



Epithelial Na⁺ channels derived from human lung are activated by shear force

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ARTICLE INFO

Article history:

Accepted 11 November 2009

Keywords:

ENaC
Epithelial Na⁺ channel
Shear force
Mechanosensitive
Pulmonary epithelium

ABSTRACT

During breathing the pulmonary epithelial cells are permanently exposed to physical forces and shear force (SF) in particular. In our present study we questioned whether the lung epithelial Na⁺ channel (hENaC) responds to shear force. For this purpose ENaC was cloned from human lung tissue, expressed in *Xenopus* oocytes and functionally characterized by electrophysiological techniques. Shear force in physiological relevant ranges was applied via a fluid stream. By the application of SF we obtained an increased inward current indicating an activation of hENaC. The SF-induced effect was reversible and interestingly, the response to SF was augmented by trypsin due to proteolytic cleavage. The direct activation of hENaC by SF was confirmed in outside-out single channel experiments. In five out of nine recordings an increased NP_0 was observed. From our observations we conclude that lung-derived hENaCs are directly activated by SF and this may represent an important feature for the regulation of pulmonary Na⁺ reabsorption and pulmonary fluid homeostasis.

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1. Introduction

The lung of air breathing vertebrates is a highly dynamic organ composed of cells that are continually exposed to mechanical forces. In mammals, the pulmonary epithelial cells, which form a barrier between the external environment and the interior of the organism, are exposed to different mechanical stimuli including distention caused by the expansion of the chest due to tidal breathing (Wirtz and Dobbs, 2000) and shear forces at the surface of the cells generated by airflow through the airways and the movement of fluid that covers the epithelial layer (Tarran et al., 2006).

Mechanical forces are important for lung function under physiological conditions. For example, fetal lung development depends on transmural pressures generated by liquid secretion into the alveolar airspace (Olver et al., 2004) and tidal breathing movements of the chest (Kitterman, 1996). Furthermore, mechanical forces influence the differentiation of alveolar epithelial cells (Dobbs and Gutierrez, 2001), the release of surfactant components (Edwards et al., 1999) and induce the release of paracrine mediators such as ATP (Grygorczyk and Hanrahan, 1997; Homolya et al., 2000). The release of ATP introduces the possibility of multiple cellular responses by the activation of purinergic receptors (Bucheimer and Linden, 2004; Burnstock, 2007).

Apart from forming the blood–air barrier, the pulmonary epithelium controls the viscosity and the volume of the fluid

that lines the epithelial surface (Matthay et al., 2002; Boucher, 2003). This is accomplished primarily through the activity of apical epithelial Na⁺ channels (ENaCs) and the basolateral Na⁺/K⁺-ATPase (Matthay et al., 2002). The concerted activity of these proteins generates transepithelial osmotic gradients, which represent the physical driving force for water movement across the epithelium. The importance of these processes is evident in patients with cystic fibrosis (Theelin and Boucher, 2007) or pulmonary edema (Matthay et al., 2002), where perturbations to alveolar ion transport are observed.

Although the function of pulmonary epithelia may be influenced by mechanical forces, little is known about the direct impact of mechanical forces on ion transport processes in general, and lung epithelial ion channels in particular. We have recently reported that exposure of native lung epithelia to increased hydrostatic pressure (5 cm H₂O) altered the behavior of epithelial cells; including alterations to ion transport processes and the activation of a Na⁺ conductance in particular (Bogdan et al., 2008). Taking this into consideration, together with the fact that mouse, *Xenopus* and rat ENaCs are activated by shear force (Satlin et al., 2001; Althaus et al., 2007), the focus of this study was to question, whether or not the human lung ortholog might also be mechanosensitive. For this approach we used α , β , and γ ENaC cloned from human lung tissue. ENaC was heterologously expressed in *Xenopus* oocytes and activity was measured by the two-electrode-voltage-clamp (TEVC) and the patch-clamp technique. Channels were mechanically challenged by the activation of a fluid stream, producing shear forces at the surface of the cell membrane.

