Expression of highly selective sodium channels in alveolar type II cells is determined by culture conditions

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Received 27 July 2000; accepted in final form 9 November 2000

Alveolar fluid clearance in the developing and mature lungs is believed to be mediated by some form of epithelial Na channels (ENaC). However, single-channel studies using isolated alveolar type II (ATII) cells have failed to demonstrate consistently the presence of highly selective Na+ channels that would be expected from ENaC expression. We postulated that in vitro culture conditions might be responsible for alterations in the biophysical properties of Na+ conductances observed in cultured ATII cells. When ATII cells were grown on glass plates submerged in media that lacked steroids, the predominant channel was a 21-pS nonselective cation channel (NSC) with a Na+/K+ selectivity of 1; however, when grown on permeable supports in the presence of steroids and air interface, the predominant channel was a low-conductance (6.6 ± 3.4 pS, n = 94), highly Na+-selective channel (HSC) with a PNa/PK > 80 that is inhibited by submicromolar concentrations of amiloride (K0.5 = 37 nM) and is similar in biophysical properties to ENaC channels described in other epithelia. To establish the relationship of this HSC channel to the cloned ENaC, we employed antisense oligonucleotide methods to inhibit the individual subunit proteins of ENaC (α, β, and γ) and used patch-clamp techniques to determine the density of this channel in apical membrane patches of ATII cells. Overnight treatment of cells with antisense oligonucleotides to any of the three subunits of ENaC resulted in a significant decrease in the density of HSC channels in the apical membrane cell-attached patches. Taken together, these results show that when grown on permeable supports in the presence of steroids and air interface, the predominant channels expressed in ATII cells have single-channel characteristics resembling channels that are associated with the coexpression of the three cloned ENaC subunits α−, β−, and γ-ENaC.

A single-channel recording; air interface; antisense oligonucleotides; epithelial sodium channels

FETAL TRANSITION TO AIR BREATHING after birth is characterized by rapid clearance of fetal lung fluid (6). Although factors that promote the sudden increase in lung fluid reabsorption at birth are largely unknown, an increase in amiloride-sensitive Na+ transport mediated by cation channels located on the apical surface of alveolar epithelial cells is considered a key step in this process. Several lines of investigation point to the presence of and a functional role for epithelial Na+ channels (ENaC) in lung epithelia, but it is not clear whether these channels are related to the ENaC that has been cloned and characterized in other epithelial tissues. In other epithelial tissues, ENaC is a heteromultimeric protein consisting of some combination of three subunits, designated α−, β−, and γ-ENaC. The exact stoichiometry of the subunits forming a channel is controversial. In rat lung, the expression of rat α-ENaC (α-rENaC) mRNA in adult and fetal alveolar type II (ATII) cells has been demonstrated by a variety of techniques (20, 42, 61). Similarly, β− and γ-subunit mRNAs have also been detected in ATII cells but are less abundant (58, 61). Evidence for a functional role for ENaC in fetal lung fluid clearance at birth comes from experiments by Hummeler et al. (28), who have shown that newborn α-subunit knockout mice failed to clear their lung fluid and died within 48 h of birth.

The coexpression of α−, β−, and γ-ENaC cRNAs in Xenopus oocytes is associated with a highly Na+-selective, 4- to 5-pS channel, that is, a highly selective cation (HSC) channel (8). Indeed, single-channel studies from several sodium-transporting epithelia have confirmed the presence of such channels (3, 19). In contrast to these studies, electrophysiological evidence for the presence of HSC channels in lung epithelia is generally lacking. With the exception of one group (58) that reported a 4-pS sodium channel in fetal distal lung epithelial cells with single-channel characteristics similar to those expected for coexpression of α−, β−, and γ-ENaC, patch-clamp studies from several laboratories, including ours (22, 31, 37, 45, 60), have shown that the predominant Na+-permeant channel in ATII cells is a nonselective cation (NSC) channel. The electrophysiological characteristics of these NSCs (high conductance, low Na+ selectivity) are quite different from the low-conductance HSCs associated with ENaC expression reported from other tissues (41, 46).

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Thus there is conflicting information about the presence and role of ENaC in distal alveolar epithelia and its role in lung fluid clearance. However, because the biochemical and molecular biological information is based on isolated tissue, whereas electrophysiological data are from cells in culture, it is possible that the process of isolation of ATII cells and in vitro culture conditions alter the electrophysiological characteristics of ion channels. It is well known that the phenotype of ATII cells in tissue culture is unstable, and a recent study has shown that airway epithelia can be maintained in a differentiated state for longer periods in vitro by use of air-liquid interface cultures (13). We therefore decided to test whether altering in vitro culture conditions would affect Na\(^{+}\) channels in the apical membranes of ATII cells. In particular, we used a steroid-containing enriched medium, grew the cells on permeable supports, and, for some of the cells, exposed the apical surface of the cells to humidified air to mimic the conditions in the lung. Depending on culture conditions, we observed two channel types. The first one was a 21-pS NSC channel similar to the one reported by us (31, 32) and others (22, 37, 45, 60), which is seen most consistently when cells are cultured in the absence of steroids and the apical surface of cultured cells is exposed to a liquid interface. The second type is a 6-pS HSC that is similar in properties to channels in the apical surface, and cells were allowed to grow with medium on the basolateral surface and air on the apical side. Alterningly, cells were cultured in an identical fashion but without the medium being drained so that cells would remain submerged. Cells were incubated in 95% air-5% CO\(_2\) and used for patch-clamp studies between 24 and 96 h after plating.

