Lung (2014) 192:935–946
DOI 10.1007/s00408-014-9640-3

The Interaction of Glucocorticoids and Progesterone Distinctively Affects Epithelial Sodium Transport

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Received: 8 April 2014 / Accepted: 12 August 2014 / Published online: 31 August 2014

Abstract
Purpose Glucocorticoids and progesterone exert stimulatory effects on epithelial Na^+ transport, including increased mRNA expression of the participating ion transporters (epithelial Na^+ channels [ENaC] and Na,K-ATPases) and their electrophysiological activity. Fetuses threatened by preterm labor may receive high doses of glucocorticoids to stimulate lung maturation and are naturally exposed to high levels of female sex steroids. However, it is still unknown how the combination of both hormones influences the epithelial Na^+ transport, which is crucial for alveolar fluid clearance.
Methods Fetal distal lung epithelial cells were incubated in media supplemented with dexamethasone and progesterone. Real-time qPCR and Ussing chamber analysis were used to determine the effects on ENaC mRNA expression and channel activity. In addition, the specific progesterone receptor antagonist (PF-02367982) and the glucocorticoid receptor antagonist mifepristone were used to identify the involved hormone receptors.
Results Both dexamethasone and progesterone increased ENaC subunit expression and channel activity. However, the combination of dexamethasone and progesterone reduced the α- and γ-ENaC subunit expression compared to the effect of dexamethasone alone. Furthermore, higher dexamethasone concentrations in combination with progesterone also significantly reduced Na^+ transport in Ussing chamber measurements. Hormone receptor antagonists showed that inhibition of the progesterone receptor increased the mRNA expression of α- and γ-ENaC, whereas mifepristone decreased mRNA expression of all ENaC subunits.
Conclusion Glucocorticoids and progesterone individually increase ENaC mRNA expression; however, the combination of both hormones decreases the stimulatory effects of dexamethasone on Na^+ transport and ENaC mRNA expression.

Keywords Glucocorticoids · Progesterone · ENaC · Epithelial Na^+ transport · Dexamethasone

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Introduction

Prior to birth, the lung has to transform from the secretory to the absorptive phenotype in preparation to air breathing. Fluid secretion [1] mediated by chloride transport across the alveolar epithelia [2] is replaced by active Na^+ transport across the alveolar epithelia leading to fluid absorption. Impairment of postnatal lung transition leads to diseases like transient tachypnea or respiratory distress syndrome (RDS) [3, 4]. Na^+ ions enter the pneumocytes through apically located epithelial Na^+ channels (ENaC) and are actively extruded by Na,K-ATPases at the basolateral membrane compartment. Water follows passively from the airspaces to the interstitium of the lung [5, 6], which is necessary for alveolar fluid clearance (AFC) and thus for postnatal survival. ENaC is composed of three subunits (α-, β-, and γ-ENaC) and is rate limiting for AFC [7, 8]. Other studies suggested that airway liquid clearance

at birth is driven by lung inflation, and ENaC mainly prevents the re-entering of liquid into the airways during expiration [9]. In any case, ENaC is crucial for postnatal survival, because knockout of α-ENaC [6] or inhibition of ENaC-mediated Na^+ currents [8] leads to RDS and lung failure. In the developing lung, different factors are responsible for the transition from secretion to absorption, including corticosteroids [10, 11], thyroid hormones [10], labor [12], and catecholamines [13].

In this study, we focused on the influence and interaction of the female sex steroid progesterone and the glucocorticoid dexamethasone on alveolar epithelial Na^+ transport. Female sex steroids are necessary for proper alveolar formation and AFC [14]. Studies showed that female preterm infants have a survival advantage [15] with less pulmonary disease severity compared to males [16, 17]. Previous experiments showed that female sex steroids led to a dose-dependent increase of Na^+ transport in fetal distal lung epithelial (FDLE) cells, demonstrated by an increased apical Na^+ transport and elevated mRNA expression of ENaC subunits [18]. In addition, glucocorticoids play a key role in lung development by increasing the transcription and activity of ENaC [11, 19] and thereby elevating Na^+ absorption [20]. Glucocorticoids, as well as female sex steroids, exert their effect via steroid receptors operating as hormone-mediated transcription factors [21, 22]. Due to their structural similarity, different types of steroid receptors can often bind to the same hormone response element, but their effect on cell metabolism might differ [23]. Increased variability is also caused by the formation of heterodimers [24, 25] and different receptor subtypes. Two progesterone receptor (PR) isoforms are known; however, in mice lung only progesterone receptor A (PR-A) was detected [22]. This isoform is often transcriptionally inactive but is able to decrease the activity of the glucocorticoid receptor (GR) [26]. In addition, for the GR two receptor isoforms are described. In human respiratory epithelial cells, the hGR-α is the predominant isoform, displaying steroid-binding activity. In contrast, hGR-β does not show ligand-binding activity [27] and inhibits the transcriptional activity of hGR-α by the formation of transcription impairing hGR-α/hGR-β heterodimers [25]. Therefore, in most cases, the hGR-β as well as the PR-A act as transcriptional repressors of target genes. In addition, it is possible for steroid hormones to bind to a different type of steroid receptor, e.g., progesterone binding to the GR [21]. The resulting effect on target gene expression might differ, because progesterone binding to the GR does not display full agonist activity [21].

Despite the importance of these hormones in lung development and transition, little is known about their interactions, although they are naturally present simultaneously. This situation is exaggerated when glucocorticoids are administered to a pregnant woman with preterm labor to accelerate lung maturation in the fetus, while placental steroid synthesis is still active. Therefore, we have investigated, in this study, how female sex steroids in combination with glucocorticoids affect epithelial Na^+ transport in FDLE cells.

Methods

Isolation and Culture of FDLE Cells

FDLE cells, a model of preterm respiratory cells, were isolated from 18 to 19 days gestational age fetuses of Sprague–Dawley rats as described previously [20, 28]. For Ussing chamber measurements, cells were seeded on Snapwell permeable supports (Costar® No. 3407, Inc., Corning, NY, 1 cm^2) at a density of 10^6 cells per insert. For RT-qPCR, cells were seeded on Transwell permeable supports (Costar® No. 3412, 2.4 cm^2) at a density of 2×10^6 cells per insert. In the first 24 h, cells were cultured in MEM + 10 % FBS, L-glutamine (2 mM, PAA Laboratories), and Antibiotic/Antimycotic agents (Life technologies) containing penicillin [100 U/ml], streptomycin [100 µg/ml], and amphotericin B [0.25 µg/ml]. On the 2nd day of culture, the medium was changed to serum-free complete medium (Cellgro, Mediatech, Herndon, VA) supplemented with different concentrations of dexamethasone (D-4902, Sigma, Germany) and progesterone (P-8783, Sigma) in concentrations based on previous experiments [18, 29]. Mifepristone (10 µM, TOCRIS bioscience, Bristol, UK) was used as PR/GR antagonist [30]. The specific non-steroidal PR antagonist PF-02367982 (150 nM) was kindly provided by Pfizer [31]. PF-02367982 exhibits potent PR antagonism and displays 100-fold selectivity over closely related members of the nuclear hormone receptor family, including GR [31]. The hormones and receptor antagonists were dissolved in ethanol. The control monolayers were treated with an equal amount of the respective solvent to exclude solvent influence on the evoked responses. Cells subjected to different experimental conditions were always age matched and derived from the same litter.

