Amiloride-insensitive Na$^+$ and fluid absorption in the mammalian distal lung

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O’Brodovich H, Yang P, Gandhi S, Otulakowski G. Amiloride-insensitive Na$^+$ and fluid absorption in the mammalian distal lung. Am J Physiol Lung Cell Mol Physiol 294: L401–L408, 2008. First published December 27, 2007; doi:10.1152/ajplung.00431.2007.—The ability of the distal lung epithelia to actively transport Na$^+$, with Cl$^−$ and water following, from the alveolar spaces inversely correlates with morbidity and mortality of infants, children, and adults with alveolar pulmonary edema. It is now recognized, in contrast to many other Na$^+$ transporting epithelia, that at least half of this active transport is not sensitive to amiloride, which inhibits the epithelial Na$^+$ channel. This paper reviews amiloride-insensitive Na$^+$ and fluid transport in the mammalian distal lung unit under basal conditions and speculates on potential explanations for this amiloride-insensitive transport. It also provides new information, using primary cultures of rat fetal distal lung epithelia and alveolar type II cells grown under submersion and air-liquid interface culture conditions, regarding putative blockers of this transport.

HOW EPITHELIA ACTIVELY TRANSPORT salt and water from the lumen to the interstitium of the lung is critical to our understanding of lung development, physiology, and pathophysiology. For normal fetal lung development to occur, there must be fluid filling the distal regions of the lung. This is physiological since the placenta can carry out gas exchange for the fetus, and most of the right ventricular output bypasses the lungs’ vasculature. However, failure to efficiently clear this fluid at birth results in transient tachypnea of the newborn, and when combined with surfactant deficiency, it results in hyaline membrane disease (reviewed in Refs. 34 and 62). During postnatal life, when there is an increase in the transvascular hydrostatic pressure gradient, an increase in the permeability of the alveolar capillary membrane to solutes, or a combination of these two processes, fluid pathologically fills the air spaces, resulting in pulmonary edema. Regardless if the patient has congestive heart failure or the adult respiratory distress syndrome (ARDS), both morbidity and mortality are related to the lungs’ ability to actively transport salt and water from the air spaces (86, 88). When a β2-agonist, which admittedly has several effects, including the ability to increase lung epithelia Na$^+$ transport, is infused into adults with ARDS, there is a decrease in lung water content, and physicians can use lower ventilator pressures to ventilate their patients (71).

The intact fetal lung was studied during the 1960s; however, it was not until the 1980s that investigators began in earnest to study the adult mammalian lung in vivo or isolated epithelia in vitro. These initial studies, combined with much work that had been done on frog skin or mammalian kidneys, resulted in a commonly held belief that all epithelial Na$^+$ transport was sensitive to amiloride. However, many studies from different laboratories have now determined that ~1/3 to 1/2 of the lung’s epithelial active transport of Na$^+$, with Cl$^−$ and water following, occurs via amiloride-insensitive conductances in the apical membrane. This review focuses on amiloride-insensitive Na$^+$ and fluid transport by the whole lung and distal lung epithelia in culture. No attempt has been made to review the amiloride-sensitive component of distal lung epithelial Na$^+$ and fluid transport or when it has been modulated (see Refs. 24 and 56) or to review epithelial ion transport by the large airways.

Amiloride-Insensitive Na$^+$ and Fluid Transport in the Fetal, Perinatal, and Adult Whole Lung

