Ca²⁺ Induced Surfactant Secretion in Alveolar Type II Cultures Isolated from the H-2K^b-tsA58 Transgenic Mouse

Paul Jennings[#], Cristina Bertocchi[#], Manfred Frick, Thomas Haller, Walter Pfaller and Paul Dietl

Department of Physiology, Innsbruck Medical University, [#]Authors contributed equally to this manuscript

Key Words

Alveolar Type II • Calcium • Cell Culture • Exocytosis • Fluorescence • Lung • Mouse • Microscopy • Surfactant

Abstract

Background/Aims: There is a need for the development of transgenic mice to elucidate molecular mechanisms in surfactant secretion. However at present very little is known about the regulation of surfactant exocytosis in murine alveolar type II (AT II) cells. **Methods:** We brought AT II cells isolated from the Immorto mouse into culture at 33°C, in the presence of interferon, to generate immortal mouse AT II cells (iMAT II). Surfactant secretion was measured using real-time fluorescence imaging. **Results:** iMAT II cells stained with lysotracker green (LTG), a dye specific for lamellar body related vesicles in rat AT II cells. Expression of densely layered structures, characteristic of LBs, was confirmed by transmission electron microscopy. Flash photolysis of caged Ca²⁺, which specifically elevates intracellular Ca²⁺ concentration ([Ca²⁺]_i), resulted in LB fusion to the plasma membrane, as analysed using the lipid staining dye FM 1-43. Purinergic stimulation with ATP (10 μM), also resulted in a rise in [Ca²⁺]_i (measured

by fura-2), which was followed by LB fusion. **Conclusions:** iMAT II cells maintain the expression of LBs over several passages. Surfactant secretion in these cells is regulated by [Ca²⁺]_i and exhibits similar characteristics to that of rat AT II cells. These cells will be beneficial in studying the impact of genetic modifications on regulated surfactant secretion.

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Introduction

The primary function of alveolar type II (AT II) cells is the synthesis and secretion of surfactant, a complex mixture of mainly unsaturated phospholipids and specific surfactant proteins (SPA-D), essential for respiration by reducing the surface tension of the air-liquid interface in the alveoli [1, 2]. In addition, surfactant facilitates fluid reabsorption from the alveolar lumen, maintains alveolar epithelium integrity and participates in host defence [3, 4]. Deficiency or dysfunction of the surfactant system causes severe diseases such as respiratory distress syndrome in infants and adults (IRDS, ARDS). Exogenous surfactant preparations are clinically used in the treatment of IRDS [5].

Surfactant is stored in specialised organelles called lamellar bodies (LBs) and is secreted into the alveolar lumen by regulated exocytosis [6-8]. A growing body of evidence exists for cytosolic Ca^{2+} as the most important factor for triggering LB exocytosis. Firstly, physical strain of AT II cells, which is considered to be the most important physiological stimulus of surfactant secretion, is Ca^{2+} dependent [9]. Secondly, various agonists, such as ATP, which stimulate surfactant secretion, do so in a Ca^{2+} dependent fashion (reviewed in [1, 10]). However, little is known about the Ca^{2+} induced fusion machinery in AT II cells due to the lack of widely available AT II cell models.

A multitude of studies performed in systems ranging from intact animals to isolated AT II cells (mostly from rat) have focused on the dynamic and complex system of surfactant metabolism, secretion and turnover [11]. In particular, short term primary cultures of rat AT II cells offer the possibility to study surfactant release on individual cells and allow biomolecular analysis of the processes involved. However, the isolation of rat type II cells is laborious, expensive and animal intensive. This technique does not lend itself well to long term studies as AT II cells dedifferentiate quickly (within 1 to 2 days). AT II cells in culture in particular lose the expression of LBs and acquire alveolar type I cell features [12-14]. Thus, the production of long-term cell cultures of differentiated AT II cells has proved difficult. One strategy which has been used successfully is to isolate AT II cells from the Immorto mouse (H-2K^b-tsA58 mouse) [15]. AT II cells isolated and cultured from the Immorto mouse have been reported to retain most of the morphological properties of AT II cells including LB expression and the production and release of the surfactant proteins, SP-A, SP-B and SP-C [15].

Here we revisited the use of the Immorto mouse to establish long term AT II cell cultures. Besides morphological studies for AT II cell characterization, we used recently developed fluorescence techniques to investigate the process of Ca^{2+} induced surfactant exocytosis in these cells, with high temporal and spatial resolution [6]. In our hands mouse AT II cells retained functionally intact LBs and exhibited regulated surfactant secretion over several passages.