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2. Materials and methods

2.1. Cloning and heterologous expression of human lung ENaC (hENaC)

The α , β and γ ENaC subunits were cloned from total RNA pools isolated from human lung tissue. The total mRNA was reverse-transcribed in cDNA using a commercial kit (M-MLV Reverse Transcriptase RNase H Minus Point Mutant Kit, Promega) and an oligo(dT)₁₂ primer. The cDNA was employed as a template in a PCR reaction (PlatinumPfx DNA Polymerase Kit, Invitrogen) using the following primers: α ENaC forward: 5'-CATGGAGGGGAACAAGCT-3', reverse: 5'-CCTTGGTGTGAGAA-CCTCTCC-3'; β ENaC forward: 5'-CCACTATGCACGTGAAGAAGTACC-3', reverse: 5'-CAGGGTTAGATGGCATCACCTCAC-3'; γ ENaC forward: 5'-CCTCAAAGTCCCATCCTCGCC-3', reverse: 5'-CCCTGCCTCAGAGCTCATCC-3'. Conditions for the PCR were: denaturation at 94 °C for 5 min, 35 cycles with 94 °C for 45 s, annealing at 55 °C for 60 s, extension at 68 °C for 2.5 min and finally termination with a final extension at 68 °C for 10 min. Amplicons of appropriate sizes (Fig. 1A) were isolated from the gels and A-tailed using an A-Addition Kit (Qiagen). Afterwards, products were cloned into the pGEM-T Easy vector (Promega). The ligation procedure was carried out according the manufacturer's protocol provided with the pGEM-T Easy vector (Promega). Vectors were then transformed into competent bacteria (*E. coli* DH5 α) and transformed clones were selected for ampicillin resistance. Plasmids were isolated (Miniprep Kit, Qiagen) and cleaved using EcoRI (α , γ subunit) and NotI (β subunit) restriction enzymes. Restricted amplicons were separated on a 1% agarose gel, and bands of appropriate sizes were isolated and purified by using a MiniElute Kit (Qiagen). For functional expression, the restricted amplicons were ligated into the pTNT expression vector (Promega), suitable for cRNA synthesis and subsequently *Xenopus* oocyte expression. Therefore, plasmids were transformed into competent *E. coli* DH5 α bacteria. Plasmids were isolated and ENaC-encoding products were confirmed by sequencing. In order to obtain cRNA suitable for oocyte injection, plasmids were linearized (BamHI) and *in vitro* reverse-transcribed (RiboMax transcription kit, Promega) according to standard protocols.

For oocyte expression, defolliculated *Xenopus laevis* oocytes of stages V and VI (Dumont, 1972) were used for cRNA injection. The hENaC-encoding cRNAs were diluted in DEPC-treated water and the cRNA for the three subunits was injected in a ratio of 1:1:1. Volumes between 9.2 and 23 nl per oocyte were injected via a microinjector (Nanoject, Drummond Scientific). The injection of 23 nl RNA solution corresponds the amount of 2.3 ng RNA for each subunit and oocyte. After injection oocytes were incubated in low-Na⁺ solution (in mM: 10 NaCl, 80 NMDG-Cl (N-methyl-D-glucamine), 1 KCl, 2 CaCl₂, 5 HEPES, 2.5 Na-pyruvate, 0.06 penicillin, 0.02 streptomycin; pH 7.4) and stored at 16 °C. Recordings were performed within 1–4 days after injection, at room temperature.

2.2. Two-electrode-voltage-clamp recordings (TEVC)

Intracellular electrodes were pulled from borosilicate glass capillaries (Hilgenberg, Germany) and filled with KCl (1 M). Oocytes were clamped to membrane voltage of –60 mV using a TEVC amplifier (Warner Instruments, USA) and transmembrane currents were recorded on a strip chart recorder (Kipp&Zonen, Netherlands). During the recordings the oocytes were superfused with Ringer's solution containing (in mM): 90 NaCl, 1 KCl, 2 CaCl₂, 5 HEPES, pH 7.4. Superfusion of the recording chamber was driven by gravity with a flow rate of ~2.5 ml/min. In order to avoid falsification of shear forces due to the continuous chamber perfusion a shield was mounted in front of the oocyte (scheme of the recording chamber

in Althaus et al., 2007). Amiloride (10 μ M, Sigma) was superfused to block hENaC currents. In order to activate membrane-located hENaCs we used trypsin (20 μ g/ml, Sigma), which is suggested to increase ENaC currents by the activation of silent channels already located in the membrane (Caldwell et al., 2004; Chraïbi et al., 1998).

2.3. Single channel patch-clamp recordings

Single channel recordings were performed in the cell-attached and in the outside-out configuration. For this purpose, cRNA-injected oocytes (treated as described above) were devitellinized and placed in a chamber containing extracellular bath solution. Cell-attached recordings were performed with bath solution containing (in mM): 145 KCl, 1.8 CaCl₂, 2 MgCl₂, 5.5 glucose, 10 HEPES (pH 7.4). The outside-out recordings were performed with bath containing (in mM): 90 NaCl, 1 KCl, 2 CaCl₂, 5 HEPES (pH 7.4). In cell-attached as well as outside-out recordings identical borosilicate glass capillaries (Hilgenberg, Germany; outer diameter 1.6 mm) were pulled to patch electrodes (2–5 M Ω resistance) and filled with pipette solution. In cell-attached recordings the following solution was used as pipette solution (in mM): 145 NaCl, 1.8 CaCl₂, 2 MgCl₂, 5.5 glucose, 10 HEPES (pH 7.4). For outside-out recordings an intracellular analogous pipette solution was used (in mM: 140 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES, 11 EGTA, pH 7.2).

