Modification of biophysical properties of lung epithelial Na\(^+\) channels by dexamethasone

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Received 29 October 1999; accepted in final form 30 March 2000

Lazrak, A., A. Samanta, K. Venetsanou, P. Barbry, and S. Matalon. Modification of biophysical properties of lung epithelial Na\(^+\) channels by dexamethasone. Am J Physiol Cell Physiol 279: C762–C770, 2000.—There is considerable interest in identifying the basic mechanisms by which dexamethasone alters ion transport across the adult alveolar epithelium. Herein, we incubated synchronized A549 cells, a human alveolar epithelial cell line, with dexamethasone (1 μM) for 24–48 h. When normalized to HPRT (a housekeeping gene), A549 β- and γ-subunit mRNA levels for the human amiloride-sensitive epithelial sodium channel (hENaC), assessed by RT-PCR, increased by 1.6- and 17-fold respectively, compared with control values (P < 0.05). These changes were abolished by actinomycin D, indicating transcriptional regulation. Western blotting studies revealed that dexamethasone also increased expression of β- and γ-hENaC protein levels. In contrast, α-hENaC mRNA increased by onefold (P > 0.05) and α-hENaC protein level was unchanged. Incubation of A549 cells with dexamethasone increased their whole cell amiloride-sensitive sodium currents twofold and decreased the K\(_{\text{D}}\) for amiloride from 833 ± 69 to 22 ± 5.4 nM (mean ± SE; P < 0.01). Single channel recordings in the cell-attached mode showed that dexamethasone treatment increased single channel open time and open probability threefold and decreased channel conductance from 8.63 ± 0.036 to 4.4 ± 0.027 pS (mean ± SE; P < 0.01). We concluded that dexamethasone modulates the amiloride-sensitive Na\(^+\) channels by differentially regulating the expression of β- and γ-subunits at the mRNA and protein levels in the human A549 cell line, with little effect on α-hENaC subunit.

ENaC; lung; whole cell currents; single channels; A549 cells; amiloride; reverse transcription-polymerase chain reaction; Western blots

THE AMILORIDE-SENSITIVE epithelial Na\(^+\) channels (ENaC) are the main pathways for Na\(^+\) ion entry in a large number of epithelial cells, including those in lung airways and alveolar epithelium, the distal nephron, colon, and kidneys (1, 13, 23, 31). The channel, which belongs to a large family of ion channels, is a heteromultimeric complex of at least three distinct but homologous subunits, named α-, β-, and γ-ENaC, which were first cloned from the colon of salt-deprived rats and human lung tissue (3, 4, 20, 40). Experiments utilizing point mutations suggested that all three subunits were involved in the channel’s pore formation (33). The subunits are characterized by two short NH\(_2\) and COOH termini, two short membrane-spanning segments, and a very large extracellular loop domain (29). The expression of the three subunits is necessary for maximal functional activity (15). The exact stoichiometry is still debated, with different groups reporting either four subunits (two α, one β, and one γ) (11) or nine subunits (three of each) in the complex (35).

There is convincing evidence for the existence of active Na\(^+\) transport across the lung alveolar epithelial type II (ATII) cells. This active transport plays an important role both in the regulation of the alveolar hypophase liquid volume and in the reabsorption of edema fluid in pathological conditions (25, 41). Expression of rat α-ENaC (α-rENaC) mRNA in adult rat ATII cells was demonstrated by Northern blot analysis (42), PCR (10), and by in situ hybridization (10, 24, 42). The β- and γ-mRNAs for rENaC were also detected in large and small airways, but they were less abundant in ATII cells compared with α-rENaC (10).

In cultured lung and airway epithelia, glucocorticoid and mineralocorticoid hormones have been shown to upregulate the electrogenic Na\(^+\) absorption (6, 32). Recently, Sayegh et al. (32) demonstrated that in a lung airway cell line (H441), dexamethasone upregulated the electrogenic Na\(^+\) transport and α-subunit human ENaC (α-hENaC) expression via activation of a hormone response element in the 5′-flanking regions of α-hENaC. This was consistent with the previous report by Champigny et al. (6) that dexamethasone increases the functional expression of the amiloride-sensitive Na\(^+\) current by enhancing transcription of all three subunits in fetal rat lung epithelial cells. However, there are no studies correlating dexamethasone-in-
duced changes in ENaC proteins with changes in functional properties of amiloride-sensitive Na⁺ channels in lung epithelial cells.

