Basolateral pressure challenges mammary epithelial cell monolayer integrity, in vitro

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Abstract Mammary gland epithelium is physiologically exposed to variations of hydrostatic pressure due to accumulation of milk and removal by suckling and mechanical milking. Integrity of the mammary gland epithelium primarily relies on the tight junction. To analyze pressure-induced effects on the tight junction, we established a modified Ussing chamber and tested the hypothesis if hydrostatic pressure on the basal side of the epithelium is able to affect barrier properties in a mammary epithelial cell model, in vitro. Therefore, a conventional Ussing chamber was modified by an additional tube system to apply hydrostatic pressure. Monolayers of the mammary epithelial cell line HC11 were mounted in the modified Ussing chambers and incubated with increasing basal hydrostatic pressure. Transepithelial resistance and short circuit current were recorded and compared to controls. Hydrostatic pressure was stably applied and incubation steps of 30 min were technically feasible,

leading to a decrease of transepithelial resistance and an increase of short circuit current in all monolayers. In a series of experiments simulating the physiological exposure time by short intervals of 5 min, these electrophysiological findings were also observed, and monolayer integrity was not significantly perturbed as analyzed by fluorescence immunohistochemistry selectively staining tight junction proteins. Moreover, electrophysiology demonstrated reversibility of effects. In conclusion, the modified Ussing chamber is an adequate method to analyze the effects of hydrostatic pressure on epithelial cell monolayers, in vitro. Both, the reduction of transepithelial resistance and the increase of short circuit current may be interpreted as protective reactions.

Keywords Barrier function · Tight junction · Ussing chamber · Hydrostatic pressure · Mammary gland · HC11

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Introduction

For analysis of transport and barrier function of epithelia, the Ussing chamber represents a powerful and widely distributed electrophysiological setup which allows the measurement of active transport processes, and clamping of potential in order to avoid the influence of transport-generated changes of electrochemical gradients (Ussing and Zerahn 1951).

Several improvements in epithelial electrophysiology have been performed, with some of the most sophisticated represented by the discrimination of epithelial versus subepithelial barrier function (Gitter et al. 1998), allowing the analysis of the epithelial barrier along the longitudinal axis of the intestine and regarding local characteristics (Markov et al. 2010, 2016). Moreover, a determination of trans- and paracellular barrier function was developed (Gitter et al. 2000), leading to the first identification of a paracellular channel (Amasheh et al. 2002). Whereas transport and barrier function can be analyzed in detail with the Ussing chamber technique, perturbation of the monolayer mostly is accomplished by the addition (or removal) of single factors, as e.g. shown for the effects of proinflammatory cytokines (Barmeyer et al. 2004), hormones (Amasheh et al. 2004), secondary plant metabolites and microbial toxins (Amasheh et al. 2010, 2012; Markov et al. 2014).

Analyses of the effects of mechanical forces often depend on flow chamber experiments applying shear stress or centrifugal forces (Brakemeier et al. 2003). These studies allow e.g. the discrimination of effects in venous and arterial endothelium (Partridge et al. 2007; James et al. 2011). Whereas these approaches can also be employed in epithelial models, information on simultaneous analysis of dynamical changes of functional properties in real time is scarce. Moreover, approaches to analyze epithelial exposure to hydrostatic pressure compared to physiological conditions in mammary glands are not available.

In mammary gland alveolar epithelium, milk accumulation and removal cause high hydrostatic pressure changes of more than 50 mmHg, which the epithelium has to stand up to (Tucker et al. 1961). To accomplish these purposes, a stable barrier integrity allowing selective and adjustable paracellular barrier function is necessary, which is provided by the tight junction. Effects of hydrostatic pressure on tight junction proteins have been studied *in vivo*, recently (Markov et al. 2012), and single milk components have been identified to affect epithelial barrier properties, *in vitro* (Dittmann et al. 2014). A first approach has been demonstrated previously, employing an Ussing chamber-based setup for the analysis of mechanical stress in amphibian lung, applying pressure of 5 cm water column (Bogdan et al. 2008). As mammary gland epithelial cells during lactation regularly can be exposed to more than 10 times higher pressure