The single channel currents were amplified by a LM/PC amplifier (List, Germany), low pass filtered at 100 Hz and acquired with 2 kHz using an Axon interface (1200 series, Axon Instruments, USA) in combination with the Axon Clampex software (Axon Instruments, USA). As a measure for single channel activity the NP_0 was determined as previously described (Althaus et al., 2007) representing the product of the number of visible channels and the open probability for each activation level. Single channel amplitudes were fitted by the Goldman–Hodgkin–Katz current equation. The single channel conductance was calculated due to the channel amplitudes obtained at –100 mV membrane potential (V_M).

2.4. Generation of shear force

In order to mechanically challenge hENaC molecules shear forces were generated via the application of a fluid stream directly directed towards the measured cell and excised membrane patch, respectively. The procedure for the application of shear force was described in detail before (Althaus et al., 2007). Briefly, for TEVC recordings, a Pasteur pipette (1 mm inner diameter) was placed in close proximity to the oocyte and the activation of a flow stream (~3 ml/min) through the Pasteur pipette produced shear forces of approximately 5 dynes/cm² at the surface of the oocyte (Althaus et al., 2007). This fluid stream was used for mechanical activation and was applied in addition to the chamber perfusion.

For the single channel recordings the patch pipette containing the excised outside-out membrane patch was moved in front of a tube (1 mm inner diameter) localized inside the recording chamber. The tube was connected with a gravity driven perfusion system (ALA Scientific Instruments, USA) and activation of flow through this system (flow rate ~0.3 ml/min) generated shear forces of ~0.5 dynes/cm² (Althaus et al., 2007).

Calculations started with the determination of the Reynolds number (Re) for each setup (whole cell or single channel recording) by the following equation:

$$Re = \frac{\theta\omega D}{\lambda}$$

where θ = density of water; ω = flow velocity; D = diameter of the perfusion pipette/tube; λ = kinematic viscosity of water. Laminar flow is predicted for $Re < 1000$. We calculated Re of 63.7 (whole cell)

