

AN INOSITOL PHOSPHATE ANALOG, INO-4995, NORMALIZES ELECTROPHYSIOLOGY IN CF AIRWAY EPITHELIA

Alexis Traynor-Kaplan,¹ Mark Moody,² and Carsten Schultz³

¹*Inologic Inc., 101 Elliot Avenue West, Suite 400, Seattle, WA 98119, USA;* ²*University of Washington, Seattle, USA;* ³*European Molecular Biology Laboratory, Meyerhofstr. 1, 69117 Heidelberg, Germany, e-mail: alexis@inologic.com*

1. INTRODUCTION

In cystic fibrosis (CF) a combination of ion transport abnormalities results in a reduced capacity to control airway surface liquid volume resulting in viscous mucus that fosters bacterial growth leading to repeated infections, lung damage and ultimately organ failure.^{1,2} CF is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein resulting in defective Cl⁻ transport and dysregulation of a host of apical ion channels. For instance, this defect is also linked to hyperabsorption of Na⁺ through the epithelial Na⁺ channel (ENaC)³⁻⁵ that underlies the abnormal physiology characterized by elevated basal short circuit current (I_{sc}) and large potential difference (PD) in CF mucosal airway epithelia.

Traditional treatments for CF have focused on managing symptoms. Physically assisted therapies such as chest percussion and postural drainage help clear mucus secretions, while three medications are now available to alleviate symptoms. Two antibiotics, Tobi[®] and the more recently tested for efficacy in CF, Azithromycin, help control infections. The other treatment on the market, Pulmozyme[®], cleaves DNA strands from ruptured neutrophils to reduce excess mucus viscosity. While these agents provide relief for some CF sufferers and have helped extend the average lifespan to 33, they do not remedy the underlying defect. Consequently, novel approaches are

still sought to extend life expectancy in CF patients. Current exploratory therapies are in various stages of preclinical and early clinical development and fall into broad categories that includes antibiotics, nutritional support, human stem cell therapy, anti-inflammatories, mucus regulation, gene therapy, CFTR protein rescue and ion channel modulators to normalize ion transport. Here we describe a novel method to normalize ion transport.

Efforts to rectify dysfunctional ion transport associated with CF have focused on modulating ENaC, CFTR, and alternate Cl⁻ channel function. There are compelling arguments for pursuing artificial activation of alternate Cl⁻ channels and regulation of ENaC to counteract CF pathophysiology.⁶ Mucosal epithelia express Cl⁻ channels other than the CFTR such as the outwardly rectifying chloride channel (ORCC), calcium activated Cl⁻ channels (CLCA) and volume regulated Cl⁻ channels (ClC_x) that are all potential targets for CF treatment. While the ORCC appears to be controlled by the CFTR and is thereby compromised in CF,⁷⁻¹⁰ active CLCA channel activity is more abundant in CF tissue.¹¹ In fact phenotypes with increased CLCA activity correlate with milder clinical manifestations.^{7,12-15} Stimulation of apical Cl⁻ secretion through the CFTR and CLCA channels is closely associated with ENaC function and Na⁺ absorption in mucosal epithelia.^{5,16-19} Thus, alternate Cl⁻ channels such as the CLCA channel and the ClC-x family may compensate for defects in CFTR function and could be utilized in a therapeutic strategy. This idea has spawned a number of clinical trials. Recently, Sucampo pharmaceuticals began testing a prostaglandin analog, SPI-8811, which targets ClC-x channels for efficacy in CF. Two other compounds in development target CLCA by elevating intracellular Ca²⁺, INS365, a PY2Y receptor agonist (Inspire Pharmaceuticals) and duramycin (Moli 1901). However, some of these latter results have been disappointing, presumably because of the inherent transience of Ca²⁺ signaling.