changes, a method had to be established to analyze effects of hydrostatic pressure of 6 kPa and more on barrier function of epithelia, *in vitro*. To simulate the situation of milk removal and the associated stress e.g. in mechanical milking (Enokidani et al. 2016) a relative under pressure had to be applied with our setup. Thus, our hypothesis was that hydrostatic pressure on the basal side of the epithelium is able to affect barrier properties in a mammary epithelial cell model, analyzed *in vitro* with our modified Ussing chamber technique.

Materials and methods

Cell lines and handling

The HC11 cell line (Ball et al. 1988) is a mouse mammary gland epithelial cell line clonally derived from the COMMA-1D cell line, (Danielson et al. 1984). These cells can be cultured for an unlimited number of passages (Williams et al. 2009). Unless stated otherwise, cells were grown in growth medium, which comprises RPMI 1640 medium (PAN, Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 100 U/mg penicillin/streptomycin per ml (Biochrom), 5 µg insulin per ml and 10 ng EGF per ml (Biochrom), and maintained in an atmosphere of 5% CO₂ at 37 °C.

Cells were seeded on 12 mm diameter PCF cell culture inserts of 0.4 µm pore size (Merck Millipore, Darmstadt, Germany), grown until confluency with growth medium and maintained without supplementation of EGF (induction medium) until they reached an age of 7 and 14 days, respectively. The transepithelial resistance (R_T) was manually measured using an EVOM (World Precision Instruments, Sarasota, FL, USA). Blank value was subtracted and data were converted to $\Omega \cdot \text{cm}^2$.

Ussing chamber

A classical commercially available Ussing chamber setup was employed as a functional prerequisite for analyses, as reported recently (Markov et al. 2014). Briefly, electrophysiological measurements were obtained by a microcomputer-controlled voltage/current clamp (supplied by Dipl.-Ing. K. Mußler, Aachen, Germany). Two KCl Gelrite™ bridges

Fig. 1 Schematic view of the modified Ussing chamber (A) A tube system (1) was linked to the reservoir (2), which could be filled with different levels of buffer solution through a connected syringe (3). The manometer (4) was linked next to the samples via a flexible tube (5); chamber (6) containing cell insert, electrodes measuring voltage (7) and short circuit current (8). During bilateral pressure incubation with 10 kPa, 15 min after addition of fluorescein, fluorescence intensity did not significantly change (B) and pH remained constant (C). Data represent mean $\pm$ SEM, ($n = 8$)

were placed near to each side of the epithelium and connected to reference electrodes for the measurement of the transepithelial potential difference (PD_T). Two platinum electrodes were placed distantly for transmitting current through the tissue. The junction potential and the fluid resistance of the buffer between the tips of the PD_T -sensing bridges were determined before the tissue was mounted and were subsequently corrected by the computer-controlled voltage clamp. The tissue was alternatively pulsed with a positive or negative pulse of 100 μA and 200 ms duration. The displacement in PD_T caused by the current pulse was measured, and the transepithelial resistance was calculated from the change in PD_T due to Ohm's law. The pulse amplitude PD_T (mV), short circuit current I_{sc} ($\mu A/cm^2$) and R_T ($\Omega \cdot cm^2$) were continuously recorded. After an equilibration period of 10 min under open-circuit conditions, the epithelia were short-circuited. Based on this setup, chambers were routed for mounting Millicell PCF permeable supports (Kreusel et al. 1991; Amasheh et al. 2002).

To apply hydrostatic pressure in the Ussing chamber, a flexible tube system was linked to the U-shaped double walled glass tube containing the bathing solution, and a manometer was attached near the chamber. The tube system could be filled with different levels of bathing solution and thus induce hydrostatic pressure at a range from 1 to 10 kPa, where 1 kPa was defined as base level (Fig. 1A). Gas flow was adjusted to the higher pressure and fluctuation of pressure produced by gas flow was minimized using thick tubes in the tube system. Ussing chamber experiments were carried out using a computer-controlled voltage clamp device.

