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Increased expression and activity of sodium channels in alveolar type II cells of hyperoxic rats

(amiloride/ethylisopropylamiloride/immunofluorescence/*in situ* hybridization/α subunit of rat epithelial Na^+ channel)

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ABSTRACT **We investigated the cellular and molecular events associated with the increase in sodium transport across the alveolar epithelium of rats exposed to hyperoxia (85% O_2 for 7 days followed by 100% O_2 for 4 days). Alveolar type II (ATII) cell RNA was isolated and probed with a cDNA for one of the rat colonic epithelial sodium channel subunits (αrENaC). The αrENaC mRNA (3.7-kb transcript) increased 3-fold in ATII cell RNA isolated from rats exposed to 85% O_2 for 7 days and 6-fold after 4 days of subsequent exposure to 100% O_2. *In situ* hybridization revealed increased expression of αrENaC mRNA transcripts in both airway and alveolar epithelial cells of hyperoxic rats. When immunostained with a polyclonal antibody to kidney sodium channel protein, ATII cells from hyperoxic rats exhibited a significant increase in the amount of immunogenic protein present in both the plasma membrane and the cytoplasm. When patched in the whole-cell mode, ATII cells from hyperoxic rats exhibited amiloride and 5-(*N*-ethyl-*N*-isopropyl)-2′,4′-amiloride (EIPA)-sensitive currents that were 100% higher compared with those obtained from air-breathing rats. Single-channel sodium currents (mean conductance of 25 pS) were seen in ATII cells patched in both the inside-out and cell-attached modes. The number and open probability of these channels increased significantly during exposure to hyperoxia. Exposure to sublethal hyperoxia up-regulated both αrENaC mRNA and the functional expression of sodium channels in ATII cells.**

Reabsorption of fluid by alveolar epithelial cells keeps alveoli dry and ensures normal gas exchange. Sodium ion(s) (Na^+) enter the apical membranes of alveolar epithelial type II (ATII) cells mainly through amiloride-sensitive ion channels and are actively transported across the basolateral membranes of these cells by the ouabain-sensitive Na^+/K^+–ATPase (1). Alveolar fluid is reabsorbed across the epithelium by the resulting osmotic gradient.

Diverse pathological conditions, including prolonged inhalation of oxidant gases, have been associated with increased production of reactive oxygen and nitrogen species and extensive injury to the alveolar epithelium, resulting in increased protein permeability, pulmonary edema, and compromised gas exchange (2, 3). Survival during oxidant stress depends on the magnitude of the oxidant load and the ability of the animals to mount appropriate defenses. Rats exposed to 85% O_2 for 7 days survive a subsequent exposure to 100% O_2 for 4 days (4)—an exposure that is lethal to normal rats. Tolerance to hyperoxia was assumed to be the result of induction of antioxidant enzymes. The demonstrated increase of Na^+ transport across the alveolar epithelium of both rats and humans exposed to sublethal oxidant stress may also play a vital role in resolving pulmonary edema and thus limiting arterial hypoxemia (5–8). Since the majority of resistance to transcellular movement of Na^+ is encountered across the apical membranes and a large fraction of this transport occurs through amiloride-sensitive ion channels, we investigated whether exposure of rats to sublethal hyperoxia (85% O_2 for 7 days) altered the functional expression of these channels in ATII cells, the most abundant alveolar epithelial cells.

METHODS

Adult male Sprague–Dawley rats (200–250 g) were exposed to 85% O_2 for up to 7 days and then to 100% O_2 for up to 4 days in environmental chambers. At indicated times, rats were removed from the chambers, heparinized, and killed with an overdose of pentobarbital (100 mg/kg of body weight).