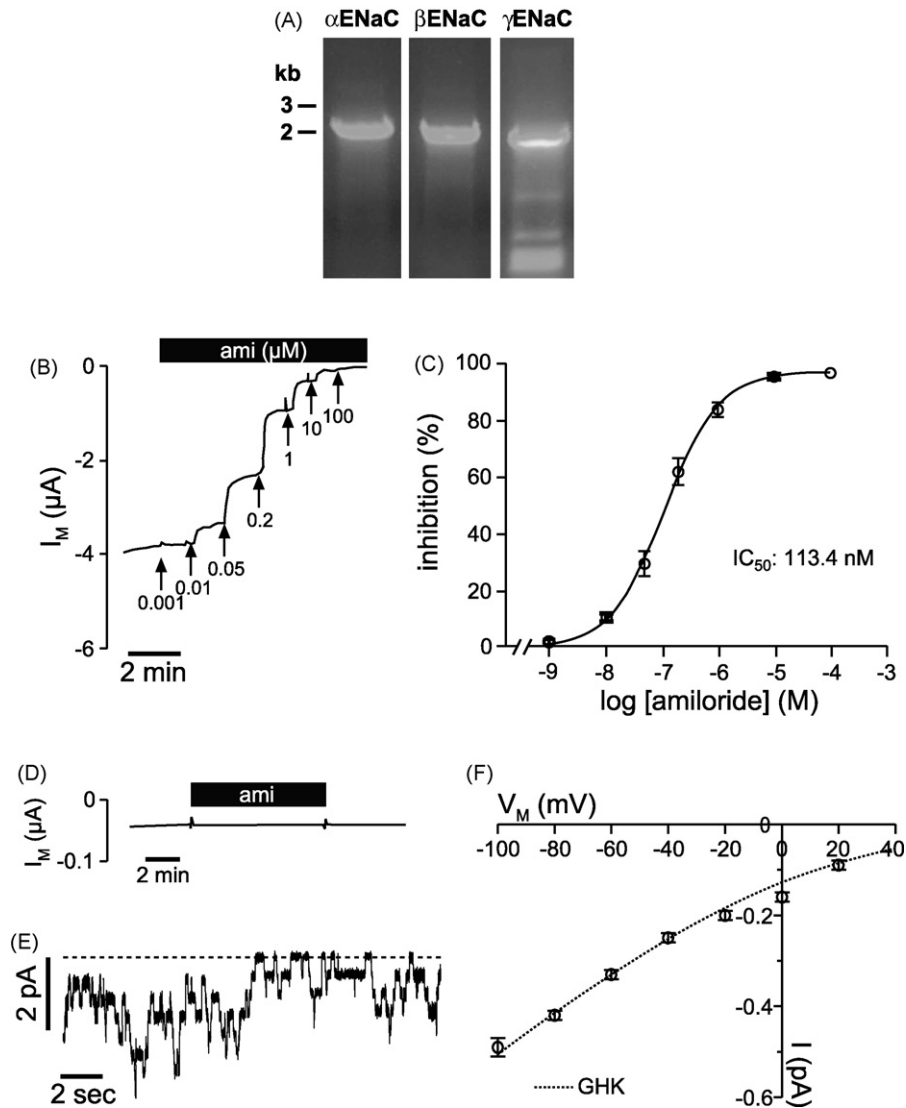


Fig. 1. Basic properties of human lung-derived ENaC (hENaC). (A) Bands of the PCR products at appropriate sizes obtained with the primers used for cloning. The expected sizes of the products were 2010 bp for the α , 1923 bp for the β and 1950 bp for the γ ENaC subunit. (B) In *Xenopus* oocytes expressed hENaC exhibited amiloride-sensitive inward current. The transmembrane current (I_M) was sensitive to increasing amiloride (ami) concentrations. (C) Half-maximal inhibitory coefficient (IC_{50}) was determined by fitting the means to the Hill-equation. Each mean (\pm SE) was determined from seven independent experiments using oocytes of two different donors. (D) Original recording of a control experiment using water-injected oocytes. In these experiments no amiloride-sensitive current was observed in response to the ENaC antagonist (ami). (E) Single channel recording in the cell-attached configuration. Ion channel activity was recorded at -100 mV, the dotted line represents baseline current (no channel activity), downward deflections correspond to the opening of epithelial Na^+ channels. (F) Current–voltage relation derived from cell-attached experiments. Data are means (\pm SE) of single channel amplitudes determined from at least three current deflections of each experiment ($n = 4$). Values were fitted by the Goldman–Hodgkin–Katz equation (dashed line; GHK: Goldman–Hodgkin–Katz).

and 6.37 (single channel). Since the flow is predicted to be laminar the drag force could be calculated by the equation:

$$F_{drag} = 0.5\theta A\omega^2 C_d$$

where θ = density of water; A = cross-sectional sphere of the oocyte; ω = flow velocity; C_d = drag-coefficient (which is ~ 1 for R_e in the range of 3–80, see Hoyer et al., 2002).

Effective shear forces (F_{shear}) were then calculated from the relation:

$$F_{shear} = \frac{F_{drag}}{\text{oocyte surface area}}$$

Due to technical reasons different setups were used for the generation of shear forces in whole cell and single channel recordings and it was not possible to use identical amounts of shear forces for both techniques elaborated in the study.

2.5. Statistical analysis

Data represent means \pm standard error of the mean (SE). The number of performed experiments is denoted with n . For each experimental approach oocytes of at least two different donors were used. Generally the paired *Student's t-test* was used to compare dependent values (e.g. before and after treatment). P values of at least <0.05 were considered as statistically significant.

3. Results

3.1. Basic properties of cloned lung hENaC

With the primers mentioned in Section 2 specific products at appropriate sizes were detected (approx. 2 kb per subunit, Fig. 1A). The products were finally cloned in an oocyte expression vector