Furthermore, an increase in intracellular Ca²⁺ does not always lead to Cl⁻ secretion. We have demonstrated that the intracellular signaling molecule, *myo*-inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P₄] “uncouples” Cl⁻ secretion from the rise in intracellular Ca²⁺ in mucosal epithelia.^{20,21} This regulatory role for Ins(3,4,5,6)P₄ has been confirmed by several investigators in various tissues including a CF pancreatic epithelial cell line, CFPAC-1.²²⁻²⁶ Therefore, we hypothesized that antagonistic analogs of Ins(3,4,5,6)P₄ may stimulate CLCA. Such molecules would be expected to act “downstream” of the rise in intracellular Ca²⁺. To test this we constructed a series of Ins(3,4,5,6)P₄ analogs and studied their effects in monolayer cultures of primary CF human nasal epithelial cells that exhibit many *in vivo* characteristics of CF mucosal epithelia. Since Ins(3,4,5,6)P₄ and constructed analogs are not membrane-permeant we masked the charged groups of these

compounds with bioactivatable, lipophilic groups, that enable cell entry.^{21,27} Once inside, the masking groups are removed and the resulting Ins(3,4,5,6)P₄ derivatives may compete for Ins(3,4,5,6)P₄ binding sites.

Elevated basal I_{sc} is a prominent characteristic of cultures of CF human nasal airway epithelia that distinguishes it from normal tissue and recent studies suggest that it is directly linked to pathophysiology of CF lung disease.⁶ Although basal I_{sc} is largely driven by Na⁺ channel activity (ENaC) it has been closely associated with both CFTR as well as flux through other Cl⁻ channels.^{5,16-19} For example, CFTR inhibits sodium absorption through ENaC such that CF epithelia exhibit a 2-3 fold increase in sodium absorption relative to normal epithelia.²⁸ Moreover, CLC coexpression with ENaC in *Xenopus* oocytes inhibits ENaC activity²⁹ and lack of lung disease in CF mice has been attributed to enhanced CLCA activity relative to human lungs.¹¹ However, the mechanism for these effects of chloride channels on ENaC remains controversial. We chose to screen our compounds for efficacy in lowering basal I_{sc} because it is convenient to measure and is particularly relevant to CF pathophysiology.

2. METHODS

2.1 Materials:

Surgically excised nasal polyps were obtained from volunteers in collaboration with Dr. Bonnie Ramsey at Children's Hospital, Seattle. Informed consent was obtained prior to receiving tissue specimens. All protocols were in compliance with institutional guidelines and approved by the Institutional Review Board at Children's Hospital in Seattle. INO-4995, inositol polyphosphates, phosphate/PM and other membrane-permeant inositol polyphosphate analogs were obtained from SiChem GmbH, Germany. All other reagents were provided by Sigma-Aldrich unless otherwise indicated.

2.2 CF Human Nasal Epithelial (CFHNE) Cell Culture

Surgically obtained nasal polyps were transported on ice in a sterile container containing a 1:1 mixture of Dulbecco's modification of minimum essential medium Eagle and Ham's F-12 nutrient medium (DMEM/F-12) (Irvine Scientific, Santa Ana, CA) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES, and 2 mM L-glutamine. CF tissue was homozygous for the Δ F508 mutation. The tissue samples were washed (5X) by suspending in 40 ml of Joklik's modification of minimum essential

medium eagle (JMEM) at 4°C, allowing the tissue to settle to the bottom of the tube, and aspirating the supernatant. The tissue was then transferred to JMEM containing 200 U/ml penicillin, 0.2 mg/ml streptomycin, 0.1 mg/ml gentamycin sulfate (Clonetics, San Diego, CA), and 0.1 µg/ml amphotericin-B (Clonetics), and 0.1% protease (Sigma), washed an additional 2X, suspended in 15 ml of JMEM in a 10 cm tissue culture dish, and incubated at 4°C for 24 hrs. The tissue samples were gently triturated, the connective tissue aseptically removed, and the remaining cell suspension centrifuged at 1000 rpm for 5 min. The supernatant was aspirated and pellet resuspended in 10ml JMEM with 0.025% trypsin-EDTA and allowed to incubate for 5 min. after which 10% fetal bovine serum (FBS) was added to deactivate the trypsin, and the cell suspension was centrifuged at 1000 rpm. The supernatant was aspirated and cell pellet resuspended in a proliferation media consisting of keratinocyte-serum free medium (KSFM)(Gibco-BRL, Grand Island, NY) containing 5 ng/ml EGF (Gibco), 50 µg/ml BPE (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine. The cell suspension was transferred to two 10 cm tissue culture dishes coated with 1 µg/cm² Vitrogen (Becton-Dickinson, Bedford, MA), incubated at 37°C in an humidified atmosphere of 5% CO₂ and 95% air. The cells were 70-80% confluent after 6 days with the media being replaced with every other day. The cells were then trypsinized using 0.025% trypsin-EDTA for 5 min. The cell suspension was collected, the trypsin deactivated with 10% FBS, and centrifuged at 1000 rpm for 5min. Cell counts were determined by hemocytometer. There was a typical yield of 3-4 x 10⁶ cells per dish. The cells (passage 1) were then cryopreserved for future experiments. Passage 1 cells thawed from cryovials were cultured until they reached 90% confluence when they were trypsinized and plated on permeable supports (passage 2) (snapwells).