Measurement of mRNA Expression

On the 5th day of culture, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer. Reverse transcription was carried out in two steps with first pre-annealing of 1 µg RNA followed by 1 h at 50 °C and 15 min at 75 °C employing $Oligo(dT)_{18}$ primers (Thermo Scientific, Erlangen, Germany) and Superscript III (Life technologies). RT-qPCR was performed

Table 1 Gene-specific primers

Gene	Forward	Reverse
α-ENaC NM_031548.2	TTCTGGGCGGTGCTGTGGCT	GCGTCTGCTCCGTGATGCGG
β-ENaC NM_012648.1	TGCAGGCCCAATGCCGAGGT	GGGCTCTGTGCCCTGGCTCT
γ-ENaC NM_017046.1	CACGCCAGCCGTGACCCTTC	CTCGGGACACCACGATGCGG
Mrps18a NM_198756.1	GCGACCGGCTGGTTATGGCT	GGGCACTGGCCTGAGGGATTAG
PR-B NM_022847.1	ATGACTGAGCTGCAGGCAAAGG	CTTCCACTCCAGAGAAAGCTCCC
PR-A/B NM_022847.1	GGCAAATCCCACAGGAGTTTGTC	CAGACATCATTTCCGGAAATTCC

in the CFX 96 Real-Time system (BioRad, Germany) with SYBR-Green (Molecular Probes, Eugene Oregon, USA) using gene-specific primers (Table 1). For the analysis of the PR, primers specific for PR-B and primers for PR-A/B were used according to Shao and colleagues [22]. Serial dilutions of target-specific plasmid DNA were used as internal standard. Calculations were done with the Bio-Rad CFX Manager. The resulting molecule concentrations were normalized to the reference gene Mrps18a (mitochondrial ribosomal protein S18A). Constant expression of Mrps18a was tested and confirmed against other reference genes (data not shown). The fold change of mRNA levels was calculated with the relative standard curve method. Melting curves and gel electrophoresis of the RT-qPCR product were routinely performed to assure the specificity of the PCR reaction.

Ussing Chamber Measurements

According to the RT-qPCR experimental setting, Ussing chamber measurements were performed on the 5th day of culture. A detailed description of Ussing chamber measurement procedures is reported elsewhere [20]. Equivalent short-circuit currents (I_{sc}) were assessed every 20 s by measuring transepithelial voltage (V_{te}) and resistance (R_{te}) using a transepithelial current clamp (Physiologic instruments, San Diego, CA, USA), and calculating the quotient $I_{sc} = V_{te}/R_{te}$. Monolayers were only included in the analysis when the transepithelial resistance (R_{te}) exceeded 300 $\Omega \cdot cm^2$ throughout the measurement. Amiloride (10 μM) (A-7410, Sigma) was applied to the apical compartment to inhibit amiloride-sensitive Na^+ transporters like ENaC, and the amiloride-sensitive I_{sc} (I_{amil} displayed as ΔI_{SC}) was calculated. After a steady state was reached, ouabain (1 mM) (O-3125, Sigma), an inhibitor of Na,K-ATPases, was added to the basolateral compartment to determine the ouabain-sensitive I_{sc} (I_{ouab} displayed as ΔI_{SC}), accordingly.

Statistical Analysis

Differences among groups incubated with hormones and controls were evaluated by the unpaired *t* test or ANOVA with Dunnett's or Tukey's post-hoc test, as appropriate, using GraphPad Prism software (GraphPad Software, Inc.).

Results

Effect of Progesterone on ENaC Subunit Expression

The mRNA expression of the ENaC subunits was measured in FDLE cells grown in media supplemented with 2.8 μM progesterone and compared to control monolayers without hormone addition. The analysis showed that the mRNA expression of the α-ENaC and γ-ENaC subunits was significantly increased by progesterone (Fig. 1). The mRNA expression of the α-ENaC subunit was elevated ninefold (Fig. 1a) and for γ-ENaC, a 12-fold increase was detected (Fig. 1c). The β-ENaC subunit did not show a significant increase by progesterone (Fig. 1b). In addition, the primers specific for PR-B mRNA expression did not detect any product (Fig. 1d). The product amplified with PR-A/B primers, therefore, represents the mRNA expression of PR-A in FDLE cells.

Effect of Glucocorticoids on ENaC Subunit Expression and Channel Activity

Next, the mRNA expression of ENaC subunits in the presence of different dexamethasone concentrations was measured (Fig. 2). The analysis revealed an increase of the mRNA expression for all three ENaC subunits in the presence of 100 nM, 300 nM, and 1 μM dexamethasone compared to control monolayers grown without dexamethasone (Fig. 2a–c). In addition, the elevation of dexamethasone from 100 nM to 300 nM further increased the mRNA expression of α-ENaC and γ-ENaC (Fig. 2a, c) but not β-ENaC (Fig. 2b). Surprisingly, exposure of FDLE cells to 1 μM dexamethasone showed that mRNA expression was not further increased but rather displayed a decrease compared with 300 nM dexamethasone for all three subunits, which was statistically significant for γ-ENaC. Comparing the fold change revealed that stimulation of mRNA expression by dexamethasone was most