Fluorescein distribution and pH-gradient in modified Ussing chamber

To evaluate stability of buffer flow and pH in the modified Ussing chambers, 10 kPa bathing solution were filled into both sides of the chambers, gassed and maintained at experimental temperature. 100 μl of a fluorescein solution (0.376 g/l) were added chamber-near using a syringe. After a time interval of 15, 30, 45

and 60 min samples of 5 ml bathing solution from near the chamber were collected. Immediately pH was measured using a pH 3110 SET 1 (WTW, Weilheim, Germany) and samples for fluorescein quantification were taken. Afterwards bathing solution was slowly refilled into the chambers, minimizing artificial flow effects. Fluorescein quantification was performed with an EnSpire (PerkinElmer, Waltham, MA, USA) and bathing solution without fluorescein was used as blank value.

Basal challenge of epithelial integrity

Cell inserts were mounted into modified Ussing chambers and 1 kPa bathing solution was filled to both sides (base level). Equilibration was performed under voltage clamp conditions until R_T was stable. In a first series of tests cells were used 7 days after seeding. Hydrostatic pressure was increased five times to the basal side of the cell inserts in steps of +1 kPa, with an incubation time of 30 min in between (30 min-challenge, 7 days). After the last incubation step (+5 kPa) the hydrostatic pressure was removed for 5 min. In a second series the same experimental protocol was used with 14 day-old cells (30 min-challenge, 14 days). In the main experimental setup, using 14 day-old cells, the incubation time between basal applications was reduced to 5 min, with an additional 5-min incubation time after equalization of pressure (5 min-challenge, 14 days). Controls were treated equally without an application of hydrostatic pressure above the base level of 1 kPa (Online Resource, Suppl. Figure 1).

Solutions and drugs

The bathing solution for Ussing chamber experiments contained (in mmol/l): 113.6 NaCl, 2.4 Na_2HPO_4 , 0.6 NaH_2PO_4 , 25 NaHCO_3 , 5.4 KCl, 5 Glucose, 1.2 CaCl_2 , 1.2 MgCl_2 , 5 HEPES. The solution was adjusted to $\text{pH } 7.4 \pm 0.1$ with HCl. Temperature was maintained at 37 °C using water bath and solution was gassed with 95% O_2 and 5% CO_2 .

Verification of tight junction proteins by immunofluorescence

For immunofluorescent detection of tight junction proteins, samples of the 5 min-challenge were taken

after the ending point of the experimental setup. Cells were immediately fixed with ethanol, then permeabilized with phosphate-buffered saline (PBS, pH 7.4) containing 0.5% Triton X-100. Unspecific proteins were blocked with 5% goat serum diluted in PBS. For detection of tight junction proteins, mouse anti-ZO-1, rabbit anti-claudin-3 and mouse anti-occludin primary antibodies, and Alexa fluor 594 goat anti-rabbit (red) and Alexa fluor 488 goat anti-mouse (green) secondary antibodies were employed (dilution 1:500); (Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Control stainings were accomplished (1) employing isotype control antibodies in accordance with the manufacturers' instructions and (2) without primary antibodies. Immunofluorescence microscopy was performed with a Leica DMI6000 B microscope (Leica, Wetzlar, Germany). Quantitative image analysis was accomplished employing Image J (Image J Software, National Institutes of Health, Bethesda, MD, USA). To accomplish this, signal intensity of monochromatic tight junction protein immunofluorescent staining images of identical size and resolution were analyzed.

Statistical analysis

Average of R_T and I_{SC} were calculated for the last minute of every incubation step and equalization time. Current direction was corrected, if necessary due to technical reasons. Data are presented as mean $\pm$ SEM (standard error of the mean) of these results. Significance was tested by the unpaired Student's *t* test and *p* values <0.05 were considered to be significant.

