Functional ion channels in pulmonary alveolar type I cells support a role for type I cells in lung ion transport

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Efficient gas exchange in the lungs depends on regulation of the amount of fluid in the thin (average 0.2 μm) liquid layer lining the alveolar epithelium. Fluid fluxes are regulated by ion transport across the alveolar epithelium, which is composed of alveolar type I (TI) and type II (TII) cells. The accepted paradigm has been that TII cells, which cover <5% of the internal surface area of the lung, transport Na^+ and Cl^- and that TI cells, which cover >95% of the surface area, provide a route for water absorption. Here we present data that TI cells contain functional epithelial Na^+ channels (ENaC), pimozide-sensitive cation channels, K^+ channels, and the cystic fibrosis transmembrane regulator. TII cells contain ENaC and cystic fibrosis transmembrane regulator, but few pimozide-sensitive cation channels. These findings lead to a revised paradigm of ion and water transport in the lung in which (*i*) Na^+ and Cl^- transport occurs across the entire alveolar epithelium (TI and TII cells) rather than only across TII cells; and (*ii*) by virtue of their very large surface area, TI cells are responsible for the bulk of transepithelial Na^+ transport in the lung.

Shortly before birth, the fetal lung converts from fluid secretion to fluid reabsorption. After birth, efficient gas exchange depends on regulation of the amount of fluid in the thin (average, 0.2 μm) liquid layer lining the alveolar epithelium (1). Alveolar flooding resulting from cardiogenic pulmonary edema or acute lung injury impairs gas diffusion across the air/blood barrier; an increase in alveolar fluid clearance restores a normal air/blood barrier. Alveolar fluid transport from alveolar to interstitial spaces, driven by active Na^+ transport across the alveolar epithelium (2), can be inhibited either by the addition of amiloride, a Na^+ channel inhibitor, to the alveolar space, or ouabain, a Na^+,K^+-ATPase inhibitor, to the vascular bed (3), suggesting that the alveolar epithelium is the major site of Na^+ transport and fluid absorption in the adult lung.

The alveolar epithelium, which covers >99% of the large internal surface area of the lung (4), is composed of two cell types, alveolar type I (TI) and type II (TII) cells. TII cells, which cover 2–5% of the internal surface area of the lung, are cuboidal cells that synthesize and secrete pulmonary surfactant. TII cells contain ion channels, including the amiloride-sensitive epithelial Na^+ channel (ENaC) (5), Na^+,K^+-ATPase (3) and the cystic fibrosis transmembrane regulator (CFTR) (6). TI cells are large squamous cells whose thin cytoplasmic extensions cover >95% of the internal surface area of the lung (7). TI cells express aquaporin 5, a water channel (8), and have the highest known osmotic water permeability of any mammalian cell type (9). The observations that TII cells contain ion channels and TI cells express aquaporins led to the paradigm that TII cells govern alveolar fluid balance by regulating Na^+ transport in the lungs, whereas TI cells merely provide a route for passive water absorption (2). Recent immunohistochemical studies demonstrating that TI cells contain ENaC and Na^+,K^+-ATPase (10, 11) and radionucleotide uptake studies showing that TI cells can transport Na^+ and K^+ (10) have raised questions about whether this

paradigm adequately describes lung ion and fluid transport. Although these newer observations are consistent with the hypothesis that TI cells play a role in active alveolar ion transport, the lack of direct electrophysiologic evidence of specific functional ion channels in TI cells has precluded general acceptance of such a concept (12). Here, we present evidence that TI cells contain functional ion channels, and compare the electrophysiological characteristics of specific ion channels in TI and TII cells. These data support the concept that TI cells play an important role in the regulation of ion and fluid balance in the lung.

Results

TI Cells Contain Highly Selective Cation (HSC) and Nonselective Cation (NSC) Channels. Patch clamp analysis of TI cells demonstrated the presence of both 4- to 6-picoSiemen (pS) HSC and 19- to 21-pS NSC channel activities (Fig. 1 *A* and *B* and Table 1). HSC channels had a positive membrane reversal potential and were inhibited by amiloride ($K_{0.5} < 50$ nM), characteristics consistent with those of HSC channels composed of α-, β-, and γENaC subunits (13). NSC channels had a unit conductance of 19–21 pS, were equally selective to Na^+ and K^+, and inhibited by amiloride ($K_{0.5} \approx 1$ mM, data not shown), characteristics consistent with NSC channels composed of αENaC alone (14, 15). The results of patch clamp studies of TI cells are shown in Table 1. Values for conductances and reversal potentials (E_R) can be found in Table 3, which is published as supporting information on the PNAS web site.