In 1990, experiments in newborn guinea pigs showed that only half of the alveolar fluid clearance (AFC) could be inhibited by intraluminal $10^{-4}$ M amiloride (64). This amiloride-insensitive AFC is influenced by development but differs between species. Lambs (77) and guinea pigs (22) have an increase in amiloride-insensitive AFC after birth, whereas the opposite occurs in rats. Folkesson et al. (25) showed that the intact fetal rat lung could, through amiloride-insensitive process, be converted to fluid absorption at days 21 and 22, but that after birth the AFC was amiloride sensitive. Developmentally dependent effects are also seen for the amiloride analog, dichlorobenzamil. Dichlorobenzamil does not block epinephrine-induced fluid absorption in fetal lambs, but it attenuates fluid absorption in 6-wk-old lambs and totally blocks absorption in 6-mo-old lambs (40, 41). The amount of amiloride-insensitive Na$^+$ transport stabilizes after birth in the guinea pig as there are similar amounts of amiloride-insensitive apical to basolateral movement of $^{22}$Na across the lung epithelia of 7- and 30-day-old animals (66).

In vivo studies in the adult lung are of particular importance. The most frequently used technique was pioneered in the early 1980s (e.g., Ref. 4) and utilizes a macromolecule within the instillate. This molecule remains within the air space as salt and water is removed, and by measuring the changes in the...
concentration of the macromolecule, AFC can be determined. Since the instillate’s oncotic pressure is reasonably comparable to the lung’s interstitial fluid, continuing AFC in the presence of amiloride indicates that the lung can absorb Na\(^+\) and water via amiloride-insensitive pathways.

A significant portion of the adult lung’s AFC is insensitive to high doses of amiloride in many different mammals. In rats, sheep, dogs, and humans, ~60–70% of the AFC is insensitive to amiloride. Mice may have lower amounts (20–40%) of amiloride-insensitive AFC (e.g., Refs. 14, 17, 29), which varies between strains (56). However, all these studies used a concentration of \(\geq 1\) mM amiloride as the measure for “amiloride-sensitive” AFC. As discussed in Pharmacological blockers of ENaC and amiloride-insensitive Na\(^+\) channels, this tremendously high dose has many effects other than its ability to block Na\(^+\) transport. Recent work has indicated that the \(K_c\) for amiloride’s inhibition of murine AFC is 2 \(\mu\)M (31). The adult guinea pig is the champion of amiloride-insensitive AFC with 3/4 of its basal AFC being amiloride insensitive (56, 60).

Amiloride-insensitive AFC has been altered in genetic experiments even though they were designed for other purposes. Mice genetically deficient in the inducible form of nitric oxide synthase have normal AFC; however, the AFC and the mice’s nasal potential difference are both entirely amiloride insensitive (29). Lung amiloride-sensitive AFC tripled in the \(\beta\)-Liddle mouse strain harboring a gain-of-function mutation (R566 stop) within the Scnn1b gene; however, there was also a doubling of the amiloride-insensitive AFC (79). Increases in amiloride-insensitive AFC do not always occur in transgenic experiments that are designed to modify lung epithelial Na\(^+\) transport: when CMV-driven transgenic overexpression of the \(\alpha\)-subunit of the amiloride-sensitive epithelial Na\(^+\) channel (ENaC) was introduced into the \(\alpha\)-ENaC knockout mouse, neonatal lung fluid clearance was rescued, but amiloride-insensitive AFC did not increase (14).

Infection can alter the amount of amiloride-insensitive AFC. When the respiratory syncytial virus infects BALB/c mice, they develop a necrotide (UTP)-dependent decrease in AFC, and their AFC loses its amiloride sensitivity without any change in the levels of mRNA encoding ENaC (9). When B6 mice are infected with mycoplasma, they experience a marked decrease in total AFC, and, to the authors’ surprise, demonstrated an increase, rather than a decrease, in AFC when amiloride was placed within the instilled fluid (31).

Amiloride-Insensitive Na\(^+\) and Fluid Transport By Monolayers of Distal Lung Epithelia Under Basal Conditions

Intact lungs are the most physiologically relevant experimental model, but they have significant limitations. It is difficult to control numerous important variables and to precisely measure Na\(^+\) and fluid transport. Accordingly, to gain further insight into these important processes, investigators have studied distal lung epithelia in primary monolayer culture. The following discusses the characteristics of these monolayers under basal conditions.