Herein we demonstrate that treatment of synchronized A549 cells, a cell line derived from human lung epithelial carcinoma cells with many of the characteristic functions of ATI cells (19), with dexamethasone resulted in a large upregulation of the γ-hENaC mRNA, together with a modest upregulation of β-hENaC mRNA. In addition, both β- and γ-hENaC protein levels were increased. Interestingly, α-hENaC mRNA and protein levels were not significantly different from control. Biophysical studies at the single channel level revealed that the treatment of synchronized A549 cells with dexamethasone increased the open probability (Pₒ) and mean open time of their amiloride-sensitive Na⁺ channel and decreased its unitary conductance. Also, dexamethasone-treated A549 cells had larger amiloride-sensitive whole cell currents that were more sensitive to amiloride than the corresponding currents in control cells. Our data provide evidence for the upregulation of the amiloride-sensitive currents in an epithelial cell line in the absence of significant changes in α-hENaC.

**MATERIALS AND METHODS**

**Cell culture.** A549 cells were purchased from American Type Culture Collection (Rockville, MD) in the 76th passage. They were cultured using a 50/50 mixture of DMEM and F-12 (DMEM-F12, Cellgro), supplemented with 1% penicillin-streptomycin and 10% fetal calf serum, plated on plastic tissue culture dishes (Corning Glass Works, Corning, NY), and incubated in a humidified atmosphere of 5% CO₂-95% air at 37°C. All measurements were conducted on cells synchronized by serum deprivation, prepared as follows. One million synchronized A549 cells prior to and following dexamethasone treatment were assessed by Western blotting studies. In some experiments, synchronized cells were incubated with 1 µM actinomycin D (100 mM stock in DMSO; GIBCO BRL) for 24 h. The cells were then washed, and the medium was replaced with serum-free medium containing dexamethasone and actinomycin D for an additional 24 h and then processed for RNA extraction.

**Western blotting studies.** Levels of α-, β-, and γ-hENaC in synchronized A549 cells prior to and following dexamethasone treatment were assessed by Western blotting studies. In some experiments, synchronized A549 cells were washed with cold PBS, incubated with 1 ml of 10 mM Tris-Cl (pH 7.4) containing 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of aprotinin, pepstatin, leupeptin, and trypsin inhibitor (buffer A; all chemicals from Sigma), and scraped using a cell scraper. The resulting suspension was sonicated for 10 s in ice and allowed to cool for 30 s; this procedure was repeated five times. More than 95% of the cells were broken as observed with light microscopy.

Sonicated cells were centrifuged at 2,000 g for 10 min at 4°C to remove nuclei, unbroken cells, and cell debris. The supernatant was collected and centrifuged at 45,000 g for 1 h. The membrane pellet was lysed by buffer A, containing 1% Nonidet P-40 (Sigma) and 150 mM NaCl and the above-mentioned protease inhibitors, for 1 h at 4°C with rotation. The suspension was centrifuged at 45,000 g for 30 min. The supernatant was then carefully removed, and its protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce) using BSA as standard. Fifty micrograms of the membrane lysate was denatured in SDS-sample lysis buffer (2% SDS, 62.5 mM Tris-Cl, pH 6.8, 5% β-mercaptoethanol, and 0.1% bromophenol blue) by boiling for 5 min. Samples were separated in 7% SDS-PAGE and were transferred to Sequi-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking with 2% BSA, the membranes were probed with an anti-α-ENaC antibody, generated by using a synthetic peptide corresponding to residues 44–57 within the NH₂-terminal intracellular domain of α-ENaC as previously described (34, 43), and then with anti-rabbit-conjugated horseradish peroxidase (HRP) and developed by enhanced chemiluminescence (ECL) using the kit provided by Pierce. In control experiments, membranes were probed with the anti-α-ENaC antibody and the synthetic peptide, followed by the secondary antibody. The anti-α-ENaC antibody and the immunizing peptide were kindly provided to us by Dr. Peter R. Smith of the Department of Physiology and Biophysics, University of Alabama at Birmingham.

To assess levels of β- and γ-hENaC, A549 proteins were separated in 7% SDS-PAGE as outlined above and transferred to Sequi-Blot PVDF membrane (Bio-Rad) in the absence of reducing agents. After blocking, the membranes were probed with polyclonal antisera against β-ENaC or γ-ENaC, followed by the secondary antibody (anti-rabbit-conjugated HRP) and developed by ECL using the kit from Pierce. The polyclonal antisera were raised as previously described and have been characterized extensively (12, 16, 48).
In control studies, membranes were incubated with preimmune sera. All other procedures were as described above.