Using the current method (11, 12) for isolation of ATII cells, we were able to obtain an ATII cell population with 95% viability as confirmed by LIVE/DEAD Euclolight Viability/Cytototoxicity Kit (Molecular Probes, Eugene, OR). The LIVE/DEAD assay kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes, calcein AM and ethidium homodimer, that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity. The assay principles are applicable to most eukaryotic cell types, including adherent cells. This fluorescence-based method of assessing cell viability can be used in place of trypan blue exclusion. The cells were 95% pure (confirmed by cell surface staining for surfactant protein C (SP-C) and aquaporin 5). Contamination by ATI cells, macrophages, or fibroblasts was <10%. Cells were used for patch-clamp experiments during the first 96 h in culture while they maintained ATII cell phenotype (see previous paragraph) and function (surfactant production by radiolabeled choline incorporation). The purity and viability of ATII cells was similar when cells were cultured on glass submerged in media and on permeable supports exposed to air interface.

**METHODS**

**Type II pneumocyte isolation and cell culture.** ATII cells were isolated by enzymatic digestion of lung tissue from adult Sprague-Dawley rats (200–250 g) by use of published techniques (12). Briefly, the rats were anesthetized with pentobarbital sodium and were heparinized (100 units/kg). ATII cells were digested by tracheal instillation of elastase (0.4 mg/ml). Lung tissue was minced in DNase type IV from bovine pancreas (1 mg/ml; Sigma, St. Louis, MO) and filtered sequentially through 100- and 20-μm nylon mesh. The purification was based on differential adherence of cells to dishes coated with rat IgG. Nonadherent ATII cells were collected, centrifuged, and seeded onto permeable supports in a highly enriched medium: 3 parts Coon’s modification of Ham’s F-12 and 7 parts Liebovitz’s L-15 with 10 μM aldosterone or 1 μM dexamethasone. The high concentration of steroid was used to ensure that, even after 5 days in culture and significant cellular metabolism, there would be sufficient steroid to activate both mineralocorticoid and glucocorticoid receptors. (For experiments in which the specific effects of steroids were investigated, lower concentrations of steroids with appropriate antagonists were used.) After isolation, some cells were allowed to grow on glass coverslips, and others were allowed to attach to a specialized culture support (38) that was optimized for patch-clamp recording and allowed the cells to grow on a permeable support (Millipore) while submerged in medium. After the cells had attached to the culture surface (this usually required 2–4 h), medium was drained from the apical surface, and cells were allowed to grow with medium on the basolateral surface and air on the apical side. Alternatively, cells were cultured in an identical fashion but...
conversion to an ATI phenotype in the first 96 h after culture. Nonetheless, we reexamined the issue under our culture conditions. Cells were cultured on glass (with fluid interface) or permeable supports (with air interface) and, in both groups, with or without dexamethasone. Cell surface staining for SP-C and aquaporin 5 was performed at 24 and 96 h. The number of cells staining for aquaporin 5 and SP-C was used to calculate the ratio of ATI to ATII cells (Table 1) (14, 57). We found that ∼5% of the cells stained for aquaporin 5 after 24 h, and a slightly larger number (although not statistically significant) were present after 96 h (the longest time we examined cells in our experiments). The balance of the cells (∼95%) stained for SP-C (Fig. 2). The percentage of cells staining with a marker for ATII cells (SP-C antibodies) and ATI cells (aquaporin 5 antibodies) showed that, during the time (24–96 h) in which we performed patch-clamp experiments, ATII cells began with and maintained an ATII phenotype for the duration of our patch-clamp experiments. Because there was no significant difference in cellular phenotype, we combined all patch data from the period between 24 and 96 h.

Solutions and drugs. All solutions were made with deionized water and then passed through a 0.2-μm filter (Gelman Sciences, Bedford, MA) before use. Bath and pipette solutions used in the cell-attached mode contained (in mM) 140 NaCl, 1 MgCl₂, 5 KCl, and 10 HEPES, pH 7.4 with 2 N NaOH. In the inside-out recordings, pipette solution was the same but the bath solution was changed to (in mM) 5 NaCl, 140 KCl, 4 CaCl₂, 5 EGTA, 1 MgCl₂, and 10 HEPES, pH 7.4 with 2 N KOH. The contents of the bath and pipette solutions were varied as appropriate for specific protocols. All chemicals were obtained from Sigma except oligonucleotides, which were generated at Emory University’s microchemical facility (EUMF).