TI Cells Contain Cyclic Nucleotide-Gated (CNG) and K^+ Channels. CNG channel activity was suggested by the existence of channels with a unit conductance of 2.9 pS, an E_R close to zero, equal selectivity of Na^+ and K^+, insensitivity to amiloride, inhibition with pimozide (300 nM), and an apparent increase in conductance in Ca^{2+}-free bath and pipette solutions (Fig. 1*D*). The activity of these channels in Ca^{2+}-free solutions was also increased by the application of membrane permeable analogues of cGMP (chloro-phenyl-thio-cGMP or 8-bromo-cGMP). K^+ channel activity was suggested by a unit conductance of 15–16 pS, a negative E_R, and barium sensitivity (Fig. 1*C*).

TI Cells Contain CFTR Channels. CFTR is an anion channel that, in airway cells, has a conductance of ≈4 pS (6) and is stimulated by cAMP. After stimulating TI cells with forskolin and isobutyl-methyl-xanthine, we could detect functional CFTR in TI cells (Fig. 1*E* and Table 1) with a conductance of ≈4 pS and an E_R consistent with the chloride reversal potential [E_R is not altered
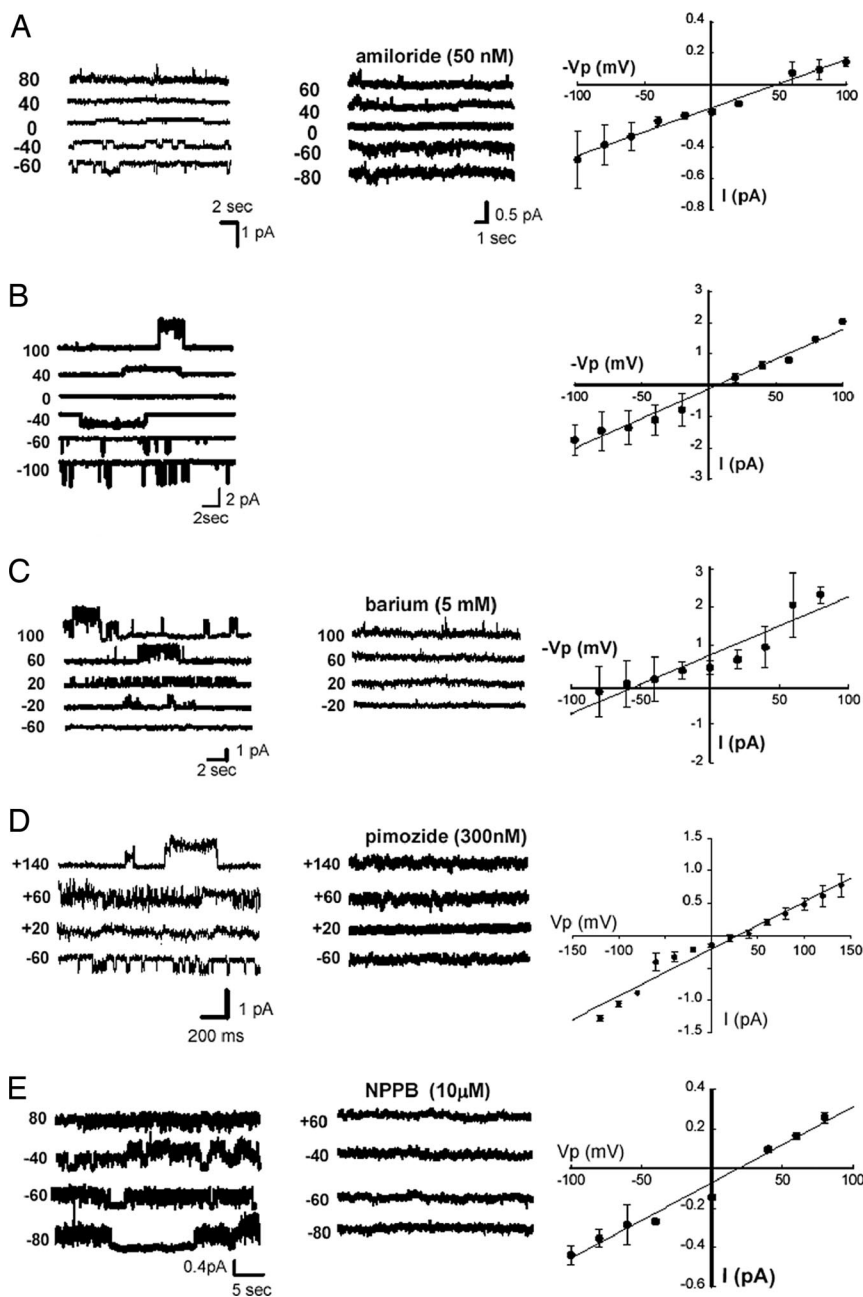