Measurements of Lung Water and Albumin Concentration in the Bronchoalveolar Lavage. Immediately after sacrifice, the chest was opened and any fluid in the chest cavity was aspirated in a syringe. Lung wet and dry weights and the hemoglobin concentrations of the right lung and of an arterial blood sample were determined as described (2, 9). In a separate group of rats, 10 ml of normal saline was slowly infused into the alveolar space via the trachea and then withdrawn. The lavage fluid was centrifuged at 250 × *g* to pellet cells, and the protein concentration in the supernatant was determined by the bicinchoninic acid technique (BCA).

ATII Cell Isolation and Culture. ATII cells were isolated by elastase digestion of lung tissue followed by Percoll centrifugation, seeded on glass coverslips or culture dishes at a density of 7.5–8.5 × 10^5 cells per cm^2, and cultured at 37°C in 5% CO_2/95% air for 24 hr in 1:1 (vol/vol) Dulbecco's modified Eagle's medium (DMEM; GIBCO)/Ham's F-12 nutrient mixture (GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and antibiotics (10).

Immunocytochemical Studies. Fixed and permeabilized ATII cells were immunostained with a polyclonal antibody raised against Na^+ channel protein purified from bovine kidney papilla (NaAb; 50 μg/ml; ref. 11) and goat anti-rabbit IgG conjugated to rhodamine. The slides were viewed with an image analysis system, and fluorescence intensity was assessed as described (10). To assess the cellular location of antigenic proteins, immunostained ATII cells were examined with a Molecular Dynamics 1000 (Sunnyvale, CA).

Patch Clamp Measurements. ATII cells were patched in the cell-attached, whole-cell, or inside-out mode by using KG-12 glass pipettes with a tip resistance of 5–10 MΩ. In all cases, current–voltage relationships across whole-cell patches were recorded under control conditions. In some cases, 1 μM amiloride or 1 μM 5-(*N*-ethyl-*N*-isopropyl)-2′,4′-amiloride

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Abbreviations: αrENaC, α-subunit of rat epithelial Na^+ channel; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-2′,4′-amiloride; ATII, alveolar type II cells; NaAb, sodium channel antibody.

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(EIPA) were added into the bath solution, and current–voltage relationships were measured again 10 min later. Single channel activity, observed in either cell-attached or inside-out patches, was expressed as NP_o, the product of the number of channels and their probabilities of being open, calculated as follows: $NP = \sum_{n=1}^{N} nt_n/T$, where N is the number of channels in a patch determined by the maximum unitary current amplitude, P_o is the probability of an individual channel being open, n is the number of simultaneously opening channels, t_n is the time of n channels opening, and T is the total recording time. Conductance was calculated as the slope of the current–voltage relationships around zero potential difference using linear regression analysis. All records were analyzed by using the PCLAMP program (Version 5.1.1; Axon Instruments, Foster City, CA).

RNA Isolation. Total RNA from lung tissue or ATII cells was isolated according to the method of Chomczynski and Sacchi (12). RNA was denatured in glyoxal and electrophoresed in 1.2% agarose gels. Northern blots were hybridized with a 3-kb *Not* I fragment of the cDNA for the α-subunit of rat epithelial Na^+ channel (αrENaC) (13). Filters were subsequently rehybridized with a ^{32}P-labeled 18S ribosomal RNA probe. Northern blots were done on nylon membranes (GIBCO) under high-stringency conditions. Autoradiogram signals were quantified with an imaging densitometer (Bio-Rad).

***In Situ* Hybridization.** *In situ* hybridization was performed as described with minor modification (14). The *Eco*RI/*Xba* I 667-bp coding region of αRENaC was subcloned into pSP72 (Promega), and its orientation and identity were verified by partial DNA sequencing. Radiolabeled sense and antisense RNA were generated by *in vitro* transcription with SP6 or T7 RNA polymerase (Promega) and uridine 5′-[α-^{32}P]triphosphate tetra(triethylammonium) salt (>1200 Ci/mmol; New England Nuclear) from the linearized plasmids.

RESULTS

ATII Cells. Each rat lung yielded about 2×10^7 cells. Alkaline phosphatase staining indicated that >85% of the cells were ATII cells. Viability, assessed by the ability of these cells to exclude trypan blue, was >95%.

