEA1), red fluorescence corresponds to DsRed-Farn. A) Unstimulated control cells, B) ATII cells after 30 min ATP stimulation (arrow heads highlight granules at which DsRed-Farn and EEA1 colocalize), C) Wortmannin treated unstimulated cells, D) Wortmannin treated cells after 30 min ATP stimulation.

sis. Compounds, which impair intracellular trafficking, were also effective in inhibiting the accumulation of DsRed-Farn on LB's surface. The treatment of cells with bafilomycin A1, an inhibitor of the vacuolar H^+-ATPase [22], abolished ATP induced DsRed-Farn uptake (RPA : 0.12 ± 0.06, N = 20). Also wortmannin and LY294002, both inhibitors of type III phosphatidylinositol 3-kinase [23-25], blocked DsRed-Farn translocation to LB´s surface (RPA 0.16 ± 0.1, N = 35 for wortmannin and 0.17 ± 0.1, N = 42 for LY294002). These results give evidence that the translocation of DsRed-Farn from the plasma membrane to LB depends on vesicular transport processes.

Uptake of DsRed-Farn into endosomal compartments

In order to elucidate the fate of endocytosed DsRed-Farn, we performed immunocytochemical experiments in DsRed-Farn over-expressing ATII cells. In these experiments, we labelled early endosomal compartments using an antibody against the early endosomal associated protein EEA1 [26]. In non-stimulated ATII cells EEA1 staining resulted in a diffuse fluorescence pattern in the cytoplasm (Fig. 6A). The stimulation with ATP for 30 min caused an increase in number and size of EEA1 positive vesicular structures compared to control cells (Fig. 6B). ATP stimulation also caused a localization of DsRed-Farn to some EEA1 positive granules (Fig. 6B), which was never observed in control cells. The ATP induced localisation of DsRed-Farn to EEA1 positive granules was significantly diminished by pre-treatment of cells with wortmannin (Fig. 6D).

Discussion

Surfactant recycling from alveolar space is a well studied mechanism and was recently shown to depend on clathrin-dependent endocytosis [3-5]. Internalized surfactant protein A is predominantly transported back to the plasma membrane for resecretion, whereas lipids translocate to LBs [4, 6, 7]. In a recent study it was shown that proteins localized at the limiting LB membrane appear at the plasma membrane and are internalized afterwards, which was interpreted as a recycling of the limiting LB membrane after exocytosis [9].

However, it is unclear if the translocation of proteins between the plasma membrane and the LB membrane is a selective process that involves specific proteins exclusively, or if plasma membrane components are translocated in a less organized way, including lipid-anchored components as well.

To address this question we transiently over expressed LAMP-3 as a green fluorescent fusion protein (LAMP-3-GFP) in combination with a red fluorescent protein extended by a peptide motif for protein farnesylation (DsRed-Farn). The over-expressed LAMP-3-GFP was distributed over the entire limiting membrane of LBs as previously shown [14]. LAMP-3 is also described as CD63 and was shown to accumulate at inner membranes of late or multi-vesicular endosomes but also to secretory organelles like Weibel-Palate bodies [27] and secretory lysosomes [28]. In ATII cells LAMP-3/CD63 was shown to co-localize with the LB membrane marker ABCA3 [29]. Therefore, the observed LAMP-3-GFP localization to the LB membrane is in good agreement

with previous studies and can be used as a marker for the limiting membrane of LBs in living alveolar type II cells. The over-expression of DsRed-Farn was used to label the inner leaflet of the plasma membrane of the cell. Farnesylation of proteins occurs as a posttranslational protein modification [30, 31]. As a result of this modification, the protein is anchored to the inner leaflet of the plasma membrane, whereas the unmodified precursors remain in the cytoplasm and were not associated with any membrane [10, 11]. Thus, DsRed-Farn fusion proteins specifically label the inner leaflet of the plasma membrane.