Fig. 1. Representative single channel activity in cell-attached patches on adult rat alveolar TI cells. TI cells were isolated and cell-attached patches formed on the apical surface of the cells as described in *Materials and Methods*. Typical records and current-voltage relationships obtained from cells with the frequency given in Table 1 are shown. (*A*) A 4- to 5-pS channel whose E_R, channel kinetics, and block by amiloride are consistent with an HSC channel (ENaC). (*B*) A 19- to 21-pS channel whose E_R and channel kinetics are consistent with an NSC channel. (*C*) A 5- to 6-pS channel whose E_R and the observation that it is completely blocked by 5 mM Ba^{2+} in the bath suggests that it is a K^+ channel. (*D*) A 7-pS nonselective cation channel blocked by 300 nM pimozide and observed in the absence of bath Ca^{2+} and with 10 μM amiloride in the pipette is consistent with a CNG channel. (*E*) A 4-pS anion channel that can be observed with either 10 μM amiloride or an impermeable cation NMDG (*N*-methyl-D-glucamine) in the pipette and can be blocked by 10 mM NPPB [5-nitro-2-(3-phenlproplyamino)benzoate], which is consistent with CFTR.

when all univalent cations in the pipette are replaced with an impermeant cation, such as *N*-methyl-D-glucamine (NMDG)]. The channel is not affected when 10 μM amiloride is in the pipette, but can be blocked by a CFTR blocker, NPPB [5-nitro-2-(3-phenylpropylamino)-benzoate] (Fig. 1*E*). In the absence of stimulation, we could not detect functional CFTR.

TI Cells Contain mRNAs for All Three ENaC Subunits and for CFTR.
Northern blotting of TI and TII cell RNA demonstrated mRNAs for α-, β-, and γENaC, α1- and β1- Na$^+$,K$^+$-ATPase (Fig. 2*A*).

Alveolar macrophages were used as a negative control for ENaC (2). TI cells contain more ENaC transcript (normalized to 18S) than do TII cells. The relative quantities of transcripts for all 3 ENaC subunits and CFTR were calculated by quantitative (Q)-PCR (Fig. 2*B*). The ratios of TI/TII mRNA content (TI cells, $n = 5$; TII cells, $n = 4$) for each ENaC subunit were: αENaC, 3.0; βENaC, 2.2; γENaC, 5.0 (Fig. 2*B*), consistent with the results found in our Northern blot analysis. CFTR mRNA was detected in TI cells via Q-PCR ($n = 6$, each cell type), although at lower levels than in TII cells; the ratio of TI/TII CFTR mRNA content was 0.2.

Table 1. Frequency of different channel types in TI cells

Channel type	Conductance, pS	No. of patches with channel	No. of channels*	Approximate density, channels per μm^2†	Total patches	Notes
ENaC (HSC)	4–5	62	109	1.49	73	Amiloride-sensitive ($K_{0.5}$ = 37 nM)
ENaC (NSC)‡	21	7	7	0.104	67	Amiloride-sensitive ($K_{0.5}$ > 2μM)
K$^+$ (Ba^{2+}-sensitive)	5–6	18	29	0.433	67	
CNG (NSC)	2–3	13	13	0.194	67	Pimozide-sensitive
CNG (NSC)§	7–8	17	22	0.468	47	Pimozide-sensitive, Ca^{2+}-free in bath and pipette
CFTR						
No stimulus	4	0	0	0	12	
Stimulated§		8	10	0.082	122	10 μM isoproterenol or 1 μM
Stimulated, NMDG§¶		1	1	0.125	8	Forskolin + 10 μM IBMX
Other cation	14	3	3	0.0448	67	Activated by depolarization

*Total number of channels observed (some patches contained more than one channel).

†The frequency is the total number of channels divided by the total number of patches. Some patches contained more than one type of channel or more than one channel of the same type. Since our patch pipettes are typically 1 μm² the frequency is approximately the number of channels per square micron.

‡We also observed two instances of a patch with a single 9-pS channel with a Na$^+$/K$^+$ selectivity of ≈4 and a frequency <3%.

§10 μM amiloride in pipette.

¶NMDG (N-methyl-D-glucamine) is an impermeant cation.