2.3 Monolayer Preparation for Ussing studies

Epithelial cells (passages 2 or 3) were prepared for Ussing Chamber and fluid transport studies using *Snapwell* permeable supports (0.4µm pore size)(Corning Costar, Cambridge, MA) coated with 1 µg/cm² Vitrogen. Cells were plated at 10⁵ cells/cm² in KSFM. After 2 days, the media was 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), non-essential amino acids (1X, Sigma), and bovine serum albumin (fatty acid-free, 3 µg/ml, Sigma). Cells were grown in the BEGM for 1 week, at which point an air-liquid interface (ALI) culture system was initiated.³⁰ The cells were grown for 2 weeks at ALI, fed every other day basolaterally until use in the Ussing chamber (usually 7-10 days).

2.4 Ussing Chamber Studies

Monolayers of CFHNE were mounted in modified Ussing chambers (Physiologic Instruments, San Diego, CA) using Ringers bicarbonate solution containing (in mM): 115 NaCl, 2.4 K₂HPO₄, 0.4 KH₂PO₄, 1.2 MgCl₂, 1.2 CaCl₂, 25 NaHCO₃, 10 glucose; unless otherwise indicated. Experiments were carried out at 37°C and the pH adjusted to 7.4 by gassing with 95%O₂/5%CO₂. After an open-circuit equilibration period of 10 min, the transepithelial PD was recorded and the monolayers 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 recorded. In preincubation experiments, compound dissolved in 100 µl of Ringers was added to the apical surface of monolayers growing on snapwells. After a 2 hour incubation at 37° C in a CO₂ incubator, the apical media with compound was removed, monolayers were washed with BEGM and returned to ALI for indicated time prior to mounting in Ussing chambers.

3. RESULTS

3.1 Acute effect of INO-4995 on amiloride-inhibitable I_{sc}

The effect of INO-4995 on the electrical properties of monolayers of human CF nasal epithelia was tested in Ussing chambers. The results depicted in Figure 1-3 show that adding 5 µM INO-4995 to the apical compartment of Ussing chambers results in a decline of I_{sc} leading to baseline over 100 minutes. This effect is not observed in monolayers treated similarly with INO-4949, an unesterified analog of INO-4995 or with the enantiomer of INO-4995, INO-4987. There was also a more gradual decline in monolayers that were not treated with compound which was not noticeably different from that of monolayers treated similarly with INO-4949, an unesterified analog of INO-4995 or with the enantiomer of INO-4995, INO-4987. Consistent with results of other investigators stimulation of purinergic receptors with 100 ATP increased I_{sc} when added after

amiloride (Figure 1). However, 100 μM ATP caused a drop in basal I_{sc} comparable to that caused by preincubation with 5 μM INO-4995 (Figure 2 and 3). Similar results have been described by other investigators who attributed the purinergic induced drop in I_{sc} to inhibition of ENaC.^{17,19} Since we suspected the gradual decline in the non-treated monolayers was due in part to the prolonged time course necessary to see a full effect, we sought alternative protocols to minimize this artifact.