Our experiments revealed that in DsRed-Farn over-expressing cells, a minor fraction of LBs showed a distinct area of red fluorescence on their limiting membrane, indicating the association of DsRed-Farn to the LB surface. Since it has been shown that protein farnesylation is accompanied by the coupling of the protein to the inner leaflet of the plasma membrane [10, 11, 30, 31], the capping of LBs by DsRed-Farn must be preceded by its translocation together with plasma membrane components. One might argue that the increase of relative pixel area (RPA) reported here could also be due to the fact that LAMP-3-GFP positive LBs are secreted in response to secretagogue stimulation, preceeding an increased uptake of DsRed-Farn by LBs, since this would also decrease the number of green fluorescent pixels to which the area of overlapping fluorescence was normalized. However, this possibility can be ruled out because stimulated LB exocytosis occurs only in a minor fraction of cells [32], in contrast to the reliable capping of LBs with DsRed-Farn. We were unable to detect significant amounts of LAMP-3 fluorescence at the plasma membrane after stimulation with secretagogues, indicating that this loss of green fluorescence from the LB membrane will only account for a minor over estimation of DsRed-Farn transport rate in stimulated ATII cells.

In order to test the effect of blockers of endocytosis on DsRed-Farn uptake in a standardized way, we measured RPA after 30 min of ATP stimulation, because this resulted in an approximately twofold increase of RPA. Phenyl arsine oxide (PAO) was shown to block clathrin-dependent endocytosis by cross-linking clathrin subunits [16-18]. In our experiments PAO was ineffective to block DsRed-Farn accumulation on LB surfaces, suggesting that ATP stimulated DsRed-Farn translocation is not clathrin-mediated. In contrast to PAO, filipin and indomethacin both effectively blocked ATP-induced DsRed-Farn capping of LBs. Filipin is reported to disrupt cholesterol rich membrane domains, which are essential for the caveolae formation [19, 20]. Indomethacin in turn is known to block the release of caveolae from the plasma membrane [21]. However, several reports stated that caveolin-1 is not expressed in ATII cells [3, 33]. Since caveolin-1 is essential for caveolae dependent endocytosis [34], caveolin-1 dependent mechanism of endocytosis can be presumed to be absent in ATII cells [3]. Therefore, it is unlikely that indomethacin acted by inhibition of this pathway. However, clathrin and caveolin independent endocytosis, which depends on cholesterol, has been reported as well [35]. These endocytotic pathways are sensitive to indomethacin [36]. Filipin also inhibits these cholesterol dependent pathways, most likely via cholesterol sequestration [35]. Thus it appears that LB capping by DsRed-Farn depends on plasma membrane uptake via clathrin and caveolae independent endocytosis. A similar uptake mechanism was recently described for ABCA3 recycling [9]. Interestingly, the induced membrane trafficking resulted in the accumulation of DsRed-Farn to discrete areas at LB surface and not in its random distribution over the entire limiting LB membrane. This implicates domain formation at LB surface of internalized plasma membrane components.

Endocytosed cargo is targeted to the early endosome, where it is designated for recycling to the plasma membrane and separated from others which have to be processed via endosomal/lysosmal pathways. Endocytosed surfactant protein A is separated from lipidic surfactant components before entering early endosome [7] and sent for resecretion back to the plasma membrane, whereas the lipidic surfactant content is further processed via endosmal/lysosomal pathways. Bafilomycin A1 was shown to prevent acidification of endosomal compartments by blocking vacuolar proton ATPases [22]. The vacuolar acidification in early endosomal organelles is considered essential for protein sorting [37]. By preventing early endosomal acidification, bafilomycin A1 prevents not only early endosomal sorting but also blocks the transport of endocytosed compounds to late endosomal organelles, whereas the uptake of particles into cells remains unaffected [38]. Here we report that bafilomycin A1 blocks the DsRed-Farn capping of LB. This result implies that the translocation of DsRed-Farn to LB surface depends on uptake and an appropriate processing in early endosomal compartments. We also demonstrate here that wortmannin and LY294002 both block the translocation of DsRed-Farn to LBs and we show that ATP induces the accumulation of EEA1 to DsRed-Farn positive vesicular compartments. This is in contrast to internalized ABCA3 proteins, which were reported not to co-

localize with EEA1 positive early endosome [7]. Thus, the internalized DsRed-Farn is processed seperately from ABCA3 proteins, which were recently discussed as a marker for LB membrane turnover [9, 39], which gives evidence that plasma membrane is transported differently from LB membrane components.