TI Cells Contain ENaC by Western Blotting and CFTR by Western Blotting and Immunohistochemistry. By Western blotting, TI cells contain α-, β-, and γENaC and CFTR protein (Figs. 3 and 4B). The ENaC antibodies (D.C.E.'s laboratory) have been extensively characterized; they detect the appropriate *in vitro* translated products and the same bands as other "verified" antibodies (13, 16, 17). ENaC undergoes extensive posttranslational processing, both by the addition of functional groups and by proteolytic cleavage (18). Such processing may result in Western blot bands presenting at different molecular weights in different cell preparations (see Table 4, which is published as supporting information on the PNAS web site), which may account for the presence of a 75- and 120-kDa band in TI cells, but an 85-kDa band in TII cells. To confirm specificity, competing peptides were used. In previous publications, the immunoprecipitated products were sequenced and found to represent the correct proteins, and selectivity of the peptides have been validated (16, 19).

TII Cells Contain HSC, NSC, and CFTR Channels. We confirmed the presence of HSC, NSC, and CFTR channels in TII cells. Patch clamp analysis of TII cells cultured on coverslips for ≈1 h demonstrated the presence of both HSC and NSC channel activity, which could be inhibited by amiloride. The presence of functional CFTR was enhanced after the addition of isoproterenol. We were unable to detect channels with electrophysiologic properties consistent with CNG channels. The frequency of each type of channel/patch in TII cells is shown in Table 2.

Discussion

In the current studies, we examined the electrophysiologic properties of ion channels in freshly isolated TI and TII cells. Previous studies focused on ion channels in cultured TII cells because techniques had been developed to isolate and culture this cell type. Because TI cells are difficult to isolate, and because cultured TII cells express some markers shared by TI cells (20), cultured TII cells have been proposed as a model for TI cells. However, TII cells cultured on tissue culture plastic undergo a change in phenotypic expression, no longer expressing important characteristics of the TII cell phenotype. However, because there are major differences in gene expression profiles between cultured TII cells and freshly isolated TII or TI cells (21), the relevance of studies performed with cultured TII cells to either TI or TII cells has been raised. For these reasons, we believed it was important to study freshly isolated TI and TII cells; cells were placed in culture only to allow adherence to the coverslips to facilitate patch clamp experiments.

Amiloride-sensitive cation channels are distinguished by distinct biophysical characteristics, including unit conductance, mean open
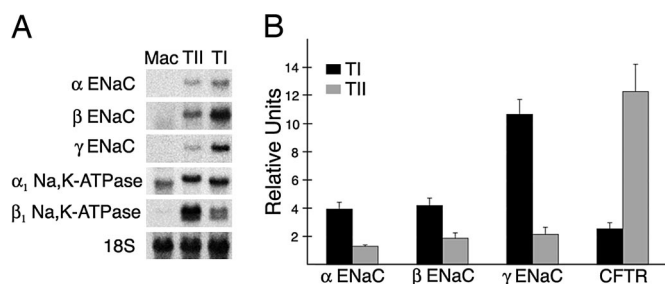
Fig. 2. Expression levels of ENaC transcripts in rat TI and TII cells. (A) Northern blots. The relative quantity of TI/TII mRNA was: αENaC, 1.3; βENaC, 2.4; γENaC, 3.6; α$_1$Na$^+$,K$^+$-ATPase, 1.0; β$_1$Na$^+$,K$^+$-ATPase, 0.3. Macrophages were used as a negative control for ENaC. (B) Q-PCR analysis. Results are expressed in relative units (n = 5 for TI, n = 4 for TII cells for ENaC analyses; n = 6 for TI and TII cells for CFTR analyses). The ratio of TI/TII mRNA content for each gene was: αENaC, 3.0; βENaC, 2.2; γENaC, 5.0 (P < 0.01 compared with TII cells for each subunit); CFTR, 0.2.
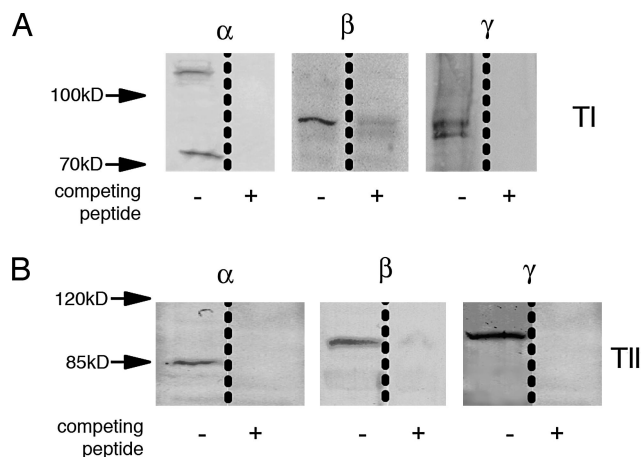
Fig. 3. Both TI and TII cells contain ENaC. Western blots for α-, β-, and γENaC in adult rat TI and TII cells show that both cell types have all three subunits and that antibody binding can be blocked by the appropriate specific peptides.
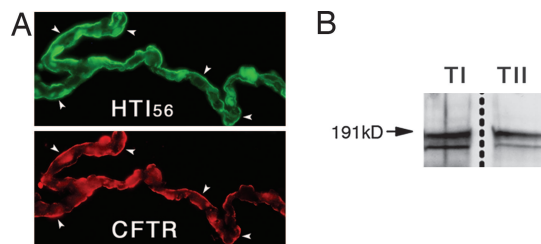
Fig. 4. Immunohistochemistry and Western blot demonstrating that CFTR is present in TI cells. (*A*) (*Upper*) Human lung tissue showing immunofluorescence (green) for HTI56 (integral membrane protein specific to human TI cells; ref. 42) showing typical apical membrane localization. (*Lower*) Immunofluorescence for CFTR (red) showing similar TI cell apical membrane. (*B*) Western blots for CFTR show protein expression in both TI and TII cells.

