Published in final edited form as:
Am J Physiol Cell Physiol. 2005 September ; 289(3): C512–C520.

Inositol polyphosphate derivative inhibits Na^+ transport and improves fluid dynamics in cystic fibrosis airway epithelia

Mark Moody[1], **Carey Pennington**[1], **Carsten Schultz**[3], **Ray Caldwell**[2], **Carlo Dinkel**[3], **Michael W. Rossi**[1], **Sharon McNamara**[5], **Jonathan Widdicombe**[4], **Sherif Gabriel**[2], and **Alexis E. Traynor-Kaplan**[1]

1 *Inologic Inc., Seattle, Washington;*

2 *University of North Carolina, Chapel Hill, North Carolina;*

3 *European Molecular Biology Laboratory, Heidelberg, Germany;*

4 *University of California, Davis, California; and*

5 *Children's Hospital, Seattle, Washington*

Abstract

Amiloride-sensitive, epithelial Na^+ channel (ENaC)-mediated, active absorption of Na^+ is elevated in the airway epithelium of cystic fibrosis (CF) patients, resulting in excess fluid removal from the airway lumen. This excess fluid/volume absorption corresponds to CF transmembrane regulator-linked defects in ENaC regulation, resulting in the reduced mucociliary clearance found in CF airways. Herein we show that INO-4995, a synthetic analog of the intracellular signaling molecule, D-*myo*-inositol 3,4,5,6-tetrakisphosphate, inhibits Na^+ and fluid absorption across CF airway epithelia, thus alleviating this critical pathology. This conclusion was based on electrophysiological studies, fluid absorption, and $^{22}Na^+$ flux measurements in CF airway epithelia, contrasted with normal epithelia, and on electrophysiological studies in Madin-Darby canine kidney cells and 3T3 cells overexpressing ENaC. The effects of INO-4995 were long-lasting, dose-dependent, and more pronounced in epithelia from CF patients vs. controls. These findings support preclinical development of INO-4995 for CF treatment and demonstrate for the first time the therapeutic potential of inositol polyphosphate derivatives.

Keywords

epithelial Na^+ channels; fluid absorption

CYSTIC FIBROSIS (CF) airway epithelia show hyperabsorption of liquid due to mutations in the gene for the CF transmembrane conductance regulator (CFTR) resulting in Cl^- channel malfunction and dysregulation of Na^+ absorption through the amiloride-sensitive epithelial Na^+ channel (ENaC) (23,45,46). ENaC, the rate-limiting step in the regulation of Na^+ absorption across epithelia, helps maintain airway surface liquid volume/depth (20). Excess fluid/volume absorption correlates with defects in ENaC regulation and plays a primary role in the reduced mucociliary clearance found in CF airways. Mucus clearance is an important defense mechanism that protects the airways from infections (22), and defective mucus

Address for reprint requests and other correspondence: A. Traynor-Kaplan, Inologic Inc., 101 Elliot Ave. West, Suite 100, Seattle, WA 98119 (e-mail: alexis@inologic.com)..

GRANTS

This work was supported by National Institutes of Health Grants 1R43 HL71401-01, 1R43 HL67615, and R44 DK52733, by Cystic Fibrosis Foundation Grant TRAYNO98WO, and by the Deutsche Forschungsgemeinschaft Schu 943/4-1.

clearance is associated with the repeated pulmonary infections in CF patients that ultimately result in organ failure.

In CF airway epithelia, ENaC-mediated Na^+ absorption is increased 100–300% (2). Initially, this was attributed to a direct molecular interaction between CFTR and ENaC. However, no such association was found and is unlikely, because the relationship between ENaC activity and CFTR function is reversed in the sweat glands. Recent studies have demonstrated that electrical potential (34), intracellular Cl^- concentration (24), or electrical coupling between Cl^- and Na^+ fluxes (16) could all mediate the inhibition. Therefore, the agents that modulate non-CFTR Cl^- channel activity, such as Ca^{2+}-activated Cl^- channels in CF tissue, could secondarily regulate ENaC.

Inhibition of Na^+ flux through ENaC is predicted to completely compensate for the CFTR defect in restoration of airway surface liquid (ASL) in a mathematical simulation of airway epithelial cells (36,37). In support of this, amiloride, an extracellular blocker of ENaC, temporarily increased mucociliary clearance in clinical trials (48). Problematically, the duration of amiloride action in vivo is very brief (15–30 min), thereby limiting its therapeutic efficacy. This transience is due in part to the diffusion and dilution of effective concentrations of extracellular amiloride. The relatively short half-life of its target, ENaC, in the plasma membrane (40) may also contribute to its brief duration of action.

Some of the >30 naturally occurring inositol polyphosphates (IPs) and phosphoinositides (18,28,32,42) are ion channel regulators (5,12–15,18,19,21,35,39,50–52). Whereas D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], a trigger for $[Ca^{2+}]_i$ release, is the most widely recognized (17), inositol tetrakisphosphates also modulate ion channel activities, including Cl^- and K^+ channels (12,18,21,50). A particular inositol phosphate, Ins(3,4,5,6)P_4, targets Cl^- channels in epithelia (5,13,14). IPs are highly charged, thereby making them unlikely to cross membranes. Thus membrane-permeant forms have been developed that enable intracellular delivery, which recapitulates the ion channel modulating activity of endogenous IPs (27,41,50).

In contrast to extracellularly acting agents directed against the extracellular domain of ion channel pores, membrane-permeant IP analogs modulate ion channel activities from inside the cell. Analogs of slowly metabolized IPs can have an extended duration of action. Therefore, they have the potential for prolonged activity in contrast to the more rapidly eliminated extracellularly active compounds. We previously observed that direct application of a membrane-permeant analog of Ins(3,4,5,6)P_3, INO-4995 (Fig. 1), to human nasal airway epithelia reduced the amiloride-inhibitable basal short-circuit current (I_{sc}) (49). This effect was long lasting but reversible after 48 h. In the current study, we investigated the ionic basis for the inhibition of I_{sc} and probed whether it corresponded to therapeutically meaningful changes in fluid flux as assessed from open-circuit fluxes of ^{22}Na. Thus, in the absence of INO-4995, the mucosa-to-serosa flux of ^{22}Na across CF airway epithelia was inhibited by amiloride, but this amiloride sensitivity was abolished by INO-4995. INO-4995 had no effects on Na^+ fluxes across non-CF airway epithelia. These effects of INO-4995 corresponded to slowing of the fluid absorption rate that portend therapeutic efficacy in CF. In related studies in a mouse nasal potential difference (PD) model, INO-4995 lowered abnormal nasal PD in gut-corrected CF mice (8), further substantiating its therapeutic potential.

METHODS

Materials

Surgically excised nasal polyps were obtained from volunteers in collaboration with Dr. Bonnie Ramsey at Children's Hospital, Seattle, WA, and Dr. Ludwig Allegra at the Northwest Nasal

Sinus Center. The patients provided informed consent before we obtained the tissue specimens. All protocols complied with institutional guidelines of, and were approved by, the Institutional Review Board at Children's Hospital in Seattle. INO-4995, IPs, phosphate/ PM, and other membrane-permeant IP analogs were obtained from SiChem. All other reagents were provided by Sigma-Aldrich unless otherwise indicated.

CF human nasal epithelial (CFHNE) and non-CF nasal epithelial (HNE) cell cultures were cultured and prepared for Ussing studies as described elsewhere (49).

Monolayer preparation for Ussing studies

Epithelial cells (*passage 2* or *3*) were prepared for Ussing chamber and fluid transport studies using Snapwell permeable supports (0.4 μm pore size; Corning Costar, Cambridge, MA) coated with 1 $\mu g/cm^2$ Vitrogen. Cells were plated at 10^5 cells/cm^2 in keratinocyte serum-free media. After 2 days, the media were changed to bronchial epithelial growth medium (BEGM), a 1:1 mixture of DMEM (MediaTech/Cellgro, Herndon, VA), and bronchial epithelial basal media (BEBM) (Clonetics/Biowhittaker, Walkersville, MD), with the following supplements: hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), bovine pituitary extract (52 μg/ml), EGF (0.5 ng/ml), all-*trans* retinoic acid (50 nM, Sigma), penicillin (100 U/ml, Sigma), streptomycin (0.1 mg/ml, Sigma), nonessential amino acids (1×, Sigma), and fatty acid-free bovine serum albumin (3 μg/ml, Sigma). Cells were grown in the BEGM for 1 wk, at which point an air-liquid interface (ALI) culture system was initiated (30). The cells were grown for 2 wk at ALI and fed every other day basolaterally until use in the Ussing chamber (usually 7–10 days).