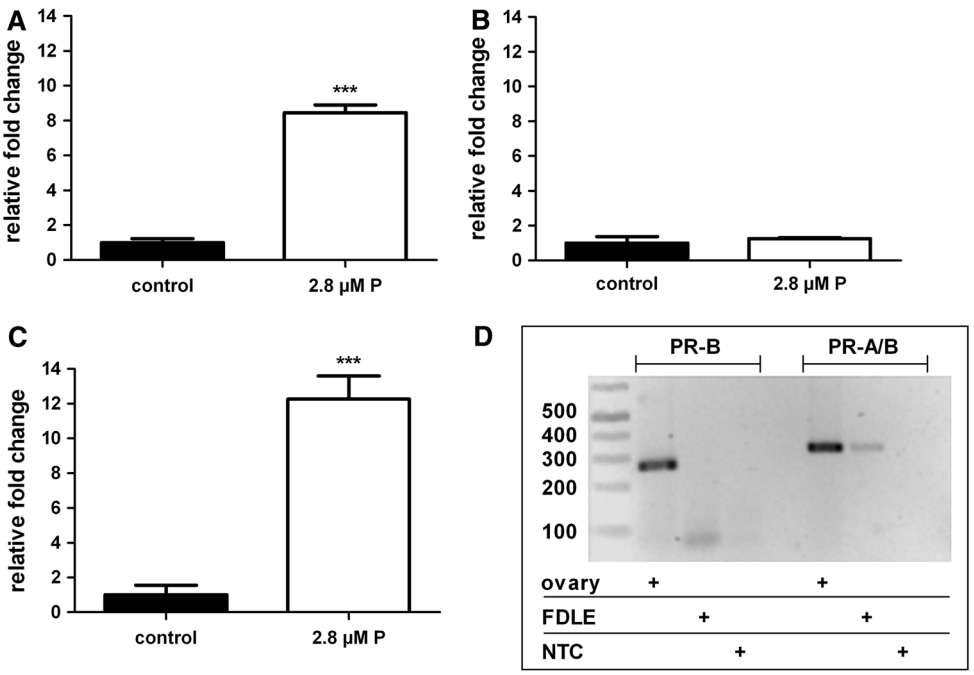

Fig. 1 Progesterone distinctively regulates ENaC subunit expression in FDLE cells. **a** α-ENaC subunit (Mean + SEM, $n = 4$, $^{***}p < 0.001$ by t test). **b** β-ENaC subunit (Mean + SEM, $n = 4$). **c** γ-ENaC subunit (Mean + SEM, $n = 4$, $^{***}p < 0.001$ by t test). **d** Ethidium bromide-stained agarose gel of PR-A and PR-B PCR products. No expression of PR-B was detected in FDLE cells; PR-A/B expression, therefore, represents the expression of PR-A in FDLE cells. Rat's ovary tissue was used as positive control. *P* progesterone, *NTC* non-template control

pronounced for α- and γ-ENaC resulting in an almost 40-fold increase induced by 300 nM dexamethasone. For β-ENaC, only a fivefold increase was achieved with 100 nM dexamethasone.

In Ussing chamber measurements, the I_{sc} (μA/cm^2) of monolayers incubated with the different dexamethasone concentrations was determined. All three dexamethasone concentrations increased the I_{sc} (Fig. 2d). In line with the mRNA expression analysis, 1 μM dexamethasone did not further increase the I_{sc}. No significant differences were seen between the different dexamethasone concentrations. In addition, I_{amil} and I_{ouab} of dexamethasone-stimulated monolayers also increased compared to control monolayers; however, no significant differences between the dexamethasone concentrations were observed.

Combined Effect of Progesterone and Dexamethasone on ENaC Subunit Expression

To determine whether the stimulating effect of dexamethasone and progesterone is additive, we combined 2.8 μM progesterone with different dexamethasone concentrations and analyzed the ENaC subunit expression. The results, however, showed no additive effect of both hormones (Fig. 3). In contrast, combining 300 nM dexamethasone with 2.8 μM progesterone resulted in a 30 % lower α-ENaC expression compared with dexamethasone alone (Fig. 3a). The same trend was detected for the combination of 100 nM and 1 μM dexamethasone with 2.8 μM progesterone. The inhibiting effect of progesterone on maximal dexamethasone stimulation was also observed in the expression of the γ-ENaC subunit, with the combination of 300 nM dexamethasone with 2.8 μM progesterone reducing mRNA expression by more than 40 % (Fig. 3c). In contrast, β-ENaC mRNA expression was not affected by the combination of dexamethasone with progesterone (Fig. 3b). The results, therefore, demonstrate no additive effect of dexamethasone and progesterone, but rather a reduction of α- and γ-ENaC subunit expression with the combination of both hormones.

Combined Effect of Progesterone and Dexamethasone on Na^+ Channel Activity

In Ussing chamber measurements, the influence of 100 nM dexamethasone in combination with 2.8 μM progesterone was analyzed and compared to monolayers incubated without steroids, 100 nM dexamethasone or 2.8 μM progesterone alone (Fig. 4a). The baseline I_{sc} was significantly increased in monolayers incubated with progesterone or dexamethasone, to almost the same extent. However, no additional increase of I_{sc} was achieved after combining progesterone and dexamethasone in the culture media. Similar effects were seen measuring the I_{amil} and I_{ouab}, which were elevated in the presence of either steroid, but no differences were observed between dexamethasone and progesterone or the combination of both. Relating to the RT-qPCR results showing a decrease of maximum dexamethasone effect after the addition of progesterone, we further determined whether higher dexamethasone concentrations in combination with progesterone reduce the maximum stimulatory effect. Therefore, 300 nM dexamethasone was