and closed times, cationic selectivity, and amiloride sensitivity. The cloning of an epithelial Na^+ channel consisting of three homologous subunits (α, β, and γ) from rat colon provided a molecular definition of one class of Na^+ channel (ENaC) (14, 22). Expression studies of various combinations of the different ENaC subunits in heterologous expression systems showed that HSC channels were composed of α, β, and γENaC subunits, and that NSC channels were composed of α subunits alone (14). The predominant amiloride-sensitive channels previously described in TII cells were NSC channels with a unit conductance of 19–24 pS and Na^+/K^+ selectivity of ≈ 1.5, and HSC channels with a unit conductance of 4–5 pS and Na^+/K^+ selectivity of >40 (23).

To determine whether TI cells contained functional ENaC, isolated TI cells were identified by their typical morphology (9) and patched in the cell-attached mode. TI cell-specific antibodies were used to confirm the identity of TI cells after patch clamping. The patches contained both 4- to 6-pS HSC channels and 19- to 21-pS NSC channels (Fig. 1 *A* and *B* and Table 1). The HSC channels were inhibited by amiloride with a $K_{0.5} < 50$ nM and had an E_R that implied a very high selectivity of the channel for Na^+ over K^+, consistent with the properties of ENaC channels composed of α, β, and γ subunits. TI cells also contained a second type of Na^+ channel, with a unit conductance of 19–21 pS, equal selectivity to Na^+ and K^+, and inhibitable by amiloride, although only at higher concentrations ($K_{0.5} \approx 1$ mM). These characteristics are consistent with NSC channels (14, 15).

TII cells examined under the same conditions also contained both HSC and NSC channels (Table 2). Culture conditions can alter the types of Na^+ channels found in TII cells. NSC channels are predominant in TII cells cultured for 12–96 h on tissue culture plastic (24), and HSC channels are predominant in TII cells cultured with an apical air interface in the presence of FBS (16), which preserves some of the characteristics of the TII

phenotype, such as surfactant protein expression (20). In the current study, TII cells cultured for brief (<24 h) periods of time contain a similar frequency of HSC channels to that observed in TI cells (Tables 1 and 2). Because culture conditions can affect ENaC expression, one might hypothesize that changes in cellular microenvironments caused by acute lung injury or pulmonary edema could alter the composition and types of functioning ion channels in alveolar epithelial cells.

TI cells contain the molecular components of the HSC and NSC channels. By Northern blotting, Q-PCR, and Western blotting, TI cells contain all three ENaC subunits. By both Northern blot (Fig. 2*A*) analysis and Q-PCR (Fig. 2*B*), TI cells contain more of all ENaC subunit transcripts than do TII cells.