Ussing chamber studies

Monolayers of HNE and CFHNE were mounted in modified Ussing chambers (Physiological Instruments, San Diego, CA) with the use of a Ringer bicarbonate solution containing (in mM) 115 NaCl, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose, unless otherwise indicated. Experiments were carried out at 37°C, and the pH was adjusted to 7.4 by being gassed with 95% O_2-5% CO_2. After an open-circuit equilibration period of 10 min, the transepithelial PD was recorded, the cells were voltage clamped at 0 mV, and the resulting I_{sc} was continuously recorded. A periodic bipolar voltage pulse monitored resistance calculated using Ohm's law. In acute experiments, drugs were added to the apical or basolateral compartment, as indicated, and the changes in response were recorded. In preincubation experiments, a compound dissolved in 100 μl of media was added to the apical surface of monolayers growing on Snapwells. After a 2-h incubation period at 37°C in a CO_2 incubator, the apical media containing compound was removed, and monolayers were washed with BEGM and returned to ALI for the indicated time before being mounted in Ussing chambers.

$^{22}Na^+$ flux

Monolayers of CFHNE were pretreated with either 20 μM INO-4995 or 2-h vehicle control for 17–22 h before being tested. Monolayers were mounted in Ussing chambers, and PD and resistance were monitored under open-circuit conditions while ion fluxes were measured as described previously (26). A quantity of 10 μCi of ^{22}Na and 45 μCi of ^{36}Cl were added to either the mucosal or serosal chamber. Aliquots (3 ml) were removed from the downhill chambers for counting at 5-min intervals. After 20 min, 100 μM amiloride was added to the mucosal chamber and aliquots were removed for an additional 20 min. Ion flux was calculated as described.

Patch clamp

Outside-out patches were configured from 3T3 cells stably expressing rat αβγ-ENaC. Patches from 3T3 containing near-silent ENaCs were activated with trypsin (4) before INO exposure. The temperature was ~23°C. The 3T3-cell bath solution contained (in mM) 150 Li^+-aspartate, 2 $MgCl_2$, 1 $CaCl_2$, and 5 HEPES, titrated to pH 7.30 with LiOH. Calculated osmolarity was 314 mosmol/l. The 3T3-cell pipette solution contained (in mM) 120 Tris-aspartate, 20 NaCl, 3 MgATP, 0.2 Na_2GTP, 0.1 $CaCl_2$, 1 EGTA, and 5 HEPES titrated to pH 7.10 with NaOH. Free [Ca^{2+}] was ~43 nM, and calculated osmolarity was 295 mosmol/l.

Apical permeabilization and Na^+-K^+-ATPase activity

Nystatin is a pore-forming antimycotic that enables permeabilization of the cell membrane to monovalent ions: Na^+, K^+, and Cl^- (23). Apical nystatin (360 μg/ml) was applied to the solution that bathed the apical compartment at the indicated time. Cytosolic levels of Na^+ (25 mM) were achieved in the bathing solutions by replacing a portion of the NaCl with NMDG-C (145 mM). After permeabilization, the increase in I_{sc} is primarily due to Na^+ movement driven by the Na^+-K^+-ATPase that is completely inhibited by basolateral application of 100 μM ouabain. The composition of the buffer was as follows (in mM): 145 NMDG-Cl, 25 Na^+, 5 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 25 HCO_3^-, 3.3 $H_2PO_4^-$, 0.8 HPO_4^{2-}, and 10 glucose, pH 7.4.

To create a K^+ gradient to evaluate for effects on basolateral K^+ channels, the buffers were as follows: basolateral compartment (in mM) 120 K-gluconate, 4 Ca-gluconate, 4 $MgSO_4$, 0.8 K_2HPO_4, 3.3 KH_2PO_4, 25 $NaHCO_3$, and 10 glucose; and apical compartment (in mM) 120 Na-gluconate, 4 Ca-gluconate, 4 $MgSO_4$, 0.8 K_2HPO_4, 3.3 KH_2PO_4, and 25 glucose.

Blue Dextran fluid transport assay

Blue Dextran (BD) is a 2,000,000-MW compound that is not absorbed by cells and does not move across tight junctions. BD stock solution was prepared with HEPES-modified Ringer buffer (HMRB) (2 mg BD/ml buffer). INO-4995 was solubilized in HMRB [pH 6.7, containing (in mM): 135 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 10 HEPES, and 10 glucose (~285 mosmol/l)] containing ~1 μM BD. The final concentration of vehicle was 0.1% [1:1, DMSO+DMSO containing 5% (wt/vol) Pluronic F-127]. Two hundred microliters of the BD (1 μM) solution containing vehicle or compound were added apically, and monolayers were incubated (Forma model 3956 set on maximum humidity setting) for 18 h. Control plates with media and impermeable membranes showed no loss of volume over 18 h under these conditions. Basolateral buffer consisted of BEGM (~300 osmol/l). After 18 h, 60 μl of the remaining apical buffer were collected for analysis. A standard concentration curve was obtained from the optical density, at 660 nm, of a serial dilution of 10 μM BD in HMRB in a 96-well plate with the use of a Packard Spectracount. The concentration of BD ([BD]) in the samples was extrapolated from the BD standard curve using Packard I-Smart software. The increase in [BD] from the starting value of 1 μM was taken as the magnitude of volume absorption. Absorption rate was calculated by dividing the Δvolume by time normalized to monolayer surface area.

RESULTS

INO-4995 inhibits sodium flux

We have previously shown that direct exposure of CF human nasal epithelial (CFHNE) monolayers to INO-4995 results in a progressive decline in amiloride inhibitable I_{sc}. Furthermore, a pulsed exposure to INO-4995 inhibits basal I_{sc} in CF airway epithelia for >24 h (49). Although I_{sc} reflects the sum of all transepithelial ion flux, the elevated basal I_{sc} in CF tissue is attributed to abnormally high levels of mucosal to serosal Na^+ absorption (29). To test

whether INO-4995 inhibition of I_{sc} was due to Na channel inhibition, we performed several steps. First, we compared the effects of INO-4995 on amiloride-inhibitable I_{sc} in human CF vs. non-CF nasal epithelium. Second, we tested whether INO-4995 inhibits I_{sc} in MDCK cells overexpressing ENaC. Third, we tested whether INO-4995 inhibits ENaC in outside-out patches from 3T3 cells overexpressing rat αβγ-ENaC. Fourth, we tested the effect of INO-4995 on transepi-thelial $^{22}Na^+$ flux under open circuit conditions. Fifth, we determined the contribution of the Na^+-K^+-ATPase to INO-4995 inhibition of I_{sc}. Finally, we determined INO-4995 effects on the fluid absorption rate across airway epithelia.

Prolonged effects of INO-4995 on CFNHE electrophysiology

We exposed monolayers apically to INO-4995 for 2 h, then washed and returned the cultures to ALI for 22 h before mounting the monolayers in Ussing chambers for testing. At this time, I_{sc} was reduced (Fig. 2*A*), demonstrating a prolonged duration of action. We repeated this experiment with multiple doses of INO-4995 to generate the dose-response curve depicted in Fig. 2*B*. INO-4995 dose-dependently inhibited the basal I_{sc} in CFHNE (EC_{50} = 10 μM; Fig. 2*B*). In addition to the vehicle controls, additional controls included pretreatment with phosphate PM ester (P_i/PM), or structurally related analogs with fewer phosphates. IP analogs that did not have the membrane-permeating protecting groups were likewise without effect. A series of membrane-permeant IP analogs ranged from having no effect to having similar effects to INO-4995 with potency corresponding to the phosphorylation pattern and the nature of the substitutions at *positions 1* and *2*. INO-4995 had no apparent deleterious effects on tight junction integrity, as resistance did not decrease (Fig. 2*C*), even with 500 μM INO-4995. In other experiments, repeated exposures over a period of 4–8 days likewise did not reveal delayed or cumulative adverse effects on monolayer integrity.