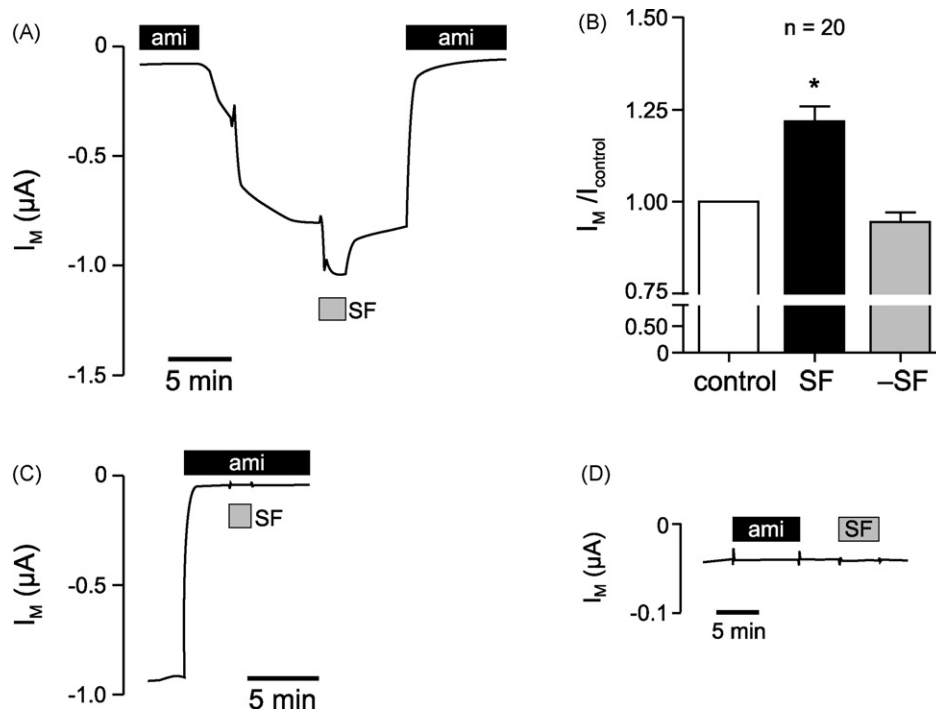


Fig. 2. Human lung ENaC expressed in *Xenopus* oocytes exposed to laminar flow producing shear forces (SF). (A) Original recording depicting the effect of SF on hENaC current. Amiloride (ami, 10 μM , application period indicated by black bar) was used to determine amiloride-sensitive hENaC. Following washout of amiloride and equilibration of the current (I_M), SF was activated (indicated by gray bar). Activation of SF stimulated I_M . The SF-activated current declined to baseline levels after SF application was stopped. (B) Summarized data (means \pm SE) obtained from experiments as shown in panel A. Measured membrane currents (I_M) were normalized with respect to the values before SF was applied (SF: shear force; -SF: after SF application; $n = 20$, $*P < 0.001$). (C) We could not detect any effect of SF in the presence of amiloride, demonstrating that the observed effect results from the activation of amiloride-sensitive channels. (D) Recording of a control experiment demonstrating that water-injected oocytes did neither respond to amiloride nor to SF.

and sequenced. Subsequent BLAST analysis identified the products as human α , β , γ ENaC subunits (SCNN1a, SCNN1b, SCNN1g; SCNN, sodium channel non-neuronal). The sequence encoding the α ENaC subunit possessed two amino acid changes, compared with the reference sequence (NCBI Nucleotide database ID: NP_001029). Both amino acid changes represent known single nucleotide polymorphisms (SNPs; NCBI SNP database ID: rs11542844 and rs2228576) and do not affect ENaC activity (Tong et al., 2006). The β and γ ENaC sequences were found to be identical compared with the reference sequences (NCBI Nucleotide database ID: NP_000327 for β and NP_001030 for γ ENaC).

Simultaneous injection of the cRNAs encoding α , β and γ ENaC (hENaC) into *Xenopus* oocytes produced robust inward currents at membrane potentials of -60 mV. The observed current was sensitive to amiloride (10 μM) and from dose–response experiments the half-maximal inhibitory coefficient (IC_{50}) was determined with 113 nM amiloride (Fig. 1B,C). In order to ensure that the observed current was due to the presence of expressed hENaC, control experiments were performed using water-injected oocytes. In these cells, no amiloride-sensitive current was observed (Fig. 1D). In cell-attached patch-clamp recordings the single channel conductance was determined with 4.9 ± 0.2 pS ($n = 5$), which matches the properties of lung hENaC channels as previously described (Voilley et al., 1994). Further, the recorded inward currents observed over the entire range of potentials tested (-100 until $+20$ mV) were consistent with a high degree of sodium selectivity (Fig. 1F).

3.2. Effect of shear force on hENaC activity

Shear force (SF) was generated by a gravity driven perfusion system. To assess whether hENaC is affected by SF, voltage-

clamped oocytes (holding potential -60 mV) were exposed to a laminar flow stream. Prior to SF exposure amiloride (10 μM) was applied to determine amiloride-sensitive baseline current. Following amiloride washout, a flow stream was activated and this resulted in an increase of the transmembrane current (I_M) of about $22 \pm 4\%$ ($n = 20$, $P < 0.001$). The SF-induced effect was observed consistently, and was completely reversible, although the decline was slower compared with the activation kinetic (Fig. 2A). No effect of shear force was obtained in the presence of amiloride, or on water-injected control oocytes (Fig. 2C, D) indicating that the observed inward current was due to the activation of hENaC.

Proteases have been described to cleave membrane-located inactive ENaCs, representing a mechanism to increase ENaC activity and thereby Na^+ -reabsorption (Rossier, 2004; Diakov et al., 2008). The present study questioned whether activation of hENaC via the protease trypsin might interfere with SF dependent activation. Assuming that proteolytic cleavage might activate ENaC through a similar gating mechanism like SF, no additional effect should be observed in response to SF. In order to assess this assumption the fluid stream was applied on hENaC expressing oocytes in the absence as well as in the presence of trypsin. Interestingly, the response to SF in the presence of trypsin was unproportionally higher (approx. 3-fold increase) compared with the current increase induced solely by trypsin (approx. 2-fold). Further, correlating the response to SF in the absence and presence of trypsin, with the ratio of the increase due to trypsin, the slope of the linear regression was 1.88 ± 0.4 ($n = 8$), indicating that trypsin facilitates the SF effect on hENaC (Fig. 3C). The effects evoked by trypsin were not obtained in water-injected control oocytes (I_M before trypsin: -55 ± 7 nA; with trypsin: -58 ± 6 nA; trypsin + SF: -56 ± 5 nA; $n = 8$).