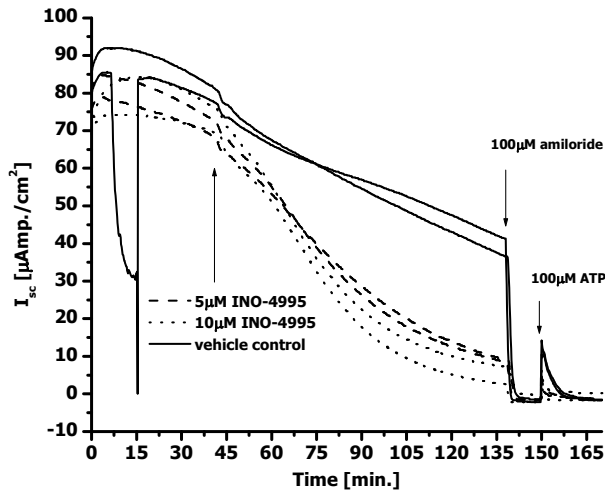


Figure 1. INO-4995 at the indicated doses and time was added to the apical compartment of Ussing chambers in which monolayers of primary CF human nasal epithelia (CFHNE) were mounted. Short circuit current (I_{sc}) was recorded. This experiment is representative of 5.

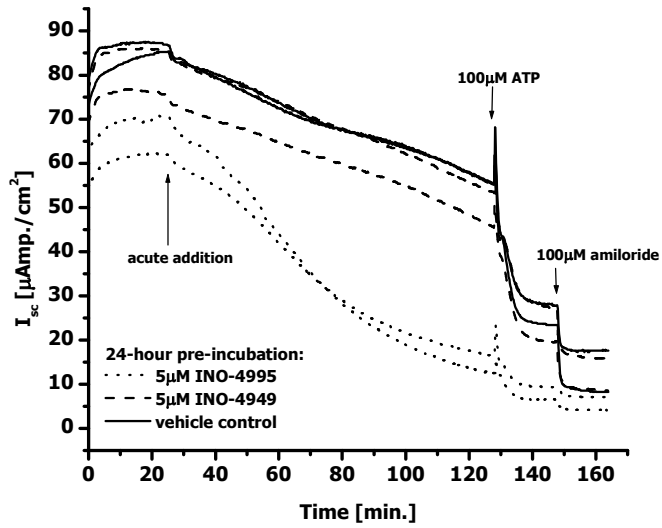


Figure 2. Comparison of the effect of INO-4995 and a de-esterified derivative, INO-4949 on basal I_{sc} .

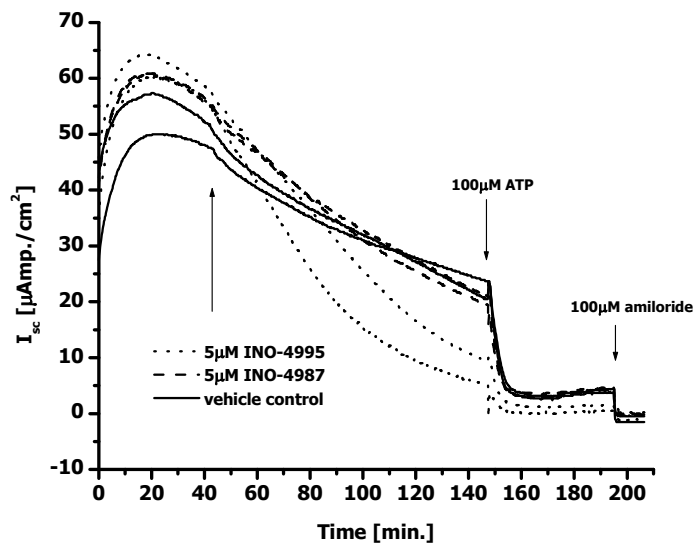


Figure 3. Comparison of the effect of INO-4995 with its enantiomer, INO-4987 on basal I_{sc} in CFHNE.