Materials and Methods

Materials

The fluorescent dyes lysotracker green (LTG), fura-2 AM, FM 1-43 and the photoactivable (caged) Ca^{2+} compound

ortho-nitrophenyl [NP]-EGTA-AM were obtained from Molecular Probes, Leiden, Netherlands. All other materials were purchased from Sigma, Vienna, Austria and were of the highest analytical grade possible.

Cell Isolation and culture

AT II cells were isolated from a single adult female H-2K^b-tsA58 transgenic mouse lung, according to a method previously described for the rat [16]. (The mouse was a kind gift from Professor Flucher, Department of Physiology, Innsbruck Medical University). In these mice the tsA58 mutant of SV40 large T antigen is controlled by the interferon-inducible Class I antigen promoter [17]. Cells were seeded on human collagen IV (5 $\mu\text{g}/\text{cm}^2$) coated cell culture dishes in DMEM with 5 mM glucose, supplemented with 7% FCS and 10 ng/ml human EGF. Cells were incubated in a 5 % CO_2 humidified incubator at 37°C. 3 days later the cells were transferred to permissive conditions to turn on the expression of simian virus 40 large tumour antigen, by supplementing the medium with 10 ng/ml mouse interferon gamma and incubation at 33°C. Under these conditions the cells continued to proliferate and were designated immortal mouse AT II (iMAT II). Cells were sub-cultured by trypsinisation and seeded on collagen coated dishes (propagation) or glass coverslips (fluorescent imaging). Cells were split usually at a 1:4 ratio. Using a resazurin based assay we estimate the doubling time to be 31 hours. Cells at passage 7 to 12 were used for experiments in this study.

Type II cell characterisation

A) Preparation for transmission electron microscopy (TEM). iMAT II cells were cultured on nitrocellulose membranes to confluence. After washing with PBS, monolayers were fixed for 30 min with 1% glutaraldehyde in PBS at pH 7.4 and postfixed (30 min) with 1% OsO_4 in sodium cacodylate (0.1M) buffer. Following dehydration in a graded series of ethanol the cell monolayers were embedded in Polybed (Polysciences, Warrington, PA, USA). After polymerization parts of the cell layers were re-embedded in flat embedding moulds for subsequent ultra-thin sectioning. Sections were cut with an ultramicrotome (Reichert OmU 3, Lecia Mikrosysteme, Vienna, Austria) followed by lead-citrate and uranyl-acetate staining and examined with a LEO EM-10 electron microscope at 80 KV.

B) Fluorescence staining of intracellular LBs. To visualize intracellular LBs we used the fluorescence dye LTG as described in detail recently [6, 18]. In brief iMAT II cells cultured on glass cover slips were incubation with 100 nM LTG for approx. 30 min in bath solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 5 mM glucose, 10 mM HEPES, pH 7.4). Images were acquired using an Axiovert 100 inverted microscope (Carl Zeiss GmbH, Göttingen, Germany) equipped with a CCD camera (Spot; Visitron, Munich, Germany).

Simultaneous measurements of surfactant secretion and fura-2 ratio

Exocytosed surfactant (i.e. LB content following LB fusion with the plasma membrane) was stained by adding 1 μM FM 1-43 (lipid specific dye) to the non-perfused bath solution [6]. Determination of LB fusion by FM 1-43 fluorescence is based

on the cell-impermeant, surfactant staining, properties of FM 1-43, resulting in localized fluorescence after LB fusion with the plasma membrane, when FM 1-43 enters lamellar body contents from the bath solution through the fusion pore [6]. Importantly, FM 1-43 is non-fluorescent in aqueous solutions, enabling fusion to be monitored in the continuous presence of the dye in the bath. In this study, exocytotic responses were assessed by counting fluorescence spots in conjunction with measuring fura-2 ratios, using an inverted microscope equipped with a 2-D-imaging system (T.I.L.L. Photonics, Gräfelfing, Germany), as previously described [8]. Cells were preincubated with fura-2 AM (1 μ M, for 15 min in DMEM at 33°C), a Ca^{2+} sensitive dye used to visualise Ca^{2+} in living cells, and fura-2 ratios were calculated by dividing emission intensities obtained at 350 and 380 nm excitation, respectively, after subtracting background fluorescence at each wavelength. Images were acquired with 40x Fluar Objective (Carl Zeiss GmbH, Göttingen, Germany) at a rate of 0,3 Hz at each excitation wavelength (350 nm and 380 nm for fura-2, 480 nm for FM 1-43) for 50 ms each at room temperature. Because there were few single exocytotic events per cell in response to a given stimulus, data from many single cells were pooled to produce the exocytosis response time histogram.