Fetal distal lung epithelium. Primary cultures of fetal distal lung epithelium (FDLE) are ambiguously, but accurately, named to reflect the fact that they are composed of epithelia from the fetal distal lung and are not a single cell type.

The initial study of FDLE mounted in Ussing chambers suggested that there was amiloride-insensitive Na\(^+\) transport. When monolayers were exposed to amiloride (10\(^{-4}\) M apically) or ouabain (10\(^{-3}\) M basolaterally), the short-circuit current \((I_{sc})\) decreased to 35% and <5%, respectively (67). Subsequent experiments, where impermeant ions were substituted for either Na\(^+\) or Cl\(^-\), showed that a significant portion of the amiloride-insensitive \(I_{sc}\) (AIC) was Na\(^+\) dependent under either postnatal (21%) or fetal (3%) oxygen concentrations (2, 63, 73). The amiloride-sensitive component of the \(I_{sc}\) (ASC) does have an inhibitor profile consistent with other Na\(^+\) transporting epithelia (IC\(50\) for amiloride = 0.3 \(\times\) 10\(^{-6}\) M, IC\(50\) for benzamil = 0.3 \(\times\) 10\(^{-7}\) M) (63). Although DIDS, an inhibitor of the HCO\(_3\)/Cl\(^-\) exchanger, Na\(^+\)/HCO\(_3\) exchanger, and other channels (e.g., Ca\(^{2+}\)-activated Cl\(^-\) channels), had a very small effect on the AIC, we were unable to find pharmacological blockers of the AIC. The \(I_{sc}\) was not affected by the Na\(^+\) glucose symport-inhibitor phosphoridzin (10\(^{-3}\) M) or the Na\(^+\)/H\(^+\) inhibitor dimethyl amiloride (DMA, 10\(^{-4}\) M) (63, 67). Although at higher doses FDLE do have EIPA-sensitive \(I_{sc}\) (apical membrane IC\(50\) = 50 \(\mu\)M) (87), studies using EIPA and numerous other inhibitors, including agents that block Cl\(^-\) secretion, similarly could not abrogate the residual amiloride-insensitive current in monolayers studied under control conditions (76). Importantly, and consistent with in vivo experiments, direct measurements of apical to basolateral transport of fluid by monolayer cultures of rat FDLE show that approximately 1/4 of the fluid transport is insensitive to 10\(^{-4}\) M amiloride (15).

Physiological and pathophysiological signals can alter the amount of AIC. FDLE cultured on mixed lung cell (MLC)-derived matrix have a significant increase in AIC under control and \(\beta\)-agonist-stimulated conditions (74). MLC-derived matrix from earlier gestational ages further decreased the FDLE’s \(I_{sc}\) sensitivity to amiloride; however, AIC did not change in FDLE grown on filters coated with type I or IV collagen, laminin, or fibronectin (74). Pathophysiological stimuli can augment the AIC by FDLE. If they are exposed to activated macrophages, a nitric oxide-dependent mechanism markedly decreases ASC with a concomitant increase in AIC (8), a significant portion of which can be blocked by flufenamic acid (12). These effects are associated with a decrease in ENaC mRNA levels and can be reversed with N-acetylcysteine (10). Similarly, exposure of FDLE to edema fluid that has been isolated from rats immediately after inducing acute congestive heart failure induces a dose-dependent increase in AIC (76), a bimodal dose-dependent increase in ASC, and an increase in the rate of amiloride-insensitive apical to basolateral fluid transport (15).