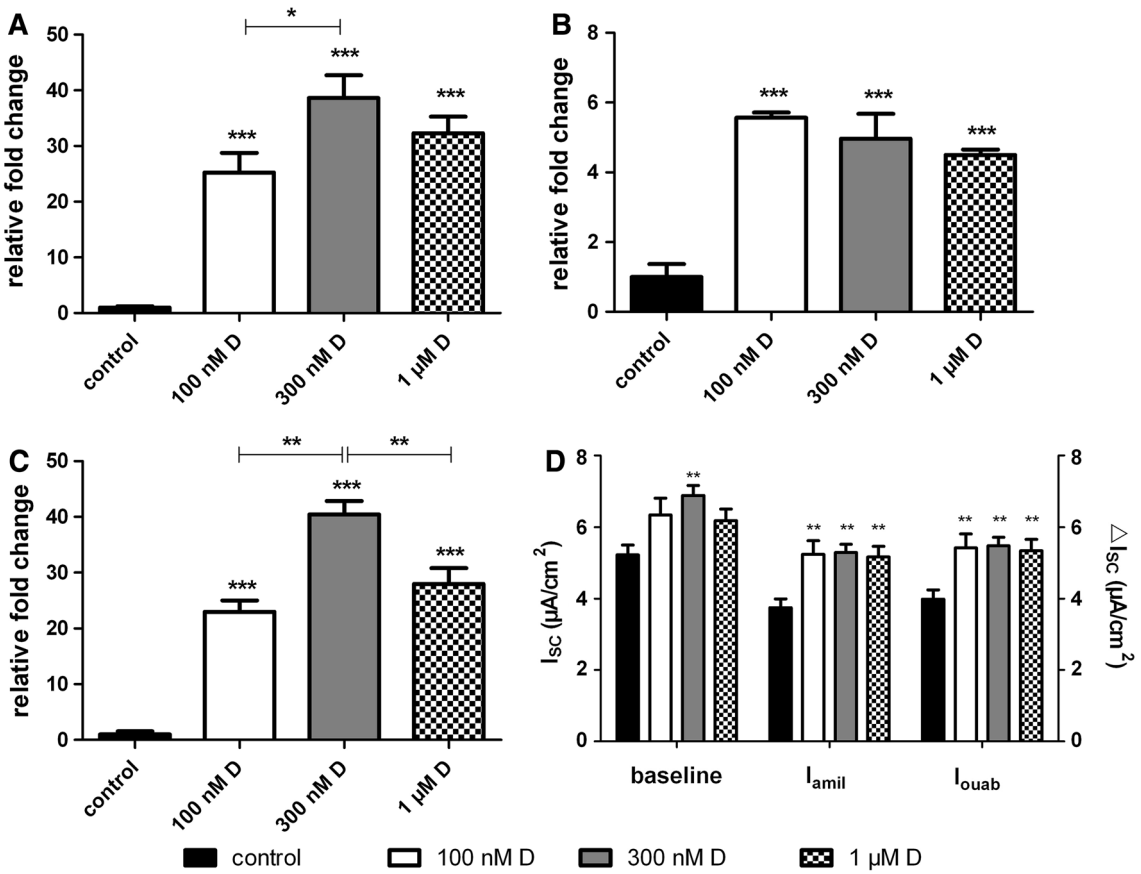

Fig. 2 Dexamethasone increases expression of all three ENaC subunits and elevates I_{SC} in FDLE cells. Monolayers were grown in dexamethasone-supplemented media with either 100 nM, 300 nM, or 1 µM. **a** α-ENaC subunit (Mean + SEM, $n = 4$, $*p < 0.05$; $***p < 0.001$ by ANOVA with Tukey's post-hoc test). **b** β-ENaC subunit ($n = 4$, $***p < 0.001$ by ANOVA with Tukey's post-hoc test). **c** γ-ENaC subunit ($n = 3$–4, $**p < 0.01$; $***p < 0.001$ by ANOVA with Tukey's post-hoc test). **d** Ussing chamber analysis of FDLE cells incubated with different dexamethasone concentrations. $I_{baseline}$ was the I_{sc} after mounting the monolayers in the Ussing chambers, I_{amil} is the current reduction caused by amiloride (10 µM), and I_{ouab} is the current reduction induced by ouabain (1 mM). (Mean + SEM, $n = 14$–19, $**p < 0.01$ by ANOVA with Dunnett's post-hoc test). *D* dexamethasone

combined with 2.8 µM progesterone and compared to the I_{sc} of monolayers incubated with 300 nM dexamethasone alone (Fig. 4b). Under these conditions, the addition of progesterone significantly reduced the I_{sc} compared to monolayers incubated with dexamethasone alone, thus supplementing the RT-qPCR results.

Combined Effect of Progesterone and Dexamethasone on ENaC Subunit Expression at Lower Concentrations

Since high concentrations of dexamethasone alone or in combination with progesterone reduced the maximum effect seen on ENaC subunit expression, we sought to determine the action of lower concentrations, i.e., 10 nM dexamethasone in combination with progesterone concentrations ranging from 0 to 1 µM (Fig. 5). A progesterone concentration of 0.28 µM alone increased the mRNA expression of all three ENaC subunits, ranging between 10 and 50 % (Fig. 5a–c). In addition, 10 nM dexamethasone alone also increased the ENaC subunit expression. The expression of α-ENaC was nearly threefold increased with 10 nM dexamethasone, which remained unchanged by an addition of 0.28 µM or 1 µM progesterone (Fig. 5a). The mRNA expression of the β-subunit was also increased by 10 nM dexamethasone, reaching a twofold level, which was also not affected by progesterone addition (Fig. 5b). The γ-subunit showed a twofold increase, similar to the β-subunit, which, however, was significantly reduced by 1 µM progesterone (Fig. 5c).

Involvement of Hormone Receptors

Finally, the involvement of hormone receptors (PR and GR) was analyzed. Mifepristone was used to antagonize the GR and PR. Furthermore, the specific PR antagonist (PF-02367982) was used, which does not interact with the GR. Thereby, it is possible to distinguish the effects mediated by GR and PR [31]. Monolayers incubated with 300 nM