Immunofluorescence Measurements. ATII cells from rats exposed to 85% O_2 for 7 days exhibited specific immunostaining that was considerably higher than the corresponding value in air-breathing rats. Mean cellular pixel intensity values (±1 SEM; n = number of cells) were as follows: air, 151 ± 23 (n = 25); 85% O_2, 297 ± 20* (n = 60); 85% O_2 plus 3–4 days in 100% O_2, 302 ± 30* (n = 30; the asterisks signify $P < 0.05$ from the air value). Cells were harvested from at least three different rats in each group. When IgG was substituted for the NaAb, mean pixel intensities were 69 ± 7; 64 ± 4; and 70 ± 2, respectively ($n \geq 30$). Confocal microscopy analysis revealed that in control ATII cells, staining was mainly confined at the level of the plasma membrane; however, in ATII cells isolated from the lungs of hyperoxic rats, significant levels of staining in cytoplasmic structures were also observed (Fig. 1).

αrENaC mRNA Expression. The αrENaC mRNA was detectable as a single 3.7-kb transcript in RNA isolated from rat lungs and ATII cells. Representative Northern blots are shown (Fig. 2 *A* and *B*). The 18S RNA autoradiograph signal remained unchanged relative to its control value for all experimental conditions. When normalized to the corresponding 18S RNA, αrENaC mRNA in total lung RNA remained at control levels following the 7-day exposure to 85% O_2 but increased consistently about 3-fold after 4 days of exposure to 100% O_2 (Fig. 3). In contrast, expression of this transcript in RNA isolated from ATII cells from rats exposed for 7 days to 85% O_2 increased 3-fold and after 4 days of subsequent exposure to 100% O_2 increased 6-fold (Fig. 3).

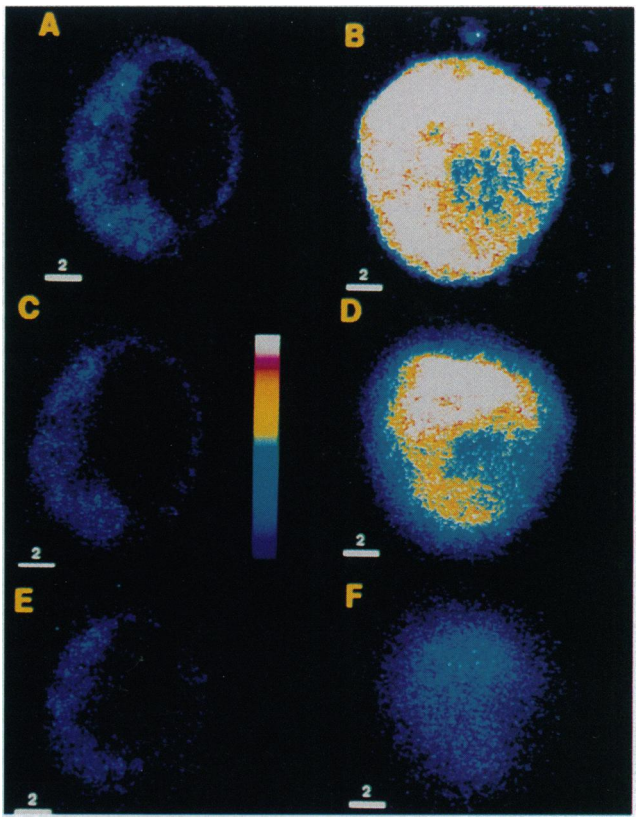

FIG. 1. Confocal microscopy images of ATII cells, isolated from either air-breathing (*A, C,* and *E*) or oxygen-breathing (7 days in 85% O_2) (*B, D,* and *F*) rats. ATII cells were immunostained as described in the text. The optical sections are 0.4 μm thick. (*A* and *B*) First optical section. (*C* and *D*) Fifth optical section. (*E* and *F*) Tenth optical section. Fluorescent intensity has been false-colored to the following scale: white>red>yellow>blue>black. The apical membrane is viewed *en face.*