Comparison of INO-4995 effects in CF vs. non-CF epithelia

We compared the dose-dependent inhibition of amiloride-inhibitable I_{sc} 22 h after incubation with INO-4995 in CF vs. non-CF tissue. INO-4995 inhibits basal I_{sc} in CF tissue but has minimal effect on tissue from non-CF donors (Fig. 2*B*). For instance, 10 μM INO-4995 inhibits I_{sc} by 45% in CF epithelial cells but only 5% in non-CF epithelial cells. This difference is independent of the magnitude of the basal I_{sc}.

INO-4995 inhibits ENaC activity in overexpressing cell lines

MDCK cells overexpressing ENaC (45) allowed more direct testing of the effect of INO-4995 on Na^+ channel activity. These cells exhibit an unusually high basal I_{sc} that is inhibitable with amiloride and therefore are a good model for CF epithelia. In this model system, as in human airway CF epithelia, ENaC activity is responsible for most of the elevated basal I_{sc}. INO-4995 (5 μM) preincubation inhibited basal I_{sc} measured 24 h later in the ENaC-overexpressing MDCK cells in much the same manner as in the CF airway epithelia (Fig. 3). The majority (91%) of the INO-4995 effect is attributable to the amiloride-inhibitable portion of the current supporting the hypothesis that INO-4995 inhibits ENaC (Fig. 3*B*).

The effect of INO-4995 on ENaC activity in outside-out excised patches was measured in 3T3 cells stably overexpressing rat αβγ ENaC subunits. Before INO-4995 exposure, the channel conductance and sensitivity to block with amiloride confirmed ENaC identity and patch configuration in this expression system (3,4). INO-4995 (20 μM) applied in the bath inhibited ENaC in this experiment in eight of nine patches but was without effect on unitary current amplitude (data not shown). A representative trace depicting single-channel transitions is depicted in Fig. 3*C*, and the change in open probability is depicted in Fig. 3, *D* and *E*.

$^{22}Na^+$ flux studies

INO-4995 effects on Na^+ flux in airway epithelia was tested under open circuit conditions. No effects on electrical parameters or ion flux were seen in initial experiments using up to 50 μM INO-4995 on healthy bovine trachea in keeping with specificity of the compound for CF tissue. In contrast, a 2-h pulsed exposure to 20 μM INO-4995 17–22 h before testing resulted in inhibition of basal open circuit PD in human CF nasal epithelia. Corresponding changes in $^{22}Na^+$ mucosal to serosal and serosal to mucosal flux are shown in Table 1. Both serosal to mucosal and mucosal to serosal amiloride inhibitable $^{22}Na^+$ flux were absent in INO-4995-treated monolayers, whereas amiloride inhibited serosal-to-mucosal ^{22}Na flux in untreated cells. These results are consistent with amiloride-sensitive fluid absorption being present normally, but being abolished by treatment with INO-4995.

In well-differentiated CFHNE monolayers, the major pathway for transcellular movement of Na^+ is apical entry via ENaC and basolateral exit via Na^+-K^+-ATPase. Consequently, apical application of amiloride (100 μM) or basolateral addition of the Na^+-K^+-ATPase inhibitor ouabain (100 μM) completely inhibited the high basal I_{sc} we observed in CFHNE cultures. To exclude the possibility that the basolateral Na^+-K^+-ATPase contributed to INO-4995 inhibition of basal I_{sc}, we tested the effect of INO-4995 on the ouabain-sensitive current in apically permeabilized monolayers. We monitored spontaneous I_{sc} for 10 min to assess the effect of INO-4995 on amiloride-inhibitable current. As shown in Fig. 4*A*, a 2-h preincubation with 10 μM INO-4995 resulted in a typical decrease of basal current measured 4 h later. Amiloride (100 μM) was added to assess the contribution of ENaC to basal I_{sc}. We then exposed the apical surface of CFHNE monolayers to nystatin, a pore-forming antimycotic that enhances permeability to monovalent ions. The Na^+-K^+-ATPase inhibitor, ouabain, completely inhibited the resulting increase in I_{sc} after apical permeabilization with nystatin. We observed no differences in INO-4995 pretreated monolayers vs. controls after amiloride addition or apical permeabilization. The results show that INO-4995 has little to no effect on ouabain-inhibitable current (Fig. 4) and supports the hypothesis that INO-4995 inhibits basal I_{sc} through modulation of ENaC activity and not Na^+-K^+-ATPase activity.

In a different protocol, we added amiloride after nystatin to determine whether the entire difference in basal I_{sc} between INO-4995-pretreated monolayers and control monolayers could be eliminated by apical permeabilization (Fig. 4*B*). Monolayers were exposed to 10 μM INO-4995 for 2 h as in Fig. 2 and Ussing chamber recordings carried out 22 h later. We monitored basal I_{sc} for 25 min, whereupon apical addition of nystatin eliminated the difference between INO-4995 pre-treated monolayers and controls after a brief period of equilibration, demonstrating that the target of the INO-4995 action was located in the apical membrane. Amiloride had very little effect when added after nystatin, but addition of ouabain brought the I_{sc} to zero, confirming the success of the nystatin permeabilization and demonstrating again that INO-4995 had no apparent effect on Na^+-K^+-ATPase.

To further probe whether there might be an effect of INO-4995 on basolateral K^+ channel activity, a K^+ gradient was applied to monolayers apically permeabilized with nystatin after pretreatment with 10 μM INO-4995 as in the experiments described above. As shown in Fig. 4*C*, the current driven by the K^+ gradient was similar in both INO-4995-treated monolayers and controls.

INO-4995 inhibits fluid absorption

To ascertain the therapeutic relevance of these electrophysiological observations, we evaluated the ability of compounds to inhibit fluid absorption measured directly. The assay consisted of monolayer cultures of polarized CF nasal epithelia exposed to an apical buffer containing test compounds and a known concentration of the nonpermeable BD, using a modification of a

protocol described by Matsui and coworkers (30). The observed absorption rates ranged from 4 to 6 $\mu l \cdot cm^{-2} \cdot h^{-1}$, consistent with values reported for CF tissue elsewhere (20). We used amiloride, a blocker of the Na^+ channel, ENaC, to assess the ability of this assay to measure relevant changes in fluid secretion. Accordingly, blocking ENaC with amiloride should significantly reduce fluid absorption. As anticipated, amiloride (continuously present for the duration of the 18-h incubation period) inhibits fluid absorption measured by the BD assay (Fig. 5*A*) in a dose-dependent fashion.

We hypothesized that because INO-4995 blocks Na^+ flux, it would inhibit fluid absorption. Testing this, we exposed well-differentiated monolayer cultures of CFHNE to an apically applied buffer containing INO-4995 and BD (Fig. 5). The EC_{50} of the effect is ~20 μM and closely parallels the effect on basal I_{sc}, suggesting that INO-4995 affects both parameters. Our results show that INO-4995 inhibits fluid absorption in CF nasal airways, resulting in greater fluid volumes on the apical surface of the epithelia, indicating that INO-4995 could be effective in treating the pulmonary defect in CF.

INO-4995 inhibition of fluid absorption persists for 42 h

The residence time of aerosolized drugs in airways is limited by expulsion, absorption, and hydrolysis. This may contribute to the short duration of action of extracellularly acting agents, such as amiloride or purinergic agonists (38,54). In contrast, membrane-permeant analogs of IP are not subject to those factors once inside the cell. Therefore, we compared the duration of the effect on fluid absorption following pulsed exposure to compounds. We compared the long-term effect of pulsed exposure to 50 μM INO-4995 (Fig. 5*C*) to that of amiloride. The data show that INO-4995 exerted prolonged effects on fluid absorption. After a 2-h exposure, INO-4995 continues to affect fluid levels up to 42 h after exposure. In contrast, amiloride effects are short lived, vanishing on removal from surface liquid so that, as expected, no residual effect of amiloride contact is detectable 42 h after exposure (Fig. 5*C*). On the basis of these results, INO-4995 is likely to be a much more effective therapeutic than amiloride due to prolonged duration of action.