Adult alveolar type II epithelium. Purified adult alveolar type II epithelium (ATII) grown in high resistance (k\(\Omega\)-cm\(^2\)) monolayer culture have, similar to FDLE (e.g., Refs. 2 and 76), an \(I_{sc}\) in the range of 3 to 6 \(\mu\)A cm\(^{-2}\) (e.g., Refs. 7 and 28). When 10\(^{-4}\) M amiloride is applied apically or 10\(^{-3}\) M ouabain is applied on the basolateral membrane of the ATII, the \(I_{sc}\) decreases to ~20% and <5%, respectively, indicating an AIC in this single cell type (7). Similar to FDLE, both amiloride (IC\(50\) = 0.85 \(\mu\)M) and EIPA (IC\(50\) = 64 \(\mu\)M) blocks \(I_{sc}\) in ATII (28).

Cheek et al. (7) measured \(22\)Na\(^+\) fluxes across ATII monolayers. Under baseline conditions, they found that the net apical to basolateral \(22\)Na flux was 5.2 \(\times\) 10\(^{-11}\) eq cm\(^{-1}\) sec\(^{-1}\),
which was comparable to the total $I_{sc}$ of $4.6 \times 10^{-11}$ eq/cm$^2$-sec$^{-1}$. These authors did not report the net $^{22}$Na flux after amiloride, perhaps because of a poor signal to noise ratio for such measurements in distal lung epithelia. However, such measurements have been carried out in tracheal epithelia where the $I_{sc}$ is ~10-fold greater than it is in ATII. Langridge-Smith et al. (52) found a significant residual apical to basolateral net $^{22}$Na$^+$ flux in bovine tracheal epithelia treated with amiloride.

Recent work has shown that there is both amiloride-sensitive and -insensitive fluid transport by ATII. Of the net apical to basolateral transport of fluid by monolayer cultures of rat ATII, 35% is amiloride insensitive, yet only 7% is not blocked by ouabain (19). In agreement with these results, our laboratory has reported that ~50% of fluid transport by rat ATII is amiloride insensitive (15). Human ATII have even greater amounts of amiloride-insensitive fluid transport from their apical to basolateral side. Fang et al. (18) found that human ATII cultured at an air-liquid interface have the majority of their basal $I_{sc}$ and apical to basal fluid transport amiloride insensitive, 70% and 57%, respectively. Extrapolation to the whole lung must be made with caution as the majority of the alveolar surface area is covered by alveolar type I epithelia (ATI), and Clara cells cover the terminal airways. However, the findings that both ATII and FDLE have amiloride-insensitive fluid absorption is consistent with previous observations in the larger airways: normal human and cystic fibrosis airway epithelia have both amiloride-sensitive and -insensitive fluid absorption (37).

**Single Channel and Cellular Ion Conductances In Alveolar Epithelia Under Basal Conditions**

Although measurement of either whole lung AFC or the $I_{sc}$ and fluid transport by epithelial monolayers can provide convincing evidence of amiloride-insensitive Na$^+$ and fluid transport in the mammalian and human lung, they do not provide much insight into the nature of the epithelia’s apical membrane channels. Apical and whole cell conductances and biophysical properties of single Na$^+$ permeant channels of rat alveolar epithelia are briefly discussed below, but readers are referred to more extensive reviews (e.g., Refs. 13 and 55).

There are three approaches to study the functional characteristics of lung epithelial ion channels: patch-clamp whole cell conductances, apical membrane conductance where the basolateral membrane has been permeabilized, and patch-clamp single channel measurements. Each has its limitations.

There have been very few whole cell current measurements of distal lung epithelia in situ or grown in primary culture. Amiloride-sensitive conductance measurements are challenging in view of the low “signal to noise ratio”: there is a large basolateral conductance that “hides” the amiloride-sensitive apical membrane conductance, which is likely only around 10–15% of the total conductance. This phenomenon may explain the results of Jiang et al. (38) where they were unable to detect amiloride-sensitive whole cell conductances in ATII under basal or $\beta_2$-stimulated conditions measured using the perforated patch technique, yet they could easily measure amiloride-sensitive $I_{sc}$ in the same cells that had been grown at an air-liquid interface. Although they observed cation currents in the ATII, it is difficult to ascribe these to amiloride-insensitive channels participating in transepithelial Na$^+$ transport. They may have arisen from divalent or other monovalent cations moving through cation channels (38).