As much as 60% of alveolar fluid clearance (AFC) is amiloride-insensitive (25). In a previous study, amiloride inhibited Na^+ uptake by $\approx 30\%$ in TI and TII cells (10), suggesting that the majority of the Na^+ uptake by both cell types is amiloride-insensitive. It has been proposed that CNG channels are responsible for the amiloride-insensitive fraction of AFC because CNG mRNA can be found in the alveolar epithelium by *in situ* hybridization (26). CNG channels show no preference for Na^+ over K^+ and are activated in the absence of Ca^{2+}; they are amiloride-insensitive but are inhibited by pimozide (27). Properties of single CNG channels are difficult to predict because channels form heteromultimers of different subunits and unit conductance is affected by the extracellular concentrations of various ions (28). In our studies, the pimozide-sensitive channels observed in TI cells are consistent with some CNG channel isoforms. We frequently observed a channel with a unit conductance of 2.5–3.0 pS and an E_R near 0 that was inhibited by pimozide (Fig. 1*D*), characteristics suggestive of a CNG channel. The unit conductance of these channels increased to 7 pS in a Ca^{2+}-free bath solution, and the open probability was increased by membrane-permeable analogues of cGMP, both consistent with CNG channels. Although we did not observe similar channels in TII cells, we cannot exclude the possibility that TII cells contain CNG channels because the expression of alternative CNG channel isoforms and/or different cellular microenvironments might make detection of these channels difficult. A previous report that TII cells contained CNG channels (29) used TII cells cultured for 2 days, consistent with other observations that culture conditions may alter channel expression. The sensitivity of lung ion transport to pimozide (25) seems likely to be due to CNG channels in TI cells. A portion of "amiloride-insensitive" AFC may also be due to a cation channel other than CNG, such as NSC channels, which are much less sensitive to amiloride ($K_{0.5} > 2$ mM) than HSC channels, and therefore may not have been completely inhibited by amiloride instilled in the upper airways.

We found similar numbers of HSC and NSC channels and many more CNG channels/patch in TI cell membranes than in TII cell membranes. In the Sprague–Dawley rat, the cell surface

Table 2. Frequency of different channel types in TII cells

Channel type	Conductance, pS	No. of patches with channel	No. of channels*	Approximate density, channels per $\mu m^{2\dagger}$	Total patches	Notes
ENaC (HSC)	4–5	185	407	1.63	249	Amiloride-sensitive ($K_{0.5} = 39$ nM)
ENaC (NSC)‡	21	34	34	0.167	204	Amiloride-sensitive ($K_{0.5} = 2.2$ μM)
K^+ (Ba^{2+}-sensitive)	5–6	0	0	0	409	None observed
CNG	2–3	0	0	0	283	None observed
CFTR						
No cAMP	8	11	16	0.127	126	NPPB-sensitive
After cAMP§		18	55	0.982	56	

*Total number of channels observed (some patches contained more than one channel).
†Frequency and density calculated as in Table 1.
‡We occasionally observed instances of a 9 pS channel with a Na^+/K^+ selectivity of 4, an amiloride $K_{0.5} \approx 800$ nM, and a frequency $<4\%$.
§cAMP was increased in cells by addition of 300 nM isoproterenol.

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area ratio of TI:TII cells is ≈ 43:1 (9). Taken together, these data suggest that there are many more Na^+ channels in TI cells than in TII cells; therefore, TI cells may be responsible for the bulk of basal Na^+ transport in the lung.

K^+ channels in alveolar epithelial cells are important in preventing cell depolarization during times of Cl^- efflux and Na^+ influx (30). Multiple types of K^+ channels (≈15) have been detected in alveolar epithelial cells via RT-PCR, but corresponding proteins have been detected for only ≈1/3 of these (30). K^+ channels are present in the apical membrane of cultured TII cells (31); there is a recent report that K^+ channels are present in TI cells *in situ*.[††] We found channels in TI cells with a unit conductance of 5–6 pS, an E_R at very negative intracellular voltages, and barium sensitivity (Fig. 1C), all characteristics of K^+ channels. We were unable to detect K^+ channels in freshly isolated TII cells. Previous studies of TII cells cultured for 5–7 days found evidence for K^+ channels (31), demonstrating again that culture conditions can alter channel expression. In addition, the α subunit of the voltage-gated K^+ channel (Kv) subfamily Kv9 can electrically silence other α subunits of the Kv2 and Kv3 subfamilies, but not the currents mediated by the α subunits of the Kv1 or Kv4 subfamilies (32), which may also explain the difficulty in detecting K^+ channels by patch clamping in TII cells.