3.1.1 Peak effect and reversibility

As it took 1-2 hours to obtain a maximal inhibition of basal I_{sc} following acute addition of INO-4995 in our early experiments (Figure 1-3), we incubated monolayers for 2 hours with compound and then returned the monolayers to air liquid interface and incubated them for 24 hours before measuring their I_{sc} in Ussing chambers. This resulted in a stable dose-dependent inhibition of basal amiloride-inhibitable I_{sc} (Figure 4). These results demonstrated that the inhibition of I_{sc} with INO-4995 is long lasting but raised the question of whether the inhibitory effect was reversible. Hence we probed the duration of basal I_{sc} inhibition by measuring electrical properties including basal I_{sc} in Ussing chambers at 8, 24, 48, and 72 hours after a two hour incubation with INO-4995. The results showed that the effect of a 2 hour incubation with 5 μM INO-4995 was virtually unchanged between 8 hours and 24 hrs after incubation (Figure 5). At 24 hours post exposure INO-4995 still shows $\sim 40\%$ inhibition of basal I_{sc} relative to control values, comparable to inhibition observed at 8 hours. The effect was reversible, however, when treated monolayers were tested 48 and 72 hrs later (Figure 5). In fact by 48 hours the inhibitory effect of INO-4995 diminished to near control values. The data demonstrates that 5 μM INO-4995 reversibly inhibits I_{sc} for 22-48 hours (Figure 5).

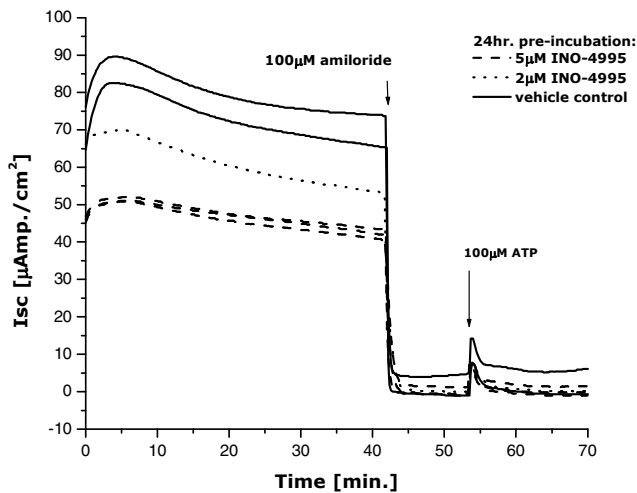


Figure 4. Comparison of the effect of 2 and 5 μM INO-4995 on amiloride-inhibitable I_{sc} 24 h after a 2 h incubation with compound.

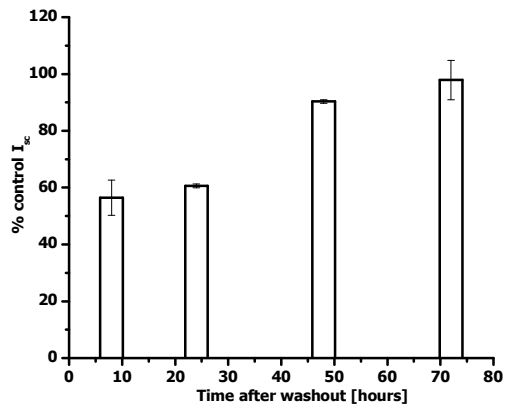


Figure 5. Time-dependent recovery of basal I_{sc} inhibition following a single dose of 5µM INO-4995 (2 h exposure): Inhibition of basal I_{sc} measured at 8, 24, 48, and 72 hours after exposure. Control=100%, n=11. Data calculated from the analysis of the I_{sc} at 10min. Mean +/-SEM of the percent inhibition.

In a separate experiment when monolayers were treated with 10 µM INO-4995 for 2 hours and I_{sc} tested 4 hours later, the degree of inhibition was comparable to the level of inhibition obtained with 10 µM INO-4995 at the 24 hour time point (data not shown) indicating that basal I_{sc} inhibition by INO-4995 remains near maximal levels for from 2 to at least 24 hours.