DISCUSSION

An increase in basal I_{sc} is a characteristic of CF airway epithelia that has been linked to Na^+ hyperabsorption, reduced ASL fluid levels, and disease severity (20,23,30). Herein we have demonstrated that a prodrug based on a derivative of $Ins(3,4,5,6)P_4$, INO-4995, reduces basal I_{sc}, Na^+ flux, and fluid absorption in human CF nasal epithelia. This effect was specific for CF tissue since INO-4995 had very little effect on I_{sc} in non-CF tissue. The inhibition is dose dependent and long lasting. INO-4995 blocked fluid absorption and I_{sc} with similar dose dependency, suggesting that the effects on Na^+, I_{sc}, and fluid absorption are interrelated. The potency of a transient exposure to INO-4995 is comparable to that of continuous exposure to amiloride in inhibiting basal I_{sc} and fluid absorption. In contrast to amiloride, INO-4995 inhibition persists for at least 42 h after the removal of compound from the extracellular compartment. In another experiment, a 2-h exposure to 100 μM INO-4995 resulted in reduced I_{sc} 4 days later (data not shown). The actions of extracellularly acting, aerosolized drugs are limited by expulsion, hydrolysis, and internalization contributing to the short-term effects of agents, such as amiloride and purinergic agonists. In contrast, membrane-permeant analogs of IPs are not subject to these factors once absorbed intracellularly by the mucosal epithelia.

The target of INO-4995, resulting in inhibition of basal I_{sc}, is located at the apical membrane. Apical membrane permeabilization of monolayers eliminated the I_{sc} differential between INO-4995 pretreated monolayers and control. There was no inhibition of basolateral Na^+-K^+-ATPase activity after INO-4995 treatment. Likewise, K^+ currents in monolayers apically permeabilized were unchanged in the INO-4995-treated monolayers in the presence of a K^+

gradient (Fig. 4*C*). These data indicate that INO-4995 inhibits Na^+ absorption through an apical, amiloride-sensitive channel, probably ENaC. This is supported by the following data. First, the elevated basal I_{sc} characteristic of CF tissue that is inhibited by INO-4995 is primarily due to absorption of Na^+ via ENaC. Second, INO-4995 inhibited the amiloride-sensitive I_{sc} in MDCK cells overexpressing ENaC where the elevated basal I_{sc} is attributable to ENaC. Third, INO-4995 inhibited current recorded via 2-electrode voltage clamp in ENaC transfected *Xenopus* oocytes (S. Gabriel, unpublished observations). Fourth, INO-4995 inhibited $^{22}Na^+$ flux in CF human epithelia. Finally, INO-4995 inhibited ENaC currents in outside-out patches from 3T3 cells overexpressing rat αβγ-ENaC.

INO-4995 does not act in the same manner as amiloride. First, while amiloride acts extracellularly, INO-4995 acts intracellularly. Second, amiloride action is short-lived and dependent on the continuous presence of the compound, whereas INO-4995 action is prolonged. Third, amiloride potency in normal HNE is similar to that in CFHNE. In contrast, INO-4995 is much more potent in CFHNE than in epithelia from non-CF donors.

INO-4995 reduced the rate of amiloride-inhibitable fluid reabsorption in CF epithelia using a BD assay. There was a component of the fluid absorption rate that was neither inhibited by amiloride nor INO-4995. This may reflect evaporative loss or an uncharacterized conductance. However, the maximum change in the rate of fluid flux with amiloride in the BD assay (2.5 $\mu l \cdot cm^{-2} \cdot h^{-1}$) is very close to the amiloride-inhibitable flux calculated from the ^{22}Na flux studies (3.3 $\mu l \cdot cm^{-2} \cdot h^{-1}$) assuming isotonicity of transported fluid. Tarran and associates (47) have shown that fluid absorption rates slow when the height of the ASL falls <20 μm. However, under the conditions of the current study, the height of the surface fluid remains well above this level during the entire experiment. Further studies are planned which could determine whether there are differential effects of INO-4995 with varying fluid levels. It should be noted that an amiloride-sensitive fluid absorption of 3 $\mu l \cdot cm^{-2} \cdot h^{-1}$ is similar to that reported by others (20) and, if unopposed, would shrink the depth of ASL by 0.5 μm/min. Therefore, if inhibiting amiloride-sensitive Na^+ absorption unmasks an opposing secretory process, then ASL depth could increase by as much as 0.5 μm/min, causing ASL to double in depth approximately every 10 to 20 min.

The key to the therapeutic potential of INO-4995 is its prolonged effect that may be due to slow metabolism of the active form, INO-4913. $Ins(3,4,5,6)P_4$ levels can remain elevated for hours (50) and are controlled by the reversible $Ins(3,4,5,6)P_4/InsP_5$ 1-kinase/1-phosphatase (15) that phosphorylates $Ins(3,4,5,6)P_4$ on the 1 position, generating $InsP_5$. The deesterified product of the prodrug INO-4995, INO-4913 (Fig. 1), cannot be directly metabolized via this pathway due to the ether-linked octyl group at the 1 position, contributing to its extended duration of its action.

We considered the possibility that INO-4995 could be acting through modulation of serine protease activity, a recently characterized path that modulates ENaC open probability (7). However, INO-4995 appears to act in a serine protease-independent manner because monolayers pretreated with 10 μM INO-4995 still exhibited reduced basal I_{sc} relative to control after the addition of trypsin (data not shown). Moreover, the addition of aprotinin, a serine protease inhibitor, further reduced basal I_{sc} in INO-4995 pretreated monolayers in an additive fashion, indicating that the two agents work through separate but complementary mechanisms.

Phosphatidylinositol (PI)3-kinase pathways can regulate ENaC surface expression in renal epithelia. For instance, Nedd4-2 regulates ENaC expression (31,43,44) and is downstream of hormone (aldosterone or insulin) triggered serum and glucocorticoid-regulated kinase (Sgk-1) and PI3-kinase signaling (1,6,11). Certain IPs interfere with PI3-kinase signaling pathways (12) and may therefore reverse PI3-kinase-mediated Sgk-1 upregulation of ENaC via Nedd4-2.

INO-4995 may directly modulate ENaC

Herein we have shown that INO-4995 inhibited ENaC open probability in excised outside-out patches. Although the concentration of INO-4995 in the bath was 20 μM, the compound would presumably have to cross the patch membrane and be deesterified to generate the active compound, INO-4913, at the site of action. Therefore, a much more detailed study is underway to determine the concentration of the relevant compound and pinpoint the site of action. INO-4995 may indirectly modulate ENaC through actions on Cl^- channels. For instance, INO-4995 is an analog of Ins(3,4,5,6)P_4, which modulates Ca^{2+}-activated Cl^- channels in multiple systems (5,19,42,50–52). We have shown that an analog of INO-4995 opens Cl^- channels in whole cell patch-clamp configuration (9), whereas a deesterified form of INO-4995, INO-4913 opens Cl^- channels in a dialyzed cell preparation (10). In addition, repeated exposure to low doses of INO-4995 enhances the I_{sc} response to ATP, suggesting an increase in Ca^{2+}-activated Cl^- channel activity (33). Others have shown that Ca^{2+}-activated Cl^- channels as well as CFTR activity can modulate Na^+ flux via electrochemical coupling (16,24,25,34). A recent study (53) suggests that both ENaC activity and expression levels are modulated by Cl^- channel-mediated changes in intracellular Cl^- levels. However, it is unclear how that mechanism could account for the inhibition of ENaC measured in excised patches described herein.

We have demonstrated that the IP analog, INO-4995, reverses physiological processes associated with pathological function in CF tissue, Na^+, and fluid absorption. Thus INO-4995 is a potential treatment for the underlying defect in fluid secretion in CF linked to morbidity and mortality. Our findings support preclinical development of INO-4995 for the treatment of CF and represent the first demonstration of the therapeutic significance of a derivative of an IP signaling molecule. In addition to a potential breakthrough in CF research, our results have much wider implications for the design of pharmaceuticals targeting intracellular IP and phosphoinositide signaling cascades.

Acknowledgements

We thank Dr. Ludwig Allegra and the staff at Northwest Nasal Sinus Center for the nasal epithelia biopsy tissue.