Procedure for single-channel recordings. Patch-clamp experiments were carried out at room temperature. The pipettes were pulled from filamented borosilicate glass capillaries (TW-150, World Precision Instruments, Sarasota, FL) with a two-stage vertical puller (Narishige, Tokyo, Japan). The pipettes were coated with Sylgard (Dow Corning) and fire polished (Narishige). The resistance of these pipettes was 5–8 MΩ when filled with pipette solution. We used the cell-attached configuration for most of our studies. After formation of a high-resistance seal (>50 MΩ) between the pipette and cell membrane, channel currents were sampled at 5 kHz with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) and filtered at 1 kHz with a low-pass Bessel filter. Data were recorded by computer with pCLAMP 6 software (Axon Instruments). Current amplitude histograms were made from stable, continuously recorded data, and the open- and closed-current levels were determined from least squares fitted Gaussian distributions. The open probability of the channels was calculated using FETCHAN in pCLAMP 6. Single-channel conductance was determined using a linear regression of unitary current amplitudes over the range of applied pipette potentials.

Voltage conventions. For cell-attached patches, voltages are given as the negative of the patch pipette potential (−Vpipette). This potential is the displacement of the patch potential from the resting potential (about −40 mV for ATII cells), and positive potentials represent depolarizations, and negative potentials represent hyperpolarizations of the cell membrane away from the resting potential. For a HSC with a sodium concentration gradient of 10 to 1 (outside to inside), the reversal potential would be +60 mV. Therefore, it would require a 100-mV positive voltage displacement (−Vpipette = +100 mV) from the resting potential to reach the reversal potential.

Antibodies for Western blotting. We had previously characterized several ENaC subunit-specific peptide antibodies in Xenopus (x) epithelial cells (52). We have now demonstrated that the same antibodies recognize ENaC subunits in mammalian epithelial cells. The anti-β antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to residues 614 (Asp) through 647 (Asn) in the β-xENaC sequence, which was conjugated to keyhole limpet hemocyanin (KLH). The anti-γ antibody is an affinity-purified polyclonal antibody against a KLH-conjugated peptide corresponding to residues 608 (Gly) through 660 (Leu) in the γ-xENaC sequence. An anti-α-xENaC antibody was generated to a 26-amino acid peptide corresponding to residues 137 through 161 of α-xENaC conjugated to KLH. Antibodies from rabbit serum were affinity purified using a peptide affinity column according to standard protocol (Pierce). The peptides were produced in the EUMF and sent to commercial laboratories, where the peptides were coupled to KLH and used to immunize at least two rabbits with each peptide. This provided at least one anti-peptide antibody specific for each subunit. These antibodies have been characterized exten-

### Table 1. Phenotype of ATII cells

<table>
<thead>
<tr>
<th></th>
<th>Without dexamethasone</th>
<th>With dexamethasone</th>
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</thead>
<tbody>
<tr>
<td><strong>Duration</strong></td>
<td><strong>ATII</strong></td>
<td><strong>ATII</strong></td>
</tr>
<tr>
<td><strong>ATII Cell Phenotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cells cultured on glass</strong></td>
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</tr>
<tr>
<td>24 h</td>
<td>4.2 ± 2.0</td>
<td>94.8 ± 2.2</td>
</tr>
<tr>
<td>96 h</td>
<td>7.4 ± 2.6</td>
<td>92.6 ± 2.6</td>
</tr>
<tr>
<td><strong>Cells cultured on permeable supports</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>4.3 ± 2.0</td>
<td>95.7 ± 2.0</td>
</tr>
<tr>
<td>96 h</td>
<td>7.0 ± 2.5</td>
<td>93.0 ± 2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed in percent. Phenotype of alveolar type II (ATII) cells cultured on permeable supports was not changed during the time frame of our patch-clamp or molecular biological studies (24–96 h). Proportion of cell types (from samples of 100 cells) is based on no. of cells staining for surfactant protein C (SP-C; ATII cells) and aquaporin 5 (ATI cells).
sively in published reports (49, 51, 62). Western blots included in our previous publications (32) show that these antibodies specifically recognize rENaC subunits. Figure 3 shows that the antibodies recognize proteins of the appropriate size in a mouse epithelial cell line (M1), a lung epithelial cell line (H1), and ATII cells and that the activity is competed by the antigenic peptide (although there was not enough of the peptide is a 50mer, which is extremely expensive to make). The antibodies also recognize competing bands of the appropriate size in the human lung epithelial cell line (A549, data not shown).

Preparation and use of antisense oligonucleotides. We used previously described methods to inhibit expression of ENaC subunits (32). Subunit-specific phosphorothioate oligonucleotides directed against the sequences around the translation start site of each subunit were produced by the EUMF according to the sequences in Table 2. In a BLAST search of GenBank, neither the sense nor the antisense oligonucleotides showed any similarity to other known sequences, and the oligonucleotides for each subunit had no significant similarity to the oligonucleotide sequences for the other subunits. Cultured ATII cells were exposed to medium containing 10 μM oligonucleotides overnight (16–18 h). Our previous experiments have shown that freshly isolated ATII cells take up oligonucleotides without the need for agents to enhance the uptake. We have previously used Western blots (quantitative densitometry) to determine that the antisense oligonucleotides do reduce the subunit protein level and that the sense oligonucleotides used as controls do not affect the protein levels (32); however, in the current results, we provide additional evidence for the efficacy of the antisense oligonucleotides. Finally, patch-clamp techniques were used to determine the effect of oligonucleotides on the expression of cation channels in apical membranes of ATII cells.