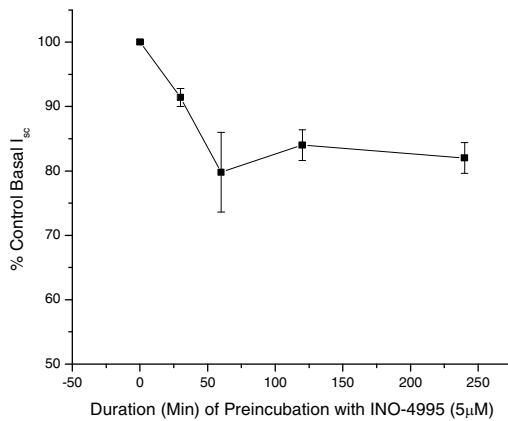


Figure 6. Effect of duration of preincubation with INO-4995 on its inhibition of basal I_{sc} in CF human nasal epithelia.

3.1.2 Optimizing incubation duration.

In order to assess the rate of entry of compound into the cells, the duration of apical incubation with INO-4995 was varied from 30 min. to 4 hrs. At respective times the buffer containing the compound or vehicle was removed and the monolayers returned to ALI culture. After 24 hrs from the time the compounds were first administered, the monolayers were mounted in Ussing chambers and the basal I_{sc} was measured.

While all incubations resulted in reduced I_{sc} , the major increase in potency occurred within the first 30-60 min, with no further change observed with longer incubations exceeding 60 min. (Figure 5).

4. DISCUSSION

CF airway epithelia are characterized by accelerated sodium absorption and attenuated response to Cl^- secretagogues acting through cyclic AMP but not calcium pathways. Although Cl^- secretion in response to secretagogues is transient, the defect in sodium absorption is constant and detectable in cells that have not been exposed to triggering agents. In addition, while the impact of the Cl^- secretory defect may be more localized, the sodium absorption defect is observed consistently throughout the airway mucosal epithelia. Therefore, the defect in sodium absorption may be as relevant to CF pathophysiology as Cl^- secretion. For this reason, we focused our initial studies on the effect of INO-4995 on parameters directly linked to the sodium absorption defect, basal amiloride-inhibitable I_{sc} that was directly proportional to the initial potential difference (PD) across the monolayers.

Addition of INO-4995 directly to the apical compartment of Ussing chambers containing CF airway epithelia resulted in a slow decline of basal I_{sc} (Figures 1-3). This effect did not occur in monolayers exposed to a de-esterified form of the drug that would not be expected to cross the membrane and gain entry to the cell (Figure 2). In addition, an enantiomer of INO-4995 was without effect (Figure 3) demonstrating stereospecificity. The I_{sc} response to the drug is gradual, and complicated analysis of early studies. This lag time may reflect the kinetics of entry and de-esterification of the prodrug to the active drug inside the cell or it may reflect other processes set in motion following release of the de-esterified product inside the cell that are necessary for I_{sc} inhibition. Further complicating interpretation is the decline in I_{sc} in untreated monolayers possibly due to nutritional deficiencies in Ringers solution or to the periodic voltage pulses used to monitor resistance. Although these are potentially interesting questions to elucidate, we wanted to first characterize the physiological action of INO-4995 and

identify a parameter that would be useful for dose/response analysis and comparison with other compounds. In order to circumvent some of the confounding variables, we exposed monolayers to compound for 2 hours in the incubator after which we returned the cells to air liquid interface culture and measured amiloride-inhibitable I_{sc} in Ussing chambers 22 hours later. This protocol resulted in a stable change in I_{sc} measurable at the 22-hour time point (Figure 4) that was more amenable to comparative analysis.

In addition, this demonstrated that INO-4995 could have a long lasting therapeutically relevant affect after a relatively brief exposure. We then sought to determine when the peak effect occurred and whether and over what time course the effect was reversible. The data depicted in Figure 5 indicate that a single 2 hour exposure to 5 μ M INO-4995 reduces I_{sc} with little change for 8-22 hours after addition. Although some inhibition was still observable after 48 hours, basal I_{sc} completely returned to normal by 72 hours. In another experiment we saw no difference between a 4 and 24-hour incubation. Therefore, based on the kinetics of the effect shown in Figure 1 and the time course data, INO-4995 inhibition of basal I_{sc} reaches its peak within the first 2 hours of exposure and remains at this level for more than 22 hours.