Results

Application of hydrostatic pressure, fluorescein distribution and pH-gradient in modified Ussing chambers

Intended levels of hydrostatic pressure were successfully induced with the modified Ussing chamber system. Prerequisite for functional measurements was a constant milieu regarding solute and ion composition. Therefore we first tested the hypothesis, that our setup provided constant conditions, without generation of gradients due to gas or fluid flow effects.

During pressure incubation with 10 kPa, 15 min after chamber-near application of a fluorescein solution into the modified Ussing chamber, fluorescein concentration did not significantly change for the next 45 min and pH remained constant (Fig. 1B, C; $n = 8$, respectively).

Transepithelial electrical resistance

In all experimental setups stepwise application of 1 kPa hydrostatic pressure to the basal side of the epithelium led to an immediate and persistent decrease of R_T , beginning after the first application. The first application induced a strong reduction of R_T , from 335 ± 36 to $56 \pm 7 \Omega \cdot \text{cm}^2$ in the 30 min-challenge, on day 7, and from 512 ± 26 to $84 \pm 25 \Omega \cdot \text{cm}^2$ in the 30 min-challenge, on day 14, ($n = 6$; Fig. 2A). In the 5 min-challenge, on day 14, a decrease of R_T from 332 ± 35 to $49 \pm 5 \Omega \cdot \text{cm}^2$ was measured at that point, ($n = 9$; Fig. 3A). The decrease started directly after application and reached a minimum very fast, after which R_T was stable or started to rise again. 5 min after pressure equalization R_T was still lower than for controls, but showed a significant increase compared to 5 kPa in the 30 min-challenge, on day 7, (from 15 ± 2 to $73 \pm 8 \Omega \cdot \text{cm}^2$) and the 5 min-challenge, on day 14, (from 22 ± 2 to $101 \pm 13 \Omega \cdot \text{cm}^2$).

Short circuit current

Stepwise increase of hydrostatic pressure by 1 kPa led to an increase of I_{SC} in all experimental series, beginning after the first application and with a maximum after the fifth application. In general, the increase started directly after application and slowed down during incubation time or reached a plateau. 5 min after pressure equalization I_{SC} was not significantly higher than controls in the 30 min-challenge, on day 14, and showed a significant decrease compared to 5 kPa in the other two approaches. In the 30 min-challenge, on day 7, I_{SC} showed a decrease after pressure equalization from 37.6 ± 8.6 to $4.9 \pm 2.5 \mu\text{A}/\text{cm}^2$ ($n = 6$; Fig. 2B) and in the 5 min-challenge, on day 14, I_{SC} decreased from $7.0 \pm 4.3 \mu\text{A}/\text{cm}^2$ at 5 kPa to $-3.5 \pm 0.8 \mu\text{A}/\text{cm}^2$ in 5 min of pressure equalization, ($n = 9$; Fig. 3B). Controls showed no significant change of I_{SC} over the experimental time in all experiments.