The EEA1 accumulation was abolished by wortmannin. Type III phosphatidylinositol 3-kinase inhibitors like wortmannin and LY294002 were shown to impair the accurate transport and processing of internalized material at different points along the endosomal/lysosomal transport route [23-25, 40, 41]. Wortmannin was recently shown to prevent homotypic fusion of early endosome [42] and to cause the dissociation of EEA1 from early endosomal vacuoles [41, 43]. Thus the observed blocking of DsRed-Farn transport from the plasma membrane to LBs by wortmannin and LY294002 can be explained by impaired processing at the early endosome. This hypothesis is supported not only by the fact that the homotypic fusion is the first wortmannin-sensitive step after internalization along the endosomal/lysosomal pathway but also by the observation that wortmannin prevents the aggregation of EEA1, which was shown in the immunocytochemical experiments.

The turnover of ABCA3 proteins in ATII cells was described to be preceded by surfactant exocytosis [9, 39]. However, the internalized ABCA3 transporters recycle back to existing LBs via endosomal organelles [39] but bypasses the early endosome [7]. In the present study, we demonstrate that DsRed-Farn is also internalized upon secretagogue stimulation into ATII cells and our results support its transport via the early endosome, which is in contrast to the previously reported transport route of ABCA3 proteins [7]. Furthermore, contrary to recycled ABCA3 proteins and internalized ambiphilic fluophores, DsRed-Farn is not incorporated into the lumen of LBs but co-localizes with LAMP-3-GFP, forming discrete domains at the limiting membrane of LBs. Lipidic material, which enters ATII cells, was shown to be transported towards LBs via a non-degradative route, which utilizes late endosome related organelles [44]. Furthermore, after secretagouge stimulation of ATII cells, ABCA3 proteins did not localize to lysosomal organelles after internalisation, but appears in LBs shortly after stimulation [39]. Thus a transport route to LBs, which is independent from classical lysosomal compartments exists in ATII cells and it is most likely, that the observed transport of DsRed-Farn described herein follows the same route, at least down stream from the early endosome. Since DsRed-Farn specifically labels the inner plasma membrane leaflet by a lipid anchor, this strongly suggests that DsRed-Farn is transported together with plasma membrane portions to be incorporated into the limiting membrane of LBs. We conclude that plasma membrane domains, which have been internalized can be transported differently from ABCA3 and converge at the limiting membrane of LBs. Why they form discrete "caps" and are not homogenously distributed within this membrane remains to be elucidated.

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References

1 Patzak A, Winkler H: Exocytotic exposure and recycling of membrane antigens of chromaffin granules: ultrastructural evaluation after immunolabeling. J Cell Biol 1986;102:510-515.

2 Rider ED, Ikegami M, Jobe AH: Localization of alveolar surfactant clearance in rabbit lung cells. Am J Physiol Lung Cell Mol Physiol 1992;263:L201-L209.

3 Stevens PA, Wissel H, Zastrow S, Sieger D, Zimmer KP: Surfactant protein A and lipid are internalized via the coated-pit pathway by type II pneumocytes. Am J Physiol Lung Cell Mol Physiol 2001;280:L141-L151.

4 Ruckert P, Bates SR, Fisher AB: Role of clathrin- and actin-dependent endocytotic pathways in lung phospholipid uptake. Am J Physiol Lung Cell Mol Physiol 2003;284:L981-L989.

5 Jain D, Dodia C, Fisher AB, Bates SR: Pathways for clearance of surfactant protein A from the lung. Am J Physiol Lung Cell Mol Physiol 2005;289:L1011-L1018.

6 Wissel H, Zastrow S, Richter E, Stevens PA: Internalized SP-A and lipid are differentially resecreted by type II pneumocytes. Am J Physiol Lung Cell Mol Physiol 2000;278:L580-L590.