We also questioned whether the duration of exposure might play a role in the magnitude of the inhibition. Longer exposures could result in diffusion of more prodrug into the cell and accumulation due to deesterification to the active drug. The data shown in Figure 6 demonstrate that the maximal effect is obtained with a 1 hour incubation when monolayers were incubated with 5 μ M INO-4995 and that no further increase in efficacy was observed with longer incubations, presumably due to breakdown of compound in extracellular buffer after 1 hour incubation.

Based on these studies we can formulate an optimal experimental protocol for evaluating INO-4995 inhibition of basal I_{sc} and comparing its effects to other compounds.

5. CONCLUSIONS

The effects of inositol polyphosphates on ion flux in CF mucosa described here suggest a novel therapeutic approach to the treatment of cystic fibrosis. These studies indicate that transient exposure to an inositol polyphosphate analog, INO-4995, causes long lasting but ultimately reversible changes in therapeutically relevant electrophysiological properties of CF human nasal epithelia. This avoids a major problem encountered with other ion channel regulators that have been advanced as potential CF treatments, limited duration of action.

REFERENCES

1. H. Matsui, B. R. Grubb, R. Tarran, S. H. Randell, J. T. Gatzky, C. W. Davis, and R. C. Boucher. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* **95**, 1005-1015 (1998).
2. H. Matsui, C. W. Davis, R. Tarran, and R. C. Boucher. Osmotic water permeabilities of cultured, well-differentiated normal and cystic fibrosis airway epithelia. *J. Clin. Invest.* **105**, 1419-1427 (2000).
3. R. Greger. Role of CFTR in the colon. *Annu. Rev. Physiol.* **62**, 467-491 (2000).
4. R. C. Boucher, M. J. Stutts, M. R. Knowles, L. Cantley, and J. T. Gatzky. Na⁺ transport in cystic fibrosis respiratory epithelia. Abnormal basal rate and response to adenylate cyclase activation. *J. Clin. Invest.* **78**, 1245-1252 (1986).
5. M. Mall, M. Bleich, J. Kuehr, M. Brandis, R. Greger, and K. Kunzelmann. CFTR-mediated inhibition of epithelial Na⁺ conductance in human colon is defective in cystic fibrosis. *Am. J. Physiol.* **277**, G709-16 (1999).
6. M. R. Knowles and R. C. Boucher. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest.* **109**, 571-577 (2002).
7. L. L. Clarke, B. R. Grubb, J. R. Yankaskas, C. U. Cotton, A. McKenzie, and R. C. Boucher. Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in Cfr(-/-) mice. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 479-483 (1994).
8. S. E. Gabriel, L. L. Clarke, R. C. Boucher, and M. J. Stutts. CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship. *Nature* **363**, 263-268 (1993).
9. M. Egan, T. Flotte, S. Afione, R. Solow, P. L. Zeitlin, B. J. Carter, and W. B. Guggino. Defective regulation of outwardly rectifying Cl⁻ channels by protein kinase A corrected by insertion of CFTR. *Nature* **358**, 581-584 (1992).
10. E. M. Schwiebert, M. E. Egan, T. H. Hwang, S. B. Fulmer, S. S. Allen, G. R. Cutting, and W. B. Guggino. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* **81**, 1063-1073 (1995).
11. B. R. Grubb, R. N. Vick, and R. C. Boucher. Hyperabsorption of Na⁺ and raised Ca(2+)-mediated Cl⁻ secretion in nasal epithelia of CF mice. *Am. J. Physiol.* **266**, C1478-1483 (1994).
12. J. M. Pilewski and R. A. Frizzell. Role of CFTR in airway disease. *Physiol. Rev.* **79**, S215-255 (1999).
13. H. J. Veeze, D. J. Halley, J. Bijman, J. C. de Jongste, H. R. de Jonge, and M. Sinaasappel. Determinants of mild clinical symptoms in cystic fibrosis patients. Residual chloride secretion measured in rectal biopsies in relation to the genotype. *J. Clin. Invest.* **93**, 461-466 (1994).
14. A. Y. Leung, P. Y. Wong, S. E. Gabriel, J. R. Yankaskas, and R. C. Boucher. cAMP- but not Ca(2+)-regulated Cl⁻ conductance in the oviduct is defective in mouse model of cystic fibrosis. *Am. J. Physiol.* **268**, C708-712 (1995).
15. R. Rozmahel, M. Wilschanski, A. Matin, S. Plyte, M. Oliver, W. Auerbach, A. Moore, J. Forstner, P. Durie, J. Nadeau, C. Bear, and L. C. Tsui. Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat. Genet.* **12**, 280-287 (1996).
16. S. K. Inglis, A. Collett, H. L. McAlroy, S. M. Wilson, and R. E. Olver. Effect of luminal nucleotides on Cl⁻ secretion and Na⁺ absorption in distal bronchi. *Pflugers Arch.* **438**, 621-627 (1999).