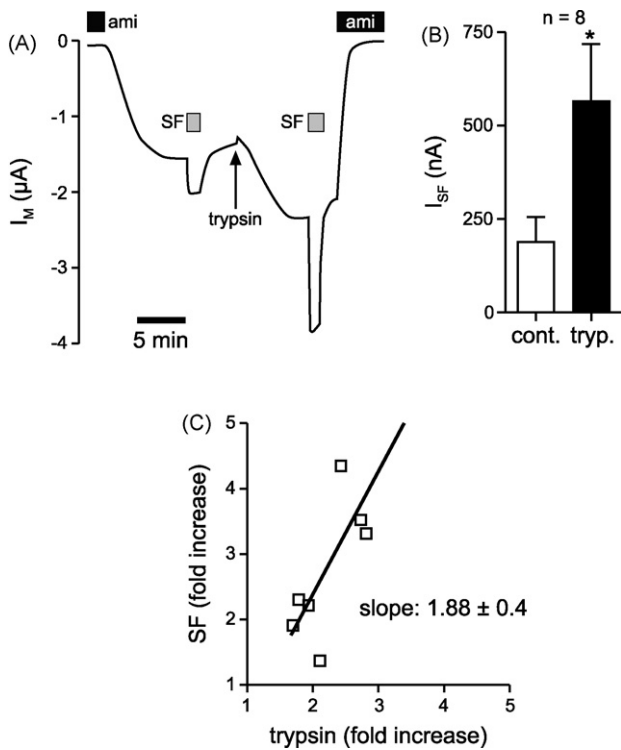


Fig. 3. Proteolytic cleavage of hENaC increases SF-induced currents. (A) Following washout of amiloride and equilibration of I_M SF-induced activation was determined in the absence as well as in the presence of trypsin (20 $\mu g/ml$). Proteolytic cleavage of hENaC resulted in an increased transmembrane current (I_M). Under these conditions the response to SF was potentiated compared to control conditions. Application of amiloride is indicated by the black bars, perfusion in order to induce shear force by gray bars. (B) Statistical analysis of SF-induced currents (I_{SF}) determined under control conditions (contr.) and after proteolytical cleavage (tryp.; $*P < 0.01$). (C) Linear regression analysis of the experiments with trypsin. Correlation of the ratios obtained by trypsin application (trypsin = I_M trypsin/ I_M before trypsin, abscissa) with the corresponding relative increase in response to SF ($SF = I_{SF\text{tryp.}}/I_{SF\text{contr.}}$, ordinate). Proportional changes (e.g. 2-fold increase by trypsin might double the response to SF) will give a slope of 1. Since the distribution of the values is shifted towards the SF effect, these data indicate that proteolytic cleavage facilitates channel sensitivity with respect to SF.

3.3. Effect of SF in outside-out patch-clamp recordings

In order to assess whether or not the shear force-mediated effect was independent of signaling mediators, single channel recordings in the outside-out configuration were performed. For this purpose, the patch pipette containing the excised outside-out membrane patch was placed in front of a tube, and placed inside the recording chamber. The patch pipette was aligned at a 90° angle with respect to the tube, and thus to the laminar flow. In these experiments, hENaC activity was recorded at -100 mV membrane potential for 1 min under control conditions (without SF), then the fluid stream was activated and ion channel activity was recorded for 1 min with SF. Recordings were terminated by the perfusion of amiloride (10 μM) to define baseline current. As a measure for hENaC activity NP_0 was determined. In five out of nine recordings an increase of hENaC activity in response to SF was observed (Fig. 4A). In these recordings NP_0 was significantly increased from 0.73 ± 0.23 to 1.2 ± 0.4 ($n = 5$, $P < 0.05$; Fig. 4B). In the remaining four experiments no obvious changes were observed in response to SF ($P = 0.2$, Fig. 4C). This phenomenon has been described previously (Satlin et al., 2001; Althaus et al., 2007), although the reasons for these inconsistent observations in the patch-clamp recordings remain to be clarified.

In addition to the channel activity, the single channel current amplitude was determined (at -100 mV) from at least three current

deflections obtained from five independent recordings. The single channel conductance of hENaC was slightly decreased compared with cell-attached recordings, but no changes were determined with respect to SF (without SF: 3.96 ± 0.13 pS; SF 3.94 ± 0.12 pA; $n = 5$).

Taken together, the results indicate that the observed increase in ion current and ion channel activity in response to SF is dependent in an activation of hENaC, which is unlikely to be mediated by soluble mediators.

4. Discussion

During ventilation pulmonary epithelial cells are exposed to shear forces at their luminal surface (Tarran et al., 2006). Different reasons might account for the appearance of shear forces: (1) the relative movement of the epithelium with respect to the luminal liquid layer (assuming that the liquid layer behaves relatively inert); (2) movement of the fluid layer induced by the airflow during in- and exhalation (Tarran et al., 2006). As a consequence of these dynamic processes, extracellular domains of integral proteins located at the apical side of pulmonary epithelial cells are exposed to shear forces. Concerning the pulmonary epithelium, different shear force induced effects are published: (1) the release of ATP and the subsequent activation of ion channels via purinergic receptors (Tarran et al., 2005); (2) impact on aquaporin expression and epithelial barrier function (Sidhaye et al., 2008) and (3) the impact of shear forces during cough (Chowdhary et al., 1999).