Methods for statistical analysis. Statistical analysis for the changes in open probability of channels and the biochemical estimations were performed using SPSS for Windows. Statistical significance between two groups was determined by paired or unpaired t-tests, as appropriate. When the comparison between more than one group was required, statistical significance was determined by one-way analysis of variance (ANOVA) followed by pairwise comparisons with the Student-Newman-Keuls test to determine significant differences between groups. The significance of differences in the observed frequency of channels between two groups of conditions was determined using a z-test for paired data or a χ² test for multiple groups. P values <0.05 were considered significant.

RESULTS

Culture support, growth medium, steroid treatment, and apical interface influence the types of Na⁺ channels expressed in apical membranes of ATII cells in primary culture. To determine the effect of in vitro culture conditions on the biophysical properties of Na⁺-permeant channels observed in apical membranes of ATII cells, we cultured the cells under several different conditions, including alternate media (standard vs. enriched), type of culture surface (glass or plastic vs. permeable support), and apical interface (fluid vs. air). Depending on the type of culture condition, we observed two types of Na⁺ channels: a 21-pS NSC channel with Na⁺-to-K⁺ permeability (PNa/PK) ratio ~1, and a HSC channel with single-channel characteristics similar to those associated with the expression of ENaC subunits in other tight epithelia. The frequency of observing these channels in different culture conditions is shown in Table 3 and Fig. 4. There was a significant increase in HSC channels (from 1 to 17%) when cells were cultured on permeable supports instead of glass coverslips. Furthermore, when aldosterone or dexamethasone was added to the culture media, a high percentage of patches had HSC channels whether the cells were grown on glass or on permeable supports. Nonetheless, the highest expression of HSC channels was observed when cells were cultured on
permeable supports in the presence of aldosterone with their apical surfaces exposed to an air interface (HSC channels were recorded from 85.4% of 164 cell-attached patches). Exposure to an air interface also led to a striking reduction in NSC channels (69% in liquid interface vs. 20% in air interface).

Because addition of aldosterone caused an increase in HSC channels, we further studied whether this effect was observable in cells grown with an air interface on permeable supports and whether the effect was mediated through mineralocorticoid or glucocorticoid receptors. Cells were first cultured on permeable supports

Figure 3. Subunit specific antibodies. Peptides were made that corresponded to a unique antigenic sequence from each subunit. Each of these antibodies was used to probe cell lysates from mouse epithelial cells (M1), a lung epithelial cell line (L2), and ATII cells in the presence (+) or absence (−) of competing antigenic peptide. In each case, the antibodies recognized one band of the appropriate molecular weight (M.W.), and the band is competed by the antigenic peptide (although there was not enough of the γ-peptide to compete on the ATII cells because the γ-peptide is a 50mer, which is extremely expensive to make). The antibodies also recognize competing bands of appropriate size in the human lung epithelial cell line (A549, data not shown). Details of antibody production are given in METHODS.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>GenBank Accession No.</th>
<th>Size, bp</th>
<th>Sense/Antisense</th>
<th>Start/End</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>X70497</td>
<td>24</td>
<td>Sense</td>
<td>70–93</td>
<td>TTCAGCTAATGATGCTGGACCAC</td>
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<td></td>
<td></td>
<td></td>
<td>Antisense</td>
<td>93–70</td>
<td>GTGTCACGATGATGCTGGACCAC</td>
</tr>
<tr>
<td>β</td>
<td>X77932</td>
<td>21</td>
<td>Sense</td>
<td>64–84</td>
<td>GTCAGCTAATGATGCTGGACCAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antisense</td>
<td>84–64</td>
<td>GTGTCACGATGATGCTGGACCAC</td>
</tr>
<tr>
<td>γ</td>
<td>X77933</td>
<td>21</td>
<td>Sense</td>
<td>85–105</td>
<td>GTGTCACGATGATGCTGGACCAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antisense</td>
<td>105–85</td>
<td>GTGTCACGATGATGCTGGACCAC</td>
</tr>
</tbody>
</table>

NSC, nonselective cation channel; HSC, highly Na⁺-selective channel. *Sequence positions are based on sequences associated with GenBank accession nos. and the original descriptions of the subunit sequences (7, 8).
with an air interface under three conditions: 1) in the presence of 300 nM aldosterone and 300 nM RU-486 (to block glucocorticoid receptors), 2) with 300 nM dexamethasone and 300 nM spironolactone (to block mineralocorticoid receptors), and 3) with 300 nM RU-486 and 300 nM spironolactone but with no steroids added. The results of these experiments are shown in Table 4.