Flash photolysis of caged Ca^{2+}

Flash photolysis of caged Ca^{2+} was performed as described previously [19]. Briefly, cells were loaded with the photoactivable (caged) Ca^{2+} compound *ortho*-nitrophenyl [NP]-EGTA-AM (10 μ M, for 30 min in DMEM at 33°C) before the onset of experiment. Caged Ca^{2+} was released using a pulsed xenon arc lamp (pulse length 0.5 ms, wavelength $\sim$ 320 to 390 nm). Since the power of the fura-2 excitation light required to obtain 2-D images was of sufficient light intensity to itself uncage the compound, we did not monitor fura-2 fluorescence prior to flash photolysis.

Statistical analysis

Results are represented as arithmetic means $\pm$ standard errors of the mean (SEMs). For statistical analysis we used an unpaired Student's t-test. $P < 0.05$ was considered as significantly different.

Results

Morphology

Immortalised mouse alveolar type II cells (iMAT II) isolated from the H-2K^b-tsA58 were cultured for 12 passages at 33°C with interferon gamma. iMAT II exhibited a cobble stone morphology, which is characteristic of epithelial cells (Figure 1 A). By phase contrast morphology numerous peri-nuclear vesicles could be observed in a large subset of the cell population as demonstrated in Figure 1 B. Examination by transmission electron microscopy demonstrated the presence of densely layered vesicular bodies, a characteristic of LB

Fig. 1. Morphology of iMAT II cells. Phase contrast morphology of iMAT II cells demonstrate characteristic cobble stone morphology (A) and at increased magnification, perinuclear localisation of vesicular structures are seen (B). Transmission electron microscopy (C), demonstrates the expression of a group of densely layered vesicular bodies (arrow) which is consistent with lamellar body morphology. Bars indicate magnification level.

structure. A representative image is given in figure 1 C. (Such structures were observed in every section, from 3 to 6 blocks of 3 independent preparations).

Fig. 2. LTG fluorescent staining pattern of iMAT II cells. iMAT II cells were incubated with LTG for 30 min and examined for fluorescent staining. On the left panels are the corresponding phase contrast images. Three different cell staining patterns were evident. Panel A demonstrates vibrant cell staining corresponding directly to large vesicles observed under phase contrast. Panel B shows many, slightly more diffuse stained vesicles, which corresponds to vesicles observed under phase contrast. Also, very small bright spots are visible which are most likely non LB related lysosomes. Panel C shows cell staining in smaller vesicles which do not correlate to structures observed under phase contrast.

Fluorescent labelling of LBs in living iMAT II cells

LTG stains acidic vesicles and has previously been demonstrated to selectively stain LB related vesicles in primary culture rat AT II cells [6]. In figure 2, 3 representative staining patterns are shown. In figure 2 A and B there is a predominant staining in large vesicles which correspond to structures observed under phase contrast microscopy. A few very small bright spots are also visible (figure 2, B). Since LTG stains all acidic compartments, fluorescent spots of such small size could also represent a lysosomal population unrelated to LBs. Figure 2 C shows a cell with vesicles staining positive for LTG, which have no obvious correlation to the

corresponding phase contrast image. These vesicles are smaller than those in figure A and B and thus may represent an earlier stage in LB development. Due to their smaller size these vesicles may be unresolved under phase contrast microscopy. Since maintenance of acidic pH is an energy dependent process this staining demonstrates that these vesicles are functionally intact [20].

LB fusion in iMAT II cells

A) Ca^{2+} -induced fusion. Flash photolysis of NP-EGTA produced a selective and rapid increase of $[Ca^{2+}]_i$ in all intact iMAT II cells ($n=150$, figure 3 C) and

Fig. 3. Ca^{2+} -induced LB exocytosis. LB exocytosis response-time histograms (right y axis) and fura-2 ratios (left y axis) triggered by flash photolysis of caged Ca^{2+} (NP-EGTA-AM) (A) or purinergic stimulation (ATP 10 mM) (B). Time = 0 denotes ultraviolet (UV)-flash, or ATP addition to bath solution. Pre-flash fura-2 ratios were not measured as the excitation light for fura-2 in our imaging system is strong enough to itself elicit photolysis of caged Ca^{2+} . In the absence of caged compounds, UV-flash had no effect (not shown). Data were pooled from 135 and 174 cells for A and B, respectively. Dotted line indicates standard errors of fura measurement. C: Percentage of cells showing an elevation of $[\text{Ca}^{2+}]_i$ with flash photolysis of caged Ca^{2+} (100 %, $n = 135$) and purinergic stimulation (70.80 ± 9.34 %, $n = 174$).