Rat FDLE have amiloride-, benzamil-, and EIPA-sensitive, but DMA-insensitive, conductances (87). Kemp and colleagues (46) have studied ATII grown on glass coverslips and found that pimozide inhibited whole cell cation conductance by ~55% (pimozide’s IC$_{50}$ = 1 $\mu$M with maximal effect at 10–30 $\mu$M), whereas 10 $\mu$M amiloride only decreased cation conductance by 25%. From this inhibitor profile, they concluded that ATII had substantial amiloride-resistant Na$^+$ conductance. Recent studies indicate the presence of amiloride-sensitive whole cell Na$^+$ currents in control, but amiloride-insensitive currents in mycoplasma-infected, murine ATII (31).

As a result of the relative ease with which patch pipettes form GΩ seals on lung carcinoma cell lines, such as A549, there are significantly more publications of whole cell current measurements in such cells. For example, approximately half of the A549 cell’s inward cation current is amiloride insensitive, and it can be modulated by altering nitric oxide synthesis (30, 85). For the reasons outlined above, the interpretation of whole cell currents is difficult, but more importantly, there is the question of using cancer cells to study normal physiology. When the A549 cell line was originally isolated in 1976, it had 64 instead of the usual 23 human chromosomes (53), yet today we are concerned about “modifier genes” significantly altering the ion transporting phenotype of native epithelia. Similarly, cancer cells have altered activity of mTOR, which has recently been shown to modulate the translation of α-ENaC mRNA (69).

Another approach is to study the apical membrane conductances after permeabilizing the basolateral membrane. This approach has been useful in the measurement of amiloride-sensitive apical membrane conductances, such as those induced by changes in ambient oxygen concentrations (2, 75) and amiloride-insensitive conductances, such as those induced by exposure to edema fluid (76). Such data should be interpreted with caution since the permeabilization alters the intracellular milieu, and measurements of amiloride-insensitive conductance assume that there has been no change in intercellular permeability.

The single channel patch-clamp technique has been more robust in the generation of new information. Evidence that some of the Na$^+$ permeant ion channels present on lung epithelial apical membranes might differ from the classic highly Na$^+$ selective, high amiloride affinity channel found in the distal nephron came from the first patch-clamp studies of FDLE (68) and ATII (21). These studies identified an amiloride-sensitive nonselective Na$^+$ channel (NSC) that was equally permeant to Na$^+$ and K$^+$. These observations provide one potential explanation why fetal lung liquid from mammals (1), including primates (65), have higher K$^+$ concentration in their lung liquid relative to plasma or that amiloride can produce a decrease in air space fluid K$^+$ in adult lungs (59).

Since these early reports, single channel studies of ATII suggested the presence of a low affinity amiloride and EIPA-sensitive moderately selective Na$^+$ channel (90).

Other laboratories, and in particular Eaton’s laboratory (reviewed in Ref. 13), have made important new observations regarding the types of Na$^+$ permeant ion channels that are present within the apical membrane of distal lung epithelia and how they are modified by culture conditions. For example, if
ATII are grown on glass supports in the absence of glucocorticosteroid (GC), the predominant channel is a 21-pS NSC, whereas if they are grown on permeable supports with GC and an air-liquid interface, then the predominant channel is the highly Na\(^{+}\) selective channel. However, even under these conditions, ATI still have NSC, and exposure to 5% oxygen, similar to fetal conditions, increases their number (33). The amiloride sensitivity of the NSC is difficult to determine as it would require progressive increments in amiloride concentration on the single channel. However, amiloride’s \(K_i\) for this NSC is believed to exceed 2 \(\mu\)M (39). This is still within the range of amiloride used in Ussing chamber or in vivo experiments (10\(^{-4}\) M and 1.5 mM in mice, see above). Johnson et al. (39) have found evidence of amiloride-insensitive, but pimozide-sensitive, very low conductance NSC (2–8 pS) in ATI. This is consistent with cyclic nucleotide-gated (CNG) NSC channels; however, this work did not include measurement of mRNA for CNG NSC channels to bolster this argument. They did not find functional evidence for CNG-NSC channels in ATII (39).