References

1. Blazer-Yost BL, Paunescu TG, Helman SI, Lee KD, Vlahos CJ. Phosphoinositide 3-kinase is required for aldosterone-regulated sodium reabsorption. Am J Physiol Cell Physiol 1999;277:C531–C536.
2. Burch LH, Talbot CR, Knowles MR, Canessa CM, Rossier BC, Boucher RC. Relative expression of the human epithelial Na^+ channel subunits in normal and cystic fibrosis airways. Am J Physiol Cell Physiol 1995;269:C511–C518.
3. Caldwell RA, Boucher RC, Stutts MJ. Neutrophil elastase activates near-silent epithelial Na^+ channels and increases airway epithelial Na^+ transport. Am J Physiol Lung Cell Mol Physiol 2005;288:L813–L819. [PubMed: 15640288]
4. Caldwell RA, Boucher RC, Stutts MJ. Serine protease activation of near-silent epithelial Na^+ channels. Am J Physiol Cell Physiol 2004;286:C190–C194. [PubMed: 12967915]
5. Carew MA, Yang X, Schultz C, Shears SB. Myo-inositol 3,4,5,6-tetrakisphosphate inhibits an apical calcium-activated chloride conductance in polarized monolayers of a cystic fibrosis cell line. J Biol Chem 2000;275:26906–26913. [PubMed: 10842174]
6. Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, Staub O. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na^+ channel cell surface expression. EMBO J 2001;20:7052–7059. [PubMed: 11742982]
7. Donaldson SH, Hirsh A, Li DC, Holloway G, Chao J, Boucher RC, Gabriel SE. Regulation of the epithelial sodium channel by serine proteases in human airways. J Biol Chem 2002;277:8338–8345. [PubMed: 11756432]

8. Drumm M, Kyle N, Moody M, Traynor-Kaplan A, Langton-Webster B. INO-4995, a candidate cystic fibrosis therapy, decreases nasal potential difference in a mouse CF model. Pediatr Pulmonol 2004;38:254.
9. Duerson K, Moody M, Schnaars A, Schultz C, Traynor-Kaplan A. Patch clamp analysis of Cl^- current stimulated by a membrane-permeant inositol polyphosphate analogue in CF nasal epithelial cells. Pediatr Pulmonol Suppl 2000;20:199–200.
10. Duerson K, Moody M, Schultz C, Traynor-Kaplan A. A desterified form of INO-4995 (INO-4913) stimulates whole cell Cl^- currents in CF human nasal epithelial cells. Pediatr Pulmonol 2004;38:241–242.
11. Eaton DC, Malik B, Saxena NC, Al-Khalili OK, Yue G. Mechanisms of aldosterone's action on epithelial Na^+ transport. J Membr Biol 2001;184:313–319. [PubMed: 11891557]
12. Eckmann L, Rudolf MT, Ptasznik A, Schultz C, Jiang T, Wolfson N, Tsien R, Fierer J, Shears SB, Kagnoff MF, Traynor-Kaplan AE. D-*myo*-Inositol 1,4,5,6-tetrakisphosphate produced in human intestinal epithelial cells in response to *Salmonella* invasion inhibits phosphoinosi-tide 3-kinase signaling pathways. Proc Natl Acad Sci USA 1997;94:14456–14460. [PubMed: 9405634]
13. Ho MW, Kaetzel MA, Armstrong DL, Shears SB. Regulation of a human chloride channel. A paradigm for integrating input from calcium, type II calmodulin-dependent protein kinase, and inositol 3,4,5,6-tetrakis-phosphate. J Biol Chem 2001;276:18673–18680. [PubMed: 11279175]
14. Ho MW, Shears SB, Bruzik KS, Duszyk M, French AS. Ins(3,4,5,6)P4 specifically inhibits a receptor-mediated Ca^{2+}-dependent Cl^- current in CFPAC-1 cells. Am J Physiol Cell Physiol 1997;272:C1160–C1168.
15. Ho MW, Yang X, Carew MA, Zhang T, Hua L, Kwon YU, Chung SK, Adelt S, Vogel G, Riley AM, Potter BV, Shears SB. Regulation of Ins(3,4,5,6)P_4 signaling by a reversible kinase/phosphatase. Curr Biol 2002;12:477–482. [PubMed: 11909533]
16. Horisberger JD. ENaC-CFTR interactions: the role of electrical coupling of ion fluxes explored in an epithelial cell model. Pflügers Arch 2003;445:522–528.
17. Irvine RF. 20 years of Ins(1,4,5)P_3, and 40 years before. Nat Rev Mol Cell Biol 2003;4:586–590. [PubMed: 12838341]
18. Irvine RF, Schell MJ. Back in the water: the return of the inositol phosphates. Nat Rev Mol Cell Biol 2001;2:327–338. [PubMed: 11331907]
19. Ismailov II, Fuller CM, Berdiev BK, Shlyonsky VG, Benos DJ, Barrett KE. A biologic function for an "orphan" messenger: D-myo-inositol 3,4,5,6-tetrakisphosphate selectively blocks epithelial calcium-activated chloride channels. Proc Natl Acad Sci USA 1996;93:10505–10509. [PubMed: 8816834]
20. Jiang C, Finkbeiner WE, Widdicombe JH, McCray PB Jr, Miller SS. Altered fluid transport across airway epithelium in cystic fibrosis. Science 1993;262:424–427. [PubMed: 8211164]
21. Jiang T, Sweeney G, Rudolf MT, Klip A, Traynor-Kaplan A, Tsien RY. Membrane-permeant esters of phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1998;273:11017–11024. [PubMed: 9556583]
22. Knowles MR, Boucher RC. Mucus clearance as a primary innate defense mechanism for mammalian airways. J Clin Invest 2002;109:571–577. [PubMed: 11877463]
23. Knowles MR, Stutts MJ, Spock A, Fischer N, Gatzy JT, Boucher RC. Abnormal ion permeation through cystic fibrosis respiratory epithelium. Science 1983;221:1067–1070. [PubMed: 6308769]
24. Konig J, Schreiber R, Mall M, Kunzelmann K. No evidence for inhibition of ENaC through CFTR-mediated release of ATP. Biochim Biophys Acta 2002;1565:17–28. [PubMed: 12225848]
25. Kunzelmann K. ENaC is inhibited by an increase in the intracellular Cl^- concentration mediated through activation of Cl^- channels. Pflügers Arch 2003;445:504–512.
26. Lee MC, Penland CM, Widdicombe JH, Wine JJ. Evidence that Calu-3 human airway cells secrete bicarbonate. Am J Physiol Lung Cell Mol Physiol 1998;274:L450–L453.
27. Li W, Llopis J, Whitney M, Zlokarnik G, Tsien RY. Cell-permeant caged InsP3 ester shows that Ca^{2-} spike frequency can optimize gene expression. Nature 1998;392:936–941. [PubMed: 9582076]
28. Majerus PW. Inositols do it all. Genes Dev 1996;10:1051–1053. [PubMed: 8654921]

29. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. Nat Med 2004;10:487–493. [PubMed: 15077107]
30. Matsui H, Randell SH, Peretti SW, Davis CW, Boucher RC. Coordinated clearance of periciliary liquid and mucus from airway surfaces. J Clin Invest 1998;102:1125–1131. [PubMed: 9739046]
31. McDonald FJ, Western AH, McNeil JD, Thomas BC, Olson DR, Snyder PM. Ubiquitin-protein ligase WWP2 binds to and downregulates the epithelial Na^+ channel. Am J Physiol Renal Physiol 2002;283:F431–F436. [PubMed: 12167593]
32. Michell RH. Inositol phosphates: a remarkably versatile enzyme. Curr Biol 2002;12:R313–R315. [PubMed: 12007430]
33. Moody M, Duerson K, Dinkel C, Pennington C, Schultz C, Traynor-Kaplan A. A membrane-permeant analog of inositol 3,4,5,6-tetrakisphosphate inhibits Na^+ absorption in CF nasal epithelia. Pediatr Pulmonol Suppl 2001;32:248.
34. Nagel G, Szellas T, Riordan JR, Friedrich T, Hartung K. Non-specific activation of the epithelial sodium channel by the CFTR chloride channel. EMBO Rep 2001;2:249–254. [PubMed: 11266369]
35. Nilius B, Prenen J, Voets T, Eggermont J, Bruzik KS, Shears SB, Droogmans G. Inhibition by inositoltetrakisphosphates of calcium- and volume-activated Cl^- currents in macrovascular endothelial cells. Pflügers Arch 1998;435:637–644.
36. Novotny JA, Jakobsson E. Computational studies of ion-water flux coupling in the airway epithelium. I. Construction of model. Am J Physiol Cell Physiol 1996;270:C1751–C1763.
37. Novotny JA, Jakobsson E. Computational studies of ion-water flux coupling in the airway epithelium. II. Role of specific transport mechanisms. Am J Physiol Cell Physiol 1996;270:C1764–C1772.
38. Olivier KN, Bennett WD, Hohneker KW, Zeman KL, Edwards LJ, Boucher RC, Knowles MR. Acute safety and effects on mucociliary clearance of aerosolized uridine 5′-triphosphate ± amiloride in normal human adults. Am J Respir Crit Care Med 1996;154:217–223. [PubMed: 8680683]
39. Renstrom E, Ivarsson R, Shears SB. Inositol 3,4,5,6-tetrakisphosphate inhibits insulin granule acidification. J Biol Chem 2002;277:26717–26720. [PubMed: 12055181]
40. Rotin D, Kanelis V, Schild L. Trafficking and cell surface stability of ENaC. Am J Physiol Renal Physiol 2001;281:F391–F399. [PubMed: 11502587]
41. Schultz C. Prodrugs of biologically active phosphate esters. Bioorg Med Chem 2003;11:885–898. [PubMed: 12614874]
42. Shears SB. The versatility of inositol phosphates as cellular signals. Biochim Biophys Acta 1998;1436:49–67. [PubMed: 9838040]
43. Staub O, Abriel H, Plant P, Ishikawa T, Kanelis V, Saleki R, Horisberger JD, Schild L, Rotin D. Regulation of the epithelial Na^+ channel by Nedd4 and ubiquitination. Kidney Int 2000;57:809–815. [PubMed: 10720933]
44. Staub O, Gautschi I, Ishikawa T, Breitschopf K, Ciechanover A, Schild L, Rotin D. Regulation of stability and function of the epithelial Na^+ channel (ENaC) by ubiquitination. EMBO J 1997;16:6325–6336. [PubMed: 9351815]
45. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC. CFTR as a cAMP-dependent regulator of sodium channels. Science 1995;269:847–850. [PubMed: 7543698]
46. Stutts MJ, Rossier BC, Boucher RC. Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics. J Biol Chem 1997;272:14037–14040. [PubMed: 9162024]
47. Tarran R, Grubb BR, Gatzy JT, Davis CW, Boucher RC. The relative roles of passive surface forces and active ion transport in the modulation of airway surface liquid volume and composition. J Gen Physiol 2001;118:223–236. [PubMed: 11479349]
48. Tomkiewicz RP, App EM, Zayas JG, Ramirez O, Church N, Boucher RC, Knowles MR, King M. Amiloride inhalation therapy in cystic fibrosis. Influence on ion content, hydration, and rheology of sputum. Am Rev Respir Dis 1993;148:1002–1007. [PubMed: 8214916]
49. Traynor-Kaplan A, Moody M, Schultz C. An inositol phosphate analog, INO-4995 normalizes electrophysiology in CF airway epithelia. Adv Exp Med Biol 2005;558:115–127.

50. Vajanaphanich M, Schultz C, Rudolf MT, Wasserman M, Enyedi P, Craxton A, Shears SB, Tsien RY, Barrett KE, Traynor-Kaplan A. Long-term uncoupling of chloride secretion from intracellular calcium. Nature 1994;371:711–714. [PubMed: 7935818]
51. Xie W, Kaetzel MA, Bruzik KS, Dedman JR, Shears SB, Nelson DJ. Inositol 3,4,5,6-tetrakisphosphate inhibits the calmodulin-dependent protein kinase II-activated chloride conductance in T84 colonic epithelial cells. J Biol Chem 1996;271:14092–14097. [PubMed: 8662902]
52. Xie W, Solomons KR, Freeman S, Kaetzel MA, Bruzik KS, Nelson DJ, Shears SB. Regulation of Ca^{2+}-dependent Cl^{-} conductance in a human colonic epithelial cell line (T84): cross-talk between Ins(3,4,5,6)P4 and protein phosphatases. J Physiol 1998;510:661–673. [PubMed: 9660883]
53. Xie Y, Schafer JA. Inhibition of ENaC by intracellular Cl^{-} in an MDCK clone with high ENaC expression. Am J Physiol Renal Physiol 2004;287:F722–F731. [PubMed: 15161604]
54. Yerxa BR, Sabater JR, Davis CW, Stutts MJ, Lang-Furr M, Picher M, Jones AC, Cowlen M, Dougherty R, Boyer J, Abraham WM, Boucher RC. Pharmacology of INS37217 [P_1-(uridine 5′)-P_4-(2′-deoxy-cytidine 5′)tetraphosphate, tetrasodium salt], a next-generation $P2Y_2$ receptor agonist for the treatment of cystic fibrosis. J Pharmacol Exp Ther 2002;302:871–880. [PubMed: 12183642]

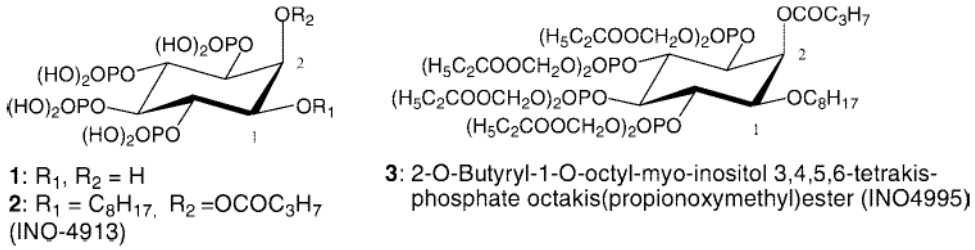

Fig. 1.

INO-4995 and INO-4913. The structure of the pro-drug, INO-4995, is depicted on the *right* side of the figure (*3*). INO-4995 ester groups are hydrolyzed by intracellular esterases releasing the active compound, INO-4913 (*2*). INO-4995 and INO-4913 are analogs of the endogenous signaling molecule, Ins(3,4,5,6)P_4 (*1*).

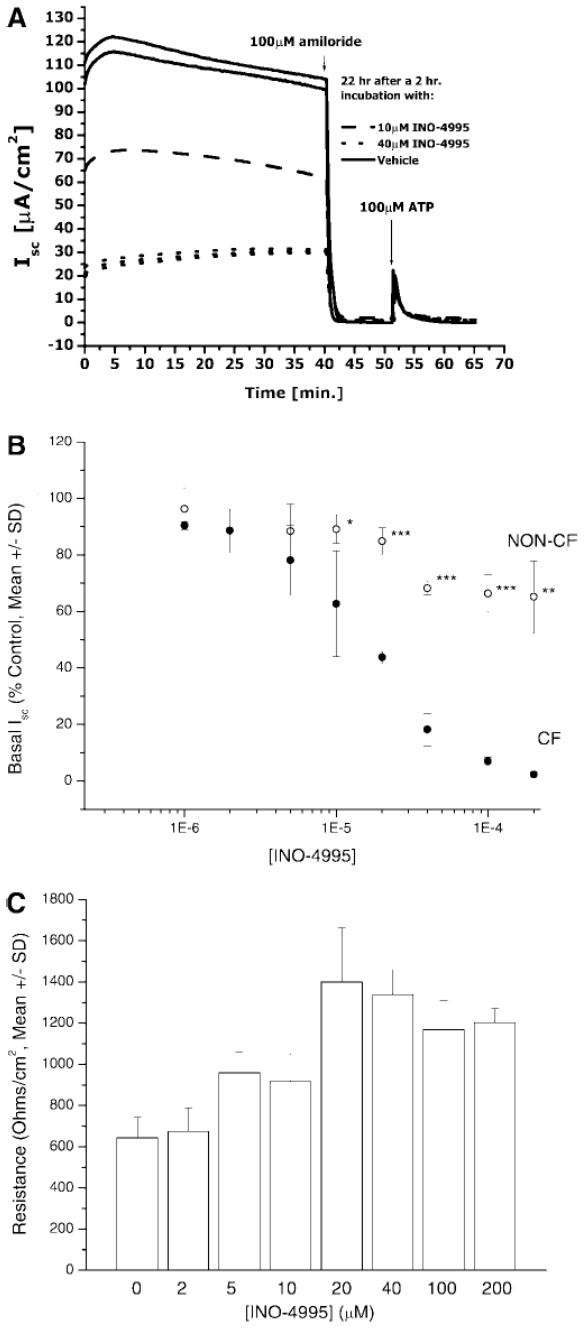

Fig. 2.