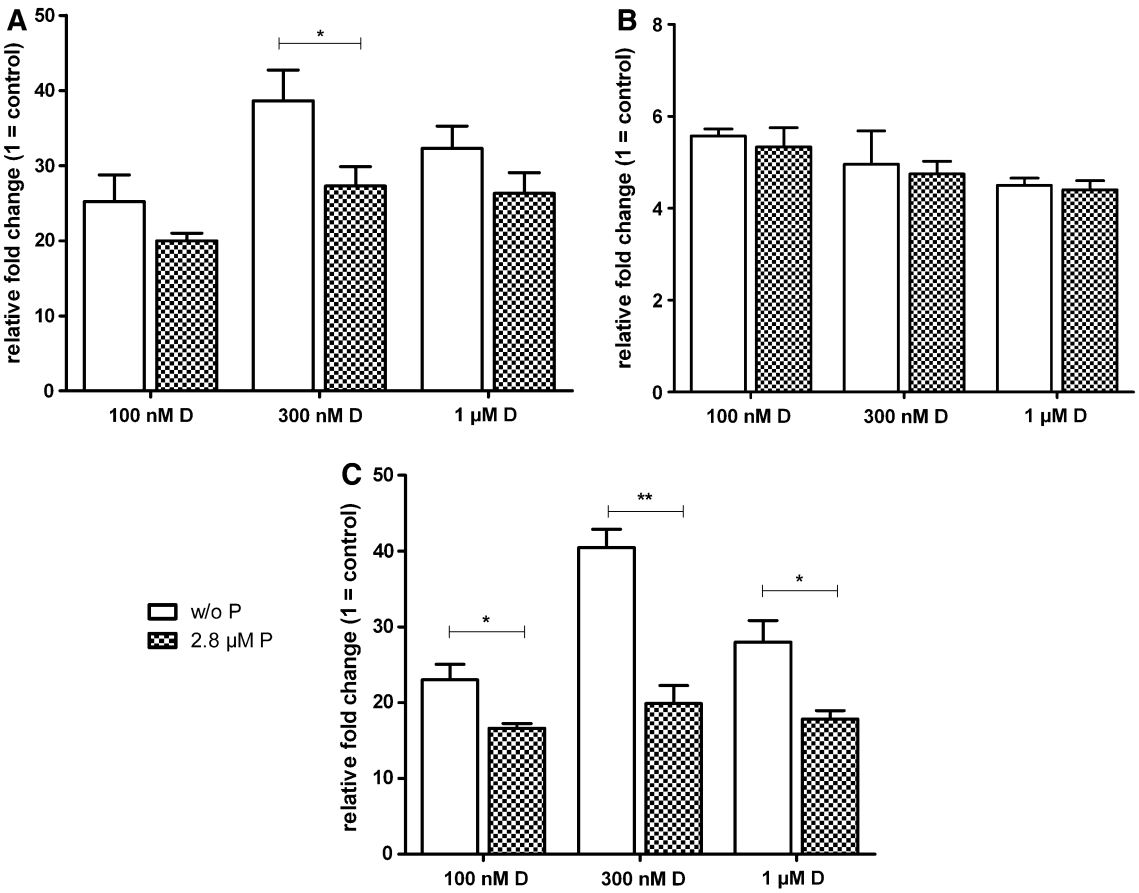

Fig. 3 The combination of dexamethasone and progesterone reduces ENaC subunit expression. Monolayers were grown in dexamethasone-supplemented media with either 100 nM, 300 nM, or 1 µM in combination with 2.8 µM progesterone. RT-qPCR analysis showed a reducing influence of progesterone on maximum dexamethasone effect. **a** α-ENaC subunit (Mean + SEM, $n = 4$, $^*p < 0.05$ by t test). **b** β-ENaC subunit ($n = 4$). **c** γ-ENaC subunit ($n = 3$–4, $^*p < 0.05$; $^{**}p < 0.01$ by t test). *D* dexamethasone, *P* progesterone

dexamethasone alone or in combination with 2.8 µM progesterone were compared, as these concentrations had caused the most pronounced effects on ENaC subunit expression and Na^+ transport. Inhibition of the PR resulted in a twofold increase of α-ENaC mRNA expression in monolayers incubated with 300 nM dexamethasone and also in monolayers incubated with 2.8 µM progesterone and 300 nM dexamethasone (Fig. 6a). However, the maximum dexamethasone effect was still reduced by progesterone even though the PR was blocked. Inhibiting both hormone receptors by mifepristone dramatically reduced α-ENaC mRNA expression. Interestingly, the difference between monolayers incubated with dexamethasone alone and in combination with progesterone disappeared after adding mifepristone (Fig. 6a). The β-subunit expression, however, reacted differently (Fig. 6b). Both the selective PR antagonist as well as mifepristone significantly reduced the β-ENaC mRNA expression independent of the presence of progesterone. There was no difference between monolayers incubated with dexamethasone alone and monolayers incubated with dexamethasone and progesterone. The γ-ENaC subunit expression increased, similar to but less pronounced than the α-ENaC expression, after the inhibition of the PR in both dexamethasone and dexamethasone/progesterone-supplemented monolayers (Fig. 6c). In addition, mifepristone also reduced γ-ENaC mRNA expression. In contrast to the α-ENaC subunit expression, the difference between monolayers incubated with dexamethasone alone and in combination to progesterone, however, persisted even in the presence of mifepristone.

Discussion

Female sex steroids and glucocorticoids stimulate alveolar epithelial Na^+ transport and thereby mediate AFC in addition to lung inflation [9, 20, 29, 32, 33]. Near-term fetal glucocorticoid levels rise and increase the transcription of ENaC subunits and prolong half life of membrane-bound ENaC channels [10, 34, 35]. In addition, the fetus is

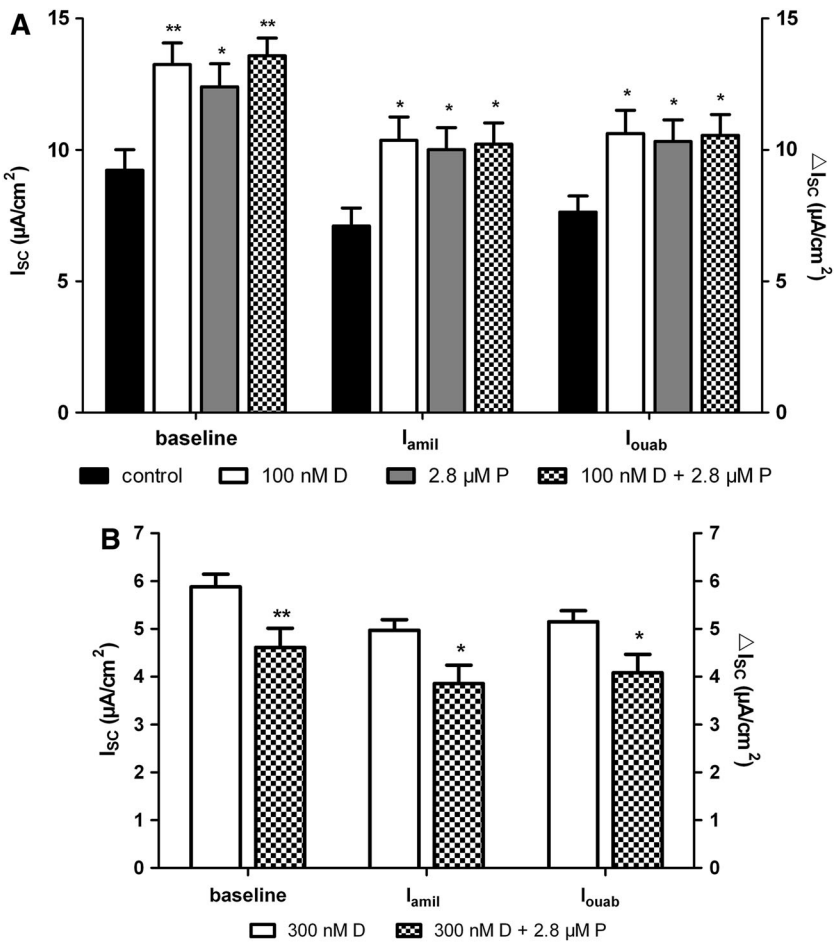

Fig. 4 Dexamethasone and progesterone influence on Na^+ transport is concentration dependent. Ussing chamber measurements were performed to analyze the influence of dexamethasone and progesterone alone and in combination on I_{sc} in FDLE cells. $I_{baseline}$ was the I_{sc} after mounting the monolayers in the Ussing chambers, I_{amil} the current reduction caused by amiloride (10 μM), and I_{ouab} the current reduction induced by ouabain (1 mM). **a** Control monolayers without hormone supplementation were compared to monolayers incubated with 100 nM dexamethasone, 2.8 μM progesterone, or both hormones in combination (Mean + SEM, $n = 8$–10, $^*p < 0.05$; $^{**}p < 0.01$ by ANOVA with Dunnett's post-hoc test). **b** Monolayers incubated with 300 nM dexamethasone alone and in combination with 2.8 μM progesterone were compared (Mean + SEM, $n = 25$–19, $^*p < 0.05$; $^{**}p < 0.01$ by t test). *D* dexamethasone, *P* progesterone

exposed to high levels of maternal sex steroids which have been shown to increase the transcription of ENaC subunits and elevate epithelial Na^+ transport [18, 29]. Although both hormones are present in the fetus at high concentrations, knowledge about the effect of both in combination is limited. We hypothesized that both hormones show additive effects, resulting in a higher increase of ENaC transcription and activity than achieved by a single hormone supplementation. However, the results showed a rather diminishing effect of a combined hormone supplementation, suggesting an inhibiting effect of progesterone on maximal glucocorticoid effect.