Stimulation of either mineralocorticoid or glucocorticoid receptors resulted in statistically significant increases in the frequency of HSC channels and decreases in NSC. However, when both types of steroid receptors were blocked, type II cells still had relatively high frequencies of HSC compared with NSC channels. Therefore, steroids appear to increase the frequency of channels, but exposure to an air interface and growth on permeable supports by itself is sufficient to promote expression of functional HSC channels.

Single-channel characteristics of channels recorded from apical membrane patches of ATII cells. The distribution of single-channel conductance of the two types of channels recorded from ATII cells can best be appreciated by comparing the channels observed under the two most extreme differences in culture conditions: cells grown on glass, under liquid, and with no steroids vs. cells grown on permeable supports, with an air interface, and with steroids. The results of this comparison are shown in Fig. 5. Details of the single-channel characteristics of the high-conductance NSC channels have been published by us previously (30, 31).

In the current study, we describe the electrophysiological characteristics of the low-conductance HSC channels that were best seen when cells were cultured on permeable supports in the presence of enriched medium and apical air interface. Figure 6 shows a recording of a single channel in a cell-attached membrane patch along with a representative current-voltage plot for this channel. At pipette potentials more negative than -40 mV, the channel had a conductance of 6.6 ± 1.0 pS (n = 94) with 140 mM NaCl in bath and pipette. Ion selectivity was determined using excised inside-out patches and solutions of varying ionic compositions. In inside-out mode (bath 140 mM potassium gluconate; pipette 140 mM sodium gluconate), patches exhibited nonlinear current voltage relationships, with a reversal of current at approximately +115 mV. For this
reversal potential, calculation with the Goldman equation (25) gives a permeability of the channel for Na\(^+\) >80 times that of K\(^-\) (\(P_{Na}/P_{K} > 80\)). The channel is sensitive to submicromolar concentrations of amiloride, with 37 nM amiloride producing a 50% decrease in channel open probability (Fig. 7). The kinetics of this channel were slow, with mean open and closed times in the range of seconds. More than one current level was observed in 98% of active patches. Thus this channel is very similar in its single-channel characteristics to the ENaC observed in other Na\(^+\)-transporting epithelia and to the one described from fetal lung epithelia by Voilley et al. (58).

**Effect of oxygen tension on channel expression.** In our experiments evaluating the effect of apical surface interface on expression and activity of ion channels, we found that the greatest increase in highly selective cation channels was seen when cells were cultured with their apical surface exposed to air interface. Because this effect could be mediated by increased availability of oxygen at the cell surface, we cultured cells overnight with an air interface on permeable supports with steroids (like group 7 in Table 3) but with only 5% oxygen. Under these conditions, the predominant channels seen in apical patches were nonselective (Fig. 8). However, when cells cultured in 5% oxygen for 24 h were exposed to 95% oxygen for 2 h, apical membrane patches returned to their previous ratio of predominantly HSC channels (Fig. 8).

**Relationship of the observed channels to the cloned ENaC.** We have previously shown (32) that NSC channels recorded from ATII cells cultured with fluid interface belong to the ENaC family of Na\(^+\) channels and are formed from \(\alpha\)-subunit protein alone or in combination with some subunit other than \(\beta\) and \(\gamma\). This conclusion was based on our studies in which we used antisense oligonucleotides directed against the \(\alpha\)-, \(\beta\)-, or \(\gamma\)-subunits of ENaC. Treatment with oligonucleotides did not cause any significant changes in open probability or conductance of HSC channels (Table 5).

**DISCUSSION**

We found that when in vitro culture conditions simulated conditions expected in vivo, the apical mem-

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**Table 4. Effect of glucocorticoids and mineralocorticoids on cation channel expression in ATII cells**

<table>
<thead>
<tr>
<th>Steroid Group*</th>
<th>Aldosterone + RU-486</th>
<th>Dexamethasone + Spironolactone</th>
<th>RU-486 + Spironolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total successful patch</strong></td>
<td><strong>37</strong></td>
<td><strong>17</strong></td>
<td><strong>26</strong></td>
</tr>
<tr>
<td>Any cation channel</td>
<td>36</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>NSC channels</td>
<td>11</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>HSC channels</td>
<td>35</td>
<td>17</td>
<td>18</td>
</tr>
</tbody>
</table>

Values are means ± SE. Three hundred nanomoles of aldosterone, dexamethasone, and the glucocorticoid receptor antagonist RU-486 and 1 \(\mu\)M aldosterone antagonist spironolactone were applied in the culture medium. Data were combined from all cells recorded between 24 and 96 h after culture. *All other culture conditions remained constant for these groups. Cells were cultured on permeable supports with an apical air interface. †Some patches had both types of channels and were, therefore, counted twice. ‡Indicates number of patches made with good seal.