**Patch clamp recordings: whole cell measurements.** For electrophysiological measurements, cells were seeded on glass cover slips, synchronized by incubation in serum-free medium, and exposed to dexamethasone as described above. Just before the start of experiments, coverslips were rinsed with standard external solution (SES) containing (in mM) 135 sodium methanesulfonate, 10 NaCl, 2.7 KCl, 1.8 CaCl₂, 2 MgCl₂, 5.5 glucose, and 10 HEPES, pH 7.4. They then were transferred to the recording chamber mounted on the stage of an inverted microscope (model IMT-2, Olympus) for patch clamp recordings. The recording chamber was continuously perfused with SES at a rate of 0.5 ml/s using a gravity-driven perfusion system.

The patch pipettes were made from Kimax50 type capillary glass tubing (Kimax) using a vertical puller (model PB-7; Narishige). They were filled with the standard internal solution (SIS; pH 7.2 at 22°C, 300 mosmol) containing (in mM) 120 potassium methylsulfonic acid, 15 KCl, 6 NaCl, 1 dimagnesium ATP, 2 trisodium ATP, 10 HEPES, and 0.5 EGTA. Only pipettes with a resistance in the 3–5 MΩ range, when filled with SIS, were used for whole cell recordings. The pipette offset potential was corrected just prior to the gigaseal formation, and series resistance and capacitance transients were compensated using the patch clamp amplifier (Axopatch 200A; Axon).

The cell membrane potential was held at −40 mV during all recordings, unless stated otherwise. Inward and outward currents were elicited by voltage protocols (from −100 to +100 mV in 10 mV steps for 450 ms), using the Clampex program (PCLAMP, Axon). The whole cell currents were digitized using a digital-to-analog and analog-to-digital converter (Digidata 1200A; Axon), sampled at 2–5 kHz, and filtered through an internal four-pole Bessel filter at 1 kHz. Current-voltage relationships were constructed from steady-state currents measured at 300 ms from the start of voltage pulses using the Clampfit program (Axon Instruments) and Origin software (Microcal Software, Northampton, MA).

To test the extent to which whole cell currents were inhibited by amiloride, we measured current-voltage relationships before and during perfusion of cells with SES containing amiloride (Calbiochem, La Jolla, CA), in concentrations ranging from 1 nM to 100 μM. The amiloride-sensitive currents were measured at a rate of 0.5 ml/s using a gravity-driven perfusion system.
were calculated by digitally subtracting the currents in the presence of amiloride from controls.

**Single channel recording.** Single channels were recorded from serum-deprived, dexamethasone-treated A549 cells patched in cell-attached mode, using pipettes with a resistance in the 5–10 MΩ range. The pipettes were coated with a thin layer of Sylgard and filled with a solution containing (in mM) 145 sodium methanesulfonate, 5 MgCl₂, 5.5 glucose, 40 mannitol, and 10 HEPES, pH 7.4. The Sylgard coating was necessary to reduce the noise and enhance the recording resolution. Prior to seal formation, cells were depolarized to 0 mV by bathing them in the following solution (mM): 145 potassium methanesulfonate, 5 MgCl₂, 40 mannitol, 10 HEPES, and 5.5 glucose, pH 7.4. Single channel activity was sampled at 2–5 kHz and filtered at 1 kHz. The data were analyzed using the Fetchan and pStat programs (PCLAMP, Axon Instruments). Single channel conductance was calculated from all event histograms, constructed as previously described (17).

**Statistics.** Results were expressed as means ± 1 SE. The Student *t*-test was used for statistical analysis between two group means. Statistical differences among multiple groups were calculated by digitally subtracting the currents in the presence of amiloride from controls.

Fig. 2. Transcriptional regulation of dexamethasone (Dex) in A549 cells. Synchronized A549 cells were treated with the indicated concentrations of dexamethasone in the presence (+) or in the absence (−) of actinomycin D (Act D, 1 μM). Total RNA (1 μg) extracted from A549 cells was used for reverse transcription, and 2 μl each of the 1st strand reaction mixture were used for amplification with human ENaC (hENaC) and also with the HPRT-gene-specific upstream and downstream primers (see text for details). Typical records were reproduced three times.