7 Wissel H, Lehfeldt A, Klein P, Muller T, Stevens PA: Endocytosed SP-A and surfactant lipids are sorted to different organelles in rat type II pneumocytes. Am J Physiol Lung Cell Mol Physiol 2001;281:L345-L360.

8 Ban N, Matsumura Y, Sakai H, Takanezawa Y, Sasaki M, Arai H, Inagaki N: ABCA3 as a Lipid Transporter in Pulmonary Surfactant Biogenesis. J Biol Chem 2007;282:9628-9634.

9 Bates SR, Tao JQ, Schaller S, Fisher AB, Shuman H: Lamellar body membrane turnover is stimulated by secretagogues. Am J Physiol Lung Cell Mol Physiol 2000;278:L443-L452.

10 Fujiyama A, Tamanoi F: Processing and fatty acid acylation of RAS1 and RAS2 proteins in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 1986;83:1266-1270.
11 Magee T, Hanley M: Protein modification. Sticky fingers and CAAX boxes. Nature 1988;335:114-115.
12 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.
13 Bertrand CA, Laboisse C, Hopfer U, Bridges RJ, Frizzell RA: Methods for Detecting Internalized, FM 1-43 Stained Particles in Epithelial Cells and Monolayers. Biophys J 2006;91:3872-3883.
14 Miklavc P, Wittekindt OH, Felder E, Dietl P: Ca^{2+}-dependent actin coating of lamellar bodies after exocytotic fusion: a prerequisite for content release or kiss-and-run. Ann N Y Acad Sci 2009;1152:43-52.:43-52.
15 Frick M, Eschertzhuber S, Haller T, Mair N, Dietl P: Secretion in Alveolar Type II Cells at the Interface of Constitutive and Regulated Exocytosis. Am J Respir Cell Mol Biol 2001;25:306-315.
16 Visser CC, Stevanovic S, Heleen VL, Gaillard PJ, Crommelin DJ, Danhof M, De Boer AG: Validation of the transferrin receptor for drug targeting to brain capillary endothelial cells in vitro. J Drug Target 2004;12:145-150.
17 Takano M, Koyama Y, Nishikawa H, Murakami T, Yumoto R: Segment-selective absorption of lysozyme in the intestine. Eur J Pharmacol 2004;502:149-155.
18 Hertel C, Coulter SJ, Perkins JP: A comparison of catecholamine-induced internalization of beta- adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells. Inhibition by phenylarsine oxide. J Biol Chem 1985;260:12547-12553.
19 Rothberg KG, Ying YS, Kamen BA, Anderson RG: Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. J Cell Biol 1990;111:2931-2938.
20 Schnitzer JE, Oh P, Pinney E, Allard J: Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. J Cell Biol 1994;127:1217-1232.
21 Smart EJ, Estes K, Anderson RG: Inhibitors that block both the internalization of caveolae and the return of plasmalemmal vesicles. Cold Spring Harb Symp Quant Biol 1995;60:243-8.:243-248.
22 Harada M, Shakado S, Sakisaka S, Tamaki S, Ohishi M, Sasatomi K, Koga H, Sata M, Tanikawa K: Bafilomycin A1, a specific inhibitor of V-type H^+-ATPases, inhibits the acidification of endocytic structures and inhibits horseradish peroxidase uptake in isolated rat sinusoidal endothelial cells. Liver 1997;17:244-250.
23 Brown WJ, DeWald DB, Emr SD, Plutner H, Balch WE: Role for phosphatidylinositol 3-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. J Cell Biol 1995;130:781-796.
24 Reaves BJ, Bright NA, Mullock BM, Luzio JP: The effect of wortmannin on the localisation of lysosomal type I integral membrane glycoproteins suggests a role for phosphoinositide 3-kinase activity in regulating membrane traffic late in the endocytic pathway. J Cell Sci 1996;109:749-762.