**Ion Channels That May Be Responsible For Amiloride-Insensitive Conductances**

How does the amiloride-insensitive movement of Na\(^{+}\) across the apical membrane occur? For reasons outlined above, it is very likely to be an ion channel rather than an exchanger or symport. Some of the potential explanations are outlined below.

An “atypical-ENaC.” An atypical-ENaC should be placed high on the list of potential explanations.

The “classic-ENaC” (5, 6) is a heteromeric complex of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC subunits, that, when active in an epithelium’s apical membrane, has a Na\(^{+}\) permeability (\(P_{Na}\)) \(\gg\) K\(^{+}\) permeability (\(P_{K}\), a very low conductance in the range of 4–8 pS, gating kinetics characterized by long closing and opening times, and is inhibited by submicromolar concentrations of amiloride (13). ENaC’s critical importance in lung alveolar epithelia fluid transport has been demonstrated by many laboratories, the most convincing studies arising from genetic experiments involving loss of function of either one of the \(\alpha\)- (32) or \(\gamma\)- (3) subunits or when there was a gain of function mutation of the \(\beta\)-subunit (79).

Different combinations of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC subunits result in dramatically different biophysical properties and channel sensitivity to amiloride or its analogs. For example, the half-inhibition constant (\(K_i\)) for amiloride can be altered by 20-fold larger when \(\alpha\)β and \(\alpha\)γ channels are studied in oocytes (57). If \(\alpha\)-ENaC subunits are expressed in the absence of \(\beta\)- or \(\gamma\)-ENaC in a transfected mouse fibroblast line, a 24-pS NSC with variable amiloride sensitivity is observed (47). However, the results of this latter study should be interpreted with caution as they only reported the results from six patches: three were sensitive and three were insensitive to amiloride. ENaC’s sensitivity to amiloride can also be modified if specific residues on any of the \(\alpha\)-, \(\beta\)-, or \(\gamma\)-subunits are mutated (43, 45, 81).

The stoichiometry of the classic ENaC channel has been debated. For example, it has been proposed that there are eight to nine subunits with more than 1 \(\alpha\)- and a minimum of 2 \(\gamma\)-subunits (16, 84). Others claim ENaC has 2 \(\alpha\)-, 1 \(\beta\)-, and 1 \(\gamma\)-subunit (23, 50). The reason for the apparently contradictory results is unknown. Since the former group studied human ENaC and the latter two groups studied rodent ENaC, one could argue that both are correct. Different stoichiometry might explain the different amounts of amiloride-insensitive AFC seen in mice and humans (see above). Recently, the crystal structure of the acid-sensing ion channel, which like ENaC is a member of the degenerin family of ion channels, has been shown to be a trimer (35). This is strong, but not conclusive, evidence that ENaC might also be a heterotrimer. Although extension of mRNA levels to stoichiometry is tenuous for several reasons, including the translational regulation of \(\alpha\)-ENaC mRNA (70), it should be noted that there are different relative amounts of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC mRNA levels in human airway (72, 83) and in different regions of the rat (20) and murine lung (80). Another potential explanation is that the stoichiometry could be altered by an insertion of an additional subunit, 6-ENaC (36).

**Other Na\(^{+}\) permeant ion channels.** CNG cation channels are permeable to monovalent and divalent cations, expressed in different tissues, including retinal rods, cones, olfactory neuroepithelium, pineal gland, aorta, testis, heart, and Na transporting epithelia, and are sensitive to blockers such as diltiazem and the amiloride analog, dichlorobenzamil (for review see Ref. 44). Using a rat model, it has been demonstrated that a significant portion of the AFC is diltiazem sensitive in terbutaline- or dibutyryl cGMP-stimulated animals, but not in rats studied under basal conditions (61). Diltiazem’s and amiloride’s effects were only partially additive, with 17% and 42% of AFC resistant to the combined inhibitors in terbutaline-simulated and dibutyryl cGMP animals, respectively.