Fig. 2 R_T (A) and I_{SC} (B) of the 30 min-challenge in 7 and 14 day old-cells compared to controls (significance indicated by asterisks) and after 5 min of pressure equalization (eq) compared to 5 kPa (significance indicated by hash signs). Bars represent mean $\pm$ SEM, ($p < 0.05$, $^{**}/\#\#$ $p < 0.01$, $^{***}/\#\#\#$ $p < 0.001$, $n = 6$). **A** In 7 day-old HC11 cells, R_T decreased from initial 335 ± 36 to $56 \pm 7 \Omega \cdot \text{cm}^2$ after the first application of 1 kPa basal hydrostatic pressure, followed by $24 \pm 2 \Omega \cdot \text{cm}^2$ at 2 kPa, $18 \pm 2 \Omega \cdot \text{cm}^2$ at 3 kPa, $17 \pm 2 \Omega \cdot \text{cm}^2$ at 4 kPa, $15 \pm 2 \Omega \cdot \text{cm}^2$ at 5 kPa, and an increase to $73 \pm 8 \Omega \cdot \text{cm}^2$ after pressure equalization. In 14 day-old cells, R_T decreased from initial 512 ± 26 to $84 \pm 25 \Omega \cdot \text{cm}^2$ at 1 kPa, followed by $28 \pm 9 \Omega \cdot \text{cm}^2$ at 2 kPa, $14 \pm 2 \Omega \cdot \text{cm}^2$ at 3 kPa, $10 \pm 2 \Omega \cdot \text{cm}^2$ at 4 kPa, $7 \pm 2 \Omega \cdot \text{cm}^2$ at 5 kPa and $23 \pm 11 \Omega \cdot \text{cm}^2$ after pressure equalization. **B** In 7 day-old cells, I_{SC} increased from $-2.7 \pm 1.5 \mu\text{A}/\text{cm}^2$ to $0.3 \pm 1.7 \mu\text{A}/\text{cm}^2$ at 1 kPa, followed by $9.3 \pm 3.4 \mu\text{A}/\text{cm}^2$ at 2 kPa, $17.0 \pm 5.3 \mu\text{A}/\text{cm}^2$ at 3 kPa, $26.5 \pm 6.0 \mu\text{A}/\text{cm}^2$ at 4 kPa, $37.6 \pm 8.6 \mu\text{A}/\text{cm}^2$ at 5 kPa and a decrease to $4.9 \pm 2.5 \mu\text{A}/\text{cm}^2$ after pressure equalization. In 14 day-old cells, I_{SC} increased from $-6.4 \pm 0.1 \mu\text{A}/\text{cm}^2$ to $-2.2 \pm 2.3 \mu\text{A}/\text{cm}^2$ at 1 kPa, followed by $7.1 \pm 6.2 \mu\text{A}/\text{cm}^2$ at 2 kPa, $21.3 \pm 12.3 \mu\text{A}/\text{cm}^2$ at 3 kPa, $36.0 \pm 18.7 \mu\text{A}/\text{cm}^2$ at 4 kPa, $49.4 \pm 24.1 \mu\text{A}/\text{cm}^2$ at 5 kPa and $24.3 \pm 21.9 \mu\text{A}/\text{cm}^2$ after pressure equalization

Fig. 3 R_T (**A**) and I_{SC} (**B**) of the 5 min-challenge in 14 day old-cells compared to controls (significance indicated by *star* signs) and after 5 min of pressure equalization (eq) compared to 5 kPa (significance indicated by *hash* signs). Bars represent mean ± SEM, ($\#p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 9$). **A** After the first application of 1 kPa basal hydrostatic pressure, R_T decreased from initial 332 ± 35 to $49 \pm 5 \Omega \cdot \text{cm}^2$, followed by $34 \pm 2 \Omega \cdot \text{cm}^2$ at 2 kPa, $27 \pm 2 \Omega \cdot \text{cm}^2$ at 3 kPa, $24 \pm 2 \Omega \cdot \text{cm}^2$ at 4 kPa, $22 \pm 2 \Omega \cdot \text{cm}^2$ at 5 kPa and an increase to $101 \pm 13 \Omega \cdot \text{cm}^2$ after pressure equalization. **B** I_{SC} increased from $-5.7 \pm 0.4 \mu\text{A}/\text{cm}^2$ to $-3.6 \pm 1.1 \mu\text{A}/\text{cm}^2$ at 1 kPa, followed by $-0.9 \pm 2.0 \mu\text{A}/\text{cm}^2$ at 2 kPa, $1.6 \pm 2.8 \mu\text{A}/\text{cm}^2$ at 3 kPa, $4.1 \pm 3.7 \mu\text{A}/\text{cm}^2$ at 4 kPa, $7.0 \pm 4.3 \mu\text{A}/\text{cm}^2$ at 5 kPa and a decrease to $-3.5 \pm 0.8 \mu\text{A}/\text{cm}^2$ after pressure equalization

Immunofluorescence microscopy

Immunofluorescence microscopy was used to examine structural condition of the HC11 monolayers after basal application of hydrostatic pressure in the 5 min-interval approach and to localize tight junction proteins, compared to controls. Integrity of all monolayers was preserved throughout the experiments unless otherwise noted. In controls, ZO-1 (Fig. 4A),