stimulated a pronounced exocytotic activity. Continuous monitoring of $[\text{Ca}^{2+}]_i$ and LB fusions revealed a temporal correlation between the decay of the Ca^{2+} -signal and the decay of triggered exocytotic activity in iMAT II cells, albeit fusion activity slightly outlasts the Ca^{2+} -signal. Mean fura-2 ratio $\pm$ SEM and the number of corresponding fusion events (%) following quick release of Ca^{2+} are illustrated in figure 3 A. The quantitative differences in fusion activity following different stimulation is illustrated in figure 5. Flash photolysis of caged Ca^{2+} elicited significantly (P value = 0.04) more fusions per cell (0.34 ± 0.13 , $n = 135$ in 5 experiments) than observed in control cells (“constitutive” = 0.02 ± 0.02 , $n = 55$ in 4 experiments).

B) Purinergic stimulation. Purinergic stimulation of rat AT II cells has been shown to elicit strong fusion activity. Stimulation of iMAT II cells with 10 μM ATP

revealed two populations of cells, that can be distinguished by their ability to elicit a Ca^{2+} -signal following purinergic stimulation (responders = 70.80 ± 9.34 %, $n = 174$ in 9 experiments). As indicated in figure 5 only responders revealed a significantly enhanced fusion response following stimulation (0.30 ± 0.08 fusions per cell, $n = 174$ in 9 experiments) compared to control cells (constitutive = 0.02 ± 0.02 , $n = 55$ in 4 experiments, P value = 0.04). Fusion events per cell in non-responders (0.09 ± 0.06 , $n = 34$ in 6 experiments) are not significantly increased (P value = 0.41), providing further evidence that vesicle fusion is linked to an elevation of $[\text{Ca}^{2+}]_i$, as previously shown in primary rat AT II cells [21]. Compared to the rapidly declining Ca^{2+} -signal elicited by flash photolysis, the ATP-induced Ca^{2+} -signal consisted of an initial peak followed by a gradual decrease towards baseline (figure 3 B). There is a slight increase in fusion activity following the initial peak, revealing a correlation

Fig. 4. Simultaneous fluorescence measurements of fura-2 and FM 1-43 in a single AT II cell. iMAT II cells were loaded with fura-2 AM (1 μ M), washed and replenished with fresh buffer containing FM 1-43 (1 μ M). Above is shown fura-2-ratio images (350/380nm) of iMAT II cells before (a) and immediately after (b) ATP stimulation (10 μ M). A fluorescence intensity scale is shown left. There is a clear increase in $[Ca^{2+}]_i$. FM 1-43 images (480 nm) are also shown before (c) and 10 min after ATP stimulation (d). The appearance of new FM 1-43 spots is indicative of LB exocytosis (arrows). In the graph the intensity of the fura-2 signal from the framed cell is plotted over time on the left y axis and the intensity of the three FM 1-43 spots from (d) are plotted on the right y axis (grey). Thus ATP causes a quick peak in $[Ca^{2+}]_i$ followed slowly by LB fusion to the plasma membrane.

to the sustained Ca^{2+} -signal (figure 3 B). A representative example of one such experiment is given in figure 4.

Discussion

Exocytosis of LBs from AT II cells is crucial to maintain a low alveolar surface tension and to facilitate lung inflation. The study of LB exocytosis is still severely hampered by a rapid change of the cell phenotype in primary cultures, resulting in a loss of secretory function. Primary cultures from mouse type II cells, which would be useful to elucidate the effects of genetic modifications, are particularly difficult to study because of a poor yield of cells during the isolation procedure. The creation of a stable *in vitro* mouse AT II model is therefore highly desirable. To this end we isolated and cultured AT II cells from a H-2K^b-tsA58 adult mouse, and used newly

developed fluorescent techniques [6, 8] to assess functional characteristics of mouse surfactant regulation.