In utero, the alveolar epithelium actively secretes Cl^- into the developing airspace, promoting fluid secretion, which in turn modulates lung growth (33); fluid in the fetal lung is cleared in the peripartum period. The importance of Cl^- transport in the adult lung is uncertain. CFTR is a cAMP-dependent Cl^- channel that regulates epithelial Cl^- and fluid secretion. It has been controversial whether CFTR is present in alveolar epithelia and whether CFTR could modulate bidirectional Cl^- flux (i.e., from the alveolar or airway space into the cell). Studies have suggested a role for CFTR in alveolar fluid transport in the adult lung (34), but only recently has there been direct evidence that functional CFTR channels are present in TII cells (6). We were able to detect both CFTR mRNA (Fig. 2B) and protein in TI cells, the latter by both immunohistochemistry and Western blotting (Fig. 4). The pattern of CFTR immunostaining in human lung is comparable with localization to the apical plasma membrane. We also found functional evidence of CFTR channels in TI cells (Fig. 1E). Isoproterenol increased CFTR channel activity in TII cell membrane patches; observation of functional CFTR in TI cells required stimulation with both forskolin and isobutyl-methyl-xanthine. CFTR undergoes rapid recycling between the plasma membrane and intracellular compartments. It is possible that different rates of CFTR recycling between these two compartments may determine the differences in observed CFTR plasma membrane activity between TI and TII cells.

TI cells contain several types of cation channels: HSC, NSC, CNG, and K^+ channels, and at least one type of anion channel: CFTR. TII cells contain HSC, NSC, and CFTR; we did not find evidence for functional K^+ or CNG channels in TII cells. Taken together, these findings suggest a revised paradigm for ion and water transport in the lung in which Na^+ transport occurs across the entire alveolar epithelium (TI and TII cells), rather than only at specialized anatomic locations (i.e., across TII cells). Fig. 5 depicts a proposed model of ion and water transport across the alveolar epithelium based on both the current results and the observations of other investigators. In this model, Na^+ is absorbed from the apical surface of both TI and TII cells via ENaC and CNG channels and transported into the interstitial space by Na^+-, K^+-ATPase. K^+ may be transported via K^+ channels located on the apical surface of TI cells or through basolateral K^+ channels in TII cells. If net ion transport is from the apical

[††]Bourke, S., Helms, M. N., Kim, K. J., Crandall, E. D., Borok, Z. & Kemp, P. J. (2004) *FASEB J.* **18**, A723 (abstr.).
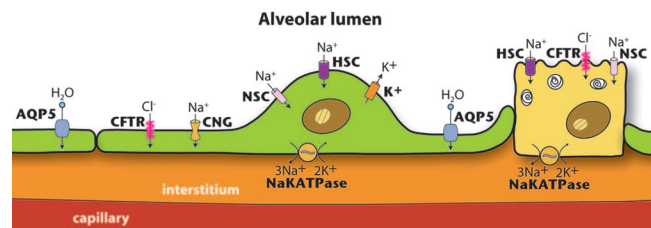
Fig. 5. Proposed paradigm of channels and transporters involved in alveolar epithelial ion and fluid transport. TI cells (green), TII cells (yellow); ion channels and transporters are labeled as they appear in the text (e.g., HSC, highly selective Na^+ channel). See text for details.

surface to the interstitium, an osmotic gradient would be created that would direct water transport either through aquaporins or by diffusion. Electroneutrality is conserved with Cl^- movement via CFTR in both TI and TII cells, and/or paracellularly through tight junctions or possibly via an as yet unidentified apical Cl^-–HCO_3^- exchanger. Because most eukaryotic cells extrude H^+ to regulate intracellular pH (35), the net effect of uptake via a Cl^--HCO_3^- exchange would be to absorb Cl^- and extrude HCO_3^-, which, when combined with H^+ in the alveolar lumen, would produce CO_2, which is exhaled. Extensive experiments characterizing the various channels and the mechanisms by which they are regulated will be necessary to understand the pathways by which anions are transported in the alveolus.

The data presented in this article lead to a revised paradigm of ion transport in the lung, in which ion transport occurs across the entire alveolar surface, rather than being limited to TII cells. Based on the ion channel characteristics of TI and TII cells and the large differences in surface area of the two cell types, it appears likely that TI cells may be more instrumental in driving bulk Na^+ transport than are TII cells. Further insight into the characterization and function of ion channels in TI cells specifically, and ion transport in the lung as a whole, will allow clarification of the pathways by which ion and water movement occur across the alveolar epithelium. Better understanding of these pathways will help delineate the mechanisms that regulate alveolar fluid balance and may provide the basis for developing strategies to prevent or treat the respiratory compromise associated with alveolar flooding.

Materials and Methods

Preparation of Rat Alveolar TI and TII Cells. The isolation and determination of cell purity of TI and TII cells from adult Sprague–Dawley rat lungs were performed as described in ref. 10. Preparations of TI cells containing <80% TI cells or with >1% TII cells were discarded, as were preparations of TII cells containing <85% TII cells or with >1% TI cells. Viabilities for both cell types were >95% (Live/Dead kit, Molecular Probes). Both cell types were plated on glass coverslips coated with fibronectin or on permeable supports (Millipore CM membranes) at a density of 1×10^6 cells per coverslip.