In the first part of this study, we have shown that progesterone increases the mRNA expression of the α- and γ-ENaC subunit, while the β-subunit expression was not stimulated by progesterone. The same differential regulation of ENaC subunits was demonstrated previously using a combination of estradiol and progesterone [29]. Estradiol, however, appeared to be less important, because it was shown before that ENaC activity was most strongly stimulated in culture media with high progesterone but very low estradiol concentrations [18]. Our present results suggest that progesterone alone is sufficient to increase ENaC expression without additional estradiol supplementation.

The analysis of dexamethasone effects showed an increase of ENaC subunit expression, thus confirming previous results [34, 36], although increasing dexamethasone concentrations revealed that the maximum effect was achieved with 300 nM dexamethasone. Moreover, a decreased mRNA expression was observed when monolayers were incubated with 1 μM dexamethasone compared to 300 nM. We speculate that this observation demonstrates the so-called squelching phenomenon. Squelching describes that excess receptor can inhibit its own transactivation as well as transcription by other transactivators [37]. This is possibly caused by limited additional factors required for transactivation which are trapped by an abundance of steroid receptors in unproductive complexes [38]. Furthermore, the observed down-regulation shown for high dexamethasone concentrations could serve as a self-protection of the cell. This is supported by the fact that excessive ENaC activity leads to diseases like Liddle's disease, an inherited form of systemic hypertension [39]. In addition, it has been shown that the administration of dexamethasone results in a down-regulation of the hGR-α mRNA

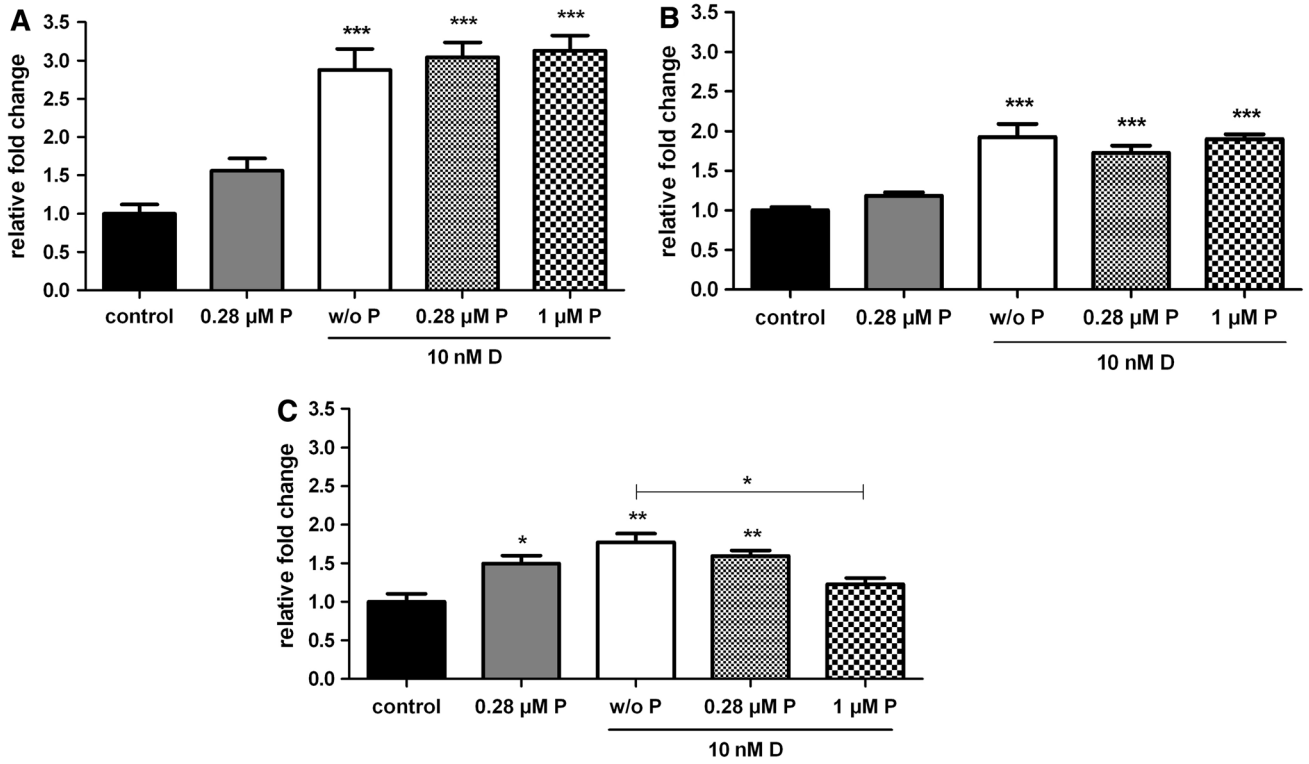

Fig. 5 The combination of dexamethasone and progesterone at low concentrations shows no additive effect. Monolayers were grown in hormone-supplemented media with either 0.28 µM progesterone, 10 nM dexamethasone, or 10 nM dexamethasone + 0.28 µM progesterone or 1 µM progesterone. RT-qPCR analysis showed no additive effect of both hormones even at low concentrations. **a** α-ENaC subunit (Mean + SEM, $n = 4$, $^{***}p < 0.001$ by ANOVA with Dunnett's post-hoc test). **b** β-ENaC subunit (Mean + SEM, $n = 4$, $^{***}p < 0.001$ by ANOVA with Dunnett's post-hoc test). **c** γ-ENaC subunit (Mean + SEM, $n = 3$–4, $^{*}p < 0.05$; $^{**}p < 0.01$ by ANOVA with Dunnett's post-hoc test). *D* dexamethasone, *P* progesterone