17. D. C. Devor and J. M. Pilewski. UTP inhibits Na⁺ absorption in wild-type and DeltaF508 CFTR-expressing human bronchial epithelia. *Am. J. Physiol.* **276**, C827-837 (1999).
18. S. J. Ramminger, D. L. Baines, R. E. Olver, and S. M. Wilson. The effects of PO₂ upon transepithelial ion transport in fetal rat distal lung epithelial cells. *J. Physiol.* **524** Pt 2, 539-547 (2000).
19. X. F. Wang, and H. C. Chan. Adenosine triphosphate induces inhibition of Na⁺ absorption in mouse endometrial epithelium: a Ca²⁺-dependent mechanism. *Biol. Reprod.* **63**, 1918-1924 (2000).
20. U. Kachintorn, M. Vajanaphanich, K. E. Barrett, and A. E. Traynor-Kaplan. Elevation of inositol tetrakisphosphate parallels inhibition of Ca²⁺-dependent Cl⁻ secretion in T84 cells. *Am. J. Physiol.* **264**, C671-676 (1993).
21. M. Vajanaphanich, C. Schultz, M. T. Rudolf, M. Wasserman, P. Enyedi, A. Craxton, S. B. Shears, R. Y. Tsien, K. E. Barrett, and A. E. Traynor-Kaplan. Long-term uncoupling of chloride secretion from intracellular calcium levels by Ins(3,4,5,6)P₄. *Nature* **371**, 711-714 (1994).
22. M. W. Ho, S. B. Shears, K. S. Bruzik, M. Duszyk, and A. S. French. Ins(3,4,5,6)P₄ specifically inhibits a receptor-mediated Ca²⁺-dependent Cl⁻ current in CFPAC-1 cells. *Am. J. Physiol.* **272**, C1160-1168 (1997).
23. W. Xie, K. R. Solomons, S. Freeman, M. A. Kaetzel, K. S. Bruzik, D. J. Nelson, and S. B. Shears. Regulation of Ca²⁺-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.* **510**, 661-673 (1998).
24. I. I. Ismailov, C. M. Fuller, B. K. Berdiev, V. G. Shlyonsky, D. J. Benos, and K. E. Barrett. 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. U.S.A.* **93**, 10505-10509 (1996).
25. M. A. Carew and P. Thorn. Carbachol-stimulated chloride secretion in mouse colon: evidence of a role for autocrine prostaglandin E₂ release. *Exp. Physiol.* **85**, 67-72 (2000).
26. M. A. Carew, X. Yang, C. Schultz, and S. B. Shears. 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.* **275**, 26906-26913 (2000).
27. C. Schultz. *Bioorg. Med. Chem.* (2003).
28. M. J. Stutts, B. C. Rossier, and R. C. Boucher. Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics. *J. Biol. Chem.* **272**, 14037-14040 (1997).
29. J. Konig, R. Schreiber, T. Voelcker, M. Mall, and K. Kunzelmann. The cystic fibrosis transmembrane conductance regulator (CFTR) inhibits ENaC through an increase in the intracellular Cl⁻ concentration. *EMBO Rep.* **2**, 1047-1051 (2001).
30. T. E. Gray, K. Guzman, C. W. Davis, L. H. Abdullah, and P. Nettesheim. Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *Am. J. Respir. Cell. Mol. Biol.* **14**, 104-112 (1996).