***In Situ* Localization of αRENaC mRNA.** The hybridization signal per cell, as counted in 500 airway epithelial cells (from three rats in each group) hybridized with the antisense probe, increased from 0.9 ± 0.2 grains per cell (air control, mean ±

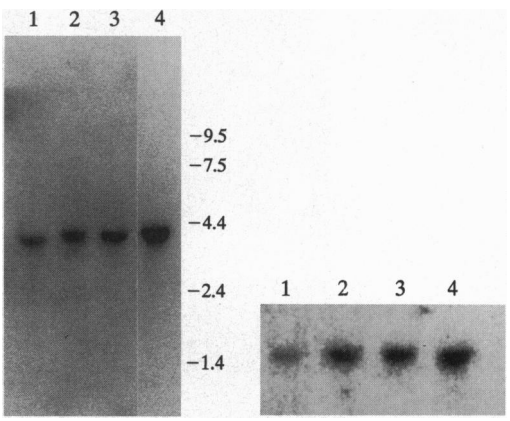

FIG. 2. Northern blots showing αrENaC mRNA expression in control and hyperoxic rat lungs. Total RNA was isolated from lungs (*Left*) or ATII cells (*Right*) from air-breathing (lanes 1) or hyperoxic rats (lanes 2–4: exposure to 85% O_2 for 7 days (lane 2) plus a subsequent exposure) to 100% O_2 for 2 days (lanes 3) or 4 days (lanes 4). Each lane represents 30 μg of RNA. An 18S ribosomal RNA probe confirmed equal loading in each lane (data not shown). The blots shown represent typical results. Three different blots of each lane were generated with RNA samples extracted from three different rats.

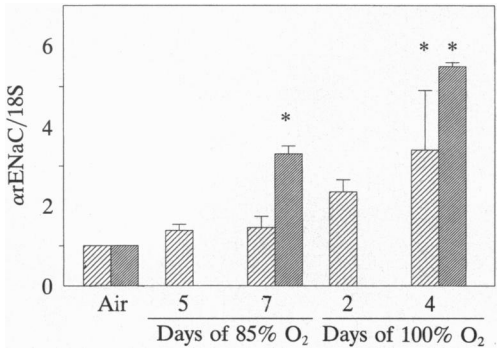

FIG. 3. Quantitation of the autoradiogram signal corresponding to the 3.7-kb transcript shown in Fig. 2 by laser densitometry in lung (▨) and ATII cells (▩). The horizontal axis shows the duration of exposure to hyperoxia, and the vertical axis shows the mean fold increase in density of the autoradiogram compared with control (assigned a value of 1-fold). Data were normalized for the corresponding 18S RNA signal in each lane and are expressed as the unitless ratio of αrENaC/18S RNA signal. Each lane of the ATII cell blot represents RNA pooled from the ATII cells of at least three different rats. Three different blots were done for both lung and ATII cell RNA with RNA samples from different rats. Values are means ± 1 SEM ($n = 3$). *, $P < 0.05$ from the corresponding control value.

1 SEM) to 4 ± 0.7 grains per cell (100% O_2 for 4 days after a 7-day exposure to 85% O_2; $P < 0.001$) (Fig. 4 *A–C*). The percentage of alveolar space-lining cells with at least 4 grains per cell increased from 7 ± 1% in the control to 25 ± 2% in the hyperoxic group (mean ± 1 SEM; $n = 400$ for each group; $P < 0.001$) (Fig. 4 *D–F*). All alveolar αrENaC-positive cells were cuboidal and located in the alveolar corners, most consistent with the appearance and location of ATII cells.