Fig. 3. Effect of dexamethasone on α-ENaC protein expression in A549 cells. Fifty micrograms of membrane lysate form synchronized and dexamethasone-treated or untreated A549 cells were separated in 7% SDS-PAGE, transferred to polyvinylidene difluoride membranes, followed by blocking and probing with anti-α-rENaC (A), anti-β-rENaC (B), and anti-γ-rENaC primary antibodies (C) and anti-rabbit horseradish peroxidase (HRP) conjugate as secondary antibody, and finally developed by enhanced chemiluminescence (ECL) reagents. Cont, control; MW, molecular mass.
RESULTS

RT-PCR. Results shown in Fig. 1A indicate the presence of α- and β-hENaC mRNAs in control A549 cells. A very small amount of γ-hENaC mRNA was detected in one of the three preparations. Exposure of A549 cells to dexamethasone (0.01–1 μM) induced dose-dependent increases in β- and γ-hENaC mRNA expression. For each experiment, values were normalized to the corresponding HPRT mRNA. Mean values (±1 SE) are shown in Fig. 1B. Exposure of A549 cells to 1 μM dexamethasone increased the relative expressions of β- and γ-hENaC mRNA by 1.6- and 17-fold from control values, respectively (P < 0.05). α-hENaC mRNA increased by onefold, but this change was not statistically significant. The dexamethasone-induced increases in β- and γ-hENaC mRNA expression were due to increased transcription, since they were not seen in the presence of actinomycin D (Fig. 2).

In Western blotting studies, the antibody against α-rENaC recognized a single 75-kDa band in both control and dexamethasone-treated A549 extracted proteins under reducing conditions (Fig. 3A). This signal was blocked completely by the immunizing peptide (data not shown). α-hENaC levels in A549 cells did not increase significantly following dexamethasone treatment. We were unable to detect β- or γ-hENaC in control membranes of synchronized A549 cells. However, following dexamethasone treatment, antibodies

against β- and γ-hENaC recognized bands in nonreduced gels at around 100 and 90 kDa, respectively (Fig. 3, B and C). No signal was seen when the β-hENaC antibody was substituted by preimmune serum (data not shown). Thus dexamethasone treatment increased the abundance of β- and γ- but not α-hENaC proteins. This is consistent with the RT-PCR data shown above.

Electrophysiology: single channel recordings. Single channel activity was seen in 23 dexamethasone-treated and 19 control cell-attached patches. A characteristic recording at −100 mV of a control cell is shown in Fig. 4A. The open time ranged from a few milliseconds to hundreds of milliseconds (395 ± 15 ms; mean ± SE), with an open probability, P_o, of 0.69 ± 0.016 for amplitude level 1 and 0.21 ± 0.02 for amplitude level 2. The mean P_o for the whole recording shown in Fig. 4A (combining the values of both levels) was 0.39 ± 0.05. Channel conductance was determined using the histogram distribution fitted to a Gaussian equation (Fig. 4B). At the patch potential of −100 mV and pipette Na⁺ concentration of 145 mM, the single channel conductance was 8.63 ± 0.036 pS (mean ± SE).

Figure 5, A and B, shows single channel activity in cell-attached patches of synchronized A549 cells treated with 1 μM dexamethasone for 24 h. A single channel with unitary conductance of 4.4 ± 0.027 pS (mean ± SE) was found. The mean unitary conductance, mean open time, and P_o before and after dexamethasone are summarized in Table 1. Dexamethasone treatment shifted the single channel reversal potential from +47 mV in control cells to +66 mV after dexamethasone (Fig. 6B). This shift is consistent
with an increase in the selectivity of the channel to Na\(^+\) ions.

**Whole cell currents.** Synchronized control and dexamethasone-treated A549 cells exhibited inward-rectifying currents (Fig. 7). We have previously shown that these currents are carried by Na\(^+\) ions (18). Perfusion of the cells with the SES containing 10 \(\mu\)M amiloride inhibited the inward (Na\(^+\)) currents rapidly. The observed effect of amiloride was totally reversible upon washout (not shown). A549 cells treated with 1 \(\mu\)M dexamethasone for 24 h showed significantly larger total and amiloride-sensitive whole cell currents compared with the corresponding values in control cells (Fig. 8). In addition, an increased sensitivity of the channels to amiloride was observed. Following incubation of the cells with dexamethasone, the inhibition constant, \(K_{0.5}\), for amiloride decreased from 833 \(\pm\) 69 nM to 22 \(\pm\) 5.4 nM (\(P < 0.01\)) (Fig. 9).

**DISCUSSION**

Data shown herein are consistent with the view that dexamethasone differentially regulates hENaC expression. RT-PCR and Western blotting studies demonstrated that treatment of A549 cells with dexamethasone for 24 h resulted in upregulation of \(\beta\)- and \(\gamma\)-hENaC mRNA and protein levels. The inhibition of the observed changes by actinomycin D, an RNA polymerase II inhibitor, indicates that dexamethasone treatment increased transcription of \(\beta\)- and \(\gamma\)-hENaC genes without altering the stability of their mRNAs. Both transcriptional and posttranscriptional mechanisms have been previously evoked in explaining the action of dexamethasone on surfactant gene regulation in fetal ATII cells (37).