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**Fig. 5. Distribution of single-channel conductances of observed channels cultured under significantly different culture conditions.** When cells were grown on glass coverslips submerged in media devoid of steroids, the predominant channels seen are NSC channels with high conductance (filled bars), but when grown on permeable filters with high concentration of aldosterone (10 \(\mu\)M) and air interface, the predominant channels are HSC channels with low conductance (open bars). Data are combined from all cells recorded between 24 and 96 h after culture. Mean conductances from the two groups are significantly different (\(P < 0.001\)).
branes of ATII cells express HSC channels with electrophysiological properties similar to channels expected from coexpression of ENaC subunits (conductance 6.6 pS, sensitivity to amiloride with $K_{0.5} = 37$ nM, $P_{Na}/P_K > 80$, slow kinetics). In particular, the presence of steroid hormones, permeable supports, and an air interface was essential for high-level expression of HSC channels. Our experiments with the use of antisense oligonucleotides to inhibit individual subunits of ENaC show that these channels are formed by at least all three subunits of ENaC ($\alpha$, $\beta$, and $\gamma$). Taken together, these studies provide strong evidence for the presence in ATII cells in vitro of HSC channels similar to those show that these channels are formed by at least all three subunits of ENaC ($\alpha$, $\beta$, and $\gamma$). Taken together, these studies provide strong evidence for the presence in ATII cells in vitro of HSC channels similar to those

Fig. 6. Single-channel characteristics of HSC channels. A: single-channel recordings from a cell-attached patch on the apical membrane of ATII cell cultured with air interface. B: current-voltage ($I-V$) relationship showing that conductance of the channel is ~6 pS. $V_p$, pipette potential. C: amplitude histogram of the patch shown in A. There was no difference in channel characteristics for cells between 24 and 96 h after initial culture.

Fig. 7. Dose-response curve for amiloride. Application of 37 nM amiloride to the extracellular side (i.e., in the micropipette) caused a 50% reduction in the open probability of the HSC channels. Data were combined from all cells recorded between 24 and 96 h after culture.

Fig. 8. Effect of reduced O$_2$ tension on the frequency of HSC and NSC channels in cells grown on permeable supports with an air interface, and the effect of O$_2$ on the incidence of NSC and HSC channels in cells grown on permeable supports in the presence of steroids. Exposure to 5% O$_2$ increased the frequency of NSC channels and decreased the frequency of HSC channels. In high O$_2$, HSC comprised almost 100% of all observable channels. Data were combined from all cells recorded between 24 and 96 h after culture.
observed when all three ENaC subunits are expressed in heterologous expression systems.

The presence of amiloride-sensitive Na\(^{+}\) transport in alveolar epithelia has been demonstrated by numerous studies using macroscopic measures of Na\(^{+}\) movement (26, 39, 40, 43). Studies have also demonstrated the functional importance of epithelial Na\(^{+}\) absorption in clearance of alveolar fluid in the developing and mature lungs (28). There is molecular and functional evidence that ENaC is present in the lung epithelial cells and that absence of α-ENaC results in failure of absorption of fetal lung fluid. The perinatal period is characterized by a rapid increase in the capacity for amiloride-sensitive Na\(^{+}\) transport in the lung (44), and there is a concomitant increase in expression of ENaC genes (54, 56). However, except for one study by Voilley et al. (58) in fetal lung cells (in which the frequency of HSC channels was not given and in which the presence of NSC channels was not reported), patch-clamp studies have failed to identify HSC channels expected from ENaC expression in apical membrane patches from type II cells. Instead, the predominant Na\(^{+}\)-permeant channels recorded from type II cells of both fetal and adult origin are mostly NSC channels that vary considerably in their single-channel characteristics from

Table 5. Effect of ENaC sense and antisense oligonucleotide treatment on frequency of HSC and NSC channels in apical membrane patches

<table>
<thead>
<tr>
<th>Sense/Antisense Oligonucleotide</th>
<th>α</th>
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<th>γ</th>
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<tr>
<td>α</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total patches made</td>
<td>20</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>Patches with any channel</td>
<td>19</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>HSC</td>
<td>19</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>NSC</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total patches made</td>
<td>19</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>Patches with any channel†</td>
<td>8</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>HSC‡</td>
<td>1</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>NSC§</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

| Values are means ± SE; n, no. of patches. ENaC, epithelial Na\(^{+}\) channel. Cells were cultured on permeable supports with an apical air interface. Data were combined from all cells recorded between 24 and 96 h after culture. *The frequency of observation of any channel, HSC channels, or NSC channels in sense-treated cells was not significantly different from the frequency of observation of the same channels in cells not treated with any oligonucleotide (group 5 in Table 2), implying that sense oligonucleotides do not affect channel expression in any statistically significant manner. †Some patches had both types of channels; e.g., for cells treated with α-antisense oligonucleotide, every patch that had a channel had an HSC channel, but 1 patch with an HSC channel also had a NSC channel. ‡The frequency of observation of HSC channels in antisense-treated cells was significantly different from sense-treated controls for any of the antisense oligonucleotides (P < 0.01 for all treatments). §Although there appears to be some trend toward a difference in treatments with β- or γ- vs. α-antisense oligonucleotides, there was actually no statistically significant difference in the frequency of NSC channels in sense- and antisense-treated cells or among cells treated with different antisense oligonucleotides.
HSC channels (41). The physiological role of the NSC channels has been questioned because of the reported high intracellular Ca\(^{2+}\) concentration required to activate this channel (37). There is recent evidence that maturation and assembly of the ENaC subunits occur slowly and that the lifetime and number of assembled ENaC channels in the plasma membrane are substantially different from the lifetime of ENaC subunits in the preapical or cytosolic pool of proteins. Therefore, detection of increased ENaC gene expression as increased mRNA or as increased total cellular ENaC subunit protein may not correlate with the expression of functional ENaC in the plasma membrane (55).