Fig. 4 Immunofluorescent staining of the 5 min-challenge in 14 day old-cells after ending point of the experiments compared to controls, ($n = 3$, respectively). **A** Integrity of the monolayer after pressure incubation shown by ZO-1 staining (green). **B** Claudin-3 (Cldn-3) (red) and occludin (Ocln) (green) were continuously located in the apicolateral membrane and showed no marked changes after pressure incubation. Nuclei were stained with DAPI (blue; bar: 20 μm). (Color figure online)

occludin and claudin-3 (Fig. 4), were continuously located in tight junctions, indicating integrity of the monolayer. After basal pressure incubation, all tight junction proteins were still located in the apicolateral membrane areas and could be detected in tight junctions after pressure incubation without marked changes in concentration or distribution, ($n = 3$), which was confirmed by quantitative image analysis,

(Online Resource, Suppl. Figure 2). For each set of experiments, negative control stainings were performed (1) employing respective recommended isotype control antibodies supplied by the same manufacturer, and (2) without primary antibodies, showing no detection of specific signals, (Online Resource, Suppl. Figure 3).

Discussion

Barrier and epithelial integrity of mammary gland monolayers primarily depend on tight junctions. The formation to lactation period in the alveoli leads to the disappearance of desmosomes and adhesive contacts between cells (Pitelka et al. 1973). Consequently, tight junction proteins determine the paracellular transport and ensure integrity of the epithelial structure. In our current study, the hypothesis was tested if hydrostatic pressure on the basal side of the epithelium is able to affect barrier properties in a mammary epithelial cell model, *in vitro*. To accomplish this, we established a modified Ussing chamber allowing the application and the monitoring of hydrostatic pressure over a time course, which is able to simulate physiological pressure changes in mammary glands.

It is known that high pressure affects epithelial tissues in different ways. Reactions of exocrine glands to high intraglandular pressures have been studied *in vivo* (Markov et al. 2012), and first results have been reported on epithelial reactions to increased hydrostatic pressure, *in vitro* (Bogdan et al. 2008). The lack of data on pressure effects of epithelial barrier properties from cell culture, and the availability of many studies on effects of intraglandular pressure, *in vivo*, can be explained by the absence of an adequate method to analyze high pressure on epithelial barrier properties, *in vitro*. *In vivo* studies normally use animal experiments to induce an accumulation of secretion and thus induce intraglandular pressure, as reported recently (Markov et al. 2012). This experimental approach has different limitations: (1) it is not possible to analyze the effects of pressure isolated from other factors like secretion, compounds and hormonal regulation, and (2) barrier properties cannot be studied dynamically during changes of pressure conditions. To accomplish this, an *in vitro* method was needed which could provide dynamic pressure application and simultaneously allow analysis of barrier

properties. Therefore, in our present study we present a modified Ussing chamber to analyze epithelial barrier properties under high pressure conditions, *in vitro*, including three series of tests.

To date, the only published Ussing chamber experiments performed with mammary epithelial cells have been reported for immortalized cell lines, namely BME-UV and HME (Quesnell et al. 2007; Palmer et al. 2011), and of cultured cells on floating collagen gels, which was technically not compatible with the current setup (Bisbee et al. 1979). Other recently developed and currently discussed approaches in different epithelial and endothelial models include high and low hydrostatic pressurization, employment of bioreactors and compressed gas cylinders (Shin et al. 2012; Chen et al. 2014; Morimoto et al. 2016), none of which are able to monitor the transport and barrier properties simultaneously, though.

Therefore, the specialized Ussing chamber was modified with a tube system to apply high levels of bathing solution and thus hydrostatic pressure, with simultaneous control of pressure by a manometer. This refined experimental approach creates a novel *in vitro* method for the study of intraglandular high pressure, which is the first relevant success of our study.