The somewhat surprising finding was the absence of a significant effect of dexamethasone on the expression of the \(\alpha\)-hENaC subunit. Exposure of confluent monolayers of an airway cell line (H441) to dexamethasone resulted in a fivefold increase in the amiloride-sensitive short-circuit current, which correlated with a fivefold increase in \(\alpha\)-hENaC mRNA levels (32). The same authors showed the presence of a glucocorticoid response element in the 5’-flanking region of the \(\alpha\)-hENaC subunit gene. In addition, Venkatesh and...
of the three subunits was increased by glucocorticoids in primary cultures of rat fetal lung epithelial cells (6, 39). The same investigators also showed that injection of dexamethasone into rats upregulated β- and γ-rENaC mRNA levels in the colon but had no effect on α-rENaC mRNA, which was already high under control conditions. From that perspective, the behavior observed in A549 cells fits best with the behavior described by Renard et al. (30) in rat distal colon. Taken together, all the experimental data mentioned above is added evidence and a clear indication of a wide spectrum of responses to dexamethasone, depending probably on factors specific to each tissue. The differential regulation of ENaC genes indicates that the mechanisms involved could be more complex than the binding of dexamethasone to its receptor and the activation of a glucocorticoid response element (GRE). Instead, the presence of various tissue-specific cofactors may be necessary for the activation to occur and may affect the response to the hormone.

The novel aspect of this study is the demonstration that the dexamethasone-induced changes in β- and γ-hENaC mRNA and protein levels altered the function of the amiloride-sensitive Na⁺ channel in A549 cells. Champigny et al. (6) showed that treatment of fetal lung epithelial cells with dexamethasone resulted in depolarization of their membranes and large increases in the amplitude of the amiloride-induced hyperpolarization, suggesting an increase in the amiloride-sensitive currents. Sayegh et al. (32) showed that treatment of H441 cells with dexamethasone increased the magnitude of the amiloride-sensitive short-circuit current. Herein we demonstrate for the first time that treatment of a human cell line, A549 cells, with dexamethasone for 24 h increased the amiloride-sensitive Na⁺ whole cell currents with only a slight increase in α-hENaC mRNA and protein levels. Single-channel measurements showed a significant increase in $P_o$ and open time of amiloride-sensitive currents (which may account for the increase in whole cell current), a shift in conductance from 8 to 4 pS, and a shift in the reversal potential of single channel current-voltage relationships toward positive values.

The observed modification of the biophysical and pharmacological properties of the amiloride-sensitive Na⁺ channel could have resulted from alterations in the stoichiometry of the α-, β-, and γ-subunits. The heterologous expression of rENaC subunits in Xenopus oocytes and in MDCK cells showed that the presence of all three subunits of the ENaC was a necessary condition for the optimal function of the channels (15, 27). Both methods of expression showed that the properties and the function of the channels were profoundly altered in absence of either the β- or γ-rENaC. The α-subunit was found to form channels (21, 27) by itself but with little or no function. Therefore the role of β- and γ-subunits seems to be regulatory, which is in the line of our findings described herein.

Previous studies have shown that injection of dexamethasone into newborn pigs ameliorated lung injury caused by hyperoxia and barotrauma (8, 9). It has been...
thought that the protective effects of dexamethasone were due to its ability to downregulate the production of inflammatory mediators. However, other studies have shown that treatment of fetal and adult ATII cells with dexamethasone also resulted in increased expression of the α1- and β1-Na-K-ATPase mRNA and protein levels (2, 5). These findings and our data lead us to speculate that dexamethasone may also decrease lung injury by upregulating Na+ transport across the injured alveolar epithelium, thus limiting the amount of lung edema (26, 42).

In conclusion, this work reports for the first time the modification of the biophysical properties of amiloride-sensitive Na+ channels in A549 cells following treatment with a corticosteroid. In addition, this work establishes the A549 cell line as a model for studying ion transport in human distal lung at the cellular level.

This project was supported by National Heart, Lung, and Blood Institute Grants HL-31197 and HL-51173 and by Office of Naval Research Grant N00014-97-1-0309. P. Barbry is supported by grants from the Centre National de la Recherche Scientifique and the French Research Ministry.

REFERENCES


30. Sayegh R, Auerbach SD, Li X, Loftus RW, Husted RF, Stokes JB, and Thomas CP. Glucocorticoid induction of epi-