Our studies show that, depending on culture conditions, two different types of amiloride-sensitive Na\(^+\)-permeant channels can be observed in apical membrane patches from both fetal and adult type II cells. The first channel is a NSC channel, observed most frequently when ATII cells are cultured on permeable supports with an air interface on their apical surface. This channel has low conductance (6.6 pS) and is highly selective for Na\(^+\). The second channel is a HSC channel, observed most frequently when cells are cultured on permeable supports with an air interface on their apical surface. This channel has low conductance (6.6 pS) and is highly selective for Na\(^+\) over K\(^+\).

**The effect of culture conditions.** As aforementioned, culture conditions are the primary determinant of the frequency with which we observe the two channel types; however, the culture conditions appear to have somewhat different effects on HSC and NSC channels. An examination of Fig. 4 and Table 3 shows that the frequency of HSC channels is equally sensitive to steroids (regardless of other culture conditions) and air interface (regardless of the presence of steroids). In fact, the frequency of HSC channels in cells grown under three conditions (group 2: with steroids, on glass, under liquid; group 6: without steroids, on permeable supports, with air interface; or group 7: with steroids, with air interface, on permeable supports) are not statistically different from one another (for the number of patches we examined) but are significantly different from all other conditions (\(P < 0.05\)). On the other hand, the highest frequency of NSC channels was observed when cells were grown on glass (statistically different from all other conditions, \(P < 0.05\)). Addition of any combination of permeable supports, steroids, or air interface significantly decreases the frequency of NSC channels, with the lowest frequency occurring when all three conditions are present (group 7). Only change of the medium type appears to have no significant effect on the frequency of either NSC or HSC channels.

**Molecular basis for HSC and NSC channels.** We have shown that despite the large differences in biophysical properties of HSC and NSC channels, both are related to the ENaC family of channels. The NSC channel is formed by the \(\alpha\)-subunit (alone or in combination with subunits other than \(\beta\) and \(\gamma\)), whereas the HSC ENaC-like channel is formed by at least \(\alpha\), \(\beta\), and \(\gamma\)-subunits.

Several other investigators have suggested that different combinations of various subunits of ENaC can form Na\(^+\) channels with different biophysical characteristics. Pyfe and Canessa (24) have recently shown that subunit composition of ENaC is a main determinant of channel kinetics. Canessa et al. (8), Ismaïlov et al. (29), and Kizer et al. (35) have all shown in whole cell measurements that \(\alpha\)-subunit alone can apparently form amiloride-sensitive cation channels, although in oocytes, currents produced by these channels are small (only \(-1\%\) of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-channels). Although addition of \(\beta\)- and \(\gamma\)-subunits alters channel properties, these subunits are, by themselves, unable to form functional channels either alone or in combination. Canessa et al. (8) showed that coexpression of \(\alpha\)- and \(\beta\)- or \(\alpha\)- and \(\gamma\)-but not \(\beta\)- and \(\gamma\)-subunits induces whole cell currents but that these reach only 10–20% of the magnitude obtained when \(\alpha\), \(\beta\), and \(\gamma\) are expressed together. The pivotal role for \(\alpha\)-subunit is further confirmed by the studies conducted by Hummeler et al. (28), in which they showed that genetically engineered mice that lacked \(\alpha\)-subunit were unable to clear fetal lung fluid and died in the early perinatal period. In contrast, newborn mice in which the \(\beta\)- or \(\gamma\)-subunit had been deleted were able to clear fetal lung fluid, albeit more slowly, and died from hyperkalemia instead (5). These results suggest that, in the absence of \(\beta\)- or \(\gamma\)-subunits, channels from \(\alpha\)-subunit alone or from \(\alpha\)- and \(\beta\)- or \(\alpha\)- and \(\gamma\)-subunits can reabsorb sodium and water from lungs at birth, although this process is less efficient. Our inhibition of ENaC subunit expression supports the idea that NSC channels can be formed from \(\alpha\)-subunits alone, whereas HSC channels require all three subunits. This effect can most easily be seen in antisense-treated cells grown under conditions (permeable supports, air interface, and added steroid) when the predominant channel is HSC (>70%). In Fig. 11, data from Table 5 are replotted as the change in both types of channels and the number of patches with no activity relative to the sense-treated cells (i.e., a value of zero would mean no difference between sense- and antisense-treated cells, \(-4\) would indicate a 4-fold decrease produced by antisense, and \(+4\) would indicate a 4-fold increase). As expected, the number of HSC channels decreases after treatment with any antisense oligonucleotide. However, treatment with \(\alpha\)-antisense reduces the frequency of all channel types, leading to a large increase in the percentage of patches in which no channel can be observed. In contrast, treatment with \(\beta\)- or \(\gamma\)-antisense actually increases the number of NSC channels, with an additional moderate increase in patches with no activity. This is exactly the pattern that is expected if \(\alpha\)-subunits are required to form any channel, but \(\beta\) and \(\gamma\) can combine with \(\alpha\) to form HSC channels, and \(\alpha\) alone can form NSC channels.