Electrophysiological Studies in ATII Cells. ATII cells were easily distinguished from macrophages because of their small granular appearance. When patched with nonsymmetrical solutions (pipette, 150 mM potassium glutamate; bath, 150 mM sodium glutamate) in the absence of free intracellular Ca^{2+}, ATII cells isolated from the lungs of rats exposed to 85% O_2 for 7 days exhibited outwardly rectified current–voltage relationships (Fig. 5). Both inward and outward currents were significantly higher than the corresponding values obtained across ATII cells isolated from air-breathing rats (Table 1).

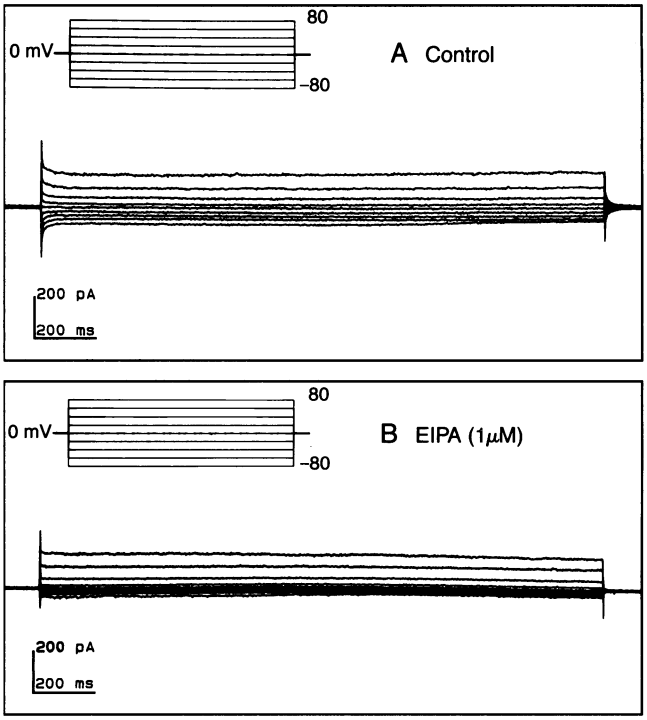

FIG. 5. Na^+ and K^+ currents recorded across an ATII cell patched in the whole-cell mode while the potential across the cell membrane was altered from −80 to +80 mV. ATII cells were isolated from the lungs of a rat that was exposed to 85% O_2 for 7 days and cultured on fibronectin-treated coverslips for 24 hr. The compositions of the pipette and bath were as follows: pipette, 150 mM potassium glutamate/5 mM Hepes/1 mM EGTA, pH = 7.2; bath, 150 mM sodium glutamate/5 mM Hepes/2 mM $CaCl_2$/1 mM EGTA, pH = 7.2. Addition of 1 μM EIPA (*B*) or 1 μM amiloride (data not shown) in the bath solution decreased the magnitude of the inward (Na^+) but not of the outward (K^+ currents) by about 50% of their control values.

Addition of either amiloride or EIPA (1 μM each) in the bath solution resulted in an identical and significant decrease of the inward (Na^+) but not the outward (K^+) currents (Table 1). Similar results were obtained from rats exposed to 85% O_2 for 7 days followed by 100% O_2 for 3–4 days. Exposure to 85% O_2