Dose dependence of the long-term effect of INO-4995 on spontaneous short-circuit current (I_{sc}) and resistance in cystic fibrosis (CF) and non-CF airway epithelia. Monolayers of differentiated CF nasal epithelial cultures were exposed to INO-4995 applied to solution bathing the mucosal membrane for 2 h. The monolayers were washed and then returned to air-liquid interface (ALI) for 22 h before being mounted in Ussing chambers for testing. In these experiments, INO-4995 inhibited the basal I_{sc} in CF human nasal airway epithelia with an EC_{50} of 10–20 μM. Vehicle [PBS containing 1% 1:1, DMSO+DMSO containing 5% (wt/vol) Pluronic F-127] present in both treatment and control for the 2-h exposure period. *A*: representative experiment contrasting basal I_{sc} in monolayers incubated for 2 h with two

different concentrations of INO-4995 vs. vehicle control and measured 22 h after exposure: 6 traces are shown, 3 from monolayers pretreated with 40 μM INO-4995 (dotted line), 1 from monolayer pretreated with 10 μM INO-4995 (dashed line), and 2 vehicle-treated controls (solid lines). *B*: comparison of the dose dependence of INO-4995 inhibition of basal I_{sc} in human nasal epithelia (CF vs. non-CF). Control CF (n = 14); control non-CF (n = 16). Differences between the means of the CF vs. non-CF samples were evaluated for statistical significance via Student's unpaired t-test. *P = 0.035; **P = 0.0012; ***P < 0.001. *C*: comparison of various doses of INO-4995 on transepithelial resistance in CF human nasal epithelia. Data are means ± SD; n = 3–17 monolayers.

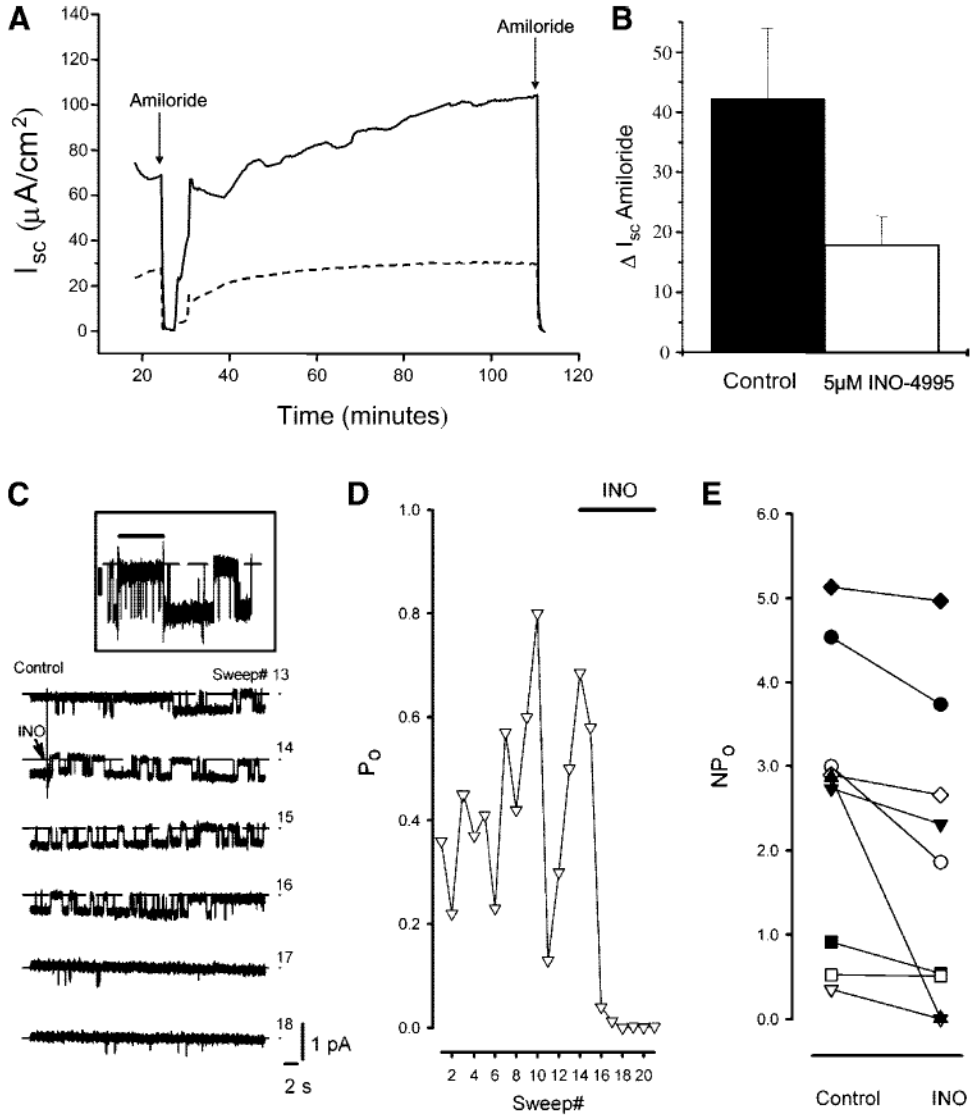

Fig. 3.

INO-4995 inhibits basal I_{sc} in Madin-Darby canine kidney (MDCK) cells overexpressing human epithelial Na^+ channels (hENaC). *A*: representative I_{sc} tracings are shown for MDCK cells incubated for 22 h in INO-4995 (100 μM; dashed line) or vehicle control (solid line). Amiloride (10^{-4} M) was added to both monolayers after baseline currents were recorded and the decrease in I_{sc} was recorded. Amiloride was then washed out of the luminal solution, and currents returned to basal values that were again sensitive to inhibition by amiloride (10^{-4} M). The amiloride-sensitive current represents ~82% of the baseline current in control cells and 91% in INO-4995-treated cells, demonstrating that the major current in both of these conditions is mediated by ENaC. *B*: summary data of the amiloride-sensitive current in INO-4995 treated monolayers (open bars) and vehicle-treated monolayers (solid bars). All values are means ± SE, $n = 8$. INO-4995 incubation results in a significantly reduced amiloride-sensitive current in these cells compared with vehicle-treated control cells ($P < 0.01$). *C*: patch-clamp recording of an excised outside-out patch from a 3T3 cell expressing rat αβγ-ENaC subunits. Channel transitions from selected consecutive traces at −60 mV are shown before (Control, *sweep 13*) and during bath application of INO (20 μM, INO, *sweeps 14–18*). An abrupt decrease in channel

activity was observed within 1.5-min drug exposure. *Inset*: recording from the same patch (−40 mV) showing bath amiloride (10 μM, horizontal line) reversibly inhibited channel activity; horizontal and vertical scale bars are 5 s and 0.2 pA, respectively. Slope conductance (7.6 picoSiemens in 150 mM Li^+) and amiloride-inhibited channel activity confirmed ENaC identity. Dashed lines through traces represent closed-channel current level. *D*: open channel diary of recording shown in *C*. Open probability (P_o) ranged from 0.13 to 0.80 (*sweeps 1-13*, control) and showed no evidence of time-dependent reduction in P_o before drug exposure (INO, see bar). *E*: summary effects of INO on ENaC activity. Channel activity (NP_o, *N* = number of channels) was reduced ~28% in the presence of INO relative to control (Control NP_o = 2.55 ± 0.56 vs. INO NP_o = 1.84 ± 0.58, means ± SE; *n* = 9, *P* = 0.042). Solid lines connect symbols of NP_o recorded before and after 1- to 5-min bath exposure to INO (20 μM) from the same patch. Experiments were performed at ~23°C.