**Role of steroids and alveolar air in promoting lung fluid reabsorption.** What role does the alveolar environment play in the composition and function of lung epithelial Na\(^+\) channels, and what physiological
Fig. 11. Effect of antisense oligonucleotides on relative numbers of HSC and NSC channels after treatment with antisense oligonucleotides to different ENaC subunits. Treatment with α-antisense (A) significantly reduced the frequency of all types of channels (P < 0.05) but greatly increased the frequency of observing patches with no channel activity (P < 0.05). Treatment with β- and γ-antisense (B) or γ-antisense (C) both significantly reduced (P < 0.05) the frequency of observing HSC channels but actually increased the frequency of observing NSC channels (P < 0.05). Both also increased the frequency of observing patches with no activity (P < 0.05). These results were most consistent with a model in which α-subunits were required for both channel types but in which β and γ were absolutely required only for HSC channels. Cells were grown on permeable supports with an air interface, steroid hormone, and enriched medium. Data were combined from all cells recorded between 24 and 96 h after culture and are expressed as relative degree of change ± SE.

However, our study shows that cells grown on permeable supports in the presence of steroids have maximum expression of HSC channels when exposed to air interface. There are several possible ways in which the presence of air interface may bring about this change. These include changes in physical forces affecting the apical membrane of the cells or an increase in the amount of available oxygen. Awayda and Subramanyam (1) and Ji et al. (33) have addressed the issue of mechanosensitivity of ENaC, but it is not clear whether ENaC function is altered in vivo by mechanical perturbations. As for the role of oxygen, several recent studies have shown that exposure of rat alveolar cells to high oxygen concentration results in upregulation of Na⁺ channel activity (4). Pitkanen et al. (47) have shown that oxygen exposure alters the bioelectric properties of fetal distal lung epithelium. There is also evidence to show that hypoxia suppresses Na⁺ channel expression and activity (48). Thus oxygen may be playing a significant role in fetal lung transition at birth. Other investigators have shown that exposure of ATI cells to air is important for ATI cell function and may promote Na⁺ transport. Dobbs et al. (14) found that ATI cells cultured on collagen gels with culture medium containing fetal calf serum and with an apical surface exposed to air retain morphological characteristics of ATI cells for a longer duration. These cells express surfactant proteins and their mRNAs and express a plasma membrane marker specific for ATI cells. Yamaya et al. (59) showed that expression of differentiated characteristics by these cells was inversely correlated to the depth of liquid overlying the apical surface of the cells. Johnson et al. (34) proposed that apical air interface promotes aerobic metabolism in ATI cells and decreases lactate levels and found increased Na⁺ transport in these cells. These studies have yet to be undertaken with fetal cells. It is possible that fetal alveoli, with a considerable amount of fetal lung fluid bathing the apical surface of alveolar epithelial cells, have low HSC channel activity but high chloride secretion into the alveoli. However, at birth, distension of alveoli with air facilitates an increase in Na⁺ transport through activation and/or increased ex-
expression of HSC channels. This exposure to air, coupled with a rise in endogenous catecholamines and steroids, which are also known to increase epithelial Na\(^+\) transport and HSC channel activity, would be expected to increase Na\(^+\) and fluid absorption. Little is known about other factors that influence this change at birth, and the speculations offered here are, at best, a small fraction of what is likely to be a very complex process.

In summary, our study suggests that under specific culture conditions, type II cells express highly selective HSC channels and that presence of \(\alpha\), \(\beta\), and \(\gamma\)-ENaC is necessary for the expression of these channels. Previously, there had been no electrophysiological evidence that ENaC-like HSC channels were present in ATII cells, raising questions about whether such channels could contribute to alveolar sodium transport. We have now shown that, at least under appropriate conditions, ENaC-like channels are the predominant channels in in vitro ATII cells. This observation must at least raise the possibility that ENaC-like channels could be present in vivo.

We thank B. J. Duke and Frank L. Harris for excellent technical support.

This research was supported by American Heart Association Grant 9950875-V, American Lung Association Grant 6-38665, Children’s Research Center at Emory University Grant 2-56036 to L. Jain, National Institutes of Health Grants R01-DK-37963 and P05-DK-50268 to D. C. Eaton, and the Center for Cell and Molecular Signaling.

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