level [27, 40] thereby reducing dexamethasone effectivity. The reason for this receptor down-regulation is still unknown, but might explain the reduction of α- and γ-ENaC mRNA expression by high dexamethasone concentrations. Some reports suggest that GR-β might be differentially regulated by glucocorticoids than GR-α (see Review [41]). One study showed an up-regulation of GR-β mRNA expression in opposition to GR-α by glucocorticoids in skeletal myoblasts [42]. Since GR-β is known as an inhibitor of hGR-α activity [25], the decrease of ENaC expression induced by high dexamethasone concentrations might be due to an increase of GR-β mRNA expression. However, this is discussed controversial, and other groups did not show an increase of GR-β but rather a decrease of both GR isoforms induced by glucocorticoids [41]. The response to glucocorticoid treatment is cell type specific [43]. A549 cells, a cell line with many characteristics of ATII cells, showed no further increase of ENaC mRNA expression between 100 and 1000 nM dexamethasone [32], but also no decrease. Furthermore, the ENaC subunits are regulated differentially by glucocorticoids. Without hormone supplementation, the γ-ENaC subunit is the most abundant subunit in FDLE cells. In the ovine lung, β-ENaC mRNA was more abundant at all gestational ages, while β-ENaC protein expression decreased with fetal maturation [44]. However, species differences between the ovine and human compared to the rodent lung in relative expression of the ENaC subunits have been noted before [33, 45]. After stimulation of FDLE monolayers with dexamethasone, the highest increase of mRNA expression was seen for the α- and γ-ENaC subunit, up to 40-fold compared to unstimulated controls. In contrast, the mRNA expression of β-ENaC was also increased by dexamethasone, yet not to the same extent and only reached a fivefold elevation. In the fetal ovine lung, it was shown that α- and γ-ENaC subunit mRNA expression peaks at the time of labor when fluid clearance has to occur and fetal cortisol levels are high [44]. As noted before, the β-ENaC mRNA expression was most abundant in that study, yet protein levels decreased during gestation [44]. Since overexpression of β-ENaC in mice decreases mucus clearance and reduces fluid in the postnatal lung resulting in a cystic fibrosis-like phenotype [46], the reduction of β-ENaC protein levels in the ovine lung and the smaller response of β-ENaC mRNA expression to glucocorticoids in the FDLE cells might be critical for postnatal lung function. Therefore, the observed expression

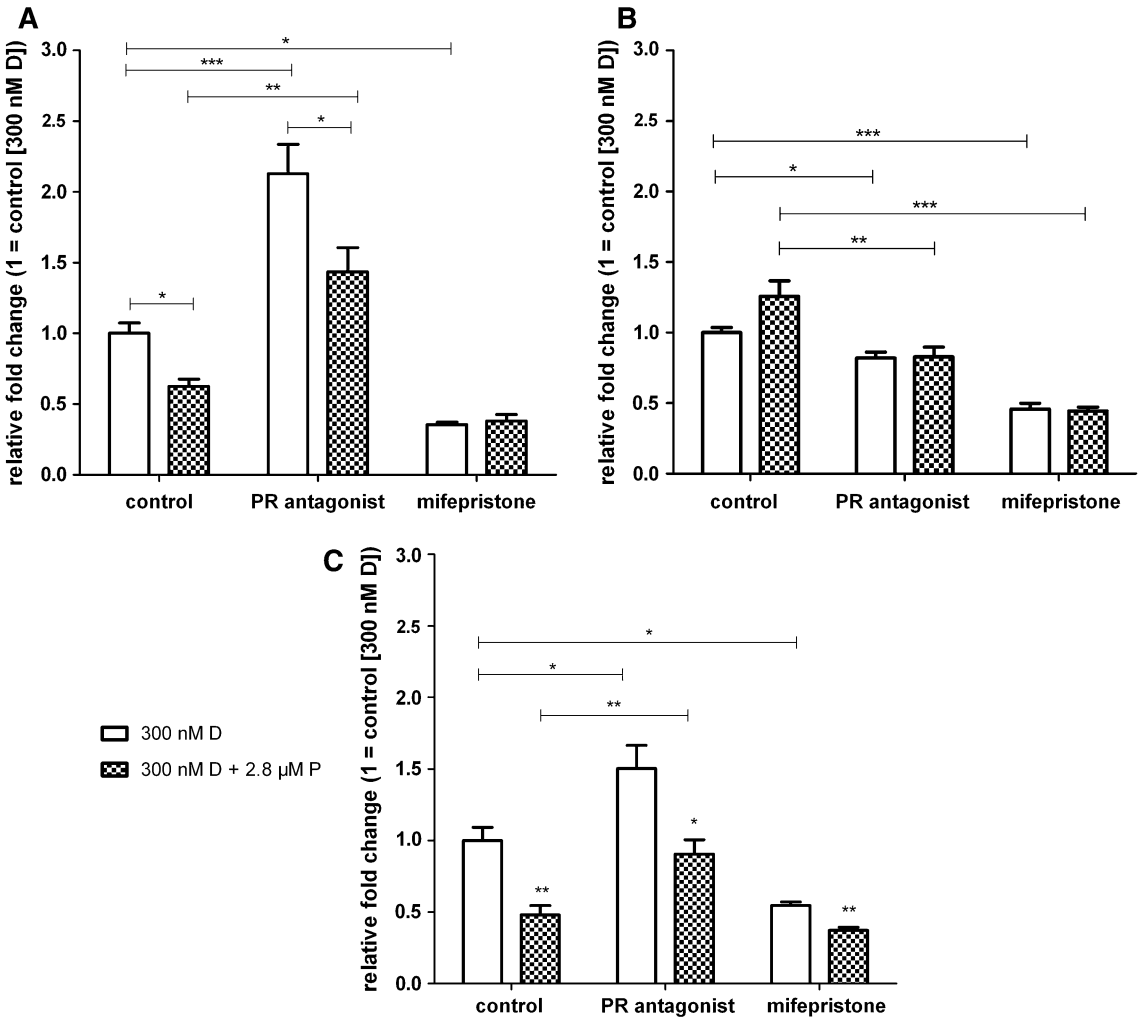

Fig. 6 PR and GR are distinctively involved in ENaC subunit expression. Monolayers were grown in 300 nM dexamethasone-supplemented media either alone or in combination with 2.8 µM progesterone. In addition, PF-02367982 was used as PR antagonist and mifepristone as GR and PR antagonist. RT-qPCR showed different responses of the ENaC subunits to the receptor antagonists. **a** α-ENaC subunit (Mean + SEM, $n = 3$–4, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by ANOVA with Dunnett's post-hoc test for the comparison of receptor antagonists or by *t* test for the comparison of dexamethasone and dexamethasone/progesterone). **b** β-ENaC subunit (Mean + SEM, $n = 4$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by ANOVA with Dunnett's post-hoc test for the comparison of receptor antagonists). **c** γ-ENaC subunit (Mean + SEM, $n = 3$–4, $^{*}p < 0.05$; $^{**}p < 0.01$ by ANOVA with Dunnett's post-hoc test for the comparison of receptor antagonists or by *t* test for the comparison of dexamethasone and dexamethasone/progesterone). *D* dexamethasone, *P* progesterone

pattern of ENaC subunits is well explained by the functional relevance of each subunit. In A549 cells, α-hENaC mRNA was not significantly elevated by dexamethasone, while γ-hENaC mRNA levels were strongly increased, accompanied by a modest up-regulation of β-hENaC mRNA expression [32]. However, A549 cells are an adult cell type, while our study and the study of the ovine lung were performed in fetal cells, which might add an additional disparity concerning the developmental stage of the cells.