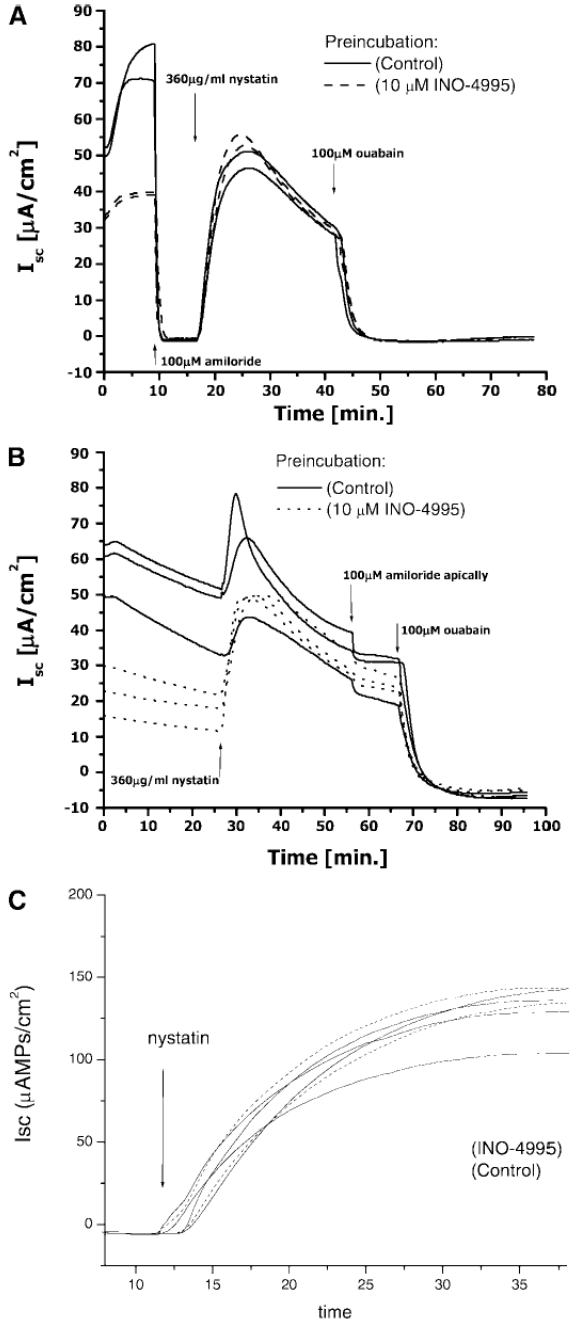

Fig. 4.

Effect of INO-4995 on basal I_{sc} in apically permeabilized monolayers. *A*: amiloride inhibits basal I_{sc} and obliterates difference between control and INO-4995-treated monolayers. I_{sc} responses in monolayers pretreated with INO-4995 are contrasted with control monolayers incubated with vehicle control. Nystatin was used to permeabilize the apical membrane, as described in METHODS. INO-4995 (10 μM) was administered to the monolayers for 2 h, removed, and cells were mounted in Ussing chambers 4 h later. Subsequent permeabilization of the apical membrane with nystatin evokes an identical shift in I_{sc} in both control (solid line) and INO-4995-treated (dashed line) monolayers. This current is completely inhibitable by blocking the Na^+-K^+-ATPase with ouabain. *B*: effect of INO-4995 is abolished after

permeabilization of the apical membrane with nystatin. Subsequent change following amiloride (100 μM) addition is indicative of effectiveness of the nystatin permeabilization. Ouabain-sensitive current is indistinguishable between INO-4995-treated (dashed line) and control (solid line) monolayers. *C*: no difference is seen with INO-4995 pretreatment (10 μM, 2 h, tested after 22 h) on I_{sc} across apically permeabilized monolayers with a basolateral to apical K^+ grandient. Amilo-ride (100 μM) was present during the experiment. Nystatin (360 = μg/ml) was added at the indicated time.

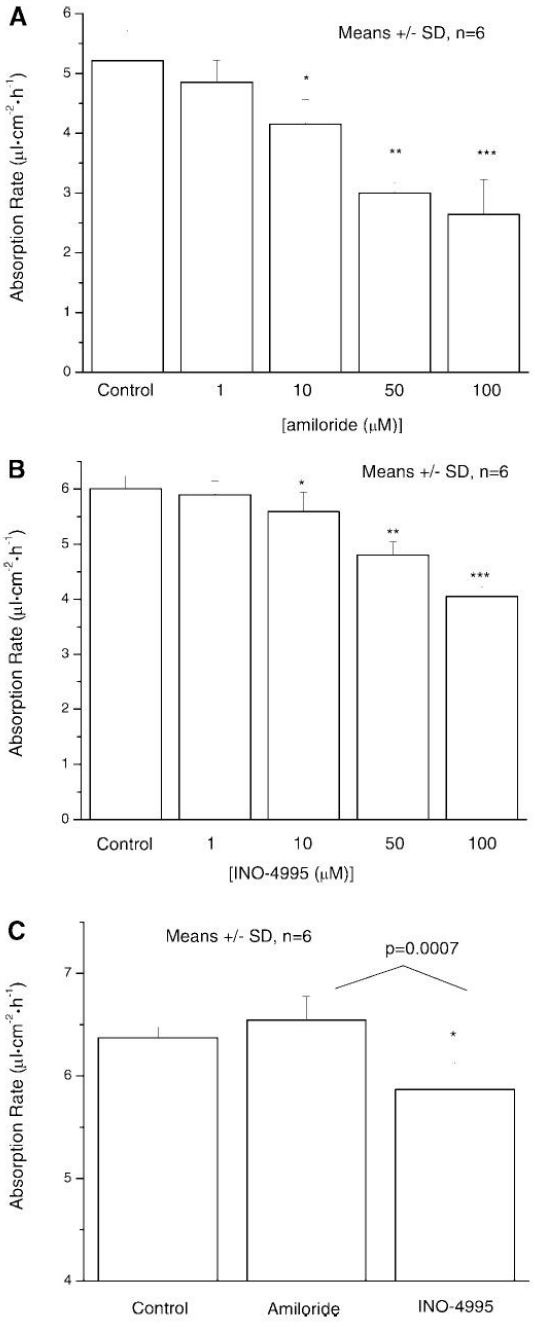

Fig. 5.

INO-4995 inhibits fluid absorption in CF airway epithelia. *A*: dose response of amiloride inhibition of absorption rate in cultured CF human nasal epithelial cells. Average absorption rates were calculated from Δ [Blue Dextran] over 18 h of amiloride exposure, as described in METHODS. Data are means ± SD ($n = 6$ for each condition). Control: vehicle. *$P < 0.05$; **$P < 0.001$; ***$P < 0.005$. *B*: dose response curve for INO-4995 inhibition of fluid absorption measured with the Blue Dextran assay, as described in METHODS. Data show the INO-4995 dose-dependent change in fluid absorption rates from the control values, means ± SD. Control: vehicle [1% 1:1, DMSO+DMSO containing 5% (wt/vol) Pluronic F-127] present during the 2-h exposure period. Differences between means of treated and control samples were evaluated

for statistical significance using Student's unpaired *t*-test: **P* = 0.038; ***P* = 5.1×10^{-6}; ****P* = 1.8×10^{-8}. *C*: comparison of the effect of a 2-h exposure with 50 μM INO-4995 vs. 50 μM amiloride on absorption rate after 42 h in cultured CFHNE. Data are means ± SD for *n* = 6 monolayers. Statistical significance (*P* = 0.0007) vs. control was determined by Student's unpaired *t*-test. Vehicle [Krebs-Ringer containing 0.1% 1:1, DMSO+DMSO containing 5% (wt/vol) Pluronic F-127] present in both treatment and control for the 2-h exposure period.

Table 1

INO-4995 inhibits ^{22}Na movement across CF airway epithelia

	Control (n = 5)		INO-4995 Treated (n = 4)	
	Before	**After amiloride**	**Before**	**After amiloride**
J_{Na}, m→s	1.62 ± 0.42	1.23 ± 0.34*	1.27 ± 0.28	1.31 ± 0.23
J_{Na}, s→m	1.10 ± 0.20	1.22 ± 0.19	1.63 ± 0.36	1.59 ± 0.33

Values are means ± SE, n, no. of cell sheets. CF, cystic fibrosis; J_{Na}, sodium flux. Fluxes are in $\mu eq \cdot cm^{-2} \cdot h^{-1}$ · CF human nasal epithelia.

* $P < 0.05$, Student's paired t-test.