At the other side, the epithelial Na^+ channel is crucial for pulmonary epithelial function. This is underlined by observations that a reduced ENaC function is associated with the formation of pulmonary edema (Hummler et al., 1996; Matthay et al., 2002) whereas a gain of function is known to produce a cystic fibrosis-like phenotype in mice (Mall et al., 2004) as well as in humans (Azad et al., 2009). Integrating these considerations, the present study hypothesized whether human lung ENaC might be regulated by SF.

4.1. Experimental evidence that hENaC is activated by shear force

To assess this hypothesis, human lung-derived ENaC (hENaC) was exposed to laminar flow producing shear force at the cell surface. For this approach, the open reading frame encoding α , β , γ ENaC was cloned from a RNA library and functionally characterized in *Xenopus* oocytes. In two-electrode-voltage-clamp experiments we found that hENaC activity was increased by SF, which neither was the case in water-injected control oocytes, nor in the presence of amiloride. This finding confirms prior studies subjecting ENaC from other species and tissue origins to SF (Satlin et al., 2001; Carattino et al., 2004; Althaus et al., 2007).

Proteases are known to activate membrane-located ENaCs by proteolytic cleavage (Rossier, 2004; Diakov et al., 2008). This modification process activates near-silent channels (low open probability, Caldwell et al., 2004) and was visible by an increased current in response to trypsin (Fig. 3A). Interestingly, the SF-induced effect was strengthened after pre-incubation with trypsin. This observation could be in general explained by an increased number of activated channels accessible to SF, assuming that uncleaved channels do not respond SF. Further there is some preliminary evidence from these experiments that proteolytic cleavage might sensitize the channels towards mechanical activation by SF, since the response to SF in the presence of trypsin was unproportionally higher compared with the current increase induced solely by trypsin. This finding contrasts prior observations with rat and *Xenopus*

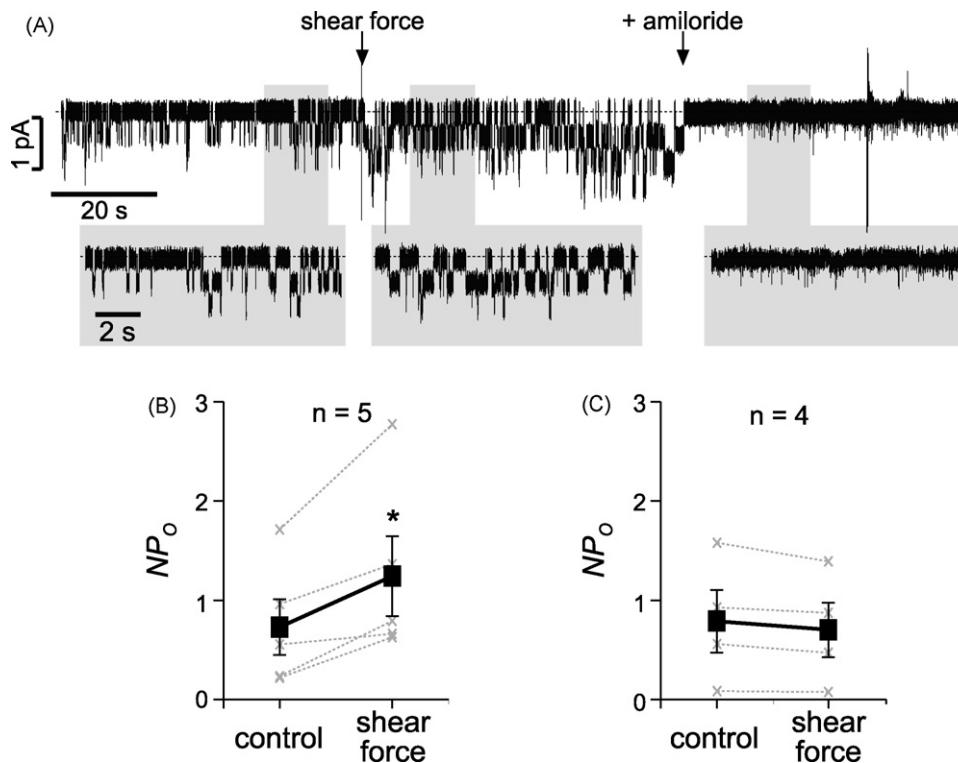


Fig. 4. hENaC activity in response to shear force. (A) Single channel recording obtained in the outside-out configuration. The pipette including the excised membrane patch was moved in front of a tube and ion channel activity was initially recorded for approximately 60 s under control conditions. Then a flow stream of extracellular bath solution through the tube was activated to produce shear force (SF) and this procedure resulted in an activation of hENaC. After additional 60 s amiloride was added to the tube perfusate leading to an obvious inhibition of hENaC activity. Dashed line marks the baseline (no channel activity) and ion channel openings are represented by downward deflections. Gray boxes depict magnifications of selected parts of the original recording. (B) As a measure for ion channel activity the relative open probability (NP_o) for each recording (gray crosses, dotted lines) was determined under control conditions and with applied shear force. Means \pm SE are plotted as black squares. In five out of nine experiments we found an increase of the NP_o (* $P < 0.05$). (C) In four experiments no significant changes were observed ($P = 0.2$).