25 Johnson EE, Overmeyer JH, Gunning WT, Maltese WA: Gene silencing reveals a specific function of hVps34 phosphatidylinositol 3-kinase in late versus early endosomes. J Cell Sci 2006;119:1219-1232.
26 Mu FT, Callaghan JM, Steele-Mortimer O, Stenmark H, Parton RG, Campbell PL, McCluskey J, Yeo JP, Tock EPC, Toh BH: EEA1, an Early Endosome-Associated Protein. J Biol Chem 1995;270:13503-13511.
27 Kobayashi T, Vischer UM, Rosnoblet C, Lebrand C, Lindsay M, Parton RG, Kruithof EKO, Gruenberg J: The Tetraspanin CD63/lamp3 Cycles between Endocytic and Secretory Compartments in Human Endothelial Cells. Mol Biol Cell 2000;11:1829-1843.
28 Griffiths GM: Secretory lysosomes - a special mechanism of regulated secretion in haemopoietic cells. Trends Cell Biol 1996;6:329-332.
29 Matsumura Y, Ban N, Ueda K, Inagaki N: Characterization and Classification of ATP-binding Cassette Transporter ABCA3 Mutants in Fatal Surfactant Deficiency. J Biol Chem 2006;281:34503-34514.
30 Fujiyama A, Tsunasawa S, Tamanoi F, Sakiyama F: S-farnesylation and methyl esterification of C-terminal domain of yeast RAS2 protein prior to fatty acid acylation. J Biol Chem 1991;266:17926-17931.
31 Fujiyama A, Tamanoi F: RAS2 protein of Saccharomyces cerevisiae undergoes removal of methionine at N terminus and removal of three amino acids at C terminus. J Biol Chem 1990;265:3362-3368.
32 Wemhoner A, Frick M, Dietl P, Jennings P, Haller T: A Fluorescent Microplate Assay for Exocytosis in Alveolar Type II Cells. J Biomol Screen 2006;11:286-295.
33 Dahlin K, Mager EM, Allen L, Tigue Z, Goodglick L, Wadehra M, Dobbs L: Identification of Genes Differentially Expressed in Rat Alveolar Type I Cells. Am J Respir Cell Mol Biol 2004;31:309-316.
34 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;293:2449-2452.
35 Nabi IR, Le PU: Caveolae/raft-dependent endocytosis. J Cell Biol 2003;161:673-677.
36 Llorente A, van Deurs B, Garred O, Eker P, Sandvig K: Apical endocytosis of ricin in MDCK cells is regulated by the cyclooxygenase pathway. J Cell Sci 2000;113:1213-1221.
37 Presley JF, Mayor S, McGraw TE, Dunn KW, Maxfield FR: Bafilomycin A1 Treatment Retards Transferrin Receptor Recycling More than Bulk Membrane Recycling. J Biol Chem 1997;272:13929-13936.
38 Bayer N, Schober D, Prchla E, Murphy RF, Blaas D, Fuchs R: Effect of bafilomycin A1 and nocodazole on endocytic transport in HeLa cells: implications for viral uncoating and infection. J Virol 1998;72:9645-9655.
39 Schaller-Bals S, Bates SR, Notarfrancesco K, Tao JQ, Fisher AB, Shuman H: Surface-expressed lamellar body membrane is recycled to lamellar bodies. Am J Physiol Lung Cell Mol Physiol 2000;279:L631-L640.
40 Christoforidis S, McBride HM, Burgoyne RD, Zerial M: The Rab5 effector EEA1 is a core component of endosome docking. Nature 1999;397:621-625.
41 Mills IG, Jones AT, Clague MJ: Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes. Curr Biol 1998;8:881-884.
42 Jones AT, Mills IG, Scheidig AJ, Alexandrov K, Clague MJ: Inhibition of Endosome Fusion by Wortmannin Persists in the Presence of Activated rab5. Mol Biol Cell 1998;9:323-332.
43 Simonsen A, Lippe R, Christoforidis S, Gaullier JM, Brech A, Callaghan J, Toh BH, Murphy C, Zerial M, Stenmark H: EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature 1998;394:494-498.
44 Wissel H, Looman AC, Fritzsche I, Rustow B, Stevens PA: SP-A-binding protein BP55 is involved in surfactant endocytosis by type II pneumocytes. Am J Physiol Lung Cell Mol Physiol 1996;271:L432-L440.