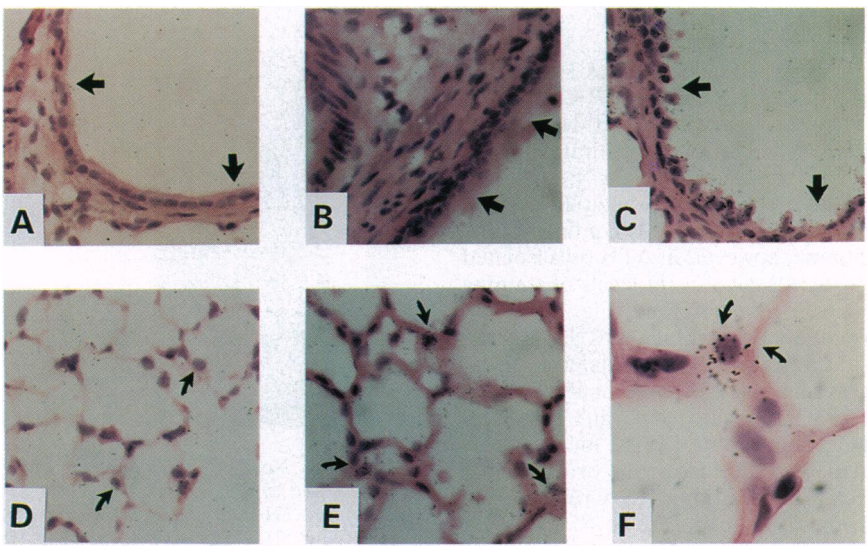

FIG. 4. Localization of αrENaC mRNA expression in lung tissue of rats that breathed either room air or excessive oxygen (85% oxygen for 7 days plus 100% oxygen for 4 days). Sections were hybridized with 3.0×10^6 cpm per slide of ^{33}P-labeled cRNA. (*A–C*) Bronchial walls. (*C*) Hyperoxia-induced expression of bronchial epithelial αrENaC mRNA as compared with expression in *A* (air control) and *B* (sense cRNA control). (*D–F*) Alveolar areas. (*E* and *F*) Induction of αrENaC mRNA in hyperoxic ATII cells (curved arrows) relative to the air control (*D*).

Table 1. Whole-cell currents across ATII cells, isolated from the lungs of rats that breathed either air or the indicated concentrations of oxygen

	Whole-cell currents, pA/20 pF			% inhibition of whole-cell currents					
				1 μM amiloride			1 μM EIPA		
O_2 intake	−80 mV	+ 80 mV	*n*	−80 mV	+80 mV	*n*	−80 mV	+80 mV	*n*
Control (air)	−150 ± 19	430 ± 5	3	—	—	—	—	—	—
85% O_2	−460 ± 35*	633 ± 14*	8	43 ± 8†	−5 ± 6	4	53 ± 4†	−14 ± 11	4
+100% O_2	−383 ± 28*	610 ± 3*	6	54 ± 10†	−16 ± 13	3	52 ± 7†	−8 ± 5	3

The buffer compositions were as shown in the legend of Fig. 5. Values are means ± 1 SEM; *n* = number of rats.
*$P < 0.05$ from the corresponding control currents (one-way analysis of variance followed by the Bonferroni modification of the *t* test).
†$P < 0.05$ by the paired Wilcoxon signed rank test (nonparametric statistics).

for 7 days resulted in a large increase in NP_o of single channels recorded in ATII cell-attached or inside-out patches (Fig. 6). No additional changes were seen in the values of these variables after reexposure of rats to 100% O_2 for 3–4 days. In all cases single-channel conductance remained unchanged from its 25 pS control value (Table 2).

Measurements of Lung Water. Exposure of rats to 85% O_2 for 7 days damaged both the microvascular endothelium and the alveolar epithelium as evidenced by the increase in extravascular lung water and alveolar protein concentration (Table 3). However, preexposed rats that subsequently were exposed to 100% O_2 for 3 days had significantly lower extravascular lung water than did rats exposed to 100% O_2 for 60 hr without prior exposure (Table 3). In addition, although the later group had large amounts of pleural fluid (range 7–10 ml), no pleural fluid was found in the preexposed rats that were subsequently exposed to 100% O_2 for 3 days.

DISCUSSION

Our results demonstrate that exposure of rats to 85% O_2 results in increased expression in both lung tissue and ATII cells of proteins antigenically related to purified epithelial Na^+ channels and of αrENaC mRNA transcripts, one of three subunits of the recently cloned amiloride-sensitive Na^+ channel (13, 15). These changes were accompanied by increased expression of functional Na^+ channels in ATII cell apical membranes, as shown by significant increases in whole-cell Na^+ currents and the appearance of multiple single-channel openings. Reexposure of these rats to hyperoxia led to a further increase of ATII cell αrENaC mRNA; however, no additional increase in Na^+ currents was seen.