The rise of mRNA expression induced by dexamethasone was accompanied by an increase of I_{amil} as shown before in different cell types [20, 32, 36]. However, the magnitude of the increase observed in our study differs from previous findings. For instance, 100 nM dexamethasone doubled the I_{amil} in H441 lung cells accompanied by a fivefold increase in α-ENaC mRNA expression [47]. In FDLE cells, we demonstrated an increase of about 40 % in I_{amil} induced by 100 nM dexamethasone accompanied by a 24-fold increase of α-ENaC mRNA expression. This increase in I_{amil} was not further elevated by increasing dexamethasone concentrations and, therefore, represents the maximal current increase inducible by dexamethasone. Differences might be explained by the subunit composition, which is important for the channel functionality. It is

known that α-ENaC is the only subunit which is able to form a functional channel by itself, and yet these channels only achieve 1 % of the maximal whole-cell current [48]. On the other hand, the presence of the α-subunit is indispensable for the assembly of a functional channel, because the β- and γ-subunits, expressed alone or in combination, are not able to induce any current [48]. This was confirmed in α-ENaC knock out mice which died within hours after birth due to defective lung fluid clearance [6]. Since the mRNA expression analysis confirmed that not all ENaC subunits are stimulated equally by dexamethasone, a smaller increase of the I_{amil} might be explicable. We did not perform Western Blot analysis of the ENaC subunit protein expression which is a limitation of this study. Therefore, we cannot exclude that translational effects might be responsible for the different magnitudes of change for the mRNA expression and current measurement results.

In the next set of experiments, we analyzed the effect of progesterone and dexamethasone in combination with ENaC mRNA expression and I_{amil} in FDLE cells. To our surprise, no additive effect was observed, but rather a diminishing effect of progesterone. The expression of α- and γ-ENaC subunit mRNA was lower in the presence of progesterone and dexamethasone than that achieved by dexamethasone alone. Therefore, progesterone, which by itself increases ENaC subunit expression, does not further increase dexamethasone-stimulated ENaC subunit expression, but reduces the effect of dexamethasone. This has not been reported before. The same effect was observed when measuring the I_{amil} in cells exposed to 300 nM dexamethasone, which was reduced by the presence of progesterone. Using lower hormone concentrations, this inhibiting effect was not as pronounced, yet still detectable in measurements of γ-ENaC. In contrast, the β-ENaC mRNA expression in the presence of dexamethasone was not affected by additional progesterone; however, as shown before, progesterone also does not affect the β-ENaC when applied alone. A possible explanation for the negative effect of progesterone on the glucocorticoid effect could be the action of one of the steroid receptors. It was demonstrated that the binding affinity of progesterone to the GR is 22 % relative to the endogenous ligand cortisol [21]. Due to the structural similarity of progesterone, it fits into the binding pocket and acts as a partial agonist at the GR [49, 50]. A partial agonist is unable to completely activate the receptor or the downstream signaling cascade compared to the full agonist. Since a partial agonist competes with the full agonist to occupy the receptor, it inhibits the effects of the full agonist and displays partial antagonist activity. This is supported by the fact that the inhibiting effect of progesterone on α- and γ-ENaC expression persisted even after blocking the PR. When the GR was blocked, however, the effect was abolished with regard to α-ENaC. Therefore, the diminishing effect of progesterone is mediated by the GR and not by the PR, and likely represents a competition of both hormones for the same receptor. The same mechanism was demonstrated for the glucocorticoid-dependent aromatase induction that was inhibited by progesterone [49]. Blocking both receptors in the last set of experiments revealed another effect that has not been demonstrated before. The mRNA expression of α- and γ-ENaC is strongly increased by inhibiting the PR by its specific antagonist. This suggests that the action of the PR negatively affects ENaC expression. To further analyze these results, we characterized the PR expressed in FDLE cells. Two isoforms of the PR are known: PR-A and PR-B. PR-A lacks 164 amino acids at the *N*-terminus compared to the full-length PR-B [26]. We were able to show the mRNA expression of PR-A, while PR-B was not expressed in FDLE cells. It has been shown before that PR-A is the predominant PR in mouse lung, and PR-B transcripts are absent in the same lung tissue [22]. It is known that PR-A acts as a dominant repressor and is able to diminish the activities of other nuclear receptors such as the GR [26]. PR-B, on the other hand, activates transcription in several tissues [51–53]. Therefore, the increase of ENaC expression observed in the presence of PR inhibition is well explicable with a reduction of PR-A-mediated GR repression. For the γ-subunit, the diminishing effect of progesterone persists even after inhibition of the GR. This suggests another pathway of progesterone-mediated inhibition of glucocorticoid action which remains yet unknown. Furthermore, the mRNA expression of all ENaC subunits displays differential regulation under receptor inhibition. As α- and γ-ENaC showed an increase of expression under PR inhibition, the β-subunit decreases, which is remarkable since progesterone showed no effect on β-ENaC expression. These results suggest that there is still a lot unknown about the interaction of these hormones and the associated receptors.

In conclusion, we have shown that each ENaC subunit is individually regulated by steroid hormones and their receptors. Furthermore, the results show that the effects obtained with dexamethasone alone differ from a combined dexamethasone and progesterone treatment, which should be considered in future analysis of glucocorticoid actions. Therefore, the study suggests that caution is necessary for the analysis of glucocorticoid effects since the presence of progesterone might alter their effect.

Acknowledgments The authors wish to thank Sylvia Taube, Maike Ziegler, and Jessica Schneider for excellent technical assistance. The specific PR antagonist PF-02367982 was kindly provided by Pfizer. All animal care and experimental procedures were approved by the responsible authority (Landesdirektion Leipzig).

Conflict of interest No Grants or conflicts of interest, financial or otherwise, are declared by the authors.

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