A cGMP-gated cation channel (CNG1) has been cloned from the rat lung, which is relatively insensitive to amiloride (11). CNG1 mRNA is expressed in whole lung, trachea, bronchi, bronchioles, and alveolar cells (11, 82). Genome analysis indicates that the cGMP-gated cation channel that was cloned from the murine kidney’s inner medullary collecting duct (42) is the \(\alpha\)1-subunit of the CNG channel, Cngal. There have been only a limited number of publications on the mRNA expression of CNG within lung epithelia.

An amiloride-insensitive (>10 \(\mu\)M) 26–29-pS NSC is abundant in acutely isolated and cultured rat and human ATII (54). However, it is not spontaneously active in cell-attached patches and requires very high intracellular Ca\(^{2+}\) (\(\geq 0.1\) mM Ca\(^{2+}\)) for activation. These features, along with the fact that it is blocked by intracellular adenosine nucleotides, the most potent being AMP, make it an unlikely candidate for an amiloride-insensitive conductance that is physiologically relevant. This channel and other transient receptor potential-activated channels are reviewed in Refs. 78 and 89.

**Pharmacological blockers of ENaC and amiloride-insensitive Na\(^{+}\) channels.** ENaC was discovered (5, 6) using a functional expression cloning technique that depended on amiloride’s ability to block epithelia Na\(^{+}\) transport. Amiloride and its many analogs each can have a quantitatively different ability to block Na\(^{+}\) channels in any given epithelia. Fortunately, they usually have similar relative potencies (phennamil = benzamil < amiloride < dichlorobenzamil < EIPA < dimethylamiloride) (48). Caution, however, must be used when assessing the amount of amiloride-sensitive and -insensitive Na\(^{+}\) and fluid transport in view of amiloride’s ability to affect many other cellular functions at higher doses. For example, when murine AFC has been measured, the instillates have
experiments as a marker of amiloride-insensitive Na⁺ in vivo (e.g., Ref. 40) and epithelial monolayer experiments. Dichlorobenzamil has been used in both.

Inability to differentiate direct effects on Na⁺ would alter intracellular homeostasis through both the blockade of Na⁺/Ca²⁺ exchange (IC₅₀ = 1 mM), which might affect calcium signaling and the blockade of protein kinase signal transduction pathways (IC₅₀ ≤ 1 mM) (48).

Dichlorobenzamil, diltiazem, and pimozide are three compounds that have shown promise as potential blockers of amiloride-insensitive Na⁺ transport. However, they all share at least one common limitation. Specifically, this lies in their inability to differentiate direct effects on Na⁺ channels, both ENaC and non-ENaC, from their indirect effects such as modifying Ca²⁺ homeostasis in whole lung or epithelial monolayer experiments. Dichlorobenzamil has been used in both in vivo (e.g., Ref. 40) and epithelial monolayer (e.g., Ref. 82) experiments as a marker of amiloride-insensitive Na⁺ transport. However, since it is an amiloride analog (48), it could directly affect ENaC. In addition, it is a potent inhibitor of the Na⁺/Ca²⁺ exchanger (IC₅₀ ≈ 10 μM) (48), and Na⁺/Ca²⁺ exchangers are found in Na⁺ transporting epithelia (26, 51).