Patch Clamp Studies. TI cells were identified by their distinctive morphologic appearance (i.e., large size, extensive cytoplasmic extensions) before patch clamping: after patch clamping, identity was confirmed by immunostaining. We used the cell-attached configuration for these studies. Cells were studied both 1 and 24 h after plating cells on coverslips or permeable supports with an air interface. The 24-h time point was chosen for practical experimental reasons, because each cell isolation took 5 h and each patch recording took ≈1 h. Results obtained at the two time points were indistinguishable. Gigaseal patch clamp methods were the same as those previously described (16) and are detailed again in *Supporting Text*, which is published as supporting information on the PNAS web site.

Northern Blot Analysis. Total RNA was extracted from freshly isolated TI cells, TII cells, or alveolar macrophages using an RNEasy kit (Qiagen), fractionated on formaldehyde–agarose gels (1%), blotted on nylon membranes, and hybridized with ^{32}P-labeled full-length cDNA probes for the α, β, or γ subunits of ENaC (kind gifts of Pascal Barbry, Centre National de la Recherche Scientifique, Paris) and for the α_1 or β_1 subunits of Na$^+$,K$^+$-ATPase (kind gifts of Jerry Lingrel, University of Cincinnati, Cincinnati). Radioactivity was quantified by PhosphorImager analysis (Storm 840, Molecular Dynamics). Results were normalized to 18S total RNA.

Real-Time Q-PCR. A real-time Q-PCR protocol (TaqMan, Applied Biosystems) was used to evaluate relative RNA expression of ENaC subunits and CFTR in TI and TII cells using the ABI Prism 7700 Sequence Detection System. Primer–probe combinations were as follows: αENaC forward 5′-GGCGCCTTCTCCTTGGA-3′, reverse 5′-CACTACAAGGCTTCCGACACTT-3′, probe 5′-CTGGGCTGTTTCTCC-3′; βENaC forward 5′-GTGACTACA-ACACGACCTATTCCA-3′, reverse 5′-CAGTTATGGATC-ATGTGGTCTTGGA-3′, probe 5′-CCTGCCTTCATTCCTG-3′; γENaC forward 5′-GGCACAATGGCCGTCTGA-3′, reverse 5′-CTTTGGTCCCAGGTGAGAACATT-3′, probe 5′-CAGC-AACCATTTCTCG-3′; CFTR forward 5′-AAGCTTGCCAAC-TACAGGAGGA-3′, reverse 5′-TCTTGCACGTTGACCTC-CACT-3′, probe 5′-TGACCAAGTTTGCAGAACAAGACAA-CACAGT-3′. Standard curves for each ENaC subunit were obtained from full-length α-, β-, or γENaC cDNAs (gifts of Pascal Barbry) of known concentration. Lung cDNA was used to generate a standard curve for CFTR. Relative amounts of mRNA were then calculated by using the comparative threshold method as recommended by Applied Biosystems with 18S total RNA as the internal control.

Western Blot Analysis. Western blot analysis for α-, β-, and γENaC in TI and TII cells was performed with antibodies that have been characterized and shown to be subunit-specific (13, 16, 17) both in the absence and in the presence of specific competing peptides as described (16). For detection of CFTR, 30 μg of both TI and TII cells were added to sample buffer and heated at 55°C for 10 min. Protein bands were resolved on a 4–12% NuPage Bis-Tris gel (Invitrogen), transferred to a nitrocellulose membrane, blocked with 5% powdered milk, and then incubated with CFTR NBD1 antibody (kind gift of David Bedwell, University of Albama, Birmingham); goat anti-rabbit IgG-HRP (Vector Laboratories) and SuperSignal West Pico chemiluminescent substrate (Pierce) were then added before autoradiography.

Immunohistochemistry. Two-micrometer cryostat sections of human lung were subjected to antigen retrieval (10) and incubation with PBS, 0.1% BSA, 0.3% Triton X-100, and 10% normal goat serum before being sequentially incubated with the following antibodies: CFTR H-182 (Santa Cruz Biotechnology); goat anti-rabbit IgG Alexa 594 (Molecular Probes); HTI56, a mouse monoclonal antibody directed against an integral membrane protein specific in human lung to TI cells (37); goat anti-rabbit IgG Alexa 488 (Molecular Probes). Images were captured with a Leica DC500 camera on a Leica Orthoplan microscope.

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