Exposure to sublethal hyperoxia has been associated with induction of the Na^+/K^+-ATPase mRNA and functional protein (7, 8). Increased levels of fluid in the alveolar space and of the various cytokines released by lung and inflammatory cells during exposure to hyperoxia may also lead to an up-regulation of ion transport proteins. Exposure of cells to higher levels of oxidants, as encountered during exposure to 100% O_2, may damage proteins by oxidizing or nitrating specific amino acids (16, 17) or even may interfere with the normal polarized distribution of transporters. For example, in ATII cells recovered from rats exposed to 100% O_2 for 4 days after exposure to 85% O_2 for 7 days, a significant amount of Na^+ channel immunoreactive protein was identified in the cell cytoplasm and thus would be unable to participate in the vectorial transport of Na^+ from the apical to the basolateral membranes.

We have previously demonstrated the presence of Na^+-selective ($P_{Na^+}/P_{K^+} > 7$), cAMP-activated, L-type (i.e., equally inhibited by amiloride, benzamil, and EIPA) ion channels in the apical membranes of ATII cells from air-breathing rats (18). Data presented herein indicate that these channels are also present in ATII cells isolated from the lungs of rats exposed to hyperoxia. Single-channel data clearly demonstrate that the higher whole-cell Na^+ currents, measured across ATII cells isolated from the lungs of rats exposed to 85% O_2 for 7 days, were due to increases in both the number of these channels and the probability of being open.

αrENaC is one of the three transcripts coding for an H-type Na^+ channel (13, 15), and has been identified in RNA isolated from a number of epithelial tissues including fetal and adult lungs and ATII cells (19). There is disagreement as to the types of Na^+ channel present in fetal ATII cells: Voilley *et al.* reported the existence of a 4-pS, amiloride-sensitive but EIPA-insensitive (H-type) Na^+ channel in rat fetal type II cells cultured for periods up to 8 days (20). Patch–clamp studies from other laboratories revealed the presence of Ca^{2+}-activated, L-type (EIPA-sensitive) but not H-type Na^+ conductances in the apical membranes of rat fetal ATII cells (21, 22). Scatchard analysis of specific [^{3}H]bromobenzamil binding to fetal rat ATII cell membranes was consistent with the presence of both H- and L-type conductive protein in these cells (21). Amiloride-inhibitable cation channels, activated by high cytoplasmic Ca^{2+} concentrations, are also present in cultured adult ATII cells (23). Our patch–clamp measurements were conducted in the presence of nanomolar concentrations of cytoplasmic Ca^{2+} to prevent the activation of this channel.

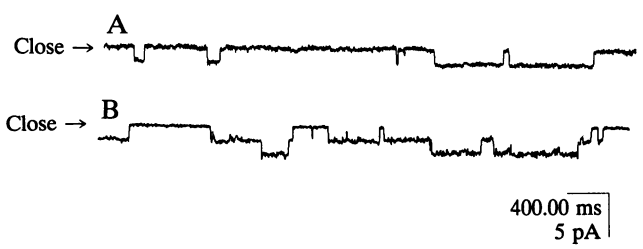

FIG. 6. Single-channel currents recorded across cell-attached patches in ATII cells isolated from the lungs of air-breathing (*Upper*) and oxygen-breathing (85% O_2 for 7 days) (*Lower*) rats. The composition of both the pipette and bath solutions was 150 mM sodium glutamate/5 mM Hepes/2 mM $CaCl_2$/1 mM EGTA, pH = 7.2. Recordings were obtained at a potential of −40 mV (reference to the bath solution). Exposure to 85% O_2 resulted in an increase of the number of active channels in each patch.