Diltiazem can partially block cGMP-stimulated changes in tracheal epithelial Iₑ (82) and at high doses has some ability to decrease AIC in edema fluid-exposed FDLE (76). However, at lower doses (1–10 μM), it blocks cGMP-activated Ca²⁺ efflux concentrations in rod outer segment membranes (49). Pimozide decreases ATII whole cell cation conductance by ~55% (IC₅₀ = 1 μM with maximal effect 10–30 μM) (46); however, pimozide has been shown to be a Ca²⁺ channel antagonist in rod photoreceptors (58). Single channel experiments do, however, provide stronger evidence that pimozide can have a direct effect on NSC. When ATII were grown on glass or permeant supports, there were 7-pS or 2–3-pS NSC that could be blocked by 300 nM pimozide both in the absence of bath Ca²⁺ or with 10 μM amiloride in the pipette, results that are consistent with a CNG channel (39).

The review of the literature revealed surprisingly little information as to these compound’s dose-response effects on the bioelectric properties of distal lung epithelia (see above). Accordingly, we have carried out experiments where we grew FDLE (15) and ATII (27) under both submersion and air-liquid interface conditions. When these monolayers were mounted in Ussing chambers, their baseline bioelectric properties were comparable to previous publications (Table 1). Diltiazem and pimozide had minimal effects on Iₑ (76). Although these data strongly suggest that, at least under baseline conditions, there are insufficient numbers of pimozide-, diltiazem-, or dichlorobenzamil-sensitive Na⁺ channels to affect bioelectric properties of intact monolayers, it does not rule out their utility. For example, the agents may be effective in vivo because the intact lung may have different FDLE and ATII phenotypes than when these cells are maintained in

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<th>FDLE Submersion</th>
<th>FDLE Air-Liquid Interface</th>
<th>ATII Submersion</th>
<th>ATII Air-Liquid Interface</th>
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<td>Transepithelial resistance, Ω·cm²</td>
<td>920±55.4</td>
<td>510±46.2</td>
<td>867±56.0</td>
<td>930±56.4</td>
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FDLE, fetal distal lung epithelium; ATII, alveolar type II epithelium.

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Fig. 1. Effects of incremental log doses of 5 apically added ion transport inhibitors (AMIL, amiloride; BENZ, benzamil; DICH, dichlorobenzamil; PIM, pimozide; and DILT, diltiazem) on the short-circuit current (Iₑ) across primary cultures of fetal and adult distal lung epithelia. A: FDLE submersion. The IC₅₀ for amiloride, benzamil, and dichlorobenzamil was, respectively, 7 × 10⁻⁷ (n = 8), 7 × 10⁻⁸ (n = 9), and 5 × 10⁻⁶ M (n = 13). Pimozide (n = 9) and diltiazem (n = 6) had minimal effects on Iₑ. B: FDLE air-liquid interface. The IC₅₀ for amiloride, benzamil, and dichlorobenzamil was, respectively, 7 × 10⁻⁷ (n = 5), 6 × 10⁻⁸ (n = 7), and 7 × 10⁻⁶ M (n = 7). Pimozide (n = 9) and diltiazem (n = 3) had minimal effects on Iₑ. C: alveolar type II (ATII) submersion. The IC₅₀ for amiloride, benzamil, and dichlorobenzamil was, respectively, 8 × 10⁻⁷ (n = 12), 1 × 10⁻⁷ (n = 12), and 2 × 10⁻⁶ M (n = 12). Pimozide (n = 12) and diltiazem (n = 8) had minimal effects on Iₑ.
primary culture or they may be effective on other cell types such as Clara cells or ATI.

Two things are clear. First, amiloride-insensitive Na⁺ transport is important in lung Na⁺ and fluid transport. Second, although some inhibitor studies have provided tantalizing promises for their ability to identify amiloride-insensitive conductances and transepithelial Na⁺ transport, they remain suspect for the above reasons. Accordingly, it is likely that significant progress in our understanding of the mammalian lung’s significant amount of amiloride-insensitive Na⁺ and fluid transport will not occur until a specific blocker of this conductance is found, and novel approaches are taken to identify the gene product that is responsible for this important physiological property of the lung.

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