By phase contrast microscopy, iMAT II cells exhibited a cobble stone morphology when confluent and many of these cells expressed large vesicular bodies (figure 1). Additionally, TEM revealed densely layered structures within these vesicles, consistent with the morphology typical for lamellar bodies (figure 1 C) [22]. Densely layered vesicular structures are the most specific sign of the phenotype of alveolar type II cells. Moreover, as LBs belong to a population of vesicles classified as “secretory lysosomes”, which maintain an acidic pH (usually below pH 6.1), we used LTG, a dye which accumulates in acidic compartments [23], for further support of our morphological findings. We found LTG staining of vesicles in iMAT II which co-localised with vesicles visible under the light microscope (figure 2 A,B). Other cells demonstrated LTG staining in smaller vesicles,

which showed no correlation to structures observed under the light microscope (figure 2 C). These cells may represent a population which are at an earlier stage in LB development. Similar LTG accumulation in LBs as those represented in figure 2 A and B has been demonstrated previously in primary rat AT II cultures [6]. These findings together provide strong support that these vesicles are LBs.

$[Ca^{2+}]_i$ is the main stimulus for LB fusion to the plasma membrane both in agonist induced [24, 25] and mechanical stretch induced [21, 26] surfactant release. In order to investigate functional aspects of surfactant regulation in iMAT II we assessed the impact of caged Ca^{2+} and purinergic stimulation on LB fusion to the plasma membrane. LB fusion to the plasma membrane was visualised in real-time using the fluorescent dye FM 1-43. This dye fluorescently stains lipids but is non fluorescent in aqueous solutions. Thus when a LB docks to the plasma membrane forming a fusion pore [18, 24], FM 1-43 in the bath solution gains access to the lipid filled LB, which is visualised as a bright fluorescent spot [6]. The FM 1-43 stained spots immediately disappeared when FM 1-43 is removed from the bath solution (not shown), thus endocytosis can be excluded as a cause for these spots. Here, cells were preloaded with the Ca^{2+} sensitive dye, fura-2 AM, to simultaneously measure $[Ca^{2+}]_i$ and LB fusion. Flash photolysis of caged Ca^{2+} (using NP-EGTA) in iMAT II cells resulted in induction of FM 1-43 fluorescent spots (figures 3a and 5). Similar results have been demonstrated in rat primary AT II cells where Ca^{2+} -triggered exocytosis of LBs occurs when $[Ca^{2+}]_i$ exceeds a threshold of approx. 320 nM [19], suggesting the involvement of a high-affinity Ca^{2+} sensor. Purinergic stimulation of iMAT II cells with ATP (10 μ M) resulted in an immediate peak in $[Ca^{2+}]_i$ in a sub-population of cells (figures 3, 4 and 5). In these Ca^{2+} responding cells, there followed the appearance and sustained fluorescence of new FM 1-43 spots. The time course of these events is similar to those previously documented in primary rat AT II cells and is consistent with the theory that Ca^{2+} induces delayed gradual fusion of LBs [8, 24]. However, the purpose of this study was to investigate functional and morphological characteristics of iMAT II cells with respect to persistent differentiation, rather than elucidating Ca^{2+} -induced LB fusion kinetics in detail, which is the topic of a subsequent study. These results represent the first functional analysis of stimulus-secretion-coupling in mouse AT II cells, in particular the coupling between $[Ca^{2+}]_i$ and LB fusion.

Fig. 5. Quantitative analysis of exocytosis. Data is expressed as LB fusion events/cell from pooled data, within 10 min of stimulation by ATP or flash photolysis of caged Ca^{2+} (0.34 ± 0.11 , $n = 135$), and without stimulation (0.02 ± 0.02 , $n = 55$). “ Ca^{2+} resp.” (0.30 ± 0.08 , $n = 174$) and “ Ca^{2+} non resp.” (0.09 ± 0.06 , $n = 34$) refer to cells with or without a Ca^{2+} response following ATP stimulation (10 μ M). * denotes P value < 0.05 .

Isolation of cells from the Immorto mouse allows the possibility to bring specific cell types into long term culture, which is otherwise difficult. In addition, the ability to generate hybrids of Immorto mice with other transgenic mice allows the study of genetic modification *in vitro*.

In conclusion, AT II cells isolated from the Immorto mouse and cultured over 12 passages, retain a level of functional differentiation that is appropriate to study surfactant secretion. iMAT II cells, morphologically resemble cultured AT II cells both at the light and electron microscopic level. They contain vesicular bodies which stain with LTG, proving that these vesicles maintain an acidic pH. Increasing $[Ca^{2+}]_i$ by either flash photolysis of caged Ca^{2+} or by purinergic stimulation results in an increase in LB plasma membrane fusion as assessed using FM 1-43, thus proving that surfactant secretion in iMAT II cells is stimulated by Ca^{2+} . The combination of long term mouse AT II cell cultures together with real-time LB imaging in living cells represents a promising tool for the elucidation of genetic factors leading to debilitating pulmonary diseases.

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