ENaC (Althaus et al., 2007) and might be interesting for future investigations.

The finding that hENaC is directly activated by shear force was additionally confirmed in outside-out patch-clamp recordings. In five out of nine experiments, we clearly observed an increased activity by the activation of SF. In the remaining four experiments no changes in NP_o were obtained (Fig. 4C). The observation that in some recordings no activation of hENaC has been observed is consistent with prior reports (Satlin et al., 2001; Althaus et al., 2007) although the reason is unknown. However, different hypothetical explanations could be reasonable: (1) suboptimal localization of the channels in the excised membrane patch with respect to flow. Similar indications were published by Palmer and Frindt (1996), which evaluated the effect of membrane stretch on ENaC using cell-attached patches; (2) the release of ATP, which has been shown to compensate the mechanical induced ENaC activation (Ma et al., 2002); (3) disruption of cytoskeletal filaments by establishing the outside-out configuration. This should be considered since it has been shown that the C-terminus of ENaC binds to F-actin (Mazzochi et al., 2006) and thus anchorage of the channel to the cytoskeleton might be necessary to assure mechanosensitivity; (4) the amount of SF used. However, it should be noted that in the single channel recordings it was impossible to use higher flow rates, since this immediately disrupted the excised membrane patches. Nevertheless we would emphasize that in the whole cell recordings a consistent and robust activation of heterologously expressed hENaC was observed and this effect was facilitated by proteolytic cleavage. Further, we suggest a direct activation of hENaC by shear forces, independent of soluble mediators. This principle is in agreement with studies demonstrating that flow induced shear force is sufficient to activate proteins via

inducing conformational changes of the protein (Schneider et al., 2007).

4.2. Physiological relevance of hENaC regulation by shear force

The shear forces applied in the present study (0.5 in single channel and 5 dynes/cm² in whole cell recordings) represent physiological relevant ranges. Tarran et al. (2006) for example predict shear forces between 0.4 and 2 dynes/cm² under resting tidal breathing for the airway epithelium. Further it has been shown, that shear forces in the range of 0.06–6 dynes/cm² can induce the release of ATP in bronchial epithelial cells (Tarran et al., 2005). Thus the magnitudes of shear forces used in our study are relevant for the situation in the lung and are sufficient to affect hENaC activity under normal breathing conditions.

Shear force induced activation of ENaC suggests the possibility of a direct, intrinsic control mechanism independent of biochemical signaling cascades. In this context, ENaC activity can be directly adapted in response to breathing activity. For example during physical exercises adaptation to the increased oxygen demand leads to increased pulmonary blood pressure as well as augmented ventilation. While the first increases the pressure gradient for water influx into the airspace, enforced ventilation provides higher amounts of shear forces. The higher the shear forces, the higher the ENaC activity to reabsorb the water influx from the airspace driven by pulmonary vasoconstriction.

There are two physical factors, which can influence the amplitude of shear force in the lung: (1) The velocity of the fluid passing the luminal cell surface depending on breathing activity. (2) The viscosity of the liquid layer covering the epithelium. The second factor could be of particular relevance for the function of pulmonary

epithelia in patients where lung/airway fluid viscosity is impaired by malfunctioning ion transport processes. This is, for example, the case in patients with cystic fibrosis and patients with pulmonary edema.

Cystic fibrosis is characterized by increased amiloride-sensitive Na^+ -reabsorption (Boucher, 2004). This phenomenon cannot be reasoned exclusively by increased shear force, but shear force might represent one factor among others which increase ENaC activity, and may therefore contribute to the severity of the disease. Alternatively, there is evidence that the resolution of pulmonary edema largely depends on efficient Na^+ -reabsorption. Integrating our findings in this context this may indicate that decreased shear forces due to a reduced viscosity of the layer lining the epithelium may contribute to decreased Na^+ -reabsorption.

In summary, we provide evidence that the human lung-derived ENaC is a mechanosensitive ion channel. This feature is of considerable relevance for the function of the lung since the lung is a highly dynamic organ and physical forces may represent a novel factor, which connects Na^+ -reabsorption directly with breathing movements.

Acknowledgements

The authors would like to thank M. Buss and S. Kristek for technical and experimental assistance. All treatments of animals were approved and overseen by the regional board Giessen as the responsible authority and were in accordance with the German Law on the Protection of Animals.

The study was supported by the German Research Foundation (DFG; grant FR 2124 to MF and WGC). MA and REM are supported by the Excellence Cluster 147 of the German Research Foundation.

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