Experiments under control conditions revealed an optimal time span of up to 4 h of experimental time after equilibration of the cells in the Ussing chamber, in which stable experimental conditions could be reached. The time intervals we used range from 5 to 25 min (in the 5 min-challenge) and from 0.5 to 2.5 h (in the 30 min-challenges), and therefore are suitable to investigate a wide range of possible time intervals. Longer basal pressure incubations of up to 4 h were not chosen because our current experiments focused on the gradual pressure increase, and since R_T of the challenged cells decreased very rapidly, we did not see a benefit in using longer time intervals. Moreover, limitations regarding viability became apparent during longer experiments, in a pilot experiment focusing on 20 h incubations (not shown). However, this study aimed to simulate relatively negative pressure, which physiologically challenges the epithelium during suckling or milking. These processes are relatively short, which led to the time interval of 5 min as a main focus.

Thus, our model most likely reflects a physiological situation when negative pressure is applied on mammary epithelium, e.g. in the process of commercial

milking as performed by milking machines (Enokidani et al. 2016). This application cannot be approached by current models regarding e.g. the application of shear stress or centrifugal force (Brakemeier et al. 2003), and thereby allows an approach to new physiological experimental questions. An example could be application in the analysis of lung physiology, which could be employed with amphibian lungs, as shown for *Xenopus leavis* (Bogdan et al. 2008; Vitzthum et al. 2015).

Basal pressure application of +1 kPa caused a strong and immediate reduction of R_T and a significant increase of I_{SC} . In the 5 min-challenge, 14 days, the timespan of 5 min between the different application steps left less time for adaption to the particular pressure level. In the 30 min-challenge, during incubation times of 30 min, an increase of R_T after the initial dropdown could be observed in some samples, which can be interpreted as a protective reaction to the higher pressure level. With additional applications R_T decreased further in all experimental setups. After the third application R_T reached a minimum near base line. These low values and the absence of regeneration over time showed that epithelia reached their limit of adjustment regarding R_T . Moreover, the increase of I_{SC} after each application in all three approaches can be interpreted as a second protective reaction, which continues when the limit of R_T reduction is reached. During establishment of the modified Ussing chamber setup we tested an experimental approach in 5 min-intervals with 7 day-cultivated cells. They reached extremely high I_{SC} -values over $1000 \mu A/cm^2$ after the third application together with the passing of bathing solution to the apical side, which showed that the epithelial barrier was impaired (data not shown). These data together with the findings in R_T led to the conclusion that with no time to adapt to the new conditions, in 7 day-old cells the limit of epithelial integrity is reached by +3 kPa hydrostatic pressure from the basal side. However, in the 30 min-challenges the incubation time of 30 min after every application enabled the cells to adapt to the situation. A comparable influence was observed in 14 days cultivated monolayers in the 5 min interval approach, and integrity of the monolayers was also verified by immunohistochemistry detecting tight junction protein expression and localization. The data of the three experimental approaches strongly suggest an adaptive

process in epithelia which adjusted barrier properties to the challenge of basal hydrostatic pressure.

On molecular level, it is known that cells have the ability to respond to mechanical stress due to the presence of membrane mechanosensitive ion channels (Sachs 1992; Hamill and Martinac 2001). These channels differ in selectivity, conductivity and other parameters, e.g. channels activated or inactivated by stretching (Morris and Sigurdson 1989) and channels responsive to the change in pressure and the curvature of the membrane (Bowman et al. 1992; Richter et al. 2014; Vitzthum et al. 2015). In cultured mammary gland cells, mechanical stimulation causes a change in membrane potential (Enomoto et al. 1987; Furuya et al. 1993). Therefore, it can be assumed that the increase of I_{SC} may be due to mechanosensitive ion channels, which would be a subject of further studies.

Conclusion

In conclusion, the modified Ussing chamber setup presented in our study adequately allows analysis of hydrostatic pressure effects on mammary epithelial cell monolayers, in vitro. The observed effects on transepithelial resistance and short circuit current may reflect adaptive and protective physiological reactions to pressure, in vivo

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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