Table 2. Single-channel variables in ATII cells

Mode	Control (air)	85% O_2	85% + 100% O_2
Cell-attached	(*n* = 4)	(*n* = 3)	(*n* = 5)
NP_o (unitless)	0.3 ± 0.07	0.8 ± 0.1*	0.9 ± 0.06*
Conductance, pS	23 ± 1	28 ± 2	23 ± 1
Inside-out	(*n* = 4)	(*n* = 5)	(*n* = 4)
NP_o (unitless)	0.3 ± 0.07	0.7 ± 0.1*	0.6 ± 0.06*
Conductance, pS	23 ± 2	22 ± 2	21 ± 2

Cells were patched in either the cell-attached or inside-out mode with symmetrical solutions (150 mM sodium glutamate). Values are means ± 1 SEM; *n* = number of rats.
*P = <0.05 compared to corresponding control value (one-way analysis of variance followed by the Bonferroni modification of the *t* test).

Table 3. Lung water and alveolar permeability to solute

Condition	*n*	Weight, g Wet	Weight, g Dry	Ratio wet/dry	Blood volume, ml	Extrav. lung water,* ml	Alveolar albumin, mg/ml
Control (air)	5	0.57 ± 0.01	0.12 ± 0.00	4.5 ± 0.07	0.06 ± 0.01	0.40 ± 0.02	0.24 ± 0.06
85% O_2, 7 days	4	0.90† ± 0.05	0.18† ± 0.01	4.8 ± 0.03	0.06 ± 0.01	0.66† ± 0.03	0.70† ± 0.08
+100% O_2, 3 days	4	1.00†‡ ± 0.06	0.21† ± 0.01	4.9 ± 0.12	0.06 ± 0.01	0.80†‡ ± 0.03	ND
100% O_2, 60 hr	5	1.3† ± 0.05	0.17† ± 0.01	6.81† ± 0.07	0.06 ± 0.01	1.0† ± 0.04	1.34† ± 0.18

Data are means ± 1 SEM; *n* = number of rats. ND, not determined.
*Extravascular lung water = (wet lung weight − blood volume) − [dry lung weight − (blood volume) × 0.15], taking 1 g of weight to occupy 1 ml.
†$P < 0.05$ compared to corresponding control value (one-way analysis of variance followed by the Bonferroni modification of the *t* test).
‡$P < 0.05$ compared to the corresponding value at 100% O_2.

A putative Na^+ channel protein has been recently isolated and immunopurified to homogeneity from ATII cells (24). This protein consists of two peptides of ≈130 and 70 kDa. Amiloride- and EIPA-blockable Na^+ channels were evident after reconstitution of these immunopurified proteins into planar lipid bilayers. The polypeptide composition of the lung Na^+ channel protein is very different from that of an H-type renal Na^+ channel (≈700 kDa) that has been purified from bovine papillary collecting ducts and A6 cells (25). However, the 135-kDa polypeptide has been identified as an amiloride-binding protein in both H- and L-type channels (25, 26). Thus, since the αrENaC may be coding for the amiloride-binding site of the Na^+ channel, it may recognize mRNA from either channel protein.

Rats preexposed to 85% O_2 and subsequently exposed to 100% O_2 had lower values of extravascular lung water compared with rats exposed only to 100% O_2. Furthermore, >95% of preexposed rats survived a subsequent exposure to 100% O_2 for 3 days, as compared with <5% of rats not preexposed. Thus, the up-regulation of Na^+ channel proteins may help to mitigate lung injury by limiting alveolar edema. It should be stressed that the development of O_2 tolerance depends upon a number of factors, including induction of antioxidant enzymes (4) and higher levels of pulmonary surfactant (27).

In conclusion, exposure of rats to sublethal hyperoxia leads to increased expression of mRNA and both antigenic and functional Na^+ channel protein that is identified on the basis of its structure–function relationship for amiloride and EIPA as belonging to the L-family of Na^+ channels. The increase in functional Na^+ channels along with the previously demonstrated increase in Na^+/K^+-ATPase (8) may enhance Na^+ reabsorption across the alveolar epithelium and may limit alveolar edema in the presence of a damaged alveolar epithelium.

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